DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING CHROMATOGRAPHIC ASSAY METHODS FOR DRUGS AND ITS COMBINED PHARMACEUTICAL FORMULATIONS ACTING ON GI TRACT

A Thesis submitted to Gujarat Technological University

For the Award of

Doctor of Philosophy

in

Pharmacy

by

Dharatiben Dilipkumar Rami

Enrollment No.:159997390002

Under supervision of

Dr. Nehal Shah

And

Dr. Ankit Chaudhary

GUJARAT TECHNOLOGICAL UNIVERSITY

AHMEDABAD

[November – 2023]

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ABSTRACT:

The gastrointestinal (GI) system encompasses the oral cavity, esophagus, stomach (both glandular and non-glandular regions), duodenum, jejunum, ileum, and colon (including cecum, colon, and rectum). This intricate digestive tract serves as the primary target for numerous commonly prescribed medications. This research endeavors to identify the most efficient UPLC, and LC-MS/MS techniques for accurately quantifying Sofalcone (SFL) in bulk, as well as in drug substances like Clidinium Bromide (CLBr), Chlordiazepoxide (CDZ), and Pantoprazole Sodium (PNT) when formulated as capsules, utilizing RP-HPLC technique.

Controlled degradation experiments were conducted to ascertain potential degradation by products that may arise during the storage of these pharmaceuticals. These standardized analytical protocols play a pivotal role in assessing the potency and purity of diverse pharmaceutical compounds. They also contribute significantly to management, tracking, and quality assurance processes.

Experiments probing the stability of these drugs under varying environmental conditions revealed crucial insights from forced degradation studies. Utilizing this data, we can establish the safest procedures for the handling and storage of these medications. Notably, under the acidic, alkaline, oxidative, photolytic, and thermal stress conditions recommended by the International Council for Harmonisation (ICH), numerous breakdown products of sofalcone were observed. Mass spectrometry is poised to further illuminate the composition of these degradation products, aiding in the development of hypotheses regarding their breakdown mechanisms and mass balance.

This PhD thesis aims to identify the most efficient analytical techniques, specifically UPLC and LC-MS/MS, for accurately quantifying SFL in dosage form and RP-HPLC for drug substances like CLBr, CDZ, and PNT when formulated as combined capsule dosage form.

Keywords: Sofalcone, Clidinium bromide, Chlordiazepoxide, and Pantoprazole sodium, Validation, Stability-Indicating Chromatographic Assay, RP-HPLC, UPLC LC-MS/MS, and GI Tract.

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-Dharati Rami

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LIST OF ABBREVIATION

PNT	Pantoprazole Sodium
CLBr	Clidinium Bromide
CDZ	Chlordiazepoxide
SFL	Sofalcone
HPLC	High-performance liquid chromatography
UHPLC	Ultra High-performance liquid chromatography
LC-MS	liquid Chromatography Mass Spectroscopy
SIAM	Stability indicating assay method
UPLC-MS	Ultra Performance Liquid Chromatography Mass Spectrometry
MRM	Multipoint Reaction Monitoring
TQ	Triple Quadrupole
DPs	Degradation products
QbD	Quality by Design
FDA	Food and Drug Administration
ICH `	International Conference on Harmonization
UPLC	Ultra-Performance liquid chromatography
MS	Mass spectrometry
FD	Forced degradation
CDSCO	Central Drugs Standard Control Organization
ACN	Acetonitrile
NaOH	Sodium Hydroxide
OPA	Ortho Phosphoric acid
BP	British Pharmacopoeia
EP	European Pharmacopoeia
LOD	Limit of detection
LOQ	Limit of quantification
NA	Not Applicable
ND	Not Detected
SD	Standard deviation
CAS	Chemical Abstract Service
IUPAC	International union of pure and applied chemistry
pKa	Dissociation constant
µm	Micro meter

mm	Milli meter
cm	Centi meter
psi	Pound per square inch
µg	Microgram
hr	Hour
ml	Milliliter
ppb	Part per billion
ppm	Part per million
mg	Milligram
i.d.	Internal Diameter
Conc.	Concentration
Sr. no	Serial No
v/v	Volume by Volume
M	Molar
N	Normal
Rf	Response factor
RSD	Relative standard deviation
DP	Degradant product
$^{\circ}C$	Degree Celsius
R^2	Correlation coefficient

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CHAPTER 1

1 Introduction

1.1 Drugs Acting on the Gastrointestinal System

Parts of the digestive system include the mouth, the stomach, the small intestine (duodenum, jejunum, and ileum), and the large intestine (cecum, colon), as well as the rectum, the anus, and the exocrine glands. The digestive system is sometimes referred to as the gastrointestinal (GI) tract (the salivary glands, the pancreas, and the gallbladder).

Some of the conditions that can be helped by medications that affect the gastrointestinal system include gastric acidity, peptic ulcers, Gastroesophageal reflux disease (GERD), bowel motility disorders (such as gastro paresis, which causes the stomach to empty slowly because some of the stomach muscles are paralysed), constipation, diarrhoea, and the prevention and treatment of nausea and vomiting [1.1, 1.2].

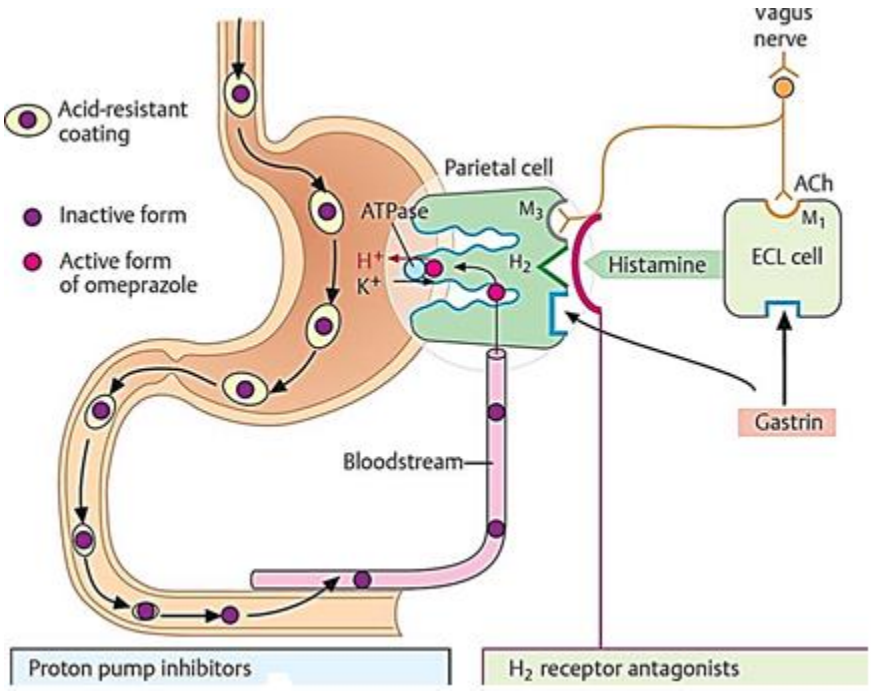


FIGURE 1.1 Gastric acid (HCl) secretion into the lumen of the stomach [1.1, 1.2].

1.1.1 Mechanism of action.

Proton pump inhibitors work by preventing the proton (H^+-K^+-ATPase) pump in the parietal cells of the stomach from functioning properly. This is due to the fact that the inside of the stomach is not being secreted with any hydrochloric acid (HCl) (Fig.1.1). Proton pump

inhibitors work to prevent the flow of H^+ and K^+ ions between parietal cells in the stomach. This movement is necessary for proper digestion. Histamine is blocked from entering the parietal lobe by antibodies that specifically target the H2 receptor. When taken on a consistent basis, studies have shown that both omeprazole and ranitidine are able to lower the amount of acid produced in the stomach. Damaging repercussions (ECL, enterochromaffin-like cell; ACh, acetylcholine). The world's events almost never play out in the manner in which people had predicted they would. They often complain of stomach aches and bouts of diarrhoea.

H^+-K^+–ATPase (the proton pump)

H^+-K^+ ATPase significant transmembrane protein called is produced in the parietal cells of the stomach. In spite of the electrochemical gradient, it actively transfers H^+ into the stomach lumen in exchange for K^+ (one H^+ for one K^+). Specifically, one H^+ is exchanged for one K^+. In order to produce usable energy, the ATP involved in this transaction must first be hydrolyzed [1.1, 1.2].

1.1.2 Gastric acid production

When the vagus nerve, histamine, or gastrin send signals to the parietal cells, they make stomach acid. Carbon dioxide (CO_2) and water are broken down in the parietal cells to make bicarbonate (HCO_3^-) and hydrogen ions (H^+) (H_2O). The H^+-K^+ ATPase pumps hydrogen ions (H^+) into the inside of the stomach. Cl is released by the parietal cells into the lumen, where it spreads out to the rest of the body. In the lumen, H^+ ions, Cl^- ions, and water come together to make hydrochloric acid (HCl) [1.1, 1.2]. The body then takes in the $HCO3^-$ that is made.

1.1.3 Gastroesophageal reflux disease (GERD)

As the oesophagus lacks a protective coating, stomach acid often runs backwards into it, producing discomfort, heartburn, and inflammation. This condition is known as gastroesophageal reflux disease (GERD). Back discomfort caused by GERD might be exacerbated worse by bending over or consuming hot beverages. High intra-abdominal pressure (produced by factors such as obesity, large meals, and tight clothes) and reduced lower esophageal sphincter (LES) tone exacerbate gastroesophageal reflux disease (GERD) (pregnancy, hiatus hernia, achalasia, fatty meals and smoking, and tricyclic and anticholinergic drugs). Antacids, H2-receptor antagonists such as cimetidine, and proton pump inhibitors are all used in the treatment of peptic ulcers (e.g., omeprazole). Similar to other prokinetic medications, metoclopromide may be used to strengthen the LES. If medicines are ineffective, surgery to tighten the LES may be required [1.1, 1.2].

1.2 Stability indicating assay method

The pharmaceutical sector faces significant challenges posed by changes in stability over the course of time and in response to a variety of environmental conditions. Stability and quality are inextricably related due to the fact that the quality of a product degrades with time and is impacted by the manner in which it is kept. The results of stability testing may be used to make estimates about how much longer something will continue to function properly or when it will become unusable. The findings of stability testing also point to a variety of other methods that may be used to keep things for an extended period of time. Your prescription or over-the-counter medication should come with a label that includes an expiration date. This date should tell you when the drug will no longer be effective. The product has not shown any indications of being damaged even when it has been cared for in accordance with the instructions. When determining how long a product may be used before it becomes unusable, it is important to store it in the appropriate circumstances, which include maintaining a consistent temperature and level of humidity. Before a release date can be determined, it is necessary to verify the reliability of everything that has been created. During the process of assessing the medicine's stability, the primary focus has been on two aspects of the product's overall quality. When administering a medication, it is essential to be aware of its potency, also known as the percentage of the active component that it contains. If there was a significant decrease in the quantity of the active component, the medicine's efficacy would be significantly diminished. The second thing that you need to perform is search for toxins that are brought on by the advanced age of the structure. This makes the procedure more difficult since you have to speculate on what the potential results of the breakdown may be. The degradation products of a medicine need to be measured if they make up 0.1% or more of the active component in the drug. Methods for tracing substances are currently being investigated. According to the US Food and Drug Administration's Stability Directive from 1987, quantitative analytical methods "that are based on the characteristic structural, chemical, or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products" are required in order to determine how much of an active ingredient is present in a drug in order to determine how much of that active ingredient is present in the drug. In accordance with the requirements of the specification, the newly developed analytical approach is able to detect the active element even in the presence of the element's breakdown products. With the help of this technology, it is feasible to determine with pinpoint accuracy the quantity of the active chemical that is present in an area where its breakdown products are also present. The acronym SIAM stands

for "validated quantitative analytical procedures." These procedures provide an accurate assessment of the active substance, as well as its breakdown products and other components of interest [1.1, 1.2]. In 1998, the Food and Drug Administration of the United States came up with these regulations for the very first time. Changes in the chemical, physical, or microbiological characteristics of the drug material or drug product must be discovered using these approaches. These changes might occur over the course of time.

1.2.1 United States-Food and Drug Administration (US-FDA) 1998

The new approach has showed some encouraging early results. Since this novel analytic approach is sensitive to just the molecules of interest, no effort is spent searching for unimportant degradation products or investigating unimportant chemicals. This technique may be used to establish time-dependent maximum and minimum limits for a pharmaceutical product's impurity and degradation product levels, as well as its concentration of an active component. It guarantees the efficacy of the treatment. Stability-indicating tests and other authorised quantitative analytical processes ensure that the same quantity of medication components and final products can be tracked throughout time. One strategy to develop stability-indicating test procedures is to subject the material to stress or other conditions that will cause it to degrade. This allows one to foretell the breakdown products and determine the test's level of specificity. There has been study into the storage breakdown process and its potential byproducts [1.3, 1.4].

1.2.2 Stability indicating assay method as Regulatory requirements

All of the quality standards, Q1A, Q2B, and Q2R1, stipulate that stability testing must be carried out with the aforementioned criteria. The recommendations established by the ICH are standards that are adhered to everywhere in the globe. They are acceptable for usage in the United States, the European Union, and Japan. According to the ICH guideline Q1A on Stability Testing of Novel Drug Substances and Products, changes in quality that are anticipated to develop during storage have an influence on the quality, safety, and efficacy of the substance. We will rely on tried-and-true approaches to ensure that these solutions are successful. In addition, the medicine and its metabolites need to be investigated in oxidative environments, at pH extremes, and at temperatures for forced breakdown that are 10 ºC higher than the accelerated temperatures (40ºC). According to ICH guideline Q1B, novel pharmaceutical substances and products have to be evaluated with the use of light. The purpose of putting anything through stress testing is to determine how stable it is by its own nature and to learn how it fails. This is done in order to facilitate the use of the suggested analytical strategies. The ICH guideline Q2R1 investigated the effectiveness of the approach

as an analytical tool in addition to analysing how the method has evolved through time. "documented assurance that offered analytical techniques are verified and acceptable for the identification and quantification of degradation products," as stated in the ICH Guideline Q3B on the Identification and Quantification of Impurities in New Drug Products (ICH Guideline Q3B on the Identification and Quantification of Impurities in New Drug Products). Specifications for drug substances and drug products may be found in ICH guideline Q6A, as are ideas for tests that can be used to determine the degree to which something is stable. The manufacturer is free to test the product based on whatever criteria they see fit, so long as they construct a complete stability-indicating profile of the product. This profile should be able to detect any changes to the identity, purity, or potency of the product. There is not a single evaluation or statistic that can determine how long these items will continue to function. Testing for stability is recommended as a prudent practise by both the World Health Organization and the European Committee for Exclusive Medicinal Products. According to the Canadian Therapeutic Products Directorate, medications that are taken often should have their stability evaluated more frequently. The United States Pharmacopeia (USP) includes guidance on stability testing in a chapter titled "Stability Studies in Manufacturing," which may be found on its website. It is necessary to use a method that demonstrates stability in order to evaluate the performance of the product samples. In addition, the World Health Organization (WHO) has mandated that stability tests must be carried out in accordance with the ICH standard (Q7A), and both the testing methodology and the stability must be evaluated [1.5, 1.6].

The purpose of stability testing is to determine how the quality of a drug or other product is affected by the passage of time as well as environmental factors such as temperature, humidity, and light. The findings of these research will assist to establish guidelines for how items should be stored, how often they should be tested, and how long they will remain effective. Registration Applications for NMEs and other drugs that are conceptually comparable are the primary focus of the ICH guideline. It describes the categories of data that need to be included in the aforementioned papers. This advice does not currently include applications that are short or shorter, modifications, clinical trial applications, or other items that are comparable. While deciding on the parameters for the guideline's tests, the meteorological conditions in the European Union (EU), Japan, and the United States were all taken into consideration. The average kinetic temperature of any location may be determined with the use of data about the climate. You need to determine the degree to which the pharmaceutical substance is stable in order to conduct a comprehensive assessment of

stability. The objective of the stability programme is determined by the stage of the manufacturing process that the medicine is currently in [1.7].

It is essential, right from the start of the product development process, to have a solid understanding of the pharmaceutical component's intrinsic stability as well as its interactions with the many excipients that are often used. Scientists are now investigating how the stability of the active component in the medication varies depending on the environment in which it is stored. It is possible to conduct tests on pharmaceutical substances and medications very fast in order to determine how stable the molecule or formulation is and what the most probable pathways are for it to degrade. The formulation team is responsible for providing the toxicology department with information on the drug's stability in the test vehicle. The preformulation stability programme receives assistance from the analytical research department. This programme is accountable for developing and verifying stability-indicating tests that will be included in NDAs. Finding a stable pharmaceutical formulation is the most critical thing that has to be done during the preclinical formulation stage. In conjunction with the IND, a sample of the drug's preclinical formulation is sent for testing (IND). The NDA will incorporate information that was gleaned from the stability programme in order to guarantee that the batches of drugs that were put through testing were risk-free. It is the responsibility of the quality control team to ensure that the authorised stability-indicating analytical technique is in satisfactory working order throughout the NDA approval process. It is assured that drug products will preserve their stability (potency) until the date that is printed on the label because of a programme that is required by the NDA called the marketed product stability programme. Tests of stability are performed on the first three commercial batches, in addition to at least one additional batch per year [1.8].

1.2.3 Development of stability indicating assay method

1.2.3.1 Critical study of the drug structure

When one examines the construction of pharmaceutical compounds, one may make educated guesses about the degree to which such molecules will be stable. A variety of functional groups, including amides, esters, lactams, and lactones, are susceptible to being degraded by water. Research is also being done on thiols and thioethers, both of which are significant molecules that are easily oxidised. You can figure out how a pharmaceutical molecule will break down, as well as how much of each component it will break down into, by looking at the functional group and at the other critical elements of the molecule.

1.2.3.2 Collection of information on physicochemical properties

In most cases, the tactic can only be chosen after exhaustive research into the many chemical and physical features of the medicine. These characteristics include the pKa value of the medication, its log P value, its solubility, its absorption rate, and its maximum wavelength. Since pH-related shifts in retention take place at pH values that are within 1.5 units of pKa, it is essential to have a solid understanding of this quantity. In order to determine the pH of the buffer solution before using it as the mobile phase, the ionisation value is applied to the solution. When choosing the sample solvent and the mobile phase, it is essential to look at the solubility data in both water and organic media first. This will help you make an informed decision.

1.2.3.3 Stress (Forced Degradation) Studies

Investigations into forced degradation include putting drug molecules through a number of different types of destruction, including as hydrolytic, oxidative, thermal, and photolytic processes.

1.2.3.4 Hydrolytic degradation

Hydrolysis is a chemical process that may allow for a more rapid breakdown of drugs over a wide pH range. Hydrolysis, which takes place when pharmaceuticals are exposed to water, results in the formation of byproducts that include a wide range of different chemical compositions. The majority of pharmaceuticals deteriorate when exposed to water, whether in the form of a solvent or as moisture in the air. For the purposes of hydrolysis research, both acidic and basic conditions will be used. It entails eliminating atoms or groups from the molecule that are not required for its functioning [1.8].

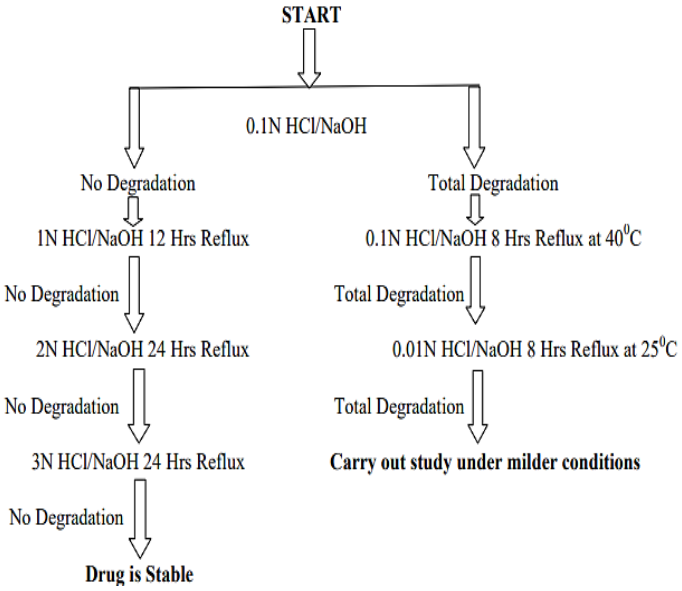


FIGURE 1.2 Hydrolytic degradation process

1.2.3.5 Oxidative degradation

When there is elemental oxygen present in the ground state, a lot of medicinal compounds go through a process called autoxidation. Free radicals were produced as a byproduct of the reaction. In order to kick off the chain reaction that occurs during autoxidation, you need a free radical initiator such as hydrogen peroxide, metal ions, or extremely minute quantities of pollution present in a medicinal component. In investigations that examine the effects of long-term exposure, oxidants like hydrogen peroxide are often used. This might result in the creation of oxidative breakdown products, which could be misunderstood for trace quantities of contamination if not taken care of properly. At temperatures no higher than 40ºC, solutions with concentrations ranging from 3% to 30% are applied for two to eight days [1.8].

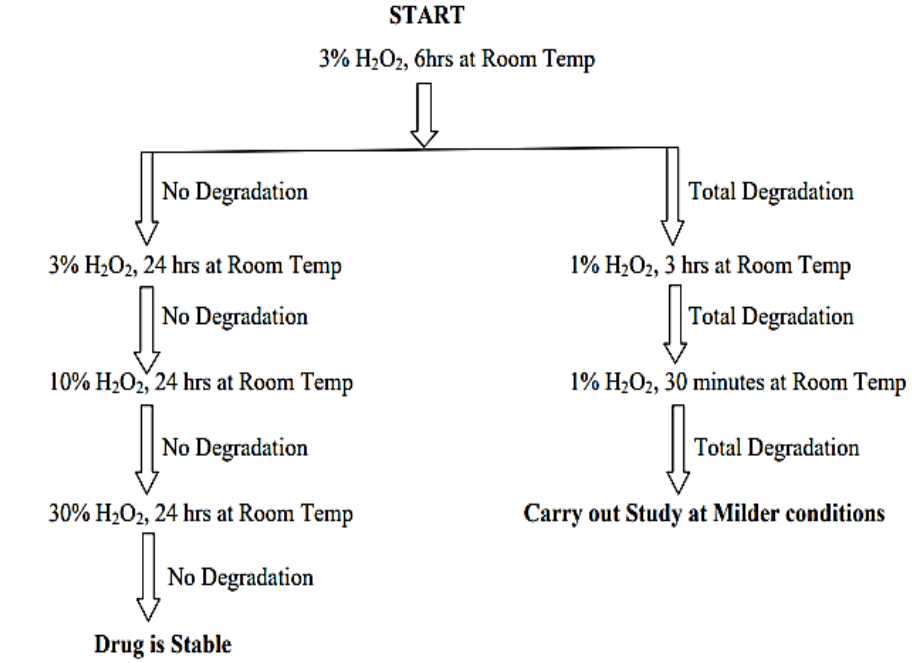


FIGURE 1.3 Oxidative degradation

1.2.3.6 Thermal degradation

The relationship between a reaction's pace and temperature is straightforward in most cases. According to the Arrhenius equation, which describes the breakdown of a material due to heat, medications degrade more rapidly at higher temperatures.

$$K = Ae^{-Ea/RT} \quad \text{(1)}$$

In this equation (1), R stands for the gas constant, which is equal to 1,987 kcal/mole, K stands for the reaction rate, A stands for the frequency factor, Ea stands for the activation energy, and T stands for the temperature in the absolute sense. Materials experience a thermal deterioration when heated over 40 ºC, namely between these temperatures. The

majority of studies are conducted over a period of one to two months at an average temperature of 70 ºC and varied degrees of low and high relative humidity.

1.2.3.7 Photolytic degradation

It's likely that pharmaceutical molecules, when exposed to light, become less stable. This is something that might happen. The pace of photodegradation is contingent not only on the amount of light that the molecule of the medicine is able to absorb but also on the intensity of the light that is incident onto the molecule from the environment. It is possible for an active pharmacological component or therapeutic product to undergo photolytic degradation when it is exposed to light with a wavelength ranging from 300 to 800 nanometers. This may take place either via the process of photolysis or by oxidation that does not need the presence of light. Isomerization, dimerization, cyclization, rearrangement, decarboxylation, and hemolytic cleavage of X-C heterobonds, N-alkyl linkages, and SO_2-C connections are examples of non-oxidative photolytic reactions. Other non-oxidative photolytic reactions include oxidative cleavage of X-C heterobonds. The oxidative photolysis process might be kicked off by either the singlet oxygen (O_2) mechanism or the triplet oxygen (O_3) mechanism. In the course of the investigation on photolytic degradation stress, the drug substance and drug products were subjected to an ultraviolet light intensity of at least 200 watt hours/m^2 and a visible light intensity of at least 1.2 million lux hours [1.9, 1.10].

1.2.3.8 Preliminary separation studies and identification of degradation products

Chromatographic methods such as Reversed Phase High Performance Liquid Chromatography (RP-HPLC), Thin Layer Chromatography (TLC), Gas Chromatography (GC), Capillary Electrophoresis (CE), Capillary Electrophoresis Chromatography (CEC), and Super Critical Fluid Chromatography (SCFC) are used to find out how many and what kinds of degradation products are made when samples are put under stress. Other chromatographic methods include Super Critical Fluid Chromatography (SFC). An RP-HPLC and a UV detector are two popular pieces of laboratory equipment that are used to analyse and quantify pollutants. Spectroscopy and elemental analysis are two of the techniques that are used in the process of determining the structures of resolved products (MS, NMR, IR, etc.). When it comes to identifying items on the internet, procedures that consist of two components, such as LC-MS, LC-NMR, LC-MS-MS, and others, are becoming more frequent.

1.2.3.9 Final method development and optimization

Validation of newly developed analytical processes is recommended by all of the following: the FDA guideline, the USP, and the ICH guidelines Q2A and Q2B. The SIAM validation

process may be broken down into two distinct phases. Testing the drug material for signs of deterioration is the first thing that is done while manufacturing a new medicine. In order to construct the Mechanism, information regarding the chemical processes through which drugs are metabolised is utilised. Before going on to other criteria, such as the accuracy, precision, linearity, and so on of the mass balance architecture, the primary emphasis at this stage of the validation process is on testing the specificity and selectivity of SIAM. The limitations that should be used for identifying and monitoring degradation products should be defined throughout the validation process. Second, the SIAM is validated by testing it on formulations or other matrices that have had excipients or other components of formulations added to them. In this particular scenario, we need simply concentrate on selection, accuracy, and precision. After a SIAM has been generated for a particular formulation, it is imperative that each validation parameter be assessed [1.11].

1.2.4 Stability Indicating HPLC Method

The stability of a pharmaceutical ingredient or finished product can be evaluated with the help of stability-indicating techniques. In order to complete an IND (Investigational New Drug) or an NDA (New Drug Application), as well as to detect when an API or drug product goes bad, it is required to have this information. Stability is the capacity of a medicine to stay performing as intended even after it has been put through a lot of testing. Before testing for stability can begin, the formation of degradation products during storage (real time, long term, and accelerated) must be isolated, identified, and measured using a method that shows stability. When an API is placed through stability testing, it is feasible to determine how environmental variables such as temperature, humidity, and light effect the product over time. The information gathered through stability studies may help us better understand the long-term impact of the environment on drugs. Stability testing can reveal how the drug degrades, what its breakdown products are likely to be, how it degrades, and how it interacts with common drug ingredients. These data will be utilised to decide how to create the product, how to package and store it, how long it will endure, and when it will expire [1.12].

1.2.4.1 Understanding of the physico-chemical properties of drug

While developing the procedure, it is essential to have a solid understanding of the physicochemical characteristics of the active pharmaceutical ingredient (API) as well as the formulations. Drug profiles, spectrum libraries, and public reports were examined for information that was required in order to manufacture new pharmaceuticals. This was done in addition to looking through other sources of information. Examining the structure of the analyte as well as any potential active sites for degradation, particularly the functional group

of those sites, enables one to ascertain whether or not the drug is susceptible to hydrolysis, oxidation, or heat. Compatibility studies check to see how the drug and the other substances function together. In turn, the investigations investigate how well the medication functions after being combined with a variety of various fillers and lubricants.

1.2.4.2 Set up Preliminary HPLC Condition

In order to devise preliminary experimental settings, we make use of either preexisting or improvised procedures, in addition to doing a literature research on the topic at hand. It is possible to validate a technique and utilise it to verify stability if the approach can demonstrate stability and is appropriate for the task at hand. In the EP/IP/USP/BP pharmacopoeias, the standard operating procedures for the pharmaceutical sector are set down. While developing tests, it's useful to consider about API features and known contaminants. It is very important to pick the right column and mobile phase. If you utilise a computer-assisted method development framework to identify columns and mobile phase combinations, you can acquire the optimal starting HPLC settings. Setting up your tests the right way from the start can save you time and trouble in the future [1.13].

1.2.4.3 Preparation of samples required for method development

In the course of stress testing, the Application Programming Interface (API) is put through a series of simulated high-pressure scenarios. This is done in order to offer granularity for the development of SIAM. Another term for the study of artificial degradation is "artificial deterioration research." Throughout the period of storage, it is possible to make educated guesses about the reactions that will take place and the byproducts that will be produced. In addition to this, the development of novel formulas, manufacturing, and packaging are all made less complicated. Early on in the development of a project, it may be challenging to collect samples that are truly representative of the whole. API stress is used in order to generate a sample that is indicative of the products that are anticipated to develop under circumstances of storage that are more common. Whether it be by the application of heat, water, oxygen, or light, the purpose of research involving forced degradation is to reduce the amount of the active pharmacological component.

1.2.4.4 Developing Separate Stability Indicating Chromatography Conditions

SIAMs for new entities need to be built with an awareness of the results of degrading systems. Chromatography must be used to separate and identify both the active pharmaceutical ingredient and the products that form when it breaks down. It is advised that a diluent ratio of 1:1 water: organic solvent be used while attempting to figure out the chromatographic conditions for a Substance. This will provide as a starting point for the

process. This will make it more probable that most of the components associated to the SIAM will dissolve, and it will also ensure that the SIAM will break down in the correct manner. The second step is to distinguish between the peaks that represent degradation products and those that represent APIs. SIAM is created by a process of trial and error to discover the best chromatographic parameters that will make it possible to detect the most distinct peaks in a collection of test samples. This procedure takes place in order to find the optimal chromatographic parameters. While forming SIAM, it is important to have accurate values for the type of solvent, the mobile phase, the pH, the kind of column, and the temperature [1.14].

1.2.4.5 Method Optimizations

The technique may be optimised for the highest possible level of productivity by tinkering with the flow rate, temperature, sample amounts, injection volume, diluents, pH (if the mobile phase contains ions), mobile phase components, and their respective ratios, as well as gradient systems. The appropriate experimental settings for stability-indicating tests will be determined by methodical examination of these properties. This will ensure that the requisite separations and sensitivities are attained.

1.2.4.6 Validation of analytical method

Validation is an essential stage in the process of conducting any analytical application since it ensures that the findings obtained are accurate and trustworthy. Yet, unlike analytical chemistry, pharmaceutical analysis has to take into consideration a variety of unique settings and situations. For instance, most analytical processes (apart from pharmacopoeial monographs) are created and applied locally. So, at the beginning, a much higher degree of competence is required in comparison to the conventional procedures. The same may be stated regarding the samples that were analysed. In pharmacological analysis, the matrix, also known as the placebo, is often consistent and well-known, and the ranges in which the sample being examined may be anticipated are typically unambiguous and not too expansive. The purpose of conducting experiments that have been meticulously planned and carried out in the name of validating an analytical method is to demonstrate that the method in question is suitable for the task that it was designed to perform. It is necessary to have analytical data that is both accurate and dependable in order to guarantee that medications are both safe and effective, as well as of a high quality. In order for a process, technique, or piece of equipment to be considered legitimate, there has to be sufficient evidence to show that it complies with standards and functions as anticipated for the purpose for which it was designed. The analytical capability of approaches is also assessed. During analytical validation, all of these

characteristics, as well as linearity, accuracy, precision, specificity, quantitation limit, detection limit, ruggedness, and robustness, are put to the test to ensure that they satisfy ICH requirements [1.15].

TABLE 1.1 Validation characteristics normally evaluated for the different types of test procedures.

Type of Analytical Procedure	**Identification**	**Testing For Impurities**		**Assay**
Characteristics		**Quantitation**	**Limit**	**Dissolution (measurement only) Content / potency**
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Intermediate precision	-	+ (1)	-	+ (1)
Specificity (2)	+	+	+	+
Detection limit	-	- (3)	+	-
Quantitation limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- Signifies that this characteristic is not normally evaluated.

+ Signifies that this characteristic is normally evaluated.

1. When reproducibility has been checked, there is no need for intermediate precision.

2. If one method of analysis isn't specific enough, it could be made up for by another method of analysis (s).

3. It might be needed sometimes.

A) Linearity

The capacity of an analytical procedure to deliver test findings that are directly connected to the quantity of analyte in a sample within a given concentration range is termed "linearity." You need a minimum of five benchmarks in order to perform the calculation, and you anticipate that these will account for between 80 and 120 % of the total. Linearity is a property of an analytical technique that describes how well it can provide findings that are proportionate to the quantity of analyte present in a sample. Examining a plot that depicts signal versus concentration or content is one method for determining whether or not linearity

exists. If there is a linear connection between the variables in question, the findings of the tests need to be analysed using the appropriate statistical procedures, such as creating a regression line using the approach that requires the fewest number of squares. Adjusting the test data mathematically before completing the regression analysis may assist demonstrate that the test findings and sample concentrations are linear? It is possible that this will occur on occasion. You can get a sense of how linear the relationship is by using the information provided by the regression line. It is essential to provide the r-squared value, as well as the slope, the y-intercept, and the residual sum of squares. It is highly recommended that the information be presented in a visual format if at all possible. Examining the distance that the actual data points are from the regression line may be of assistance when attempting to get a better understanding of how linear the data is. Using at least five distinct concentrations will provide the most accurate results when attempting to determine linearity.

B) Range

The linearity studies define the required range. It is the interval between the upper and lower concentration (amounts) of analyte in the sample. The analytical technique delivers adequate degrees of precision, accuracy, and linearity within the range. The interval between the highest and lowest analyte concentrations in a sample for which it has been demonstrated that the analytical technique has an acceptable degree of precision, accuracy, and linearity is known as the range of an analytical procedure. The required range is dependent on the intended application of the analytical process. Table 1.3 outlines the validation criteria that are commonly used to establish the lowest operational range of an analytical technique. [1.16].

C) Accuracy

What this indicates is that the value that was measured for the analyte in the sample is quite close to what the real value is. There are various techniques to determine how how exact something is.

1. After doing an analysis on a sample whose concentration is already known, the first step in determining the concentration is to compare the findings of the two samples. In this situation, the value is measured against the gold standard.

2. The second innovation is a more effective use of placebos in the healing process. This technique involves adding a predetermined quantity of a pure active ingredient to a formulation that has been left "blank," analysing the results, and then comparing them to the concentration that had been predicted.

3. The standard procedure for determining anything involves first analysing a sample, and then repeating the previous steps while also include a certain quantity of the unadulterated form of the active component. The difference in results between the two experiments may be attributed to the different concentrations of the pure active component that were used. By contrasting the outcomes of two experiments in which the active component was utilised in its unadulterated form. Tests must be done on at least three different concentrations (50, 100, and 150%) to determine out how accurate it is. The degree to which an analytical approach "corresponds to the value that is recognised as either a conventional true value or an acceptable reference value" is one way in which the degree to which it is accurate may be evaluated. Due of this, it is frequently given as an excellent example of a true statement. Quantitative approaches need a minimum of nine measurements to be carried out over the range of interest. One example of this would be carrying out three repetitions at three distinct concentrations. Helpful measurements include the confidence interval, the standard deviation from the established true value, and the percentage of recovery [1.16].

D) Precision

When the same homogeneous sample is tested multiple times under identical conditions, the test results should be comparably consistent. This shows that the analytical technique is accurate. Two-way accuracy both throughout the day and between days. The two sorts of days are intraday and interday. To determine the relative standard deviation, statistical methods and at least six identical experiments are required. Analytical accuracy is measured by the spread of findings received from repeated measurements on the same homogeneous sample under controlled settings. In terms of accuracy, we may differentiate between three levels: repeatability, intermediate precision, and reproducibility. Using actual samples to improve precision is preferred. As parameters, you must calculate the standard deviation, the relative standard deviation (coefficient of variation), and the confidence interval for each level of precision. Repeatability refers to the degree to which results may alter analytically over a short period of time while preserving otherwise similar operational parameters (within-assay, intra-assay). It is essential to note that additional random factors within laboratories, such as different days, analysts, equipment, etc., may influence the intermediate accuracy of the procedure [1.16, 1.17].

E) Limit of Detection (LOD)

The smallest amount of an analyte in a sample that can be identified by an analytical method, but which may not necessarily be able to be measured, is referred to as the limit of detection. LOD can be calculated by the formula as follows.

$$LOD = 3.3 \times \sigma/s \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

"The detection limit of an analytical process is the smallest amount of analyte that can be found in a sample. Even though it is impossible to put an exact number on this amount, it is still referred to as the detection limit. The lowest concentration of analyte in a sample that can be quantitatively measured with appropriate precision and accuracy is referred to as the quantitation limit of a specific analytical method. This limit is set by the provided analytical procedure. Since the ICH guideline explains how to calculate the Limit of detection and Limit of quantification, there is no need to perform these calculations once more.

F) Limit of Quantitation (LOQ)

The smallest amount of the analyte in the sample that can be quantitated with a specified level of accuracy under specific experimental circumstances is known as the limit of quantitation. Quantitative assays employ this parameter to identify tiny levels of chemicals in complicated combinations. Its main use is to find small amounts of an active ingredient, byproducts of degradation, or impurities. The LOQ calculation uses the following formula.

$$LOQ = 10 \times \sigma/s \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

As part of the approval process for commercialising a drug, regulatory agencies demand detailed profiles of the substances and finished products' impurities. Safety standards are connected to toxicological investigations of both the active chemical and the byproducts that are created when it is made and broken down. It is very important to demonstrate that impurity profiles are within the ranges considered in toxicological studies. The amount of degradation products should be kept to a minimum, and it is also very important to demonstrate that [1.18, 1.19].

G) System Suitability testing

Part of the system appropriateness test includes comparing the test chromatogram to a reference chromatogram. The form of the peak, its breadth, and the resolution of the base line are all examined. The system suitability test When putting up a report, a number of parameters need to be established. Quantity of theoretical plates (efficiency), separation (relative retention), resolution, tailing factor, and relative standard deviation are all instances of these measurements. It is possible to get these characteristics by calculating them based on the retention and breadth of a peak or peaks that are already known.

H) Specificity

The degree to which an analytical method is able to detect the active ingredient in question can be used as a measure of a drug's specificity. By employing a placebo, the amount of interference may be assessed. If available, it may be assessed by measuring the API in a

sample including contaminants or degradation products. If this option is unavailable, the evaluation may be completed using an alternate technique. If API-related chemicals are unavailable, stress or forced drug degradation are used to generate degradation products. Specificity is defined as the capacity to assess one analyte clearly in the presence of additional components that are expected to be present. Common examples of things that may be classified under this category are degradation byproducts, matrices, and pollutants. Additional supporting analytical processes may make up for an individual procedure's lack of specificity(s). It is crucial to be able to detect the difference between identical chemical compounds when recognising both positive and negative samples. For chromatographic assays and impurity testing, it is possible to put enough impurities or degradants into the relevant matrix or to use samples that have already broken down [1.20].

For the aim of the assay, it is possible to establish that the result is unaffected by the substance that was spiked. To remove impurities, the matrix elements or the impurities themselves must be isolated. By comparing the output with another independent analytical procedure, specificity may also be demonstrated. In the case of chromatographic separation, resolution factors should be defined for crucial separations. Diode array detection (DAD) and mass spectrometry are two of the most popular choices for checking peak homogeneity (MS). A great deal of discussion and debate has occurred regarding the appropriate way to label this validation criterion. In contrast to the ICH, the majority of other analytical groups refer to this as "selectivity," while the "ultimate degree of selectivity" is known as "specificity" in the IUPAC's terminology. Despite this argument, there is a universal understanding that specificity/selectivity is the basic basis of every analytical approach. If there is not enough selectivity, all the other performance metrics are meaningless. In contrast to the process of chemical analysis, which involves studying and evaluating each analytical step in isolation, the process of pharmaceutical analysis involves performing a wide variety of control tests in order to evaluate a batch. Hence, the total degree of selectivity sought may be achieved by the mutual improvement of the performance of these several analytical methods. An assay that was conducted using a titration that was less selective and therefore contained impurities that had the same functional groups, for example, may be validated (or rectified) by using a selective impurity determination that was performed using LC. This determination was carried out in order to remove impurities that shared the same functional groups. Specificity has to be considered right from the start of the method development process, with the characteristics of the analyte and the sample being taken into consideration. The (adequately) selective determination of the analyte can be accomplished through

sufficient sample preparation, separation, and/or detection, as appropriate in each case. In most circumstances, a hybrid strategy integrating numerous methodologies will be devised [1.21].

I) Robustness

The term is used to describe the robustness of an analytical method against even minor changes. System suitability is established by analysing the effect of various operating conditions on chromatographic outcomes like peak retention, resolution, and efficiency. It's safe to presume that an analytical method will hold up well under typical conditions of use if it can withstand well considered shifts in a few of its parameters without compromising its precision. A warning should be included in the procedure or the analytical conditions should be strictly controlled if the measured values are sensitive to variations [1.7]. The resolution test is only one example of a system appropriateness parameter that should be built as part of the robustness assessment to guarantee that the analytical technique always yields reliable results. Common variations include;

- ✓ Influence of variations of pH in a mobile phase,
- ✓ Influence of variations in mobile phase composition,
- ✓ Different columns (different lots and/or suppliers),
- ✓ Temperature,
- ✓ Flow rate

J) Ruggedness

It is the capacity of an analytical technique to consistently provide the same findings, regardless of the environment in which it is applied or the person carrying out the analysis. It is examined by a different analyst in a separate laboratory, and then by a third analyst in the same laboratory, but using entirely unique techniques [1.22].

1.2.5 Importance of Stability-Indicating Analytical Methods in pharmaceutical analysis

In order to conduct stability studies and arrive at reliable conclusions on the product's stability, it is essential to get analytical data from stability samples of a high quality. Scientists are able to keep track of any potential changes that may take place over the course of time and in response to varying storage circumstances if they examine the degree to which a product or substance is stable. In order to demonstrate that something is reliable, we shall only employ approaches that can be relied upon. According to the International Conference on Harmonization (ICH), there are a few different approaches to determining the degree to which a medicine is stable. These procedures need to take into consideration the

identification of the drug, the purity of the drug, and the potency of the drug. When it comes to developing stability-indicating technologies, one of the most significant challenges is the difficulty in obtaining samples that have already degraded for the sake of research and development. It is essential to gather samples that have undergone modifications in real time, and these samples have to demonstrate all of the significant shifts that take place during storage. Because the stability of the product is dependent on such a wide range of variables, including the parameters of the manufacturing process, the quality of the excipients, and environmental factors such as humidity and temperature, this cannot be done. As a result, lengthy development times are required. For the purpose of developing technologies that can determine how stable a medicine is, scientists working in the pharmaceutical business make use of samples that are designed to degrade in a certain manner. Whether or not forced degradation testing can accurately predict real deterioration is the subject of a large number of research.

Official stability tests are carried out on pharmaceuticals three times: once when they are being manufactured, once while they are being registered, and once just before they are released into circulation. These research contribute to ensuring that medication will be available for a significant amount of time in the future. Maintain a high level of manufacturing quality throughout the commercialization process, and provide assistance for any adjustments that must be made to the website or the product. The process of registering a product involves evaluating both the product's quality and its potential marketability. Data about the drug's stability are required to be included in the registration dossier for both the drug substance and the drug product. This allows the shelf life to be determined, as well as the optimal method for storing the substance. R&D analysts play a highly significant role in the process of developing new pharmaceuticals since it is their responsibility to ensure that the drugs continue to be pure, effective, safe, and of high quality throughout the whole length of time that they are intended to be on the market. The laboratory for quality control makes extensive use of analytical procedures that were developed at the laboratory for analytical research and development. When regulatory bodies have given their stamp of approval to a medical product, analysts in the QC and QA departments examine each batch to ensure that it satisfies the standards that have been established. It is essential to keep a careful check on the manufacturing process as well as the list of components in order to maintain the product's quality and guarantee its safety if there are even minute changes to any of these. Changes in the chemical and physical characteristics of the drug substance and drug products may be detected using stability indicating procedures that have been validated. This enables an

accurate and interference-free evaluation of the active ingredient, degradation products, and other components of interest. [1.23, 1.24].

1.3 Forced Degradation Studies for Drug Substance & Drug Product

It is typical practise to make a medicine break down in order to get additional information about how the drug does so. Mass balancing is an essential aspect of this inquiry since it demonstrates that the decrease in the amount of the parent medication is the source of the rise in the number of degradation products or impurities. A universal detection technique may not be able to provide an accurate evaluation of all degradants since contaminants can take a wide variety of forms and exhibit a wide variety of chemical and physical characteristics. We are going to employ a mass detector and a photodiode array (also known as a PDA) in conjunction with one another in order to overcome the issues associated with detecting and quantifying degradants (MS). With the use of orthogonal detection, we will demonstrate how co-elutions affect computations involving the mass balance. MS and UV are both useful tools for determining whether or not the peak purity has been achieved and for determining whether or not the API and its breakdown products have been successfully separated in the final separation. Whether we are speaking about co-elutions or species that don't change colour, we may learn more about the mass balance and the breakdown process by using MS data. This is the case regardless of the situation. In the process of discovering and developing novel small-molecule medicines, forced degradation testing is often seen as an essential stage. High-Performance Liquid Chromatography (HPLC) or a single analytical technique that can differentiate between the peak of the degradant and the peak of the drug substance or drug product is used in stress testing, which is also referred to as forced degradation. This demonstrates the level of detail required to establish a technique for analytically determining stability. New pharmaceutical components and/or pharmacological formulations need to undergo stability testing that complies with ICH standards before an estimated shelf life can be calculated for them (Q1A). Investigations demonstrating the product's capacity to maintain its integrity throughout storage are needed for certain FDA submissions [1.25].

Stability at accelerated and moderate rates, as well as temperature regulation at normal temperature It is common practise to conduct stability tests prior to estimating the length of a pharmaceutical component's or medical product's shelf life. The completion of rapid studies typically takes approximately six months, while stability testing may take anywhere from twelve to twenty-four months when conducted at temperatures in the middle of the temperature range and in environments that are tightly regulated. While conducting stability

tests, it is necessary to take into consideration the risk that the active therapeutic component or the finished pharmaceutical product might deteriorate and give off undesirable byproducts known as contaminants. This is done to determine the molecule's stability when it is left to its own devices. The primary component is subjected to a number of strains in order to induce the formation of contaminants as part of the forced degradation testing procedure. When the impurities have been produced, they must next be separated from the parent molecule and from one another before proceeding. It is suggested that the shelf life of new medicinal components and/or pharmacological formulations be determined by using the results of forced degradation experiments to estimate the degradant or decomposed impurities that would occur during stability testing. This would be done in order to figure out how long the new components and/or formulations will remain effective.

During a study of forced degradation, a variety of analytical techniques and instruments may be used in order to locate and analyse the whole set of degradant chemicals that may be present. The pharmaceutical industry makes extensive use of high-performance liquid chromatography with ultraviolet (UV) detectors and high-performance liquid chromatography (HPLC) with photodiode array (PDA) detectors to develop and test stability-indicating technologies. Both of these detectors are used with high-performance liquid chromatography. It is possible to determine the structure of the degradants by the use of nuclear magnetic resonance (NMR) spectroscopy, gas chromatography with mass spectrometer (GC-MS), and high performance liquid chromatography with mass spectrometer (LC-MS) [1.26].

1.3.1 Important Applications of the Forced Degradation Study

Analytical investigations, such as forced degradation, will be used by pharmaceutical companies in order to develop stability-indicating procedures as a component of their regulatory filings with the FDA. The findings of the study have a variety of applications, including the following:

1. Developing and validating stability-indicating procedures in accordance with ICH recommendations.
2. To determine the structure and toxicity of a substance, as well as to specify degradants or contaminants.
3. To present the product's shelf life without real-time stability data.
4. To prevent interference, adjust formulations and pick placebos for therapeutic products.
5. Justify contaminants that are process-related or decomposition products.

6. To aid in determining the underlying cause during out-of-specification (OOS)/laboratory investigations.
7. To accompany submissions of the drug master file, ANDA/NDA, and IND to the FDA.

1.3.2 Selection and Procedures of Forced Degradation Condition

According to the recommendations established by the ICH and the conventional practises used in the industry, the forced degradation of a sample should typically be carried out at the same time as a control sample that is subjected to varying degrees of stress. Acids, bases, peroxide, high temperatures, and ultraviolet radiation are some of the substances that fall under this category. It is hypothesised that the various stressful circumstances would result in a decline of anywhere from 5 to 30 percent. This is due to the fact that there are no indicators that everyone believes point to a fall in manufacturing. The aim of the stress testing degradation is to get the state of affairs up to the level of predictability that is indicated by the temperature in the control room. As the pace at which a substance degrades varies, it is essential to determine the optimal circumstances or reagent concentrations for the reaction. A mass balance should be supplied for the whole of the degradation research, and after taking into consideration the analytical error margins, it should be extremely near to 100%. While conducting research on mass balance, it is essential to determine the quantities of the various types of degradants and impurities that are present. For the purpose of the research on forced degradation, any lot that wasn't submitted to the regulators may be utilised. If there are many dosage strengths of the same placebo, but each one has a different quantity of the active pharmaceutical ingredient (API), the dose strength that contains the smallest amount of API need to be selected. It is necessary to demonstrate that all placebos were purposefully designed to have a lower effect size before comparing them to one another. Both the placebo and the active pharmaceutical ingredient need to be shown throughout the whole of the drug product degradation inquiry in order for the true degradation pathways to be located. Even though there are several sorts of placebos to account for the extensive variety of medicinal product strengths, all of them have to be investigated in research on decline [1.27].

1.3.3 Characterizations and Mass Balance of the Forced Degradation Study

It is essential to conduct the analyses of the diluent, the placebo, and the control sample solutions using the same appropriate analytical methods. It is strongly suggested that, in the course of the forced degradation, the following standards be used in order to determine what constitutes an acceptable outcome:

- Every one of these issues should reduce quality by between 5 and 30 percent.

- It is essential that the primary peak be easily distinguishable from the diluent, placebo, recognised peaks, and degradant peaks produced by the drug's degradation.
- There should be no commingling of peaks around the primary summit.

Empower 3 and similar pieces of software may be used to assess whether or not a peak is pure based on whether or not the purity threshold is higher than the purity angle. The graphic that follows illustrates how cutting-edge software can produce a three-dimensional depiction of each peak in a chromatogram, which is helpful for determining the purity of individual peaks.

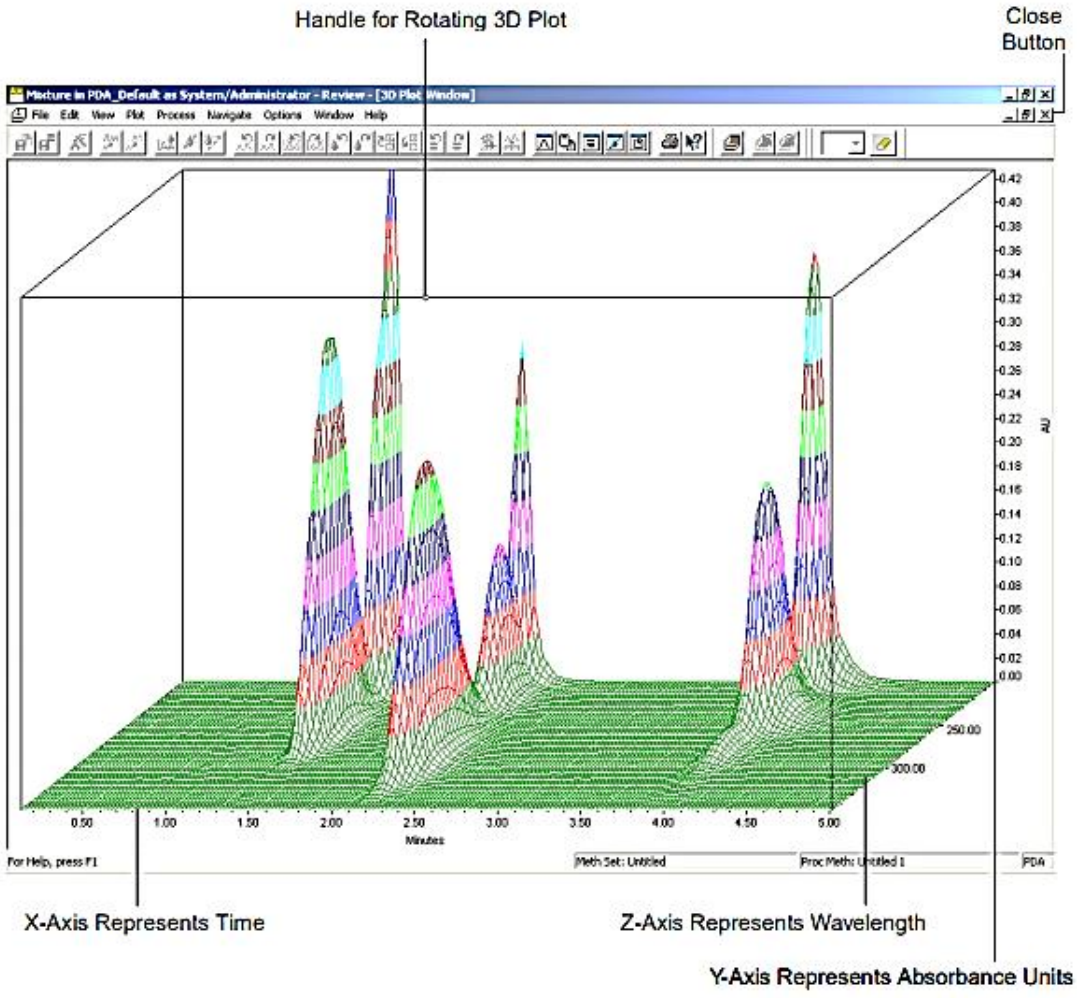


FIGURE 1.4 3D picture of all the peaks from each chromatogram

The use of external forces to cause deterioration may lead to the formation of several additional degradant peaks, each of which is an offshoot of the primary peak. After the completion of the study, the amount of main peak deterioration attributable to active degradation will be known. The FDA has designated the formula that is shown below as the official criterion by which degradation in the pharmaceutical industry is to be measured.

Degradation Efficiency (%)

$$= 100 - \frac{Acid\ sample\ main\ peak\ area}{control\ sample\ main\ peak\ area} x \frac{control\ Spl.Wt}{Acid\ Spl.Wt} x100 \dots\dots\dots\dots\dots (4)$$

Examine the mass-energy-balance for each degradant peak using the degradation percentage. The mass balance should be very near to 100%, given the study's margin of error. Mass spectrometry techniques such as gas chromatography mass spectrometry nuclear magnetic resonance spectroscopy and high performance liquid chromatography may be used to determine the composition and abundance of the degradants. Find out what a degradant is by using cutting-edge analytical techniques like LC-NMR. To remove impurities from filtrate or waste generated during API production, preparative high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), or column chromatography may be used [1.28].

1.4 Development of Stability-Indicating Methods Incorporating Force Degradation Study

During the inquiry of forced degradation, it's feasible that a greater number of degradation byproducts will be produced than the ACC originally anticipated. It is essential that while developing an analytical technique, consideration be given to both samples that have undergone alteration as well as recognised contaminants. Since these samples represent the worst-case situation, it is essential that all peaks, including the original peak, the degraded peak, and any known contaminants, be simple to distinguish from one another. If it is presently not able to differentiate between the peaks, the technique of analysis may need to be modified. The creator of this analytical framework drew from a wide variety of sources, including but not limited to books, articles, and their own personal experience. Practical HPLC Method Development, written by R. Snyder, is widely regarded as a seminal piece of literature within the industry. Users might benefit from having a better understanding of how to better separate peaks. An analytical method must be developed in order for the method to be deemed a method that indicates stability. This approach must be capable of examining all samples of stability and distinguishing all peaks from samples that are growing worse. When it is shown that an analytical technique can be utilised for what it was designed to do, method validation (in accordance with ICH principles Q2 (R1) Validation of Analytical Processes) is considered to have been successfully completed. If a sample's analysis reveals peaks that don't appear in any forced degradation scenario, then the sample was presumably contaminated either during manufacture or analysis. This is the most likely explanation for

the finding. Both on location in the field and back in the lab, sample analysis can benefit from a process called forced degradation [1.29-1.31].

1.4.1 High pressure liquid chromatography (HPLC)

The linear dynamic choice of HPLC/UPLC is sufficient to allow for the estimation of active pharmaceutical ingredients and impurities in the same chromatogram using different detector types. This enables its use on fully computerised instruments, which in turn provides continuous quantitative precision and accuracy. The reliability of HPLC and exceptional UPLC is enhanced by careful consideration of column chemistry, which also adds to the broad chemical application of HPLC. High-pressure liquid chromatography is a member of the family of liquid-solid chromatography techniques (HPLC). This complex instrumental procedure may trace its roots back to a technique known as adsorption chromatography. The two most prevalent kinds of chromatography are called normal-phase chromatography and reverse-phase chromatography. It is possible to differentiate between polar and non-polar chemical combinations that do not evaporate with the help of this substance. Because of its understatement, this strategy has shown to be effective. It serves as the standard by which all other approaches to evaluating medical treatments are judged. Before carrying out certain chromatographic tests, the FDA and the USP both advise doing a system appropriateness review first. The evaluation of the HPLC technique involves looking at chromatographic parameters that are considered standard. There is information given on the retention period and the capacity factor (k'). The Components of a Resolving Statement (d) the number of potential plates, represented by the letter d e) the answer to the question that was posed in the resolving statement. Coefficient of tailing and symmetry of peaks [1.32].

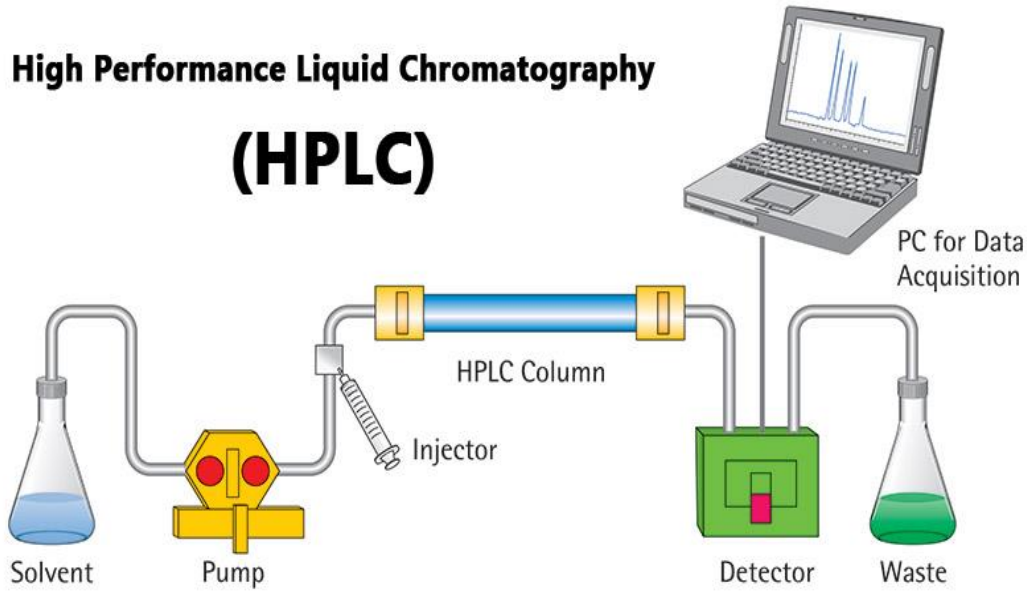


FIGURE 1.5 High pressure liquid chromatography [1.32]

Retention time: It is the length of time that passes from the moment that the sample is injected till the moment that the chromatographic peak emerges.

Capacity factor: Capacity factor is a factor that indicates where a sample peak will be located on a chromatogram. It is unique for a certain chemical. The temperature, the stationary phase, and the moving phase are some of the things that may have an effect on K'etc.

$$K'_1 = {}_tR_1\text{-}t_0/t_0 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(5)}$$

$$K'_2 = {}_tR_2\text{-}t_0/t_0 \ldots\ldots\ldots\ldots\ldots\ldots \text{(6)}$$

Separation factor: A separation factor may be defined as the ratio that exists between two capacity factors.

$$\alpha = K'_1/\ K'_2 = {}_tR_1\text{-}t_0/\ tR_2\text{-}t_0 \ldots\ldots\ldots\ldots\ldots\ldots \text{(7)}$$

Resolution factor: A measurement of how well two bands are separated from one another is referred to as the resolution factor. Overlapping bands have modest RF values. The height and width of two peaks are used as measuring sticks for determining it.

$$R_F = 2(t_2\text{-}t_1)/W_1+W_2 \ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(8)}$$

Number of theoretical plates: How well the packing and mass transport mechanisms function in a column may be determined by looking at the number of theoretical plates. It may be necessary to use a bigger "n" in order to differentiate the more difficult sample from the other ones.

$$N=16[{}_tR/W]^2 = 5.54[{}_tR/W_{1/2}]^2 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(8)}$$

Symmetry factor: The symmetry of a peak may be determined by measuring 10% of its height, where A is the distance between the beginning of the chromatographic peak and the peak maximum and B is the distance between the peak maximum and the beginning of the chromatographic peak. The optimal value is 1, which indicates that both A and B are equal.

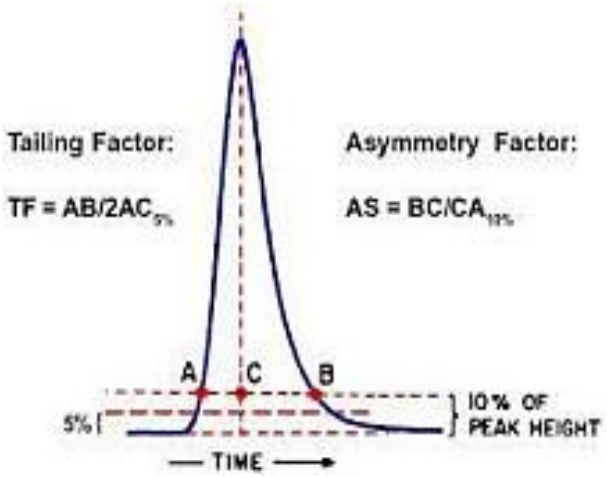


FIGURE 1.6 Diagrammatic presentation for Symmetry and Tailing factor

Tailing factor: A measure of the symmetry of the peaks is referred to as the tailing factor, abbreviated as T. It has a value of 1 for peaks that are completely symmetrical, and its value increases as the strength of the tailing increases.

Tailing Factor = $A_{B/2}$ AC……………………………. (9)

1.4.1.1 HPLC Instrumentation

The basic parts of the HPLC set up are:

a) Solvent delivery systems

b) Sample injection system

c) Columns and ovens

d) Detectors

e) Data acquisition system

a) Solvent Delivery systems:

The pump on a contemporary liquid chromatograph receives solvent, buffer solution, or a mix of the two from a reservoir that is located on the instrument. Any liquid chromatography (LC) pumping system worth its salt will be resistant to solvents and able to handle pressures of several thousand pounds per square inch. Moreover, the system will be capable of handling higher flow rates. The perfect LC pumping system will be able to work with a wide variety of solvents, will be easy to use and maintain, will be able to draw from a sizable external reservoir, and will deliver solvent in an accurate, precise, and pulse-free manner over a wide flow rate range. These are the essential characteristics of the perfect LC pumping system. The use of gradient elution does not call for a significant amount of adjusting time either. Using one or more pumps in a modern pumping system allows for the creation of a gradient between two or more solvents of varying concentrations. [1.32].

b) Sample Injection system:

To guarantee the highest possible level of productivity, the sample is injected into the pressurised column in the form of a pointed column plug using the injection technique.

c) Columns and Ovens:

A chromatographer's knowledge of the chemical and physical interactions between samples, mobile phase, and stationary phase is crucial for making rapid decisions on which phases to use.

d) Detectors:

A qualitative as well as quantitative assessment is provided by the detector by way of an electrical signal that is sent to the recorder and data system. The RI and UV/PDA detectors included in LC instruments make up the majority of the market share, followed by

fluorescence, ELSD, conductivity, and electrochemical detectors. A photometer that has a very small sample cell makes up the UV light detector. It is quite similar to the typical cell that is used in UV-Visible spectrophotometers, with the exception of the cell's construction. It is possible for LC UV detectors to include scanning and ratio recording, two characteristics that are typical of more sophisticated spectrophotometers. At this point in time, the Light Diode Array Detector is the detector that the LC most strongly recommends (DAD). At the same time, the DAD displays a chromatogram (intensity versus time) in two dimensions (D), as well as a spectrum in three dimensions (D) (intensity versus wavelength). This concept may also be referred to as spectrochromatography. With a more in-depth examination of the data, new insights into the complexities of co-elution, the locations of merged peaks, and information on peak purity are uncovered. The conventional UV detector uses a deuterium lamp as its light source since it generates adequate light between 190 and 400 nanometers. For measurements in the visible region (400-700 nm), tungsten-halide lamps with a higher power output are often used. In spite of this, operations using HPLC often make use of wavelengths less than 400 nm. After passing through an ultraviolet (UV) transmission flow cell that is attached to the column, the light from the lamp is then allowed to impinge on a diode, the intensity of which is measured [1.33].

e) Data acquisition system:

The data system compiles a chromatogram by analysing the data received from the detectors and then displaying the results. The efficiency of the system is improved when users connect through modem. They look at chromatograms, do mathematical calculations, examine statistical data, and store information. In addition, the data systems are responsible for controlling the many systemic factors. Both an isocratic and a gradient method of supplying solvents are viable options for today's high-performance liquid chromatography systems, which may be either completely integrated or made up of discrete sections. As their name indicates, modular systems are constructed from a variety of components that are assembled into a single unit to form the system as a whole. It may be advantageous to use modular systems since it is simpler to replace components for the purposes of testing and maintenance. Nevertheless, this flexibility may be seen as a disadvantage in regulated laboratories since maintaining validation and certification compliance may be challenging. When it comes to managing solvents and samples, integrated systems may be superior to modular ones since the components of integrated systems may share resources such as power, data, and fluid controls. Current integrated systems are designed to make advantage of sample management and solvent management in order to increase precision and accuracy,

reduce the amount of time needed for the injection cycle, and provide users with a diverse array of detection possibilities. The high-performance liquid chromatography system may either be modular or integrated, and the method of solvent administration can be isocratic or gradient [1.34].

In the pharmaceutical industry, high-pressure or high-performance liquid chromatography is often used for the purpose of analysing a broad variety of sample types. It is the gold standard for testing the purity of drugs, measuring the effectiveness of novel formulations, monitoring the development of synthetic processes, determining the efficacy of large-scale production runs, and determining the efficacy of changes to already existing medical items.

During the process of developing a method, it is essential to pay close attention to the following, in particular:

1. Techniques that would eliminate all known and unknown byproducts of breakdown as well as impurities in the substance.
2. The qualification and validation of instruments in order to fulfil the requirements of regulatory agencies.
3. Verifying the viability of HPLC procedures before implementing them on a larger scale.

Before to constructing a technique, it is more vital to have a solid understanding of the sample to be used and the purpose of the development.

The following is a list of potential guiding goals for the development of a stability indicating assay and impurity technique for pharmaceutical goods.

- **For Impurities method:**

i) Ensuring that all contaminants are kept distinct from one another and from the components that are really doing the work.

ii) Eliminating any and all contaminants that are present in the medicinal product's constituent parts (placebo).

iii) It is much simpler to prepare samples when the appropriate diluents are used.

iv) Keep run times as minimal as feasible without negatively impacting the method's ability to get the job done.

- **For Assay method:**

i) Separating the active compounds from any contaminants and any substances that are serving as a placebo.

ii) Straightforward methods for the preparation of samples, which include selecting appropriate diluents.

iii). Keep run times as minimal as feasible without negatively impacting the method's ability to get the job done.

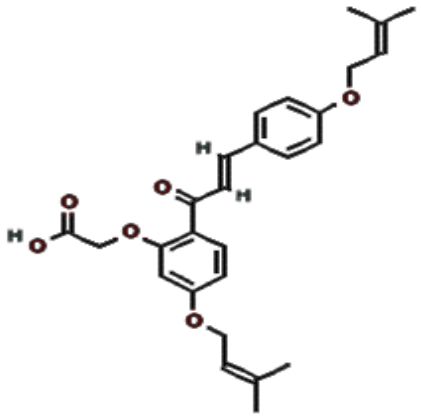
1.4.2 Hyphenated Techniques

Methods that combine chromatography and spectroscopy are used to form the hyphenated techniques. These approaches are utilised jointly. Spectroscopy is a method that may be used to determine the nature of the components after chromatographic separation of the components. In between the two processes was an interphase that existed. The process of analysing samples is simplified when the terms "separation" and "identification" are used in conjunction with one another. Methods denoted with hyphens are superior in terms of sensitivity, specificity, accuracy, and precision, and they are also simpler to dissect. The most common types of analytical techniques employed today are GC-MS, LC-MS, LC-FTIR, and LC-NMR, while CE-MS is also becoming more popular [1.34].

1.5 Drug Profile

1.5.1 Sofalcone

TABLE 1.2 Drug Profile of Sofalcone [1.35-1.36]

Name	Sofalcone
Structure	
IUPAC Name	2-[5-(3-methylbut-2-enoxy)-2-[(E)-3-[4-(3-methylbut-2-enoxy)phenyl]prop-2-enoyl]phenoxy]acetic acid
Molecular Formula	$C_{27}H_{30}O_6$
Molecular Weight	450.5
CAS Number	64506-49-6
Wavelength	220 nm
Physicochemical Properties	
Appearance	Solid
Color	Light Yellow
Solubility	Chloroform (Slightly), Ethyl Acetate (Slightly)
Melting Point	143-144°C
pKa Value	3.22
Log P	4.94
Refractive Index	1.4870 (estimate)
Pharmacological & Therapeutically Properties	
Therapeutic Category	Investigated for use/treatment in gastroenteritis and ulcers.
Mechanism of action	A protective impact on the mucosa may be achieved by inhibiting the enzyme that is responsible for breaking down prostaglandins. It has also been discovered that sofalcone inhibits the pathogenic

	factor of *H. pylori*, in addition to having an antibacterial impact on *H. pylori* itself. Directly killing *H pylori*, blocking urease, and reducing the likelihood of the organism adhering to stomach epithelial cells are all benefits of taking sofalcone.
Therapeutic Use	Anti-Ulcer Agents
Usage and dosage	Oral: 100mg, 3 times a day.
Adverse reaction	Occasional constipation, thirst, heartburn, etc.
Pharmacokinetics	Upon oral administration of the medicine, it was swiftly absorbed, and the peak concentration in the blood lasted for nearly some entire hours after reaching its lowest point. After 12 hours, which is the length of time represented by the half-life, there was almost no plasma left in the blood. The primary metabolites are produced in the body when the isoprene side chain of the chalcone skeleton is oxidised, and the -unsaturated bond of the chalcone skeleton is reduced. After forty-eight hours, 6% to 8% of urine will include metabolites of the oxidation of isoprene chain.

TABLE 1.3 Toxicity of Sofalcone [1.35-1.36]

Organism	Test Type	Route	Dose	Effect
Rat	LD_{50}	Intraperitoneal	1680 mg/kg	Behavioral: changes in sleep duration (including changes in righting reflex); gastrointestinal: hypermobility, diarrhoea; skin and appendages (skin): hair; other skin and appendages (skin): other skin conditions
Rat	LD_{50}	Subcutaneous	3900 mg/kg	Additional alterations in the sense organs and special senses include olfaction, bleeding in the sense organs and special senses including the eye, and pulmonary, thoracic, or respiratory abnormalities. respiratory depression

Organism	Test Type	Route	Dose	Effect
Rat	LD_{50}	Intravenous	105 mg/kg	Behavioral changes include somnolence (usually low activity), behavioural changes including convulsions or an influence on the seizure threshold, and pulmonary, thoracic, or respiratory behavioural changes including other variations.
Mouse	LD_{50}	intraperitoneal	609 mg/kg	While people sleep, their sense organs and other senses, except the eye, as well as their behaviour, might alter (including change in righting reflex)
Mouse	LD_{50}	subcutaneous	1130 mg/kg	Sense organs and special senses: other: the eye; behavioural: changes in sleep duration (including alterations in the righting reflex); pulmonary, thoracic, or respiratory: respiratory depression;
Mouse	LD_{50}	intravenous	131 mg/kg	Respiratory depression; behavioural convulsions or impact on seizure threshold; lungs, thorax, or other parts of the body

TABLE 1.4 Chemical and Physical Properties of Sofalcone

Property Name	Property Value
Molecular Weight	450.5
XLogP3-AA	6.2
Hydrogen Bond Donor Count	1
Hydrogen Bond Acceptor Count	6
Rotatable Bond Count	12
Exact Mass	450.20423867
Monoisotopic Mass	450.20423867
Topological Polar Surface Area	82.1 $Å^2$

Property Name	Property Value
Heavy Atom Count	33
Formal Charge	0
Complexity	706
Isotope Atom Count	0
Defined Atom Stereocenter Count	0
Undefined Atom Stereocenter Count	0
Defined Bond Stereocenter Count	1
Undefined Bond Stereocenter Count	0
Covalently-Bonded Unit Count	1
Compound Is Canonicalized	Yes

1.5.1.1 Marketed Preparation

Sofalco Capsules:

(CDSCO approval: 02/06/2008)

Marketed by: Sunpharma

Composition:

Sofalcone ….100mg

Excipients……. q.s.

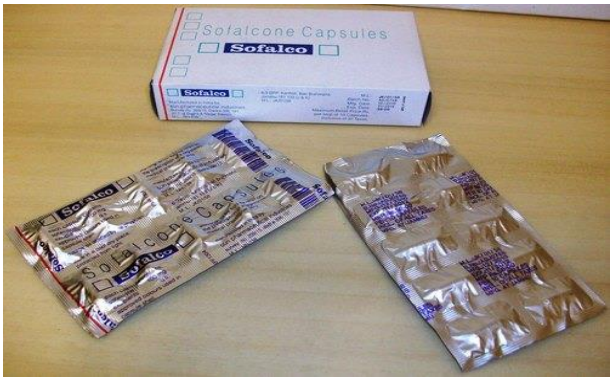


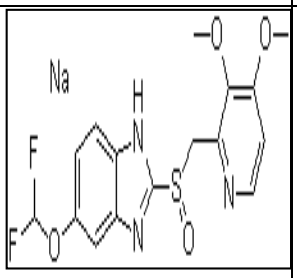
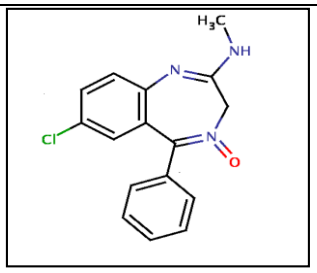
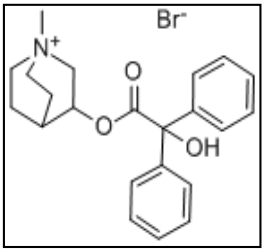
FIGURE 1.7 Sofalcone Capsules

1.5.1.2 Indication and Usage

For the treatment of gastrointestinal mucosal lesions brought on by acute gastritis or a rapid worsening of chronic gastritis, including erosion, bleeding, redness, and swelling.

1.5.2 Pantoprazole Sodium, Chlordiazepoxide and Clidinium Bromide

TABLE 1.5 Drug Profile of Pantoprazole Sodium, Chlordiazepoxide and Clidinium Bromide [1.38 to 1.47]

Sr. No	Parameter	Pantoprazole-sodium	Chlordiazepoxide	Clidinium bromide
1.	Category	Anti-ulcer Agents, Proton-pump Inhibitors	Anti-anxiety Agents, Hypnotics and Sedatives, Benzodiazepines, Adjuvants, Anesthesia GABA Modulators	Antispasmodics, Anticholinergic Agents, Parasympatholytics , Antiarrhythmic Agents,
2.	Structure			
3.	IUPAC name	(RS)-5 (Difluoromethoxy) -2[(3,4dimethoxypyridin-2 yl)methylsulfinyl]-1*H*-benzo[d]imidazolesodium	7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine-4-oxide	3-[(2-hydroxy-2,2-diphenylacetyl)oxy]-1-methyl-1 azabicyclo[2.2.2]octan-1-ium bromide
4.	Chemical name	5-(Difluoromethoxy)-2-(((3,4-dimethoxy-2-pyridinyl)methyl) sulfinyl)-1*H*-benzimidazole sodium	7-Chloro-*N*-methyl-5-phenyl-3*H*-1,4-benzodiazepin-2-amine 4-oxide	3-hydroxy-1-methylquinuclidinium bromide benzilate

Sr. No	Parameter	Pantoprazole-sodium	Chlordiazepoxide	Clidinium bromide
5.	Molecular Formula	$C_{16}H_{14}F_2N_3NaO_4S$	$C_{16}H_{14}ClN_3O$	$C_{22}H_{26}BrNO_3^+$,
6.	Molecular weight	405.35g/mol	299.75 g/mol	352.447 g/mol
7.	CAS No	138786-67-1	58-25-3	3485-62-9
8.	Approval Status	Approved Drug	Approved Drug	Approved Drug
9.	State and Appearance	White or almost white powder	Almost white or light yellow, crystalline powder	Crystalline powder that is either white or very close to white in colour, practically odourless, and optically inactive.
10.	Melting Point	139-140 °C	236-236.5 °C	240-241°C
11.	Solubility	It dissolves easily in water and ethanol (96%), but almost not at all in hexane.	To a very negligible degree soluble in water, very little soluble in ethanol (96%/1:50), soluble in chloroform (1:6250), and soluble in ether (1:130).	It dissolves in water and alcohol, but not in ether or benzene very well.
12.	Pka Value	pKa_1 3.92[Pyridine] and PKa_2 8.19	pKa-4.76	pKa-13.73
13.	Official Status	Official in IP, USP, BP	Official in IP, USP, BP	Official in USP

Sr. No	Parameter	Pantoprazole-sodium	Chlordiazepoxide	Clidinium bromide
14.	Mechanism of action	Long-lasting suppression of stomach acid production is achieved by the use of a benzimidazole derivative, which reacts with the (H^+/K^+)-ATPase enzyme to prevent its normal function.	Anxiolytic and acting as an agonist at a specific benzodiazepine receptor, chlordiazepoxide has a neuroinhibitory effect. Chlordiazepoxide and GABA interact to alleviate patient anxiety by increasing the transmission of chloride ions across neuronal membranes and stimulating benzodiazepine receptors.	It inhibits acetylcholine's muscarinic actions at parasympathetic postganglionic neuroeffector sites. It is used for the treatment of peptic ulcer disease and to ease the pain of IBS, diverticulitis, and colicky abdominal pain.
15.	Indication and Usage	Zollinger-syndrome, Ellison's *Helicobacter pylori* infection, reflux esophagitis, and peptic gastric and duodenal ulcers	Alcohol withdrawal syndrome; muscle soreness; mild to severe sadness; treatment of depression with anxiety;	Reduce propulsive contractions and relax gut smooth muscle to alleviate gastrointestinal distress after eating, including gas, bloating, and a sense of urgency to defecate.

1.5.2.1 Marketed Preparation

Product Name: Ulrax

Marketed by: Mission Research Laboratories Pvt. Ltd.

FIGURE 1.8 Ulrax Capsules

Content:

Each hard gelatin capsule contains:

Pantoprazole Sodium	20.0 mg
Chlordiazepoxide	5.0 mg
Clidinium Bromide	2.5 mg
Excipients	q.s.
Packing:	10*10

1.5.2.2 Indication and Usage

The goal of this research proposal is to come up with a way to test the stability of the following pharmaceutical compounds:

1. Ulrax Capsule (Drug License authority: April 28, 2010, Getting Drug License authority from Uttarakhand, listed State Name in CDSCO as State Drug Controller) Mission Research Laboratories Pvt. Ltd. was in charge of marketing.

Medicine Overview of Ulrax 20 mg/5 mg/2.5 mg Capsule [1.44 -1.47]

- **Uses of Pantoprazole:**

Acid reflux, stomach ulcers, intestinal ulcers, and heartburn are all conditions that are treated with pantoprazole.

How the 20 mg/5 mg/2.5 mg Ulrax pill functions:

An inhibitor of the proton pump is pantoprazole (PPI). It works by lowering stomach acid levels, which relieves acid-related indigestion and heartburn.

- **Uses of Chlordiazepoxide:**

Acid reflux, stomach ulcers, intestinal ulcers, and heartburn are all conditions that are treated with pantoprazole.

How the 20 mg/5 mg/2.5 mg Ulrax pill functions:

Introduction

An inhibitor of the proton pump is pantoprazole (PPI). It works by lowering stomach acid levels, which relieves acid-related indigestion and heartburn.

- **Uses of Clidinium:**

Abdominal discomfort may be treated with clidinium.

Implementation of Ulrax 20 mg/5 mg/2.5 mg Capsule:

Clidinium relaxes the muscles in your intestines and stomach to produce its effects (intestine). It prevents unexpected muscular contractions (spasms). It also reduces bloating, pain, cramps, and discomfort throughout the process.

- **Rationale of combination drugs:**

Alcohol withdrawal symptoms, heartburn, insomnia, stomach or intestinal ulcers, excessive stomach acid secretion, stomach acid backflow, and other conditions are treated with Ulrax capsules.

1.6 Rational of the Project

The chemical stability of pharmaceutical compounds is significant because it impacts how safe and effective the medicine is. This is because the chemical stability of pharmaceutical compounds may change over time. According to the FDA and ICH recommendations, in order to determine how the quality of a drug substance or drug product shifts over time and in reaction to changes in the environment, you need data from stability testing. Since it helps you determine how to create it and package it, as well as how to keep it, and how long it will survive, knowing how stable a molecule is being necessary for the paperwork required by regulatory agencies.

- **Sofalcone**

Sofalcone is used for the treatment of stomach ulcers as well as the preservation of the stomach lining. A great number of bioanalytical methods have been developed with the intention of quantifying sofalcone. In spite of the fact that we are aware that it breaks down into well-characterized degradation products when exposed to the ICH-recommended acidic, alkaline, oxidative, photolytic, and thermal stress conditions, no study using stability-indicating HPLC or UHPLC techniques has been carried out. We were able to get a more in-depth understanding of these degradation products, as well as their composition, via the use of mass spectrometry. We next applied LC-MS/MS to estimate the route of degradation and the Mass Balance. The majority of individuals believe that multicomponent dosage forms are successful because they function in the body in a variety of different ways than single-component ones. Analytical chemists have a difficult time developing an assay technique due to the fact that there are a variety of dosage forms, each of which has its own unique challenges, and there is a great deal of pharmacological components on the market. It is difficult to provide an accurate estimate of how much of each medicine is included in these multicomponent dosage forms since it is difficult to extract or separate the individual pharmaceuticals.

- **Pantoprazole Sodium, Chlordiazepoxide and Clidinium Bromide**

Pantoprazole sodium, chlordiazepoxide and clidinium bromide is used to treat Irritable Bowel Syndrome, which is the most prevalent and dangerous condition and is characterised by cramping, stomach discomfort, bloating, constipation, and diarrhoea. The United States Pharmacopoeia, the British Pharmacopoeia, and the Indian Pharmacopoeia each recognise the official status of chlordiazepoxide, pantoprazole sodium, and clindamycin bromide, respectively. Analyzing clodinium bromide, pantoprazole, and chlordiazepoxide on their own or in combination with other medications may be done using a variety of techniques,

including spectrophotometry, high-performance liquid chromatography (HPLC), and high-performance thin-layer chromatography (HPTLC). Nonetheless, it has been shown that clodinium bromide and chlordiazepoxide may be successfully analysed using just a select few analytical procedures, such as HPLC and HPTLC. As far as we know, there is no published chromatographic method that uses the ICH stress testing strategy for these three drug combinations when they are taken with their breakdown products.

1.7 Objectives of Work

Based on the rationale of the study, we have aimed the following objectives:

1. Validated stability-indicating UHPLC method for the estimation of sofalcone in pharmaceuticals and the identification of its degradation products by LC–MS.
2. Force Degradation with Mass Balance Investigation of Sofalcone in Pharmaceuticals
3. Development and validation of an RP-HPLC method for the estimation of sofalcone in bulk drug and formulations, with forced degradation studies.
4. Development and validation of a quick RP-HPLC technique for the measurement of Clidinium bromide, chlordiazepoxide, and sodium pantoprazole in their combination capsule dose form.
5. Stability-indicating RP-HPLC technique development and validation for the quantification of Clidinium bromide, Chlordiazepoxide, and pantoprazole sodium in bulk medicines and their formulations, along with forced degradation investigations.

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CHAPTER 2

2 Literature review

2.1 Literature Review of Sofalcone

1. Sofalcone, which was identified by Wang et al., originated from the *Sophora subprostrata* plant, which is used in traditional Chinese medicine. In the treatment of stomach and duodenal ulcers, the mucosal protectant sofalcone is used. Using a liquid-liquid extraction process, sofalcone and indomethacin, which serve as the laboratory's internal standards, were removed from 0.5 ml of human plasma or urine samples. For the purpose of determining the method's level of dependability, we considered its specificity, sensitivity, linearity, repeatability, accuracy, and consistency. The new assay method for sofalcone exhibited a high level of precision and accuracy, as shown by the fact that it was possible to obtain a linear calibration curve with R2 greater than 0.99 between 0.5 and 500 ng/ml for both plasma and urine samples. The LLOQ has been set at 0.5 ng/ml. Despite an accuracy that ranges from 96.21 to 107.33% on average, the intra- and inter-day variations of the present assay were found to be within 13.77% for low concentration quality control (QC) samples and 8.7% for other QC samples. This was determined despite the fact that the average accuracy ranged from 96.21 to 107.33%. According to the results of a number of tests and the conditions under which they were stored, the samples were reliable for at least one month. After that, the created approach was applied to the task of determining the amounts of sofalcone in clinical samples. The pharmacokinetics of sofalcone in plasma and urine were characterised based on the results of this study. A clinical pharmacokinetic investigation was conducted with healthy Chinese adults, and it was found that the present technique was effective in its application. This demonstrates that the method is capable of detecting sofalcone in human plasma or urine samples in a speedy manner while maintaining a high degree of sensitivity [2.1].
2. As sofalcone is used to treat ulcers, Wen et al. developed a high performance liquid chromatography technique that is user-friendly, fast, accurate, and reliable in its results. To determine the sofalcone concentration in plasma that had been extracted with ethyl acetate under acidic conditions, we made use of HPLC, which was outfitted with a C18

column and a mobile phase consisting of methanol and 0.1% formic acid in an aqueous solution in the ratio of 80:20. A concentration range of 0.01 to 5.0µg/ml was used in the process of developing linear calibration curves for sofalcone in human plasma. In human plasma, the LOQ for this test was shown to be 10 ng/ml. It was determined that the measurements of the plasma were accurate to within 15%. After being drawn, approximately 85 % of the blood was recovered. In pharmacokinetic studies, this method for the detection and quantification of sofalcone was utilised, and it was found to be successful [2.2].

3. Han and his colleagues employed a method known as liquid chromatography-electrospray ionisation tandem mass spectrometry (LC-ESI/MS/MS) to discover a method that was sensitive as well as selective for measuring sofalcone and its active metabolite in human plasma. Plasma samples were placed onto 96-well plates, and with the assistance of sample handling equipment, they were then spiked with solutions of sofalcone metabolites (internal standard). After adding 0.5 ml of acetonitrile to the 96-well plate and stirring the plasma samples for 30 seconds, the plate was inverted and stored at room temperature. It was only possible to measure sofalcone and its metabolite up to a maximum concentration of 2 ng/ml using a sample volume of 0.2 ml. It was determined whether or not the approach could be replicated by analysing ten sets of samples with values ranging from two to one thousand ng/ml. Studies conducted to validate the method demonstrated that the test had a high degree of both precision and accuracy. This approach yielded fruitful findings when applied to the investigation of the movement of sofalcone and its active metabolite through the plasma of human subjects [2.3].
4. Protein precipitation and liquid chromatography-tandem mass spectrometry were the two methods that Kim and colleagues used to develop a method that was both sensitive and accurate for detecting sofalcone in human plasma. Plasma samples were plated on 96-well plates using the aforementioned sample-handling devices, and then spiked with an internal standard solution (d3-sofalcone). After shaking the plasma samples for thirty seconds in a 96-well plate that contained half a millilitre of acetonitrile, the supernatant was centrifuged, transferred to a new 96-well plate, and sprayed with liquid nitrogen at a temperature of 40ºC until it disappeared. It was only after the addition of the mobile phase to the dried residue that it was rendered useable once again. The PerkinElmer Multi PROBE II HT and the TOMTEC Quadra 96 workstations were used in order to carry out all sample transfers and protein precipitation in a mechanical fashion. For the purpose of

determining the method's degree of repeatability, five samples were analysed using nine distinct quality control (QC) levels, ranging from 2 ng ml all the way up to 1000 ng ml. After going through the process of technique validation, the test demonstrated a high degree of precision and accuracy in its results. Because of the high sample throughput offered by this method, it was chosen to carry out a pharmacokinetic investigation of sofalcone in human plasma, which was ultimately fruitful [2.4].

5. Lee et al. developed a sensitive and selective method to quantify sofalcone and its active metabolite in human plasma using liquid chromatography-electrospray ionisation tandem mass spectrometry. Using an automated sample handling system, d3-sofalcone and its metabolite solutions were added to plasma samples on 96-well plates (internal standard). The plasma samples on the 96-well plate were then mixed for 30 seconds in 0.5 ml of acetonitrile. The supernatant was centrifuged, transferred to another 96-well plate, and evaporated in a nitrogen mist at 40 ºC. A C18 column equipped with a reversed phase was used to separate the residue from the mobile phase. The lowest quantity of sofalcone and its metabolite that could be detected in a 0.2 ml sample was 2 ng/ml. Ten replicate samples ranging in concentration from 2 to 1000 ng/ml were analysed, proving the method's reusability. The test has been shown to be accurate and precise in experimental settings. Sofalcone and its active metabolite in human plasma were investigated using this technique [2.5].
6. A novel family of amphiphilic Sofalcone compounds with antibacterial peptidomimetic characteristics was discovered, manufactured, and tested by Lin and colleagues. Compound 14, which had two arginine residues, showed the greatest amount of potential since it did not harm the cells, was effective against Gram-positive bacteria such as MRSA, and had a mild impact on the blood cells. Compound 14 was effective against a broad variety of bacteria, was not affected by a wide range of salinities, and swiftly eliminated bacteria by rupturing their cell membranes. A mouse model of keratitis induced by Staphylococcus aureus ATCC29213 responded well to treatment with compound 14, as well. Compound 14 may represent an innovative kind of antibiotic that, in light of what we now know, has the potential to eradicate Gram-positive bacteria that have developed resistance to more conventional antibiotics [2.6].
7. Using an *in vitro* model, Sarosiek et al. investigated how the novel antiulcer medication Solon (Sofalcone) altered the thickness of gastric mucus, how effectively it allowed hydrogen ions to pass through, and how readily pepsin was able to break it down. 7. After being preincubated with Solon, the isolated gastric mucus had a significantly

increased viscosity. Its impact became more pronounced as more of it was present, reaching a high of 230% for solon at a concentration of 2.2 h 10-1M. Studies on permeability found that a mucus concentration of 2.2 to 10-2M solon was 32% more effective in delaying the entrance of hydrogen ions, while a concentration of 2.2 10-1M solon was 43% more effective. The medicine had no effect on the consistency of albumin's thickness or the efficiency with which it blocked the movement of hydrogen ions. According to the results of the peptic activity test, solon slowed down the rate at which albumin and mucus were broken down. Up to 1.0 to 10-5M, the concentration of solon was related to the rate at which peptic activity was halted. This was the case when 60% of mucus proteolysis and 45% of albumin proteolysis were stopped. The *in vitro* findings presented here imply that solon enhances the integrity of the stomach mucosa and may aid in the healing of ulcers. It does this by lowering the level of peptic erosion of the mucus layer, which causes it to become thicker and more effective at preventing hydrogen ions from passing through [2.7].

8. Kim et al. investigated how sofalcone activates the nuclear factor-erythroid 2 (NF-E2) p45-related factor 2 (Nrf2)-heme oxygenase (HO)-1 pathway to protect cells and halt inflammation. We also investigated whether Sofalcone's ability to prevent colitis in the animal model required activation of this pathway. Increased nuclear accumulation of the transcription factor Nrf2 was required for sofalcone to increase HO-1 protein production in human colon cancer cells. Sofalcone also formed Michael adducts with nucleophilic thiol compounds. Degraded sofalcone (SFCR) showed no chemical or biological activity and was inert in the Michael reaction. A biotinylated sinalbinol coupled to the cytoplasmic repressor of nuclear factor erythroid 2-related factor 2 (Nrf2), Kelch-like ECH-associated protein 1. (KEAP1). To prevent binding, we employed sofalcone and a thiol molecule instead of SFCR. Couchelcone treatment also resulted in the dissolution of the Nrf2-KEAP1 complex. Sofalcone alleviated dinitrobenzene sulfonic acid-induced colitis in rats by reducing inflammation and increasing colonic Nrf2 and HO-1 nuclei. The protective effects of sofalcone against colon damage and inflammation were significantly attenuated in the presence of a HO-1 inhibitor [2.8].
9. The effects of sofalcone, an antiulcer medication, on stomach mucus were studied by Piotrowski et al. Two groups of rats were given vehicle or 100 mg/kg of sofalcone twice daily for three days. The GI mucosa was analysed physically and chemically when the rats were killed 16 hours after the last treatment. After using sofalcone, the mucus gel expanded by 23%, while sulfo and sialomucin levels increased by 54% and 25%,

respectively. The mucus's capacity to slow down H^+ rose by 16%, its viscosity doubled, and its gel component became 39% less water resistant as a consequence of these modifications. The percentage of fat, carbs, and proteins in mucus all rose by 18%, 30%, and 10%, respectively, after being exposed to sofalcone. High molecular weight mucus glycoprotein levels in the mucus gel of the animals increased to 50% in the sofalcone group, while they were only approximately 30% in the control group. The data demonstrates that sofalcone enhances the gastric mucosal barrier's protective mucus qualities [2.9].

2.2 Literature Review of Pantoprazole Sodium, Chlordiazepoxide and Clidinium Bromide.

1. In order to identify both clidinium bromide (CLI) and chlordiazepoxide(CHLOR) in a combination pharmaceutical product, Pathak et al. developed and validated a stability-indicating reverse-phase high-performance liquid chromatography approach. Phenomenex Luna C18 (250 mm×4.6 mm, i.d., 5 µm) column is used for separations at room temperature with a mobile phase consisting of potassium dihydrogen phosphate buffer (0.05M, pH 4.0 adjusted with 0.5% orthophosphoric acid), methanol, and acetonitrile (40:40:20, *v/v/v*). 1 ml/min of flow rate is used at a wavelength of 220 nm for detection. The method's linearity, precision, accuracy, system applicability, and resilience have all been verified in separate experiments. Use of the technique using commercial formulations that have been proved to be stable under circumstances that accelerate deterioration demonstrates its efficacy. The approach was successful in distinguishing between the drug and its metabolites in real-world samples. Medicines and stress tests both employ similar ingredients in tablet form [2.10].
2. Nickerson developed capillary electrophoresis (CE) to locate Ro 5-5172, a non-UV-absorbing byproduct of clidinium bromide. The electrophoresis buffer consisted of a solution of sodium phosphate and bromobenzyltrimethylammonium. The periods at which analytes move may be recorded more precisely after being cleaned with sodium hydroxide, water, and a fresh capillary electrophoresis buffer. Furthermore, dependable findings were achieved by using an internal marker to ensure that the injection volume and migration time were consistent across runs. Standard deviations of less than 1% were discovered in both the migration time ratio and the peak area ratio for Ro 5-5172 when compared to the internal standard. Ro 5-5172 has a detection threshold lower than 0.01%. Consistent results were found when a batch of clidinium bromide medication was analysed using both the CE technique and thin layer chromatography [2.11].

3. Amin et al. devised a method for locating clidinium bromide (Clid) and sertraline hydrochloride in tablet and bulk form through the use of spectrophotometry (Sert). The purpose of the research was to find an efficient, low-cost, and precise method of performing visible spectrophotometry. In aqueous buffered pH 3 solution, they form an ion-pair complex with bromocresol green (BCG), bromophenol blue (BPB), and bromothymol blue (BTB). Spectrophotometry is used to extract the colourful byproducts at the wavelengths where each complex has maximum spectral absorption. Experimentation how-to guide. The concentration range from 1-30µg/ml was significantly correlated, as determined by Beer-Lambert analysis. The detection and measurement range, apparent molar absorption, and Sandell sensitivity were all calculated. Ringbom's suggested concentration range of 2-27µg/ml was used to make the analysis more precise. Clidinium bromide and sertraline hydrochloride concentrations in pharmaceutical formulations can now be measured with high precision, independent of the effects of commonly used excipients. The advantages of this method include its sensitivity and its simplicity. Since the drugs under study are made of compounds that don't absorb much UV light, this method is ideal for locating them [2.12].
4. Reversed phase high performance liquid chromatography was used by Sharma et al. to develop a method that was not only straightforward but also accurate and dependable when it came to measuring the same quantity of clidinium bromide, chlordiazepoxide, and dicyclomine hydrochloride. A Chromatopak C-18 column (250 mm×, 4.6mm i.d., 5 µm particle size) was used to separate the three drugs. The column's flow rate was 0.8 ml/min. The mobile phase consisted of 0.1% triethylamine dissolved in water at a pH of 7.4 adjusted with 5% *o*-phosphoric acid and acetonitrile in the ratio of 30:70 *v/v*. The search used a wavelength of 210 nm to locate it. It was determined how long three different medications remained in the body using a series of tests. The retention times of CNB, CHZ and DCH were found to be 7.457 min, 4.400 min and 3.397 min respectively.. Following a series of tests, it was discovered that the method in question was linear, accurate, and precise, in addition to having a low LOD and a high LOQ [2.13].
5. To search for trace levels of clidinium bromide (Clid) in pharmaceutical formulations and actual materials, Khayoon and Yonis utilise dispersive liquid-liquid microextraction (DLLME) and UV-Vis spectrophotometry. Clid and bromocresol green may form ion pairs in aqueous solution with the aid of a citrate buffer. The coloured material was extracted using a mixture of 800 litres of acetonitrile and 300 litres of chloroform. Spectrophotometry was used to identify the sediment phase at a wavelength of 420 nm.

The primary variables affecting DLLME's effectiveness have been modified. Sandal's detection and sensitivity have been calculated, both on the high and low ends of the spectrum. Reference materials, medicines, human urine, and serum enrichment factors were all calculated. Clid may be detected in both natural and synthetic drug samples using this approach [2.14].

6. Amira and her colleagues devised two spectrophotometric methods to investigate the interactions of clidinium bromide with other drugs in binary and ternary mixtures. In the first strategy, participants were split into two groups, one of which was given chlordiazepoxide and the other trifluoperazine, and asked to discover the drug. An other strategy for quantifying clidinium bromide in a combination including the compound's chlordiazepoxide metabolite and a third chemical was to devise a method for measuring the quantity of clidinium bromide. The amount of clidinium bromide was determined using this technique. To do so, we employ the first derivative of the ratio spectrum in conjunction with observations taken at zero-crossing wavelengths. The absorption spectrum of the mixture was divided by that of chlordiazepoxide to get the ratio spectra. The clidinium concentration was determined using a calibration curve. Several synthetic mixes and dosage formulations were discovered by following the procedures we discussed before [2.15].
7. Simple, fast, and selective RP-HPLC techniques with UV detection were developed by Haggag et al. for the simultaneous determination of carvedilol, hydrochlorothiazide, chlordiazepoxide hydrochloride, and mebeverine hydrochloride (Mixture I) (Mixture II). To chromatographically separate the two solutions, we employed an RP-C8 (octylsilyl) analytical column. Acetonitrile, 0.05 M disodium hydrogen phosphate triethylamine, pH 2.5 was used as the mobile phase, and a 247 nm detector was used to examine Sample I. The pH 4.0 mobile phase was composed of acetonitrile, 0.05 M disodium hydrogen phosphate, and water. The detector was tuned to 220 nm. Analyte concentrations were calculated using peak areas. In less than six minutes, we were able to rule out both of the possible outcomes. The effectiveness and dependability of the proposed HPLC procedures were evaluated using statistical tests for linearity, range, precision, accuracy, selectivity, robustness, LOD, and LOQ. Commercial tablet dosage forms were analysed using conventional HPLC techniques, and no interference peaks from routinely used pharmaceutical adjuvants were found [2.16].
8. It was shown by Patel et al. binary mixture of imipramine HCl and chlordiazepoxide that was determined by three different methods. The first involved determination of

imipramine HCl and chlordiazepoxide using the first derivative spectrophotometric technique at 219 and 231.5 nm over the concentration ranges of 1-20 and 2-24 µg/ml with mean accuracies of 99.47 +/- 0.78 and 101.43 +/- 1.20%, respectively. The second method utilized RP-HPLC with methanol-acetonitrile-0.065 M ammonium acetate buffer (45 + 25 + 30, v/v/v, pH adjusted to 5.6 +/- 0.02 with phosphoric acid) as the mobile phase pumped at a flow rate of 1.0 mL/min. Quantification was achieved using UV detection at 240 nm over concentration ranges of 0.25-4.0 and 0.1-1.6 microg/mL, with mean accuracies of 101.17 +/- 0.56 and 100.67 +/- 0.40% for imipramine HCl and chlordiazepoxide, respectively. The third method was HPTLC with carbon tetrachloride-acetone-triethylamine (pH 8.3; 6 + 3 + 0.3, v/v/v) as the mobile phase. Quantification was achieved with UV detection at 240 nm over concentration ranges of 50-600 and 20-240 ng/spot with mean accuracies of 99.51 +/- 0.59 and 100.59 +/- 0.84% for imipramine HCl and chlordiazepoxide, respectively. The suggested procedures were checked using prepared mixtures, and were successfully applied for the analysis of pharmaceutical preparations. The accuracy and precision of the methods were confirmed when the standard addition technique was applied. The results obtained by applying the proposed methods were statistically analyzed. [2.17].

9. Mebeverine hydrochloride (MEB) and chlordiazepoxide (CPZ) may be separated and quantified with great precision according to the work of Heneedak et al., who developed a method utilising reversed-phase high-performance liquid chromatography. Both the purified MEB and the CPZ capsules found in stores were free of the principal CPZ contaminants and their breakdown products. We used a Phenomenex® Luna C18 (250mm × 4.6mm i.d., 5m ps) analytical column and a gradient mobile phase system consisting of (A) water and (B) methanol. In this example, the peak area was calculated using UV detection at 254 nm. Oxidative degradation, acidic hydrolysis, and basic hydrolysis all put stress on MEB and CPZ. Impurities in CPZ or byproducts of MEB decomposition did not mask the chemicals' true identities. In this sense, the suggested approach is analogous to a stability experiment [2.18].
10. To discover a mixture of trifluoperazine HCl and chlordiazepoxide, Patel and Patel et al. (10) pumped 1.0 ml/min of methanol: water (97:03, *v/v*). UV detection at 262 nm has an average accuracy of 101.050.47 for concentrations between 0.1 and 1µg/ml, and 98.970.33 for values between 0.5 and 5µg/ml. After it was determined that the excipients in tablets wouldn't interfere with chromatography, the technique was applied to them as

well. The standard method of addition had no effect on the efficiency of the procedure [2.19].

11. Sujatha et al. 11 ensured that their Reverse Phase High Performance Liquid Chromatography method for detecting amitriptyline HCl and chlordiazepoxide in tablet dose form was user-friendly, cost-effective, selective, precise, and accurate, all of which are required by the International Council for Harmonization (ICH). The drug concentration in the sample was determined using an isocratic YMC Collimated C8 (250×4.6mm, 5µ) column. Column was maintained at 40°C, mobile phase was Orthophosphoric Acid and Methanol (pH adjusted to -2 with Orthophosphoric Acid), and detector was tuned at 253 nm. Amitriptyline HCl had a retention time of 2.502 seconds, whereas chlordiazepoxide's was 5.176 seconds. For the exam, the percentiles for scores of 100% and 99.999% were respectively 100 and 99.99%. The proposed approach satisfies all of the ICH criteria for accuracy, precision, linearity, range, specificity, and robustness. This process was used to create tablets of amitriptyline hydrochloride and Chlordiazepoxide [2.20].
12. Clidinium bromide (CDB), chlordiazepoxide (CDZ), and dicyclomine hydrochloride (DICY) are all computed concurrently in the study by Doki et al., and their respective bulk and combination tablet dose forms are explained in detail. At a flow rate of 1.0 ml/min, potassium di hydrogen phosphate buffer (0.05M, PH 4.0 adjusted with 0.5% ortho phosphoric acid) was injected onto a Kromasil C18 (250mm×4.6 mm id, 5µm) column for chromatographic separation. We measured retention periods of 7.457 minutes for clidinium bromide, 4.400 minutes for chlordiazepoxide, and 3.397 minutes for dicyclomine hydrochloride. Precision, linearity, LOD, LOQ, robustness, and ruggedness were all evaluated to ensure the proposed approach satisfied ICH standards. Clidinium bromide, chlordiazepoxide, and dicyclomine hydrochloride were all measured in their respective bulk and combination tablet dosage forms using the previously developed and proven technique [2.21].
13. For simultaneous detection of chlordiazepoxide (CDO) and mebeverine HCl (MBV) in the presence of the CDO impurity (2-amino-5-chlorobenzophenone, or ACB) and the MBV degradation product, El-Shaheny et al. developed a simple, rapid, and sensitive RP-HPLC approach (veratric acid, VER). Isocratic mode, 1 ml/min flow rate, BDS Hypersil phenyl column with 4.5mm× 250 mm, 5µm particles size, and a mobile phase consisting of 0.1 M potassium dihydrogen phosphate and triethylamine were utilised to achieve the separation in 9 minutes. The mobile phase pH was adjusted to 4.5 using

orthophosphoric acid, and UV detection at 260 nm was used. The procedure for vetting guests out was meticulous. The recommended procedure was utilised to calculate the amounts of CDO and MBV present in the pills, with mean % recoveries of 99.75 ± 0.62 and 98.61 ± 0.38, respectively. The findings of the proposed approach were compared to those of a comparable HPLC method using the Student t-test and the variance ratio F-test. The byproducts of MBV breakdown were determined by infrared (IR) and mass spectrometry studies [2.22].

14. In order to correctly, rapidly, and selectively identify mebeverine hydrochloride (MVH) and chlordiazepoxide (CDZ) in a binary combination, Michael et al. 14 employed reversed phase high performance liquid chromatography (HPLC) and high performance thin layer chromatography with UV detection. Column: ACE-126-2546 AQ C-18 (250×4.6 mm i.d., 5µm particle size); isocratic mode; mobile phase: 25 mM ammonium acetate buffer: acetonitrile (60:40, *v/v*); pH adjusted to 3±0.2 with hydrochloric acid; flow rate: 1.0 ml/min; detection wavelength: 260 nm. The MVH retention time was determined to be 7.23±0.01minutes, whereas the CDZ retention time was determined to be 3.85±0.01 minutes. We scanned at 222 nm using Wincats Software and a Camag TLC scanner. Our mobile phase was an 8:4 (*v/v*) mixture of ethyl acetate and methanol. Coated silica gel 60F_{254} was used as the stationary phase. MVH's RF values were found to be 0.26±0.02, whereas CDZ's were found to be 0.73±0.01. Many aspects of analytical validation were investigated in this work. We compared our data to the data from the official protocols and found no statistically significant differences using the student t-test, the F-test, and the one-way analysis of variance [2.23].
15. Both Ali and Singh have developed and validated an RP-HPLC technique for the rapid, sensitive, accurate, and cost-effective measurement of amitriptyline in tablet form and pure chlordiazepoxide. The stages included using a Waters C18, 250 mm× 4.6 mm (5µm) reverse-phase column with a mobile phase ratio of 56:24:20 *v/v/v* to determine the amounts of amitriptyline and chlordiazepoxide. In methanol, acetonitrile, and water, 0.2M orthophosphoric acid has a pH of 4.5. The constant infusion rate of 1 ml/min for the mobile phase was maintained. The concentrations of chlordiazepoxide and amitriptyline were respectively 10 and 320 ng/ml and 50 and 1600 ng/ml when measured at 240 nm. It took 15 minutes to finish the split. The typical quantity of recovered chlordiazepoxide was 0.98% *w/w*, whereas the typical amount of recovered amitriptyline was 98.46 ±0.47 % *w/w*. Coefficients (R^2) of 0.9998% were found for both amitriptyline and chlordiazepoxide. Amitriptyline HCl had a Limit of Detection (LOD) of 25 ng/ml, a

LOQ of 83 ng/ml, and an LOE of 4.5 ng/ml. Both amitriptyline HCl (97.47 ± 0.58 % *w/w*) and chlordiazepoxide (95.29 ± 0.59 % *w/w*) had a relative standard deviation (RSD) of 2% both within and between days. Both components in solid tablet dosage forms may now be routinely measured with the help of the new techniques. They are user-friendly, fast, precise, dependable, and inexpensive [2.24].

16. Forced degradation stability studies were conducted on chlordiazepoxide and amitriptyline hydrochloride using RP-HPLC by Boobalan et al. Bondapak C18 column (300mm × 3.9 mm, 10 µm particle size), wavelength detector, methanol buffer, acetonitrile, and THF (50:20:30 *v/v/v*) (254nm). Since it conforms to ICH guidelines, this technique may now be used. The validation parameters' sensitivity, linearity range, recovery, and resilience were analysed. The linearity coefficient (R^2) was 0.99999 for the concentration range of chlordiazepoxide, while it was 0.9998 for the concentration range of amitriptyline hydrochloride. Chlordiazepoxide has a LOD of 0.258µg/ml, whereas amitriptyline hydrochloride has a LOD of 0.283µg/ml. Chlordiazepoxide recovery ranged from 98.75% to 98.99%, whereas amitriptyline hydrochloride recovery was in the 99.66% to 99.49% range. Calculating the dosages of chlordiazepoxide and amitriptyline hydrochloride using the proposed approach is simple and reliable. As a result, a technique for gauging stability was developed. Pharmaceutical formulations and samples from mass manufacturing may both be put through their paces using this technique. The novel approach was superior to the previously described one because it required less time for retention and more clearly distinguished between mobile phase components [2.25].
17. Chlordiazepoxide• HCl and other impurities were detected in capsule and tablet forms by Roberts and Delaney using a quantitative high-performance liquid chromatographic approach. The octadecylslane column was utilised with methanol and water as the mobile phase. The ability to distinguish between elements is facilitated by the absorption of 254 nm. Three separate injections of chlordiazepoxide and chlordiazepoxide•HCl showed a variation of less than 1%. All capsule and tablet formats have a recovery rate of 99.2% or higher from authentic samples [2.26].
18. Chlordiazepoxide and amitriptyline HCl are found in three distinct methods by Patel and Patel. The first derivative spectrophotometric approach was used to estimate the concentrations of amitriptyline HCl and chlordiazepoxide at 219 and 230 nm, with mean accuracies of 100.9±0.87 and 99.2±1.0%, respectively, spanning concentration ranges of 120 and 224µg/ml. For the second technique, a reversed-phase high performance liquid

chromatographic instrument was utilised with orthophosphoric acid as the mobile phase and methanol, acetonitrile, and 0.065 M ammonium acetate buffer (50:20:30, *v/v/v*) as the stationary phase. The pH was maintained at 5.5 ± 0.02 during the whole procedure. Mean accuracy ranges for UV detection at 240 nm were from 100.55±0.62 for concentration ranges of 0.25 to 4µg/ml and from 100.71±0.81% for concentration ranges of 0.1 to 1.6µg/ml. The third technique was the use of HPTLC on tablets. After separating the medicines, the densitometer was used to determine the 240 nm spot sizes. A mixture of carbon tetrachloride, acetone, and triethylamine (6:3:0.2) (*v/v/v*) was employed as the mobile phase, and the components were separated using Merck thin layer chromatographic aluminium silica gel 60 F254 sheets. The linear range for chlordiazepoxide was 20–240 ng/spot, whereas that for amitriptyline hydrochloride was 50–600 ng/spot. The approaches may find usage in the pharmaceutical sector since the excipients utilised in tablet production did not result in any chromatographic interferences. Research confirms the accuracy of the quantitative approaches employed to identify the chemicals [2.27].

19. Chlordiazepoxide (CDZ) was shown to be poisonous to plants by Soentjens-Werts et al. due to the presence of an N4-oxide group. CDZ photoisomerizes to produce the unusual byproduct oxaziridine at 350 nm. Using acetonitrile as the solvent, heating the irradiated solutions to 10ºC, and irradiating them for 70 to 90 minutes was determined to be the most effective approach to irradiate CDZ during cytotoxicity experiments, with concentrations ranging from 12.2 to 152.0 µg/ml. Methods for determining CDZ photodegradation sequence kinetic parameters. The first reversible or irreversible order was selected for each situation using Akake's rule. The amount of damage induced by CDZ and oxaziridine radiation was calculated using a backwards HPLC technique. Demoxepam's oxaziridine and 2-amino-5-chlorobenzophenone were effectively extracted from the drug's water of synthesis, and 2-amino-5-chlorobenzophenone was also extracted. Using the experimental irradiation settings, we isolated oxaziridine with a purity of 98% from CDZ. The study of molecules in the pharmaceutical industry may benefit from this HPLC technique [2.28].
20. Patel D and Patel JK developed and demonstrated three accurate, rapid, and selective procedures for determining chlordiazepoxide and mebeverine hydrochloride concentrations in medicines. The first derivative spectrophotometric analysis is the first technique to consider. We considered and adjusted for every environmental factor that may have an impact on the outcome. For the second technique, we will employ HPTLC

to analyse the densitometric characteristics of the unbroken drug spots at 220 nm. This allows the separation of chlordiazepoxide (R_f = 0.43 ± 0.04) and mebeverine hydrochloride (R_f = 0.72 ± 0.02). As a mobile phase, a mixture of chloroform, methanol, and ammonia (9.5:0.5:0.1, (*v/v/v*)) was employed to separate substances on silica gel plates. We found a linearity range of 200-1200 ng/spot for mebeverine hydrochloride and 100-600 ng/spot for chlordiazepoxide. The third technique includes a C18 reversed-phase column, a mobile phase of potassium dihydrogen phosphate buffer (0.05 M, pH 4.0 adjusted with 0.5% orthophosphoric acid), methanol, and water (30:50:20, *v/v/v*), and UV detection at 260 nm. Peak area suggests a 5-minute analysis time. These approaches' dependability and precision were shown by their use of the additive standard method. The ICH guidelines were followed throughout the validation procedure. The efficacy of the methods was analysed statistically [2.29].

21. High pressure liquid chromatographic (HPLC) was developed by Strojny et al. to detect chlordiazepoxide and its metabolites in plasma in a time- and labor-saving manner. Chlordiazepoxide and its metabolites are extracted from plasma using a pH 9 buffer and dissolved in diethyl ether for the experiment. Chlordiazepoxide concentrations in plasma as low as 50–100 ng/ml may be recovered with a 5% loss. (S.D.). Chlordiazepoxide and its metabolites in the plasma were examined after an oral dose of chlordiazepoxide HCl [2.30].
22. Amitriptyline and chlordiazepoxide capsules were studied by Abuirjeie et al. Both substances were detected using high-performance liquid chromatography and first-derivative spectrophotometry, as stated by the researchers. To separate amitriptyline, chlordiazepoxide, and diazepam, a Micro pack MCH-5 (CI8) column was used in conjunction with a mobile phase consisting of acetonitrile/water (50:50) at pH 3 and 0.01 M sodium-n-octane sulphate (internal standard). The medications were accessible at a room temperature and a wavelength of 230 nm. The first derivative spectrophotometric trough amplitudes at 245 nm and 282 nm were utilised to distinguish amitriptyline from chlordiazepoxide. We evaluated commercial capsules as well as lab-made mixes containing varying concentrations of both medications using this cutting-edge method. Examination of the data using t-test and F-test confirmed the accuracy of the findings. [2.31].
23. For the analysis of chlordiazepoxide in pharmaceutical formulations and in bulk pharmaceuticals, Shrivastav et al. developed a dependable reverse-phase HPLC approach. Acetonitrile and dipotassium hydrogen phosphate anhydrous buffer (pH 6.8)

were eluted isocratically on a Phenomenex Luna C18 stationary phase to create this technique (250× 4.6 mm, 5µm). To ensure the system was linear across a large range of values, its usability, precision, and accuracy were all tested to ensure compliance with ICH requirements. Both the LOD and LOQ were discovered in this study. How long the current solutions would hold up was also investigated. In order to determine how much chlordiazepoxide was included in a novel formulation that also included an excipient, a newly constructed and proven technique was utilised. Results showed recoveries from 97.0 to 101.0% using this strategy. Chlordiazepoxide was identified as a substance that could be measured using the technique (99.7 %). Chlordiazepoxide in bulk medications and formulations may now be tested rapidly using a linear and reliable RP-HPLC technique [2.32].

24. To determine the concentration of pantoprazole in enteric-coated tablets, dissolving solution, and human plasma, Emami et al. developed a sensitive, specific, and user-friendly HPLC approach. The technique built upon the success of a liquid-liquid extraction. In order to separate the compounds, a -Bondapak C18 HPLC column was utilised, along with a mobile phase of 0.01 M sodium hydrogen phosphate solution/acetonitrile (60:40 *v/v*) at pH 7.4 ± 0.1 at a flow rate of 1.5 ml/min. Plasma samples were analysed with omeprazole serving as an internal reference. The half-lives of omeprazole and pantoprazole in the body were respectively 7 and 10 minutes. Pantoprazole showed a straight calibration curve with an average recovery of 85.5% when evaluated in human plasma. In no case did accuracy exceed 14.4%, and on average it was 15.8%. The analysis was performed using a reversed-phase column and UV detection in an isocratic HPLC run. The detection limit we employed in this investigation, 25 ng/ml, was nearly 20 times lower than that used in previous research. Pantoprazole levels in plasma samples from healthy volunteers were measured [2.33].
25. Pandey et al., investigated the stability of bulk pantoprazole using a high-performance liquid chromatography (HPLC) technique that Pandey et al. established with a short run time. The medication was separated from potential contaminants and breakdown products using a Hypersil ODS column with a gradient of 0.01 M phosphate buffer at pH 7 and acetonitrile as the eluent, with a detection wavelength of 290 nm. The selected flow rate was 1 ml/m^{-1}. The technique had a 0.043$\mu g/ml^{-1}$ LOD and a 0.147$\mu g/ml^{-1}$ LOQ. The data was reliable, linear (r^2=0.999), fixable (97.9-103%), and robust. A reliable test is one that remains consistent in the face of deliberate manipulation. Pantoprazole degradation is accelerated in the presence of acidic, oxidative, and photolytic stress. High

pH, heat, and humidity didn't seem to affect the medicine much. There were no issues with the excipients when this method was used to create drug formulations [2.34].

26. Manasa et al. demonstrate that RP-HPLC may be utilised to determine the concentrations of pantoprazole sodium and mosapride citrate in a pharmaceutical preparation. Using a flow rate of 1 ml/min of orthophosphoric acid and a mobile phase consisting of 0.007M sodium phosphate buffer (pH 4) and 40 mM acetonitrile, the analysis was performed in isocratic mode on a 5 µm, 4.6 mm × 150 nm Hypersil BDS column. Using an ultraviolet detector, we determined that the absorbance was 278 nm. Pantoprazole sodium and mosapride citrate had retention periods of 2,803 and 5,167 minutes, respectively, when this technique was utilised. Linearity, precision, accuracy, specificity, ruggedness, and robustness were evaluated according to International Council for Harmonization system suitability standards (ICH). Both pantoprazole and mosapride were shown to have a recovery rate of 99.77% 0.23% and 99.44% 0.50%, respectively. The pharmacokinetics of both pantoprazole and mosapride were linear throughout a wide concentration range, with the former being at 5-30µg/ml and the latter at 1.9-11.4µg/ml. Mosapride citrate and pantoprazole sodium have a LOD and LOQ of 0.1599 and 0.1790µg/ml, respectively. As it satisfies all ICH validation criteria, this approach may be utilised to estimate both pantoprazole sodium and mosapride citrate simultaneously in combination pharmaceutical formulations [2.35].
27. Prasanna Kumar et al. developed and validated a high performance liquid chromatographic technique for determining the presence of pantoprazole sodium and Lansoprazole in commercially available drugs. The compounds were separated by passing them down a 5 × 150 × 4.6 mm Inertsil C18 column at a flow rate of 1.0 ml/min, with UV detection set to 230 nm. Phosphate buffer (pH 7.0) was used as part of the mobile phase at a ratio of 60:40 (*v/v*). The half-life of sodium pantoprazole in the body was 2.538 minutes, whereas that of lansoprazole was just 2.017 minutes. Verifying the linearity of the procedure (correlation coefficient: 0.999). This research demonstrates that pantoprazole sodium and Lansoprazole may be quantified with high reproducibility using a single mobile phase and reversed-phase liquid chromatography [2.36].
28. Pantoprazole (PNT) concentrations in pharmaceutical dosage forms may be determined using a straightforward reversed-phase HPLC approach, as first described by Rao et al. and subsequently verified by other studies. The best way to proceed: The proposed RP-HPLC technique for isocratic separation utilises a (5) Hibar 250-4, (6) Li Chrospher 100 RP-18 column with a cap on one end. In this experiment, the mobile phase flow rate was

1 ml/min, and the output wavelength was 288 nm. Methanol and water (by volume) made up 80% of the mobile phase. Results: The retention durations of both the pure medication and the PNT formulation ranged from 3,558 minutes to 3,575 minutes. Between 0.5 and 400µg/ml, the provided approach yielded linear results. The sum collected may be refunded in full (99, 240, and 17%). Data on intermediate accuracy from a wide variety of experimental conditions were analysed using the F-test and t-test at a 95% confidence level, and the computed value was determined to be inadequate. The LC approach presented here is a quick and easy way to pinpoint the PNT with high sensitivity and precision. The LC technique was developed and validated; it is applicable to the analysis of both unadulterated and adulterated PNT [2.37].

29. Saini et al. eveloped a method for measuring pantoprazole in Multiparticulate dose form that is easy to use, specific, precise, cost-effective, and repeatable by using high pressure liquid chromatography. HPLC-grade acetonitrile and methanol were used as the mobile phase, while a gradient-capable Shimadzu Octa decyl Silane (ODS) C18 column was used as the stationary phase. Beer-Lambert compatibility between 5 and 25µg/ml has been shown for the following procedure. The statistical analysis and the recovery tests confirmed the investigation's findings. The proposed strategy was shown to be statistically reliable due to its low standard deviation (SD). Successful testing of commercially available 40 mg pantoprazole solid dosages has been reported [2.38].
30. The stability of a product containing itopride hydrochloride and pantoprazole was investigated by developing and validating a reversed-phase high-performance liquid chromatography (HPLC) technique, which was done by Gupta et al. With an apparent pH of 5.0 and UV detection at 289.0 nm, phosphate buffer and acetonitrile (55:45, *v/v*) constitute the mobile phase of choice for the RP-HPLC technique. For this study, we used the suggested technique to examine the effects of thermal, photolytic, hydrolytic, and oxidative stress on PAN, ITH, and their combination pharmaceutical formulation. Linearity was shown for both the PAN and ITH methods here across their whole 4-20µg/ml and 15-75µg/ml ranges, respectively. When comparing PAN and ITH recovery rates, the former was 100.02 while the latter was 99.88. By observing for the lack of peaks that co-eluted with the primary peaks of the medications' chromatographic peak purities, we were able to determine the test method's specificity for assessing PAN and ITH in the presence of degradation products. Combination drugs are an example of an area where the suggested quality control technique might be useful [2.39].

31. An easy-to-use, sensitive, and precise high performance liquid chromatographic technique for the detection of Pantoprazole sodium and Lansoprazole was developed and validated by Reddy et al. Chemical analysis of medications used in industry is performed using this technique. The chemicals were isocratically separated by passing them over a C18 column (Use Inertsil C18, 5, 150 mm × 4.6 mm) at a flow rate of 1.0 ml/min and using UV detection at 230 nm. Phosphate buffer (pH 7.0) was used as part of the mobile phase at a ratio of 60:40 (v/v). Pantoprazole sodium's retention time was 2.538 minutes, whereas that of Lansoprazole was 2.017 minutes. A linear progression was seen throughout the procedure (correlation coefficient: 0.999). This research shown that both Pantoprazole sodium and Lansoprazole may be tested using sensitive and selective reversed-phase liquid chromatography using a single mobile phase [2.40].
32. Thanikachalam et al. employed an HPLC approach that indicated the drug's stability, and it was tested to see whether it could be used to detect domperidone and pantoprazole in tablets. The optimal mobile phase for this HPLC technique is a 20:33:47 (*v/v/v*) combination of methanol, acetonitrile, and a 20 mM buffer of dipotassium hydrogen phosphate and phosphoric acid at pH 7.0. The column's internal diameter is 4.6 millimetres and its overall length is 5 metres (length). The observed flow rate is 1.19 ml m-1. UV detection at 285 nm was utilised to quantify domperidone concentrations between 0.5 and 5 g ml^{-1} and pantoprazole concentrations between 1 and 10 g ml^{-1} based on peak area and linear calibration curves (R^2 > 0.999 for both medications). Method accuracy, precision, linearity, low detection and quantitation limits, and reproducibility were all validated throughout the process. There was no evidence that the procedure used altered the effectiveness of the pills' contents. Dry heat, oxidation, acid, base, and neutral hydrolysis were the stresses applied to domperidone, pantoprazole, and their combination medication. The stressed samples were then put through their paces using the suggested approach. The stability of these medications over time in commercial and wholesale contexts might be evaluated using the suggested approach due to the possibility of separating the drug from its breakdown products. [2.41].
33. A fixed-dose combination of meloxicam and pantoprazole was developed and evaluated by Ahmad et al. Fixed-dose combos of pantoprazole and meloxicam have been more precisely calculated and verified with the use of RP-HPLC. The medicines were separated in a mobile phase consisting of a phosphate buffer/acetate solution (30:70, *v/v*), pH 3.4, 1.0 ml/min flow rate, and 25 °C. As the molecule had a 310 nm wavelength, it was simple to locate. The half-lives of meloxicam and pantoprazole were 6 and 9

minutes, respectively. The linearity of the detector was verified by testing at concentrations ranging from 0.1 mg/l to 200 mg/l. The overall correlation between the two approaches is 0.9999. Typically, the percentage of success was between 98% and 102%. All of the specifications set out by the International Council for Harmonization were adhered to. Regular testing of the drug formulation using the procedure described in is warranted given the widespread use of meloxicam and pantoprazole combined. [2.42].

34. In order to separate the Rac-pantoprazole (PAN) enantiomers, Tanaka et al. developed a direct, simple, and isocratic reversed-phase HPLC technique using cellulose-based chiral stationary phases (Chiralcel OD-R and Chiralcel OJ-R). The chiral benzimidazole sulfoxides rac-omeprazole (OME) and raclansoprazole (LAN) were also investigated due to their structural similarities. The enantiomers of rac-LAN were most easily separated using Chiralcel OD-R. Chiralcel OJ-R proved to be the most effective method for isolating the rac-PAN and rac-OME enantiomers. For rac-PAN and rac-OME, acetonitrile worked best as an organic modifier on Chiralcel OJ-R, whereas methanol worked best on Chiralcel OD-R. Enantiomer resolution was unaffected by decreasing retention by increasing column temperature or buffer concentration. Separation factors of 1.26 and 1.13 were achieved for rac-PAN and rac-OME enantiomers on a Chiralcel OJ-R column, while 1.16 was achieved for rac-LAN enantiomers. An acetonitrile and sodium perchlorate solution (50 mM) was utilised as the mobile phase in these analyses. [2.43].
35. Using a high-throughput parallel HPLC-MS/MS approach, Wang et al. demonstrated how to distinguish between enantiomers of pantoprazole. It took just 4.5 minutes to separate the two enantiomers of pantoprazole using a Chiralcel OZ-RH column. The results of all the enantiomer tests tended linearly, and the assays were reliable and precise enough to be used routinely. Chiral pharmacokinetic analysis in beagle dogs demonstrated its efficacy and shown its applicability to high-throughput studies. Finally, there is an HPLC-MS/MS test that is quite comparable to the Pantoprazole test. This technique has been validated for PK investigations and can accommodate almost twice as many samples [2.44].
36. Abd El-Hay et al. designed and evaluated a precise high-performance liquid chromatographic approach for the simultaneous detection of pantoprazole (PNT) and piroxicam (PIR) as an NSAID in tablets, capsules, and other medication formulations. Separation of PAN and PIR by chromatography was optimised using response surface

approach (RSM). At room temperature, an ODS-3 Inertsil C18 column was used in conjunction with a mobile phase consisting of acetonitrile and 48.09 mM phosphate buffer (pH 4.92; 53.61:46.39, *v/v*) (250 mm ×4.6 mm i.d., 5 µm). Time spent storing the PAN was 4,217 minutes, while the PIR was 6,249 minutes. Results for PNT were linear between 5 and 30µg/ml when the novel technique was applied, while findings for PIR were linear between 2.5 and 15µg/ml. This approach may be used to efficiently compute PNT and PIR simultaneously even in quality control laboratories with limited resources [2.45].

37. An HPLC-based technique for quantifying sodium pantoprazole was developed and validated by Raffin et al. 37. Everything was completed correctly and meticulously. A medication solution was tested for stability during dissolution by storing it in a 7.4-pH phosphate buffer, away from light, and at room temperature for 22 days. After 6 hours, pantoprazole's effectiveness had decreased by less than 5%, and its half-life was 124 hours [2.46].
38. Rahic et al. came up with and tested a simple, reliable, and accurate approach to locate pantoprazole in pantoprazole pellets. The evaluation of pantoprazole in pantoprazole pellets is not covered by the current pharmacopoeias (USP, BP). A C8 column with an internal diameter of 250 mm × 4.6 mm and a flow rate of 2 ml/min was used for this separation in water. The wavelength of 290 nanometers was chosen as the cutoff for the UV detector's sensitivity. Based on ICH guidelines, the procedure is considered safe and effective. The technique that the active component in pantoprazole pellets was determined has been proved to be accurate and dependable [2.47].
39. To differentiate between different types of sodium pantoprazole, Liu et al. They developed a technique called RP-HPLC. This was accomplished using a Lichrospher ODS C_8 column (4.6 mm× 200 mm 5 µm). The mobile phase included 35% acetonitrile, 65% Na_2HPO_4, 0.1% n-octylamine in 0.1% NaH_3PO_4, and H_3PO_4. The pH of the mobile phase was 7.0. One millilitre was being dispensed each minute. The detection was made at a 254-nm wavelength. The temperature within the shaft was a comfortable 35 degrees. In general, things were improving rapidly. The rates of reproductive success (RSDs) were high. This strategy is convenient in that it is straightforward, rapid in its completion, and reliable [2.48].
40. Both domperidone and pantoprazole were discovered in otc medications by Sivakumar et al. using reversed-phase HPLC. Twenty experiments were conducted to develop mathematical models, with the mobile phase composition, buffer molarity, and flow rate

serving as the independent variables. The first-peak retention factor, the resolution, and the retention times all played roles in the analysis. Best conditions for separating the substances under consideration were predicted by fitting the experimental responses into a second-order polynomial and optimising all six responses simultaneously. The optimal mobile phase for the experiment was determined to be 1.19 ml/min of methanol, acetonitrile, and potassium hydrogen phosphate. Under such ideal circumstances, we could distinguish between baselines with a resolution of at least 2.0 in under 6 minutes. The approach was able to predict values for all parameters that were within a small margin of error of the experimental results. The increased assay condition was evaluated for specificity, linearity, accuracy, and precision according to ICH recommendations [2.49].

41. To investigate the photolytic and oxidative mechanisms that cause pantoprazole (PNT) to degrade in a stress-dependent manner, Al Bratty and coworkers developed a reversed-phased high-performance liquid chromatography-diode array detection (HPLC-DAD) technique. PNT degradation products were removed from the sample by passing it down a C18 column submerged in a mobile phase of methanol and water (60:40, *v/v*; pH 3.0) at a flow rate of 1 ml/min. Between 5 and 25µg/ml, the concentration ranges with the highest linear regression coefficient of 0.9995 was found. The technique proved reliable, with a limit of detection of 0.25µg/ml, a standard deviation of less than 0.5% for repeatability, and a standard deviation of less than 1.5% for intermediate precision. PTZ stress sample analysis revealed that nine of the eighteen identified degradation products were present in both photolytic and oxidative degradations. Eleven distinct impurities were discovered when azobis isobutyronitrile was oxidised. Three of these are superior to PTZ in terms of water resistance. UV light was discovered to disintegrate into eight different compounds, whereas visible light disintegrates into only seven. We also tested the long-term stability of an injectable form of pantoprazole sodium using the same methodology. There were no new contaminants discovered in the formulations, however three well-known pollutants were detected in both stress situations. Spike study demonstrates that frequent byproducts of PTZ injectable formulations include sulfone, N-oxide, and N-oxide sulphide. [2.50].
42. Pantoprazole (PNT), Domperidone (DPD), and Drotoverine(DRT) were all examined in an HPLC stability assay developed and validated by Mishra et al. The rates were as follows: 1 ml/min for PNT, 2.5 ml/min for DPD, and 1 ml/min for DRT. For this chromatographic separation, we utilised a mobile phase consisting of 20 ml of methanol,

33 ml of acetonitrile, and 1 ml of 0.02M dipotassium hydrogen phosphate (pH 7.0). PNT (290 nm), DRT (240 nm), and DPD (320 nm) were shown to be the optimal wavelengths (240 nm). Studies on the test's linearity, precision, accuracy, recovery, and specificity were conducted as recommended by ICH. The retention times for PNT, DPD, and DRT were 2.50, 6.01, and 11.80 minute s. Limits of Detection (LODs) were determined to be 0.01721µg/ml for PNT, 0.0115µg/ml for DPD, and 0.0212µg/ml for DRT. It was determined that 0.0573µg/ml was the LOQ for PNT. The LOQ for DPD was 0.0385µg/ml and for DRT it was 0.0706µg/ml. DPD had a linear range of 0.125 to 8µg/ml (n=7), whereas PNT and DRT had a range of 0.25 to 16µg/ml. The coefficient of correlation in a cross-sectional study was determined to be 1. The method's selectiveness for pharmaceutical formulations was shown using forced degradation testing [2.51].

43. Ondansetron hydrochloride, granisetron hydrochloride, and pantoprazole sodium were all put through their paces in a high performance liquid chromatography study. The drug ketorolac tromethamine used as the reference for comparison. Ondansetron or Granisetron hydrochloride and pantoprazole sodium were effectively separated using a Hypersil BDS-C18 column and isocratic elution of a mobile phase consisting of acetonitrile: 10 mM acetate buffer: trimethylamine (20:80:0.5, *v/v/v*), pH 3.5. In order to distinguish between pantoprazole sodium and ondansetron hydrochloride and pantoprazole sodium and Granisetron hydrochloride, a dual wavelength setup was created at 290 and 305 nm. Pantoprazole sodium and ondansetron hydrochloride or granisetron hydrochloride may be measured using the standard technique [2.52].
44. Levo sulpiride (LSP) and pantoprazole sodium sesquihydrate (PNT) in capsules were measured using a rapid, accurate, and user-friendly RP-HPLC technique developed by Kothapalli et al. It is common practise to use prokinetic drugs containing PNT, as well as proton pump inhibitor drugs containing LSP. At a flow rate of 1.0 ml min-1, a 0.02 M potassium dihydrogen o-phosphate solution (pH-4 adjusted with *o*-phosphoric acid) was used for the chromatographic separation on a Thermo BDS C18 column (250 mm × 4.6 mm, 5.0 particle size). From 8 to 48µg/ml^{-1} of pantoprazole sodium and 7.5 to 45µg/ml^{-1} of levo sulpiride were measured linearly and quantitatively at 238 nm. The approach has been evaluated and found to be true in terms of its LOD, LOQ, robustness, specificity, linearity, accuracy, precision, and LOD. To ensure that the proposed method is up to ICH standards, it has been refined and tested. [2.53].
45. To determine whether or not gastro-resistant capsules with delayed release contain pantoprazole, Marques et al. created a straightforward liquid chromatographic method

involving a reverse-phase column, a 75:25 mixtures of acetonitrile and water as the mobile phase, a flow rate of 1 ml/min, and a detector with a wavelength of 290 nm. We analysed the selectivity, precision, linearity, and reproducibility. The pellets and the acid used to break them down are irrelevant to the procedure. Between 2.0 to 18.0µg/ml^{-1} was the linearity range. The average RSDs for accuracy ranged from 0.92% to 2.00% throughout all seven days of the week. Between 93.48% and 105.150% of the typical dosage of pantoprazole was detected in the tablets. Patients administered pantoprazole pellets had a 96.27-102.87% likelihood of survival [2.54].

46. Using reverse phase high performance liquid chromatography, Gurupadayya and Sama developed a simple, sensitive, fast, accurate, and repeatable approach for detecting clopidogrel and pantoprazole in rat plasma. Using a C8 (250× 4.6 mm, 5 µm) column and a mobile phase containing 0.03M potassium dihydrogen ortho phosphate buffer (pH 3) and acetonitrile combined in a 40:60 (*v/v*) ratio, we separated a variety of compounds at a flow rate of 1.2 ml/min. The analyte was monitored using a UV detector set to 240 nm. With this technique, I was able to achieve a 2.6-minute pantoprazole retention time and an 8.2 minute clopidogrel retention time. Throughout a 10- to 50-fold concentration range, the suggested approach is effective for both clopidogrel and pantoprazole. Numerous experiments were utilised to demonstrate the method's validity; they included tests for system appropriateness, linearity, precision, LOD/LOQ, sensitivity/specificity, accuracy (recovery), robustness, stability, and forced degradation (specificity). The formulation has been modified for usage on animals. It has been shown that the pharmacokinetic parameters area under the curve (AUC), maximum concentration (Cmax), and time to maximum effect (Tmax) are all statistically significant [2.55].
47. For the simultaneous determination of levosulpiride (LVS) and pantoprazole sodium, Arige et al. developed and validated an isocratic reversed-phase high-performance liquid chromatographic technique (PNT). The separation was carried out at a flow rate of 1.0 ml/min using a Symmetry C18 column with a mobile phase consisting of 60 parts methanol, 20 parts acetonitrile, and 1-part phosphate buffer pH.9 (150 mm× 4.6 mm I.D., 5 µm particle size). At 294 nm, the PDA was detected. The half-lives of levosulpiride and pantoprazole were 3,516 and 4,869 minutes. Calibration curves for Pantoprazole Sodium (24-56µg/ml) and Levosulpiride (75-450µg/ml) were linear (R^2 = 0.9999). This procedure was used to validate linearity, precision, accuracy, robustness, and toughness. When applied to Pantoprazole Sodium and Levosulpiride, the suggested technique provided a reliable estimation of the combined daily dosage. Scientific evidence

demonstrates that the recommended approach is superior in these regards. The proposed approach is suitable for regular quality control analysis of Pantoprazole Sodium and Levosulpiride bulk and tablet dosage forms, as shown by the high percentage of recovery and low percentage of RSD. [2.56].

48. To determine which ingredients in commercial pharmaceutical goods are naproxen and pantoprazole, Kumar et al. developed and validated a straightforward, sensitive, and accurate HPLC approach. All of the substances were separated using a BDS Hypersil C-18 reversed-phase column, acetonitrile and mixed phosphate buffer (pH 6.92) as the mobile phase at a 45:55 (*v/v* %) ratio, a flow rate of 1.0 ml/min, and 290 nm detection. A straight line emerged in the range of 20 µg and 120 g. Naproxen and pantoprazole had correlation values of 0.997 and 0.995, respectively, when the computed technique was utilised. As a result of the intervention, the success rate was between 99.67 and 101.39 percent. It seems that capsule dosage formulations of naproxen and pantoprazole might be tested using this approach due to its high recovery rate and low relative standard deviation [2.57].
49. An HPLC approach developed and validated by Vidyadhara et al. enables rapid, sensitive, and accurate detection of naproxen and pantoprazole in commercial pharmaceutical goods. A BDS Hypersil C-18 reversed-phase column and a mobile phase of 45:55 (*v/v* %) acetonitrile and mixed phosphate buffer were used to effectively separate the compounds at a flow rate of 1.0 ml/min at a detection wavelength of 290 nm (pH 6.92). The scale was consistent from 20 g to 120 g. Naproxen and pantoprazole were shown to have correlations of 0.997 and 0.995, respectively. Overall, the success rate ranged from 99.67 to 101.39 %. Naproxen and pantoprazole capsules were evaluated, and the results showed a high rate of recovery and a low relative standard deviation [2.58].
50. Therapeutic dose forms of pantoprazole sodium and cinitapride hydrogen tartrate were simultaneously detected using an RP-HPLC technique developed and validated by Macharla and Bairam et al. Since it is simple, accurate, low-cost, and fast, this technology is ideal for routinely assessing the quality of manufactured and raw pharmaceuticals. Using UV detection at 264 nm and a flow rate of 1.0 ml/min, a combination of methanol and 0.1% *v/v* triethylamine (pH 6) was pumped down a Thermoscientific BDS Hypersil C18 (250 4.6 mm, 5 l) column for chromatographic separation. Calculations revealed that the half-life of pantoprazole is 4.73 minutes and that of cinitapride is 2.86 minutes. It is clear that this approach is robust across a large

concentration range due to the linearity observed for Cinitapride (R^2 = 0.9922) and Pantoprazole (R^2 = 0.9974) between 0.5 and 1.3µg/ml. Using the standard HPLC technique, the concentrations of both pure cinnapride and pantoprazole and their tablet forms were determined, and the findings were found to be in excellent accord with the claim. The simplicity, precision, exactness, and focus of this procedure have made it effective for evaluating pharmaceutical and bulk formulations [2.59].

51. An easy, specific, accurate, and precise method of simultaneously testing levosulpiride and pantoprazole sodium in capsule form was developed by Khanage et al. They accomplished this using a C18 (HiQ Sil C18, 250 mm × 4.6 mm, particle size 5 µm) column in reverse phase. The sample was analysed using a UV-2075 plus detector at 249 nm, a mobile phase of methanol, and a 5 mM ammonium acetate buffer, after the pH was adjusted to 4.0 using glacial acetic acid. Both levosulpiride and pantoprazole were retained for 2.29 and 4.30 minutes, respectively. Specificity, linearity, precision, accuracy, robustness, and system applicability were all achieved, as required by the International Council for Harmonization (ICH). The method was linear for a large concentration range (from 5 to 25µg/ml) for both levosulpiride and pantoprazole sodium (correlation values of 0.998 and 0.9999, respectively). The fact that all of the levosulpiride and 99.91 % of the pantoprazole were recovered demonstrates the reliability of the procedure. When testing repeatability, the standard deviation was significantly lower than 2%. This technique was successfully used to the dosing of both Pantoprazole Sodium and Levosulpiride tablets [2.60].
52. An easy, accurate, and high-performance liquid chromatographic technique for the concurrent detection of mosapride and pantoprazole in pharmaceutical dosage form was developed by Siddartha et al. for the 52nd submission. A solution of orthophosphoric acid was diluted with phosphate buffer (55:45 *v/v*) to bring the pH of the mobile phase up to 3.0. It was decided to use the Isocratic mode on five 150 mm 4.6 mm Altima columns. The wavelength was 260 nm and the flow rate was 1.1 ml/min. Researchers determined that mosapride and pantoprazole remained in the system for 2.39 and 3.19 minutes, respectively. Pantoprazole and mosapride have comparable linear ranges (20-120µg/ml). Mosapride had a recovery rate of 99.22% to 100.09%, whereas pantoprazole had a rate of 98.02% to 99.98%. This procedure was used to tablets containing both mosapride and pantoprazole to determine their individual dosages. [2.61].
53. Abdelhameed and Afifi A rapid, sensitive, and accurate HPLC-DAD technique for quantifying pantoprazole (PTZ) and etodolac (ETD) in rat plasma for therapeutic drug

monitoring has been developed and validated. To separate the analytes on a Waters Symmetry C18 column, we ran a 6-minute, 0.8-ml/min^{-1} gradient from eluent A (phosphate buffer pH 4.0) to eluent B (acetonitrile, 55:45 *v/v*). Analytes that were eluted were detected at 254 nm using a photodiode array detector. The approach was linear for PNT across the concentration range of 0.1 to 15 µg/ml^{-1} and for ETD over the range of 5 to 50 µg/ml^{-1}; the computed detection threshold for PTZ is 0.033 µg/ml^{-1}, and for ETD it is 0.918 µg/ml^{-1}. The linearity, accuracy, precision, and selectivity of the approach were statistically validated, as required by ICH recommendations. Repeatability studies showed a high degree of consistency with an intraday repeatability of 7.76% for PTZ and an interday repeatability of 7.50% for ETD [2.62].

54. An isocratic reversed-phase high performance liquid chromatographic technique for simultaneous determination of amitriptyline hydrochloride and pantoprazole sodium (PNT) in bulk and capsule dose forms was developed and validated by Shaikh and Jadhav. The analyte was separated using a cosmosil 18 (250 mm ×4.6mm, 5 µm) column, a UV-visible detector, Workstation software, and a flow rate of 0.8 ml/min through reversed-phase high-performance liquid chromatography. The mobile phase was a methanol and phosphate buffer solution. The wavelength of 244 nm was used to make the discovery. A 3.21-minute difference was found between the half-lives of amitriptyline hydrochloride and pantoprazole sodium. Calibration plots were used to determine that amitriptyline hydrochloride and pantoprazole sodium both had linearities of 0.9995 and 0.9997, respectively. This procedure was used to validate linearity, precision, accuracy, robustness, and toughness. Both amitriptyline hydrochloride and pantoprazole sodium tablets were successfully produced using the suggested procedure. Scientific evidence demonstrates that the recommended approach is superior in these regards. The proposed approach seems to be suitable for regular quality control analysis of bulk and capsule forms of Pantoprazole Sodium and Amitriptyline Hydrochloride due to its high recovery and low % RSD [2.63].
55. Souri et al. investigated the degradation of pantoprazole under stress and developed an HPLC method for detecting the drug in the presence of its metabolites. Pantoprazole was rather stable in basic conditions but degraded in acidic, oxidising, heating, and light conditions. Utilizing a Nova-Pak C_{18} column and a 25:75 acetonitrile and 10 mM KH_2PO_4 (pH 7.4) mobile phase, breakdown products of pantoprazole were isolated from the parent molecule. UV radiation was detected at a wavelength of 290 nm. The technique was linear between 1 and 50 µg/ml of pantoprazole ($r^2 > 0.999$). Results for

accuracy varied between 0.7% and 3.1% across days and weeks. Use the method described in to figure out how much pantoprazole is in pills or solvents. [2.64].

56. Using bovine serum albumin (BSA) as the chiral selector in capillary zone electrophoresis, Sivakumar et al. were successful in separating the enantiomers of pantoprazole sodium, omeprazole, and lansoprazole. Before separating the three medicines with similar structures, crucial experimental parameters had to be fine-tuned. The effects of 1-propanol concentration as an organic modifier and BSA content on separation were investigated. The chiral resolution improved with increasing BSA concentration but the equipment lost sensitivity. It was feasible to distinguish between enantiomers only in the pH range of 7-8. The optimal pH balance between enantio-resolution and peak shape was observed at 7.4. By adding 1-propanol to the buffer system, the resolution and shape of the analytes' peaks were made better. Sodium pantoprazole validation demonstrates that the improved procedure is mature enough for routine usage. [2.65].
57. HPLC employing a C_{18} column and UV detection at 285 nm was used by Hanif et al. to separate pantoprazole sodium sesquihydrate (PSS) and domperidone maleate (DM). The optimal conditions for the test were deemed to be a pH 4.0 phosphate buffer and acetonitrile combination flowing at a rate of 1 ml/min. With a retention duration of fewer than seven minutes and a high degree of specificity, PSS and DM may be differentiated in this scenario. Regression values of 0.999 and 0.9994 were found for PSS and DM, respectively. From 1.56 µg/ml up to 25 µg/ml, this demonstrates that the approach is linear. The percentage recovery ranges for both PSS and DM were between 97.60 and 99.20% and between 96.32 and 98.80%, respectively. The suggested approach was sensitive and specific, and it could be used to monitor both PSS and DM in raft-forming bilayer tablets in real time [2.66].
58. Chlordiazepoxide (CDZ) and clidinium bromide (CDB) were both successfully quantified in pharmaceutical formulations and pure samples using an accurate and sensitive RP-HPLC method established by Ashour et al. The separation was carried out at 25 ºC using a Nucleodur C_8 (250 × 4.6 mm i.d., 5 μm particle size) column. A 0.1 M solution of CH_3CN-MeOH-NH_4OAc (30:40:30, *v/v/v*) was used as the mobile phase at a flow rate of 1.0 ml/min^{-1} with a detection wavelength of 218 nm. Almotriptan (ALT) was used as the reference standard within the study. The proposed procedure was tested, and found to be linear, accurate, precise, low-error, high-quality, and resilient. The method showed excellent linearity throughout the concentration ranges of 2.5-300.0 and

3.0-500.0 µg/ml^{-1} for CDB and CDZ, respectively. Recovery ranged from 100.40 to 103.38 % for CDB and from 99.98 to 105.59% for CDZ. CDB's LOD and LOQ were 0.088 and 0.294 µg/ml^{-1}, whereas CDZ's were 0.121 and 0.403 µg/ml^{-1}, respectively. When applied to the determination of CDB and CDZ in combination dosage forms, the proposed method yielded accurate findings that agreed with the label claim [2.67].

59. Fayez Stability was evaluated in the presence of the alkali-induced breakdown product of chlordiazepoxide (CDZ) and clidinium bromide (CDB). Signifying a phase shift Using UV detection, both high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC)-densitometric methods have been developed and validated (DEG). It was possible to isolate the medications from the degradation product. For the RP-HPLC method, the stationary phase was ACE126-2546 AQ C-18 (250 × 4.6 mm i.d., 5µm particle size) column at 25°C, in an isocratic mode, using mobile phase containing a mixture of 25 mM ammonium acetate (pH 5.4): acetonitrile in the ratio of (20:80, *v/v*), at the fow rate of 1.0 mL min^{-1} and UV detection was performed at 222 nm. The retention times for CDZ and CDB were (4.13 ± 0.01) and (8.5 ± 0.01) min, respectively.

    For the TLC-densitometric method, the separation was performed using a stationary phase of precoated Silica Gel G/UV254 and mobile phase composed of a mixture of ethyl acetate: methanol: ammonia (8:3:1, *v/v/v*) and scanned at 222 nm. The R_f values were (0.79 ± 0.02) and (0.11 ± 0.01) for CDZ and CDB, respectively. The linearity graphs for CDZ and CDB, respectively, were found to be linear over (0.5– 40) µg ml^{-1} 1 and (2–45) µg ml^{-1} with mean percentage recoveries (99.69 ± 0.836) and (99.28 ± 1.838) for RP-HPLC method and (1–14) µg band^{-1} and (0.5–10) µg band^{-1} with mean percentage recoveries (100.00 ± 0.782) and (100.19 ± 1.010) for TLC-densitometric method. A comparative study of different analytical validation parameters such as accuracy, precision, specifcity and robustness were conducted. The obtained results were statistically compared with those of the offcial and reported methods; using Student's t test, F test and one-way ANOVA, showing no signifcant difference with high accuracy and good precision. The proposed RP-HPLC method was also used to study the kinetics of the alkaline hydrolysis of clidinium bromide that was found to follow pseudo-frst order kinetics. The t1/2 was 8.5729 min while k (the degradation rate constant) was 0.0808353 min^{-1} [2.68].

60. Toral et al. established a simple and uncomplicated first derivative spectrophotometric method for the simultaneous determination of clidinium bromide and chlordiazepoxide in pharmaceutical products. Acetonitrile was used as a solvent to extract the drugs from

the formulations, and direct derivative spectrophotometry was used to analyse the samples. The concentrations of chlordiazepoxide and clidinium bromide may be calculated concurrently using either the graphical technique or the zero-crossing method, respectively. Chlordiazepoxide (0.740–12.0 mg/l) and clidinium bromide (0.983–21.62 mg/l) concentration profiles were linear. There are many parts in commercial pharmaceuticals that are completely safe. These pills were found in tablets using the indicated method [2.69].

61. Quick difference spectrophotometric procedures for chlordiazepoxide and demoxepam in chlordiazepoxide formulations were developed by Davidson et al. to address the lack of specificity in the approved spectrophotometric tests. The procedures are based on the differences in absorbance at 269 nm between equimolar solutions of chlordiazepoxide at pH 8 and pH 3, and between equimolar solutions of demoxepam at pH 13 and pH 8. Both chlordiazepoxide and demoxepam require techniques that are specific for either substance in the presence of 2-amino-5-chlorobenzophenone or other substances, such as other medications or formulation excipients. When commercial dosage forms of chlordiazepoxide were analysed, some older samples were discovered to have levels of demazepam above the pharmacopoeial guidelines [2.70].

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CHAPTER 3

3 Identification of Active Pharmaceutical Ingredients

3.1 Materials and Methods

Melting point, infrared spectroscopy, and ultraviolet visible spectroscopy were used to identify API.

Reagent and chemicals

Sofalcone, clidinium bromide, chlordiazepoxide, and pantoprazole sodium were all of the highest grade (purity > 98%) and were utilised as external standards. sofalcone was purchased in this instance from Zeta Scientific LLP in Mumbai. pantoprazole sodium was provided by Aum research Labs in Ahmedabad as a free sample, while clidinium bromide and chlordiazepoxide were provided by Ontop Pharmaceuticals Pvt. Ltd. in Bangalore.

Instrumentation

Microbalance: Mettler Toledo XPE-26, Water Purification System: Milli-Q-water system by Merck, Melting Point Apparatus: VMP-D, FT-IR: Model: Shimadzu 8400S, UV Visible Spectrophotometer: Shimadzu UV Spectrophotometer UV-1700, and

3.1.1 Sofalcone

3.1.1.1 FT-IR Spectrum

Infrared light was captured via FT-IR spectroscopy. The spectrum of the medications was found to overlap with the spectrum used as a benchmark.

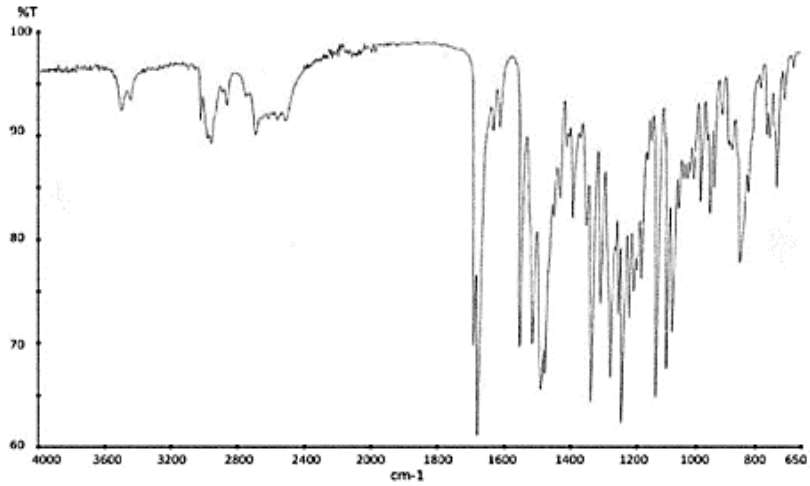

FIGURE 3.1.Reference IR Spectrum of sofalcone [3.7]

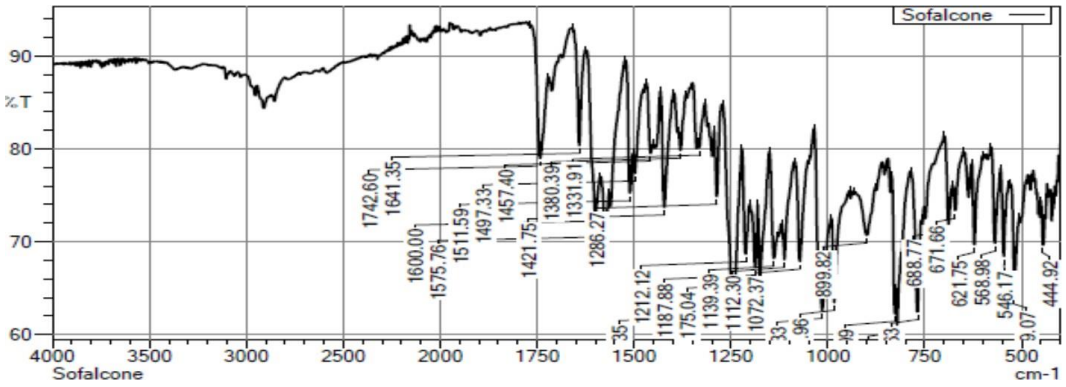

FIGURE 3.2 Recorded IR Spectrum of sofalcone

TABLE 3.1 Interpretation of FT-IR Spectrum of sofalcone

Functional Group	Characteristic Peak	Observed Peak
N-H (asymmetric str.)	3500-3400	3475.49
C-H str.	2950-2840	2947.03, 2927.74
O-H str.	3500-2500	2655.80, 2478.36
C=O str.	1680-1600	1658.67, 1649.02
N-H bending	1560-1510	1519.80
C=C	1600-1400	1487.01
CH_3 bending	1465-1440	1458.08, 1450.37
O=C-O-C (aromatic)	1310-1250	1305.72
=C-O-C (asymmetrical)	1275-1200	1253.64, 1209.28
C-O=C str.	1085-1150	1107.06

3.1.1.2 Melting point determination

Capillary analysis with a melting point device has established the API melting point.

Drug Name	Reported Melting Point	Observed Melting Point
sofalcone	~ 145°C	143-148°C

3.1.1.3 UV Spectroscopy

Sofalcone (20 µg/ml) UV Spectrum were recorded in methanol and water to determine max.

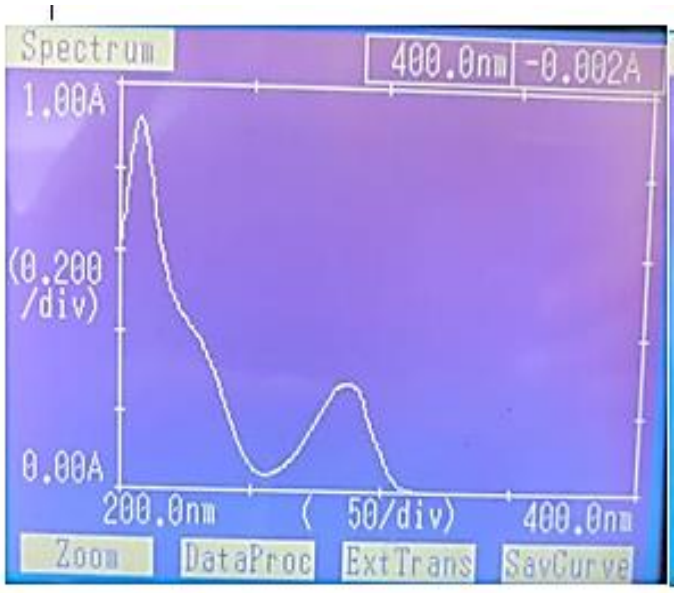

FIGURE 3.3 UV Spectrum of sofalcone in methanol (20 µg/ml) Wavelength: 350 nm, Absorbance: 0.278

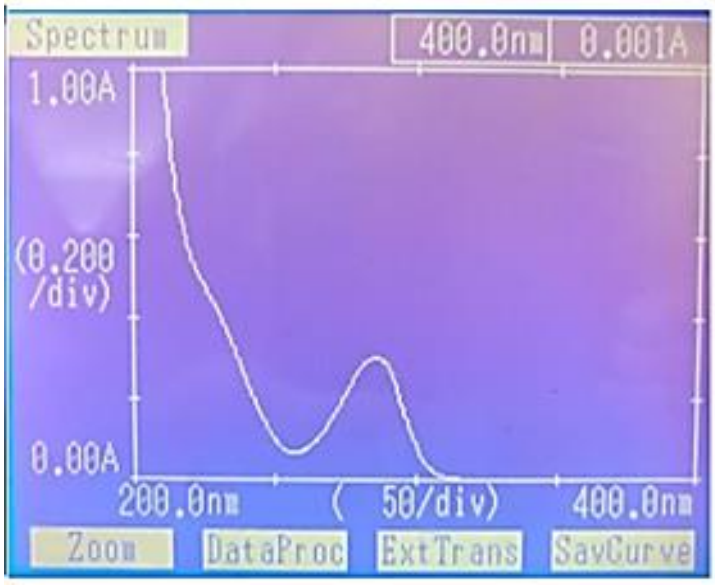

FIGURE 3.4 UV Spectrum of sofalcone in Water (20 µg/ml) Wavelength: 348 nm, Absorbance: 0.30

3.1.2 Clidinium bromide

3.1.2.1 FT-IR Spectrum

A pharmaceutical pellet formed by compressing KBr (Spectroscopic Grade) between 7 and 10 tonnes in a pellet press. The FT-IR measured frequencies between 400 and 4000 cm^{-1}. The IR Spectrum of CLBr were compared to reference Spectrum of CLBr. As a result, there was no doubt that these medications were genuine.

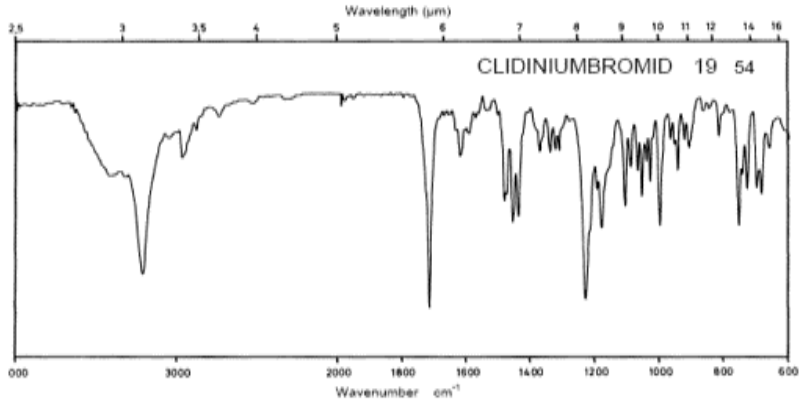

FIGURE 3.5 Reference IR Spectrum of clidinium bromide [3.6]

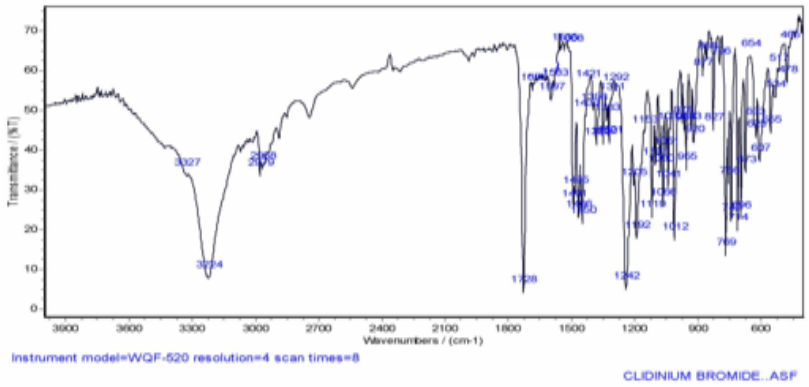

FIGURE 3.6 Sample FT-IR Spectrum of clidinium bromide

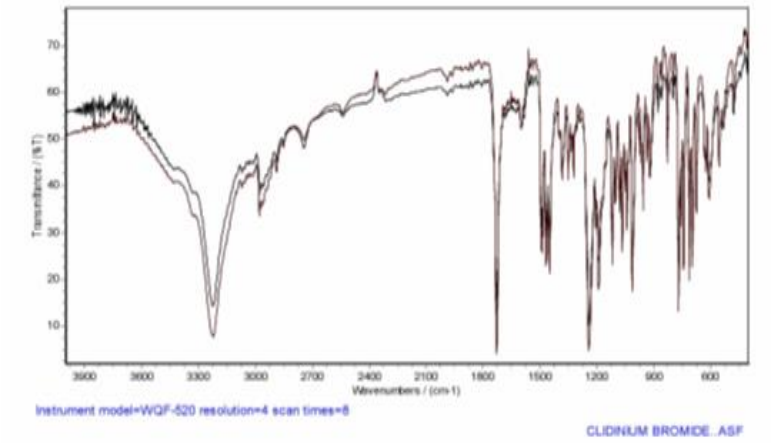

FIGURE 3.7 Comparison of IR spectrum of chlordiazepoxide (Gratis sample with Reference sample)

TABLE 3.2 Interpretation of FT-IR Spectrum of clidinium bromide

Sr. No.	Functional Group	Standard wave Number cm^{-1}	Observed wave Number cm^{-1}
1	Ar-OH Stretching (Phenyl group.)	3400-3200	3456
2	Aromatic rings -C=C stretching	1600	1611
3	Quaternary Amine C-N stretching	1350-1000	1365
4	Ar-COO- (Ester group.)	1100-1035	1087
5	C-Br (Bromide group.)	650-510	645,553

3.1.2.2 Melting point determination

Capillary analysis with a melting point device has established the API melting point.

Drug Name	Reported Melting Point	Observed Melting Point
clidinium bromide	240 ºC – 241ºC	240.5 ºC – 241 ºC

Results from an investigation of the melting point of a free sample confirmed its authenticity.

3.1.2.3 UV Spectroscopy

Clidinium bromide (20 µg/ml) UV Spectrum were recorded in methanol and water, and the maximum absorbance (max) was noted.

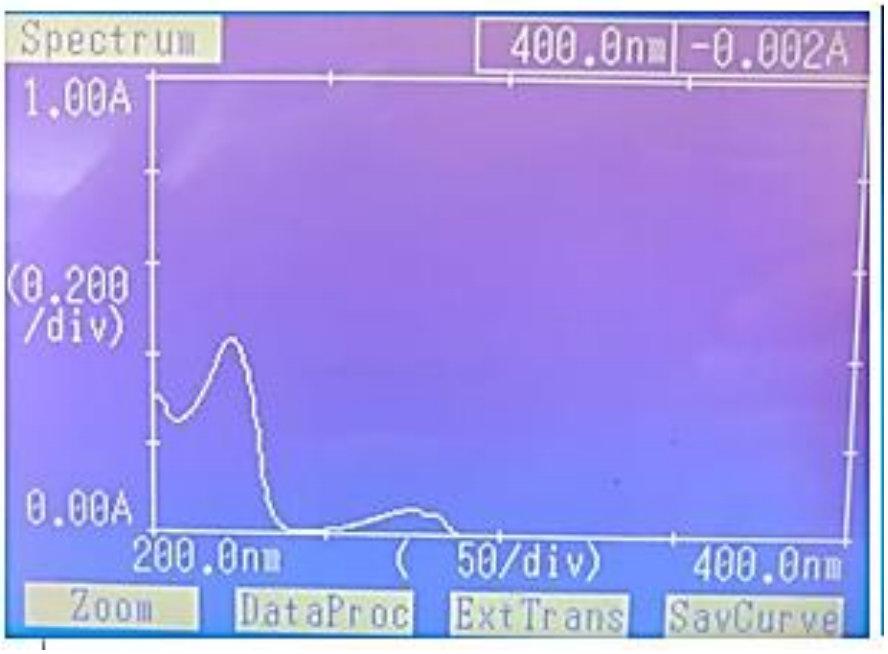

FIGURE 3.8 UV Spectrum of clidinium bromide in methanol (20 µg/ml) Wavelength: 223 nm, Absorbance: 0.435

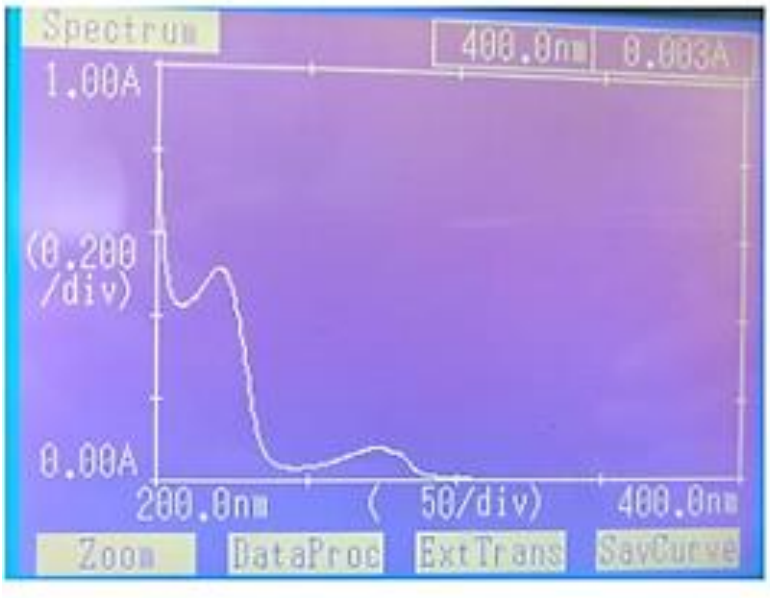

FIGURE 3.9 UV Spectrum of clidinium bromide in Water (20 µg/ml) Wavelength: 221 nm, Absorbance: 0.516

3.1.3 Chlordiazepoxide

3.1.3.1 FT-IR Spectrum

To create a pharmaceutical pellet, KBr (Spectroscopic Grade) is combined with a hydraulic pellet press that can exert pressures between 7 and 10 tonnes. The FT-IR measured frequencies from 400 cm^{-1} to 4000 cm^{-1}. They compared CDZ's IR Spectrum to their gold standard, the CDZ IR spectrum. These details established beyond any reasonable doubt that the capsules were genuine.

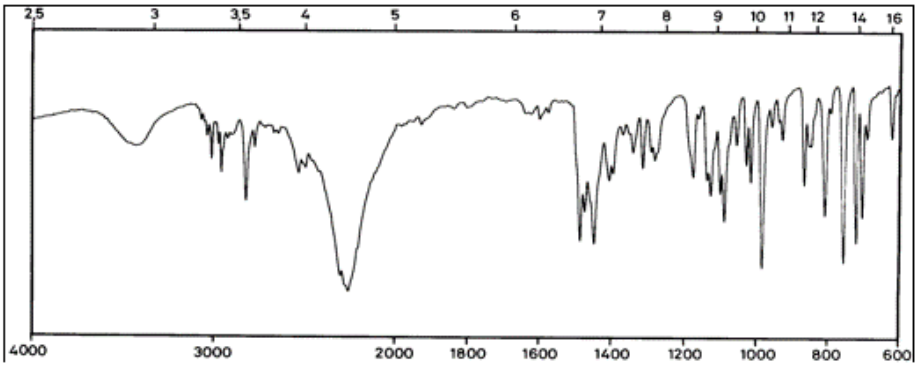

FIGURE 3.10 Reference IR Spectrum of chlordiazepoxide[3.6]

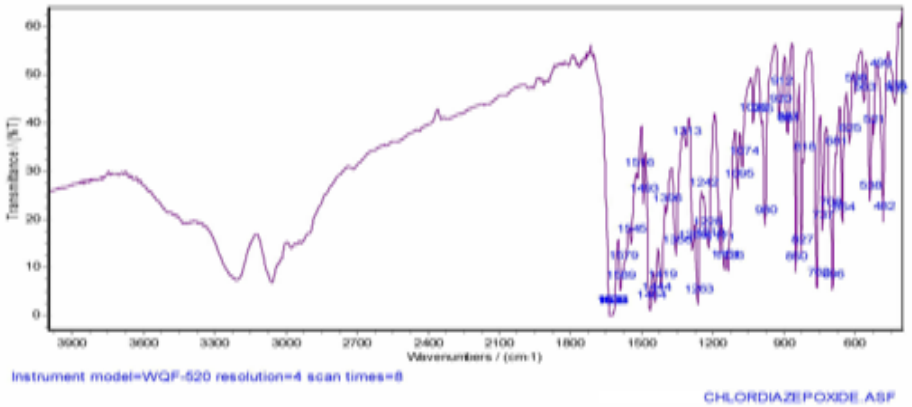

FIGURE 3.11 Sample FT-IR Spectrum of chlordiazepoxide

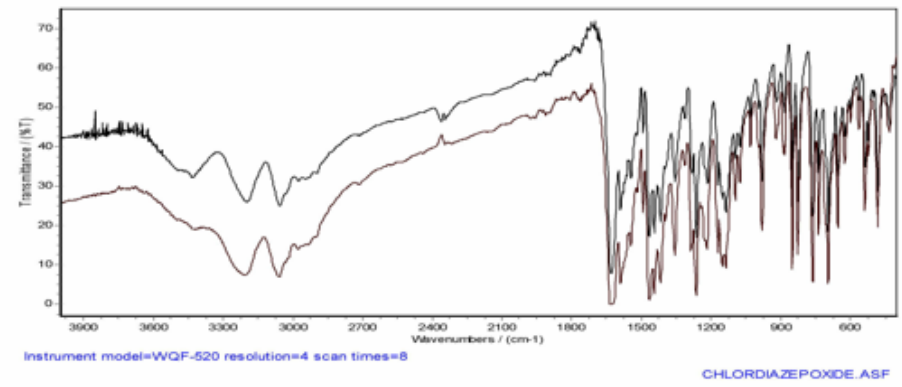

FIGURE 3.12 Comparison of IR spectrum of chlordiazepoxide (Gratis sample with Reference sample)

TABLE 3.3 Interpretation of FT-IR Spectrum of chlordiazepoxide

Sr. No.	Functional Group	Standard wave Number cm^{-1}	Observed wave Number cm^{-1}
1	$-NH_2$ Stretching	3500-3300	3315
2	=C-H stretching	3050-3010	3033
3	Aromatic rings -C=C stretching	1600	1629
4	Aromatic Nitro Compound-N=O	1550-1490	1539
5	Amines C-N stretching	1350-1000	1369
6	Acid hydrochlorides	1100-1035	1087
7	-C-Cl	730-550	725

3.1.3.2 Melting point determination

The Melting Point of API has been determined by the Capillary method using the Melting point apparatus.

Drug Name	Reported Melting Point	Observed Melting Point
chlordiazepoxide	236 ºC - 236.5 ºC	236 ºC - 236.5 ºC

3.1.3.3 UV Spectroscopy

UV Spectrum of chlordiazepoxide (20 µg/ml) was taken in methanol, and λ_{max} was observed.

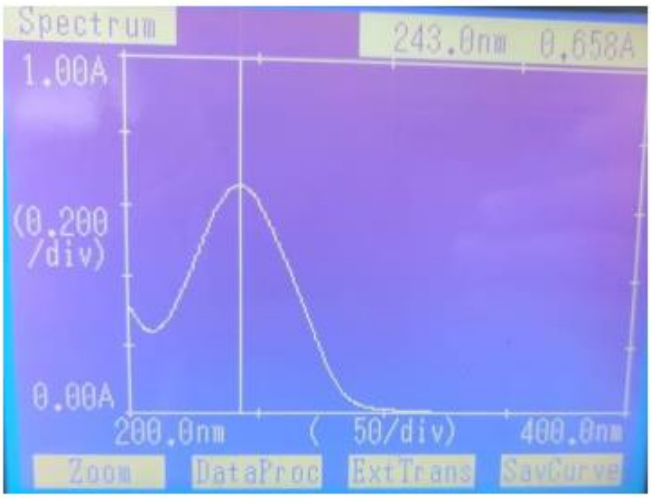

FIGURE 3.13 UV Spectrum of chlordiazepoxide in methanol (20 µg/ml) Wavelength: 243 nm, Absorbance: 0.658

3.1.4 pantoprazole sodium

3.1.4.1 FT-IR Spectrum

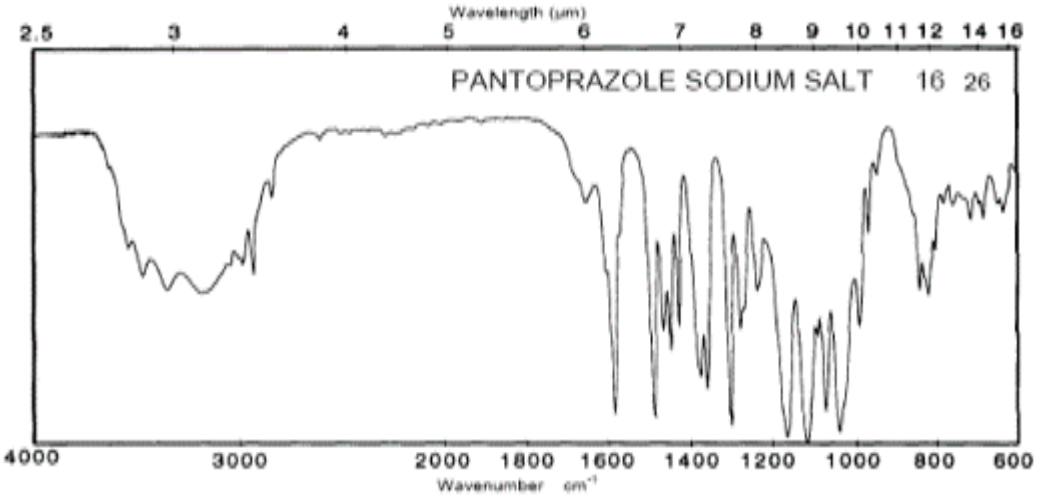

FIGURE 3.14 Reference IR Spectrum of Pantoprazole

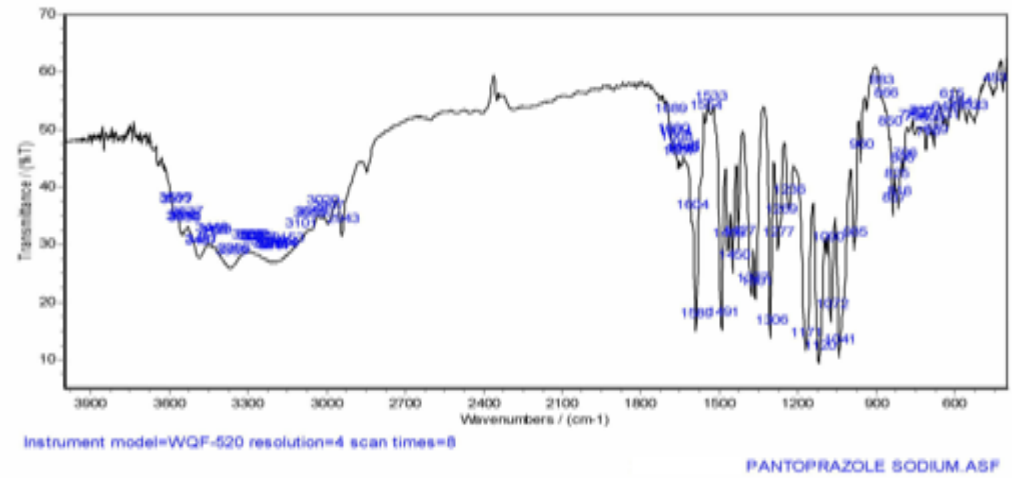

FIGURE 3.15 Sample FT-IR Spectrum of Pantoprazole

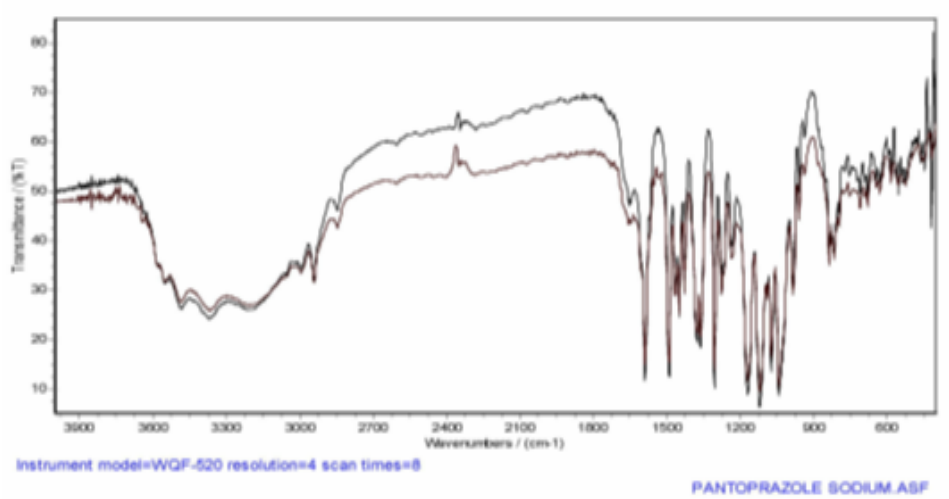

FIGURE 3.16 Comparison of IR spectrum of pantoprazole sodium (Gratis sample with Reference sample)

TABLE 3.4 Interpretation of FT-IR Spectrum of Pantoprazole

Sr. No.	Functional Group	Standard wave Number cm^{-1}	Observed wave Number cm^{-1}
1	Amines N-H	3500-3000	3453
2	Aromatic Rings =C-H	3050-3010	3028
3	Imines C=N	1690-1640	1685
4	Ar-C=CH=O	1680	1681
5	Sulfoxide S=O	1050	1053
6	C-F	1400-1000	1100
7	C-N	1350-1000	1339
8	Ether C-O	1300-1000	1291

3.1.4.2 Melting point determination

The Melting Point of API has been determined by the Capillary method using the Melting point apparatus.

Drug Name	Reported Melting Point	Observed Melting Point
pantoprazole sodium	139 ºC -140 ºC	138 ºC - 140 ºC

From the melting point study of the gratis sample it was concluded that the samples were found to be authentic.

Summary:

A pharmaceutical pellet formed by compressing KBr (Spectroscopic Grade) between 7 and 10 tonnes in a pellet press. The FT-IR measured frequencies between 400 and 4000 cm^{-1}. Comparisons were made between the IR Spectrum of PNT, CDZ, and CLBr and their respective reference standards. They inspected both the PNT IR Spectrum and their customary IR spectrum to find differences. The infrared spectroscopy data for PNT, CDZ, and CLBr are presented in the correct sequence. As a result, there was no doubt that these medications were genuine.

3.1.4.3 Pantoprazole

UV Spectrum of pantoprazole (20 µg/ml) was taken in methanol and Water, and λmax wasobserved.

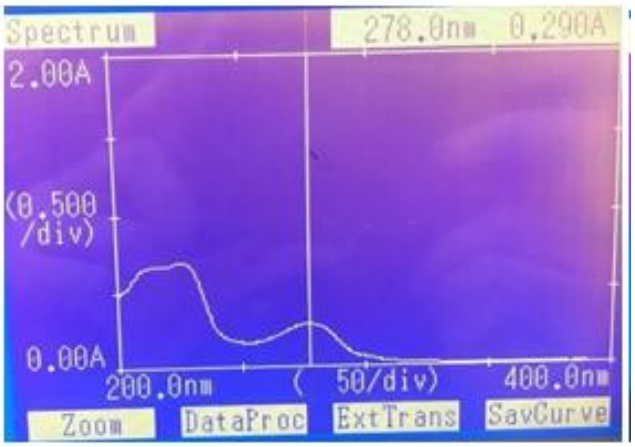

FIGURE 3.17 UV Spectrum of pantoprazole in methanol (20 µg/ml) Wavelength: 277 nm, Absorbance: 0.658 226.5 nm, Absorbance: 0.700

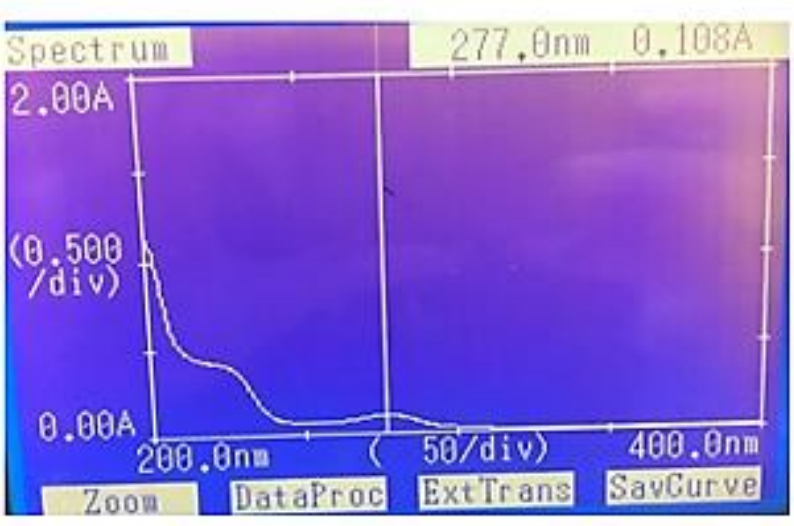

FIGURE 3.18 UV Spectrum of pantoprazole inWater (20 µg/ml) Wavelength: 276.5 nm, Absorbance: 0.109

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CHAPTER 4

4 Validated stability-indicating RP-UHPLC method for the estimation of sofalcone in drugs, reconciling mass balance in force degradation studies and LC-MS identification of its degradation products:

4.1 Material and methods

4.1.1 Reagent and chemicals

Zeta Scientific LLP. - Mumbai was the source for our supply of sofalcone. The other chemicals were purchased from Merck Specialty Private Limited and were of HPLC quality.

- **Procurement of Drug**

FIGURE 4.1 sofalcone were procured from Zeta Scientific LLP. – Mumbai

4.1.2 Instruments and Equipments

TABLE 4.1 Instruments and Equipments

Sr. no.	Name	Brand/Model	Manufacturer/Supplier
1.	Auto Sample	G7129B	Agilent Tech. Ltd.
2.	Column Oven	G7116B	Agilent Tech. Ltd.
3.	Detector	G7115A	Agilent Tech. Ltd.
4.	UHPLC System	1290 Infinity 11	Agilent Tech. Ltd.
5.	Mass System	6470 LC/TQ	Agilent Tech. Ltd.
6.	Pump	G6470A	Agilent Tech. Ltd.
7.	Software	Mass Hunter 10.0	Agilent Tech. Ltd.
8.	HPLC Column	Persuit Diphenyl Column	Agilent Tech. Ltd.
9.	Analytical Balance Digital	AUX 220	Shimadzu
10.	Micropipette (2-20 µL, 10-100 µL)	Research Plus	Eppendorf
11.	Centrifuge	CPR 24 Plus	Remi
12.	FT-IR	IR-Spirit	Shimadzu
13.	UV Spectrophotometer	UV-1900	Shimadzu
14.	Ultra Sonicator	LMUC6	Labman
15.	Vortex Shaker	CM-101	Remi
16.	Melting Point Apparatus	-	Gallenkamp
17.	Water Purification System	Integral 3	Millipore Merck
18.	Refrigerator	LG 308 L 3 Star	LG Electronics

TABLE 4.2 List of Glassware and Apparatus

Sr.no.	Name	Brand/Model	Manufacturer/Supplier
1.	Volumetric Flask	Appropriate Volume	Borosil Glassware Ltd.
2.	Glass Beaker	Appropriate Volume	Borosil Glassware Ltd.
3.	Pipette's	Appropriate Volume	Borosil Glassware Ltd.
4.	Measuring Cylinder	Appropriate Volume	Borosil Glassware Ltd.

5.	Centrifuge Tube	Appropriate Volume	Tarson Product Ltd.
6.	Glass Bottle	Appropriate Volume	Borosil Glassware Ltd.
7.	Auto Sampler Vials	-	Agilent

TABLE 4.3 Filters and column:

Sr. No.	Name	Make
1	0.22 µm PVDF membrane filter (P/ No.: GVWP04700)	Millipore
2	0.45 µm Nylon syringe filter (P/ No.: SENN0602MNXX106)	mdi
3	0.45 µm pre-filter + PVDF syringe filter (P/ No.: SYVG0602MNXX104)	mdi
4	0.45 µm pre-filter + PTFE syringe filter (P/ No.: SYTG0602MNXX104)	mdi
5	Eclipse Plus C_{18} (150mm × 4.6mm, 5µm) (P/ No.: 00F 4040-E0)	Phenomenex

4.1.3 Optimized Chromatographic Conditions

TABLE 4.4 Optimized Chromatographic condition:

Parameters	Optimized condition
Chromatographic Mode	Reverse Phase
Elution mode	Gradient
Mobile phase composition	Mobile Phase A: Water (0.1% Formic Acid) Mobile Phase B: Ammonium Acetate in methanol Mobile Phase A : Mobile Phase B (Initial 30:70)
Column	Agilent Eclipse Plus C18 RRHD (100mm × 2.1mm, 1.8µm)
Flow rate	0.300 ml/min
Detection wavelength	350 nm
Injection volume	2.00 µL
Run time	20 minutes
Retention Time (min)	5.3 minutes

Parameters	**Optimized condition**		
Sample concentration	100 µg/ml		
Mobile Phase	Time (mins)	Mobile Phase A: Water (0.1% Formic Acid)	Mobile Phase B: Ammonium Acetate in methanol
	2	30	70
	3	20	80
	5	20	80
	6	15	85
	8	15	85
	9	10	90
	12	10	90
	13	5	95
	15	5	95
	15.10	30	70
	20	30	70

4.1.4 Preparation of solutions

4.1.4.1 Blank (Diluent)

Acetonitrile: water (20:80) (Dissolve sample in 5ml THF; top up with Diluent to make final volume).

- **Solution A**

0.1% Formic Acid

Mix 1 ml of formic acid into 1 litre of water. Put the mixture through a filter with a 0.45 µm Nylon membrane.

- **Solution B**

Ammonium Acetate in methanol

Ammonium Acetate (6.8 g) should be dissolved in 950 ml of water. Use a diluted Glacial Acetic Acid solution to get the pH level down to 5.6 ± 0.05. Here, 50 ml of methanol was added and well combined. Put the mixture through a filter with a 0.45 µm Nylon membrane.

- **Mobile Phase**

Mix 30 % of Solution A with 70 % of Solution B. Degas for 10 minutes with a sonic bath.

4.1.4.2 Standard Solution (100 µg/ml)

Add 5 ml of tetrahydrofuran to a 100 ml volumetric flask containing 100 mg of the working standard. It has to be diluted with 10 ml of liquid. The standard is diluted with diluent to the necessary concentration, then mixed well once it has cooled to room temperature and been fully dissolved using sonication.

4.1.4.3 Sample solution: (Prepare sample in duplicate) (100 µg/ml)

We purchased commercially available sofalcone formulations. Careful measurements were taken of both 20 capsules and a solid substance of the same weight. Exactly 100 mg of sofalcone was weighed and transferred to a 100 ml volumetric flask. Tetrahydrofuran, in the amount of 5 ml, must be added. For about ten minutes, sonicate the ingredients. The next step is to sonicate 95 ml of diluent on and off for an hour. Let the flask come to room temperature before adding the diluent, diluted solution, and mixing. Remove the first 3-4 ml of the filtrate after passing the solution through a 0.45 µm Pre-filter + PTFE filter. After thoroughly mixing the diluent, pipet 10 ml of the clear filtrate solution into a 100 ml volumetric flask and dilute to volume with the diluent. Make an example out of this solution.

4.1.5 Force Degradation study:

The forced degradation study was carried out on placebo, sofalcone API and sofalcone Capsules. The samples will be subjected for acid degradation, base degradation, oxidation degradation, hydrolysis degradation, photolytic degradation, humidity degradation and thermal degradation. For each degradation study prepare a blank accordingly.

4.1.5.1 API Solution (Prepare control API solution in duplicate) (100 µg/ml)

100 mg of sofalcone will need to be weighed and moved into a 100 ml volumetric flask. Add 5 ml of tetrahydrofuran. Process at high speed for about 10 minutes. For an hour, mix the sonicated diluent every so often. Let the flask cool to room temperature before adding the diluent, diluted solution, and mixing. After putting the solution through a 0.45 µm Pre-filter + PTFE filter, take out the first 3–4 ml of the filtrate. After putting 10.0 ml of the clear filtrate

solution into a 100 ml volumetric flask with a pipette, the diluent is added to make up the volume. The solution is then mixed. This answer could be used as a model.

4.1.5.2 Preparation of forced degradation solutions for placebo, API and Capsules

4.1.5.2.1 Acid degradation

Using exact weighing, put 100 mg of API sofalcone pellets, 100 mg of placebo pellets, and sample pellets into three 100 ml volumetric flasks. Add 5 ml of tetrahydrofuran. It is best to sonicate for about 10 minutes. Then, sonicate 95 ml of diluent for 60 minutes while mixing it every now and then. Add the right amount of hydrochloric acid that has been diluted. If you don't want the test value to drop by more than 20%, you should keep the flask in a water bath or on a room-temperature work surface. When it has cooled down, you can neutralise it with a 1% solution of sodium hydroxide. After using the diluent to get the right concentration, combine. A 0.45 µm filter must be used to filter the solution. If you use PTFE as a pre-filter, you should throw away the first 3–4 ml of the filtrate. After pipetting 10.0 ml of the filtrate into a 100 ml volumetric flask, the filtrate is diluted to volume with the diluent and mixed well. Think about this answer as a model.

4.1.5.2.2 Base degradation

The sample pellets, placebo pellets, and active ingredient (API) sofalcone pellets must all weigh 100 mg. Bring the measured weights over to three volumetric flasks of 100 ml each. Tetrahydrofuran, in the amount of 5 ml, must be added. In a nutshell, ten minutes of sonication. Next, for 60 minutes, sonicate 95 ml of diluent on and off. Put in the right amount of sodium hydroxide solution. Keep the flask as close to room temperature as feasible to avoid a drop in test value of more than 20%. Once it has cooled, dilute it with hydrochloric acid until the two solutions are equal. Mix after diluting to a smaller amount. After filtering the solution using a 0.45 µm Pre-filter + PTFE filter, discard the first 3-4 ml of the filtrate. The filtrate solution is pipetted into a volumetric flask with a capacity of 100 ml. The next step is to add the diluent and stir until the volume reads 100 ml. This answer may be used as an example.

4.1.5.2.3 Oxidation degradation

The API of sofalcone, placebo pellets, and sample pellets should all be weighed to the closest 100 mg. Add the ingredients measured in grammes to three 100-milliliter volumetric flasks. Tetrahydrofuran (5 ml) must be added. Sonicate for about 10 minutes to thoroughly blend ingredients. Then, sonicate 95 ml of diluent intermittently for 60 minutes. Add a suitable

concentration of hydrogen peroxide solution. If you want to ensure that your test result does not decrease by more than 20%, place the flask at room temperature on the benchtop. Following dilution, mix the reduced volume. After passing the solution through a 0.45 µm Pre-filter + PTFE filter, remove the first 3 to 4 ml of the filtrate. Pipette 10.0 ml of the clear filtrate solution into a 100-ml volumetric flask. Next, dilute it with the solvent until the volume reaches 100 ml and mix.

4.1.5.2.4 Photo degradation: UV

Three Petri plates were prepared with 5 g of placebo pellets, 10 capsules of 100 mg sofalcone, and 2,000 mg of sofalcone API. Put the plates in the photo stability chamber, which is irradiated by about 200 watts per square metre, to monitor their degradation. Consider the following in this context: sofalcone (100 mg), Placebo (100 mg), and Sample (100 mg) Pellets. Add the ingredients measured in grammes to three 100-milliliter volumetric flasks. 5 ml of tetrahydrofuran must be added. Sonicate for about 10 minutes to thoroughly blend ingredients. Then, sonicate 95 ml of diluent intermittently for 60 minutes. Maintain the flask as much as possible in a water bath at the right temperature or on a work surface at room temperature to avoid a 20% decrease in test results. Following dilution, mix the reduced volume. After passing the solution through a 0.45 µm Pre-filter + PTFE filter, remove the first 3 to 4 ml of the filtrate. Pipette 10.0 ml of the clear filtrate solution into a 100-ml volumetric flask. Next, dilute it with the solvent until the volume reaches 100 ml and mix. You may use this response as an example.

4.1.5.2.5 Thermal degradation

In three separate Petri dishes, bake for three hours at 80°C a combination of 5 g of placebo pellets, 40 capsules containing 100 mg of sofalcone, and 2,000 mg of sofalcone API. On the scales, put 100 mg pellets of sofalcone, 100 mg pellets of placebo, and 100 mg pellets of test pellets (100 mg). Put the components into three 100 ml volumetric flasks in the correct quantities. Add 5 ml of tetrahydrofuran. The duration of sonication should be about 10 minutes. Next, sonicate 95 ml of diluent for sixty minutes while intermittently mixing. As much as feasible, flasks should be stored at room temperature on a workbench to prevent a 20% decrease in test value. When the desired quantity of diluent has been added, the mixture is mixed. After filtering the solution with a 0.45 µm Pre-filter + PTFE filter, discard the first 3–4 ml of the filtrate. 50 ml of the clear filtrate solution should be poured into a 50 ml volumetric flask. Consider this response as an example.

4.1.5.2.6 Procedure for treated sample injection

Set up the HPLC column by running a full gradient programme over it after letting the mobile phase settle for at least an hour. Each of the five µl of blank, standard, and sample solutions must be injected separately. Degradation experiments using acid, base, oxidation, hydrolysis, photo, humidity, and heat (single) should be performed on API Solution (Control), blank, placebo, API, and sample solutions, respectively, in a chromatograph. Write down how much of a shift there was in the peak region for sofalcone on the chromatogram. sofalcone represents the epitome of cleanliness.

4.1.5.2.7 Acceptance Criteria

i) The peak for sofalcone should be easily discernible without any confounding noise from blank, placebo, or degradation products.

ii) All of the deteriorated samples should meet the peak purity standards for sofalcone (Peak purity = Purity angle Purity threshold).

4.1.6 LC-MS Method Validation

4.1.6.1 System Suitability

System Suitability is assessed by injecting six replicate (n=6) and% relative standard deviation is measured which has to be less then 10%. The result of RSD injection shown in result and discussion section.

4.1.6.2 Specificity

The Specificity of the method was carried out by comparing the chromatograms of blank, standard mixture and test solution. There was not any interference in the standard mixture chromatogram of the other chromatograms.

4.1.6.3 Linearity

Analytical linearity refers to the ability of a method to provide results that are directly (or after a well-defined mathematical transformation) proportional to the concentration of an analyte in a sample. In addition, the approach has been used to evaluate a mixture's uniformity and consistency. Using five different concentrations ranging from 50% to 150% of the test concentration, do the linearity test. Dilute the prepared sofalcone standard solution (Solution C) to provide concentrations that are 50, 80, 100, 120, and 150 % of the concentration being tested in order to ensure linearity.

4.1.6.3.1 Preparation of solutions

Prepare blank (diluent) and standard solution as per given in Methodology (Test Procedure).

4.1.6.3.2 Sofalcone standard solution for linearity (Solution C):

After carefully weighing 100 mg of sofalcone, it is necessary to add 30 ml of diluent to a 100 ml volumetric flask. The standard is diluted with diluent to the required concentration, then thoroughly mixed using sonication once it has cooled to room temperature and been completely dissolved. Prepare the linearity standard solution by serially diluting the sofalcone standard solution (Solution C) according to Table 4.6. Do three injections per level.

TABLE 4.5 Preparation of Linearity standard solutions

Sr. No.	Level	% concentration of sofalcone w.r.t test concentration (µg/ml)	Added Solution C (in ml)	Dilution Volume (in ml)	Concentration of sofalcone (in ppm)
1	Level 1	50	2.5	50	50
2	Level 2	80	4	50	80
3	Level 3	100	5	50	100
4	Level 4	120	6	50	120
5	Level 5	150	7.5	50	150

4.1.6.3.3 Procedure

Allow at least an hoursfor the mobile phase and HPLC column to mix before conditioning the column with a single full gradient. Mix 5 µL of the linearity standard solution, the method standard solution, and the blank on a chromatograph. Note the shift in peak area for sofalcone on the chromatogram. The concentration and average reaction area should be plotted. Compute and record the regression line's R-squared value, slope, Y-intercept, and %Y-intercept.

4.1.6.3.4 Acceptance Criteria

The correlation coefficient (‘R’) value should not be less than 0.99 over the working range.

4.1.6.4 Accuracy

A technique is considered accurate if the test result it produces is near to the actual value. Often, the quantity of analyte recovered indicates the degree of accuracy. To all intents and purposes, accuracy is a measurement of the analytical method's precision. Examine the accuracy at 50%, 100%, and 150% of the concentration range of interest. By injecting a

known quantity of sofalcone into the placebo, analysing the sample, and determining how much sofalcone was recovered, the effectiveness of the procedure will be evaluated.

4.1.6.4.1 Preparation of solutions

Prepare blank (diluent) and standard solutions according to Methodology's instructions (Test Procedure). Produce placebo solution according Specification's instructions.

4.1.6.4.2 Accuracy sample preparation

After correctly weighing the pellets with the proper instrument, gently place them in a 500 ml volumetric flask. One pellet contains about 100 mg of sofalcone. Add 25 ml of tetrahydrofuran. The duration of sonication should be about 10 minutes. Afterwards, add 280 ml of Diluent and continue sonicating for an additional 60 minutes while periodically agitating the container. Use a magnet to remove the magnetic bar from the volumetric flask with caution. The magnetic bar in the flask's stopper must be cleaned. When the flask is at room temperature, the diluent may be added, it can be mixed, and it can be diluted. The solution must be filtered using a 0.45 µm Pre-filter. When PTFE is used as a pre-filter, the first 3–4 ml of filtrate must be discarded. Using a pipette, 5 ml of the clear filtrate solution should be transferred to a 50 ml volumetric flask. Then, include the diluent until the desired concentration is obtained. Consider this response as an example. Make three copies of the solutions and inject them into a single copy at each degree of research precision.

TABLE 4.6 Preparation of sample solutions for accuracy study

Sr. No.	Level	(%) Spiked	Conc. from formulation (µg/ml)	Standard Conc. Added	Concentration of sofalcone (in ppm)
1	Level 1	50%	50	50	50
2	Level 2	100 %	100	50	100
3	Level 3	150 %	150	50	150

4.1.6.4.3 Procedure

Before conditioning with a single full gradient procedure, you should let the mobile phase mix with the HPLC column for at least an hour. Put 5 µl of the placebo solution, the standard solution made according to the procedure, the accuracy sample solution, and the blank solution into a chromatograph separately. Take note of the area of the peak where sofalcone

shows up on the chromatogram. Find out the percentage of recovery, the average recovery, and the RSD at each level. Make a chart that shows how much money has been given compared to the total amount.

4.1.6.4.4 Acceptance Criteria

i) sofalcone's individual and average recoveries should fall in the range of 98.0% to 102.0%.

ii) sofalcone's mean overall recovery rate is anticipated to be between 98.0% and 102.0%.

iii) The maximum allowable RSD (% RSD) for sofalcone is 2.0%.

4.1.6.5 Precision:

Accuracy is the degree to which repeated measurements of the same homogenous sample under the same conditions provide reliable findings.

4.1.6.5.1 System precision:

You may check the precision of the system by injecting six replicates of the standard solution from the same UPLC vial in line with the test methodology.

4.1.6.5.1.1 Preparation of solutions:

Prepare blank (diluent) and standard solution as per given in Methodology (Test Procedure).

4.1.6.5.1.2 Procedure:

In order to condition the column with a single full gradient, the mobile phase and UPLC column must be well mixed for at least an hour. Inject 10 µL of both the standard solution and the blank solution into the chromatograph, as directed. Take careful note of the chromatogram and determine the percentage of change in the peak region of sofalcone.

4.1.6.5.1.3 Acceptance Criteria:

i) Tailing Factor: The peak area of sofalcone from the first injection of the standard solution should not have a tailing factor more than 2.0.

ii) Theoretical plates: sofalcone theoretical plates from the first standard solution injection should be more than 2000.

iii) RSD: sofalcone peak area from six duplicate injections of standard solution should not have a relative standard deviation more than 2.0%.

4.1.6.5.2 Method precision (Repeatability):

The reliability of the results was assessed by their ability to be replicated under the same or similar conditions within a reasonable time frame. Six separate sample solutions will be

prepared from the same batch of capsules to test the Method Precision of sofalcone 100 mg Capsules.

4.1.6.5.2.1 Preparation of solutions:

Prepare blank (diluent), standard solution and sample solution as per given in Methodology (Test Procedure).

4.1.6.5.2.2 Procedure:

Allow at least an hoursfor the mobile phase and UPLC column to mix before conditioning the column with a single full gradient. In accordance with the instructions, add 10 µL of sample, standard, and blank solutions to the chromatograph. Note the shift in peak area for sofalcone on the chromatogram. Determine the mean assay value, standard deviation (RSD) of the six measurements, and individual assay values.

4.1.6.5.2.3 Acceptance Criteria:

i) Individual and mean % assay values should fall within the specified range.

ii) The RSD of six measurements should not exceed 2.0%.

4.1.6.5.3 Intermediate precision:

Accuracy was within the laboratory variance even when a different analyst used the same sample set but on a different day with different UPLC equipment and a different column of the same brand. The technique calls for six separate sample solutions to be made from the same batch of samples on separate days by separate analyzers using separate HPLC equipment and columns of the same brand. Moderate care will be used throughout production of sofalcone 100 mg Capsules.

4.1.6.5.3.1 Preparation of solutions:

Prepare blank (diluent), standard solution and sample solution as per given in Methodology (Test Procedure).

4.1.6.5.3.2 Procedure:

In order to condition the column with a single full gradient, the mobile phase and UPLC column must be well mixed for at least an hour. 10 µl each of the blank solution, the standard solution, and the sample solution should be injected into the chromatograph. Write down how much of a shift there was in the peak region for sofalcone on the chromatogram. Find the average, standard deviation, and individual assay results for a set of six measurements. Find the numeric difference between the intermediate precision and method precision means from the test data.

4.1.6.5.3.3 Acceptance Criteria:

1. Each person's test value, as well as the average, must be within the specified range.
2. There should not be more than a 2.0% RSD between each set of six conclusions.
3. Mean percentage assay results from the method precision study and the intermediate precision study should not deviate by more than 2.0% in absolute terms.

4.1.6.6 Robustness:

Change each chromatographic parameter on purpose and observe its influence on the assay and the test to see whether the system is suitable. To test the effectiveness of 100 mg sofalcone Capsules, prepare a sample solution in accordance with the protocol. Using the chromatographic settings and test method variables listed below, analyse the sample solution. Check whether the system is suitable for each variable condition and calculate the assay result.

4.1.6.6.1 Preparation of solutions:

Prepare blank (diluent), standard solution and sample solution (in triplicate) as per given in Methodology (Test Procedure).

1. Change in Column oven temperature (+ 5°C) of 25°C

High column oven temperature (HCT): 30°C

Low column oven temperature (HCT): 20°C

2. Change in Wavelength (± 2 nm) of 350 nm

High Wavelength (HW): 352 nm

Low Wavelength (LW): 348 nm

3. Change in Flow Rate (0.05 ml/min) of 0.2 ml/min

High Flow Rate (HFR): 0.25 ml/min

Low Flow Rate (LFR): 0.35 ml/min

4.1.6.6.2 Procedure:

In order to condition the column with a single full gradient, the mobile phase and HPLC column must be well mixed for at least an hour. A chromatograph requires the injection of 2 µl of each a blank, a standard, and a sample solution. Write down how much of a shift there was in the peak region for sofalcone on the chromatogram. By comparing the percentage of test results received under each different scenario to the percentage of assay results obtained with process accuracy, the % RSD and overall % RSD may be calculated.

4.1.6.6.3 Acceptance criteria:

i) Tailing Factor: The peak area of sofalcone from the first injection of the standard solution should not have a tailing factor more than 2.0.

ii) Theoretical plates: sofalcone theoretical plates from the first standard solution injection should be more than 2000.

iii) RSD: sofalcone peak area from six duplicate injections of standard solution should not have a relative standard deviation more than 2.0%.

iv) RSD of % assay the relative standard deviation (RSD) of test findings from three sample solutions for each different condition should not exceed 2.0%.

v) Overall RSD of % the relative standard deviation (RSD) of all test findings, including those obtained under each different condition, should be less than 2.0%.

4.1.6.7 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were calculated using the following formulae, which were derived from the ICH recommendations.

$$LOD = 3.3 \times a/ S$$

$$LOQ = 10 \times a/ S$$

Where, a is standard deviation of y intercepts & S is the slope regression line of calibration curve.

4.1.6.8 Analysis of Marketed Formulation

It was chosen to get sofalcone (Sofalco) from a local pharmacy since it was a commercially available medicine. The pill was crushed and diluted to produce a 100 mg/ml solution of sofalcone, which was then analysed by chromatography. The optimal RT was also utilised to examine the chromatogram for undesirable peaks resulting from formulation excipients. This verified the method's specificity.

4.2 Result and discussion

4.2.1 Selection of wavelength

As the greatest absorbance of sofalcone is at 350 nm, this is the wavelength that was used for the estimate.

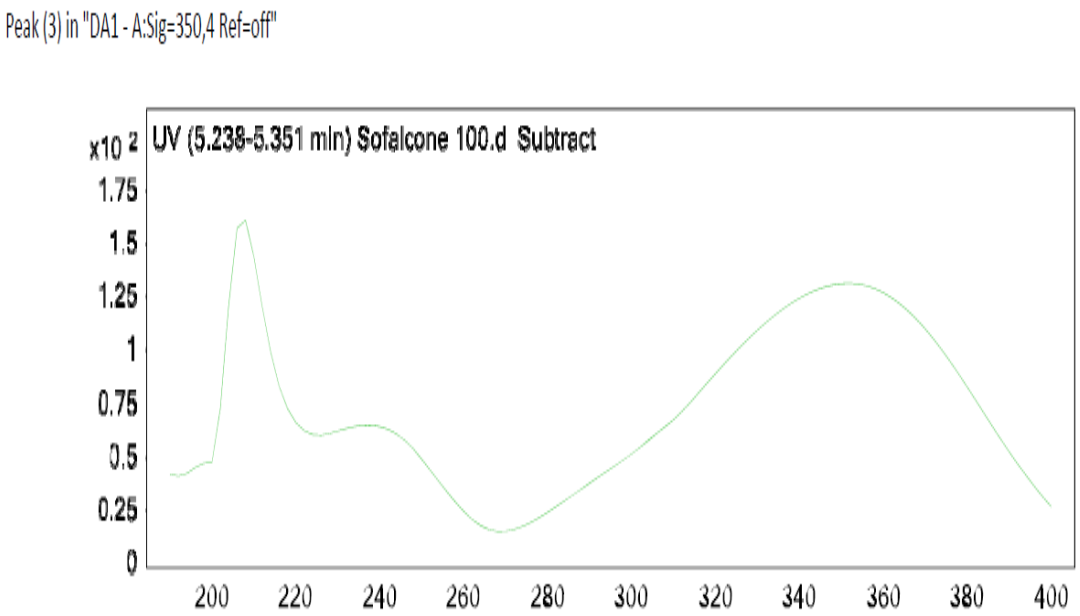


FIGURE 4.2 UV Spectrum of taken from LC-MS Chromatogram between 5.238-5.321 retention time.

The Optimum wavelength selected for the estimation was 350 nm where sofalcone give maximum absorbance.

4.2.2 LC-MS/MS Method development

Acceptable choice some of the things that can change chromatography and mass parameters are the type of drug, its molecular weight, its solubility, and its volatility. Here, a number of tests are done to find out which chromatographic and mass parameters are the best. From the chromatograms that were made, column efficiencies, capacity factors, and asymmetry factors were calculated. We used conditions with the best possible resolution, symmetry, and capacity factor to make this estimate.

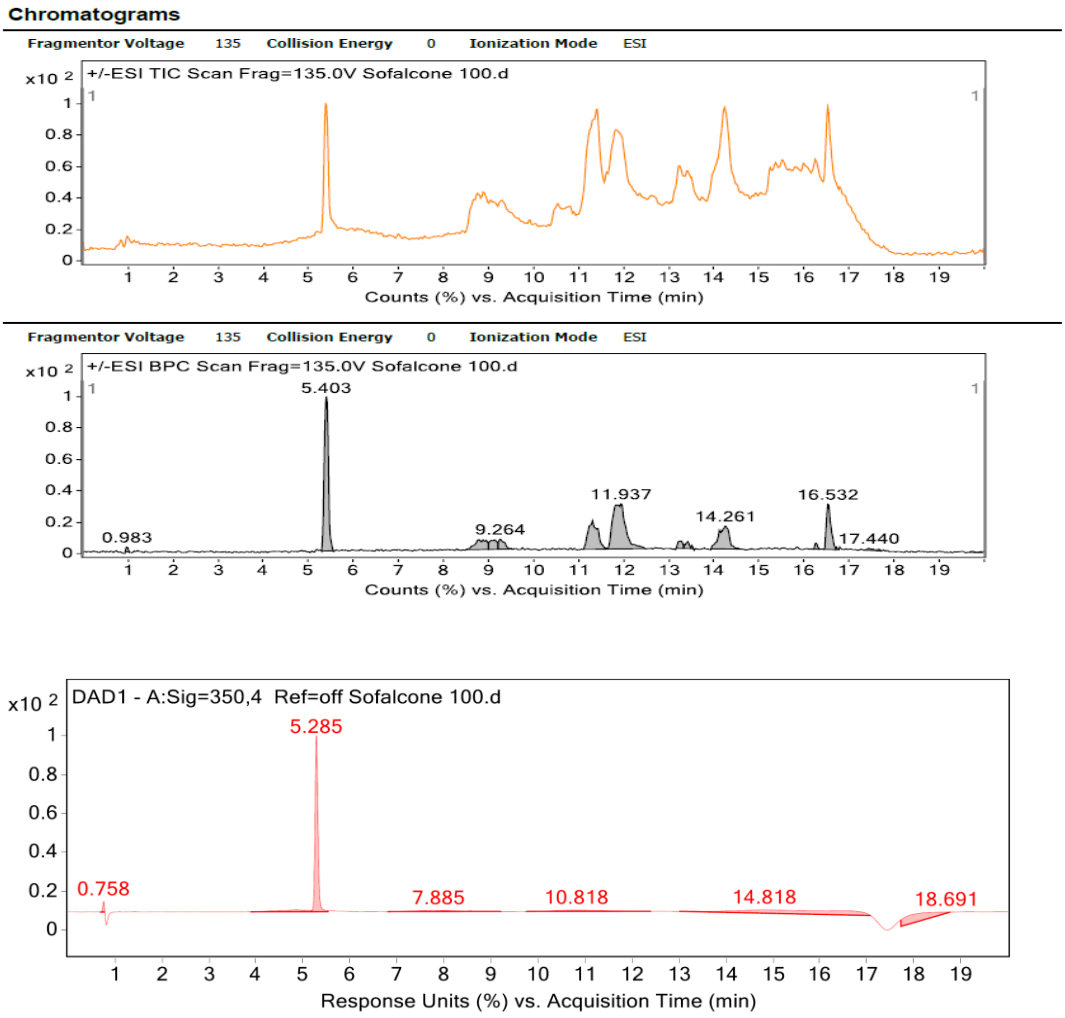
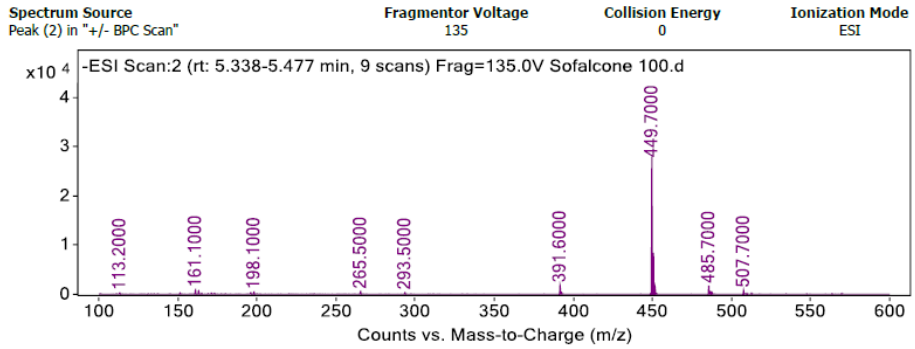
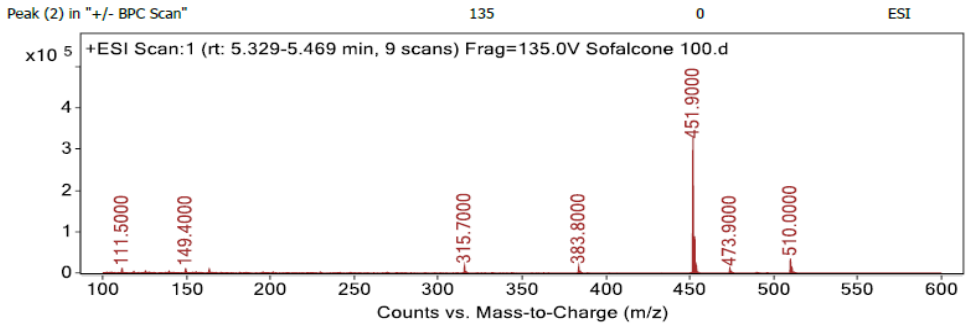


FIGURE 4.3 Optimized chromatogram of standard solution containg 100 ppb sofalcone using mobile phase Mobile Phase A: Water (0.1% Formic Acid) Mobile Phase B: Ammonium Acetate in methanol



Peak List

m/z	z	Abund
161.1		884.76
163.1		715.5
265.5		599.5
391.6	1	1862.54
449.7	1	27954.56
450.7	1	8451.38
451.7	1	1522.19
485.7	1	1775.66
486.7	1	552.03
507.7		871.57



Peak List

m/z	z	Abund
111.5		10819.74
149.4		9964.91
315.7		16178.25
383.8	1	14866.16
451.9	1	327459.94
452.9	1	88793.3
453.9	1	16699.17
473.9		10158.09
510	1	34691.14
511	1	10243.61

FIGURE 4.4 Mass Spectrum of sofalcone (Q1 with positive and negative ionization modes)

TABLE 4.7 Mass Identification of sofalcone

Standard	Fragmentor (m/z)
Sofalcone	1- 451.9 2- 449.7

4.2.3 Mass Balance study with Forced degradation:

In the forced degradation trial, 100 mg capsules of sofalcone, its active pharmaceutical ingredient (API), and a placebo were used. The samples were examined for their resistance to acid, base, oxidation, hydrolysis, photolysis, humidity, and heat. Preparing a blank properly for each degrading study.

- **API Solution (Control):**

After being weighed, 100 mg of sofalcone was poured into a 100 ml volumetric flask. Five ml of tetrahydrofuran were put in. In a nutshell, ten minutes of sonication. The sonicator was turned on after 95 ml of diluent were added, and it shook intermittently for 60 minutes. Before adding water and stirring, let the flask warm to room temperature. The solution was filtered using a PTFE + µm Pre-filter, and the first 3-4 ml of the filtrate were thrown away. In a volumetric flask with a 50 ml capacity, I transferred 5 ml of the clear filtrate solution, diluted it to volume with the diluent, and gave it a good stir. This solution was provided as an example of a suitable solution.

- **Preparation of forced degradation solutions for Placebo, API and Capsules:**

4.2.3.1 Acid degradation:

We distributed 100 mg of sofalcone API, 100 mg of placebo pellets, and 100 mg of sample pellets into three 100 ml volumetric flasks based on their relative weights. Tetrahydrofuran (5 ml) must be added. Sonicate for about 10 minutes to thoroughly blend ingredients. The mixture was sonicated for 60 minutes and shaken for 30 minutes after 95 ml of diluent was added. Added was a 1 ml (1N) solution of hydrochloric acid. Leave the flask at room temperature for 1, 2, or 3 hours. When the solution has cooled, neutralise it by adding a solution of 1 N sodium hydroxide. Diluted to the appropriate concentration with the use of a solvent. The components were separated using a 0.45 µm filter. After PTFE pre-filtration, the first three to four ml of filtrate should be discarded. A volume of 10.0 ml of the clear filtrate solution was pipetted into a 100 ml volumetric flask, and the contents were diluted with the diluent to equal the capacity of the flask. This answer was provided as an example.

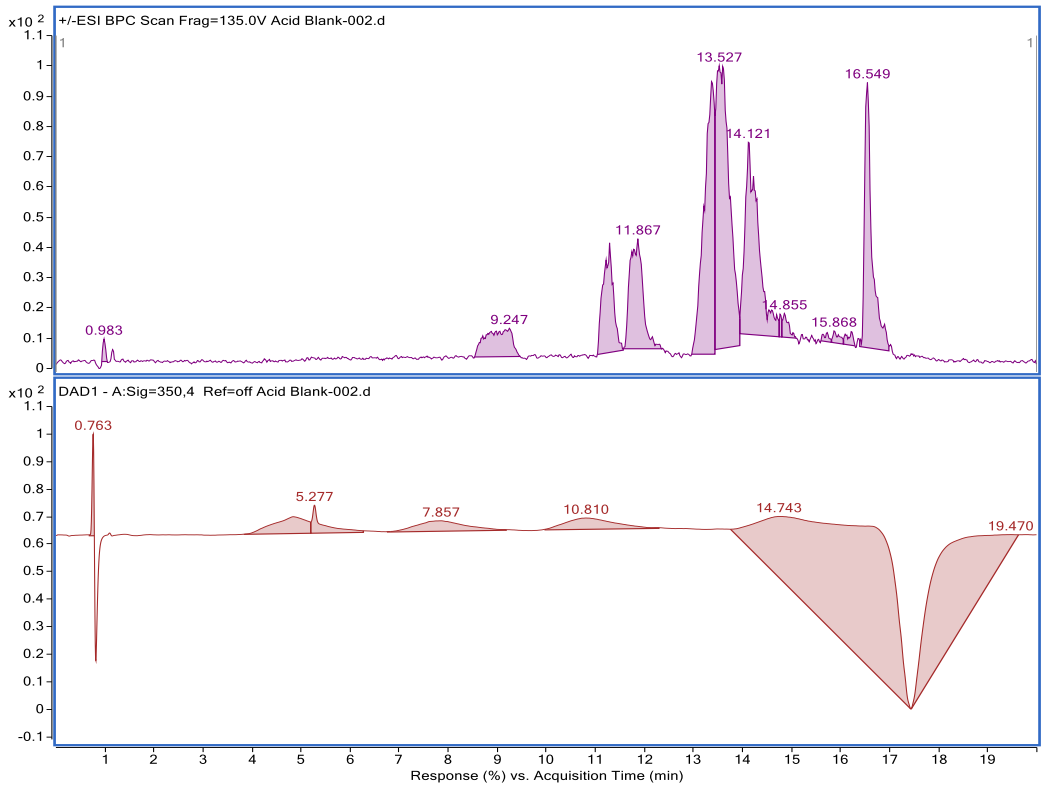


FIGURE 4.5 Chromatogram of blank used for Acid degradation

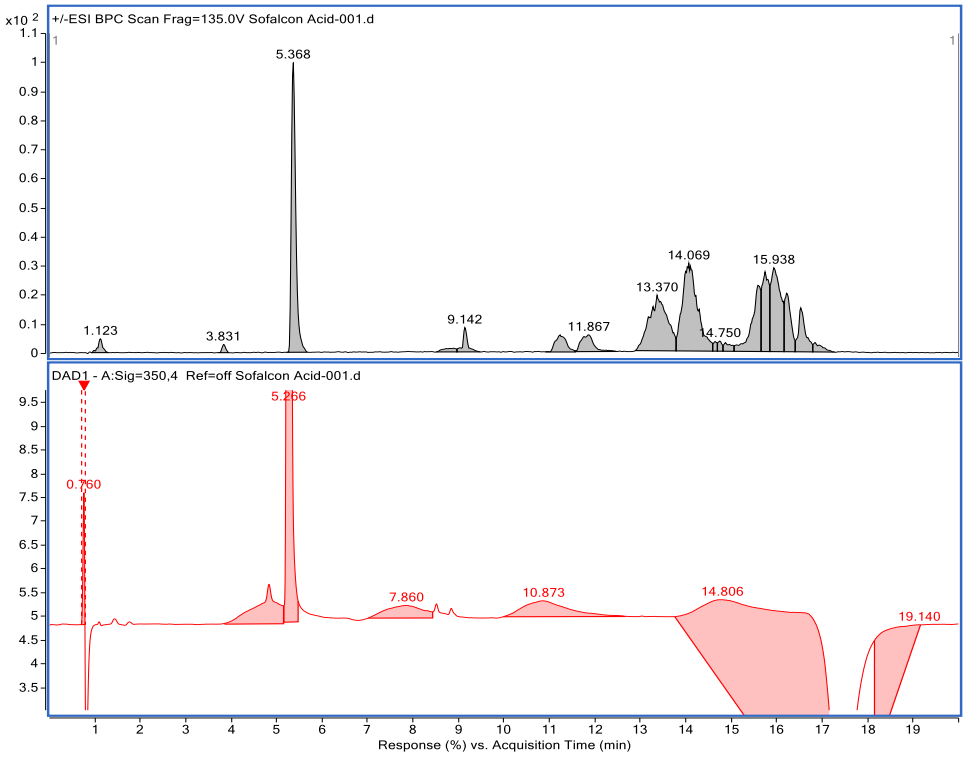


FIGURE 4.6 Chromatogram of sample under 1N 1ml HCl at 3Hr

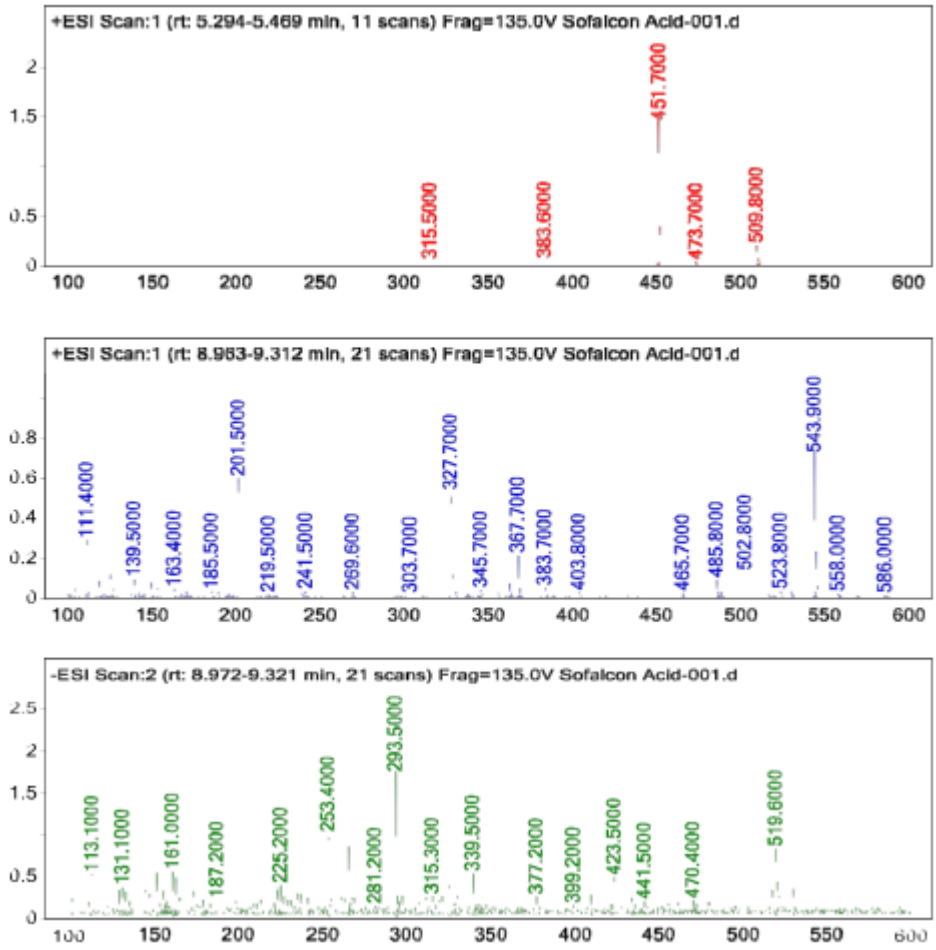


FIGURE 4.7 Mass Spectrum of sofalcone and Degradant after Acid degradation

Retention Time (min)	RRT*	Molecular Weight	% Area
5.368		450.70	85.00%
9.142	0.58 RRT	294.50	15.9%

*RRT-Relative retention time

% Total Impurities = % Impurity Observed × (MP ÷ MD)

Where: MP= Molecular weights of a parent drug

MD= Molecular weight of Degradant

= 15.9 × 450.70/294.50

=24.33 % Total Impurities.

4.2.3.2 Base degradation:

In three individual 100 ml volumetric flasks, we weighed out 100 mg of sofalcone API, 100 mg of placebo pellets, and 100 mg of sample pellets. Tetrahydrofuran, in the amount of 5 ml, must be added. In a nutshell, ten minutes of sonication. The mixture was sonicated and agitated for an hoursafter 95 ml of diluent was added. Include 1 millilitre of a 1 N sodium hydroxide solution. For three hours, the flask was kept at Room temperature. The material was cooled and neutralised using a solution of 1N hydrochloric acid. Mixed and diluted with a suitable diluent to the required strength. The solution was filtered using a PTFE + 0. 45 µm Pre-filter, and the first 3-4 ml of the filtrate were thrown away. A volume of 10.0 ml of the clear filtrate solution was pipetted into a 100 ml volumetric flask, and the contents were diluted with the diluent to equal the capacity of the flask. This solution was provided as an example of a suitable solution.

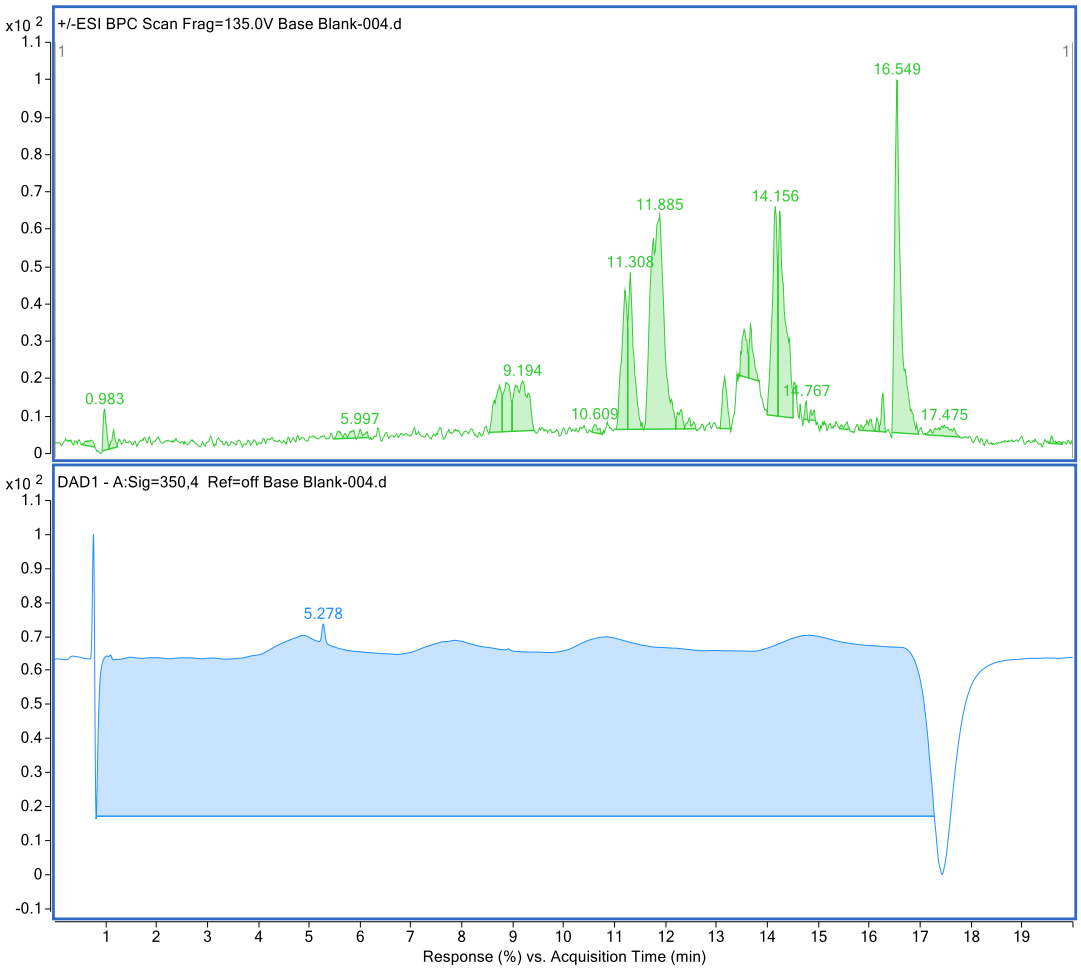


FIGURE 4.8 Chromatogram of blank used for Alkali degradation

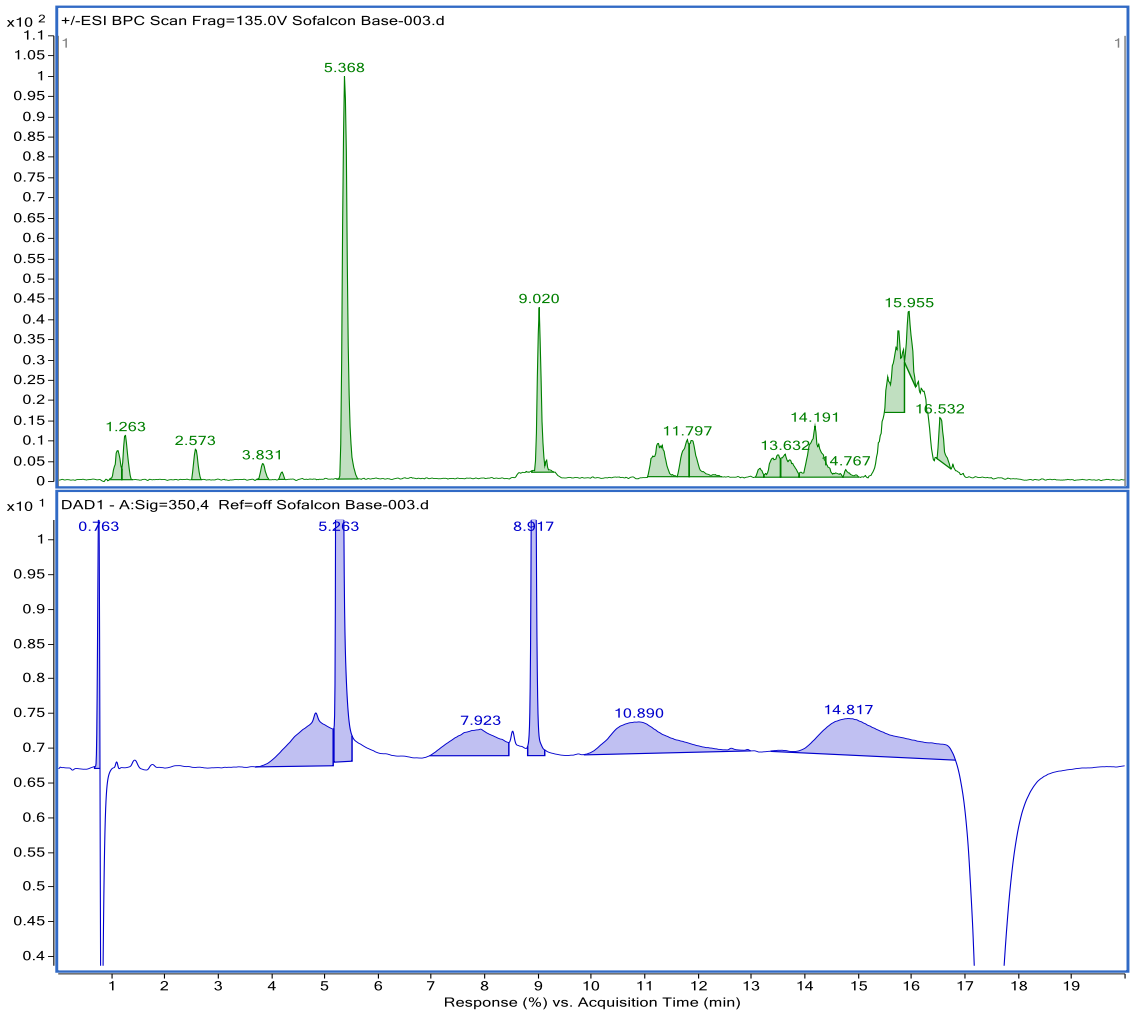


FIGURE 4.9 Chromatogram of sample under 1N 1ml NaOH

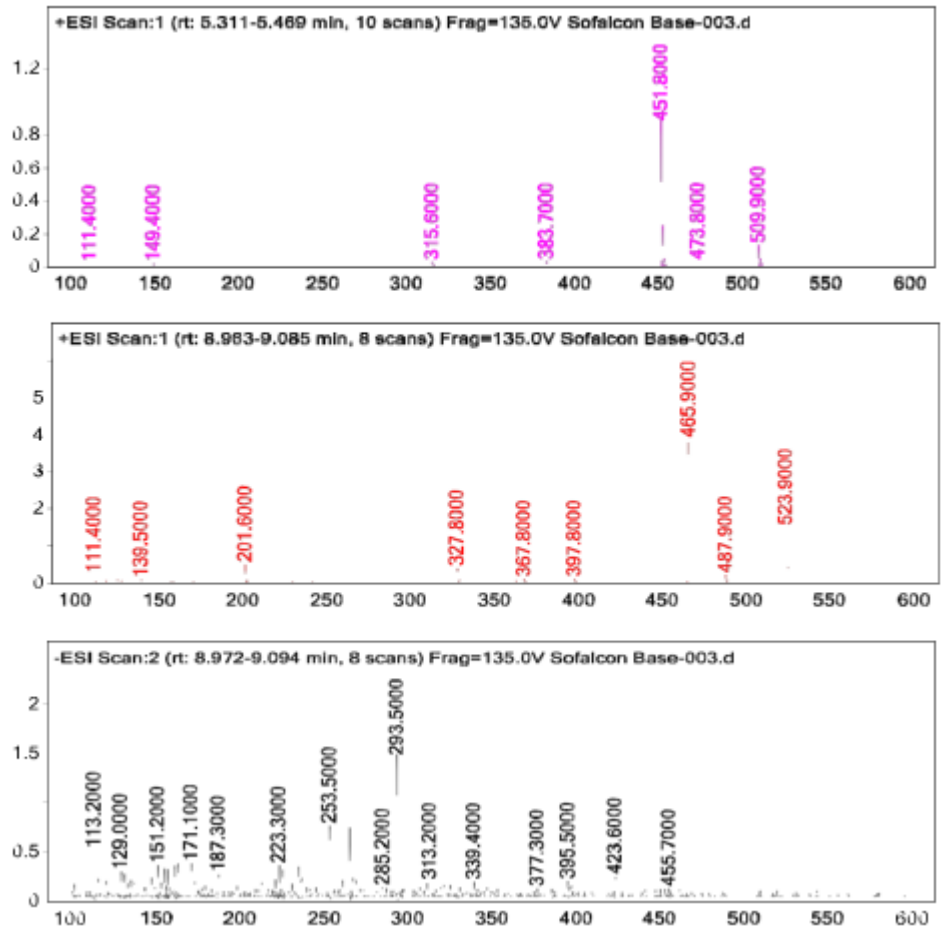


FIGURE 4.10 Mass Spectrum of sofalcone and Degradant after Base degradation

TABLE 4.8 Retention Time of sofalcone

Retention Time (min)	RRT*	Molecular Weight	% Area
5.228	-	450.80	95.71%
9.87	0.52 RRT	294.50	2.14%

*RRT-Relative retention time

% Total Impurities = % Impurity Observed × (MP ÷ MD)

Where:

MP= Molecular weights of a parent drug

MD= Molecular weight of Degradant

= 2.14 × 450.70/294.50

=3.27 % Total Impurities

4.2.3.3 Oxidation degradation:

There was 100 mg of API sofalcone, 100 mg of placebo pellets, and 100 mg of sample pellets in each of the three 100 ml volumetric flasks. Add 5 ml of tetrahydrofuran. Allow about 10 minutes to sonicate. Next, put 95 ml of the diluent in the container and sonicate it for 60 minutes while shaking it every so often. The flask was kept at room temperature for three hours. Then watered down to the right strength. PTFE + 0.45 µm. The solution was filtered with a pre-filter, and the first 3–4 ml of the filtrate were thrown away. Before the filtrate solution was pipetted into a volumetric flask, it was diluted with the diluent to 50 ml and mixed well. This response was used as a model.

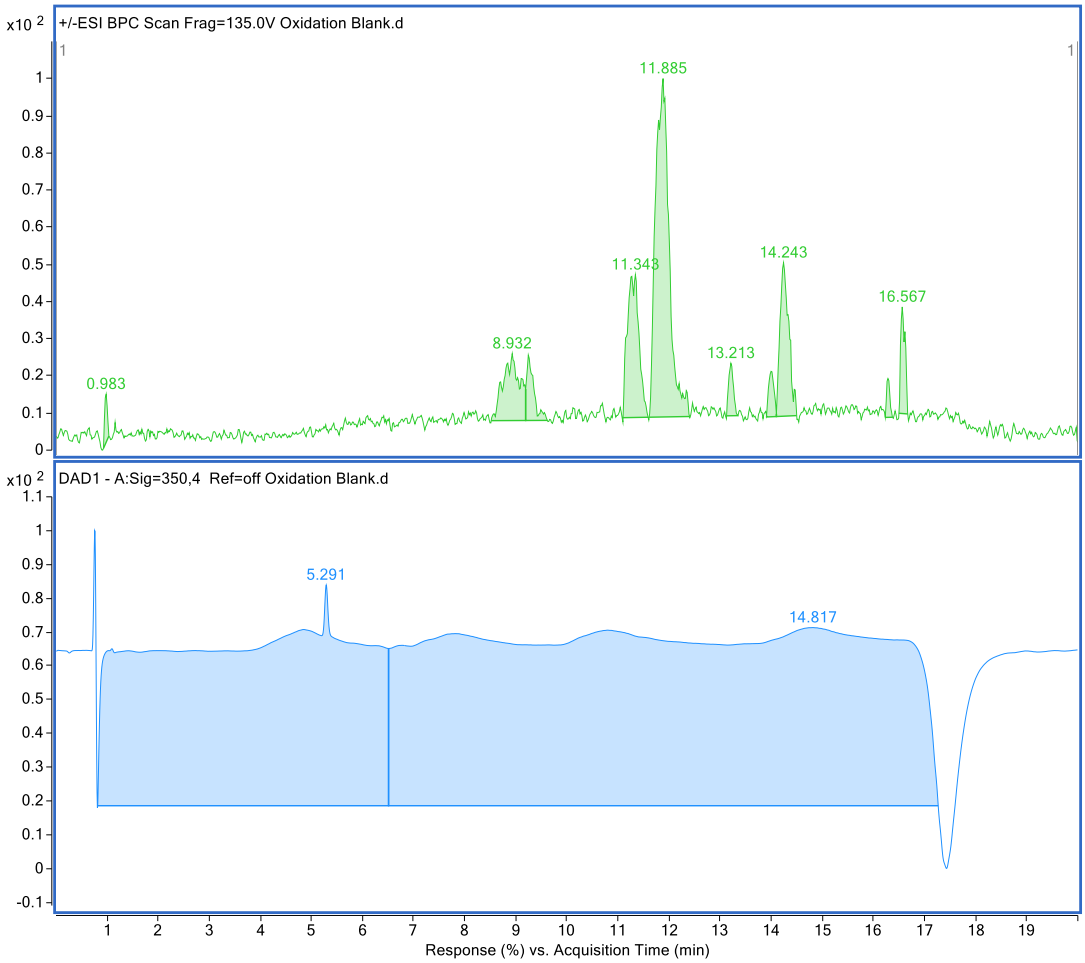


FIGURE 4.11 Chromatogram of blank used for Oxidative degradation

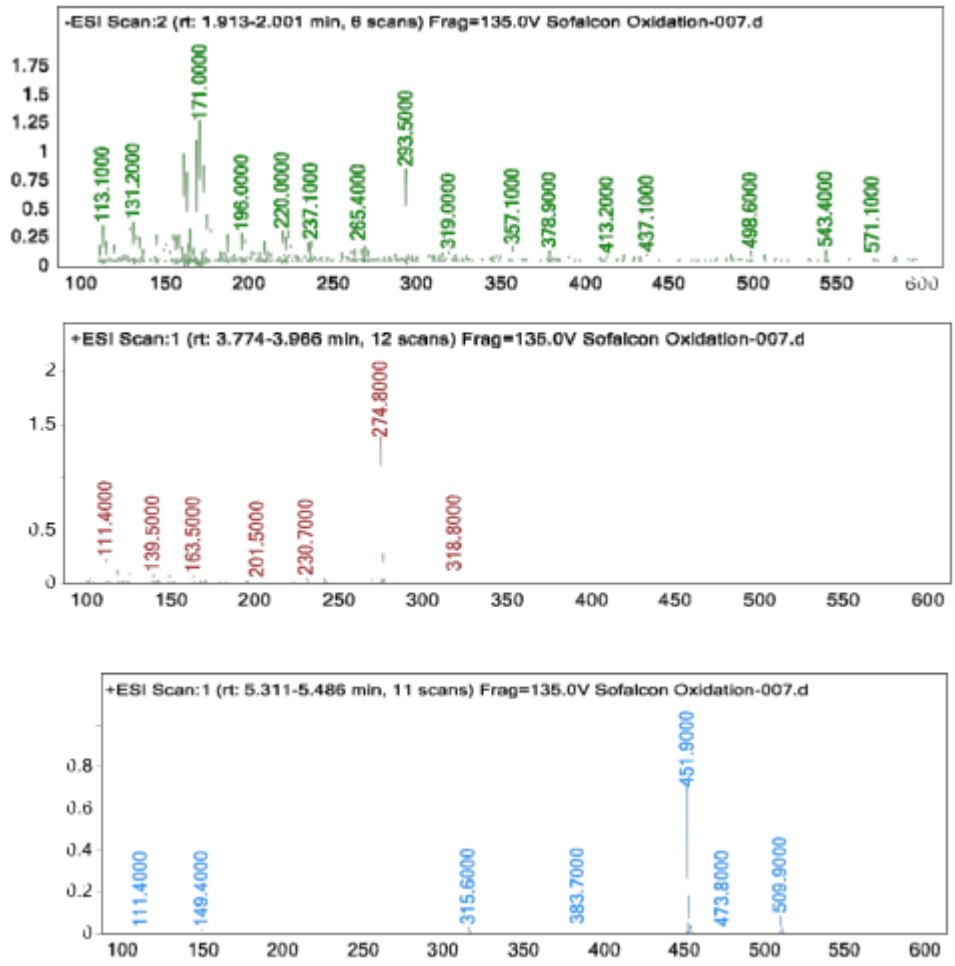


Figure 4.12 Mass Spectra of sofalcone and Degradant after oxidation degradation

Retention Time (min)	RRT	Molecular Weight	% Area
5.368	-	450.80	92.47%
1.123	4.780 RRT	294.50	1.05%
3.848	1.395 RRT	273.80	5.21%

% Total Impurities = % Impurity Observed × (MP ÷ MD)

Where: MP= Molecular weights of a parent drug

MD= Molecular weight of Degradant

= 5.21 × 450.80/273.80

=8.57 % Total Impurities

4.2.3.4 Photo degradation: UV

Three Petri dishes containing placebo pellets, 20 sofalcone 100 mg Capsules, and 2,000.21 mg sofalcone API were put in a light stability room with about 200 watt-hours per square metre of light intensity. Three 100 ml volumetric flasks each contained 100 mg of API sofalcone, 100 mg of placebo pellets, and 100 mg of sample pellets. Add 5 ml of tetrahydrofuran. Sonicate for about 10 minutes to thoroughly blend ingredients. Three hours were spent exposing the flask to ultraviolet light. Diluted to the appropriate concentration with the use of a solvent. PTFE + 0.45 µm the solution was filtered using a pre-filter, and the top 3–4 ml of the resultant filtrate were discarded. Pipetting 10.0 ml of the filtrate solution into a 100ml volumetric flask, the volume was then adjusted by adding the diluent. This reaction served as a model for my own.

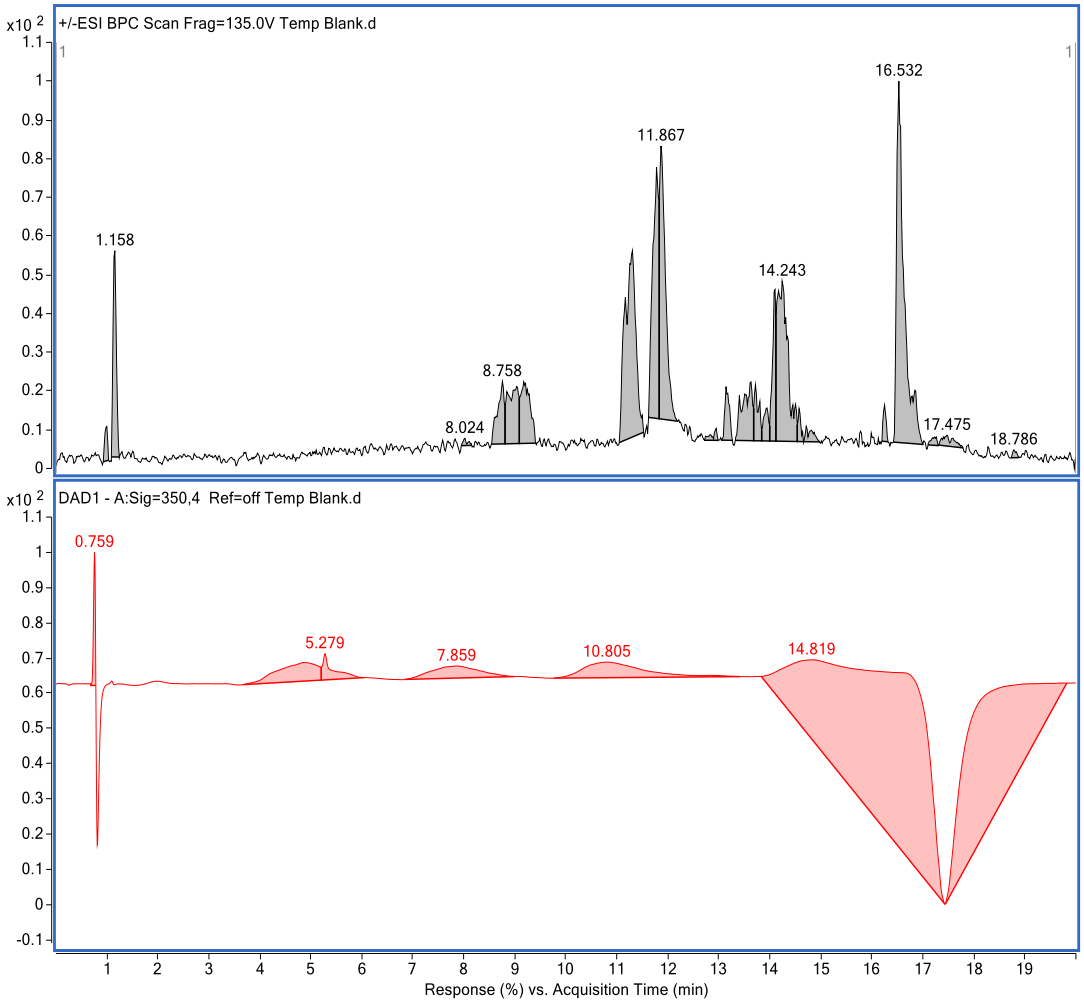


FIGURE 4.13 Chromatogram of Blank under UV light 3 Hours

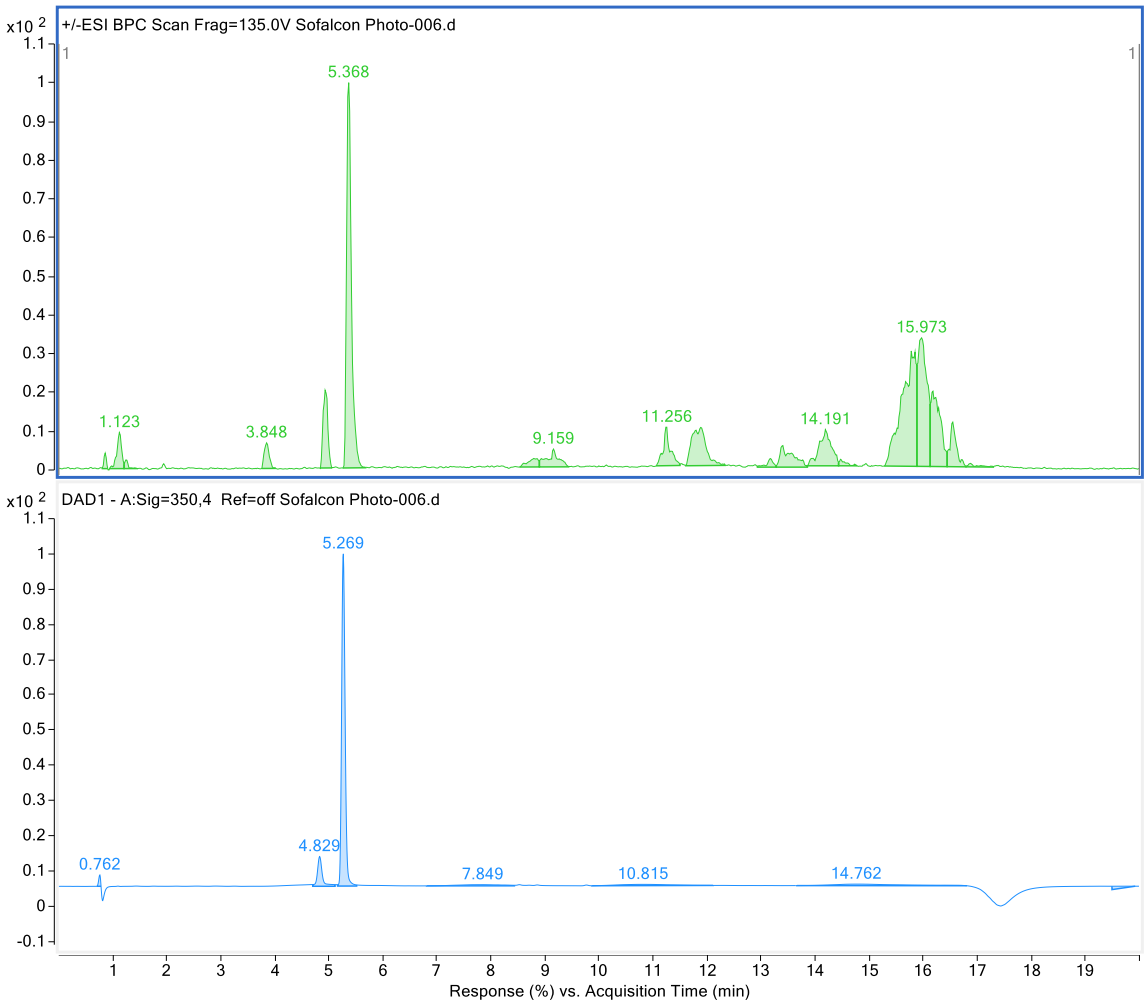


FIGURE 4.14 Chromatogram of sample under UV light 24 Hours

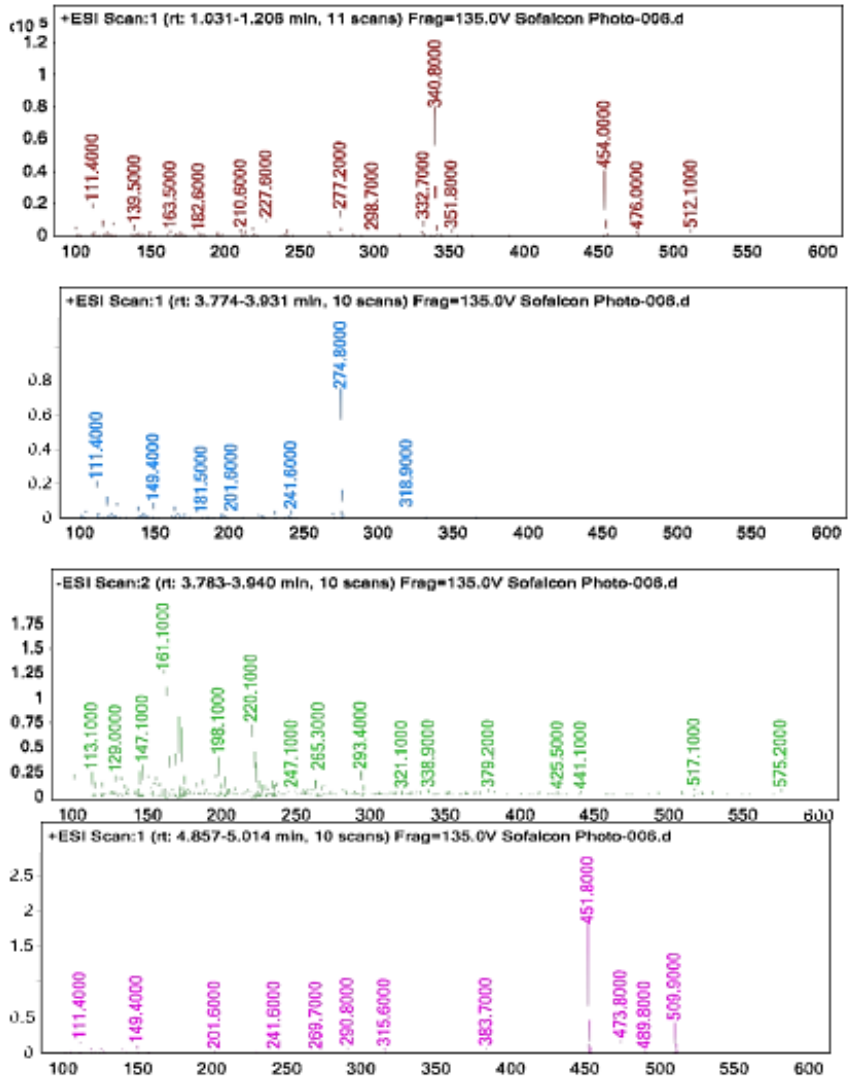


FIGURE 4.15 Mass Spectra of sofalcone and Degradant after photo degradation

Retention Time (min)	RRT	Molecular Weight	% Area
5.368	-	450.80	66.92%
3.848	1.395 RRT	273.80	19.73%

% Total Impurities = % Impurity Observed × (MP ÷ MD)

Where: MP= Molecular weights of a parent drug

MD= Molecular weight of Degradant

= 19.73 × 450.80/273.80

=32.48 % Total Impurities

4.2.3.5 Thermal degradation:

At 105 ºC for 24 hours, we watched the deterioration of placebo pellets, 40 sofalcone 100 mg capsules, and 2,000 mg of sofalcone API. The 100 mg of sofalcone API, 100 mg of placebo pellets, and 100 mg of sample pellets were each weighed and then transferred to their own 100 ml volumetric flasks. Tetrahydrofuran, in the amount of 5 ml, must be added. In a nutshell, ten minutes of sonication. For three hours, the flask was heated in an 80°C water bath. Mixed and diluted with a suitable diluent to the required strength. PTFE + 0.45 µm the first three to four ml of the filtrate after filtering the solution through a pre-filter were thrown away. In a volumetric flask with a 100 ml capacity, I transferred 10 ml of the clear filtrate solution, diluted it to volume with the diluent, and gave its good stir.

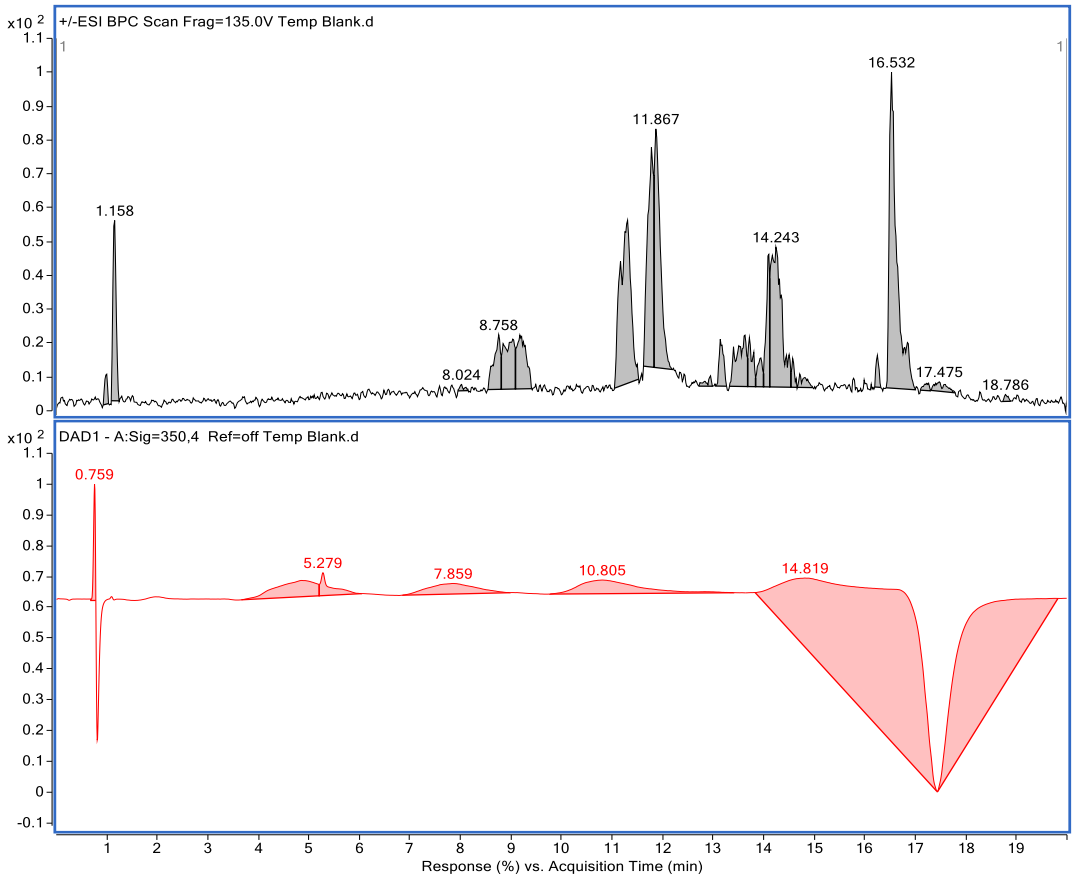


FIGURE 4.16 Chromatogram of blank for Thermal degradation

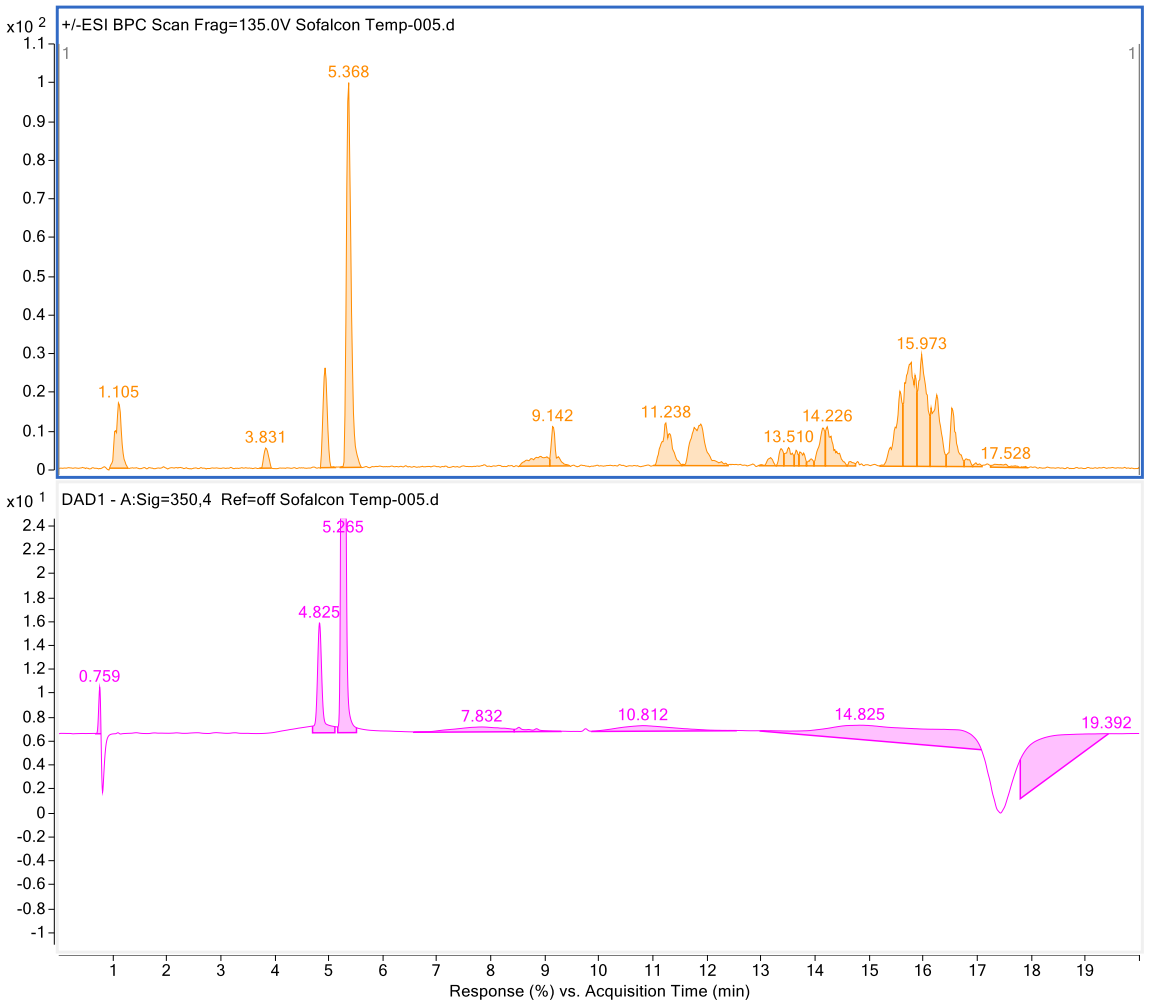


FIGURE 4.17 Chromatogram of sample at 80°C for 30 min

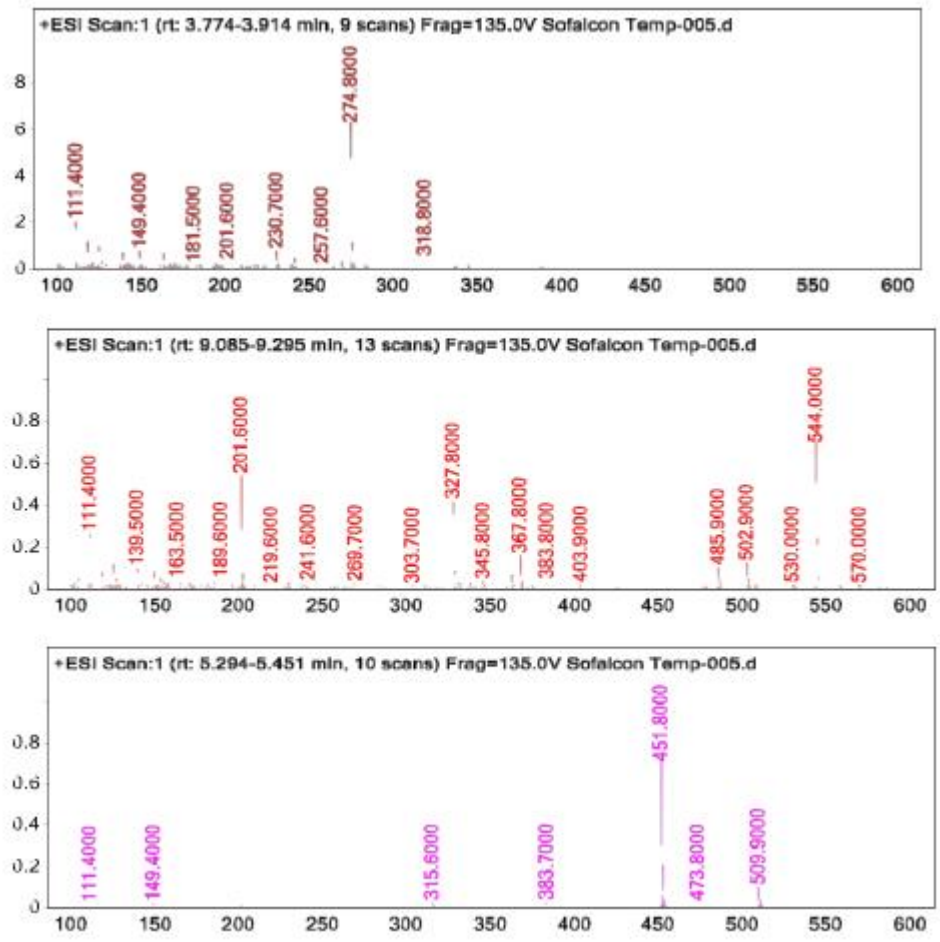


FIGURE 4.18 Mass Spectra of sofalcone and Degradant after thermal degradation

Retention Time (min)	RRT	Molecular Weight	% Area
5.368	-	450.80	97.01%
3.831	1.400 RRT	273.80	2.12%

% Total Impurities = % Impurity Observed × (MP ÷ MD)

Where: MP= Molecular weights of a parent drug

MD= Molecular weight of Degradant

= 2.12 × 450.80/273.80

=3.49 % Total Impurities

4.2.3.5.1 Acceptance Criteria:

i) The peak for sofalcone should be easily discernible without any confounding noise from blank, placebo, or degradation products.

ii) All of the deteriorated samples should meet the peak purity standards for sofalcone (Peak purity = Purity angle < Purity threshold).

4.2.3.5.2 Observation:

i) The sofalcone peak is clearly distinguishable from the blank, placebo, and degradation products.

ii) All of the deteriorated samples meet the criterion for sofalcone peak purity (Peak purity = Purity angle < Purity threshold).

4.2.3.5.3 Conclusion:

All the results are well within the acceptance criteria; hence method is specific.

- **Result of Mass Balance Study:**

In mass balance, the loss of a parent drug is proportionate to the increase in degradation products. One quality control check for analytical procedures is the capacity to verify that all degradation products can be adequately recognised and do not impact the amount of the parent drug (i.e., stability indicating methods). Regulatory authorities use mass balance to guarantee that all degradants have been examined and that an appropriate analytical technique has been used to determine stability.

TABLE 4.9 Analysis of Reconciling Mass Balance in Force Degradation studies

Conditions	Unspecified impurity at RRT 0.58	Unspecified impurity at RRT 4.7	Unspecified impurity at RRT 1.39	% Total impurities	Assay (%)	Mass Balance (% Total impurities + % Assay) (%)	Mass Balance wrt to as such sample
As such (Unstressed Sample)	ND	ND	ND	0	102.5	102.5	NA
Acid degradation (1N HCl, RT, 3 Hours)	8.9	ND	ND	24.33	77.67	102.0	99.5
Base degradation (1N NaOH, RT, 3 Hours)	2.14	ND	ND	3.27	95.71	98.98	96.6
Oxidation degradation (5ml 30 % H_2O_2, RT , 1 Hours)	ND	1.55	5.21	8.57	92.47	101.04	98.6
Thermal degradation (80°C, 3 hours)	ND	ND	2.12	3.49	97.01	100.5	98
Photo Degradation (UV light for 3hours)	ND	ND	19.73	32.48	66.92	99.4	97

4.2.4 Stability-indicating property

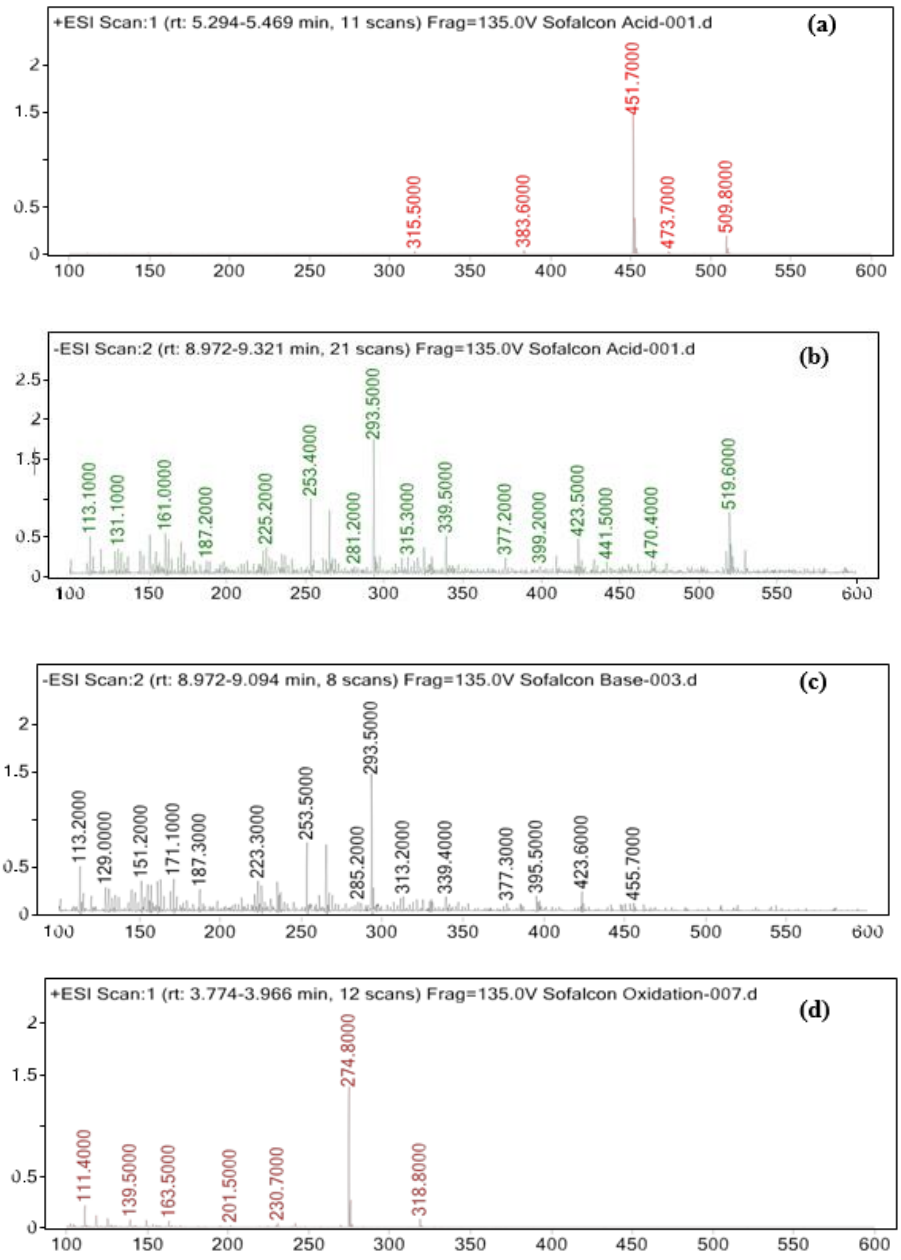
Samples of standard sofalcone were degraded in acid, alkali, oxidation, heat, and light to determine stability indicators. Many reagents, concentrations, and time periods were used during optimisation of the degradation experiments to breakdown sofalcone at room temperature in the most efficient way. sofalcone degrades by 24.33 % in 1 N HCl and 3.27 % after three hours in an acidic solution. Using this peak, we determined that the acidic degradation product of sofalcone produced at an RT value of 0.58. This appears to suggest that sofalcone degrades more quickly in acidic environments. After three hours in 1 N NaOH, alkalinity decreases by 3.27 %. An alkaline degradation product was formed with an RRT of 0.52. Degradation of sofalcone by 3 % hydrogen peroxide is 8.57 %. After 3 hours, it decomposes into 2.78 and 1.39 RRT-valued byproducts. It was simple to recognise and differentiate the degradation products from the typical peak. After 24 hours, one of the breakdown products of 3% H_2O_2 has an RRT value of 1.39 and has degraded 32.48 % of sofalcone by photolysis. sofalcone was stable after being heated to 80 ^oC for 30 minutes.

4.2.5 Analysis of degradation products

Both the standard and processed test solutions were injected directly into the MS in full scan mode (Q1 with positive and negative ionisation modes) with a mobile phase consisting of water (0.1% FA)-Ammonium acetate in methanol (30:70, *v/v*) in order to identify the degradation products by LC MS/MS. Each impact took 25 eV of energy, and the whole experiment lasted twenty minutes. The mass spectra of the main peaks were shown using the positive ionisation mode of ESI, and the mass to charge ratio (m/z) was used to determine the degradation products. The m/z values of the major components of conventional sofalcone mass spectra are 293.50, 201, 327, 543, 274.8, and 465. sofalcone has a maximum molecular ion mass of 451.7 m/z. The m/z value of the breakdown products was always 293.50, regardless of whether the sample was acidic, alkaline, or oxidising. Values of 293.50 and 274.8 m/z were found in the mass spectra of a second product that had been broken down by oxidation and light. m/z 293.50 (impurity 1) and m/z 274.8 (impurity 2) likely correspond to the molecular formulas $[C_{16}H_{20}O_5]^+$ and $[C_{15}H_{14}O_5]^+$, respectively, if Benzoic acid [M-$C_7H_6O_2$] is removed from the (acryloyl) phenoxy moiety.

Due to the constant and tiny discrepancy between the two sets of numbers, it is probable that the mobile phase used for LC MS/MS promotes sofalcone protonation in the positive mode. The results showed that under acidic, alkaline, and oxidative conditions, m/z 293.50 was the

primary degradation product while m/z 274.8 was the secondary degradation product. Results from this study showed that acid hydrolysis, photolysis, and oxidative degradation all yielded the same results regardless of temperature or pH. Figure 4.20 depicts the predicted chemical compositions and structures of all degradation products. The first UHPLC-MS/MS method for detecting and measuring sofalcone and its metabolites has been developed.



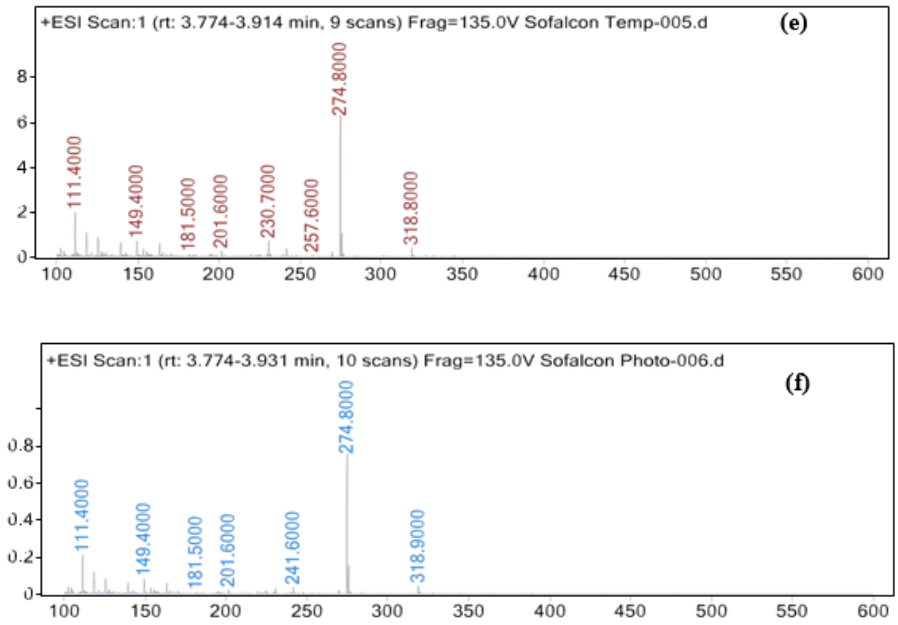


FIGURE 4.19 Mass spectra of (a) standard sofalcone and degradation product of (b) acidic (1 N HCl), (c) basic (1 N NaOH), (d) oxidative (30% H_2O_2), (e) thermal (80°C), and (f) Photo (UV Light)

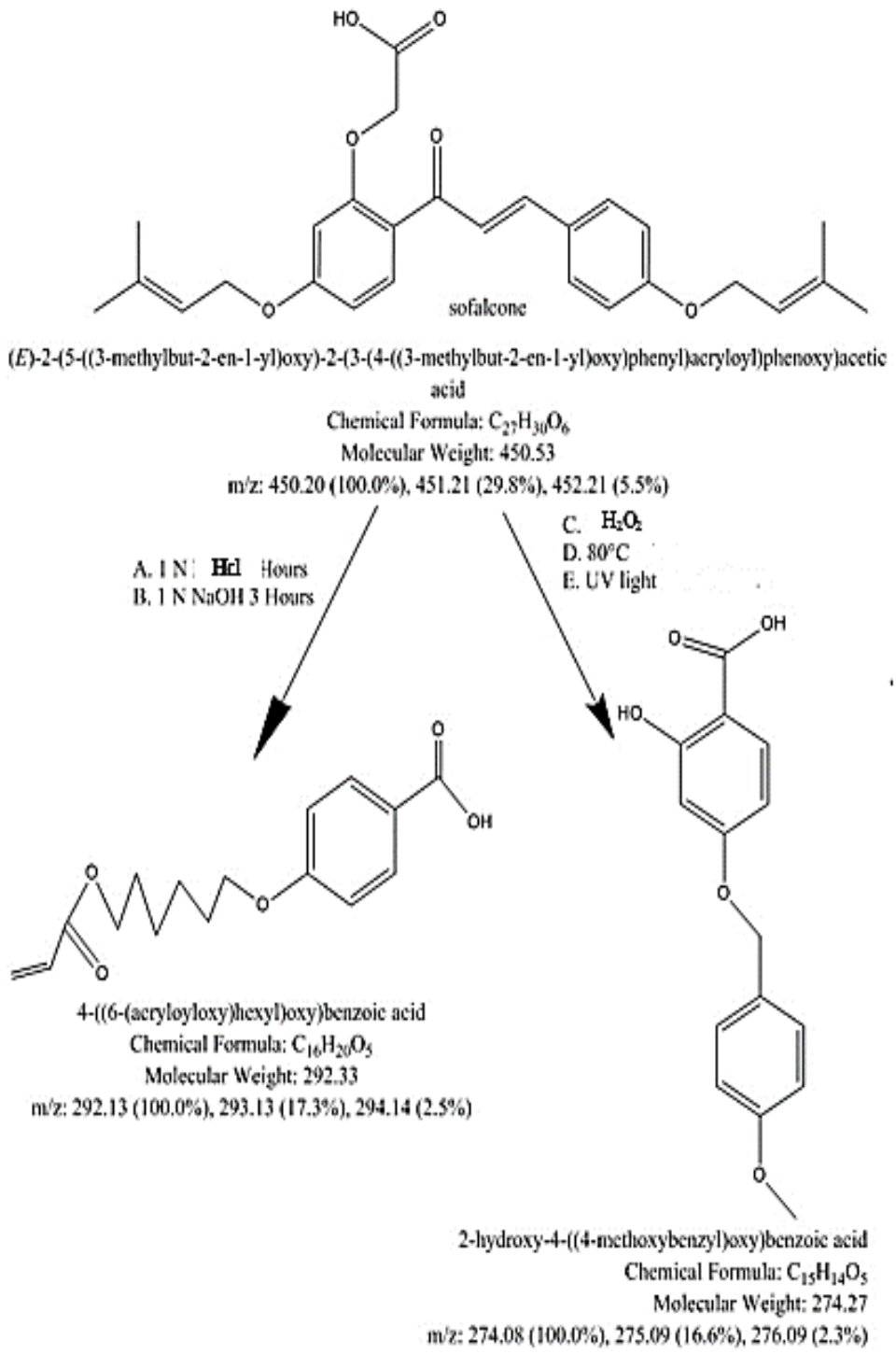


FIGURE 4.20 The proposed degradation pathway of sofalcone in different stress conditions

4.2.6 Method Validation

4.2.6.1 Specificity:

Specificity is the ability of an assay to reliably measure the analyte even when other factors are present. Degradants, matrices, and contaminants are common examples.

4.2.6.1.1 Check for blank, placebo and impurities interference:

The chromatograph was performed with blank, standard, placebo, sample, placebo mixed with known impurities and sofalcone, sample mixed with known impurities and sofalcone, and identification solutions. Assess the discrimination of the approach to determine whether the sofalcone peak is influenced by the use of a placebo. The obtained data are shown in Table 4.8.

TABLE 4.10 Retention time and purity data for sofalcone

Sample Name	Retention Time (Min)	Purity Angle	Purity Threshold	Peak Purity
Blank				
sofalcone	ND	NA	NA	NA
Standard solution				
sofalcone	5.269	0.105	0.282	Pass
Placebo solution				
sofalcone	ND	NA	NA	NA
Sofalcone Capsules 100 mg				
Sample solution_1				
sofalcone	5.285	0.345	0.728	Pass
Placebo spiked solution with sofalcone				
Sofalcone	5.241	0.716	0.812	Pass

ND: Not Detected NA: Not Applicable

4.2.6.1.2 Acceptance Criteria:

i) Blank, placebo, and contaminants should not interfere with the retention time of the sofalcone peak.

ii) Peak purity criterion (Peak purity = purity angle < purity threshold) must be met for the peak of sofalcone in the standard solution, sample solution, placebo spiked solution with known impurities, and sofalcone and sample spiked solution with known impurities.

4.2.6.1.3 Observation:

i) There is no interference seen at the peak retention time of sofalcone owing to blank, placebo, or contaminants.

ii) Peak purity requirements (Peak purity = purity angle < purity threshold) are met for the peak of sofalcone in the standard solution, the sample solution, and the placebo solution injected with sofalcone.

4.2.6.1.4 Conclusion:

All findings are well within the threshold for approval; hence, the procedure is specific.

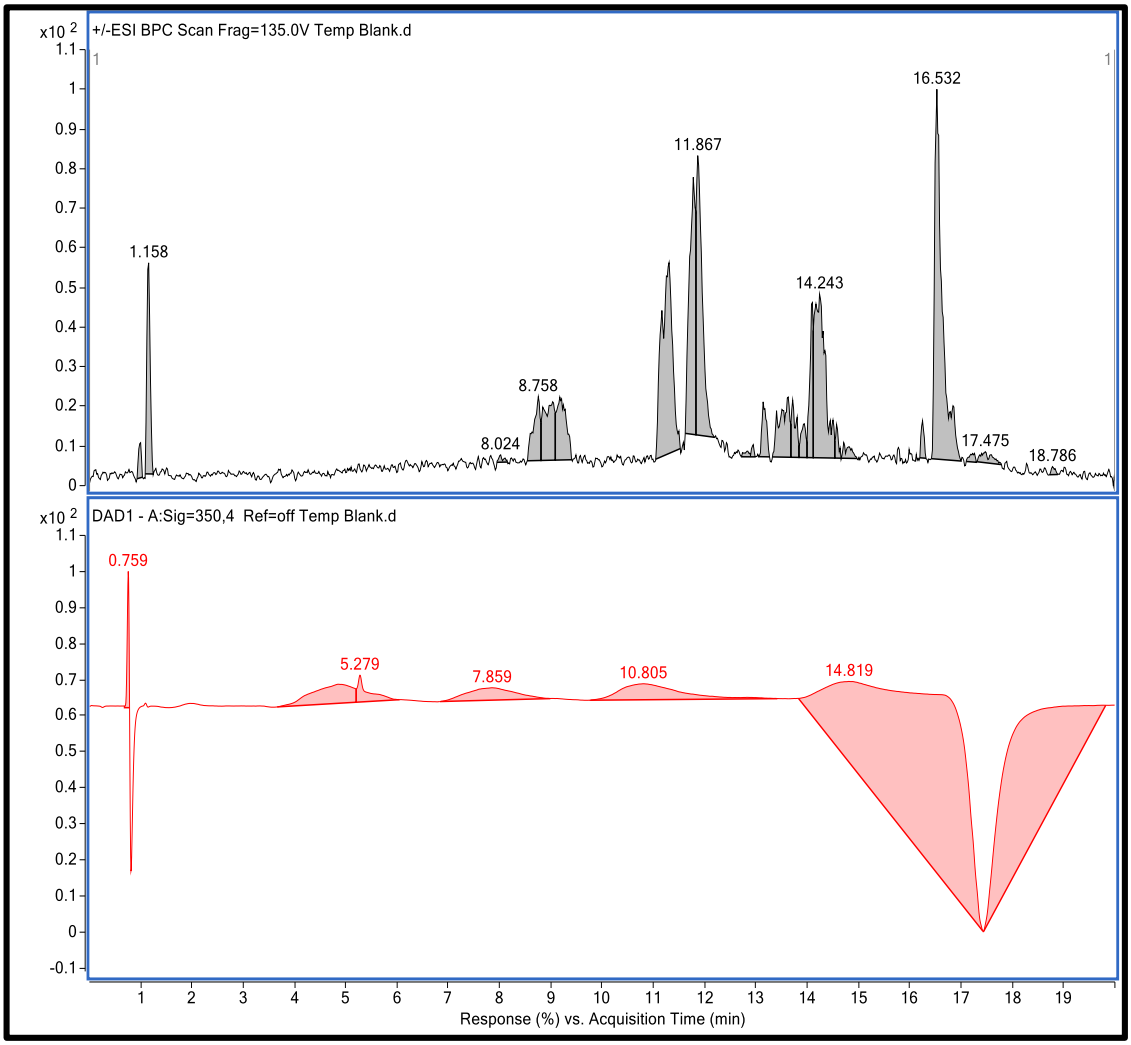


FIGURE 4.21 Chromatograms of Blank

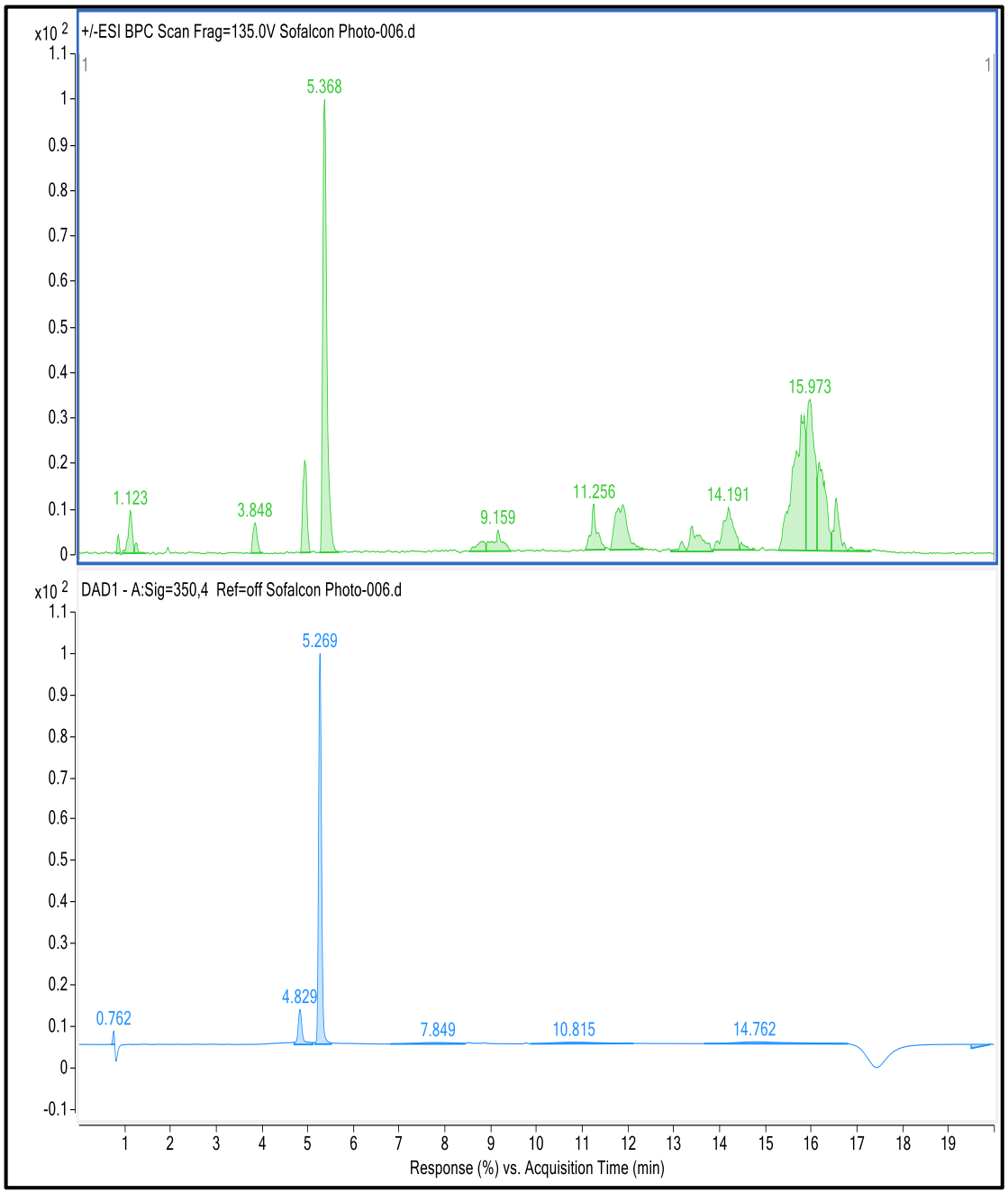


FIGURE 4.22 Chromatograms of Standard

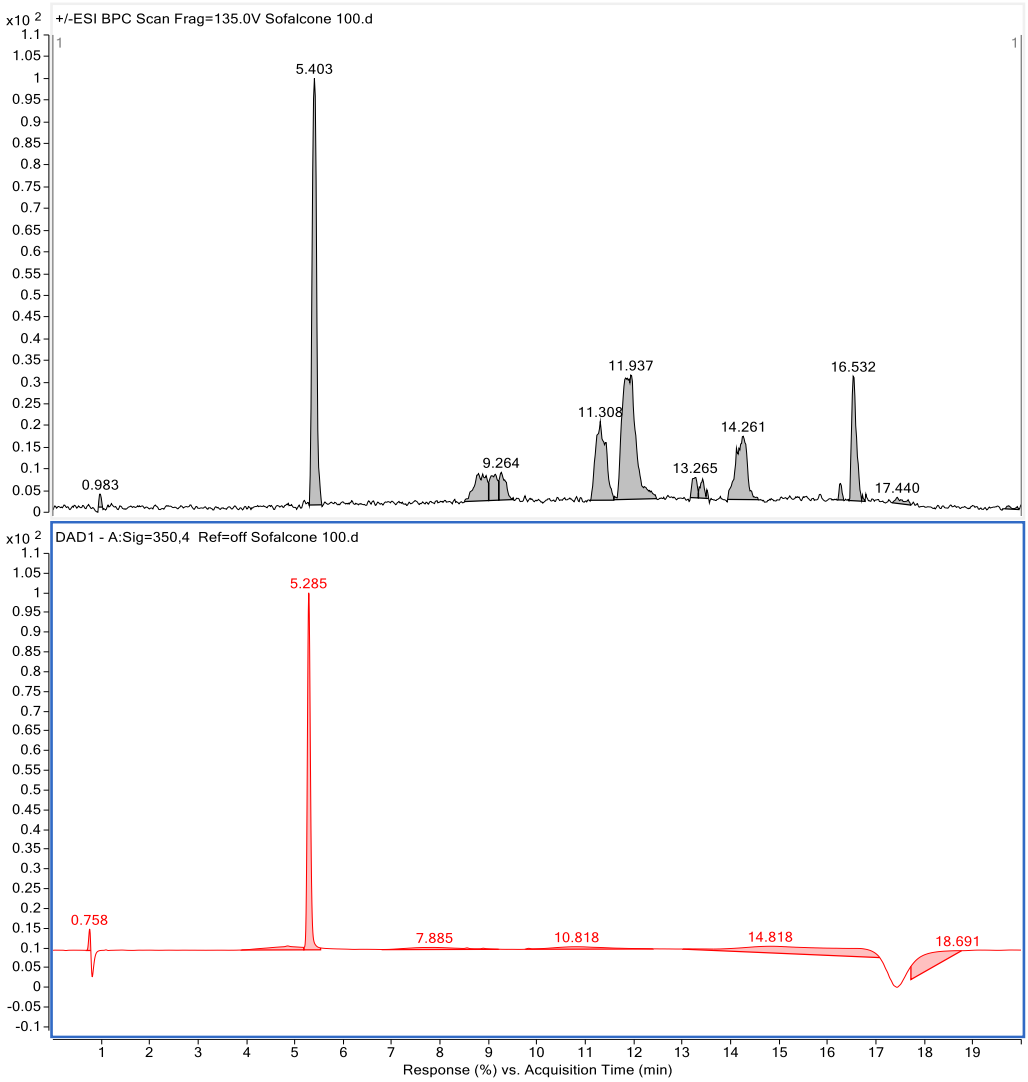


FIGURE 4.23 Chromatograms of Samples

4.2.7 Validation Summary

Sr. No.	Validation Parameter	Results	Acceptance Criteria
4.1.1	**Specificity**		
4.1.1.2	**Check for blank, placebo and impurities interference**		
	Interference	During the highest retention time of sofalcone, no interference was seen from blank, placebo, or contaminants.	Blank, placebo, and impurity peaks should not interfere with the retention period of the sofalcone peak.

	Peak purity	The standard solution, the sample solution, the placebo spiked with a known impurity, and the sofalcone and sample spiked solution all meet the peak purity criterion (Peak purity = purity angle purity threshold).	The peak of sofalcone in the standard solution, the sample solution, the placebo spiked solution with known impurities, and the sofalcone and sample spiked solution with known impurities must meet the peak purity requirements (Peak purity = purity angle purity threshold).
4.1.2	**Forced degradation: Check for blank, placebo and degradation products interference**		
	Interference	The peak of sofalcone may be clearly seen without any observable interference from blank, placebo, or degradation products.	The peak for sofalcone should be easily discernible without any confounding noise from blank, placebo, or degradation products.
	Peak purity	All of the deteriorated samples satisfy the peak purity criterion for sofalcone peak (Peak purity = Purity angle Purity threshold).	All of the deteriorated samples should meet the peak purity standards for sofalcone (Peak purity = Purity angle Purity threshold).

<table>
<tr><td>4.1.3</td><td>Linearity</td><td colspan="4">R^2 = 0.9954, which is the correlation coefficient.</td><td>Across the operational range, the value of the correlation coefficient ('R') must be greater than 0.99.</td></tr>
<tr><td rowspan="6">4.1.4</td><td rowspan="6">Accuracy</td><td>Level</td><td>% Conc.</td><td colspan="2">Mean % Recovery</td><td>% RSD</td><td rowspan="6">Sofalcone's individual and average recoveries should fall in the range of 98.0% to 102.0%.
The expected range of mean recovery for sofalcone is 98.0–102.0%.
The maximum allowable RSD (% RSD) for sofalcone is 2.0%.</td></tr>
<tr><td>1</td><td>50</td><td colspan="2">100.34</td><td>0.0527</td></tr>
<tr><td>2</td><td>100</td><td colspan="2">100.04</td><td>0.0153</td></tr>
<tr><td>3</td><td>150</td><td colspan="2">100.42</td><td>0.0903</td></tr>
<tr><td colspan="2">Overall % Recovery</td><td colspan="3">100.34</td></tr>
<tr><td colspan="2">Overall % RSD</td><td colspan="3">0.23</td></tr>
<tr><td>4.1.5</td><td colspan="6">Precision</td></tr>
<tr><td>4.1.5.1</td><td>System precision</td><td colspan="4">Tailing factor = 0.9
Theoretical plates = 3190</td><td>Tailing factor: The peak area of sofalcone from the first injection of the standard solution should not have a tailing factor more than 2.0.
Theoretical Plates: sofalcone theoretical plates from the first standard solution injection should be more than 2000.</td></tr>
</table>

		% RSD = 0.76	**RSD:** sofalcone peak area from six duplicate injections of standard solution should not have a relative standard deviation more than 2.0%.
4.1.5.2	**Method precision**	**% Assay of sofalcone 100 mg Capsules:** Sample 1 = 99.95 Sample 2 = 101.18 Sample 3 = 99.24 Sample 4 = 100.44 Sample 5 = 99.09 Sample 6 = 99.55 Mean % assay = 99.91 RSD of six determinations = 0.79%	The average and median assay percentages must be within the specified range. There should not be more than a 2.0% RSD between each set of six conclusions.
4.1.5.3	**Intermediate precision**	**% Assay of sofalcone 100 mg capsules:** Sample 1 = 100.92 Sample 2 = 100.28 Sample 3 = 100.67 Sample 4 = 100.00 Sample 5 = 101.20 Sample 6 = 100.53 Mean % assay = 100.51 RSD of six determinations = 0.80% Absolute difference = 0.6	The average and median assay percentages must be within the specified range. There should not be more than a 2.0% RSD between each set of six conclusions. The mean % assay results from the method precision study and the intermediate precision

			study should not deviate by more than 2.0 in absolute terms.
4.1.8	**Solution Stability**	**Standard Solution:** Up to 78 hours at room temperature, the relative standard deviation of sofalcone peak area was 0.7%. **Sample Solution:** Absolute difference in the % assay value of sofalcone obtained in sample solution at initial and at 50 hours at room temperature = 1.6	When comparing standard solutions acquired at different intervals of time, the relative standard deviation of sofalcone peak area should not exceed 2.0%. There should not be more than a 2.0% absolute difference between the assay values of the sample solution taken at the beginning and the end of each time period.

4.2.8 Analysis of Marketed Formulation

Under the optimal chromatographic conditions, a solution of a commercially available formulation (100 µg/ml) was injected, and a recovery of 100.91% was achieved.

TABLE 4.11 Analysis of Marketed Formulation

Formulation	Labelled amount (mg)	Amount found (mg)	%Label claim ± SD Assay (n=3)	%RSD
Sofalcone	100	100.25	100.91 ± 0.763	0.756
		100.75		
		101.75		

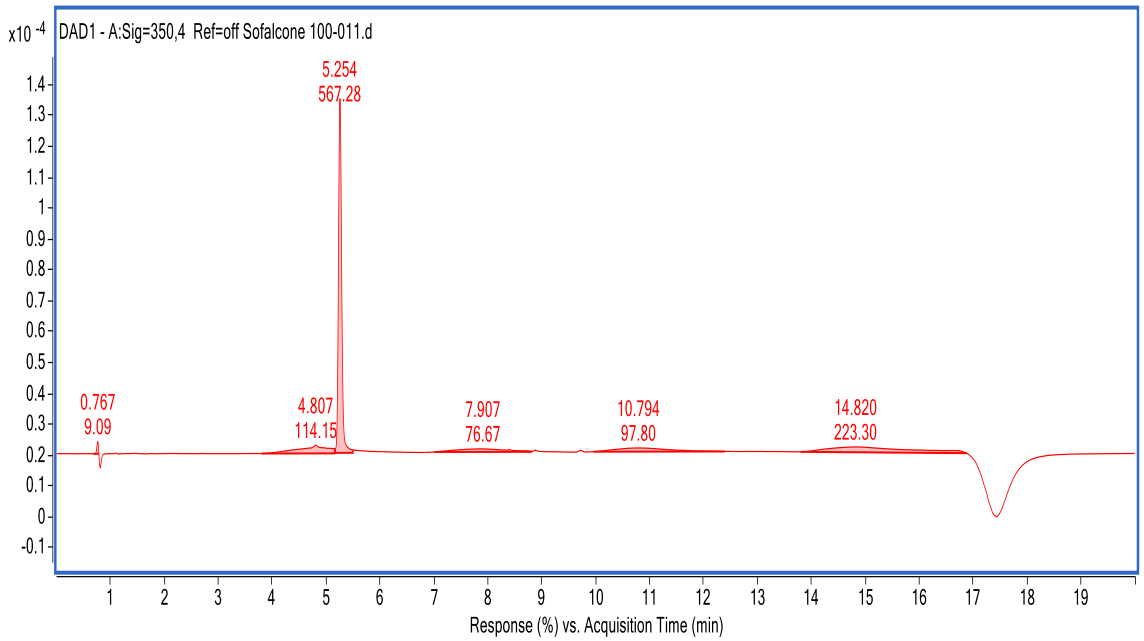


FIGURE 4.24 Chromatogram of marketed formulation with optimized chromatographic conditions

4.3 Summary of validated Stability-indicating RP-UHPLC method

All metrics, including plate count (found to be >4000) and tailing factor (found to be <2), were determined to be within the specified limit despite the fact that variation in flow rate altered the retention period of the primary peak.

- **Acceptance criteria:**

i) Tailing Factor: The sofalcone peak observed after the first injection of the standard solution should have a tailing factor of no more than 2.0.

ii) Theoretical plates: A theoretical plate count of less than 2000 for the sofalcone peak after the first injection of the standard solution is unacceptable.

iii) RSD: Six duplicate injections of the standard solution should not result in a relative standard deviation of the sofalcone peak area more than 2.0%.

iv) The relative standard deviation (RSD) for test findings from three different sample solutions for each different condition should not exceed 2.0%.

v) The relative standard deviation (RSD) of all test findings, including those obtained under each different condition, should be less than or equal to 2.0%.

- **Conclusion:**

Results for wavelength, temperature, and flow rate changes are all within acceptable ranges.

CHAPTER 5

5 RP-HPLC method development and validation for the estimation of sofalcone in bulk drug and formulations with forced degradation studies

5.1 Material and Methods

5.1.1 Reagents and chemical

Through Zeta Scientific LLP. In Mumbai, we were able to get sofalcone. Everything else was HPLC-grade and purchased from Merck Specialty Private Limited.

5.1.2 Instruments and Equipments

Sr. No.	Instruments	Model no.	Manufacturer
1	HPLC	1260 Infinity II	Agilent
2	HPLC Column	Eclipse Plus C18 (150mm × 4.6mm, 5µm)	Agilent
3	Detector	Photo Diode Array	-
4	FT-IR	IR Spirit	Shimadzu
5	UV-Visible Spectrophotometer	UV- 1900	Shimadzu
6	pH meter	EQ-610	Lab Line
7	Ultra Sonicator	LMUC 6	-
10	Water purification system	-	Mili- Q
11	Analytical Weighing Balance	ME204/A04	Shimadzu
12	Centrifuge	-	Remi

5.1.3 Optimized Chromatographic conditions

Mode	:	Isocratic
Column	:	Eclipse Plus C_{18} (150mm × 4.6mm, 5µm) (or) equivalent
Injection Volume	:	5 µL
Flow rate	:	1.0 ml / minute
Wavelength	:	UV 348 nm
Column oven Temperature	:	25°C
Sample Temperature	:	25°C
Retention Time (min)	:	About 4.7 Minutes
Run Time	:	10 Minutes
Needle wash	:	Mixture of Acetonitrile and Water in the ratio of 90:10.
Seal wash	:	Mixture of Acetonitrile and Water in the ratio of 10:90.
Mobile Phase		Mobile Phase A: Ammonium Acetate buffer + Triethylamine (pH 5.6 Adjusted with Glacial Acetic Acid) Mobile Phase B: Acetonitrile Mobile Phase A : Mobile Phase B (50:50)
Diluent		Water: Acetonitrile (20:80) (Sample dissolved in 5ml THF and adjust volume with Diluent)

5.1.4 System Suitability

i) Tailing factor: The sofalcone peak observed after the first injection of the standard solution should have a tailing factor of no more than 2.0.

ii) Theoretical Plates: A theoretical plate count of less than 2000 for the sofalcone peak after the first injection of the standard solution is unacceptable.

iii) RSD: Six duplicate injections of the standard solution should not result in a relative standard deviation of the sofalcone peak area more than 2.0%.

5.1.4.1 Procedure for sample injection:

Allow at least an hoursfor the mobile phase and HPLC column to mix before conditioning the column with a single full gradient. 5 µL volumes of the blank, reference, and sample solutions

should be injected to the chromatograph according to the table below. Mark the chromatogram and compute the peak area that sofalcone occupies.

TABLE 5.1 Injection sequence

Sr. No.	Sample name	No. of injections
1	Blank	1
2	Standard solution	6
3	Sample solution_1	1
4	Sample solution_2	1
5	Standard solution (Bracketing)	1

5.1.5 Preparation of solutions

5.1.5.1 Blank (Diluent)

Mix a combination of Water and Acetonitrile in the proportion of 50:50 *v/v* (Sample dissolved in 5ml THF and adjust volume with Diluent).

5.1.5.2 Buffer Solution (pH 5.6 ± 0.05)

- **Phosphate Buffer solution**

Dissolve 6.8 grammes of ammonium acetate in one thousand ml of water. Adjust pH to 5.6 ± 0.05 using a weak solution of glacial acetic acid. Use a 0.45 µm Nylon membrane filter for filtration.

- **Mobile Phase**

Create a combination of 50 % buffer solution and 50 % acetonitrile. Sonicate for 10 minutes to degas.

5.1.5.3 Standard Solution (100 µg/ml)

Working standard or main reference standard (10 mg) must be weighed accurately and put to a 100 ml volumetric flask. 5 ml of tetrahydrofuran is poured into the tube. It requires 10 ml of fluids to become less potent. The standard is diluted with diluent until the desired concentration is reached. After being cooled to ambient temperature and entirely dissolved using ultrasonication, it is thoroughly blended.

5.1.5.4 Sample solution: (Prepare sample in duplicate) (100 µg/ml)

At least 20 capsules should be weighed after the pellets have been removed. Put everything together and figure out how much the filling weighs on average. The pellets, each containing around 100 mg of sofalcone, should be properly weighed using a reputable scale and deposited in a 100 ml volumetric flask. Tetrahydrofuran, in the amount of 5 ml, must be

added. In a nutshell, ten minutes of sonication. Finally, sonicate 95 ml of diluent on and off for 60 minutes. Let the flask come to room temperature before adding the diluent, diluted solution, and mixing. Use a 0.45 µm filter to purify the solution. Discard the first 3–4 ml of the filtrate after PTFE prefiltration. A volumetric flask of 100 ml is filled with the crystal-clear filtrate solution. The next step is to add the diluent until you have a total volume of 100 ml. This answer may serve as an example.

5.1.6 Forced degradation

Forced degradation research will compare API, 100 mg capsules, and placebo. pH, alkalinity, oxidation, hydrolysis, photolysis, humidity, and thermal breakdown tests will be performed on the samples. Make a void for every piece of research that demeans you.

5.1.6.1 API Solution (Prepare control API solution in duplicate) (100 µg/ml)

Put 100 ml of water into a 100 ml volumetric flask, and then add 100 mg of sofalcone by weight. Tetrahydrofuran, in the amount of 5 ml, must be added. In a nutshell, ten minutes of sonication. Finally, sonicate 95 ml of diluent on and off for 60 minutes. Let the flask come to room temperature before adding the diluent, diluted solution, and mixing. Use a 0.45 µm Pre-filter to purify the solution. Discard the first 3–4 ml of the filtrate after PTFE prefiltration. A volumetric flask of 100 ml is filled with the crystal-clear filtrate solution. The next step is to add the diluent until you have a total volume of 100 ml. Take a cue from this sample solution and use it as a guide.

5.1.6.2 Preparation of forced degradation solutions for placebo, API and Capsules

5.1.6.2.1 Acid degradation

Accurately weigh three sets of 100 pellets: active ingredient sofalcone (100 mg), placebo (100 mg), and sample (100 mg). Fill three 100 ml volumetric flasks with the weighted objects. Tetrahydrofuran, in the amount of 5 ml, must be added. In a nutshell, ten minutes of sonication. Finally, sonicate 95 ml of diluent on and off for 60 minutes. Hydrochloric acid solution, at the proper concentration, should be added. Flasks should be kept in a water bath at the appropriate temperature or on a work surface at room temperature as much as possible to avoid a 20% drop in assay value. When it has cooled, neutralise it with a sodium hydroxide solution of the same strength. Mix after diluting to a smaller amount. When filtering a solution using a 0.45 µm Pre-filter + PTFE filter, discard the first 3-4 ml of the filtrate. A volumetric flask of 100 ml is filled with the crystal-clear filtrate solution. The next step is to add the diluent until you have a total volume of 100 ml. Take a cue from this sample solution and use it as a guide.

5.1.6.2.2 Base degradation

Active ingredient pellets of sofalcone (100 mg), placebo pellets of sofalcone (100 mg), and sample pellets of sofalcone (100 mg) must all be weighed to the nearest 0.1 mg. put the measured quantities of the chemicals into three 100-ml volumetric flasks. Add 5 ml of tetrahydrofuran. Process for about 10 minutes at a rapid rate. Next, sonicate 95 ml of diluent for 60 minutes with occasional stirring. Add the necessary quantity of sodium hydroxide solution. The test result should not decrease by more than 20%, thus maintain the flask's temperature at room temperature on the workstation. After it has cooled, neutralise it with a solution of similar-strength hydrochloric acid. Combine after diluting to the required volume using a diluent. After passing the solution through a 0.45 µm Pre-filter + PTFE filter, remove the first 3 to 4 ml of the filtrate. After pipetting 10.0 ml of the filtrate solution into a 100 ml volumetric flask, it is diluted to volume with the diluent and then mixed. This response may serve as an example.

5.1.6.2.3 Oxidation degradation

Weigh three sets of 100 pellets with precision: the active component sofalcone (100 mg), the placebo (100 mg), and the sample (100 mg). Fill three volumetric flasks of 100 ml with the weighted items. Five ml of tetrahydrofuran must be added. In brief, 10 minutes of ultrasonication. Lastly, sonicate 95 ml of diluent intermittently for sixty minutes. The proper concentration of hydrogen peroxide solution should be applied. The test result should not decrease by more than 20%; thus, maintain the flask in a water bath at the appropriate temperature or on a work surface at room temperature as much as possible. Combine after diluting to a lesser concentration. When filtering a solution with a 0.45 µm Pre-filter + PTFE filter, throw away the first 3 to 4 ml of the filtrate. A 100 ml volumetric flask is filled with the transparent filtrate solution. The next step is to add diluent until the total volume reaches 100 ml. Using this example response as a model and guidance.

5.1.6.2.4 Photo degradation: UV

Three Petri plates were prepared with 5 g of placebo pellets, 10 capsules of 100 mg sofalcone, and 2,000 mg of sofalcone API. The plates should be exposed to roughly 200 watts of light per square metre in a photo stability chamber. You may monitor their deterioration in this way. Here are some things to consider: Each pellet contains 100 mg of active pharmaceutical ingredient (API) sofalcone, 100 mg of placebo, and 100 mg of sample. Fill the three 100 ml volumetric flasks with the appropriate quantities of the various components. THF to the tune of 5 ml. Ten minutes of sonication time is recommended. After that, sonicate 95 ml of diluent

for 60 minutes while stirring occasionally. If you don't want to lose more than 20% of your test value, keep your flasks in a temperature-controlled water bath or on a work surface at room temperature. To adjust the volume, add water and stir. The first three to four ml of a solution filtered through a 0.45 µm Pre-filter + PTFE filter should be discarded. Then, you'll need to transfer 10 ml of the filter solution into a 100 ml volumetric flask. Next, dilute it with the solvent until it has the same volume as the original. You have provided a valid response.

5.1.6.2.5 Thermal degradation

Three Petri dishes containing 5 g of placebo pellets, 10 capsules containing 100 mg of sofalcone, and 2,000 mg of sofalcone API were created. Check after 24 hours at 105 ºC to determine whether they have disintegrated. Here are some considerations: Pellets containing 100 mg of sofalcone, 100 mg of placebo, and 100 mg of sample. Add the ingredients measured in grammes to three 100-milliliter volumetric flasks. Tetrahydrofuran (5 ml) must be added. Sonicate for about 10 minutes to thoroughly blend ingredients. Then, sonicate 95 ml of diluent intermittently for one hour. Maintain the flask as much as possible in a water bath at the right temperature or on a work surface at room temperature to avoid a 20% decrease in test results. Following dilution, mix the reduced volume. Filter the solution with a 0.45 µm Pre-filter + PTFE filter and preserve the first 3 to 4 ml of the filtrate. Pipette 10.0 ml of the filtrate solution into a 100-ml volumetric flask. Once the volume has reached 100 ml, add the diluent and mix well.

5.1.6.2.6 Procedure for treated Sample injection

Let the mobile phase to settle for at least an hour, and then run a full gradient programme over the HPLC column to prepare it for use. Each of the 5 µl of blank, standard, and sample solutions need to be injected separately. The chromatograph should be set up to test the blank, placebo, API, and sample solutions after they have been subjected to acid, base, oxidation, hydrolysis, sunlight, humidity, and heat (single degradation). Make some notes on the chromatogram and calculate the percentage change in the peak area for sofalcone. sofalcone represents the epitome of cleanliness.

5.1.6.2.7 Acceptance Criteria

i) The peak for sofalcone should be easily discernible without any confounding noise from blank, placebo, or degradation products.

ii) Peak purity criteria (Peak purity = Purity angle < Purity threshold) should pass for sofalcone peak in all the degraded samples.

5.1.7 Method Validation

5.1.7.1 Specificity

Specificity refers to the clarity with which the analyte is evaluated in the presence of objects that you would expect to be in the sample matrix. Typical examples include contaminants, degradants, matrices, and so on.

5.1.7.1.1 Check for blank, placebo and impurities interference

To test the effectiveness of the method, both known contaminants and a placebo may be introduced to the sample. Test for the influence of the blank, the placebo, and any known contaminants on the sofalcone peak.

5.1.7.1.2 Preparation of solutions

Blank (diluent) solutions, standard solutions, and sample solutions (in duplicate) must be made for sofalcone 100 mg Capsules (Test Procedure).

5.1.7.1.3 Placebo solution

Drop each placebo pellet into a volumetric flask that holds 100 ml after establishing its weight using a credible scale; each pellet is about equivalent to 100 mg of sofalcone. Add 5 ml of tetrahydrofuran to the bottle. Use a sonic blender for around 10 minutes to thoroughly combine the items. Next, sonicate 95 ml of the diluent for 60 minutes while sporadically blending. Carefully use a magnet to pry off the magnetic bar from the volumetric flask. Remove any debris from the magnetic bar within the flask's stopper. Wait until the flask has cooled to room temperature before adding the diluent and mixing the solution. While filtering the solution, use a filter with a micron size of 0.45. After PTFE prefiltration, the first 3–4 ml of filtrate may be thrown away. A 100-ml volumetric flask needs 10.0 ml of a clear filtrate solution pipetted into it. Following that, add the diluent drop by drop until you reach 100 ml and stir the solution until it is uniform throughout. Learn from this response and use it as a template for your own.

5.1.7.1.4 Placebo spiked solution with sofalcone

Put the placebo pellets, which weigh about the same as 100 mg of sofalcone, in a 100 ml volumetric flask using a trustworthy scale. Mix 5 ml of tetrahydrofuran API with 100 mg of sofalcone API. Sonicate for about 10 minutes to thoroughly blend ingredients. Then, sonicate 95 ml of diluent intermittently for one hour. Carefully remove the magnetic bar from the volumetric flask using the magnet. Clean the magnetic bar in the cork of the flask. Let the flask to reach room temperature before adding the diluent, diluting, and mixing. Filter the solution with a 0.45 µm Pre-filter + PTFE filter and preserve the first 3 to 4 ml of the filtrate.

Pipette 10.0 ml of the filtrate solution into a 100-ml volumetric flask. Once the volume has reached 100 ml, add the diluent and mix well. Using this reaction as a model for your own.

5.1.7.1.5 Procedure

Allow at least an hoursfor the mobile phase and HPLC column to mix before conditioning the column with a single full gradient. Individual 5 µl injections of a blank, standard solution, sample solution produced according to method, placebo solution, individual identification solution, placebo solution with known contaminants added, and sofalcone are needed. Calculate the % change in sofalcone peak area based on the chromatogram. At sofalcone, the purity reaches its apex.

5.1.7.1.6 Acceptance Criteria

i) Blank, placebo, and contaminants should not interfere with the retention time of the sofalcone peak.

ii) For each of the four solutions (standard, sample, placebo, and sofalcone and sample treated with known contaminants), the peak of sofalcone must meet the peak purity criteria (Peak purity = purity angle < purity threshold).

5.1.7.2 Linearity

The linearity of an analytical method is defined as its ability to provide test results that are proportional, either directly or via a well-defined mathematical transformation, to the concentration of an analyte in a sample. The test protocol was also utilised to examine the homogeneity and consistency of the mixture. Five concentrations ranging from 50 to 150 % of the test concentration must be utilised to determine linearity. Prepare dilutions of the sofalcone standard solution (Solution C) at approximately 50%, 80%, 100%, 120%, and 150% of the test concentration.

5.1.7.2.1 Preparation of solutions

Prepare blank (diluent) and standard solution as per given in Methodology (Test Procedure).

5.1.7.2.2 Sofalcone standard solution for linearity (Solution C)

Using a 100 ml volumetric flask, weigh out about 100 mg of sofalcone and add 30 ml of diluent. When the standard has been dissolved with the help of sonication and cooled to room temperature, it can be diluted with diluent to the desired concentration and mixed well. Make the linearity standard solution by injecting three copies of a diluted version of the sofalcone standard solution (Solution C), as shown in Table 5.2.

TABLE 5.2 Preparation of Linearity standard solutions

Sr. No.	Level	% concentration of sofalcone w.r.t test concentration (µg/ml)	Added Solution C (in ml)	Dilution Volume (in ml)	Concentration of sofalcone (in ppm)
1	Level 1	50	2.5	50	50
2	Level 2	80	4	50	80
3	Level 3	100	5	50	100
4	Level 4	120	6	50	120
5	Level 5	150	7.5	50	150

5.1.7.2.3 Procedure

Allow at least an hoursfor the mobile phase and HPLC column to mix before conditioning the column with a single full gradient. Mix 5 µL of the linearity standard solution, the method standard solution, and the blank on a chromatograph. Calculate the % change in sofalcone peak area based on the chromatogram. Compare the concentration to the mean area response using a line graph. Notate the values for the sum of squares (RES), slope of the regression line, Y-intercept, %Y-intercept, and slope of the regression line.

5.1.7.2.4 Acceptance Criteria

The correlation coefficient ('R') value should not be less than 0.99 over the working range.

5.1.7.3 Accuracy

How closely test findings resemble the true value is a measure of how accurate an analytical technique is. Accuracy is most often reported as a percentage of the analyte actually detected. Accuracy is often used to evaluate the efficacy of an analytical method. Do the precision evaluation at three separate concentrations (i.e., 50 %, 100 %, and 150 % of the test concentration) between 50 % and 150 %. To test the reliability of this method, sofalcone may be injected into the placebo, analysed, and the amount recovered calculated.

5.1.7.3.1 Preparation of solutions

Prepare blank (diluent) and standard solution as per given in Methodology (Test Procedure). Prepare placebo solution as per given in Specificity.

5.1.7.3.2 Accuracy sample preparation

Put the placebo pellets, which weigh about the same as 100 mg of sofalcone, in a 100 ml volumetric flask using a trustworthy scale. Tetrahydrofuran (5 ml) must be added. Sonicate

for about 10 minutes to thoroughly blend ingredients. Then, sonicate 95 ml of diluent intermittently for one hour. Carefully remove the magnetic bar from the volumetric flask using the magnet. Clean the magnetic bar in the cork of the flask. Let the flask to reach room temperature before adding the diluent, diluting, and mixing. Use a 0.45 µm filter to filter the solution. After PTFE pre-filtration, the first three to four ml of filtrate should be discarded. Pipette 10.0 ml of the filtrate solution into a 100-ml volumetric flask. Once the volume has reached 100 ml, add the diluent and mix well. You may use this response as an example. Make three replicas of the responses for the purpose of studying accuracy, and insert one copy at each step.

TABLE 5.3 Preparation of sample solutions for accuracy study

Sr. No.	Level	(%) Spiked	Conc. from formulation	Standard Conc. Added	Concentration of sofalcone (in ppm)
1	Level 1	50 %	50	50	50
2	Level 2	100 %	100	50	100
3	Level 3	150 %	150	50	150

5.1.7.3.3 Procedure

Mobile phase and HPLC column should be well mixed for at least an hoursbefore conditioning the column with a single full gradient. Each of the following solutions (standard, placebo, accuracy sample, and blank) should be injected into the chromatograph at a volume of 5 litres. Make some notes on the chromatogram and calculate the percentage change in the peak area for sofalcone. Calculate the RSD, mean recovery, and percentage of recovery for each level. Make a chart that shows how the amounts after addition and subtraction compare.

5.1.7.3.4 Acceptance Criteria

i) sofalcone's average and median recoveries should be between 98 % and 102 %.

ii) sofalcone's average rate of success in treating insomnia should range from 98.0 % to 102.0 %.

iii) The maximum acceptable RSD (as a percentage) for sofalcone is 2 %.

5.1.7.4 System precision

The precision of an analytical method is defined by the consistency of results obtained from repeated measurements of the same homogeneous material under the same conditions. To assess the precision of the system, you should inject six copies of the standard solution from the same HPLC vial, as per the test protocol.

- **Preparation of solutions**

Prepare blank (diluent) and standard solution as per given in Methodology (Test Procedure).

- **Procedure**

Let the HPLC column acclimatise to the mobile phase for at least an hoursbefore conditioning with a single full gradient procedure. In accordance with the protocol, fill the chromatograph with 10 µL of both the standard solution and the blank solution. Collect data from a chromatogram and calculate the sofalcone peak area.

- **Acceptance Criteria**

i) Tailing Factor: No more than a tailing factor of 2.0 should be seen in the sofalcone peak after the first injection of the standard solution.

ii) Theoretical plates: A theoretical plate count of less than 2000 for the sofalcone peak after the first injection of the standard solution is unacceptable.

iii) RSD: Six duplicate injections of the standard solution should not result in a relative standard deviation of the sofalcone peak area more than 2.0 %.

5.1.7.4.1 Method precision (Repeatability)

Reproducibility under controlled conditions and time limitations served as a quantitative measure of the results' dependability. For the Method Precision analysis, a total of six sample solutions must be made from the same batch of sofalcone 100 mg Capsules.

- **Preparation of solutions**

Prepare blank (diluent), standard solution and sample solution as per given in Methodology (Test Procedure).

- **Procedure**

Mobile phase and HPLC column should be well mixed for at least an hoursbefore conditioning the column with a single full gradient. Keep through with the process and load 10 µL of the sample, standard, and blank solutions into the chromatograph. Make some notes on the chromatogram and calculate the percentage change in the peak area for sofalcone. Learn the average assay result, the standard deviation of the six observations, and the assay values themselves.

➢ **Acceptance Criteria**

i) Individual and mean % assay value should be within specification limit.

ii) The RSD of six determinations should not be more than 2.0 %.

5.1.7.4.2 Intermediate precision

The accuracy was within the laboratory variance even when another analyst used the identical set of samples and HPLC equipment on a different day. The technique calls for six separate sample solutions to be made from the same batch of samples on separate days by separate analyzers using separate HPLC equipment and columns of the same brand. Moderate care will be used throughout production of sofalcone 100 mg Capsules.

➢ **Preparation of solutions**

Prepare blank (diluent), standard solution and sample solution as per given in Methodology (Test Procedure).

➢ **Procedure**

Allow at least an hoursfor the mobile phase and HPLC column to mix before conditioning the column with a single full gradient. Follow the instructions carefully and put 10 µL of the blank solution, the standard solution, and the sample solution into the chromatograph. Calculate the % change in sofalcone peak area based on the chromatogram. In this instance, we must determine the average assay value, the RSD of the six measurements, and the individual assay values. Calculate the absolute value of the difference between the mean % test results from the method precision study and the intermediate precision study.

➢ **Acceptance Criteria**

1. Individual and mean percent assay values should fall within the specified range.

2. The RSD of six determinations must not exceed 2.0%.

3. The absolute difference between the mean % assay results from the method precision study and the intermediate precision study should not exceed 2.0.

5.1.7.5 Solution stability

To ensure that the operation is carried out correctly, the standard and sample solutions need to be prepared and stored at room temperature. Return to this page on a regular basis to check on the status of the solution. To determine the % RSD of the sofalcone peak area in the reference solution, compare the % assay results for the sample solution at various periods.

➢ **Preparation of solutions**

Prepare blank (diluent), standard solution and sample solution (in duplicate) as per given in Methodology (Test Procedure).

➢ **Procedure:**

Mobile phase and HPLC column should be well mixed for at least an hoursbefore conditioning the column with a single full gradient. At different points throughout the process, you'll need to add 5 µL of blank solution, standard solution, and sample solution to the chromatograph. Make some notes on the chromatogram and calculate the percentage change in the peak area for sofalcone. Find the relative standard deviation (RSD) as a percentage for the sofalcone's peak area response. Calculate the percentage change in the assay value of the sample solution between the beginning of the interval and the end of the interval.

➢ **Acceptance Criteria**

i) When comparing standard solutions acquired at different intervals of time, the relative standard deviation of sofalcone peak area should not exceed 2.0 %.

ii) There should not be more than a 2.0 % absolute difference between the assay values of the sample solution taken at the beginning and the end of each time period.

5.1.7.6 Robustness

See how changing each chromatographic parameter has an impact on the assay result and the test to check whether the system is suitable. Prepare the sample solution for the 100 mg sofalcone Capsules as directed in the method. Using the chromatographic settings and test method variables listed below, analyse the sample solution. Check whether the system is suitable for each variable condition and calculate the assay result.

➢ **Preparation of solutions:**

Prepare blank (diluent), standard solution and sample solution (in triplicate) as per given in Methodology (Test Procedure).

1. Change in Column oven temperature (+ 5°C) of 25°C

High column oven temperature (HCT): 30°C

Low column oven temperature (HCT): 20°C

2. Change in Wavelength (± 2 nm) of 348 nm

High Wavelength (HW): 350 nm

Low Wavelength (LW): 346 nm

3. Change in Flow Rate (0.1 ml/min) of 1.0 ml/min

High Flow Rate (HFR): 1.1 ml/min

Low Flow Rate (LFR): 0.9 ml/min

➢ **Procedure:**

Mobile phase and HPLC column should be well mixed for at least an hoursbefore conditioning the column with a single full gradient. Each of the blank, standard, and sample solutions from the variable conditions must be 5 µL in volume for use in the chromatograph. Make some notes on the chromatogram and calculate the percentage change in the peak area for sofalcone. Compare the assay findings from each changing condition to the assay results from the method precision to calculate the RSD and the overall RSD.

- **Acceptance criteria:**

i) Tailing Factor: No more than a tailing factor of 2.0 should be seen in the sofalcone peak after the first injection of the standard solution.

ii) Theoretical plates: A theoretical plate count of less than 2000 for the sofalcone peak after the first injection of the standard solution is unacceptable.

iii) RSD: Six duplicate injections of the standard solution should not result in a relative standard deviation of the sofalcone peak area more than 2.0 %.

iv) The relative standard deviation (RSD) of test findings from three sample solutions for each different condition should not exceed 2.0 %.

v) The relative standard deviation (RSD) of all test findings, including those obtained under each different condition, should be less than or equal to 2.0 %.

5.1.7.7 System suitability

Each set of validation parameters must begin with a check of the system's appropriateness. Verify that the system meets all methodological requirements.

- **Acceptance criteria:**

i) Tailing Factor: No more than a tailing factor of 2.0 should be seen in the sofalcone peak after the first injection of the standard solution.

ii) Theoretical plates: A theoretical plate count of less than 2000 for the sofalcone peak after the first injection of the standard solution is unacceptable.

iii) RSD: Six duplicate injections of the standard solution should not result in a relative standard deviation of the sofalcone peak area more than 2.0 %.

5.2 Result and discussion

5.2.1 Selection of wavelength

As the greatest absorbance of sofalcone is at 348 nm, this is the wavelength that was used for the estimate.

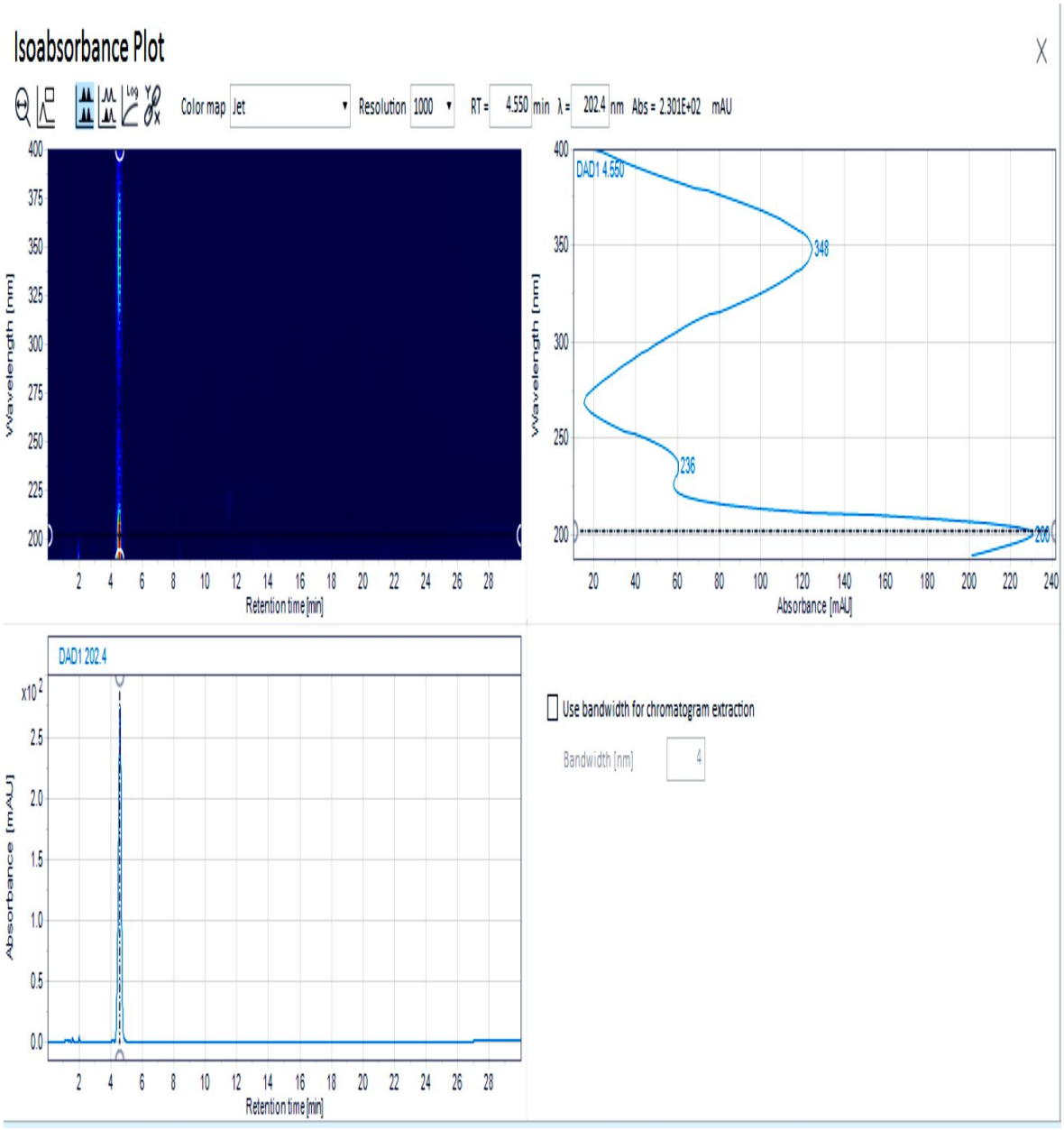


FIGURE 5.1 Isoabsorbance plot at 348 nm (λ max of sofalcone)

5.2.2 Effect of ratio of mobile phase

Mobile phase	Proportion ratio (% *v/v*)	Retention Time (min)	Justification	Detection Wavelength [nm]
Water: Acetonitrile	50:50	-	Broad peak with splitting	348
Water : Acetonitrile	70 : 30	-	Broad peak with tailing	348
Water : Acetonitrile	30:70	-	Broad Peak	348
Water: Acetonitrile	10:90	-	Peak with tailing	348
Water: methanol	50:50	-	Broad Peak	348
Buffer : methanol	20:80	2.702	Good Peak but less retention time	348
Buffer: methanol	30:70	12.726	Good Peak but more retention time	348
Buffer: methanol	25:75	2.969	Good Peak but less retention time	348
Buffer: Acetonitrile	50:50	2.702	Good Peak Observed	348

The following mobile phases were used to chromatograph a mixed standard solution of sofalcone (100 g/ml):

Preparation of mobile phase:

Mobile Phase A: To generate this mobile phase, combine 7.7 g of ammonium acetate with 995 ml of water (0.1M). Mix with 1 millilitre of tri-ethylamine. Glacial acetic acid was then used to bring the pH level up to 5.64. Then filter using a filtering unit.

5.2.3 Method Development

Sonication is used to remove air bubbles from mobile phase A, which consists of a 0.1 M Ammonium Acetate buffer containing 1 ml Triethylamine (pH 5.6 adjusted with Glacial Acetic Acid). A mobile phase B of 50% acetonitrile at 1 ml/min flow rate is used. In table 5.3, you can see the employed isocratic wavelength and several other relevant factors. Figure 5.1 illustrates the chromatograms that indicate this.

5.2.4 Forced degradation

The forced degradation studies used 100 mg sofalcone Capsules, sofalcone API, and a placebo. Acidity, alkalinity, oxidation, hydrolysis, photolysis, humidity, and thermal breakdown tests were performed on the materials.

- **API Solution (Control)**

We weighed out 100 mg of sofalcone and poured it into a 500 ml volumetric flask. Five ml of tetrahydrofuran were put in. In a nutshell, ten minutes of sonication. The sonicator was turned on after 95 ml of diluent were added, and it shook intermittently for 60 minutes. Before adding water and stirring, let the flask warm to room temperature. The solution was filtered using a PTFE + 0.45 µm Pre-filter, and the first 3-4 ml of the filtrate were thrown away. After transferring 10.0 ml of the clear filtrate solution with a pipette into a 100 ml volumetric flask, I diluted the solution with the diluent until it was the same volume as the flask. This solution was provided as an example of a suitable solution.

- **Preparation of forced degradation solutions for Placebo, API and Capsules**

5.2.4.1 Acid degradation

We distributed 100 mg of sofalcone API, 100 mg of placebo pellets, and 100 mg of sample pellets into three 100 ml volumetric flasks based on their relative weights. Tetrahydrofuran (5 ml) must be added. Sonicate for about 10 minutes to thoroughly blend ingredients. The mixture was sonicated for 60 minutes and shaken for 30 minutes after 95 ml of diluent was added. Added was a 1 ml (1N) solution of hydrochloric acid. Leave the flask at room temperature for 1, 2, or 3 hours. When the solution has cooled, neutralise it by adding a solution of 1 N sodium hydroxide. Diluted to the appropriate concentration with the use of a solvent. The components were separated using a 0.45 µm filter. After PTFE pre-filtration, the first three to four ml of filtrate should be discarded. A volume of 10.0 ml of the clear filtrate solution was pipetted into a 100 ml volumetric flask, and the contents were diluted with the diluent to equal the capacity of the flask. This answer was provided as an example.

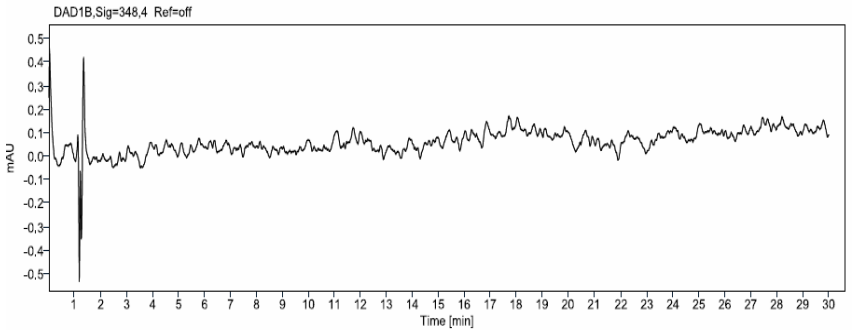


FIGURE 5.2 Chromatogram of blank used for Acid degradation

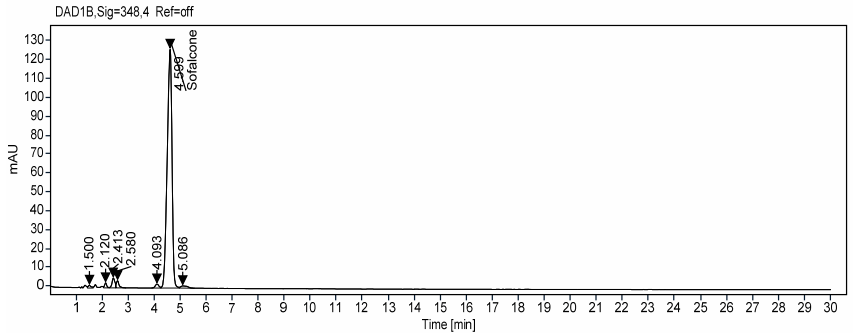


FIGURE 5.3 Chromatogram of sample under 1N 1ml HCl at 1Hr

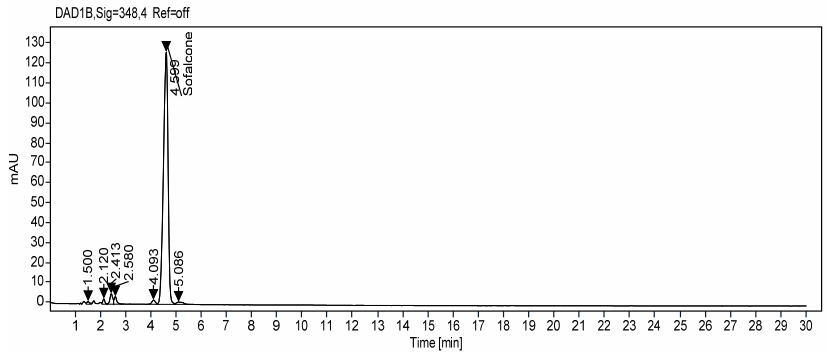


FIGURE 5.4 Chromatogram of sample under 1N 1mL HCl at 2 Hr.

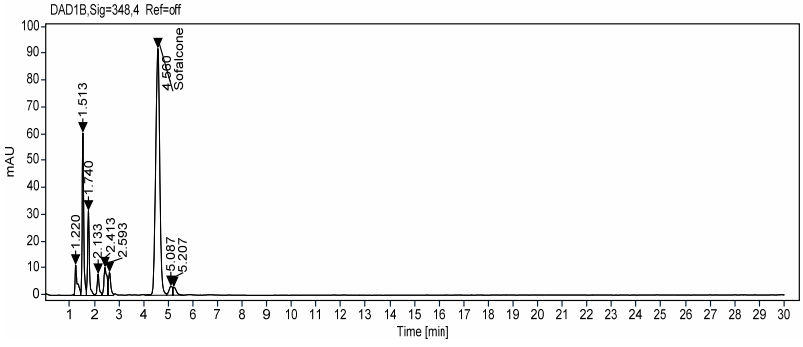


FIGURE 5.5 Chromatogram of sample under 1N 1mL HCl at 3 Hr.

Drug name	Condition	Area	% Degradation
Sofalcone	At 1hrs.	1719.39	8.9306
	At 2 hrs.	1612.32	14.6016
	At 3 hrs.	1055.36	44.1016

5.2.4.2 Base degradation

In three individual 100 mL volumetric flasks, we weighed out 100 mg of Sofalcone API, 100 mg of placebo pellets, and 100 mg of sample pellets. Tetrahydrofuran, in the amount of 5 ml, must be added. In a nutshell, ten minutes of sonication. The mixture was sonicated and agitated for an hour after 95 mL of diluent was added. Include 1 millilitre of a 1 N sodium hydroxide solution. For three hours, the flask was heated in an 80°C water bath. The material was cooled and neutralised using a solution of 1N hydrochloric acid. Mixed and diluted with a suitable diluent to the required strength. The mixture was filtered via a 0.45 µm filter. Discard the first 3–4 mL of the filtrate after PTFE prefiltration. After transferring 10.0 mL of the clear filtrate solution with a pipette into a 100 mL volumetric flask, I diluted the solution with the diluent until it was the same volume as the flask. Used this solution as an example.

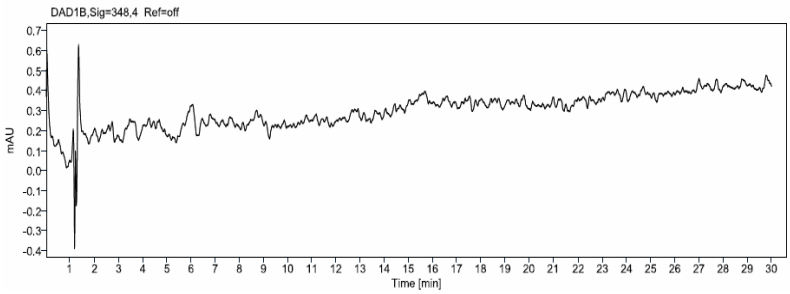


FIGURE 5.6 Chromatogram of blank used for Alkali degradation

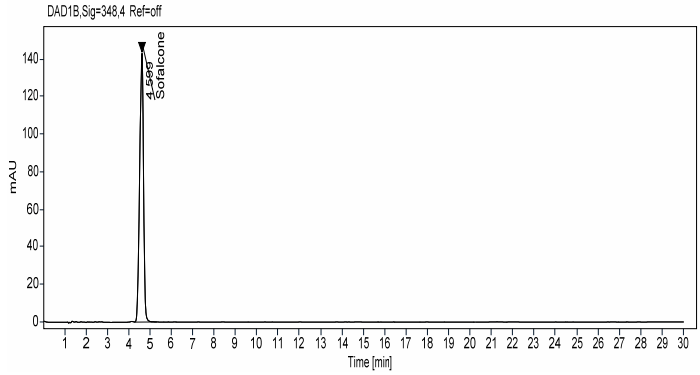


FIGURE 5.7 Chromatogram of sample under 1N 1mL NaOH

Drug name	Condition	Area	% Degradation
Sofalcone	At 3hrs.	1802.82	4.5116

5.2.4.3 Oxidation degradation

We distributed 100 mg of Sofalcone API, 100 mg of placebo pellets, and 100 mg of sample pellets into three 100 mL volumetric flasks based on their relative weights. Tetrahydrofuran (5 mL) must be added. Sonicate for about 10 minutes to thoroughly blend ingredients. The mixture was sonicated for 60 minutes and shaken for 30 minutes after 95 mL of diluent was added. The addition of 3% hydrogen peroxide to a 1mL solution. In a water bath, the flask was heated to 80 degrees Celsius for three hours. Diluted to the appropriate concentration with the use of a solvent. The components were separated using a 0.45 µm filter. After PTFE pre-filtration, the first three to four millilitres of filtrate should be discarded. A volume of 10.0 mL of the clear filtrate solution was pipetted into a 100 mL volumetric flask, and the contents were diluted with the diluent to equal the capacity of the flask. This answer was provided as an example.

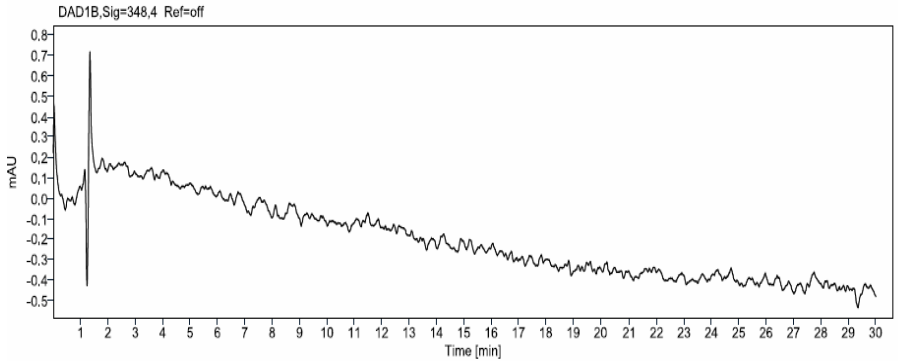


FIGURE 5.8 Chromatogram of blank used for Oxidative degradation

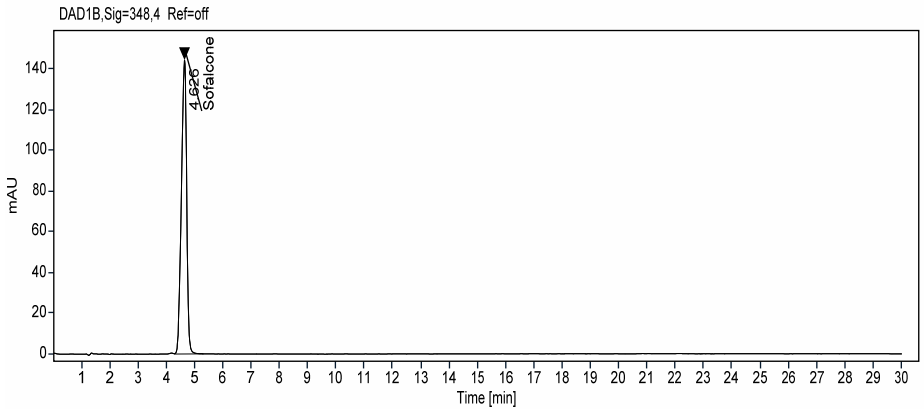


FIGURE 5.9 Chromatogram of sample under 1 mL 3 Hrs. 3% H2O2

Drug name	Condition	Area	% Degradation
Sofalcone	At 3hrs.	1869.85	0.9613

5.2.4.4 Photo degradation: UV

Observing the stability of placebo pellets, 20 capsules of 100 mg sofalcone, and 2,000.21 mg of the API under 200 watts per square metre of light. We distributed 100 mg of Sofalcone API, 100 mg of placebo pellets, and 100 mg of sample pellets into three 100 mL volumetric flasks based on their relative weights. Tetrahydrofuran (5 mL) must be added. The mixture was sonicated for 60 minutes and shaken for 30 minutes after 95 mL of diluent was added. Sonicate for about 10 minutes to thoroughly blend ingredients. Three hours were spent exposing the flask to ultraviolet light. Diluted to the appropriate concentration with the use of a solvent. PTFE + 0.45 µm the solution was filtered using a pre-filter, and the top 3–4 mL of the resultant filtrate were discarded. A volume of 10.0 mL of the clear filtrate solution was pipetted into a 100 mL volumetric flask, and the contents were diluted with the diluent to equal the capacity of the flask. This answer was provided as an example.

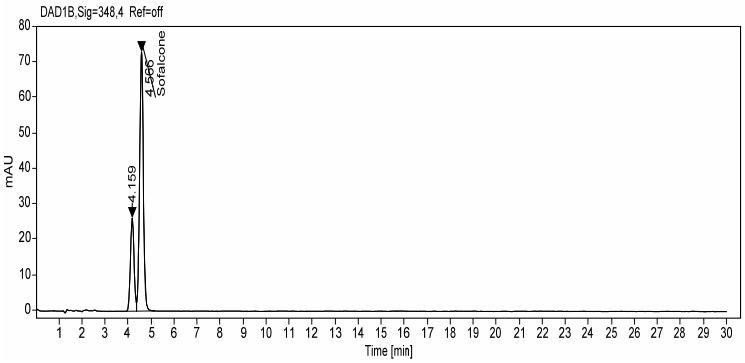


FIGURE 5.10 Chromatogram of sample under UV light 3 Hours

Drug name	Condition	Area	% Degradation
Sofalcone	24 hours	815.11	56.8268

5.2.4.5 Thermal degradation:

Transferred placebo pellets, 20 Sofalcone 100 mg Capsules and 2000.34 mg Sofalcone API into a three different petri dishes and keep in the oven at 80°C for 3 hours for degradation. Weighed placebo pellets (equivalent to about 100 mg of Sofalcone), sample pellets (equivalent to about 100 mg of Sofalcone) and 100 mg of Sofalcone API and transferred into three different 100 mL Twenty (2000) Sofalcone 100mg Capsules. The degradation of 34

mg of Sofalcone API and placebo pellets was tested by placing them in three different Petri dishes and baking them at 80°C for three hours. In three individual 100 mL volumetric flasks, we weighed out 100 mg of Sofalcone API, 100 mg of placebo pellets, and 100 mg of sample pellets. Tetrahydrofuran, in the amount of 5 ml, must be added. In a nutshell, ten minutes of sonication. For three hours, the flask was heated in an 80°C water bath. Mixed and diluted with a suitable diluent to the required strength. PTFE + 0.45 µm the first three to four millilitres of the filtrate after filtering the solution through a pre-filter were thrown away. After transferring 10.0 mL of the clear filtrate solution with a pipette into a 100 mL volumetric flask, I diluted the solution with the diluent until it was the same volume as the flask. Used this solution as an example.

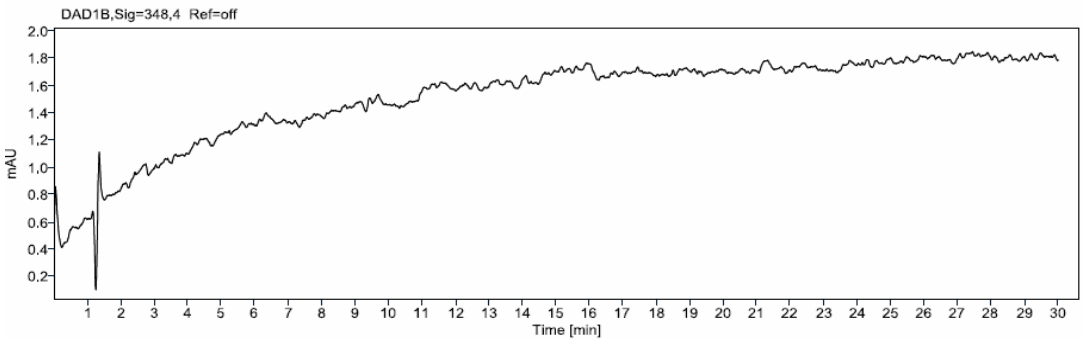


FIGURE 5.11 Chromatogram of blank for Thermal degradation

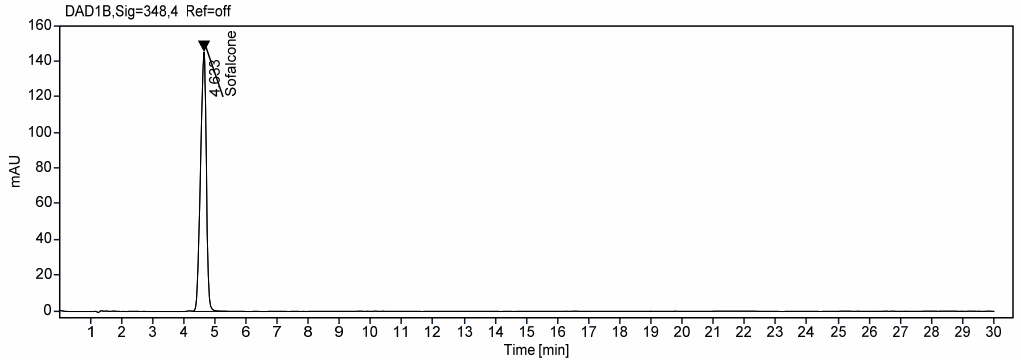


FIGURE 5.12 Chromatogram of sample at 80°C for 30 min

Drug name	Condition	Area	% Degradation
Sofalcone	At 30 min	1882.03	0.3162

TABLE 5.4 Forced degradation and peak purity data for Sofalcone in sample solution

Parameter	Condition	% Degradation	Peak purity	Peak purity pass/ fail
		Sofalcone	Sofalcone	
Acid	At 1Hrs.	8.9306	999.9	True
	At 2 Hrs.	14.6016	999.9	True
	At 3 Hrs.	44.1016	999.9	True
Alkali	At 3Hrs.	4.5116	999.9	True
Oxidation	At 3Hrs.	4.5116	999.9	True
Thermal	30min 80° C	0.3162	1000.0	True
Photolytic	UV Light 24 Hours	56.8268	999.9	True

- **Acceptance Criteria:**

i) The sofalcone peak should be free of noise from blank, placebo, and degradation products.

ii) All of the deteriorated samples should meet the peak purity standards for sofalcone (Peak purity = Purity angle < Purity threshold).

- **Observation:**

i) No observable interference with the sofalcone peak is present from blank, placebo, or degradation products.

ii) All of the deteriorated samples meet the criterion for sofalcone peak purity (Peak purity = Purity angle < Purity threshold).

- **Conclusion:**

All the results are well within the acceptance criteria; hence method is specific.

5.2.5 Method Validation

5.2.5.1 Generation of calibration curves

Allow at least an hoursfor the mobile phase and HPLC column to mix before conditioning the column with a single full gradient. Inject 5 µL of each of the blank, reference, and sample solutions into the chromatograph in the order stated in the table below. In the chromatograph, the size of the sofalcone peak should be quantified and documented.

TABLE 5.7 Injection sequence

Sr. No.	Sample name	No. of injections
1	Blank	1
2	Standard solution	6
3	Sample solution_1	1
4	Sample solution_2	1
5	Sample solution_3	1
6	Standard solution (Bracketing)	1

5.2.5.2 Specificity

In the presence of additional chemicals that are likely to be present in the sample matrix, the ability to accurately test for the analyte is known as its "specificity." Contaminants, degradants, the matrix, etc., might all fall within this category.

5.2.5.3 Check for blank, placebo and impurities interference

Several sorts of solutions were made and injected into the chromatograph to see what would happen: blank, standard, placebo, sample, placebo combined with known impurities and sofalcone, and sample mixed with known impurities and sofalcone, and identification solutions. Determine whether the blank placebo has an effect on the sofalcone peak and the discriminatory power of the procedure. The data uncovered is shown in Table 5.6.

TABLE 5.8 Retention time and purity data for sofalcone

Sample Name	Retention Time (Min)	Purity Angle	Purity Threshold	Peak Purity
Blank				
Sofalcone	ND	NA	NA	NA
Standard solution				
Sofalcone	4.751	0.105	0.282	Pass
Placebo solution				
Sofalcone	ND	NA	NA	NA
Sofalcone Capsules 100 mg				
Sample solution_1				
Sofalcone	5.273	0.125	0.278	Pass
Placebo spiked solution with sofalcone				
Sofalcone	5.271	0.106	0.282	Pass

➢ **Acceptance Criteria:**

i) The peak retention time for sofalcone should be free of blank, placebo, and impurity interference.

ii) Standard solution, sample solution, placebo spiked solution with known impurities, and sofalcone and sample spiked solution with known impurities must all meet peak purity standards (Peak purity = Purity angle < Purity threshold) for sofalcone peak.

➢ **Observation:**

i) The peak retention time of sofalcone is independent of blank, placebo, and impurity retention times.

ii) The reference solution, the sample solution, and the placebo with sofalcone added all meet the peak purity standards (Peak purity = Purity angle < Purity threshold).

➢ **Conclusion:**

All the results are well within acceptance criteria; hence method is specific.

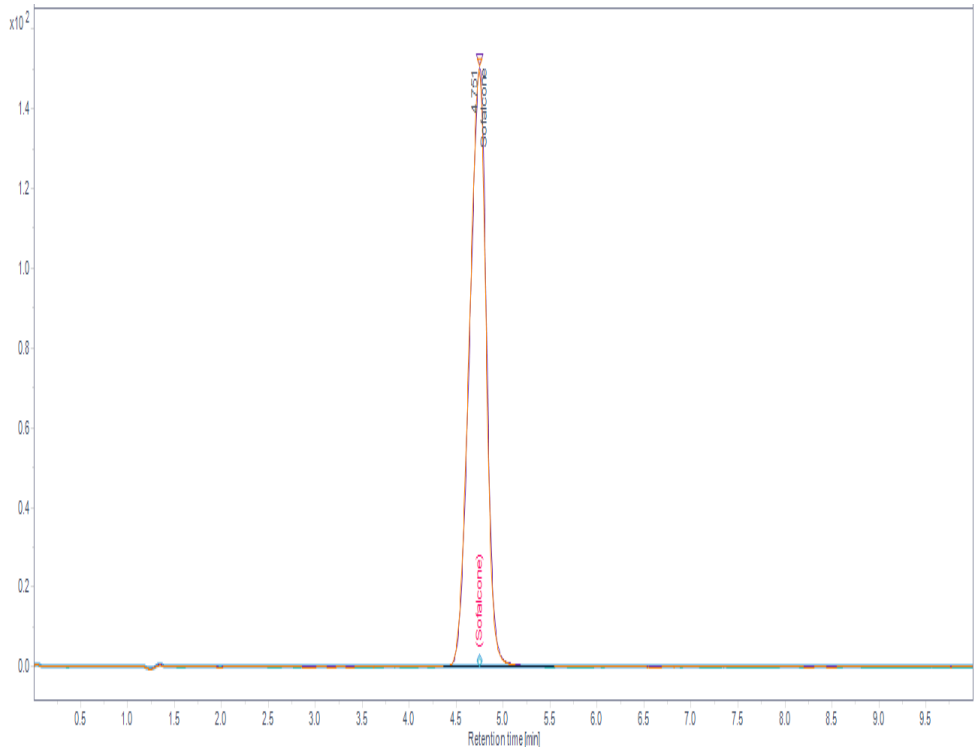


FIGURE 5.13 Specificity

5.2.5.4 Linearity

Analytical linearity refers to the ability of a method to provide results that are directly (or after a well-defined mathematical transformation) proportional to the concentration of an

analyte in a sample. From 50% to 150% of the test concentration, five linearity levels were analysed. To create concentrations of 50%, 80%, 100%, 120%, and 150% of the test concentration, the standard solution for sofalcone (Solution C) was linearized and diluted. After that, we injected each linearity standard solution and a blank standard solution into a chromatograph. The perfect spot of sofalcone's sensitivity. We determined the sum of squares, the regression line slope, the Y intercept, the %Y intercept, and the slope of the regression line for the remaining data. A graph was made displaying the concentration as a function of the average peak area. The found data is tabulated for your convenience in Table 5.7.

TABLE 5.9 Linearity study data for sofalcone

Weight (Mg) of sofalcone	**Volume up to with Diluent (a)**	**Volume to be taken from (a)**	**Final Volume with Diluent**	**Concentration in ppm (µg/ml)**
100.2	100	2.5	50	50.1
100.2	100	4	50	80.16
100.2	100	5	50	100.2
100.2	100	6	50	120.24
100.2	100	7.5	50	150.3

TABLE 5.10 RSD data for sofalcone

Concentration (µg/ml)	**Area Mean ± S.D. (n=3)**	**RSD**
50	935.78 ± 43.218	0.00225
80	1501.59 ± 16.985	0.060988
100	1852.72 ± 124.963	0.064314
120	2248.06 4± 4463.049	0.031222
150	2807.48 ± 721.017	0.019236

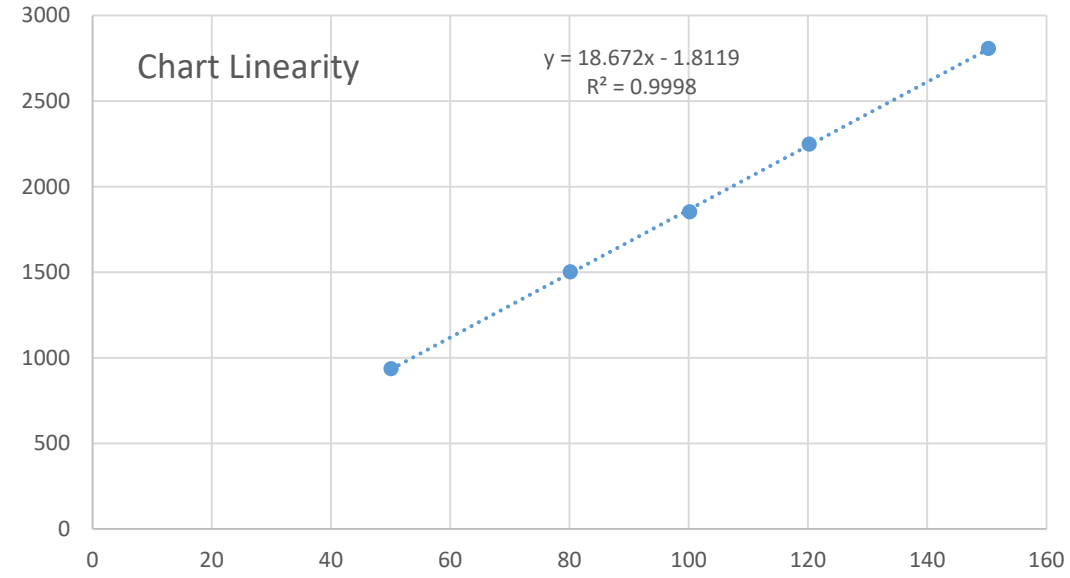


FIGURE 5.14 Linearity graph of sofalcone concentration (ppm) vs. Mean peak area

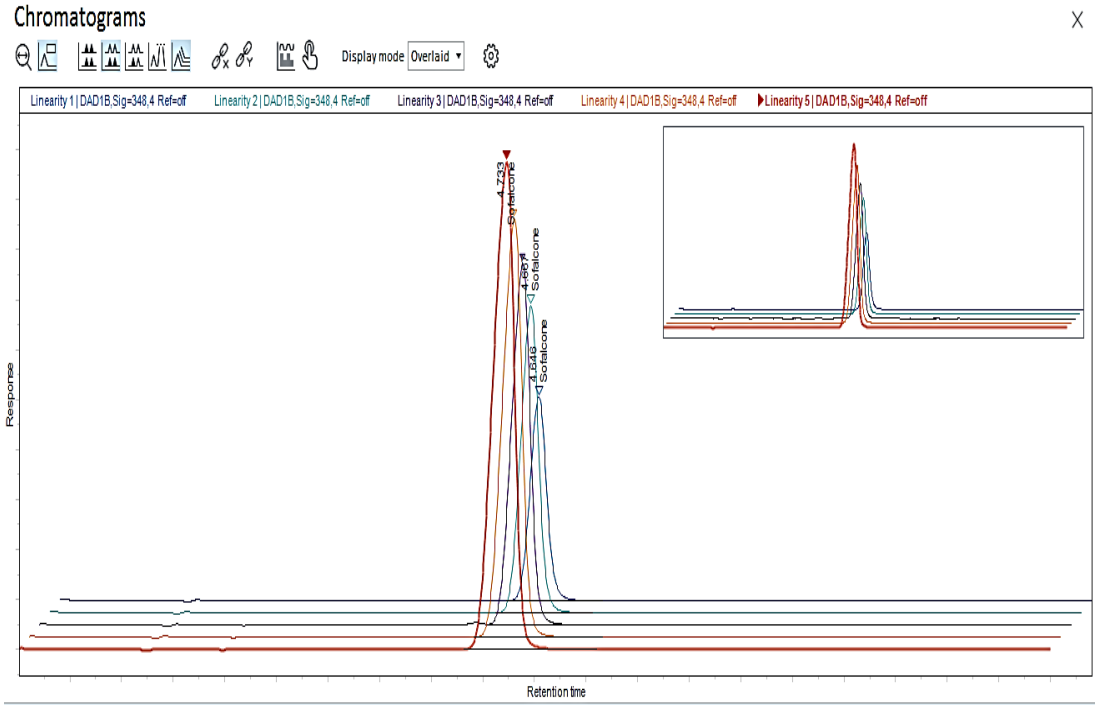


FIGURE 5.15 Linearity Overlain chromatogram of 50-150 µg/ml sofalcone

- **Acceptance Criteria:**

The correlation coefficient ('R') value should not be less than 0.99 over the working range.

- **Observation:**

The correlation coefficient ('R') value = 0.99997

- **Conclusion:**

It can be seen from the linearity graph that the region of the peak response to sofalcone increases as its concentration grows. Hence, the approach is Linear.

5.2.5.5 Accuracy:

How closely test findings resemble the true value is a measure of how accurate an analytical technique is. Accuracy is most commonly reported as a percentage relative to a standard addition of a known analyte. Accuracy is often used to evaluate the efficacy of an analytical method. To test the accuracy of the procedure, sofalcone was injected into the placebo in a predetermined amount. On a chromatograph, different sample solutions such as a "blank," "standard," "placebo," and "precision" were introduced. Percentage recovery, mean percentage recovery, and total percentage RSD of sofalcone were calculated at 50%, 100%, and 150% of the test concentration. Table 5.8 displays the data that was compiled.

TABLE 5.11 Accuracy study data of sofalcone

Level	Concentration of sofalcone w.r.t test concentration (%)	Amount of Sofalcone Added (mg)	Amount of Sofalcone recovered (mg)	% Recovery	Mean % Recovery	% RSD	Overall % recovery and overall % RSD
1	50	99.60	100.90	101.3	101.2	0.2	Overall % recovery = 100.8 Overall % RSD = 0.4
		99.75	101.02	101.3			
		99.75	100.73	101.0			
2	100	199.33	201.15	100.9	100.9	0.2	
		199.28	201.23	101.0			
		199.22	200.64	100.7			
3	150	298.77	300.41	100.6	100.4	0.3	
		298.74	298.80	100.0			
		298.83	300.29	100.5			

➢ **Acceptance Criteria:**

i) sofalcone's average and median recoveries should be around 102 %.

ii) The average recovery for sofalcone should be between 98.0% and 102.0%, according to the manufacturer.

iii) sofalcone's total RSD as a whole must be less than 2%.

➢ **Observation:**

Individual recovery at each level is found within 98.0% to 102.0%.

➢ **% Mean recovery:**

Level	% Concentration	Mean % Recovery	% RSD
1	50	101.2	0.2
2	100	100.9	0.2
3	150	100.4	0.3
Overall % Recovery		**100.8**	
Overall % RSD		**0.4**	

➢ **Conclusion:**

All results are found well within the acceptance criteria. Hence method is accurate.

➢ **Accuracy graph of sofalcone amount added vs. amount found**

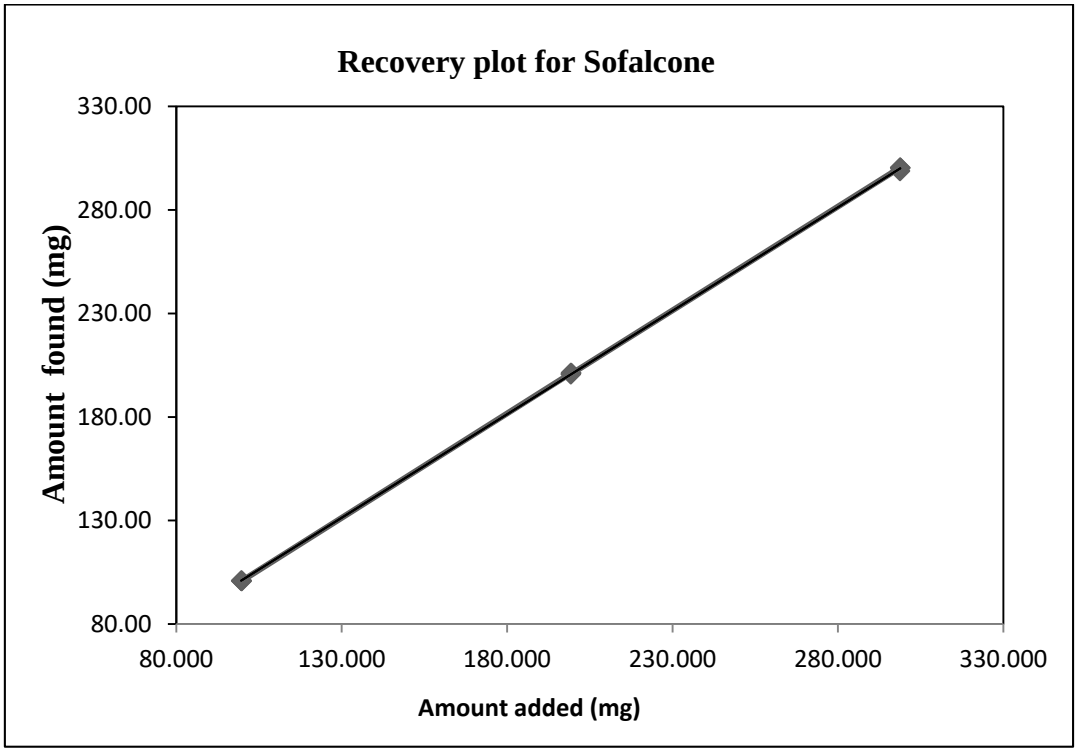


FIGURE 5.16 Recovery plot for sofalcone

5.2.5.6 Precision:

One strategy to assess the accuracy of an analytical technique is to compare the results of several measurements made under controlled conditions on the same homogeneous sample.

5.2.5.6.1 System precision

The accuracy of the system was tested by injecting six duplicates of the standard solution from the same HPLC vial, following the test technique. The gathered information is detailed in Table 5.9.

TABLE 5.12 System precision data for sofalcone

Sr. No.	Replicates of Standard Solution	Peak area of sofalcone
1	Standard solution - 1	1901.76
2	Standard solution - 2	1899.76
3	Standard solution - 3	1901.20
4	Standard solution - 4	1898.62
5	Standard solution - 5	1900.62
6	Standard solution - 6	1899.98
Mean		**1900.32**
SD		**1.12**
% RSD		**0.06**
Tailing Factor		**0.9**
Theoretical plates		**2896.28**

- **Acceptance criteria:**

i) Tailing factor: The tailing factor of the sofalcone peak measured after the first injection of the standard solution should be less than 2.0.

ii) Theoretical plate: sofalcone peak theoretical plates produced after the first injection of the standard solution should be more than or equal to 2000.

iii) RSD: sofalcone peak area from six duplicate injections of standard solution should not vary by more than 2.0% relative standard deviation (RSD).

- **Conclusion:**

All results are found well within the acceptance criteria. Hence, the system is precise.

5.2.5.6.2 Method precision (Repeatability)

When the same conditions were used for a very short time frame, reproducibility indicated how reliable the results were. The chromatograph was used to test the effectiveness of the method on six different samples of sofalcone 100 mg Capsules. Table 5.10 displays the calculated % assay, the mean % assay, and the % RSD.

TABLE 5.13 Method precision data of sofalcone 100 mg Capsules

Sofalcone		
Sr no.	**Conc.(µg/ml)**	**Assay**
1	100	99.58679
2	100	99.86375
3	100	99.37359
4	100	99.13049
5	100	99.03845
6	100	99.06251
Mean	99.3426	
SD	0.3311	
% RSD	0.333291	

- **Acceptance Criteria:**

i) The assay value (both the average and the individual's) must be within the specified range.

ii) Two % RSD or less should separate any six separate findings.

Conclusion:

All results are found well within the acceptance criteria. Hence method is precise.

5.2.5.6.3 Intermediate precision

As indicated in the section on repeatability, a second analyst on a different day with the same set of samples using a different HPLC machine, column, and brand displays intermediate accuracy within the laboratory variance. Six sample solutions of sofalcone 100 mg Capsules were subjected to tests of intermediate precision by three different analyzers on three consecutive days. Use several HPLC systems with comparable columns to analyse sample solutions. In method precision and intermediate precision experiments, calculated% assay, mean% assay, and absolute difference of mean assay values were detected. The obtained data may be shown in Tables 5.11 and 5.12.

TABLE 5.14 Intermediate precision data of sofalcone 100 mg Capsules

Sample No.	% Assay
1	100.20
2	99.70
3	99.90
4	99.40
5	100.40
6	100.00
Mean	**99.9**
SD	**0.36**
% RSD	**0.36**

TABLE 5.15 Absolute difference between mean assay values obtained from the method precision and intermediate precision study for sofalcone 100 mg Capsules

Sample Name	Method Precision	Intermediate Precision	Absolute difference
	Mean % Assay		
Sofalcone 100 mg Capsules	99.3	99.9	0.6

➢ **Acceptance Criteria:**

1. Individual and average assay values should fall within the specified range.

2. The RSD of six measurements should not exceed 2.0%.

3. The absolute difference between the mean % assay results from the method precision study and the intermediate precision study should not exceed 2.0.

➢ **Conclusion:**

All results are found well within the acceptance criteria. Hence method is precise and rugged.

5.2.5.7 Range

The analyte concentration (in terms of quantity) in a sample that has been proven to fall within the demonstrated precision, accuracy, and linearity of the analytical technique is referred to as the range of the method. The range of the method may be found in the table below. In most cases, the range will make use of the same measuring method (% or parts per million) as the analytical data.

➢ **Acceptance Criteria:**

The linearity, accuracy, and precision validation data should be used to determine the range before establishing the range itself.

➢ **Observation:**

Based on the findings of the linearity research, the accuracy study, and the precision study, the acceptable range for the sofalcone assay technique has been determined to be between 50% and 150% of the test concentration.

➢ **Conclusion:**

The established range for sofalcone assay method is 50% to 150% with respect to test concentration.

5.2.5.8 Solution stability

Procedures included making standard and sample solutions, keeping them at room temperature, and checking on them often. Using a series of standard solution injections and sample solution tests, the total % RSD of the sofalcone peak area was calculated and compared to the original % assay result at a number of time points. The data collected is shown in 5.13.

TABLE 5.16 Solution stability data of sample solution at room temperature for sofalcone 100 mg Capsules

Time Interval	% Assay		Average % Assay	Absolute Difference in assay value
	Sample - 1	Sample - 2		
Initial	99.0	98.8	98.9	NA
7 Hours	99.0	98.9	98.9	0.0
13 Hours	99.4	99.1	99.2	0.3
19 Hours	99.7	99.5	99.6	0.7
26 Hours	100.0	99.6	99.8	0.9
32 Hours	100.0	99.7	99.8	0.9
41 Hours	100.4	100.1	100.3	1.4
50 Hours	100.8	100.3	100.5	1.6
59 Hours	101.7	100.4	101.1	2.2*
68 Hours	101.2	100.5	100.9	2.0
76 Hours	101.2	100.7	100.9	2.0

➢ **Acceptance Criteria:**

i) There should not be more than a 2.0% difference between samples of the reference solution for sofalcone taken at various times.

ii) The % assay value of the sample solution at the beginning and at the end of each time period should not be more than 2.0 percentage points apart.

➢ **Observation:**

Sample Solution:

Absolute difference in the % assay values of sofalcone obtained at initial and at 50 hours at room temperature = 1.6

➢ **Conclusion:**

The sample solution is stable up to 50 hours at room temperature.

5.2.5.9 Robustness

The chromatographic parameters were manipulated on purpose to conduct the test. It was feasible to see the effects of these changes on the system suitability parameters and the assay result as a percentage by infusing the standard solution and the sample solution, respectively. Table 5.14 displays the data collected. Variables including wavelength (±2nm), temperature (±5 °C), and flow rate (±0.1) were individually varied by two units to perform a robustness study.

TABLE 5.17 System suitability data of robustness study

Parameter	Optimized	Used	Retention Time (RT), Min	Plate Count	Tailing Factor
Wavelength (± 2nm)	348nm	350	4.681	1896.28	0.87
		346	4.685	1858.04	0.83
Temperature (±5°C)	25°C	20ºC	4.693	1889.18	0.88
		30 °C	4.630	1889.63	0.85
Flow rate (ml/min)	1.0	0.9	4.238	1719.87	0.88
		1.1	5.131	2097.64	0.85

5.2.6 Validation Summary

Sr. No.	Validation Parameter	Results	Acceptance Criteria
4.2.1	**Specificity**		
4.2.1.1	**Check for blank, placebo and impurities interference**		

Sr. No.	Validation Parameter	Results	Acceptance Criteria
	Interference	Blank, placebo, and impurity peaks did not interfere with the retention period of the sofalcone peak.	The peak retention time for sofalcone should be free of blank, placebo, and impurity interference.
	Peak purity	Standards, samples, placebos, and solutions containing both sofalcone and known contaminants all meet the peak purity criterion (Peak purity = purity angle < purity threshold).	Standard solution, sample solution, placebo spiked solution with known impurities, and sofalcone and sample spiked solution with known impurities must all meet peak purity standards (Peak purity = purity angle < purity threshold) for sofalcone peak.
4.2.1.2	**Forced degradation: Check for blank, placebo and degradation products interference**		
	Interference	Nothing resembling blank, placebo, or degradation products is seen interfering with the sofalcone peak.	The peak for sofalcone should not be masked by background noise from blank, placebo, or degradation products.
	Peak purity	The sofalcone peak meets the peak purity requirement (Peak purity = Purity angle < Purity threshold) in all the deteriorated samples.	All of the deteriorated samples should meet the peak purity standards for sofalcone (Peak purity = Purity angle < Purity

<table>
<tr><th>Sr. No.</th><th>Validation Parameter</th><th colspan="4">Results</th><th>Acceptance Criteria</th></tr>
<tr><td></td><td></td><td colspan="4"></td><td>threshold).</td></tr>
<tr><td>4.2.2</td><td>Linearity</td><td colspan="4">Correlation coefficient (‘R’) : 0.999</td><td>Across the operational range, the value of the correlation coefficient ('R') must be greater than 0.99.</td></tr>
<tr><td rowspan="6">4.2.3</td><td rowspan="6">Accuracy</td><td>Level</td><td>% Conc.</td><td>Mean % Recovery</td><td>% RSD</td><td rowspan="6">Sofalcone's individual and average recoveries should fall in the range of 98.0% to 102.0%.
Sofalcone's mean overall recovery rate is anticipated to be between 98.0% and 102.0%.
The maximum allowable RSD (% RSD) for sofalcone is 2.0%.</td></tr>
<tr><td>1</td><td>50</td><td>100.45</td><td>0.05</td></tr>
<tr><td>2</td><td>100</td><td>100.04</td><td>0.03</td></tr>
<tr><td>3</td><td>150</td><td>100.54</td><td>0.02</td></tr>
<tr><td colspan="2">Overall % Recovery</td><td colspan="2">100.34</td></tr>
<tr><td colspan="2">Overall % RSD</td><td colspan="2">0.23</td></tr>
<tr><td>4.2.4</td><td colspan="6">Precision</td></tr>
<tr><td>4.2.4.1</td><td>System precision</td><td colspan="4">Tailing factor = 0.9</td><td>Tailing factor: It is unacceptable to have a tailing factor of more than 2.0 for the sofalcone peak produced from the first injection of the standard solution.
Theoretical Plates: sofalcone theoretical</td></tr>
</table>

Sr. No.	Validation Parameter	Results	Acceptance Criteria
		Theoretical plates = 3180 % RSD = 0.76	plates from the first standard solution injection should be more than 2000. **RSD:** sofalcone peak area from six duplicate injections of standard solution should not have a relative standard deviation more than 2.0%.
4.2.4.2	**Method precision**	**% Assay of sofalcone 100 mg Capsules:** Sample 1 = 99.9 Sample 2 = 99.9 Sample 3 = 99.4 Sample 4 = 99.1 Sample 5 = 99.0 Sample 6 = 99.1 Mean % assay = 99.3 RSD of six determinations = 0.3%	Assay values, both on an individual and average basis, must fall within a predetermined range. There should not be more than a 2.0% RSD between each set of six conclusions.
4.2.4.3	**Intermediate precision**	**% Assay of sofalcone 100 mg capsules:** Sample 1 = 100.2 Sample 2 = 99.7 Sample 3 = 99.9 Sample 4 = 99.4 Sample 5 = 100.4 Sample 6 = 100.0 Mean % assay = 99.9	Assay values, both on an individual and average basis, must fall within a predetermined range. There should not be more than a 2.0% RSD between each set of six conclusions. Mean percentage assay

Sr. No.	Validation Parameter	Results	Acceptance Criteria
		RSD of six determinations = 0.4% Absolute difference = 0.3	results from the method precision study and the intermediate precision study should not deviate by more than 2.0 in absolute terms.
4.2.7	**Solution Stability**	**Standard Solution:** Up to 78 hours at room temperature, the relative standard deviation of sofalcone peak area was 0.7%. **Sample Solution:** The initial and 50-hoursroom-temperature sofalcone sample solution test values are different by an absolute value of 1.6	Sofalcone peak area in the standard solution acquired at different times should not deviate by more than 2.0% from the mean. The % assay value of the sample solution before and after each time period must not deviate by more than 2.0 in absolute terms.

5.3 Summary of Developed Stability Indicating RP-HPLC method

All metrics, including plate count (found to be >4000) and tailing factor (found to be ± 2), were determined to be within the specified limit despite the fact that variation in flow rate altered the retention period of the primary peak.

- **Acceptance criteria:**

i) Tailing Factor: The sofalcone peak at the first standard solution injection should have a tailing factor of no more than 2.0.

ii) Theoretical plates: No less than 2000 theoretical plates of the sofalcone peak should be seen after the first injection of the standard solution.

iii) RSD: Six duplicate injections of the reference solution should provide a peak area for sofalcone with a relative standard deviation of no more than 2.0%.

iv) RSD of % assay the relative standard deviation (RSD) for test findings from three different sample solutions for each different condition should not exceed 2.0%.

v) Assay findings should have a relative standard deviation (RSD) of not more than 2.0% as a consequence of technique accuracy and each different circumstance.

- **Conclusion:**

The results are found well within acceptance criteria with respect to Change in wavelength, temperature, Flow rate.

CHAPTER 6

6 Development and validation of a rapid RP-HPLC method for the determination of clidinium bromide, chlordiazepoxide and pantoprazole sodium in their combined capsule dosage form

6.1 Material and Methods

6.1.1 Reagents and chemical

Sr. No.	Name	Manufactured by / Supplied by
1	Chlordiazepoxide	Ontop pharmaceuticals Pvt. Ltd. , Bangalore
2	Clidinium Bromide	Ontop pharmaceuticals Pvt. Ltd. , Bangalore
3	pantoprazole sodium	Aum research Laboratories, Ahmedabad

All other chemicals were of analytical grade and procured from Merck Specialties Private Limited.

6.1.2 Instruments and Equipments

Sr. No	Instruments Name	Instruments No.	Model	Make
1	Infra-red spectrophotometer	--	Infra 3000A FT-IR Model	Analytical Technologies Limited
2	UV-Visible double beam spectrophotometer	A114548	UV 1800	Shimadzu, Kyoto, Japan
3	pH METER	--	CL 180	Chemiline Digital pH meter
4	Analytical Balance	KE-129	K-EA 210	K-Roy Instrument Pvt. Ltd.
5	Melting Point Apparatus	DDPC/210/09-10	--	Vijay laboratory furnisher
6	Ultrasonic Bath sonicator	--	UC 3000	PEI

7	High Performance Liquid Chromatography Instrument (HPLC)			
	HPLC Instrument 1:	--	Ezchrom 2006	Agilent 1260 Infinity Quaternary LC
	HPLC Instrument -2	--	Clarity software	Analytical Technologies Limited

6.1.3 Selection of chromatographic condition

Size, solubility, and molecular composition all play a role in determining the optimal HPLC method to utilise (ionic, ionizable, or neutral molecule). The polarity of the medications in this investigation dictates which of three chromatographic methods reversed phase, ion-pair, or ion-exchange must be used. For the preliminary separations, we employed reversed phase HPLC since it is a convenient and adaptable technique. The effects of the mobile phase's pH, flow rate, and solvent ratio were examined to determine the optimal chromatographic conditions. Column efficiencies, capacity factors, and asymmetry factors were determined from the chromatograms that were taken. In order to generate a reliable forecast, we focused exclusively on the most favourable scenarios in terms of resolution, symmetry, and capacity factor.

6.1.4 Selection of detection wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. In the present study individual drug solutions of 20 µg/ml CLBr, 40 µg/ml CDZ and 160 µg/ml PNT were prepared in solvent mixtures of 50 ml 0.4%TEA, 30 ml Methanol and 20 ml Acetonitrile (pH 6.0 adjusted with Orthophosphoric acid). These drug solutions were than scanned in the UV region of 200 - 400 nm and the overlay spectrum was recorded.

6.1.5 Effect of ratio of mobile phase

The following mobile phases were used to chromatograph a mixed standard solution comprising 25 µg/ml of CLBr, 50 µg/ml of CDZ, and 200 µg/ml of PNT:

Mobile phase	**Proportion ratio (*v/v*)**	**Justification**	**Detection Wavelength [nm]**
Methanol : Water	50:50 (*v/v*)	Not well separated	220
Acetonitrile : Water	50 : 50 (*v/v*)	Not well separated	220
0.05 M Phosphate Buffer: methanol : Acetonitrile (pH 3.0 with *O*-Phosphoric acid)	40:40:20 (*v/v/v*)	Broad in 3rd peak and no proper resolution	220
0.05 M Phosphate Buffer: methanol : Acetonitrile(pH 3.0 with *O*-Phosphoric acid)	50:30:20 (*v/v/v*)	Proper resolution but not good peak shape with Tailing in 1st peak and 3rd peak	220
Water: methanol : Acetonitrile(pH 6.0 with *O*-Phosphoric acid)	50:30:20 (*v/v/v*)	Good Resolution but not sharp peak	220
0.4%TEA in Water: methanol : Acetonitrile(pH 6.0 with *O*-Phosphoric acid)	50:30:20 (*v/v/v*)	three symmetrical peaks with good resolution	220

A cellular phase of 0.4% was identified as necessary for testing and refinement. It can be broken down into 50 % acetonitrile, 30 % orthophosphoric acid, and 20 % water to form TEA. As it produced three symmetrical peaks with high resolution, this blend was regarded adequate. The best conditions for chromatography are shown below.

Sr. No	Parameter	Condition
1	Mobile Phase	0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v*)
2	Pump mode	Isocratic
3	Stationary phase	Sunshell_Coreshell Column C18, (100 × 4.6 mm i.d), Particle size 2.6 nm
4	Flow rate (ml/min)	1.6
5	Run time (min)	10min
6	Volume of Injection (µl)	10.0
7	Detection wavelength (nm)	220nm
8	Retention time (min)	CLBr: 1.318 PNT: 2.434 CDZ:4.691
9	Diluent	Mobile Phase

6.1.6 Estimation of CLBr, CDZ and PNT by RP-HPLC Method

6.1.6.1 Preparation of Mobile Phase:

The mobile phase consisted of 500 ml of a 0.4% TEA solution in double-distilled water, 300 ml of methanol, and 200 ml of acetonitrile (v/v). The ultimate pH of the mobile phase was adjusted by adding orthophosphoric acid after organic solvents were combined (pH 6).

6.1.6.2 Standard CLBr stock solution (500 µg/ml)

After being precisely weighed, 12.5 mg of CLBr was added to a 25 ml volumetric flask, where it was dissolved in and diluted with methanol to the appropriate concentration.

6.1.6.3 Standard CDZ stock solution (1000 µg/ml)

CDZ (25.0 mg) was accurately weighed, then added to a 25 ml volumetric flask, dissolved in methanol, and diluted to the appropriate concentration with methanol.

6.1.6.4 Standard PNT stock solution (4000µg/ml)

PNT (100.0 mg) was accurately weighed and then dissolved in and diluted with methanol to fill a 25 ml volumetric flask.

6.1.6.5 Mixed standard stock solution of CLBr, CDZ and PNT

Standard stock stock solutions of 100 µg/ml CLBrµ, 200 µg/ml CDZ, and 800 µg/ml PNT were prepared by combining 10 ml aliquots of the respective stock solutions in a 50 ml volumetric flask and then filling to the mark with mobile phase.

6.1.7 Calibration curve for CLBr, CDZ and PNT

A 10-ml volumetric flask was charged with the correct amount of an aliquot from a mixed standard stock solution of CLBr, CDZ, and PNT. The concentrations of CLBr, CDZ, and PNT in the solution were as follows: 10, 15, 20, 25, and 30 µg/ml; 80, 120, 160, 200, and 240 µg/ml; and 240 µg/ml. The combined standard solution was chromatographed under the same conditions as the other solutions. Each solution was filtered through a 0.45 µm membrane before being utilised. Calibration curves were constructed by plotting the mean peak area vs drug concentrations for all three drugs. Linear equations were derived from these calibration curves.

6.1.8 Determination of CLBr, CDZ and PNT from combined dosage form

6.1.8.1 Sample preparation

We purchased commercially available forms of ULRAX. Careful measurements and weights were taken of twenty pills and a solid material of the same mass. A volumetric flask of 25 ml was precisely filled with 12.5 mg CLBr, 25 mg CDZ, and 100 mg using the given weights. The medication was dissolved by adding 15 ml of methanol to the volumetric flask and sonicating the mixture for 20 minutes. The filtered solution was transferred to a 25ml volumetric flask after being processed using Whattman filter paper (0.45 µm). A solution containing 500 µg/ml of CLBr, 1000 µg/ml of CDZ, and 4,000 µg/ml of PNT was prepared by shaking the flask and adding the appropriate quantity of methanol. We prepared a solution containing 20 µg/ml of CLBr, 40 µg/ml of CDZ, and 160 µg/ml of PNT by adding 2 ml of this aliquot to a 50 ml volumetric flask and filling the remainder with methanol. The outcome was analysed using the provided procedure.

6.1.8.2 Estimation of CLBr, CDZ and PNT in combined dosage form

Using mobile phase at a flow rate of 1.6 ml/min, the prepared sample solution was chromatographed for 10 minutes. The quantities of three medications were determined by analysing the chromatograms and measuring the peak areas.

6.1.9 Method Validation

A blank sample matrix was ingested, followed by samples spiked at three different quantities (50, 100, and 150% of the original quantity) to evaluate the method's dependability. For each concentration, three independent chromatographic runs were conducted to estimate the average recoveries.

6.1.9.1 Precision

To determine the test's reproducibility, six identical test samples were measured. The findings of a research on the accuracy of CLBr, CDZ, and PNT throughout the day and between days are shown in terms of CV. The quantities of CLBr, CDZ, and PNT were measured three times on the same day and three times on three subsequent days as part of this investigation.

6.1.9.2 Linearity

Standard stock solutions of CLBr, CDZ, and PNT were diluted to the appropriate concentrations and aliquoted into a 10-ml volumetric flask. The concentrations of CLBr, CDZ, and PNT in the solution were as follows: 10, 15, 20, 25, and 30 µg/ml; 80, 120, 160, 200, and 240 µg/ml; and 240 µg/ml. The mixed standard solution was chromatographed under the same circumstances (n=6) as before. Each solution was filtered through a 0.45 µm membrane before being utilised. Calibration curves were constructed by plotting the mean peak area vs drug concentrations for all three drugs. Linear equations were derived from these calibration curves.

6.1.9.3 Specificity and Selectivity

Quantitative detection of analytes in the presence of other compounds in the sample matrix is referred to as "specificity." Selectivity, on the other hand, allows one to qualitatively pinpoint the analyte amongst other potential confounding factors in the sample matrix.

6.1.9.4 Detection limit and Quantitation limit

Under the ICH guideline, there are a few different approaches that may be used to determine the detection and quantitation limits. A visual examination, calculating the signal-to-noise ratio, calculating the standard deviation of the response, and calculating the slope of the

calibration curve are the components that make up these tests. The third method was used to arrive at the LOD and LOQ values for this inquiry, which were found to be 3.3 σ /S and 10 σ /S, respectively. In this case, stands for the standard deviation σ of the y-intercepts of the regression lines, and s refers to the slope of the calibration curve.

6.1.9.5 Robustness

The approach's usefulness was established by monitoring test solutions after implementing small, controlled changes to the analytical conditions. Using the suggested method, we were able to lower the flow rate to 1.6 ± 0.2 ml/min and raise the mobile phase pH to 6.0±0.2. Retention period CV values were consistently below 2%.

6.1.9.6 System suitability

Experiments on the feasibility of the system showed that the suggested method could provide enough resolution and high repeatability between the peaks of interest. Liquid chromatography requires system compatibility testing as part of the process. Peak area, theoretical plates (N), resolution (R), and tailing factors of each solute were analysed using six replicate injections of newly produced standard solutions to assess the system's efficacy (T). A peak resolution (R) of more than 2.0 between two adjacent peaks for three analytes, a theoretical plate number (N) of at least 2,000 for each peak, and a USP tailing factor (T) of less than 1.5 were all required for CLBr, CDZ, and PNT systems. The results show that the suggested layout meets these needs while staying within the established restrictions.

6.1.9.7 Solution stability

We monitored the solution's absorbance over the course of 24 hours at 4-hoursintervals using several analysts and the same equipment to determine the solution's consistency over time.

6.2 Results and Discussion

6.2.1 Selection of chromatographic condition

Both the peak parameter and the resolution of the chromatographic run were maximised. At the early step in selecting the mobile phase, many ratios of methanol-water and acetonitrile-water were tried, but the resolution of the three drugs was not sufficient. After then, several other combinations of acetonitrile, methanol, and water were tested, all at a pH of 6.0. There's a lot going on here, which is great, but the peaks aren't very well formed. To enhance the peak forms of amine medicines, I diluted TEA in water to a concentration of 0.4%. Three symmetrical peaks were seen between CLBr and CDZ (with values of 9.21 and 14.05, respectively) and between CDZ and PNT (with a flow rate of 1.6 ml/min) in a mixture of 0.4%TEA, methanol, and Acetonitrile at a pH of 6 adjusted with orthophosphoric acid (50:30:20 *v/v*). There was a significant difference in retention times between CLBr (1,318 minutes), CDZ (2,434 minutes), and PNT (4,691 minutes). CLBr had an asymmetry factor of 1.37, CDZ was 1.15, and PNT was 0.99. To determine the optimal detection wavelength, we applied the system suitability parameter to the whole UV spectra of CLBr, CDZ, and PNT. Studies indicated that the medicines were well absorbed at 220 nm.

HPLC INSTRUMENT- 2

Make: waters (India) Pvt. ltd.

Detector: water 996PDA detector

Injector: Rheodyne 7725i

Pump : 515 Hplc pump

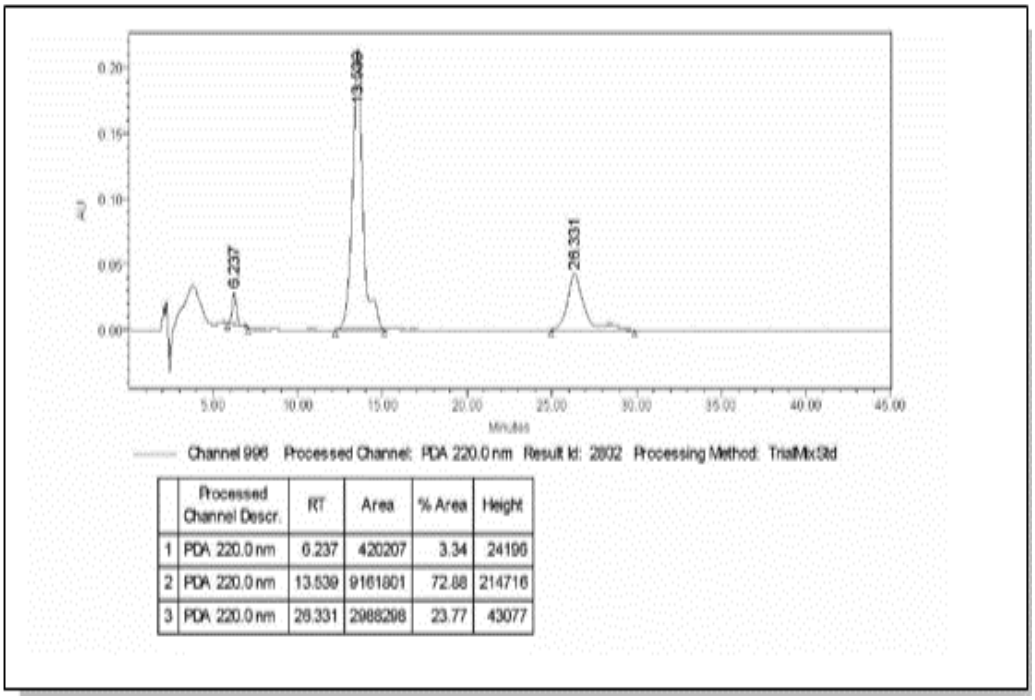
Column: JNJ ODS BP, Column C18, and 250 × 4.6 mm (5 µm)

Software: Millennium software

Detection wavelength: 220nm

Injection volume: 20 microliter

Flow rate: 1ml/min



	Processed Channel Descr.	RT	Area	% Area	Height
1	PDA 220.0 nm	6.237	420207	3.34	24196
2	PDA 220.0 nm	13.539	9161801	72.88	214716
3	PDA 220.0 nm	26.331	2988298	23.77	43077

FIGURE 6.1 Chromatogram of standard solution containing 25 µg/ml CLBr, 50 µg/ml CDZ and 200 µg/ml PNT using mobile phase 0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v/v*)

HPLC INSTRUMENT -3

Make: Analytical Technologies Limited

Detector: UV 3000 scanning spectrophotometer

Injector: S 5200 sample injector

Pump : P3000 Plus HPLC pump

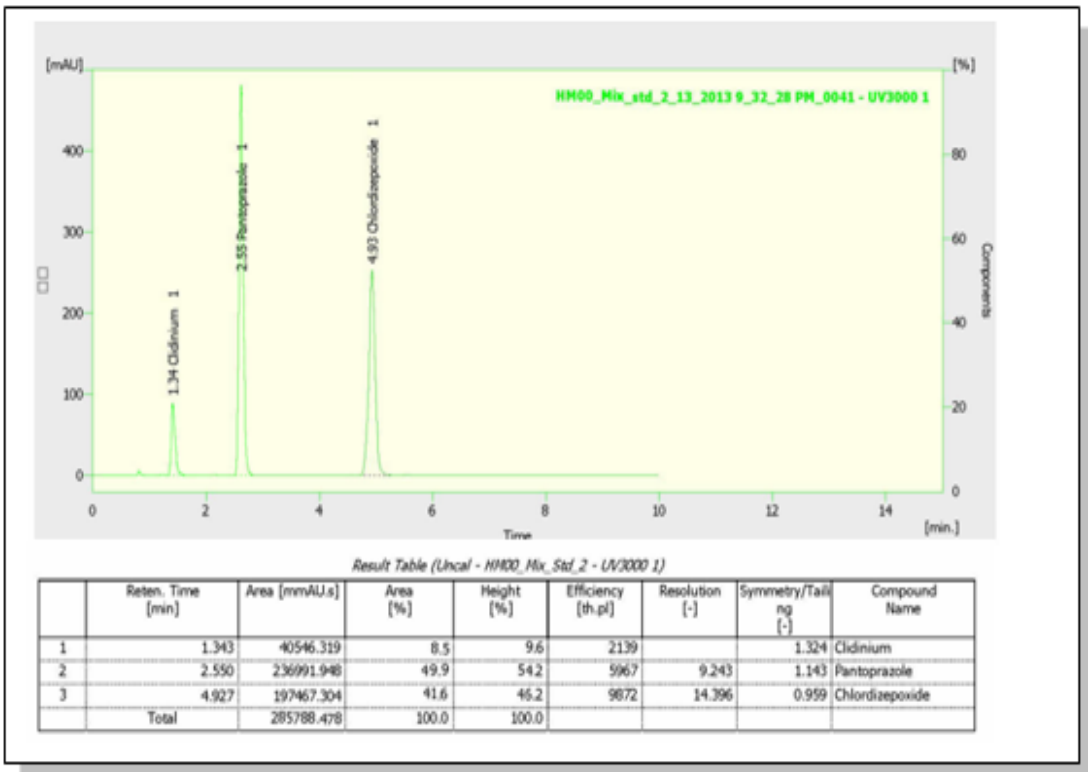
Column: Sunshell_Coreshell ODS- 2.6µm (4.6mm × 100mm)

Software: Clarity software

Detection wavelength: 220nm

Injection volume: 10 microliter

Flow rate: 1.6ml/min



Result Table (Uncal - HM00_Mix_Std_2 - UV3000 1)

	Reten. Time [min]	Area [mmAU.s]	Area [%]	Height [%]	Efficiency [th.pl]	Resolution [-]	Symmetry/Tailing [-]	Compound Name
1	1.343	40546.319	8.5	9.6	2139		1.324	Clidinium
2	2.550	236991.948	49.9	54.2	5967	9.243	1.143	Pantoprazole
3	4.927	197467.304	41.6	46.2	9872	14.396	0.959	Chlordiazepoxide
	Total	285788.478	100.0	100.0				

FIGURE 6.2 Chromatogram of standard solution containing 100 µg/ml CLBr, 200 µg/ml CDZ and 800 µg/ml PNT using mobile phase 0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v/v*)

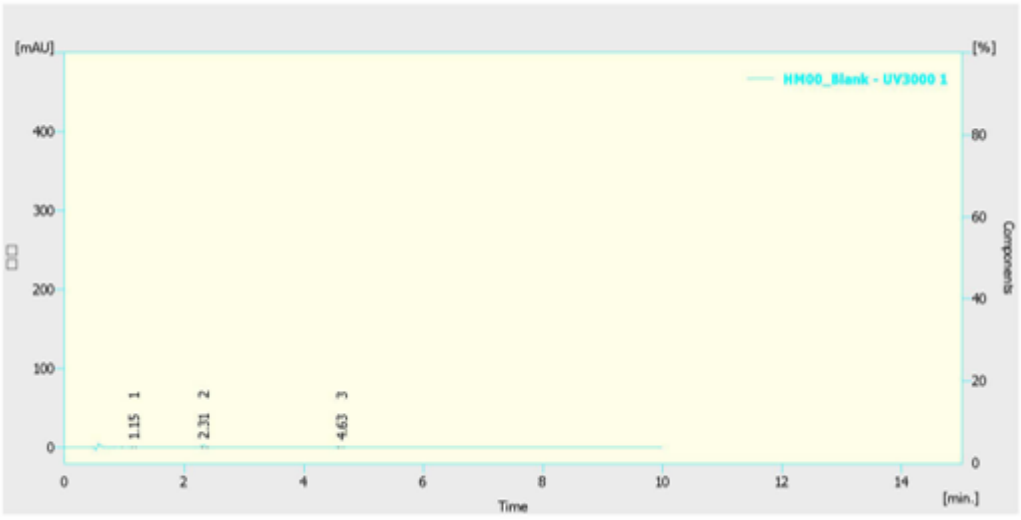


FIGURE 6.3 Chromatogram of Blank solution containing mobile phase 0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v/v*)

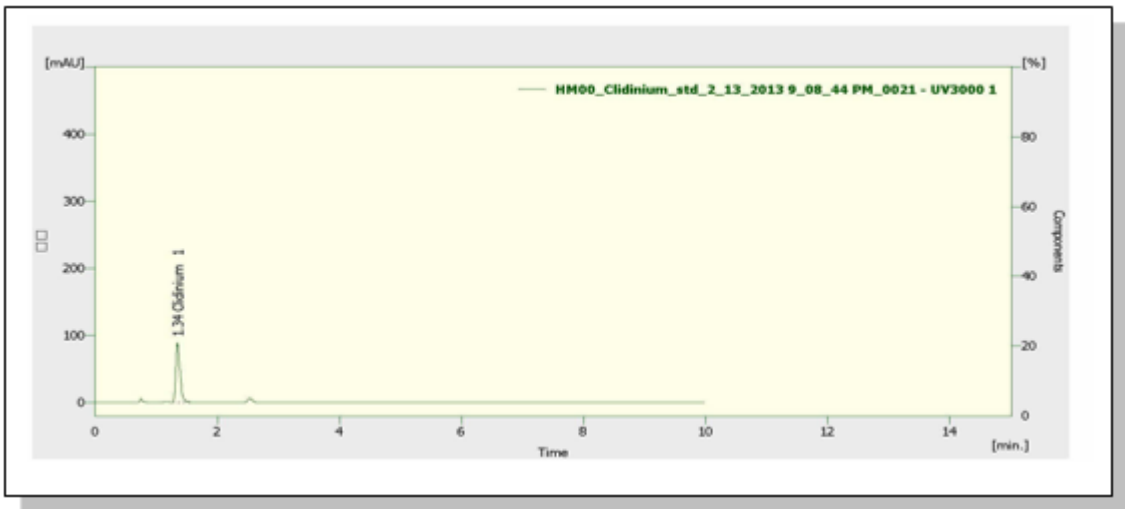


FIGURE 6.4 Chromatogram of standard solution containing 100 µg/ml CLBr using mobile phase 0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v/v*)

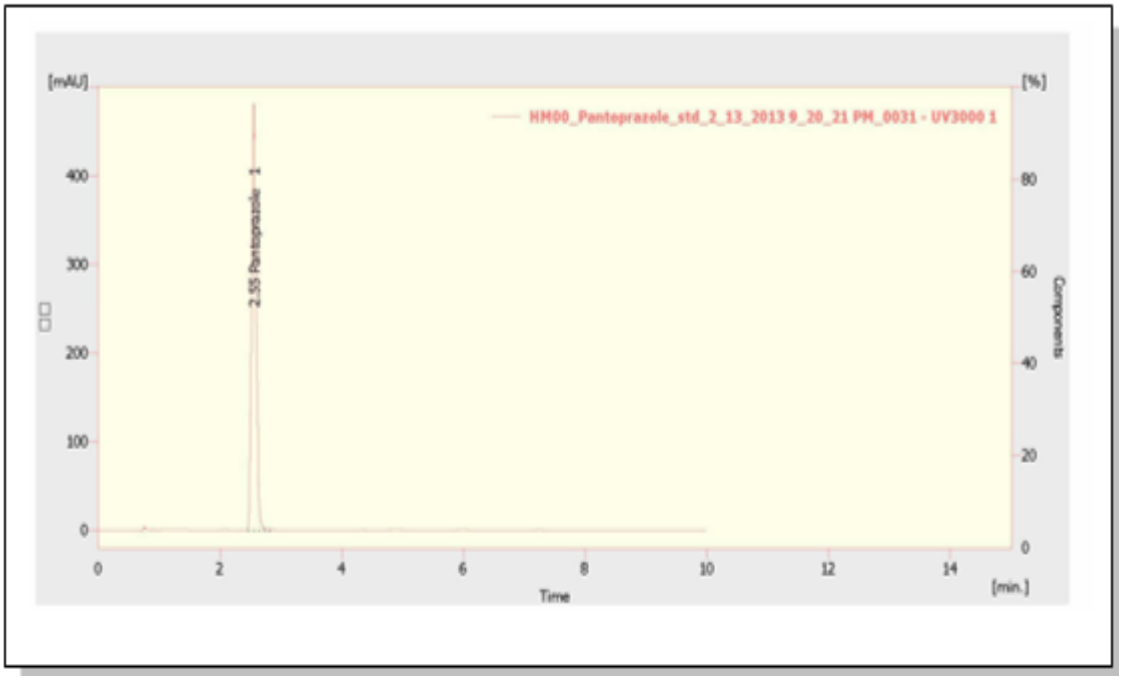


FIGURE 6.5 Chromatogram of standard solution containing 800 µg/ml PNT using mobile phase 0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v/v*)

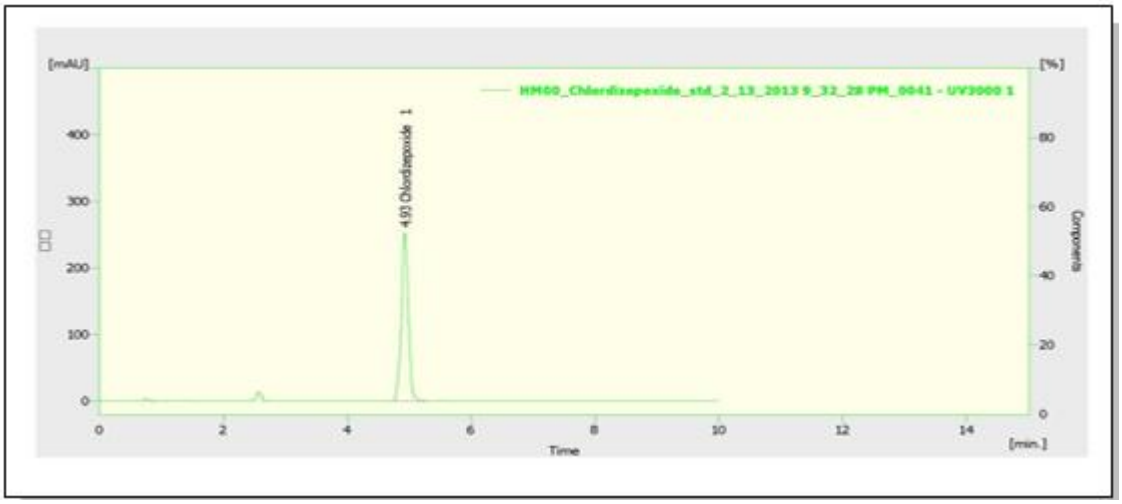


FIGURE 6.6 Chromatogram of standard solution containing 200 µg/ml CDZ using mobile phase 0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v/v*)

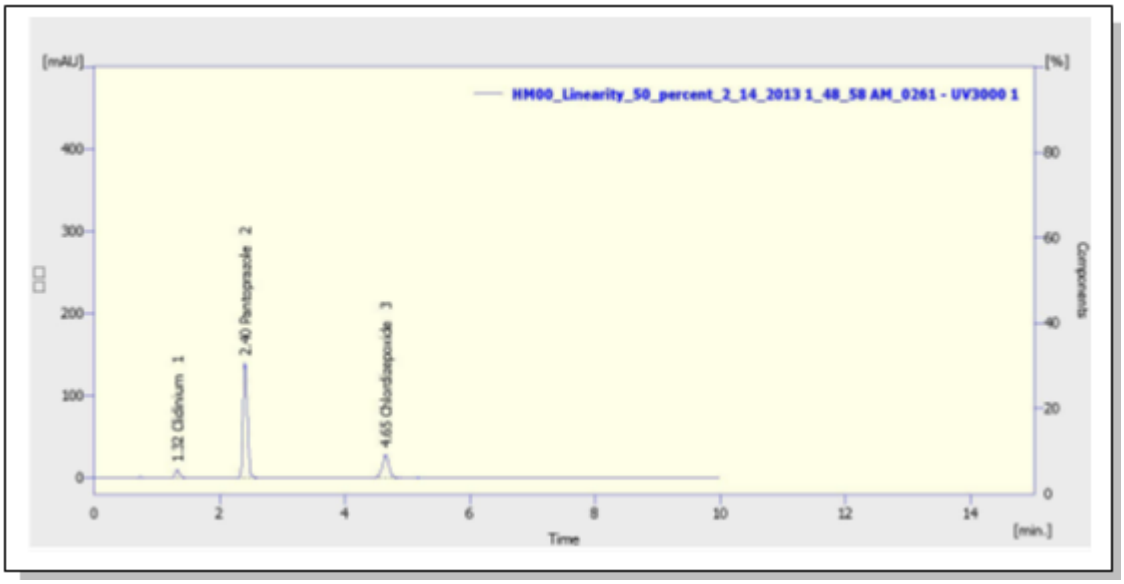


FIGURE 6.7 Chromatogram of standard solution containing 10 µg/ml CLBr, 20 µg/ml CDZ and 80 µg/ml PNT using mobile phase 0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v/v*)

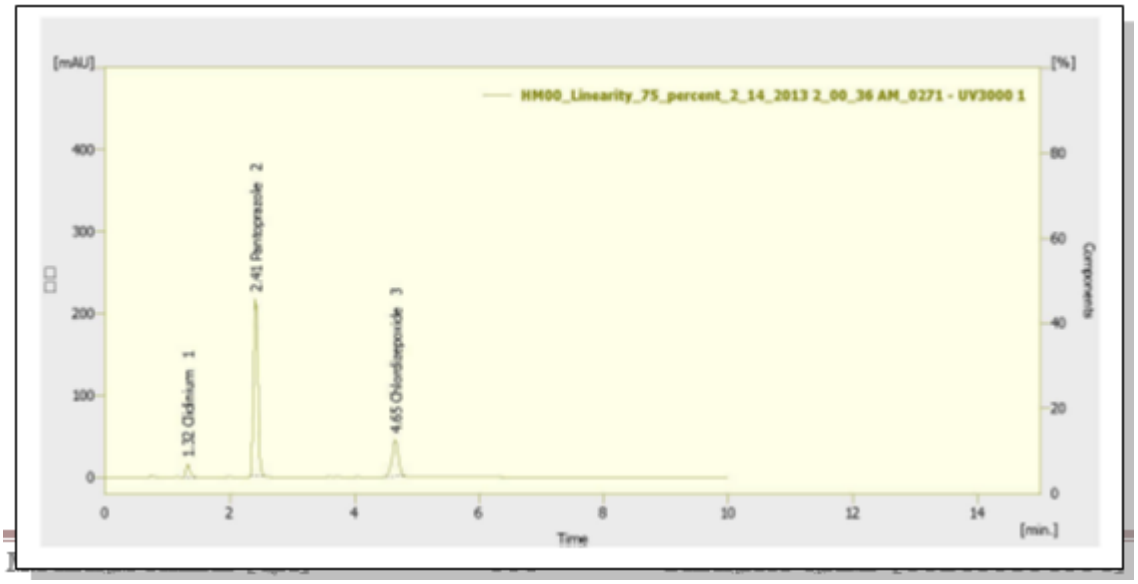


FIGURE 6.8 Chromatogram of standard solution containing 15 µg/ml CLBr, 30 µg/ml CDZ and 120 µg/ml PNT using mobile phase 0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v/v*)

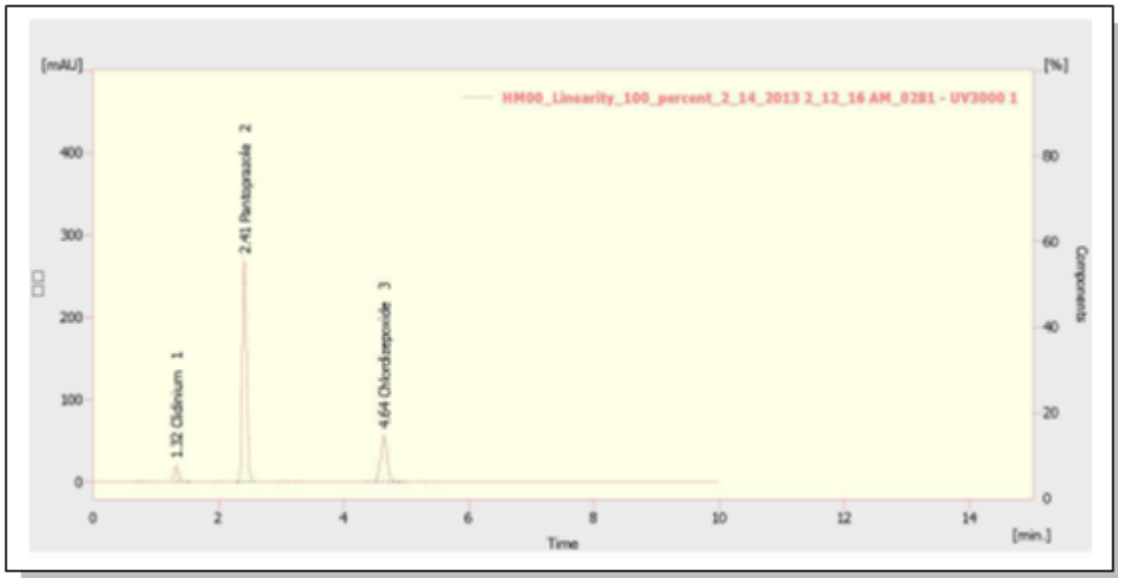


FIGURE 6.9 Chromatogram of standard solution containing 20 µg/ml CLBr, 40 µg/ml CDZ and 160 µg/ml PNT using mobile phase 0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v/v*)

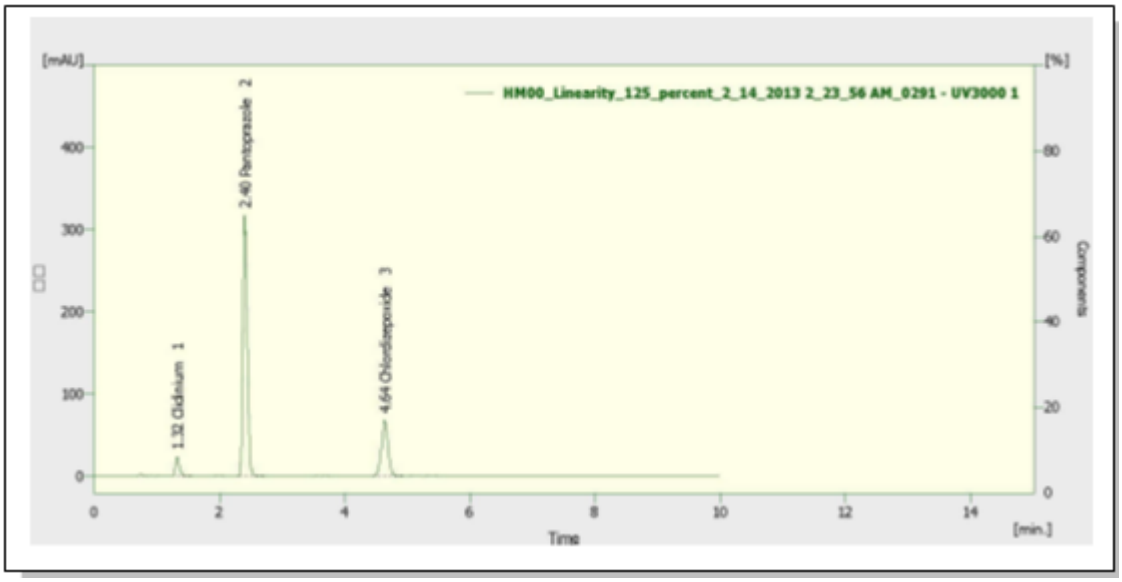


FIGURE 6.10 Chromatogram of standard solution containing 25 µg/ml CLBr, 50 µg/ml CDZ and 200 µg/ml PNT using mobile phase 0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v/v*)

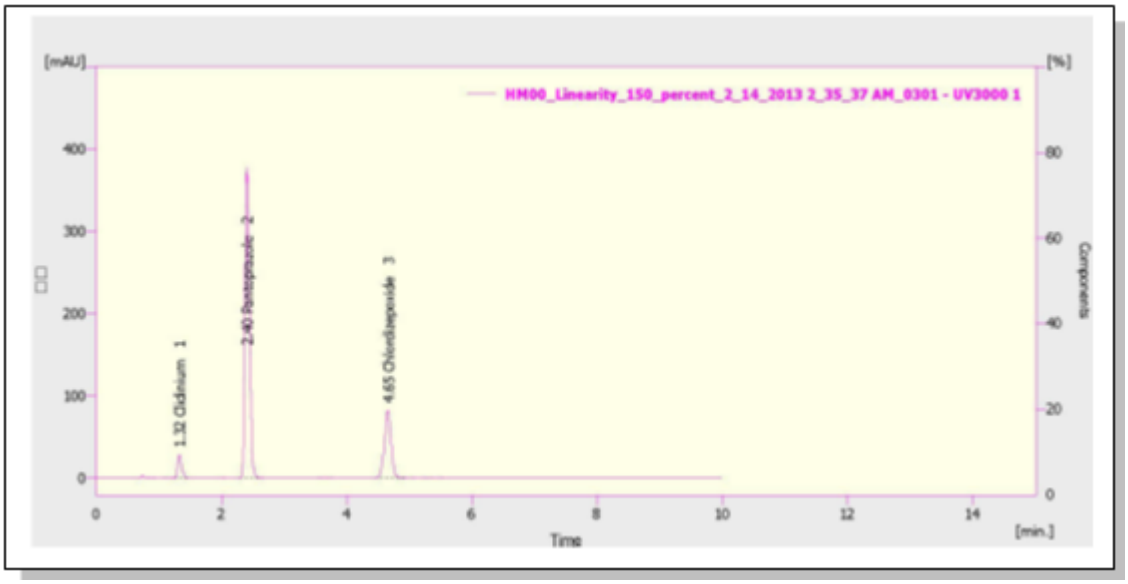


FIGURE 6.11 Chromatogram of standard solution containing 30 µg/ml CLBr, 60 µg/ml CDZ and 240 µg/ml PNT using mobile phase 0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v/v*)

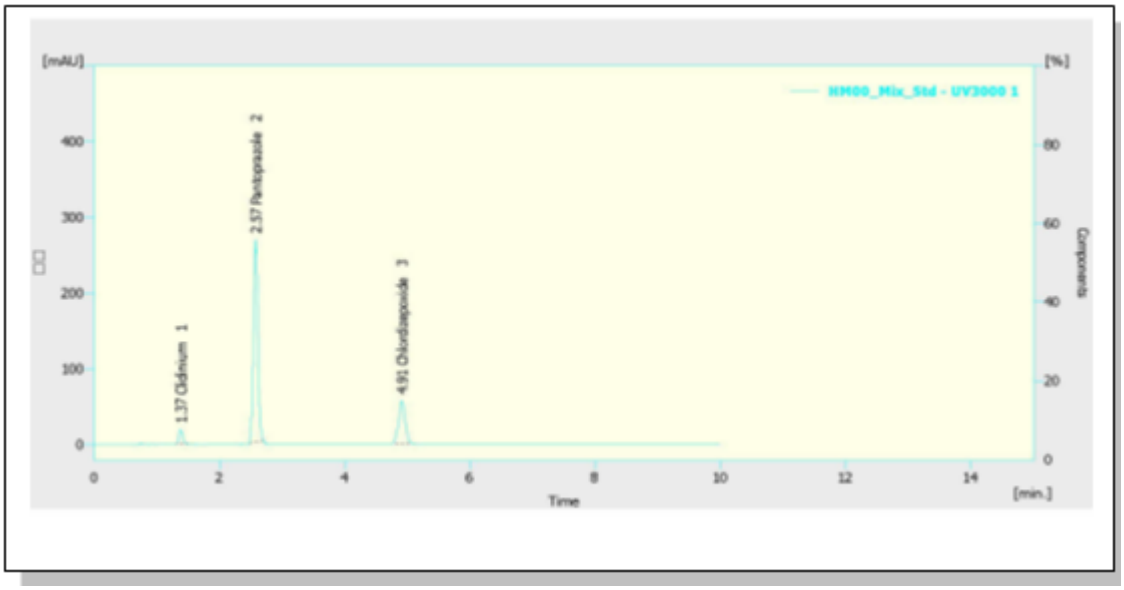


FIGURE 6.12 Chromatogram of Capsule formulation containing 20µg/ml CLBr, 160 µg/ml PNT and 40µg/ml CDZ using mobile phase 0.4% TEA: methanol: Acetonitrile (pH-6 adjusted by OPA) (50:30:20 *v/v/v*).

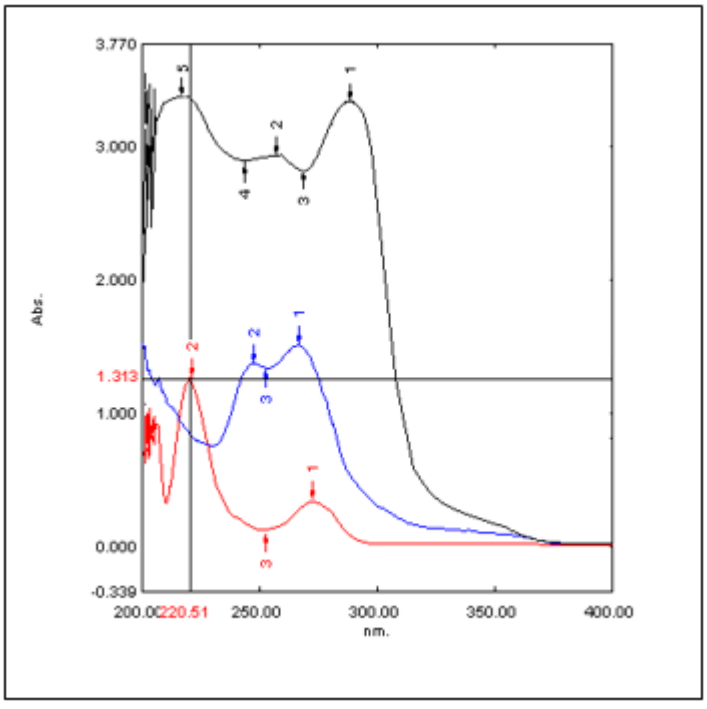


FIGURE 6.13 Overlain Spectra of 10 µg/ml CLBr, 20 µg/ml CDZ and 160 µg/ml PNT in Mobile Phase

6.2.2 Calibration curve for CLBr, CDZ and PNT

TABLE 6.1 Result of calibration readings for CLBr by HPLC method

Concentration (µg/ml)	Area Mean ± S.D. (n=6)	C.V.
10	4617.33 ±8.33	0.18
15	6631.17± 8.83	0.13
20	8343.45 ± 10.08	0.12
25	10304.67± 9.15	0.088
30	12408.59 ± 12.63	0.101

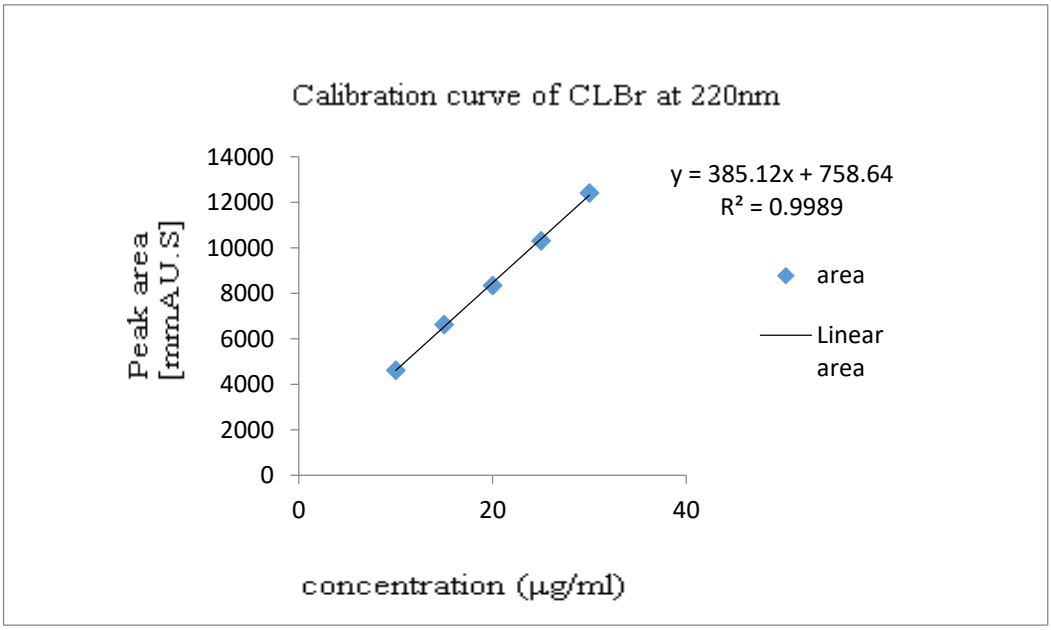


FIGURE 6.14 Calibration curve of CLBr by HPLC method

TABLE 6.2 Result of calibration readings for PNT by HPLC method

Concentration (µg/ml)	Area Mean ± S.D. (n=6)	C.V.
80	65477.01 ±14.55	0.022
120	99881.65± 20.72	0.020
160	126986.32±15.06	0.011
200	150547.09± 20.53	0.013
240	182768.69± 19.86	0.010

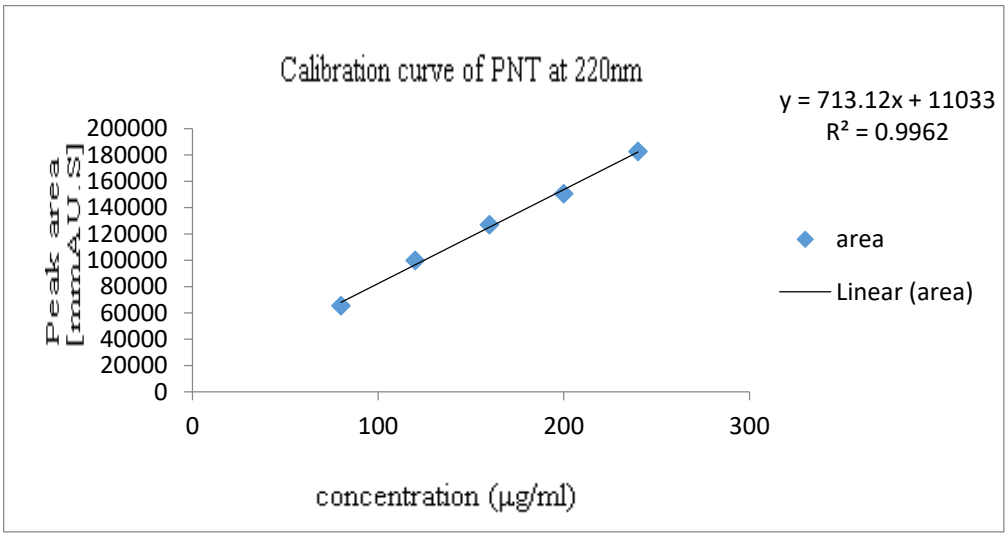


FIGURE 6.15 Calibration curve of PNT by HPLC method

TABLE 6.3 Result of calibration readings for CDZ by HPLC method

Concentration (µg/ml)	Area Mean ± S.D. (n=6)	C.V.
20	20528.96±21.00	0.102
30	30937.13± 13.74	0.044
40	41412.01±11.30	0.027
50	50025.04± 10.27	0.020
60	61787.06± 6.48	0.010

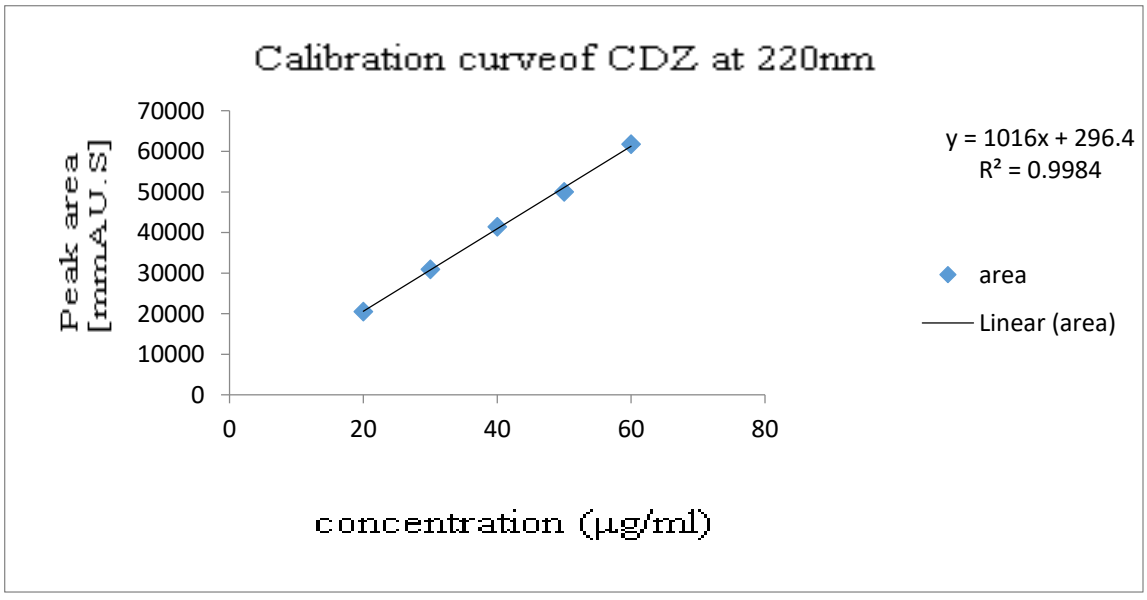


FIGURE 6.16 Calibration curve of CDZ by HPLC method

6.2.3 Validation of the Developed HPLC Method

The HPLC procedure was developed and validated. Using the HPLC technique, we calculated the linear range, correlation coefficient, limit of detection and quantitation, and standard deviation for CLBr, CDZ, and PNT (Table 6.5). The accuracy was calculated once the recovery was made. Recoveries of 99.34% to 99.71% for CLBR, 99.66% to 100.21% for CDZ, and 99.76% to 99.97% for PNT indicated that the method was successful (Table.6.6-6.9). The three drugs were evaluated based on their consistency and variation from day to day. The intraday CLBr C.V. varied from 0.90 to 1.56, the interday ranged from 0.65 to 0.93, the CDZ intraday C.V. ranged from 0.23 to 1.23, the PNT intraday C.V. ranged from 0.43 to 1.04, and the PNT interday C.V. ranged from 0.31 to 0.78. As no problems were found while testing the medications with the excipients present, the approach was reliable. The procedure was trustworthy even though the flow rate was only able to be adjusted by 1.6+0.2 ml/min and the pH of the mobile phase was only able to be adjusted by 6+0.2. Throughout the retention period as a whole, all of the C.V. values that were less than 2% are shown in

TABLE 6.4 System suitability results of the proposed method

Compound	**N**	**R**	**T**	**C.V. of**	
				***t*R**	**Peak area**
CLBr	2133		1.37	1.09	0.74
PNT	5756	9.21	1.15	1.76	0.78
CDZ	9402	14.05	0.99	1.53	0.45
Required limits	*N*> 2000	*R*>2	*T* < 1.5	C.V < 2%	

Where, *N*: theoretical number of plates; *R*: resolution; *T*: USP tailing factor; *t* R: retention time; C.V: Coefficient Variation for retention time or peak areas obtained from six replicate injections (instrument precision).

TABLE 6.5 Statistical Data for CLBr, PNT and CDZ by HPLC method

Parameter	**CLBr**	**PNT**	**CDZ**
Linear range (µg/ml)	10-30	80-240	20-60
Slope	385.1	713.1	1016
Intercept	758.6	11033	296.4
SD of Slope	0.8687	0.1471	0.4082

SD of Intercept	18.2012	20.0574	21.5566
Limit of Detection (µg/ml)	0.1559	0.0928	0.0700
Limit of Quantitation (µg/ml)	0.4725	0.2812	0.2121

TABLE 6.6 Determination of Accuracy

% Level	**Amount Added (µg/ml)**			**Mean of Amount Recovered (µg/ml) (n=3)**			**% Mean Recovery ± S.D [n=3]**		
	CLBr	**PNT**	**CDZ**	**CLBr**	**PNT**	**CDZ**	**CLBr**	**PNT**	**CDZ**
50	10	80	20	9.93	79.81	20.04	99.63± 0.3064	99.87± 0.2030	99.77± 0.2705
100	20	160	40	19.96	159.96	39.87	99.70 ± 0.3538	100.05± 0.1011	99.88 ± 0.1934
150	30	240	60	29.86	239.91	59.79	99.89± 0.2289	99.97± 0.0602	99.93± 0.1588

TABLE 6.7 Accuracy of CLBr

Level	**Set**	**Amt. Of Drug Added (µg/ml)**	**Amount Recovered (µg/ml)**	**% Recovery**	**Mean % recovery**	**S.D of Recovery**
50%	**1**	10	9.832	98.32	99.34	0.9913
50%	**2**	10	10.03	100.3		
50%	**3**	10	9.94	99.40		
100%	**1**	20	19.82	99.10	99.71	0.5346
100%	**2**	20	20.01	100.05		
100%	**3**	20	20.06	100.0		
150%	**1**	30	29.82	99.40	99.55	0.6145
150%	**2**	30	29.71	99.03		
150%	**3**	30	30.07	100.23		

n =3 determination

TABLE 6.8 Accuracy of PNT

Level	Set	Amt. Of Drug Added (µg/ml)	Amt Recovered (µg/ml)	% Recovery	Mean % recovery	S.D of Recovery
50%	**1**	80	79.85	99.81	99.76	0.3402
50%	**2**	80	80.06	100.07		
50%	**3**	80	79.52	99.40		
100%	**1**	160	160.09	100.075	99.97	0.3701
100%	**2**	160	160.04	100.033		
100%	**3**	160	159.76	99.8		
150%	**1**	240	240.06	100.025	99.96	0.0818
150%	**2**	240	239.69	99.87		
150%	**3**	240	239.99	99.99		

n =3 determination

TABLE 6.9 Accuracy of CDZ

Level	Set	Amt. Of Drug Added (µg/ml)	Amt Recovered (µg/ml)	% Recovery	Mean % recovery	S.D of Recovery
50%	**1**	20	19.85	99.25	100.21	0.8607
50%	**2**	20	20.10	100.5		
50%	**3**	20	20.18	100.9		
100%	**1**	40	39.83	99.575	99.69	0.5346
100%	**2**	40	40.11	100.27		
100%	**3**	40	39.69	99.225		
150%	**1**	60	60.12	100.2	99.66	0.7405
150%	**2**	60	59.98	99.96		
150%	**3**	60	59.29	98.81		

n =3 determination

TABLE 6.10 Repeatability Data for CLBr

Conc. (µg/ml)	**10 (µg/ml)**	**15 (µg/ml)**	**20 (µg/ml)**	**25 (µg/ml)**	**30 (µg/ml)**
Area	4610.56	6631.42	8341.88	10308.63	12415.93
	4632.14	6628.96	8349.12	10316.22	12417.47
	4619.52	6641.84	8355.18	10298.12	12386.14
	4612.41	6635.21	8331.21	10311.48	12412.65
	4610.25	6615.48	8332.16	10302.11	12418.14
	4619.12	6634.14	8351.14	10291.47	12401.25
Mean	4617.33	6631.17	8343.45	10304.67	12408.59
S.D	8.33	8.83	10.08	9.15	12.63
C.V	0.18	0.13	0.12	0.088	0.10

TABLE 6.11 Repeatability Data for PNT

Conc. (µg/ml)	**80 (µg/ml)**	**120 (µg/ml)**	**160 (µg/ml)**	**200 (µg/ml)**	**240 (µg/ml)**
Area	65475.07	99891.55	126997.53	150552.94	182760.85
	65449.15	99885.54	126999.15	150549.56	182754.74
	65482.45	99895.36	126990.25	150531.63	182743.52
	65480.24	99879.95	126963.69	150514.84	182769.87
	65484.62	99896.21	126971.36	150564.21	182795.65
	65490.47	99841.26	126995.95	150569.38	182787.54
Mean	65477.00	99881.65	126986.32	150547.09	182768.69
S.D	14.55	20.72	15.06	20.53	19.86
C.V	0.022	0.020	0.011	0.013	0.010

TABLE 6.12 Repeatability Data for CDZ

Conc. (µg/ml)	**20 (µg/ml)**	**30 (µg/ml)**	**40 (µg/ml)**	**50 (µg/ml)**	**60 (µg/ml)**
Area	20512.77	30936.04	41402.72	50021.64	61786.21
	20569.65	30948.51	41421.25	50031.24	61798.18
	20524.65	30935.25	41400.51	50025.65	61779.58
	20531.54	30911.48	41412.54	50039.85	61787.63
	20518.65	30944.26	41429.24	50022.69	61781.92
	20516.48	30947.25	41405.84	50009.15	61788.84
Mean	20528.96	30937.13	41412.01	50025.04	61787.06
S.D	21.0014	13.7493	11.3052	10.2732	6.4860
C.V	0.10	0.044	0.027	0.020	0.010

TABLE 6.13 Repeatability of sample application data for CLBr, PNT and CDZ

Conc. (µg/ml)	**AREA**		
	CLBr 10(µg/ml)	**PNT 20(µg/ml)**	**CDZ 80(µg/ml)**
1	8292.42	128172.14	40864.51
2	8270.35	128170.01	40832.69
3	8250.22	128173.25	40897.61
4	8277.35	128154.29	40868.80
5	8243.69	128127.43	40845.16
6	8277.41	128140.42	40850.04
Mean	8268.57	128156.26	40859.8
S.D	18.3498	19.0562	22.7069
C.V.	0.22	0.014	0.055

(n=6 determination)

TABLE 6.14 Precision data for CLBr

Conc. (µg/ml)	Intraday (Area ± SD)	C.V.	Inter day (Area ± SD)	C.V.
10	4449.66±69.83	1.56	4463.29± 41.52	0.93
20	8348.79± 379.72	0.49	8396.25±67.12	0.79
30	11532.44± 186.72	0.90	11574.54±76.32	0.65

(n=3 determination)

TABLE 6.15 Precision data for PNT

Conc. (µg/ml)	Intraday (Area ± SD)	C.V.	Inter day (Area ± SD)	C.V.
80	64571.26± 675.83	1.04	64620.56±510.23	0.78
160	126995.31± 815.03	0.64	126885.59±401.62	0.31
240	181386.09± 791.81	0.43	181420.12±628.31	0.34

(n=3 determination)

TABLE 6.16 Precision data for CDZ

Conc. (µg/ml)	Intraday (Area ± SD)	C.V.	Inter day (Area ± SD)	C.V.
20	20379.700± 352.53	1.72	20412.32±251.23	1.23
40	41385.15± 383.12	0.92	41325.78±96.35	0.23
60	59687.26± 611.26	1.024	59724.36±524.21	0.87

(n=3 determination)

TABLE 6.17 Robustness study for CLBr, PNT and CDZ

Parameter	Variation	CLBr 20(µg/ml)		PNT 160(µg/ml)		CDZ 40(µg/ml)	
		Area± SD [n=3]	%Assay (Mean)	Area± SD [n=3]	%Assay (Mean)	Area± SD [n=3]	%Assay (Mean)
Flow rate	1.6ml/min	7595.05 ± 12.56	100.72	125889.61 ± 19.62	100.44	40497.66 ±41.23	100.40
	1.5ml/min	8341.88 ± 65.36	100.59	126997.53 ± 5.69	99.85	41402.72 ±74.23	100.34
	1.7ml/min	8446.063 ± 19.36	100.49	131429.38 ±9.69	100.24	39803.63 ±69.25	100.39
Mobile phase ratio	50:30:20	7715.73 ± 21.63	99.16	126542.47 ±10.29	99.09	40217.68 ±48.21	99.08
	50:29:21	8341.88 ± 54.21	100.49	126997.53 ±18.63	100.24	41402.72 ±63.25	100.39
	50:31:19	8649.929 ± 20.36	99.28	134431.59 ±26.63	98.89	41945.48 ±70.14	98.84
Wavelength	220	8423.69 ± 15.20	100.36	125963.59 ±25.21	100.77	41589.25 ±20.36	101.30
	218	7962.58 ± 16.23	99.16	125986.36	99.09	41258.69	99.08

				±12.25		±21.63	
	222	8269.23 ± 32.02	100.72	131589.29 ±58.12	100.44	42157.34 ±28.56	100.40
pH of Mobile Phase	6.0	8163.95 ± 29.63	99.28	123694.23 ±15.69	98.89	42178.11 ±24.69	98.84
	6.1	7963.95 ± 41.27	99.16	135891.26 ±51.36	99.09	41025.29 ±22.68	99.08
	5.9	8147.97 ± 12.56	100.59	129848.26 ±19.65	99.85	41578.98 ±21.69	100.34

The LOD for CLBr, CDZ and PNT was found to be 0.15µg/ml, 0.070µl/ml and 0.092µg/ml respectively. Summary of validation parameters is tabulated in [Table 6.18].

TABLE 6.18 Summary of validation Parameters of HPLC

Parameters	CLBr	PNT	CDZ
Recovery %	99.34 – 99.71	99.76 – 99.97	99.66-100.21
Repeatability (C.V., n=6)	0.22	0.014	0.055
Precision (C.V.)			
Intra - day (n=3)	0.90 – 1.56	0.43 – 1.04	0.92-1.72
Inter - day (n=3)	0.65 – 0.93	0.31 – 0.78	0.23-1.23
Robustness	Robust	Robust	Robust
Solvent stability	Suitable for 24hr	Suitable for 24Hrs	Suitable for 24hr

6.2.4 Analysis of marketed formulation

Marketed formulation was analyzed by the proposed method and assay result of marketed formulation was shown in (Table 6.19).

TABLE 6.19 Assay result of marketed formulation

Formulation	Drug	Amount Taken (µg/ml)	Amount Found (µg/ml) (n = 3)	Labelled claim (mg)	Amount found per Tablet (mg)	% Label claim ±SD
Ulrax (capsule)	CLBr	20	20.72	2.5	2.59	100.75 ± 0.9755
	CDZ	40	40.58	5	5.19	100.44 ± 0.3931
	PNT	160	160.61	20	20.25	100.29 ± 1.1993

6.3 Summary of developed RP-HPLC method

The validated HPLC approach has been proven to be a simple, specific, accurate, precise, and repeatable way to identify CLBr, CDZ, and PNT in combination capsules. The suggested method is a successful means of facilitating cooperation between CLBr, CDZ, and PNT. In less than 5 minutes, this technology allows for a quantitative analysis of three different medicinal dose forms. This is a major benefit for regular analysis since it reduces the amount of solvent needed. The validity of the procedure provided here was determined by comparing it to the ICH-Q2 (R1) validation criteria. A number of characteristics of the system were evaluated for appropriateness, specificity, linearity, LOD, LOQ, intra-day and inter-day precision, and accuracy during the validation trials. The shown procedure may be utilised to evaluate the efficacy of CLBR, CDZ, and PNT in combination dose forms.

CHAPTER 7

7 RP-HPLC method development and validation for the estimation of clidinium bromide, chlordiazepoxide and pantoprazole sodium in bulk drug and formulations with forced degradation studies

7.1 Material and Methods

7.1.1 Reagents and chemical

Sr. No.	Name	Manufactured by / Supplied by
1	chlordiazepoxide	Ontop pharmaceuticals Pvt. Ltd. , Bangalore
2	Clidinium bromide	Ontop pharmaceuticals Pvt. Ltd. , Bangalore
3	pantoprazole Sodium	Aum research Laboratories, Ahmedabad

All other chemicals were of analytical grade and procured from Merck Specialties Private Limited.

7.1.2 Instruments and Equipments

Sr. No	Instruments Name	Instruments No.	Model	Make
1	Infra-red spectrophotometer	--	Infra 3000A FT-IR Model	Analytical Technologies Limited
2	UV-Visible double beam spectrophotometer	A114548	UV 1800	Shimadzu, Kyoto, Japan
3	pH METER	--	CL 180	Chemiline Digital pH meter
4	Analytical Balance	KE-129	K-EA 210	K-Roy Instrument Pvt. Ltd.
5	Melting Point Apparatus	DDPC/210/09-10	--	Vijay laboratory furnisher

6	Ultrasonic Bath sonicator	--	UC 3000	PEI
7	High Performance Liquid Chromatography Instrument (HPLC)			
	HPLC Instrument 1:	--	Ezchrom 2006	Agilent 1260 Infinity Quaternary LC
	HPLC Instrument -2	--	Clarity software	Analytical Technologies Limited

7.1.3 Filters and column

Sr. No.	Name	Make
1	0.22 µm PVDF membrane filter (P/ No.: GVWP04700)	Millipore
2	0.45 µm Nylon syringe filter (P/ No.: SENN0602MNXX106)	mdi
3	0.45 µm pre-filter + PVDF syringe filter (P/ No.: SYVG0602MNXX104)	mdi
4	0.45 µm pre-filter+ PTFE syringe filter (P/ No.: SYTG0602MNXX104)	mdi
5	Eclipse Plus C18 (150mm × 4.6mm, 5µm) (P/ No.: 00F 4040-E0)	Phenomenex

7.1.4 Optimized Chromatographic conditions

Sample name :	pantoprazole + clidinium bromide + chlordiazepoxide
Sample ppm :	160+20+40 ppm
Column :	Zorbax SB Phenyl (150*4.6mm, 3.5µ)
	Make: Agilent
	Stationary Phase: Phenyl
Flow rate :	1.5ml/min
Column temperature :	30°C

Wavelength :	230 nm
Injection volume :	20µL
Run time :	10 minutes
Diluent :	Water : Acetonitrile_65:35% *v/v*
Mode :	Isocratic
Mobile phase :	MP-A: Sodium perchlorate buffer
	MP-B: Acetonitrile

7.1.5 System Suitability Criteria

A chromatogram and a table detailing the retention periods of clidinium bromide, chlordiazepoxide, and sodium pantoprazole may be found in the results section.

i) Tailing factor: The tailing factor for the CLBr, CDZ, and PNT peaks generated by the first injection of the standard solution should not exceed 2.0.

ii) Theoretical Plates: At least 2000 theoretical plates of the CLBr, CDZ, and PNT peaks should have been collected from the first standard solution injection.

iii) RSD: After six replicate injections of standard solution, the relative standard deviation of the peak areas for CLBr, CDZ, and PNT should not exceed 2.0%.

7.1.6 Preparation of solutions

Blank (Diluent):

Water: Acetonitrile (20:80)

Buffer Solution:

Sonication was used to remove air bubbles before adding 2 ml of 60% perchloric acid in 1 litter of water, 0.5 ml of TEA, and adjusting the pH to 4.0 with diluted NaOH.

Mobile Phase:

Have ready a 50:50 combination of buffer solution and acetonitrile. Degas by sonicating for ten minutes.

7.1.6.1 Standard CLBr stock solution (500 µg/ml)

A 25 ml volumetric flask was filled with precisely weighed CLBr (12.5 mg), which was then dissolved and diluted with methanol to the appropriate concentration.

7.1.6.2 Standard CDZ stock solution (1000 µg/ml)

A 25 ml volumetric flask was filled with precisely weighed CDZ (25.0 mg), which was then added, dissolved, and diluted with methanol to the appropriate concentration.

7.1.6.3 Standard PNT stock solution (4000µg/ml)

PNT (100.0 mg) was accurately weighed, transported to a 25 ml volumetric flask, and then dissolved and diluted with methanol to the appropriate concentration.

7.1.6.4 Mixed standard stock solution of CLBr, CDZ and PNT

Standard stock solutions of 100 µg/ml CLBr, 200 µg/ml CDZ, and 800 µg/ml PNT were prepared by combining 10 ml aliquots of the respective stock solutions and then filling the remaining space in a 50 ml volumetric flask with mobile phase to the mark.

7.1.7 Calibration curve for CLBr, CDZ and PNT

The appropriate quantity of an aliquot from a mixed standard stock solution of CLBr, CDZ, and PNT was added to a 10-ml volumetric flask. A solution was created with CLBr concentrations of 10, 15, 20, 25, and 30 µg/ml, CDZ concentrations of 20, 30, 40, 50, and 60 µg/ml, and PNT concentrations of 80, 120, 160, 200, and 240 µg/ml. Under the same circumstances as the other solutions, the combined standard solution was chromatographed. Before being used, every solution was filtered via a 0.45 µm membrane. By graphing the average peak area vs. the concentrations for all three medications, calibration curves were generated. Using these calibration curves, linear equations were developed.

7.1.8 Determination of CLBr, CDZ and PNT from combined dosage form

7.1.8.1 Sample preparation

The commercially available forms of ULRAX were bought by us. Careful measurements were taken of twenty pills and the equal amount of a solid substance. The precise weights of 12.5 mg CLBr, 25 mg CDZ, and 100 mg were used to fill a 25-ml volumetric flask. The medication was dissolved by adding 15 ml of methanol to the volumetric flask and shaking it for 20 minutes. The filtered solution was placed in a 25ml volumetric flask after being filtered via Whattman filter paper (0.45 µm). A solution of 500 µg/ml of CLBr, 1000 µg/ml of CDZ, and 4000 µg/ml of PNT was obtained by progressively adding methanol to the shaking flask. Two ml of the aliquot was placed in a 50 ml volumetric flask, and the remaining volume was filled with methanol to generate a solution containing 20 mg/ml of CLBr, 40 mg/ml of CDZ, and 160 mg/ml of PNT. What happened was dissected using the suggested method.

7.1.8.2 Estimation of CLBr, CDZ and PNT in combined dosage form

During 20 minutes, we chromatographed the pre-mixed sample solution in a mobile phase. The concentrations of three medicines were determined by measuring their peak areas in the chromatogram.

7.1.9 Forced Degradation

7.1.9.1 Acid Hydrolysis

By weighing 20 whole capsules, we were able to calculate the mean net quantity. Twenty capsules were opened, the contents powdered, and the resulting 20 mg of clidinium bromide, 40 mg of chlordiazepoxide, or 160 mg of sodium pantoprazole was measured in a 100 ml volumetric flask. 5 cc of 0.1N HCl was added after the mixture had been at room temperature for an hour. The environment was stabilised when 5 ml of 0.1N NaOH was added. Sonicate it for an hour, spin it at 5,000 revolutions per minute for twenty-five minutes, and then filter it through a syringe filter. The volume may be adjusted with diluent if necessary. The same procedure was used to store 0.1 N HCl in a volume of 1 ml for 2 hours at room temperature.

7.1.9.2 Alkali Hydrolysis

The average net weight of twenty whole capsules was determined by weighing twenty capsules. Twenty crushed capsules provide a powder containing 20 mg of clidinium bromide, 40 mg of chlordiazepoxide, or 160 mg of sodium pantoprazole per 100 ml. Measuring flask. After one hoursat room temperature, 5 ml of 0.1N sodium hydroxide is added. Using 5 ml of 0.1N HCl, the acid was neutralised. Sonicate it for ten minutes, spin it at 5,000 revolutions per minute for twenty-five minutes, and then filter it using a syringe filter. If required, the volume may be adjusted using diluent. The same procedure was performed using 5 cc of 0.1 N NaOH at room temperature for two to three hours.

7.1.9.3 Oxidative Hydrolysis

20 capsules were weighed to get an average net weight; next, 5 ml of 0.3% hydrogen peroxide were added to the powder from the capsules, and the mixture was added to a 100 millilitre volumetric flask. The mass of the flask was recorded. After that, we let the mixture sit for three hours at room temperature. To attain the appropriate concentration, add diluent, sonicate the mixture for 10 minutes, centrifuge it at 5000 rpm for 25 minutes, and then filter it through a syringe filter. To further dilute the filtrate, up to 10 ml of the diluent is added to 3 ml of the initial solution. The same methods were used to assess the results of exposing 5 ml of 0.3% H_2O_2 to room temperature for 6 and 9 hours.

7.1.9.4 Thermal Degradation

The average net weight of twenty whole capsules was determined by weighing twenty capsules. The powder from 20 capsules was weighed to be equivalent to 20 mg of clidinium bromide, 40 mg of chlordiazepoxide, or 160 mg of pantoprazole sodium, and then baked at 60 ºC for one hour. 70% dilution was followed by sonication for 10 minutes, centrifugation at 5000 rpm for 25 minutes, then filtering using a syringe filter. (The same method was used for the 3-hour, 60° C condition.)

7.1.9.5 Photo Degradation

Twenty whole capsules were weighed to get an average net weight. clidinium bromide, chlordiazepoxide, and sodium pantoprazole each weigh 20 mg/ml, while the powder from 20 capsules weighs 160 mg/ml. Following that, the powder was left out in the sun for 30 minutes (a non-repeatable process). It was sonicated for 10 minutes and centrifuged at 5000 rpm for 25 minutes after being given 70% of the diluent.

✓ **Acceptance Criteria:**

i) There should be no confounding of the PNT, CDZ, and CLBr peaks by blank, placebo, or degradation products.

ii) Degraded samples of PNT, CDZ, and CLBr should all meet peak purity standards (Peak purity = Purity angle < Purity threshold).

7.1.10 Method Validation

The method's linearity, precision, accuracy, LOD, LOQ, specificity, and robustness were all evaluated in compliance with ICH Q2 (R1) recommendations.

7.1.10.1 Generation of calibration curves

The HPLC column has to be set up with the mobile phase for at least an hoursbefore conditioning using a single full gradient procedure. Following the order listed in the table below, inject 5 µL of the blank, reference, and sample solutions into the chromatograph. Use the chromatograph to determine the peak area response to CLBr, CDZ, and PNT.

TABLE 7.1 Injection sequence

Sr. No.	Sample name	No. of injections
1	Blank	1
2	Standard solution	6
3	Sample solution_1	1

4	Sample solution_2	1
5	Sample solution_3	1
6	Standard solution (Bracketing)	1

7.1.10.2 Linearity

✓ **Standard stock solution**

Dissolved in methanol at the concentrations indicated in a volumetric flask of 25 ml were 12.5 mg of CLBr, 25 mg of CDZ, and 100 mg of PNT. A solution comprising 500 µg/ml CLBr, 1000 µg/ml CDZ, and 4000 µg/ml PNT was obtained by shaking the flask while progressively adding methanol. By adding 10 ml of this aliquot to a 25 ml volumetric flask and filling it with methanol, we were able to create a solution containing 200 µg/ml CLBr, 400 µg/ml CDZ, and 1600 µg/ml PNT.

Standard stock solutions of CLBr, CDZ, and PNT were diluted to the appropriate concentrations and aliquoted into a 10-ml volumetric flask. After a significant volume change caused by methanol, solutions of CLBr (5, 10, 15, 20, 25, and 30 µg/ml), CDZ (10, 20, 30, 40, 50, and 60 µg/ml), and PNT (40, 80, 120, 160, 200, and 240 µg/ml) were prepared. The mixed standard solution was chromatographed under the same circumstances (n=6) as before. Each solution was filtered through a 0.45 µm membrane before being utilised. Calibration curves were constructed by plotting the mean peak area vs drug concentrations for all three drugs. Linear equations were derived from these calibration curves.

7.1.10.3 Specificity

Specificity refers to the clarity of assessing the analyte in the presence of predicted components of the sample matrix. Typically, they are composed of a degradant, matrix, and contaminant. Researchers determined the sensitivity of the approach by applying it to a reference medication and sample. By comparing the RF and spectra of the band, we were able to confirm that the sample included CLBr, CDZ, and PNT.

7.1.10.4 Accuracy

Spiking the sample at 50%, 100%, and 150% concentrations was used to assess the reliability of the procedure. As the blank sample matrix was being eaten, this was done. After doing three separate chromatographic runs at each concentration, we were able to calculate the average recoveries.

7.1.10.5 Method Precision

The precision of an analytical method is defined by the consistency of results obtained from repeated measurements of the same homogeneous material under the same conditions.

7.1.10.6 System Precision

Six injections of the standard solution from the same HPLC vial will be used to determine the system's accuracy, as required by the test protocol.

- **Preparation of solutions:**

Prepare blank (diluent) and standard solution as per given in Methodology (Test Procedure).

- **Procedure:**

The HPLC column has to be set up with the mobile phase for at least an hoursbefore conditioning using a single full gradient procedure. Following the protocol, inject 10 µL of both the standard solution and the blank solution into the chromatograph. Measure the heights of the peaks for CLBr, CDZ, and PNT on the chromatogram.

- **Acceptance Criteria:**

i) Tailing Factor: When using a standard solution for the first time, the tailing factor of the CLBr, CDZ, and PNT peaks should not exceed 2.0.

ii) Theoretical plates: A minimum of 2000 theoretical plates for the CLBr, CDZ, and PNT peaks should be acquired after the first injection of the standard solution.

iii) RSD: Standard solution injections should be repeated six times, and the relative standard deviation of the CLBr, CDZ, and PNT peaks should not exceed 2.0%.

7.1.10.7 Method Precision (Repeatability)

When the same conditions were used for a very short time frame, reproducibility indicated how reliable the results were. To apply the method's precision to CLBr, CDZ, and PNT 100 µg capsules, six separate sample solutions will be prepared from the same sample batch.

- **Preparation of solutions:**

Prepare blank (diluent), standard solution and sample solution as per given in Methodology (Test Procedure).

- **Procedure:**

The HPLC column has to be set up with the mobile phase for at least an hoursbefore conditioning using a single full gradient procedure. Keep through with the process and load 10 µL of the sample, standard, and blank solutions into the chromatograph. Create a chromatogram and measure the area of the peaks for CLBr, CDZ, and PNT to see how they

react. Find the average, standard deviation, and individual assay results for a set of six measurements.

- **Acceptance Criteria:**

i) Individual and mean % assay value should be within specification limit.

ii) The RSD of six determinations should not be more than 2.0%.

7.1.10.8 Intermediate precision:

Accuracy was within the laboratory variation even when a different analyst used the same sample set but on a different day with different HPLC equipment and a different column of the same brand. The method calls for preparing six different sample solutions from the same sample lot on different days by a different analyst using a different HPLC system and a different column of the same brand in order to establish intermediate precision for 20 µg/ml of CLBr, 40 µg/ml of CDZ, and 160 µg/ml of PNT.

- **Preparation of solutions:**

Prepare blank (diluent), standard solution and sample solution as per given in Methodology (Test Procedure).

- **Procedure:**

The HPLC column has to be set up with the mobile phase for at least an hoursbefore conditioning using a single full gradient procedure. A total of 10 µL of each the blank solution, the standard solution, and the sample solution should be injected into the chromatograph. Create a chromatogram and measure the area of the peaks for CLBr, CDZ, and PNT to see how they react. Find the average, standard deviation, and individual assay results for a set of six measurements. Compare the intermediate precision test results' mean percentage to the method precision test results' mean percentage and get the absolute difference.

- **Acceptance Criteria:**

1. The assay value (both the average and the individual's) must be within the specified range.
2. The relative standard deviation (RSD) of six separate findings must be less than 2.0%.
3. There should not be more than a 2.0-point discrepancy between the mean-% assay results found in the method precision study and the intermediate precision research.

7.1.10.9 Solution Stability

The standard and sample solutions must be prepared and stored at room temperature following the procedure. Examine the outcome based on the response at various time intervals. The % assay values at several intervals in the sample solution are compared to the % relative standard deviation of the CLBr, CDZ, and PNT peak regions in the standard solution.

- **Preparation of solutions:**

Prepare blank (diluent), standard solution and sample solution (in duplicate) as per given in Methodology (Test Procedure)**.**

- **Procedure:**

The HPLC column has to be set up with the mobile phase for at least an hoursbefore conditioning using a single full gradient procedure. Input 5 litres of each the blank solution, the standard solution, and the sample solution to the chromatograph at the appropriate times. Create a chromatogram and measure the area of the peaks for CLBr, CDZ, and PNT to see how they react. Find the % RSD for the peak area responses of CLBr, CDZ, and PNT in the standard solution. Find the minimum and maximum absolute differences between the first test result and subsequent assay results obtained at different times for the sample solution.

- **Acceptance Criteria:**

i) There should not be more than a 2.0% difference between samples of the standard solution for CLBr, CDZ, and PNT taken at various times.

ii) The percentage assay value of the sample solution obtained at the beginning and at the end of each time period must not deviate by more than 2.0 percentage points.

7.1.10.10 Robustness

To evaluate whether the system is suitable, try adjusting the chromatographic parameters listed below one at a time to observe how it affects the assay result and the test. Sample solutions for 100 mg CLBr, CDZ, and PNT capsules should be prepared in accordance with the procedures in order to evaluate its effectiveness. Using the chromatographic settings and test method variables listed below, analyse the sample solution. Check whether the system is suitable for each variable condition and calculate the assay result.

- **Preparation of solutions:**

Prepare blank (diluent), standard solution and sample solution (in triplicate) as per given in Methodology (Test Procedure).

1. Change in Column oven temperature (+ 5°C) of 25°C

   High column oven temperature (HCT): 30°C

   Low column oven temperature (HCT): 20°C

2. Change in Wavelength (± 2 nm) of 348 nm

   High Wavelength (HW): 350 nm

   Low Wavelength (LW): 346 nm

3. Change in Flow Rate (0.1 ml/min) of 1.0 ml/min

   High Flow Rate (HFR): 1.1 ml/min

   Low Flow Rate (LFR): 0.9 ml/min

- **Procedure:**

The HPLC column has to be set up with the mobile phase for at least an hoursbefore conditioning using a single full gradient procedure. The chromatograph calls for 5 µL of each of the blank, standard, and sample solutions across all conditions. Create a chromatogram and measure the area of the peaks for CLBr, CDZ, and PNT to see how they react. Assay results from each changing condition are compared to method precision assay findings to calculate the RSD and total RSD.

- **Acceptance criteria:**

i) Tailing Factor: When using a standard solution for the first time, the tailing factor of the CLBr, CDZ, and PNT peaks should be less than 2.0.

ii) Theoretical plates: Standard solution peak theoretical plates for CLBr, CDZ, and PNT should be greater than or equal to 2000 at 1st injection.

iii) RSD: Peak areas for CLBr, CDZ, and PNT from six duplicate injections of standard solution should not vary by more than 2.0% relative standard deviation (RSD).

iv) The relative standard deviation (RSD) of the test findings from three sample solutions for each different condition should not exceed 2.0%.

v) Assay results produced from procedure precision and each modified condition should have a relative standard deviation (RSD) of not more than 2.0%.

7.1.10.11 LOD-LOQ

Calibration curve was achieved and the help of intercept and slope LOD and LOQ were calculated.

Limit of Detection (LOD): In order to calculate the SD of the intercepts (responses), the linearity curve equation was used. Limit of detection (LOD) for the substance was calculated

using the formula suggested by the International Committee for Harmonization (ICH) guideline:

LOD = 3.3× SD (σ) / Slope……. (4)

- **Limit of Quantitation(LOQ):**

The following equation, specified by the International Conference on Harmonization (ICH) guideline, was used to determine the drug's limit of quantitation (LOQ):

LOQ = 10 × SD (σ) / Slope……. (5)

7.1.10.12 Dosage form Analysis

Using mobile phase chromatography on the produced sample solution, we were able to determine the concentrations of all three medications.

7.2 Result and discussion

7.2.1 Selection of wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. In the present study individual drug solutions of 20 µg/ml CLBr, 40 µg/ml CDZ and 160 µg/ml PNT were prepared in solvent mixtures of 50 ml 0.4%TEA, 30 ml Methanol and 20 ml Acetonitrile (pH 6.0 adjusted with Orthophosphoric acid). These drug solutions were than scanned in the UV region of 200 - 400 nm and the overlay spectrum was recorded.

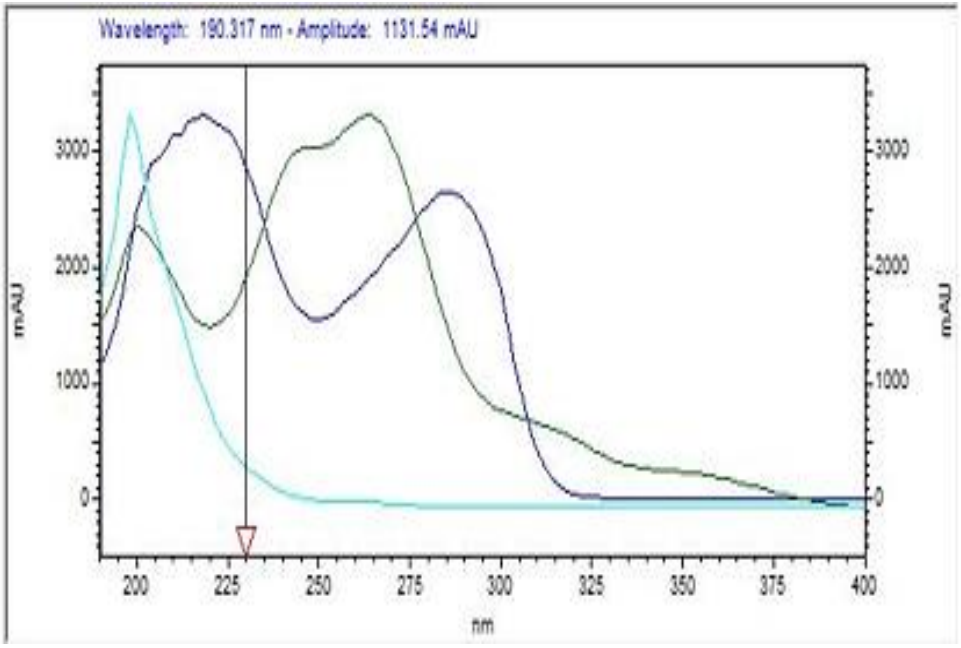


FIGURE 7.1 Overlain Spectra of 10 µg/ml CLBr, 20 µg/ml CDZ and 160 µg/ml PNT in Mobile Phase.

7.2.2 Effect of ratio of mobile phase

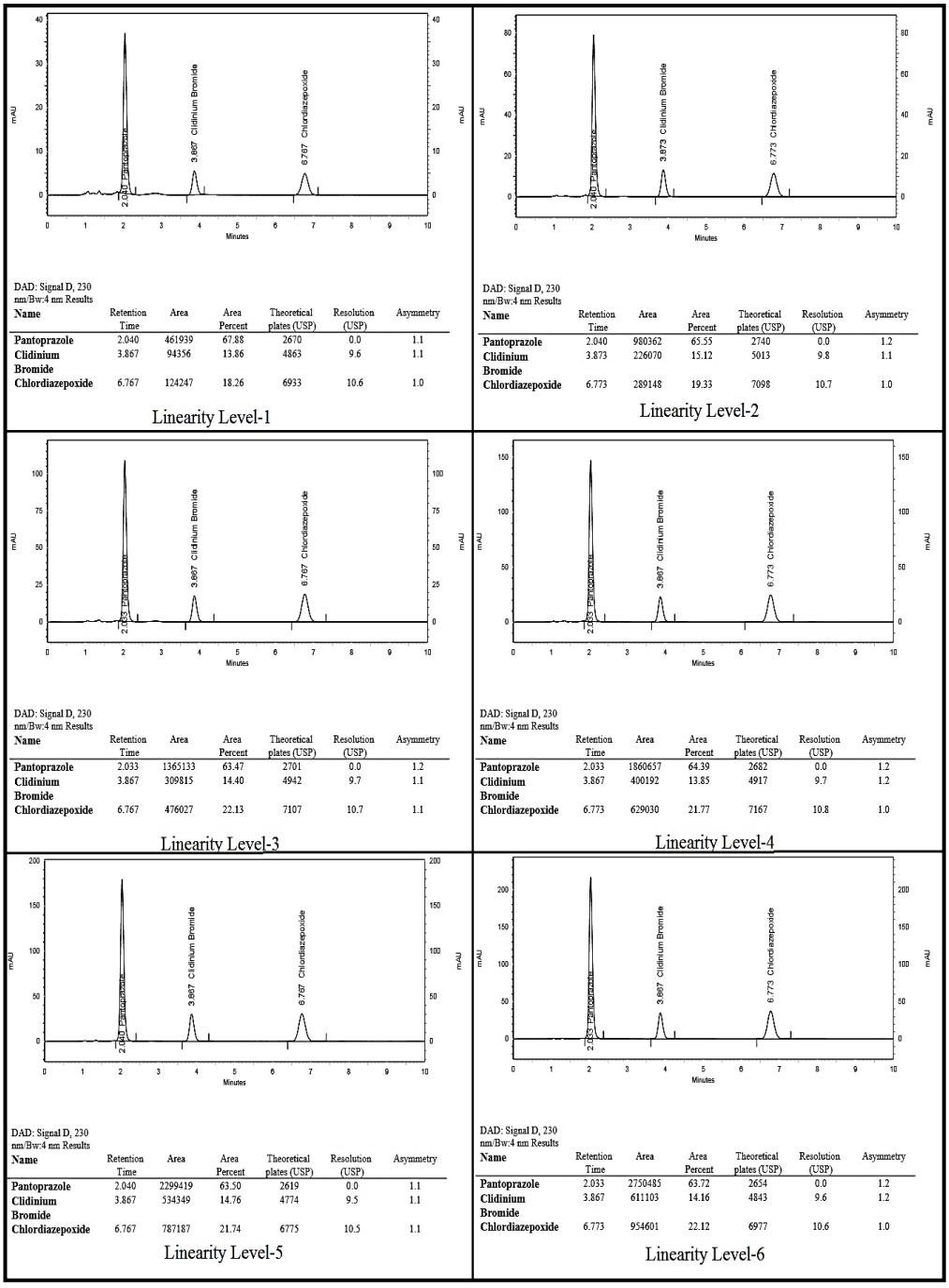
The mixed standard solution containing 25 µg/ml of CLBr, 50 µg/ml CDZ and 200 µg/ml of PNT were chromatographed using following mobile phases:

Mobile phase	Proportion ratio (*v/v*)	Justification	Detection Wavelength [nm]
Methanol : Water	50:50(*v/v*)	Not well separated	230
Acetonitrile : Water	50 : 50(*v/v*)	Not well separated	230
0.05 M Phosphate Buffer: water : Acetonitrile (pH 3.0 with *O*-Phosphoric acid)	40:40:20 (*v/v/v*)	Extensive in the third peak, with no adequate resolve	230
0.05 M Phosphate Buffer: Water : Acetonitrile(pH 3.0 with *O*-Phosphoric acid)	50:30:20 (*v/v/v*)	Good resolution, but not great peak form; the first and third peaks both have tailing.	230
Sodium Perchlorate Buffer: Acetonitrile(pH 4.0)	65:35 (*v/v*)	Good Resolution	230
Buffer Preparation: 2 ml of Perchloric acid-60% in 1 litter of water, 0.5 ml of TEA, pH adjusted to 4.0 with diluted NaOH, mixed and degassed it by sonication.)			

7.2.3 Method Development

The Condition used was Zorbax SB Phenyl (150 mm × 4.6 mm, 3.5 µm) column, mobile phase A is Sodium perchlorate buffer (2 ml of perchloric acid-60% in 1 liter of water, 0.5 ml of TEA, pH adjusted to 4.0 with diluted NaOH, mixed and degassed it by sonication.) and mobile phase B is Acetonitrile (65:35) at 1.5 ml/min flow rate. With the Wavelength of 230nm.Used isocratic and other necessary parameter are described in table 7.1.4. The chromatograms for this are shown in figure 7.6. Mobile phase A is Sodium perchlorate buffer

(2 ml of perchloric acid-60% in 1 litre of water, 0.5 ml of TEA, pH adjusted to 4.0 with diluted NaOH, mixed and degassed by sonication), and the condition utilised was Zorbax SB Phenyl (150 mm 4.6 mm, 3.5 m) column and Acetonitrile (65:35) at a flow rate of 1.5 ml/min makes up mobile phase B. has a 230 nm wavelength. The relevant isocratic parameters and others are provided in Table 7.1.4. The corresponding chromatograms are shown in Figure 7.6.



DAD: Signal D, 230 nm/Bw:4 nm Results

Name	Retention Time	Area	Area Percent	Theoretical plates (USP)	Resolution (USP)	Asymmetry
Pantoprazole	2.040	461939	67.88	2670	0.0	1.1
Clidinium Bromide	3.867	94356	13.86	4863	9.6	1.1
Chlordiazepoxide	6.767	124247	18.26	6933	10.6	1.0

Linearity Level-1

DAD: Signal D, 230 nm/Bw:4 nm Results

Name	Retention Time	Area	Area Percent	Theoretical plates (USP)	Resolution (USP)	Asymmetry
Pantoprazole	2.040	980362	65.55	2740	0.0	1.2
Clidinium Bromide	3.873	226070	15.12	5013	9.8	1.1
Chlordiazepoxide	6.773	289148	19.33	7098	10.7	1.0

Linearity Level-2

DAD: Signal D, 230 nm/Bw:4 nm Results

Name	Retention Time	Area	Area Percent	Theoretical plates (USP)	Resolution (USP)	Asymmetry
Pantoprazole	2.033	1365133	63.47	2701	0.0	1.2
Clidinium Bromide	3.867	309815	14.40	4942	9.7	1.1
Chlordiazepoxide	6.767	476027	22.13	7107	10.7	1.1

Linearity Level-3

DAD: Signal D, 230 nm/Bw:4 nm Results

Name	Retention Time	Area	Area Percent	Theoretical plates (USP)	Resolution (USP)	Asymmetry
Pantoprazole	2.033	1860657	64.39	2682	0.0	1.2
Clidinium Bromide	3.867	400192	13.85	4917	9.7	1.2
Chlordiazepoxide	6.773	629030	21.77	7167	10.8	1.0

Linearity Level-4

DAD: Signal D, 230 nm/Bw:4 nm Results

Name	Retention Time	Area	Area Percent	Theoretical plates (USP)	Resolution (USP)	Asymmetry
Pantoprazole	2.040	2299419	63.50	2619	0.0	1.1
Clidinium Bromide	3.867	534349	14.76	4774	9.5	1.1
Chlordiazepoxide	6.767	787187	21.74	6775	10.5	1.1

Linearity Level-5

DAD: Signal D, 230 nm/Bw:4 nm Results

Name	Retention Time	Area	Area Percent	Theoretical plates (USP)	Resolution (USP)	Asymmetry
Pantoprazole	2.033	2750483	63.72	2654	0.0	1.2
Clidinium Bromide	3.867	611103	14.16	4843	9.6	1.2
Chlordiazepoxide	6.773	954601	22.12	6977	10.6	1.0

Linearity Level-6

FIGURE 7.2 Chromatogram of standard solution containing 20 µg/ml CLBr, 40 µg/ml CDZ and 160 µg/ml PNT using mobile phase Sodium Perchlorate Buffer: Acetonitrile (pH-4 adjusted by diluted NaOH) (65:35 *v/v*)

7.2.4 Chromatographic Conditions

Under optimal chromatographic circumstances, the drug resolution between pantoprazole sodium and clidinium bromide was determined to be 9.7, whereas the resolution between clidinium bromide and chlordiazepoxide was 10.8. The stationary phase was a Column Zorbax SB Phenyl (150mm × 4.6mm, 3.5m) and the mobile phase A was Sodium perchlorate buffer (2% perchloric acid in a Sodium hydroxide solution). In addition, a flow rate of 1.5 ml/min is permitted for mobile phase B, which is acetonitrile (65:35).

7.2.5 System suitability

All validation parameters began with a system appropriateness evaluation. Table 7.2 provides information on system appropriateness.

TABLE 7.2 System suitability results of the proposed method

Compound	N	R	T	C.V. of	
				t_R	Peak area
PNT	2732.2	0	1.18	0.188202	0.125234
CLBr	5205.6	9.9	1.1	0.078128	0.289642
CDZ	7824.2	11.16	1	0.039624	0.388539
Required limits	N > 2000	R > 2	T < 1.5	C.V < 2%	

Where,

N: theoretical number of plates;

R: resolution;

T: USP tailing factor;

t_R: retention time;

C.V: Coefficient Variation for retention time or peak areas obtained from six replicate injections.

7.2.6 Forced degradation study in formulation

During the force degradation studies, the combination of pantoprazole sodium, clidinium bromide, and chlordiazepoxide in capsule dosage form was found to be stable under acidic,

basic, peroxide, and thermal stress conditions. However, it was found to be unstable under the sunlight (Photo) stress condition.

- **Preparation of solutions:**

The average net weight of twenty whole capsules was determined by weighing twenty capsules. Twenty capsules' contents were ground and weighed into a 100 ml volumetric flask to provide 20 mg of clidinium bromide, 40 mg of chlordiazepoxide, and 160 mg of sodium pantoprazole. I sonicated a total of 70 ml of diluent for 30 minutes while my hands sometimes shook. Let the solution to reach room temperature before adding the volume and adjusting the concentration. After spinning the solution at 5000 rpm for 5 minutes, the liquid that came to the surface was collected.

TABLE 7.3 Sample prepaeations

Sample Preparation						
API	**Wt eq. to**	**Flask (ml)**	**Stock Conc. (In PPM)**	**ml Withdrawn from Stock**	**Diluted up to(in ml)**	**Final Conc. (In PPM)**
PNT	160	100	1600	5	50	160
CDZ	40	100	400	5	50	40
CLBr	20	100	200	5	50	20

		PNT		**CLBr**		**CDZ**	
		Area	**% Assay**	**Area**	**% Assay**	**Area**	**% Assay**
As such Sample	Set-1	976730	101.6	225010	101.3	287510	101.0
	Set-2	981698	102.1	225745	101.6	288478	101.4
	Average		101.8		101.4		101.2

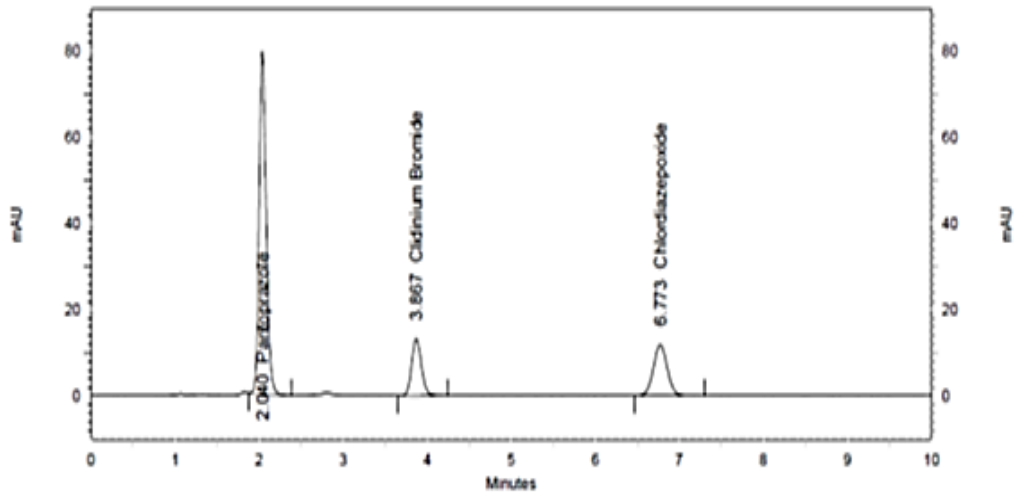


FIGURE 7.3 Chromatogram of untreated sample solution-1 containing PNT, CLBr & CDZ

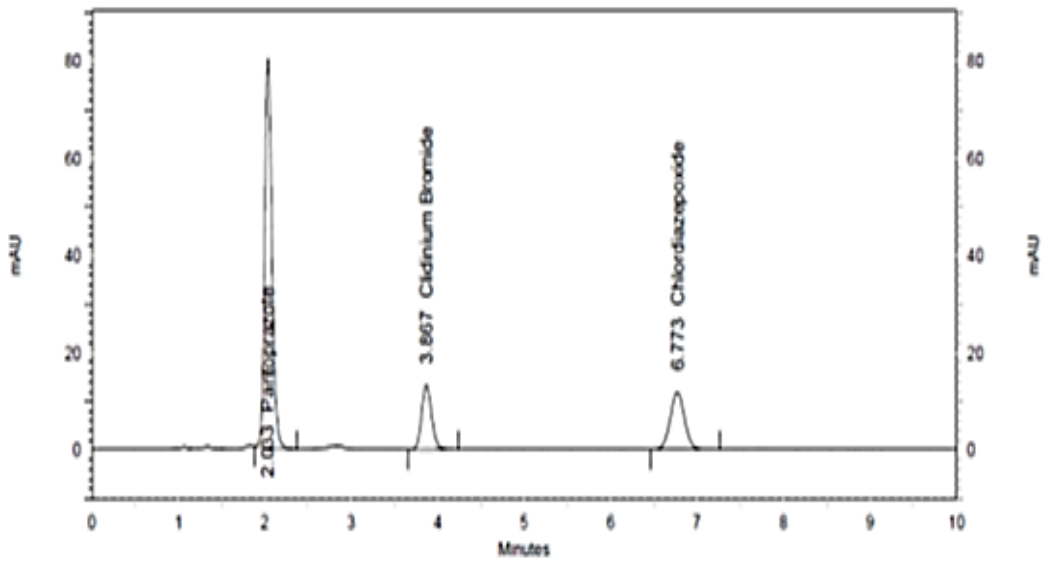


FIGURE 7.4 Chromatogram of untreated sample solution-2 containing PNT, CLBr & CDZ

7.2.6.1 Acid degradation

As mentioned in section 7.2.2, acid hydrolysis was conducted, and the percentage of degradation was determined according to table 7.3. Figures 7.16 and 7.17 depict the chromatogram of the sample.

TABLE 7.3 Forced degradation study on sample solution containing PNT, CDZ & CLBr using 0.1 N HCl solution after 1 hoursand 2 hours.

Parameters	Ulrax Capsule	Retention time (min)	Peak area	Resolution	% Assay	% Degradation
Acid Treatment (At 1hour)	PNT	2.040	961341	-	100.0	1.9
		2.720	8463	2.8		
	CLBr	3.873	220192	4.3	99.1	2.3
	CDZ	6.773	283558	11.2	99.6	1.6
Acid Treatment (At 2hour)	PNT	2.040	959249		99.7	2.1
		2.730	8463	2.8		
	CLBr	3.872	219262	4.3	98.7	2.8
	CDZ	6.783	278548	11.2	97.9	3.3

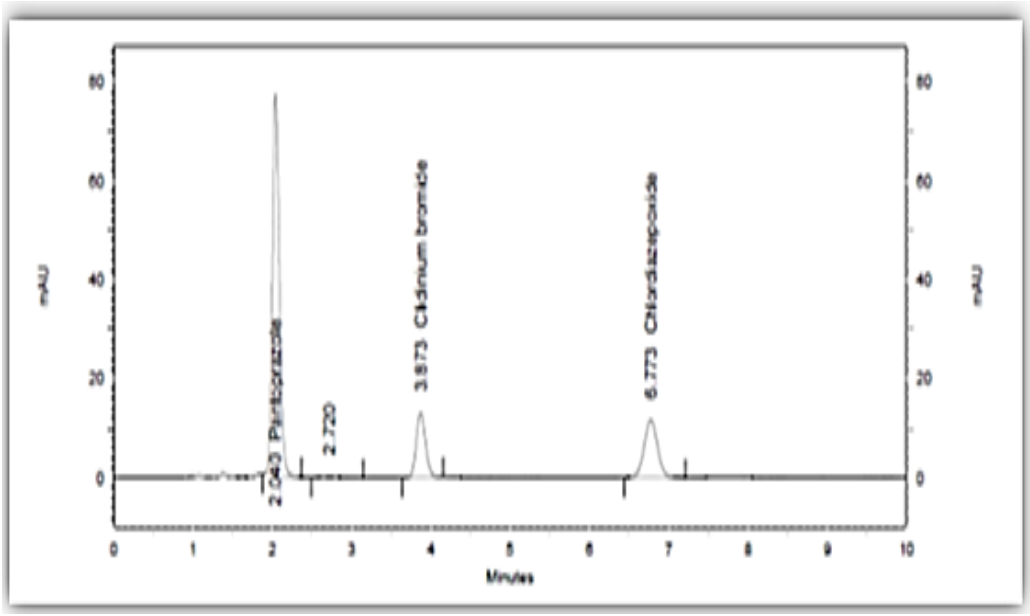


FIGURE 7.5 Chromatogram of Forced degradation study on sample solution containing PNT, CDZ & CLBr using 0.1 N HCl solution after 1 hour

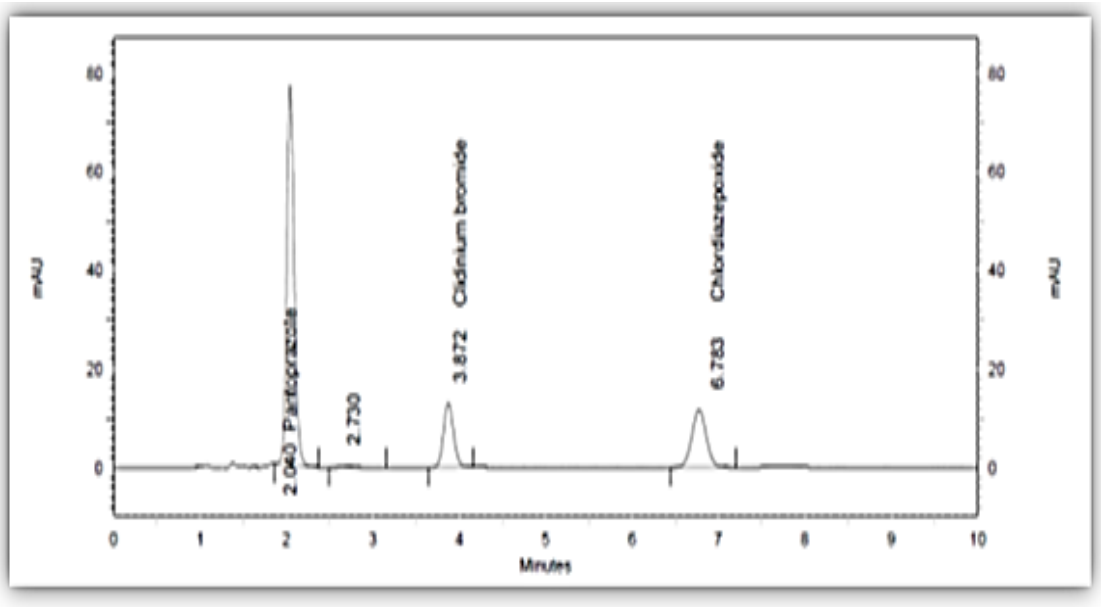


FIGURE 7.6 Chromatogram of Forced degradation study on sample solution containing PNT, CDZ & CLBr using 0.1 N HCl solution after 2 hour

7.2.6.2 Alkali degradation

As described in section 7.2.3. Alkali hydrolysis was performed and % degradation was found to be as per table No. 7.4, Chromatogram of sample was given in figure 7.18 and 7.19.

TABLE 7.4 Forced degradation study on sample solution containing PNT, CDZ & CLBr using 0.1 N NaOH solution after 1, 2 and 4 hour.

Parameters	Ulrax Capsule	Retention time (min)	Peak area	Resolution	% Assay	% Degradation
Alkali Degradation (at 1hr)	PNT	1.827	4468	-	101.6	0.3
		2.040	976730	1.6		
		2.807	16098	3.2		
	CLBr	3.867	226002	3.9	101.7	-0.3
	CDZ	6.773	288616	11.2	101.4	-0.2
		7.760	2947	2.4		
Alkali Degradation (at 2hr)	PNT	1.827	5243	0.0	101.3	0.6
		2.040	973850	1.6		
		2.876	18246	3.2		
	CLBr	3.863	224550	3.9	101.1	0.4

	CDZ	6.760	286675	11.1	100.7	0.5
		7.780	3247	2.4		
Alkali Degradation (at 4hr)	PNT	1.827	9463	0.0	100.7	1.1
		2.040	968320	1.6		
		2.807	33249	3.2		
	CLBr	3.827	211864	3.9	95.4	6.1
	CDZ	6.767	272564	11.2	95.8	5.4
		7.770	6978	2.4		

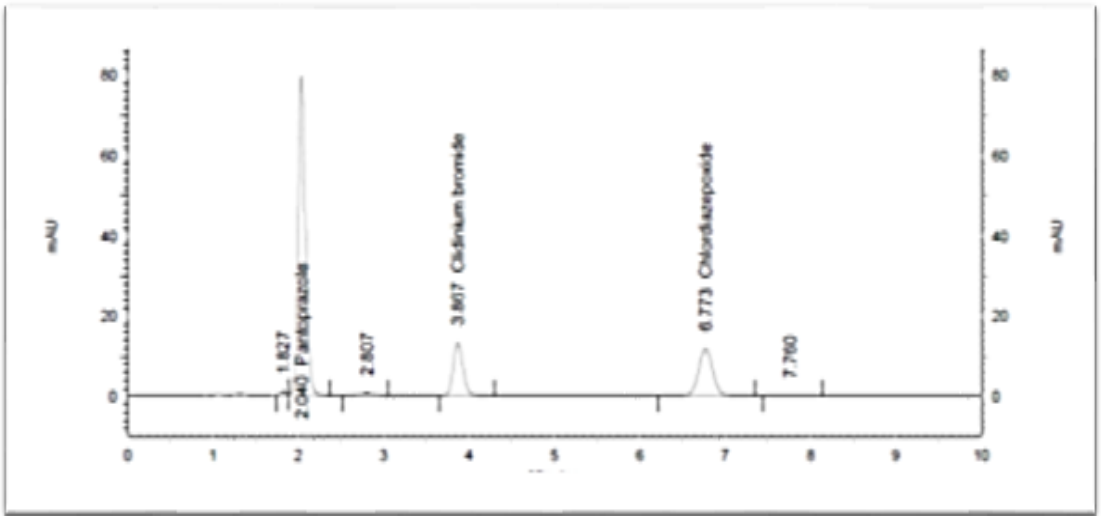


FIGURE 7.7 Chromatogram of Forced degradation study on sample solution containing PNT, CDZ & CLBr using 0.1 N NaOH solution after 1 hour

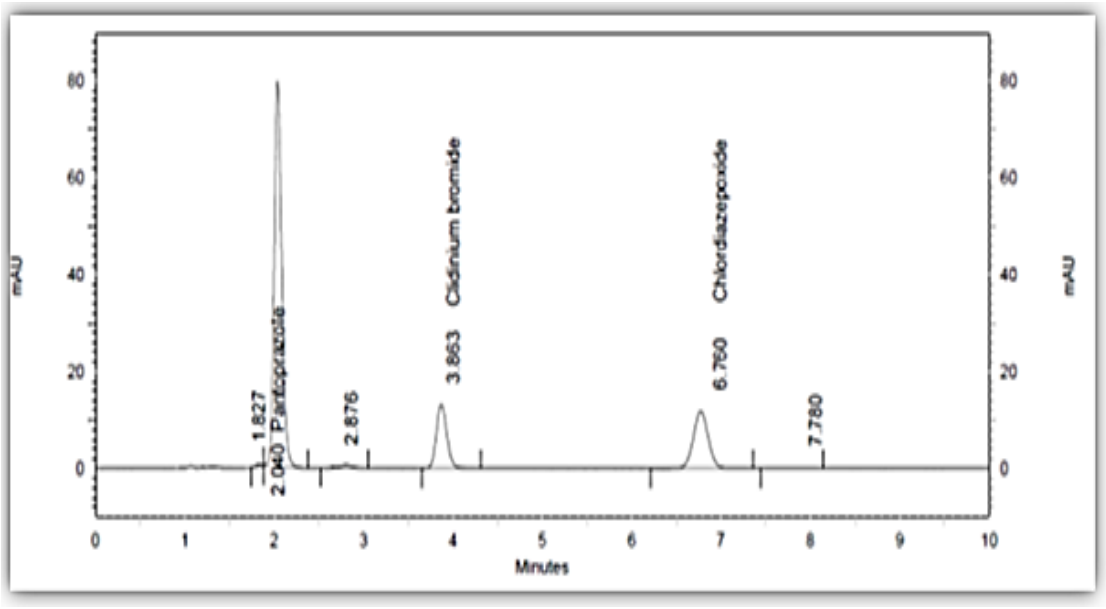


FIGURE 7.8 Chromatogram of Forced degradation study on sample solution containing PNT, CDZ & CLBr using 0.1 N NaOH solution after 2 hour

7.2.6.3 Peroxide Degradation

As described in section 7.2.4. Oxidative hydrolysis was performed and % degradation was found to be as per table No. 7.5, Chromatogram of sample was given in figure 7.21 and 7.22.

TABLE 7.5 Forced degradation study on sample solution containing PNT, CDZ & CLBr using 0.3% H_2O_2 solution after 3, 6 and 9 hour

Parameters	Ulrax Capsule	Retention time (min)	Peak area	Resolution	% Assay	% Degradation
Peroxide Degradation (At 3 hr.)	H_2O_2	1.373	8110	0.0		
	PNT	2.033	959442	5.2	99.8	2.1
		2.653	11299	2.4		
	CLBr	3.867	220546	4.2	99.3	2.2
	CDZ	6.773	285486	11.2	100.3	0.9
		7.613	29204	1.9		
Peroxide Degradation (At 6 hr.)	H_2O_2	1.373	8205	0.0		
	PNT	2.033	951564	5.2	98.9	2.9
		2.667	19862	2.4		
	CLBr	3.853	213782	4.2	99.3	5.2

	CDZ	6.713	280416	11.2	100.3	2.7
		7.673	35652	1.9		
Peroxide Degradation (At 9 hr.)	H_2O_2	1.373	9985	-		
	PNT	2.033	29924	5.2	98.4	3.4
		2.655	204561	2.4		
	CLBr	3.861	204561	4.2	92.1	9.4
	CDZ	6.753	275568	11.2	96.8	4.4
		7.613	42616	1.9		

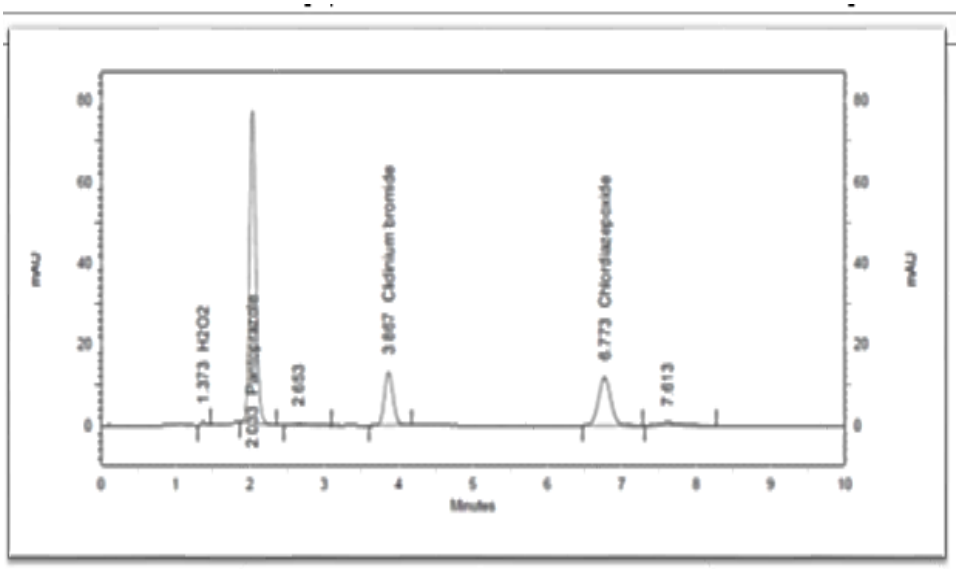


FIGURE 7.9 Chromatogram of Forced degradation study on sample solution containing PNT, CDZ & CLBr using 0.3% H_2O_2 solution after 3 hour

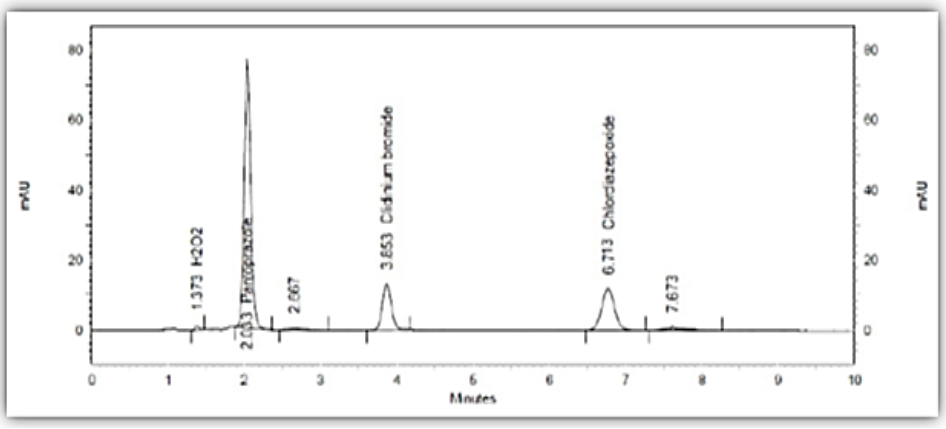


FIGURE 7.10 Chromatogram of Forced degradation study on sample solution containing PNT, CDZ & CLBr using 0.3% H_2O_2 solution after 6 hour

7.2.6.4 Thermal Degradation

As described in section 7.2.5. Oxidative hydrolysis was performed and % degradation was found to be as per table No. 7.6, Chromatogram of sample was given in figure 7.24 and 7.25.

TABLE 7.6 Forced degradation study on sample solution containing PNT, CDZ & CLBr using 60°C in water bath solution after 1 and 4 hour

Parameters	Ulrax Capsule	Retention time (min)	Peak area	Resolution	% Assay	% Degradation
Thermal Degradation (at 1hr)	PNT	2.040	962286	-	100.1	1.8
	CLBr	3.867	223592	9.8	100.6	0.8
	CDZ	6.767	284517	11.1	100	1.2
Thermal Degradation (at 4hr)	PNT	2.040	959574	-	99.8	2.0
	CLBr	3.866	221565	9.8	99.7	1.7
	CDZ	6.767	281259	11.1	98.8	2.4

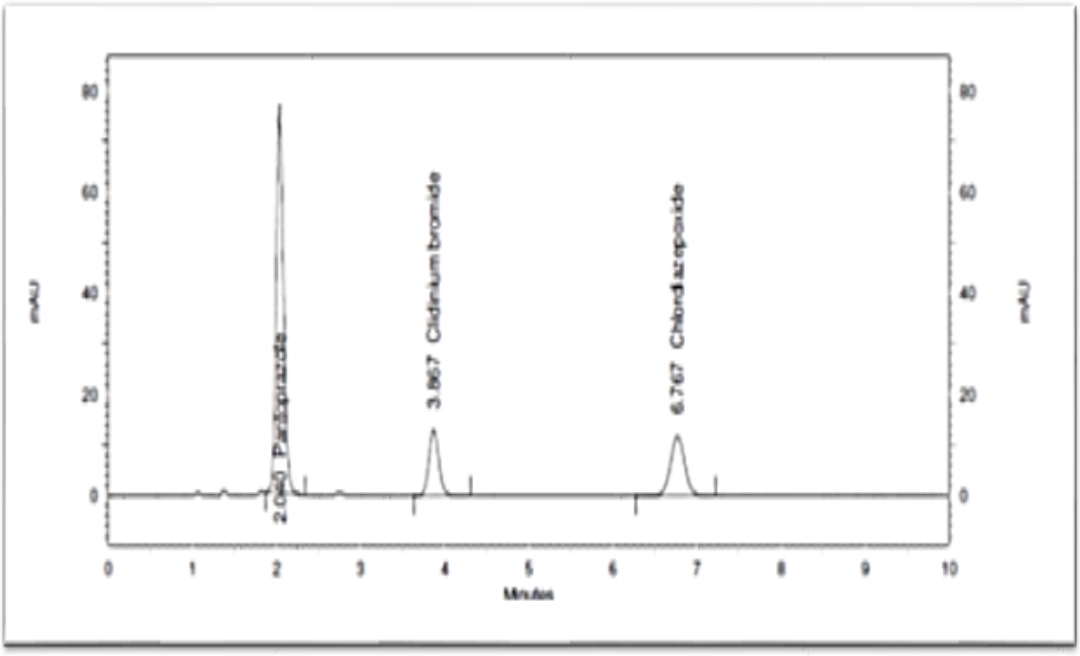


FIGURE 7.11 Chromatograph of Forced degradation study on sample solution containing PNT, CDZ & CLBr using 60°C in water bath solution after 1 hour

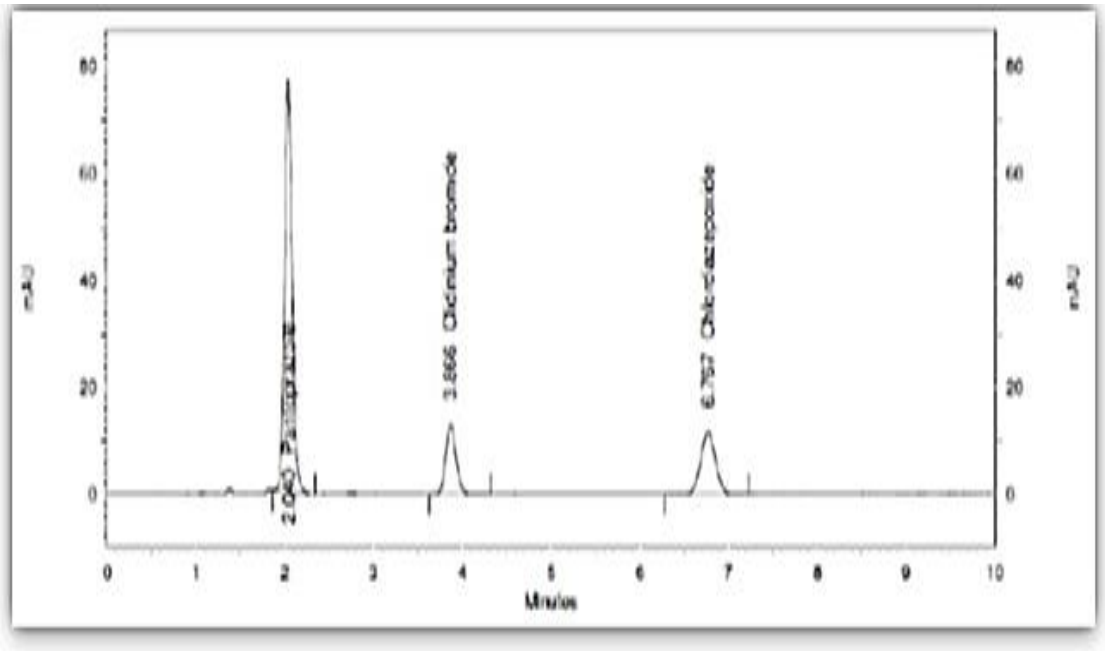


FIGURE 7.12 Chromatograph of Forced degradation study on sample solution containing PNT, CDZ & CLBr using 60°C in water bath solution after 4 hour

7.2.6.5 Photo Degradation

TABLE 7.7 Forced degradation study on on sample solution containing PNT, CDZ & CLBr using Sun light 30min.

Parameters	API	Retention time (min)	Peak area	Resolution	% Assay	% Degradation
Sun light 30min	PNT	2.040	790980	-	82.2	19.6%
		2.867	5598	3.3		
	CLBr	3.873	199352	0.0	89.7	11.7%
	CDZ	6.767	282158	0.0	99.1	2.1%

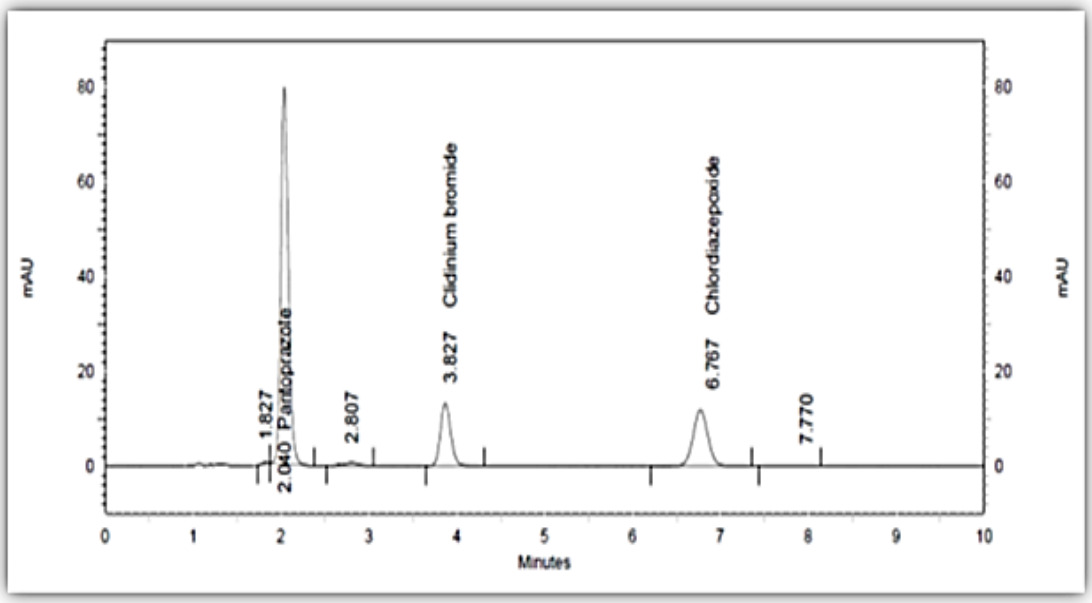


FIGURE 7.13 Chromatogram of Forced degradation study on sample solution containing PNT, CDZ & CLBr using 30 min solution for 30 min

7.2.7 Forced degradation study in Bulk Drugs

Each unique API of pantoprazole sodium, clidinium bromide, and chlordiazepoxide underwent a forced degradation investigation. For ideal chromatographic conditions, all samples were prepared in the same way, using the same mobile phase. They were then tested for the effects of heat, humidity, light, oxygen, and various acids and bases. Any demeaning research requires a well prepared blank.

TABLE 7.8 Force Degradation study on pantoprazole sodium (160 PPM) at Room temperature

Sr. No.	Condition applied	Area	% Assay	% Degradation	Remark
1	Untreated Sample	976730	102.1	---	---
2	HCl Treated	813941	85.49	14.51	Degradation products
3	NaOH Treated	880972	92.1	7.9	
4	H_2O_2 Treated	766062	80.03	19.97	Degradation products
5	Thermal Treated (80°C)	881272	88.07	8.4	
6	UV Light Treated	765062	82.2	19.6%	Degradation products

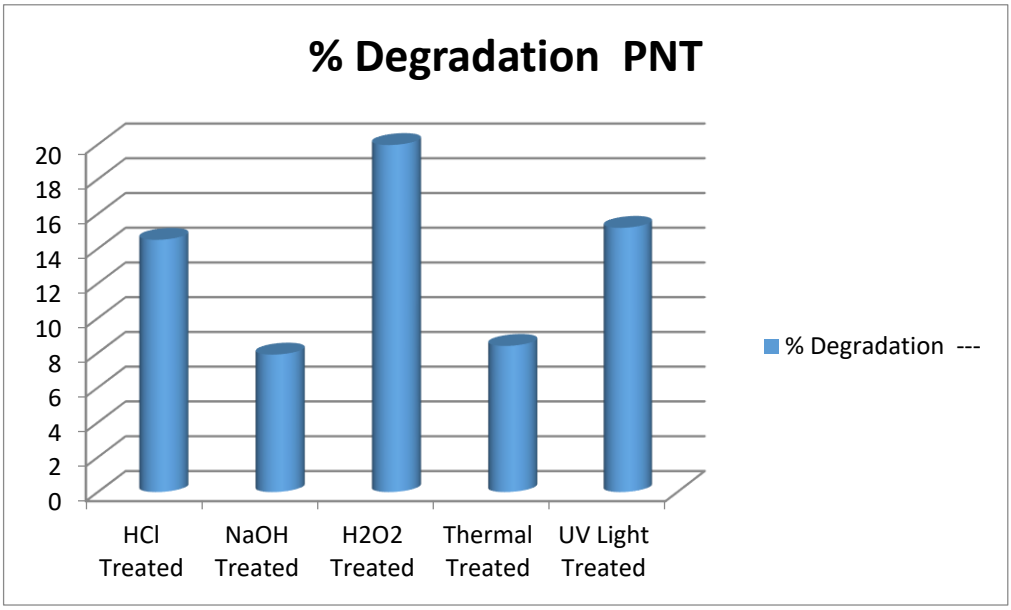


FIGURE 7.14 % Degradation PNT Drug Substance

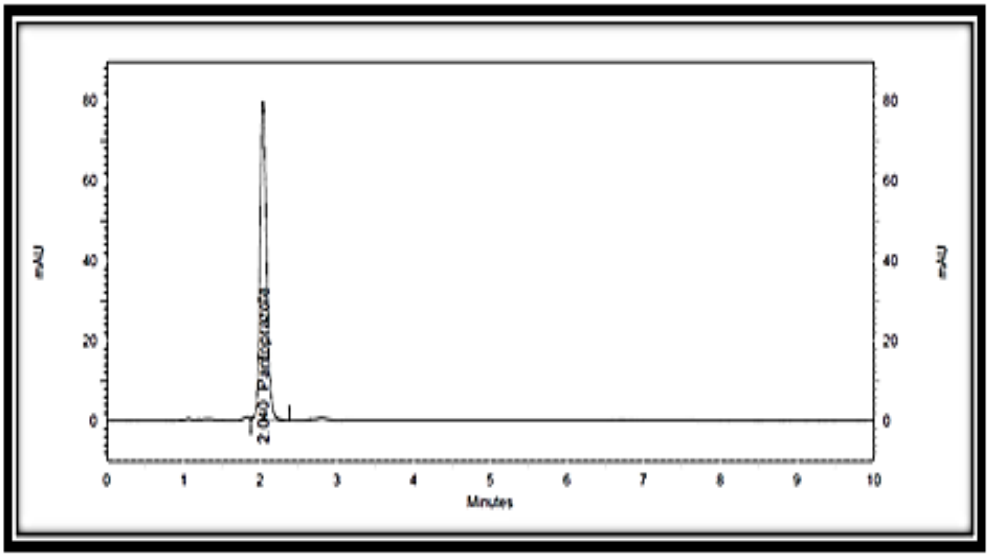


FIGURE 7.15 Chromatogram of untreated sample solution containing PNT Drug Substance

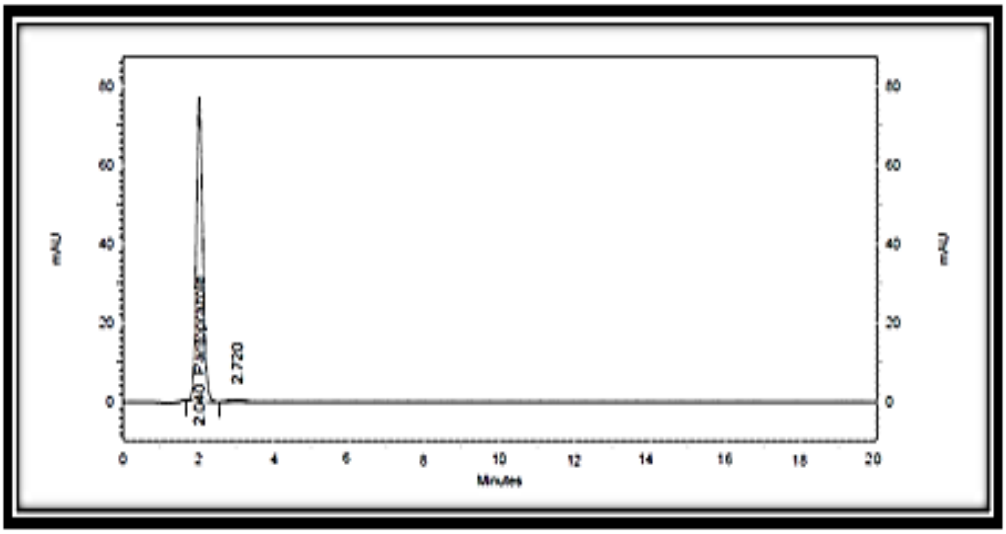


FIGURE 7.16 Chromatogram of Forced degradation study on PNT using 0.1 M NaOH solution after 1 hour

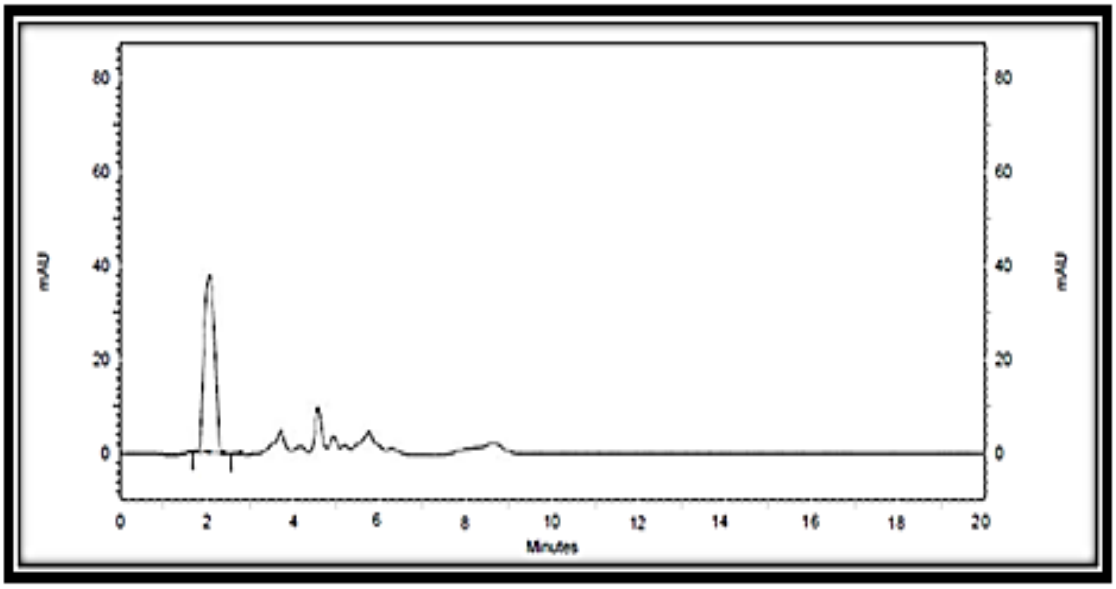


FIGURE 7.17 Chromatogram of Forced degradation study on PNT using 0.1 M HCl solution after 2 hour

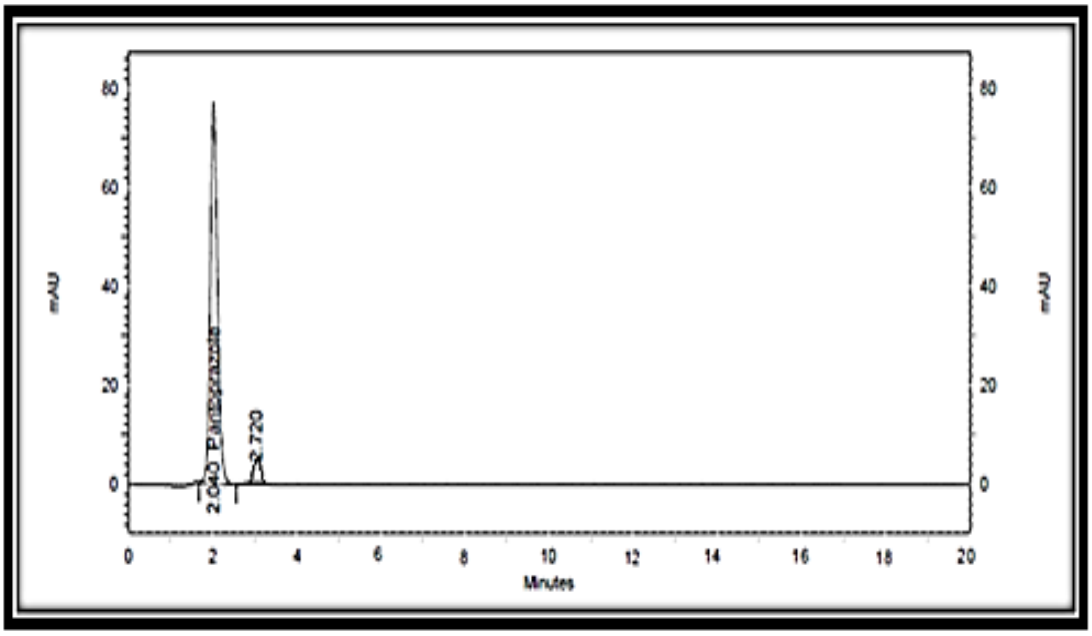


FIGURE 7.18 Chromatogram of Forced degradation study on PNT using 60ºC in water bath solution after 4 hour

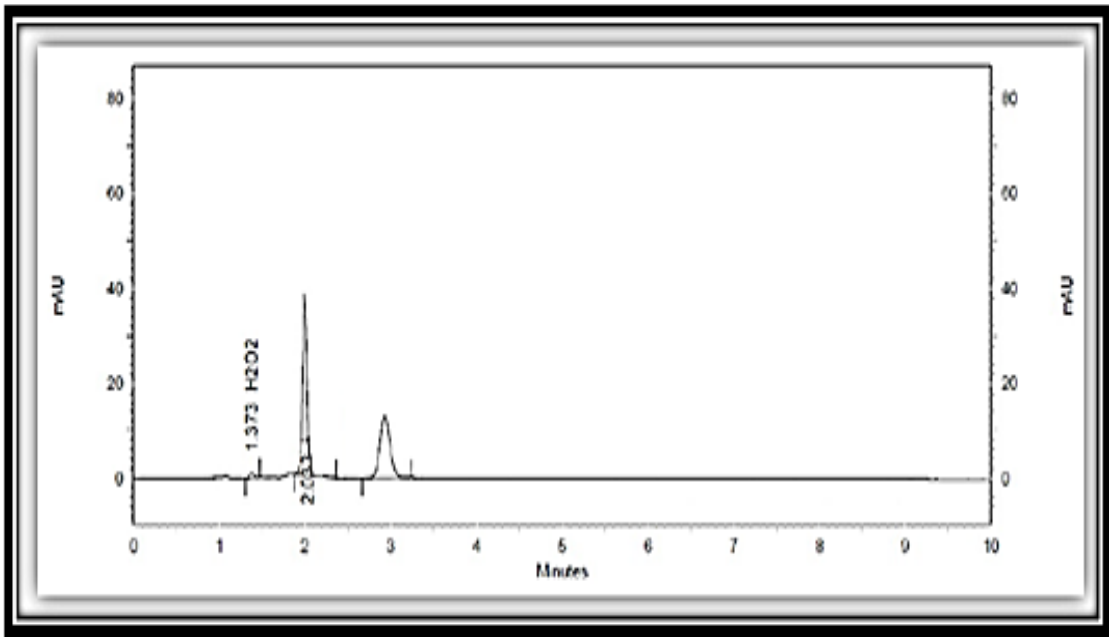


FIGURE 7.19 Chromatogram of Forced degradation study on PNT using 0.3% H_2O_2 solution after 9 hour

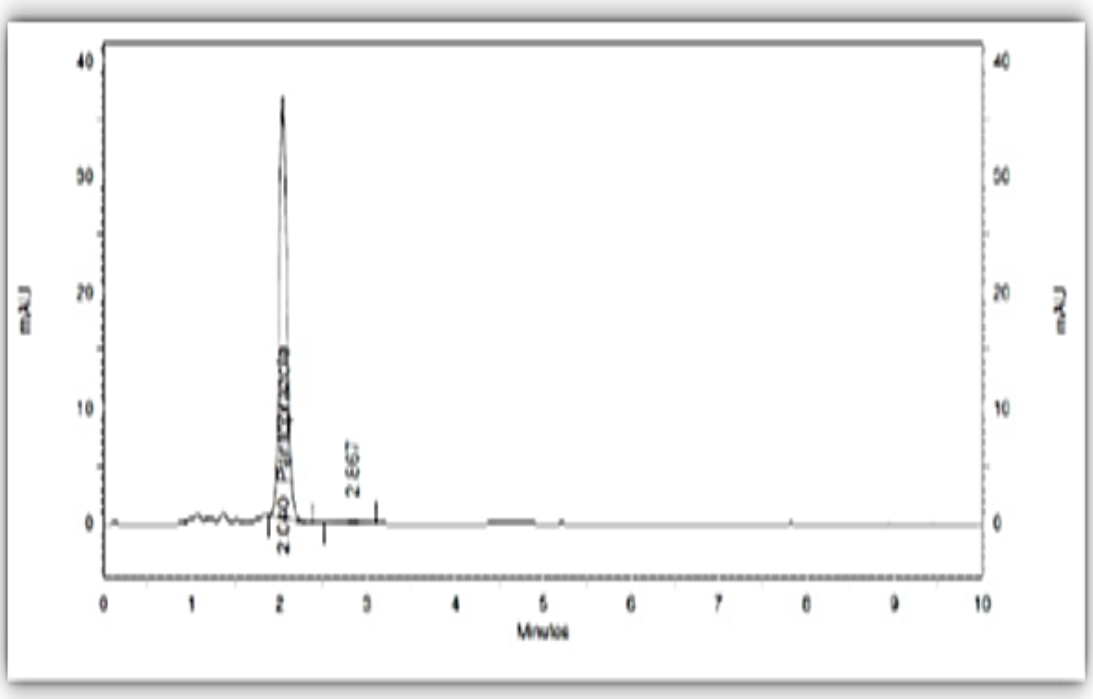


FIGURE 7.20 Chromatogram of Forced degradation study on API containing PNT using Sun light 2 Hr.

TABLE 7.9 Force Degradation study on clidinium bromide (20 PPM) at Room temperature

Sr. No.	Condition applied	Area	% Assay	% Degradation	Remark
1	Untreated Sample	225745	101.6	---	---
2	HCl Treated	219252	98.7	2.8	---
3	NaOH Treated	211864	95.4	6.1	---
4	H_2O_2Treated	204561	92.1	9.4	---
5	Thermal Treated (80°C)	221565	99.7	1.7	---
6	UV Light Treated	199352	89.7	11.7%	Degradation products

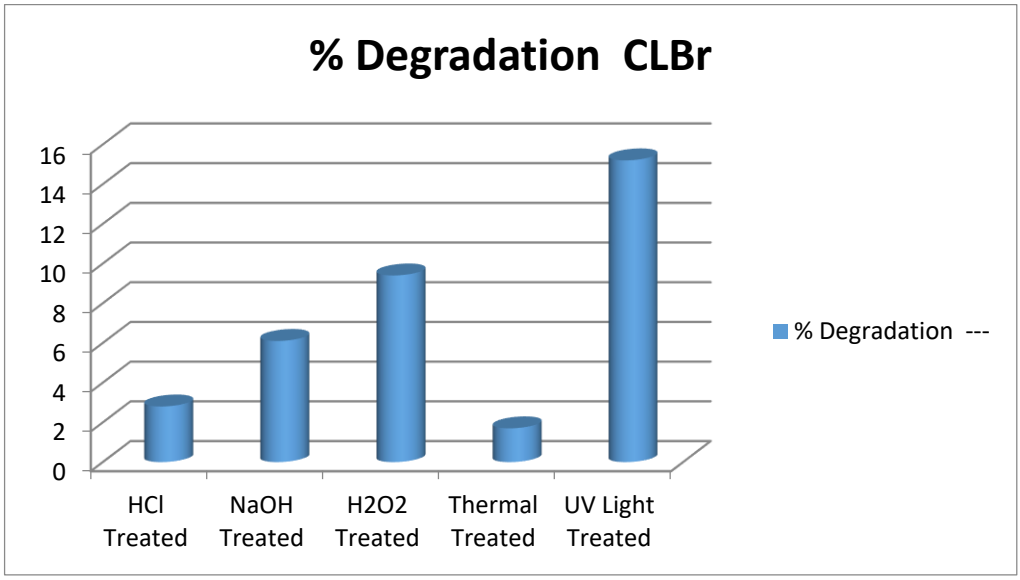


FIGURE 7.21 Force Degradation study on clidinium bromide

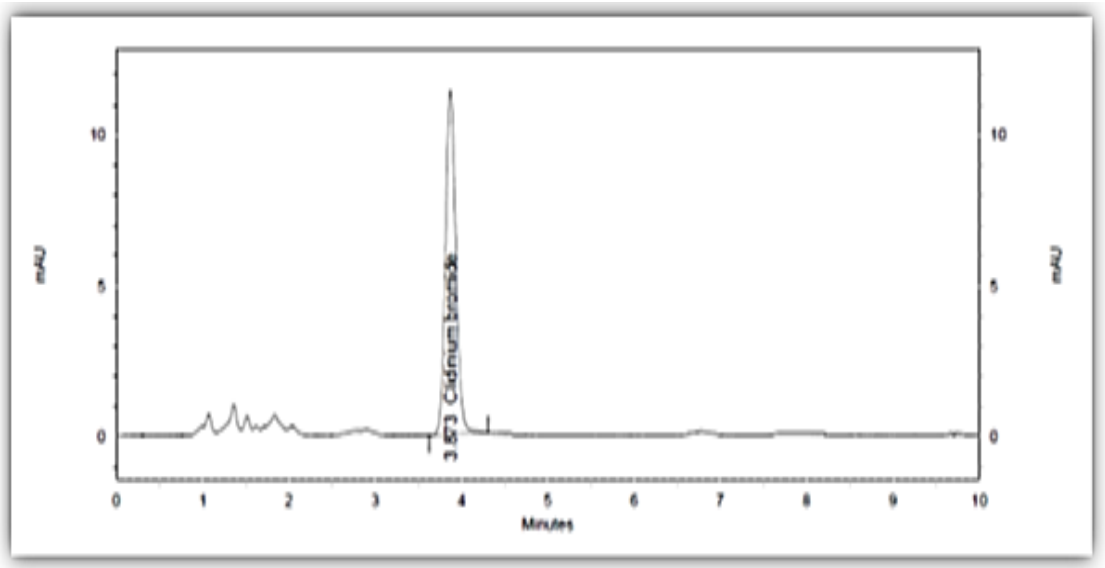


FIGURE 7.22 Chromatogram of Forced degradation study on API containing CLBr using Sun light 2 Hr.

TABLE 7.10 Force Degradation study on chlordiazepoxide (40 PPM) at Room temperature

Sr. No.	Condition applied	Area	% Assay	% Degradation	Remark
1	Untreated Sample	288478	101.4	---	---
2	HCl Treated	278548	97.9	3.3	---
3	NaOH Treated	272564	95.8	5.4	---
4	H_2O_2 Treated	275568	96.8	4.4	---
5	Thermal Treated (80ºC)	281259	98.8	2.4	---
6	UV Light Treated	282158	99.1	2.1%	---

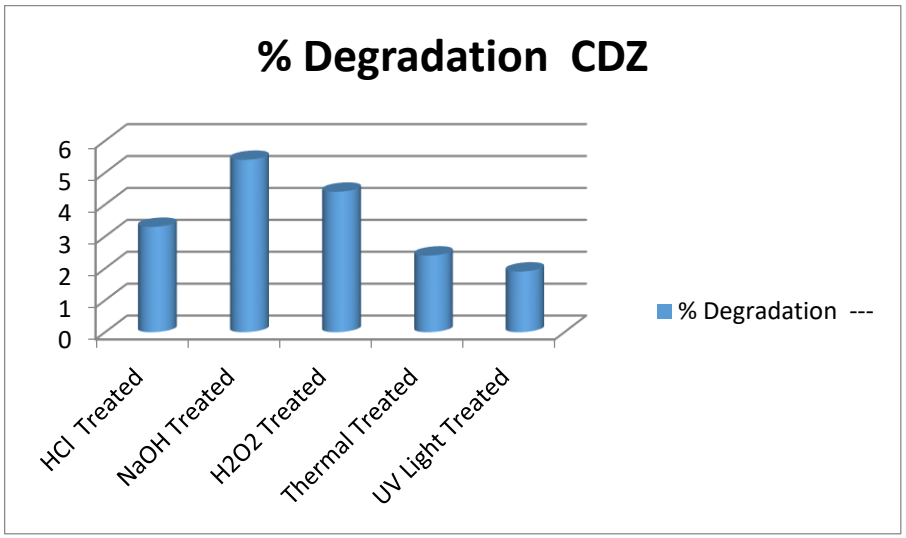


FIGURE 7.23 Force Degradation study on chlordiazepoxide

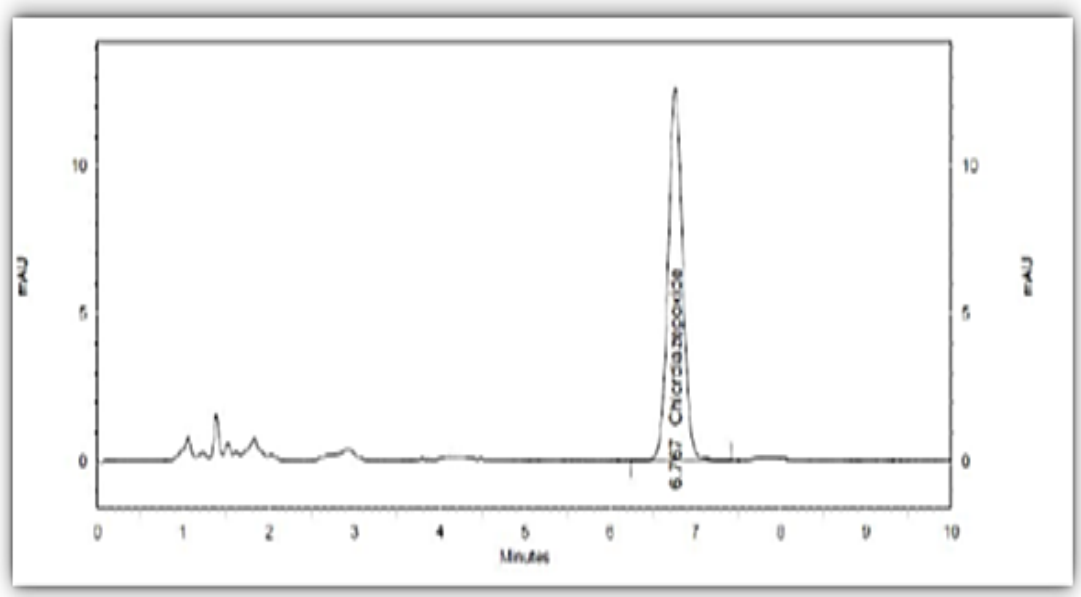


FIGURE 7.24 Chromatogram of Forced degradation study on API containing CDZ using Sun light 2 Hr.

7.2.8 Method Validation

7.2.8.1 Linearity

For an analytical technique to be considered linear, it must provide test findings that are proportionate, either directly or through a well-defined mathematical transformation, to the concentration of an analyte in the sample.

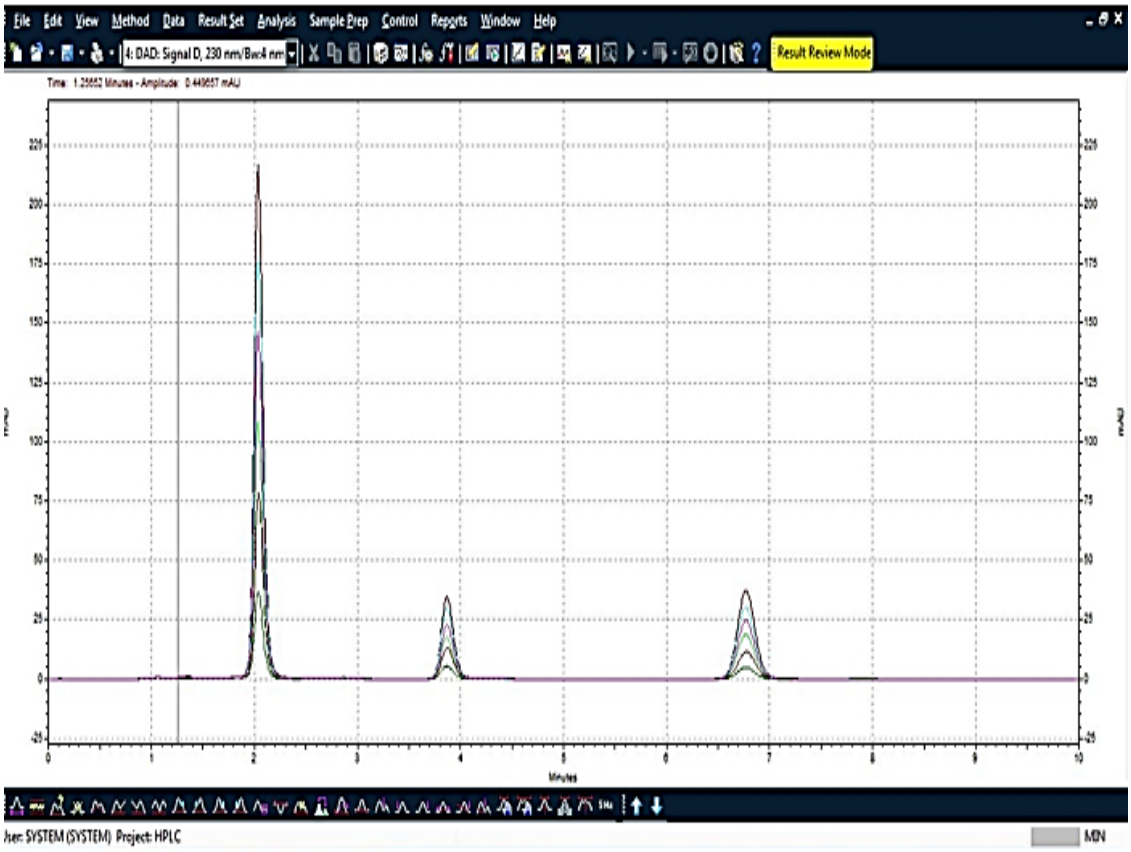


FIGURE 7.25 Linearity Overlain chromatogram of 5-30 µg/ml CLBr, 40-240 µg/ml PNT, and 10-60 µg/ml CDZ at 230 nm.

TABLE 7.11 Calibration readings for PNT by HPLC method

Concentration (µg/ml)	Area Mean ± S.D. (n=3)	C.V.
40	460961 ± 10.39	0.00225
80	979806 ± 597.5651	0.060988
120	1363588 ± 876.9734	0.064314
160	1860273 ± 580.8144	0.031222
200	2299135 ±442.2503	0.019236
240	2743596 ± 3786.263	0.138004

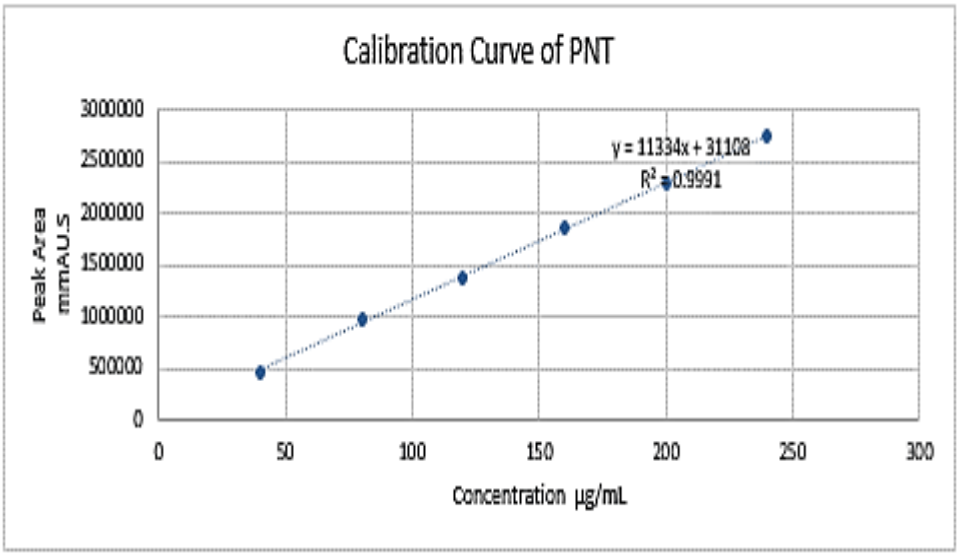


FIGURE 7.26 Calibration curve of PNT by HPLC method

TABLE 7.12 Calibration readings for CLBr by HPLC method

Concentration (µg/ml)	Area Mean ± S.D. (n=3)	C.V.
5	94029.33 ± 565.8033	0.601731
10	227369.3 ± 1424.194	0.626379
15	308855.7 ± 830.807	0.268995
20	400180 ± 551.098	0.137713
25	532912.3 ± 1244.19	0.23347
30	615620 ± 1496.492	0.243087

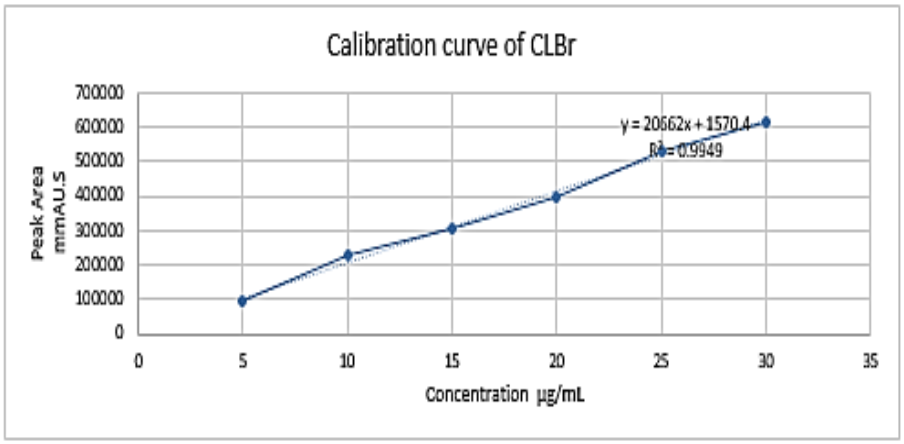


FIGURE 7.27 Calibration curve of CLBr by HPLC method

TABLE 7.13 Calibration readings for CDZ by HPLC method

Concentration (µg/ml)	Area Mean ± S.D. (n=3)	C.V.
10	124794 ± 540.1361	0.432822
20	289551.3 ± 414.412	0.143122
30	475573.7 ± 509.6649	0.107168
40	627628 ± 1615.722	0.257433
50	785683.7 ± 1339.962	0.170547
60	955660.7 ± 839.2767	0.087822

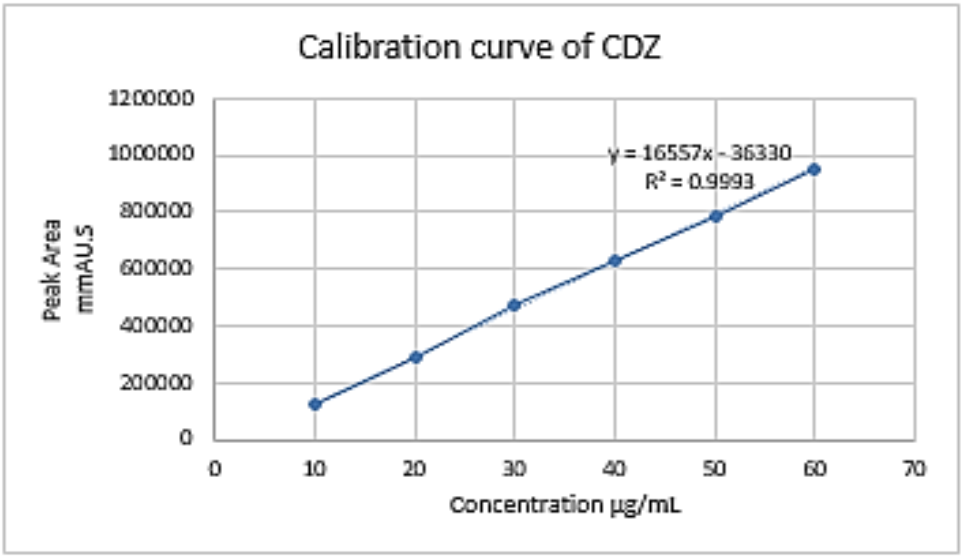


FIGURE 7.28 Calibration curve of CDZ by HPLC method

7.2.8.2 Accuracy

The accuracy of an analytical method is the degree to which a test result is close to the real value. Most of the time, the accuracy is given as a percentage of a known analyte addition. Accuracy, which is true in most cases, is a way to measure how accurate an analytical procedure is.

TABLE 7.14 Determination of Accuracy

% Level	Amount Added CLBr (µg/ml)	Mean of Amount Recovered CLBr (µg/ml) (n=3)	% Mean Recovery ± S.D [n=3] CLBr	Amount Added PNT (µg/ml)	Mean of Amount Recovered PNT (µg/ml) (n=3)	% Mean Recovery ± S.D [n=3] PNT	Amount Added CDZ (µg/ml)	Mean of Amount Recovered CDZ (µg/ml) (n=3)	% Mean Recovery ± S.D [n=3] CDZ
50	10	10.47	99.20 ± 0.78	80	81.01	99.10 ± 0.97	20	20.27	100.90 ± 1.34
100	20	20.14	99.09 ± 0.55	160	160.87	101.18 ± 0.43	40	41.79	100.70 ± 0.72
150	30	29.79	99.23 ± 0.47	240	239.85	101.52 ± 0.18	60	60.87	99.62 ± 0.32

TABLE 7.15 Accuracy of CLBr

Level	Set	Amt. Of CLBr Drug Added (µg/ml)	Amt Recovered (µg/ml)	% Recovery	Mean % recovery	S.D of Recovery
50%	1	10.00	9.95	99.50	99.56	0.2416
50%	2	10.00	10.10	101.00		

50%	3	10.00	10.02	100.20		
100%	1	20.00	19.92	99.60	99.86	0.3132
100%	2	20.00	19.99	99.95		
100%	3	20.00	20.06	100.30		
150%	1	30.00	30.03	100.10	100.03	0.1332
150%	2	30.00	29.89	99.63		
150%	3	30.00	30.16	100.53		

TABLE 7.16 Accuracy of PNT

Level	**Set**	**Amt. Of PNT Drug Added (µg/ml)**	**Amt Recovered (µg/ml)**	**% Recovery**	**Mean % recovery**	**S.D of Recovery**
50%	**1**	80	80.23	99.53	99.76	0.3402
50%	**2**	80	79.81	100.31		
50%	**3**	80	80.14	99.22		
100%	**1**	160	160.34	99.84	99.97	0.3701
100%	**2**	160	160.02	100.01		
100%	**3**	160	160.00	99.85		
150%	**1**	240	239.95	100.05	99.96	0.0818

150%	**2**	240	239.82	99.95		
150%	**3**	240	240.19	99.92		

n =3 determination

TABLE 7.17 Accuracy of CDZ

Level	Set	Amt. Of Drug Added (µg/ml)	Amt Recovered (µg/ml)	% Recovery	Mean % recovery	S.D of Recovery
50%	**1**	20	19.95	99.75	100.39	0.5346
50%	**2**	20	20.21	101.05		
50%	**3**	20	19.92	99.60		
100%	**1**	40	39.63	99.075	99.56	0.2556
100%	**2**	40	40.35	100.875		
100%	**3**	40	39.74	99.35		
150%	**1**	60	59.82	99.70	99.10	0.4569
150%	**2**	60	60.17	100.283		
150%	**3**	60	59.42	99.033		

n =3 determination

7.2.8.3 Precision

The accuracy of an analysis is measured by how closely the results of many measurements taken on the same homogenous sample under the same conditions agree with one another.

TABLE 7.18 Precision data for CLBr

Conc. (µg/ml)	Intraday (Area ± SD)	C.V.	Inter day (Area ± SD)	C.V.
10	5518.33± 86.86	1.57	5505.86± 49.09	0.89
20	10504.41± 478.77	0.47	10538.04± 83.82	0.79
30	14565.66± 235.07	0.81	14600.82± 95.46	0.65

TABLE 7.19 Precision data for PNT

Conc. (µg/ml)	Intraday (Area ± SD)	C.V.	Inter day (Area ± SD)	C.V.
80	100203.82± 1051.23	1.05	100301.12± 765.34	0.76
160	197984.95± 1272.46	0.64	197874.77± 616.42	0.31
240	282584.68± 1235.47	0.44	282630.18± 972.42	0.34

TABLE 7.20 Precision data for CDZ

Conc. (µg/ml)	Intraday (Area ± SD)	C.V.	Inter day (Area ± SD)	C.V.
20	16875.89±146.82	0.87	33301.98±315.49	0.95
40	16899.23±201.56	1.19	48072.59±321.68	0.67
60	33289.22±231.68	0.70	48055.27±423.52	0.88

TABLE 7.21 Repeatability of sample application data for CLBr, PNT and CDZ

Conc. (µg/ml)	AREA		
	CLBr 20(µg/ml)	PNT 160(µg/ml)	CDZ 40(µg/ml)
1	10504.47	197987.90	16856.99
2	16952.11	191634.20	15638.80
3	18654.29	199689.70	17234.77
4	10831.48	206013.50	16324.36
5	13024.93	207895.60	16944.85
6	12093.17	193918.20	17913.25

Mean	12,905.48	200,388.33	16641.37
S.D	2938.628	7,471.515	716.0367
C.V.	0.227	0.037	0.039

(n=6 determination)

Table 7.22 Repeatability of sample application data for CLBr, PNT and CDZ

	CLBr (20 µg/ml)	PNT (160 µg/ml)	CDZ (40 µg/ml)
1	10504.47	197987.90	16856.99
2	6952.11	151634.20	11638.80
3	8654.29	179689.70	14234.77
4	10831.48	226013.50	18324.36
5	13024.93	267895.60	20944.85
6	15093.17	303918.20	23913.25
Mean	10646.9867	219621.9333	17194.4367
S.D	3643.4976	45000.5425	3818.3896
C.V.	0.342	0.205	0.222

7.2.8.4 Robustness

The chromatographic parameters were manipulated on purpose to conduct the test. Infusing a standard solution and a sample solution allowed us to see the effects of the adjustments on the system suitability parameters and the percentage assay result. The results of the survey are shown in Table 7.16.

TABLE 7.23 Robustness study for PNT, CLBr & CDZ

Parameter	Variation	CLBr 20(µg/ml)		PNT 160(µg/ml)		CDZ 40(µg/ml)	
		Area± SD [n=3]	%Assay (Mean)	Area± SD [n=3]	%Assay (Mean)	Area± SD [n=3]	%Assay (Mean)
Flow rate	1.4ml/min	221188± 10.5	98.5	962566± 8.2	101.1	282614± 6.7	100.50
	1.5ml/min	221195± 8.8	99.2	985257± 11.4	98.8	286556± 4.3	100.00
	1.6ml/min	220290± 7.2	100.1	965774± 9.6	99.6	283733± 5.9	100.19
Oraganic Composition	64:36	222196± 12.1	99.8	985102± 7.9	99.9	284496± 8.3	99.34
	65:35	222196± 8.9	100.3	961872± 11.2	98.7	282400± 6.2	100.39
	66:34	220192± 6.5	101.5	981351± 13.5	98.1	283558± 9.7	99.8
Column temperature	29	223779± 9.2	100.6	962566± 10.1	100.2	284575± 11.9	100.3
	30	221188± 8.7	99.9	962566± 7.3	101.8	283478± 10.5	101.5
	31	220546± 6.9	100.4	959627± 9.8	100.7	284720± 12.2	100.65

7.2.8.5 Statistical Data for CLBr, PNT and CDZ by HPLC method

Parameter	CLBr	PNT	CDZ
Linear range (µg/ml)	5-30	40-240	10-60
Slope	20662	11334	16557
Intercept	1570	31108	36330
SD of Slope	0.57735	15.82193	6.928203
SD of Intercept	402.8652	322.1661	330.8217
Limit of Detection (µg/ml)	0.06434	0.0938	0.06593
Limit of Quantitation (µg/ml)	0.1949	0.2842	0.1998

7.2.8.6 Assay result of marketed formulation

Formulation	Drug	Amount Taken (µg/ml)	Amount Found (µg/ml) (n = 3)	Labelled claim (mg)	Amount found per Capsule (mg)	% Label claim ±SD
Ulrax (capsule)	CLBr	20	21.72	2.5	2.34	100.35 ± 0.9897
	CDZ	40	39.58	5	5.10	100.04 ± 0.3567
	PNT	160	160.45	20	20.05	100.19 ± 1.1876

7.3 Summary of Developed Stability Indicating RP-HPLC method

TABLE 7.24 Summary of validation Parameters of HPLC

PARAMETERS	CLBr	PNT	CDZ
Recovery %	99.09 – 99.23	99.10– 101.52	99.62-100.90
Repeatability (C.V., n=6)	0.227	0.037	0.039
Precision (C.V.) Intra - day (n=3) Inter - day (n=3)	0.47 – 1.57 0.65 – 0.89	0.44 – 1.05 0.31 – 0.76	0.70-1.19 0.67-0.95

PARAMETERS	CLBr	PNT	CDZ
Solvent stability	Suitable for 24hr	Suitable for 24Hrs	Suitable for 24hr

- ✓ The developed RP- HPLC approach is capable of resolving all peaks associated with the degradation of both pharmaceuticals, allowing for a complete study of the drugs in the presence of their breakdown products.
- ✓ The nature of this HPLC technique indicates stability.
- ✓ It was determined that the devised RP-HPLC technique was straightforward, sensitive, specific, and accurate.
- ✓ Degradation percentages ranged from 5% to 30% when subjected to force. As a result, this technique may be used for the regular study of medicines in both their commercially-available formulation and in bulk form.

CHAPTER 8

8 CONCLUSION

In this investigation, we successfully established and validated several analytical techniques for the estimation of sofalcone and the combination of clidinium bromide, chlordiazepoxide, and pantoprazole sodium in bulk drugs and formulations.

First, a validated RP-UHPLC technique for determining sofalcone concentrations in medical goods was created. If the medicine degrades in any manner while on the market, our technique can detect and eliminate the degradation products. In degradation research, our mass-balance reconciliation provides insight on the degradation process and the stability of the drug under various stress situations. LC-MS is capable of identifying degradation products, hence bolstering the prediction validity of the approach.

Using forced degradation studies, we designed and validated an RP-HPLC technique for measuring sofalcone in formulations and bulk medicines. As this procedure is trustworthy, it may be used to routinely test the quality of sofalcone in its many forms.

The correct capsule dosages of clidinium bromide, chlordiazepoxide, and pantoprazole sodium have been estimated via a fast RP-HPLC approach. The procedure was shown to be precise and effective; it may now be included into ordinary quality assurance testing.

Utilizing forced degradation experiments and RP-HPLC, we have developed and validated a method for measuring clidinium bromide, chlordiazepoxide, and pantoprazole sodium in bulk medications and formulations. With this technology, any degradation products that the medicine may produce while on the market could be reliably recognised.

Thus, the analytical techniques created and validated in this work might be used across a range of formulations to assure effectiveness and help in quality assurance and control of the right medications. Optimal conditions for handling and keeping pharmaceuticals might be determined with the use of data gained through forced degradation tests. This research emphasises the necessity for pharmaceutical quality assurance studies into forced deterioration, validation of analytical methods, and method development.

Appendix-1

List of Publications

1. **Dharati Rami**, Nehal J. Shah, (2021) Development and Validation of a Rapid rp-hplc method for the determination of clidinium bromide, chlordiazepoxide and pantoprazole sodium in their combined capsule dosage form, *world journal of pharmacy and pharmaceutical sciences, 12(12),* ISSN 2278-4357, pp 1687-1701. https://doi.org/10.20959/wjpps202112-20619

2. **Dharati Rami**, Nehal J. Shah, Ankit Chaudhry, (2022) RP-HPLC method development and validation for the estimation of sofalcone in bulk drug and formulations with forced degradation studies, *Asian Journal of Pharmacy and Pharmacology,* 8(1), ISSN 2455-2674, pp 18-25. https://doi.org/10.31024/ajpp.2022.8.1.4

3. **Dharati Rami**, Nehal J. Shah, (2023) Validated Stability-indicating RP-UHPLC method for the estimation of sofalcone in drugs, Reconciling Mass Balance in Force Degradation studies and LC-MS identification of its degradation products" *International Journal of Pharmaceutical Sciences and Research*, Vol. 14(6), E-ISSN: 0975-8232; P-ISSN: 2320-5148, pp. 2930-2938. https://doi.org/10.13040/IJPSR.0975-8232.14(6).2930-38