DEVELOPMENT AND VALIDATION OF LC AND LC-MS/MS METHODS FOR ANALYTICAL AND BIOANALYTICAL APPLICATIONS OF ACTIVE PHARMACEUTICAL INGREDIENTS

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IN

CHEMISTRY

BY

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DEPARTMENT OF CHEMISTRY

NATIONAL INSTITUTE OF TECHNOLOGY WARANGAL

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Dedicated
To
My Beloved Family

CERTIFICATE

This is to certify that the thesis entitled "**Development and Validation of LC and LC-MS/MS Methods for Analytical and Bioanalytical Applications of Active Pharmaceutical Ingredients"** that is being submitted by **Mr. Vijendar Reddy K** for the award of **Ph. D. degree in Chemistry,** National Institute of Technology, Warangal (Telangana), has carried out research work under our guidance and supervision.

It is also certified that he has not submitted the same in part or in full to any other University or Institute for the award of a diploma or any degree.

Dr. Raghu Chitta

Thesis Supervisor

Date: 09-03-2023

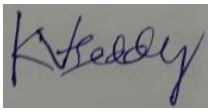
Place: NIT Warangal

Dr. M. Raghasudha

Thesis Co-Supervisor

DECLARATION

I hereby declare that this thesis entitled **"Development and Validation of LC and LC-MS/MS Methods for Analytical and Bioanalytical Applications of Active Pharmaceutical Ingredients"** which is being submitted by me under the supervision of **Dr. Raghu Chitta** and Co-supervision of **Dr. M. Raghasudha,** Department of Chemistry, National Institute of Technology-Warangal (Telangana), India. I declare that this work is original and has not been submitted for any degree/diploma of this or any other university/institute.



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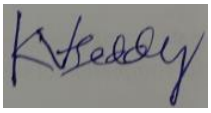


(**Vijendar Reddy Karla)**

ABBREVIATIONS

λ Max	Absorbance maximum (nm)
ACL	Adenosine triphosphate-citrate lyase
ACN	Acetonitrile
AD	Atopic dermatitis
ADME	Absorption, distribution, metabolism, and excretion
APCI	Atmospheric pressure chemical ionization
API	Active pharmaceutical ingredients
APPI	Atmospheric pressure photo ionization
ASCVD	Atherosclerotic cardiovascular disease
BPA	Bempedoic acid
CBE	Crisaborole
C_{max}	Maximum plasma concentration
CV	Coefficient of variation
CVD	Cardiovascular disease
DPs	Degradation products
ELX	Eluxadoline
ESI	Electrospray ionization
EZE	Ezetimibe
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
ICH	International conference on harmonization
IS	Internal standard
KOH	Potassium hydroxide
LC	Liquid chromatography
LDL-C	Low-density lipoprotein cholesterol
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
LQC	Lower quality control concentration
MeOH	Methanol
MQC	Medium quality control concentration

MRM	Multiple Reaction Monitoring mode
NaOH	Sodium hydroxide
OPA	Orthophosphoric acid
PCM	Procainamide hydrochloride
PD	Pharmacodynamics
PDA	Photodiode Array
PK	Pharmacokinetics
QC	Quality control
QqLIT	Quadrupole-linear ion trap
RFX	Rifaximin
RT	Room temperature
TEA	Triethylamine
TFA	Trifluoroacetic acid
T_{max}	Time of maximum plasma concentration
ULOQ	Upper limit of quantification

CONTENTS

Chapter-I: Importance of LC and LC-MS in Analytical and Bioanalytical applications

Chapter-I

Importance of LC and LC-MS in Analytical and Bioanalytical Applications

1.1 Introduction

Today's pharmaceutical sector relies heavily on analytical chemistry. The separation, identification, and measurement of chemical components in both natural and manmade materials are the subjects of this study. Analytical chemistry is employed in the complete process in pharmaceutical industry. This includes drug discovery, marketing, study of physicochemical properties of drugs, formulations to determine the quality as well as stability of drug products for ensuring the safety of the drug.

For the discovery, and manufacture of pharmaceuticals, analytical technique development and validation are crucial. Combination products are pharmaceuticals that combine the therapeutic effects of two or more medications into a single product with the goal of meeting unmet patient requirements. The analytical chemist working on the development and validation of appropriate analytical procedures may face alarming difficulties as a result of these pharmaceutical goods. The quality control laboratories make use of official test methods obtained from these processes in ensuring the uniqueness, purity, effectiveness, and performance of drug products.

The major role of the bio analysis is to assess the overall ADME (absorption, distribution, metabolism, and excretion) characteristics of the new chemical entities. The advancement of drug discovery and development benefits greatly from the use of bioanalytical tools. Body fluids such blood, plasma, serum, urine, and tissue are examined to gauge how well the medication candidate is absorbed and eliminated. Accurate analysis is especially challenging due to stability issues and interferences from endogenous components in the biological matrix. Complete separation of analytes from matrix components is necessary for methods developed to analyze pharmacokinetic study samples. The performance of a bioanalytical assay can be improved by eliminating interferences from the matrix and concentrating the analyte of interest by going through complex sample preparation steps. Bioanalytical techniques instigate in separation of drugs from sample matrix. The main purpose of sample preparation is to remove undesired matrix components that can interfere with the analysis. Thus, the performance of a bioanalytical assay can be improved.

The safety and effectiveness of pharmaceutical products can be ensured by maintaining the quality of the pharmaceuticals during the specified shelf lives of the products. Their shelf life is determined with the help of standardized storage conditions which include controlled temperature and humidity thus translating them into accepted product lifetimes. Analysis of stability samples must be carried out with the use of stability-indicating analytical techniques. To quantitatively assess a reduction in the amount of active pharmaceutical ingredient (API) in the drug product as a result of degradation process, stability-indicating analytical techniques are applied. Stress testing is required to explain the inherent stability properties of the active

chemicals in conformity with the updated ICH (International Conference on Harmonization) guideline, "Stability Testing of New Drug Substances and Products" (Q1AR2). It urges the establishment of degradation pathways as well as the identification of the degradation products generated under various conditions.

Forced degradation studies are most useful in formulating, understanding the drug excipient interactions, manufacturing and packing. Hence, the degradation studies play an important role in pharmaceutical industry.

1.1.1 Drug discovery

Drugs and pharmaceuticals contribute significantly to the advancement of human civilization by treating numerous diseases. The majority of drugs used today are made synthetically. Drug discovery is a specific strategy that pharmaceutical companies use to achieve their goal of attaining a competitive edge by using science and technology in a definite environment.[1-3] To improve the effectiveness of the discovery process and minimize the lead time, drug development and clinical research require a significant amount of information from various disciplines. With increased in-depth understanding of diseases, it is difficult and more exciting to develop drugs that are both safe and effective. It requires 12-15 years to develop a new drug including the time of its initial stages of discovery to the time at which it may be used to treat patient.[4] After satisfying all the requisites, eventually, One or two compounds eventually obtain approval for every 5,000–10,000 compounds that enter the research and development (R&D) pipeline.

Identification of a new drug begins with detailed investigation of a specific disease or disease of concern. The process of identification and investigation may be attempted in the pharmaceutical research laboratories but may also be conducted in academic institutions, government research institutes, small "boutique" pharmaceutical companies, or in a blend of these. Due to the complexity of medical research today, most of pharmaceutical companies now do the majority of their research in collaboration with others.

The research towards a potential drug is usually initiated when a definite receptor or target in the body is identified. Targets may be of different types: a specific cell type, enzyme, gene, pathway, or process. It is assessed that pharmaceutical companies currently have more than 500 targets under investigation**.**

As soon as a target has been selected, the pharmaceutical industry and some academic institutions have rationalized a number of early steps to recognize compounds that have appropriate characteristics to make satisfactory drugs. Various stages of drug discovery are schematically represented in figure 1.1.

1.1.2 Stages of drug discovery and development

- Selection of medical condition to be treated
- Identification of the drug target
- Target validation
- Identification of a lead compound (s)
- Lead optimization
- Product characterization
- Formulation and development
- Preclinical investigation
- Application for an investigational new drug
- Clinical trials
- Application of new drug
- New drug approved by regulatory bodies

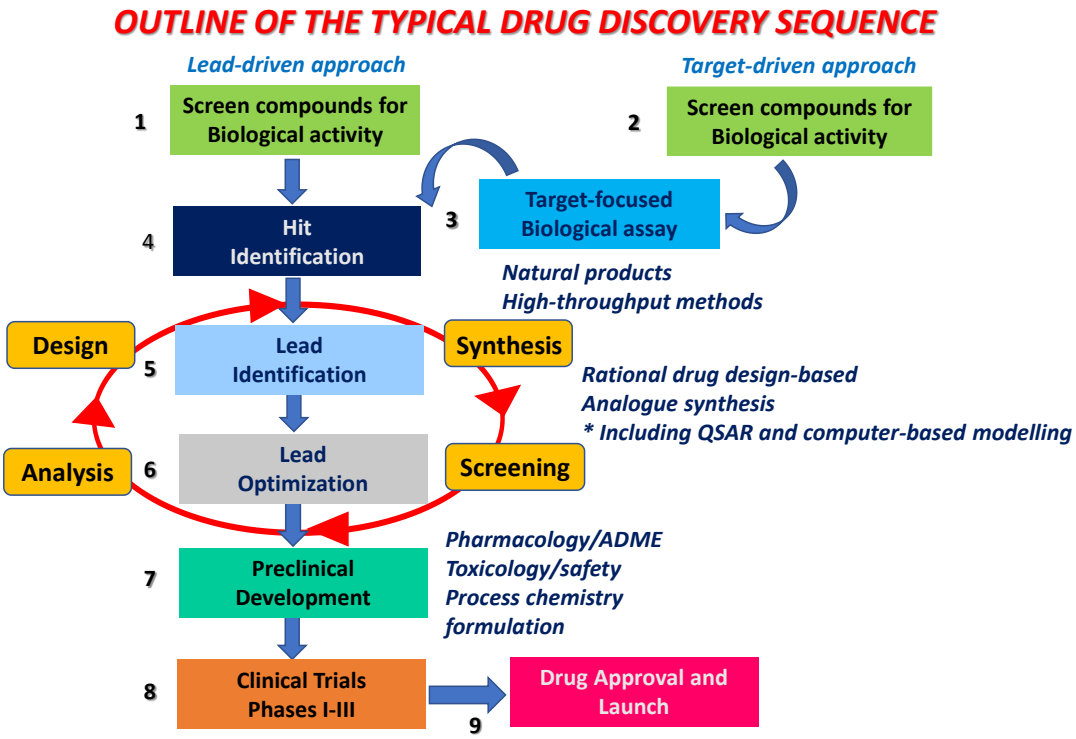


Figure 1.1. Schematic representation of the various stages of drug discovery

Following the selection of a promising therapeutic target, scientists want to identify the molecules that interact with the target to produce the desired biological outcomes. A hit compound is a substance that displays the desired activity in an assay for high-throughput screening (HTS). The process of hit discovery and hit-to-lead selection requires the development of pharmacologically appropriate screening assays. By altering the screening standards to make it easier to choose the most promising molecule for further development, lead compounds are select from a pool of hits. The secondary tests used for lead selection can

screen the physicochemical, ADME (absorption, distribution, metabolism, and excretion), and off-target effects.[5,6] Inexpensive analytical methods are necessary to effectively deal with considerable number of compounds having potential worth as drug targets. The most analytical technologies used in drug discovery include the below mentioned aspects:

- Analytical approaches applied to HTS
- Utilizing various extraction procedures to extract and purify medicines and their metabolites from biological samples including urine, blood, and tissues etc.
- Identification and interpretation of novel drug metabolites in biological matrices
- Development of impurity profiles, and determination of enantiomeric purity
- Assessing of pharmacokinetic/ pharmacodynamics (PK/PD) parameters
- Development of instrumental techniques for the assays of new chemical entity (NCE)

Analytical methods involving automation, robotics and miniaturization, in conjunction with other parallel techniques, have resulted in the development and discovery of new drugs.[7]

1.2 Importance of bioanalytical and ADME in drug discovery

Over the last few years, a change in the approach of the drug discovery process has happened emphasizing the necessity for the very early optimization of the pharmacokinetic features of the NCEs. To maintain a high degree of quality, the lead optimization requires fast sample turnover for the origination of early ADME and PK data (figure 1.2). Pharmacokinetics are often regarded as being important in the process of discovering and developing new drugs. This is because a number of prospective drugs have been abandoned after commencing clinical investigations as a result of insufficient ADME/PK research. The ADME/PK study is therefore typically included in the discovery process.[8-13]

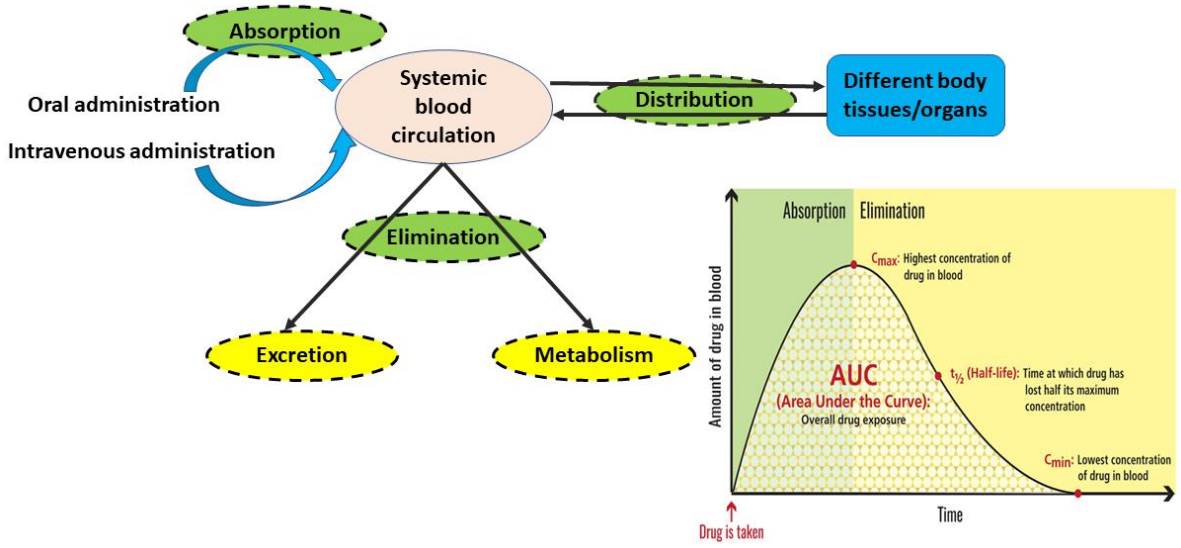


Figure 1.2. Schematic representation of the ADME/PK study

The difficulty in the field of bioanalysis is to develop quantification techniques that are quicker and more accurate while yet maintaining a high throughput while accounting for matrix interference and method resilience. Data on bioavailability, bioequivalence, pharmacokinetics, and toxicokinetics are evaluated and interpreted using bioanalytical method development and validation used for the quantitative determination of drugs and their metabolites in biological fluids such as blood, serum, plasma, urine, and tissues. Sensitive and precise bioanalytical techniques are crucial in the research of pharmacokinetics and toxicokinetics.

Every successful drug development procedure requires reliable data that can be obtained by the specific, sensitive, selective, and repeatable detection of a drug and its metabolites in biological samples.[14,15] The discipline of bioanalysis will have many exciting opportunities to develop in the future by improving its cost-effectiveness, environmental impact, throughput of assays, sensitivity, specificity, accuracy, and efficiency.[16-22] Yet more, standards prescribed by regulatory bodies for method development and validation define the limits of both speed and quality. The bioanalysis community offers a platform for the fast dissemination of original research and insightful reviews of all significant recent and relevant findings, including

- Mass spectrometry and other important detection methods
- Sample preparation and analyte extraction
- Chromatography and separation sciences
- Method development and validation
- Data analysis and statistics
- New apparatus and machinery
- Novel bioanalytical techniques
- Compliance and regulatory matters
- Efficiency and automation in laboratories

1.3 Sample preparation

In a bioanalytical investigation, sample preparation is a crucial step in the examination of medicines and metabolites. Complex matrices are frequently thought of as being present in bioanalytical samples. Prior to separation, detection, and quantification, their analysis requires sufficient sample preparation. The sample pretreatment procedure takes a long period.[23,24] The objective of sample preparation is to offer a treated sample that will result in better research findings. The fundamental idea behind sample preparation is to change the actual matrix into one that is appropriate for analysis using an analytical technique. Complex matrices frequently require for a more focused approach to sample preparation than, say, pharmaceutical solutions. To give a sample that has been processed and will produce better analytical results is the goal of sample preparation.[25] The goal of sample pretreatment optimization should be to increase

selectivity, increase sample throughput, and fully automate the process while concurrently lowering costs and raising analytical quality.

1.3.1 Sample preparation techniques

Some of the sample preparation techniques are

- Liquid-liquid extraction
- Solid phase extraction
- Protein precipitation
- Column-switching
- Solid phase micro extraction
- Ultra-filtration
- Dried plasma spots
- Drug conjugates hydrolysis
- Direct HPLC injection
- Derivatization
- Dried blood spot technique

The main sample preparation techniques are:

1.3.1.1 Liquid-Liquid Extraction (LLE)

LLE is a method for separating the components of a combination using water and an organic immiscible solvent. An analyte's LLE depends on how it divides into an immiscible organic phase and an aqueous phase. Here, an appropriate organic solvent can be used for extraction. The pH, ionic strength, and type of organic solvent used in the aqueous solution all have an impact on how the analyte is distributed across the two phases.[26-28] The LLE method is less expensive, quicker, and simpler over previous approaches. By using the multiple continuous extraction method depicted in figure 1.3, the majority of the drugs can be recovered to an extent of 90%. Support membrane extraction, liquid phase micro extraction, and single drop liquid phase micro extraction are just a few of the complicated technologies that have been developed to replace simple LLE procedures.

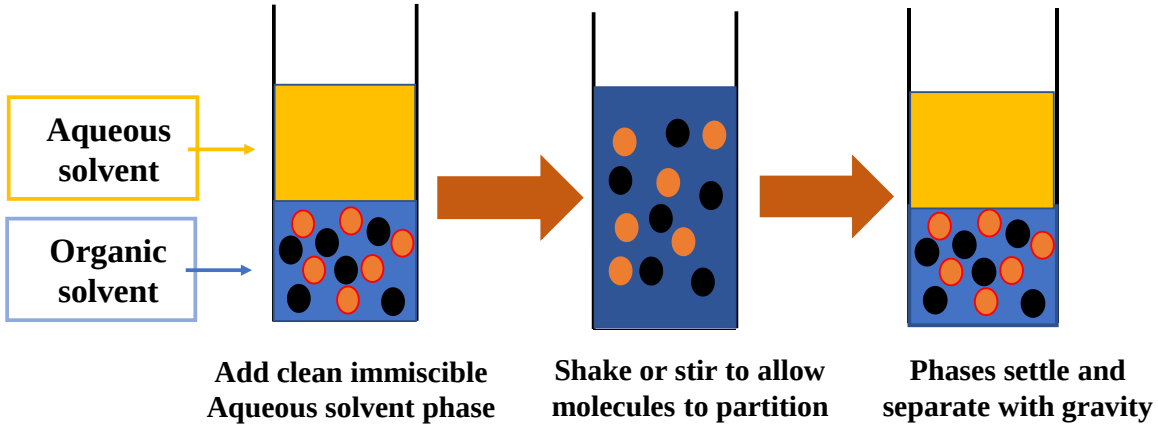


Figure 1.3. Liquid-Liquid Extraction (LLE) process

1.3.1.2 Solid-Phase Extraction (SPE)

In SPE, the analytes are divided into portions of liquid and solid.[29] SPE is among the most simple, efficient, and flexible sample preparation techniques for separating one or more analytes from a complex matrix. In most cases, intervening substances are removed by rinsing off the solid adsorbent, and an eluting solvent is subsequently used to desorbate the analytes. SPE is possible with a variety of sorbents and formats, and the systems are simple to automate. Normal-phase, reversed-phase, ion exchange, restricted access median (RAM), and molecularly imprinted polymers are among examples (MIPs).[30-32] The full SPE process is displayed in figure 1.4.

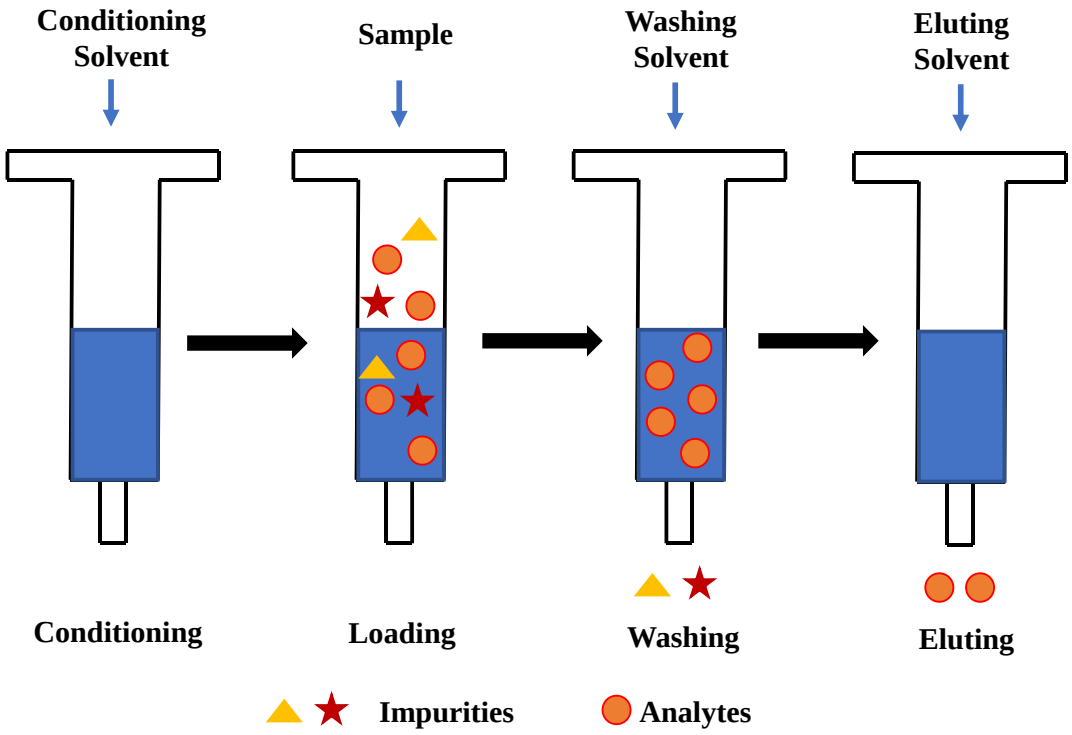


Figure 1.4. Solid-Phase Extraction (SPE) process

1.4 Chromatography

Chromatography was discovered in 1903 by a Russian botanist, Tswett, during the separation and isolation of various plant pigments.[33] Tswett called his newly discovered phenomena as "Chromatography" which is derived from Greek which means "colour writing".[34] This chromatographic method was not recognized earlier until L.S. Palmer and C.D here independently published about the similar separation process.[35] Chromatography is accomplished by distributing a mixture's chemical contents through a moving mobile phase and a stationary phase. The elements are carried by the mobile phase, which can be a liquid or a gas, as it flows through the stationary phase, which can be a solid or a liquid supported on a solid. The components with greater interactions with the stationary phase move more slowly in comparison to components with weaker interactions through the column.The separation of the mixture's components is brought on by this variation in flow speeds.

1.4.1 Steps involved in chromatography

- A substance or substances adhering to or remaining on the stationary phase
- Adsorption separation by mobile phase
- Elution is a process that recovers the separated substances by continuously flowing mobile phase.
- Qualitative and quantitative evaluation of the eluted compounds

1.4.2 Chromatography methods

The use of stationary and mobile phases allows for the classification of chromatographic techniques. Various chromatographic methods include the following:

- **Adsorption chromatography**

This chromatography method employs a solid stationary phase and a liquid or gaseous mobile phase (silica gel or any other silica-based packing). The solute is adsorbed on the surface of the solid particles. The separation of distinct analytes is accounted for via equilibration between the stationary and mobile phases.

- **Partition chromatography**

By selectively partitioning between a liquid stationary phase placed on the surface of a solid support, analytes can be separated. In the zone between the stationary liquid and the mobile phase, the solute establishes equilibrium.

- **Ion-exchange chromatography**

An opposite charge to the ions in the sample is present on the surface of the fixed bed. This strategy is frequently used with ionic or ionisable materials. Electrostatic force attracts

oppositely charged ions of a solute to the stationary phase. The aqueous buffer that serves as the mobile phase's pH and ionic strength determine how long the elution process lasts.

Size exclusion chromatography

- Effective method that works well with high-molecular-weight species
- Small (10 m) silica or polymer particles that make up column packings have a consistent pore network that allows solute and solvent molecules to diffuse through it
- The molecules are efficiently trapped and removed from the flow of the mobile phase in the pores
- The analyte molecules' typical residence time is governed by their effective size

1.4.3 High performance liquid chromatography (HPLC)

HPLC is a key analytical technology used across all phases of drug discovery, development, and manufacture in the modern pharmaceutical industry. The drug discovery and development paradigm leads to the development of robust analytical HPLC separation processes customised to each development group (i.e., early drug discovery, drug metabolism, pharmacokinetics, process research, pre formulation, and formulation). In the later half of the 1960s, HPLC's methodology was created. A sophisticated method of column liquid chromatography is high performance liquid chromatography. The components of a mixture are separated via HPLC using a liquid mobile phase.[36,37] We inject the sample, which is in solution form, into a liquid mobile phase in HPLC (figure 1.5). The components of the sample are divided based on how well they can partition between the mobile phase and stationary phase as the sample is moved through a packed or capillary column by the mobile phase. The separation of molecules that are polar, ionic, metabolic products, thermally unstable, and non-volatile is best accomplished by HPLC.

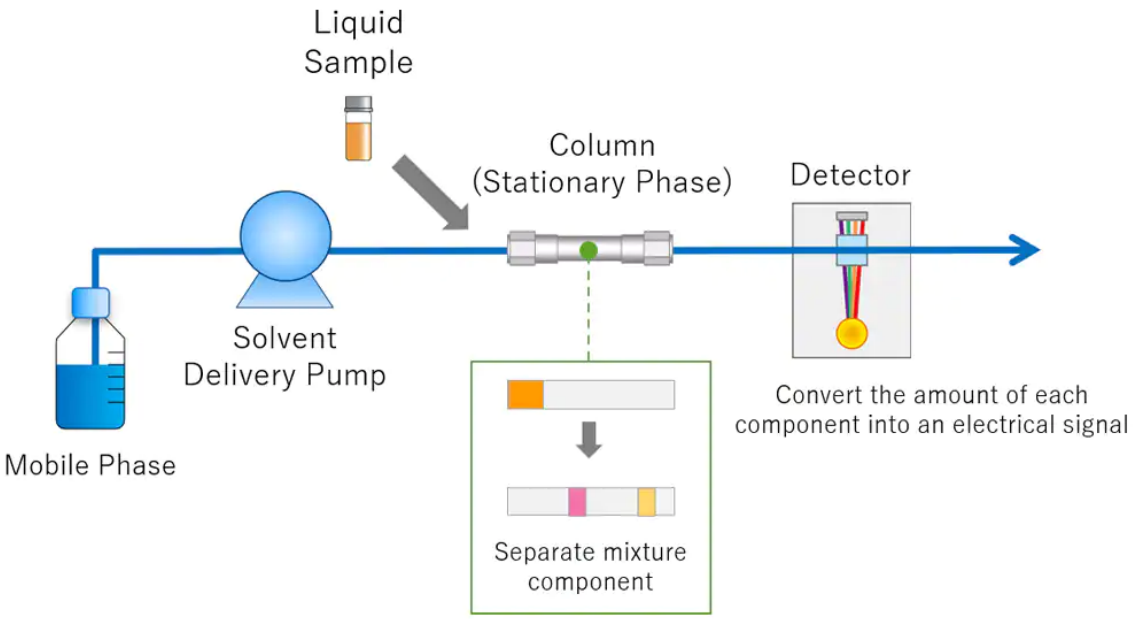


Figure 1.5. Schematic representation of HPLC instrumentation

A typical HPLC instrumentation consist of the following main compounds

Apparatus

This system operates in an isocratic mode, which means that a single solvent or combination is pumped throughout the analysis. Gradient elution can be achieved for various measurements by progressively changing the solvent composition.

Solvent reservoirs

Reservoirs for solvents are used to store enough mobile phases for the system to run continuously.

Pumps

Pumps ensure that the mobile phase moves through the system consistently and continuously. Modern HPLC pumps feature an online degassing mechanism and enable regulated solvent mixing from various reservoirs. Modern pumps also have specific filters to prevent any suspended particles from entering the mobile phase.

Sample Injector

A sample injector is used to inject the sample into the mobile phase stream that is coursing through the column. The majority of contemporary injectors are autosamplers, which enable programmed injectors of various sample volumes to be removed from the vials in the auto sampler tray.

Columns

The separation of the analytes in the mixture is actually performed by this component, which is the important part of the HPLC system. In a column, the mobile phase and stationary phase are in contact, generating an interface with large surface. The majority of recent chromatography development was focused on creating various approaches to improve this interfacial. The importance of column temperature cannot be overstated; it influences solvent viscosity, sample solubility, and diffusion. Air circulation is used to maintain a column's temperature continuously.

Detector

This tool is used to continuously record certain physical characteristics of column effluent. The primary purpose of the detector is to identify the sample's constituent parts by keeping track of the mobile phase as it leaves the column. The analyte's presence in the detector flow-cell results in a change in absorbance. A change in effluents is detected by the detectors and converted into an electric signal that the data system records. Different HPLC detectors are employed in the examination of various sample types to find solutes with various chemical nature. The desirable characteristics for detectors used in HPLC should be:

- Should be affordable
- Extremely sensitive
- Good repeatability and stability
- Response to the solute linearly
- Minimized baseline noise
- High usability and dependability
- The detector should be non-destructive
- Responses should be independent of mobile phase composition
- Ability to reveal information on the solute's identity
- A fast reaction time independent of flow-rate

The following are the most popular liquid chromatographic detectors:

Ultraviolet detectors/Absorbance detectors

The ultraviolet (UV) detector is an incredibly popular detector for HPLC analysis. The material inside a flow cell absorbs light when flow cell is directed at a specific wavelength. There is a decrease in light intensity because the amount of light that enters the flow cell is greater than the amount that exits. By using an absorbance detector, the decrease in light intensity is gauged. There are three typical types of absorbance detectors. Below is a discussion about them.

- **Fixed wavelength detector**

  The device continuously monitors the absorbance of only one wavelength (usually at 254 nm).

- **Variable wavelength detector**

  With the help of a deuterium source and a monochromator, the variable wavelength detector enables the selection of a specific wavelength in the UV-vis spectrum for selective detection.

- **Photodiode array detector (PDA)**

  The Photodiode Array Detector (PDA), a common UV detector, works by simultaneously observing a solute's absorbance at a number of distinct wavelengths. An achromatic lens system collimates the light from the broad emission source so that all of it can traverse the detector cell and directly onto the holographic grating. The sample is exposed to the light from the lamp's entire spectrum in this manner. A diode array is allowed access to the scattered light from the grating. There may be hundreds of diodes in the array, and a computer routinely analyzes the output from each diode and stores the sampled data on a hard drive. Any diode's output can be selected at the conclusion of the run, and a chromatogram can be made using the UV wavelength that was incident on that particular diode. Real-time recording of one diode's output during chromatographic development results in a real-time chromatogram. It has been observed that a spectrum can be generated by recording the time of a certain peak and then retrieving the output of every diode at that precise moment. For the evaluation, identification, and quantification of peak purity, the detector generates spectral and absorbance data. Figure 1.6 depicts the PDA detector's schematic diagram.

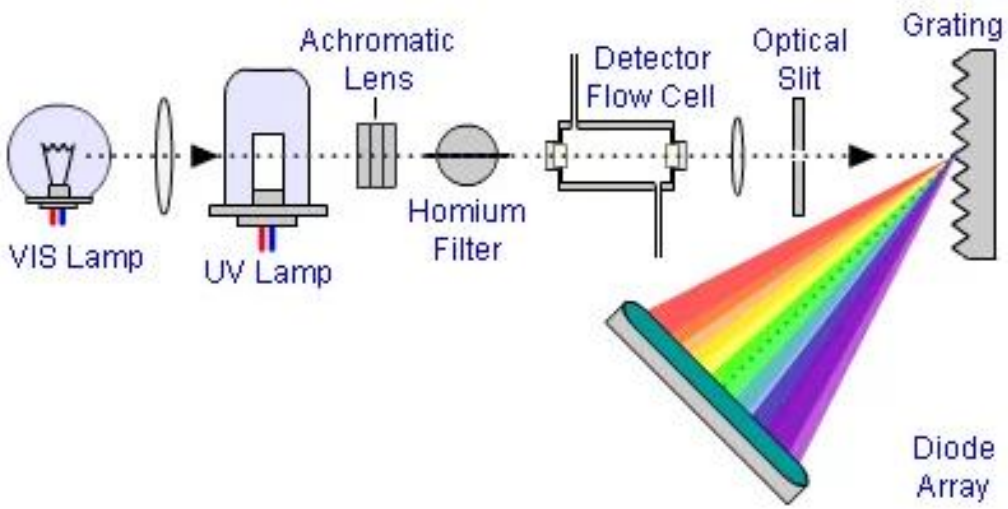


Figure 1.6. Schematic diagram of PDA detector

Refractive index detector (RID)

Refractive index detectors (RIDs) are helpful for various substances that are not UV-absorbing. In RID, a glass plate positioned at an angle separates the reference and sample segments,

causing incident beam bending for two different solutions. This can identify variations in the refractive indices of mobile phase and the column eluent. It is appropriate for all analytes, including inorganic ions, fatty acids, sugars, and alcohols. Thus, it is known as a universal detector. The drawback of the RI detector is that gradient analysis cannot be performed by it.

Electrochemical detector (ECD)

A number of tests, including those for phenols, thiols, neurotransmitters, catecholamines, aminoglycosides, and carbohydrates, use the very sensitive and selective electrochemical detection for HPLC method. The electrolysis of electroactive species at an electrode under the right voltage conditions is the basis for the detection concept, and the presence of such solutes can be determined by observing the current generated.

Fluorescence detector (FLD)

Fluorescence detectors excel in sensitivity and specificity when compared to other HPLC detector types. Fluorescence detectors examine the light emitted from excited atoms in an analyte to gather data from a solution that was obtained from an HPLC column. Despite the fact that many chemicals naturally glow, solutions without fluorescence can nevertheless be evaluated in this way by adding a fluorescent derivative. Pharmaceuticals, natural products, clinical samples, and petroleum products can all be analysed with FL detectors.

Recorder

Recorder keeps track of the signals from detectors as a deviation from the baseline. The constitution of the mobile phase, temperature, flow rate, injection volume, and other operating parameters are all controlled by software in modern HPLC systems, which are computer-based.

1.4.4 HPLC types

The following HPLC variants are dependent on the process's phase system (stationary):

1.4.4.1 Normal phase HPLC (NP-HPLC)

They are sometimes referred to as absorption chromatography or normal-phase chromatography. Polarity is used in this method to separate analytes. It has a non-polar mobile phase and a polar stationary phase. Thus, the popular mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures, whereas silica typically serves as the stationary phase. The method is employed for chiral compounds, class separations, geometric isomers, cis-trans isomers, and water-sensitive substances.

1.4.4.2 Reverse phase HPLC (RP-HPLC)

Since it operates on the theory of hydrophobic interactions, the longer a material is kept, the more nonpolar it must be. The mobile phase is an aqueous, moderately polar substance, such as mixtures of water, acetonitrile, or methanol, in contrast to the stationary phase, which is nonpolar (hydrophobic) in nature. Ionic, non-polar, polar, and polar molecules can all be prepared using this technique.

1.4.5 Applications of HPLC

The HPLC has a variety of potential uses. Resolution, chemical identification, and compound quantification are all types of data that HPLC may collect. It also aids in chemical separation and filtration.

1.4.5.1 Applications in the pharmaceutical industry

- To regulate drug stability
- A study of the tablet form of pharmacological dosing
- Control of pharmaceutical quality

1.4.5.2 Applications in the environment

- Identification and detection of phenolic compounds in water
- Pollutant biomonitoring

1.4.5.3 Forensic applications

- Detection of drug concentrations in biological samples
- Detecting steroids in urine, blood, and other body fluids
- Textile dye forensic investigation
- Detecting cocaine and other illicit narcotics in blood, urine, etc.

1.4.5.4 Applications in food and flavours

- Quality evaluation of water and soft drinks
- Fruit juice sugar analysis
- Analyzing the polycyclic substances found in vegetables
- Evaluation for preservation

1.4.5.5 Clinical test applications

- Examination of the urine and blood for antibiotics
- Biliverdin and bilirubin analysis in liver diseases
- Identifying endogenous neuropeptides in the brain's extracellular fluid

1.5 Hyphenated separation techniques used in pharmaceutical analysis

In the past few years, Identification and quantification of pharmaceutical impurities and assessment of their potential toxicological profile is a very challenging and tedious process as

very often, these impurities are present at very low concentrations in pharmaceutical products.[38] Hence, for accurate identification of impurities which are present at trace amounts, highly sophisticated and sensitive analytical techniques are required.[39] There are numerous methods for detecting impurities in drugs. The conventional method entails the separation of impurities, synthesis of impurities based on knowledge of synthetic routes and the chemistry involved, followed by structural analysis of impurities or DPs, and fractionation or isolation by preparative LC to obtain a sufficient amount of impurities for their structural characterization by mass spectrometry or NMR studies. But there are a few limitations to these conventional approaches. The process is often time-consuming and becomes tedious if impurities are present at very low concentrations in the sample. Some impurities or degradants in a sample are unstable and hence their identification becomes a harder task.[39]

All these shortcomings of conventional approaches have led to an increased focus towards hyphenated techniques. Several hyphenated techniques, including as LC-MS, GC-MS, LC-NMR, ICP-MS, and CE-MS have been used in the analysis of pharmaceutical drugs. LC-MS is made by connecting liquid chromatography with highly sensitive mass spectrometry. LC-MS and its modifications are commonly used to characterise impurities or degradants.[40] LC-MS is an extremely potent hyphenated analytical technique that couples the separation ability of LC with the detection ability of MS. Drug impurities, including by-products and degradation products, can be detected and identified using the LC-MS technique.

1.5.1 Liquid chromatography-mass spectrometry (LC-MS)

For the majority of assays employed during different stages of drug development, the preferred analytical technique is high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS or LC-MS/MS). The mass spectrometer has advanced as the primary technique for assessing mixtures in pharmaceutical research and development, working in combination with chromatographic separation techniques in the form of LC-MS and LC-MS/MS. The generation of mass spectral data by mass spectrometry yields essential information on the molecular weight, structure, identity, quantity, and purity of the material. The results of qualitative and quantitative analysis are more precise and trustworthy when combined with mass spectral data. For the majority of compounds, a mass spectrometer is far more sensitive and selective than any other LC detector. An LC-MS can also be used to evaluate compounds without chromophores. Without enhancing the chromatographic conditions, the components in an unresolved chromatogram are also recognised. Once a method for determining specific impurities using HPLC and UV detection was developed, it could be applied to LC-MS. While setting up an effective LC-MS technique for routine use, several aspects should be taken into consideration.[41] In LC-MS, non-volatile buffers such as phosphates, citrates, and borates should

be avoided. Ammonium formate and ammonium acetate are the best buffers for LC-MS analysis.[42] In general, all mass spectrometers work in four steps: putting in the sample; ionising it to turn neutral molecules into ions in the gas phase (ionisation method); utilising a mass analyzer to sort the gas phase ions according to their mass-to-charge ratios; and then identifying the separated ions. Ion source and mass analyzer are important components of a mass spectrometer. Various ionization methods have been developed over the years. Figure 1.7 depicts a mass spectrometer.

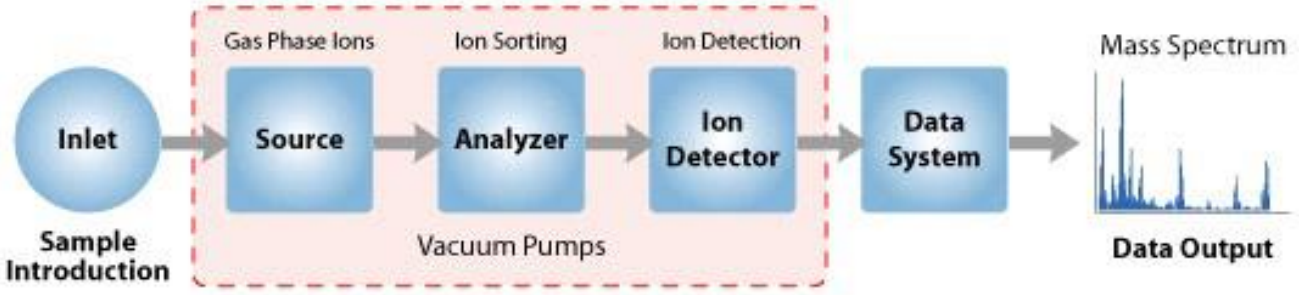


Figure 1.7. An illustration of the typical LC-MS process.

1.5.2 Ionization source

The ionisation source connects the LC to the mass spectrometer. The main benefit of the various ionisation sources is the ability to transfer the analytes to the appropriate gas phase while evaporating the mobile phase. In order to do mass analysis, it was necessary to ionise and shift from high or atmospheric pressures where chromatographic separation was done to low pressure. Below is a description of various ionisation sources used in mass spectrometry.

1.5.2.1 Electrospray ionization (ESI)

Before the analyte enters the mass spectrometer, electrospray produces analyte ions from solution. In the existence of a powerful electrostatic field, hot drying gas, and atmospheric pressure, the LC eluent is squirted (nebulized) into a chamber. The analyte molecules further split apart due to the electrostatic field. The solvent was transformed into droplets by the hot drying gas and allowed to evaporate. The charge concentration in the droplets grew as they shrank. Ions eventually desorb into the gas phase when the cohesive forces are overcome by the equivalent charge ion repulsive force. These ions are attracted to and enter the mass analyzer by a capillary sampling aperture. Proteins, peptides, and oligonucleotides are examples of samples that become multicharged and can be examined using electrospray ionisation. Figure 1.8 presents a schematic depiction of this electrospray ionisation.

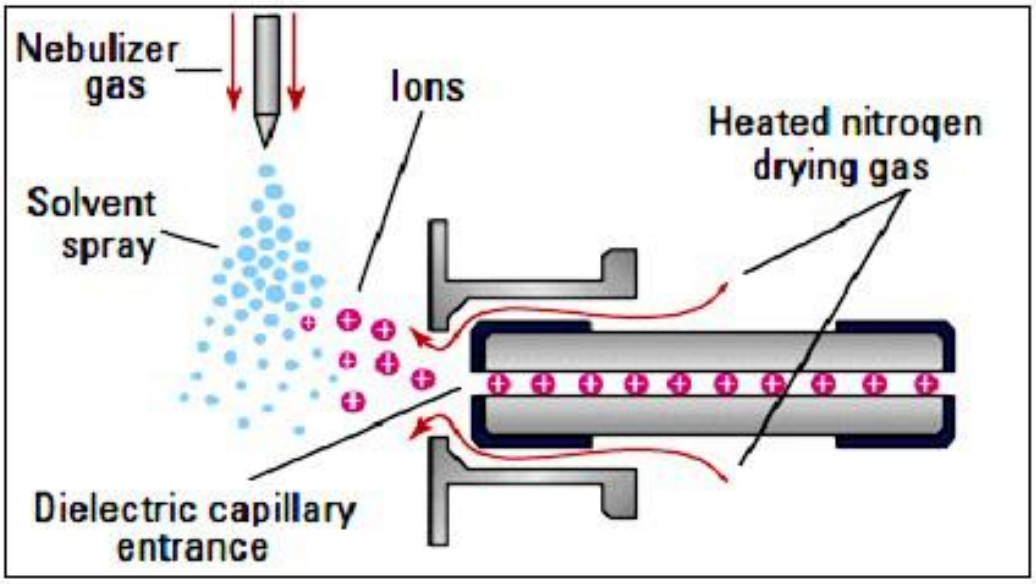


Figure 1.8. Schematic representation of an electrospray ionisation source

1.5.2.2 Atmospheric pressure chemical ionization (APCI)

Atmospheric pressure chemical ionization (APCI) is a widely used ionization technique in mass spectrometry. Due to its ability to analyse relatively non-polar substances and its ability to produce ions directly from solution, APCI has also grown to be a significant source of ionisation. Similar to electrospray, the APCI probe introduces the liquid effluent directly into the ionisation source. In APCI, a heated (often 250 °C–400 °C) vaporizer is used to spray the LC eluent. The liquid evaporates as a result of the heat. Electrons released from a corona needle ionise the resultant gas phase solvent molecules. Chemical ionisation is the process by which the solvent ions charge-transfer to the analyte molecules. Figure 1.9 is a diagrammatic depiction of this concept.

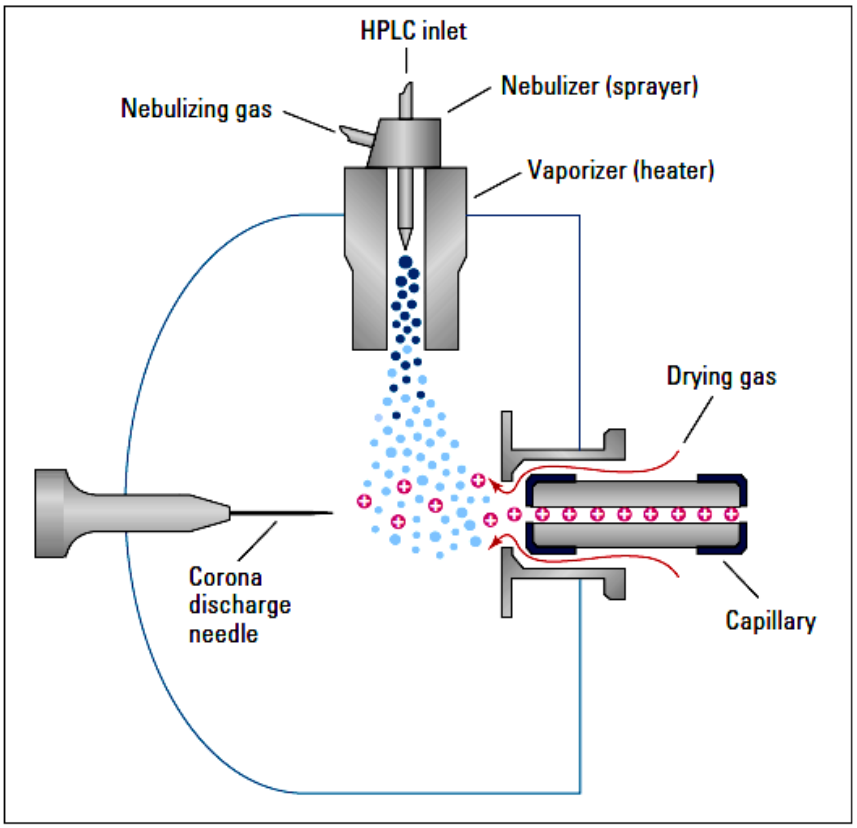


Figure 1.9. Diagrammatic representation of chemical ionization

1.5.2.3 Atmospheric pressure photo ionization (APPI)

A fairly new method is atmospheric pressure photo ionisation (APPI) for LC/MS. In APCI, a vaporizer transforms the LC eluent to the gas phase. Photons are produced by a discharge lamp within a constrained range of ionisation energies. The energy range was carefully designed to ionise as many analyte molecules as feasible while reducing ionisation of solvent molecules. The resulting ions enter the mass analyzer through a capillary sampling aperture. APPI can be used on moderately polar compounds such as polycyclic aromatics, steroids, etc. and is less recognized than ESI or APCI. A diagrammatic illustration of the photoionization caused by air pressure can be found in figure 1.10.

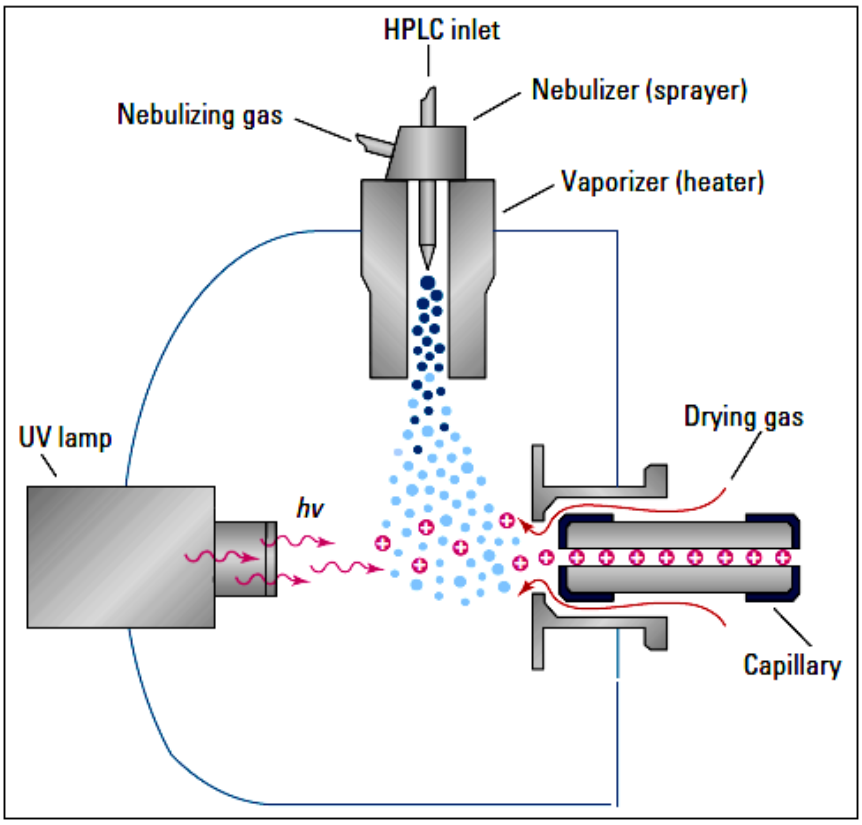


Figure 1.10. Diagram of an atmospheric pressure photo ionization

1.5.3 Mass analysers

As was previously mentioned, the ions generated in the ion source are accelerated into the vacuum interface, where they are examined. Only four of the many different types of mass analyzers are regularly employed in mass spectrometers linked to HPLC systems. The following is a brief explanation

1.5.3.1 Quadrupole mass analyzer

Four identically spaced parallel rods (quads) form a quadrupole MS, which is oriented on the central axis. Along the polar axis, ions are introduced. By applying various voltages to the various quadrupoles, it is possible to accelerate the ions at lower voltages while creating conditions where only detect ions with a specific m/z ratio can traverse to an ion detector. The simplest and most affordable mass analyzers are often quadrupoles.

Quadrupole mass spectrometers can be used to scan across a range of m/z values or to measure one or a few given ions in a mixture. They are frequently employed for targeted, quantitative applications. They can support MS and MS/MS applications, depending on its configuration. In a triple-quadrupole mass spectrometer, for example, you can choose the ions you want to study in the first quadrupole, smash them against a gas in the second quadrupole, and then

analyse the fragmented ions in the third quadrupole. Figure 1.11 provides a visual representation of a quadrupole mass analyzer.

- Quadruple mass analyzers can function in several modes:
- **Scanning mode (scan)**: In scanning mode, the mass analyzer examines a variety of mass-to-charge ratios.
- **Selected ion monitoring mode (SIM)**: SIM mode is far subtler than scan mode but only provides information on a smaller number of ions because the mass analyser only checks a small number of masses to charge ratios.
- **Multiple reaction monitoring mode (MRM)**: MRM mode is appropriate when there are plenty of quadruples. Since the first analyser transmits user-selected specific ions and the second analyser analyses user-selected specific fragments emerging from these ions, both analysers in this case are static. A thorough identification and characterization of the material under consideration is required before conducting this kind of experiment.

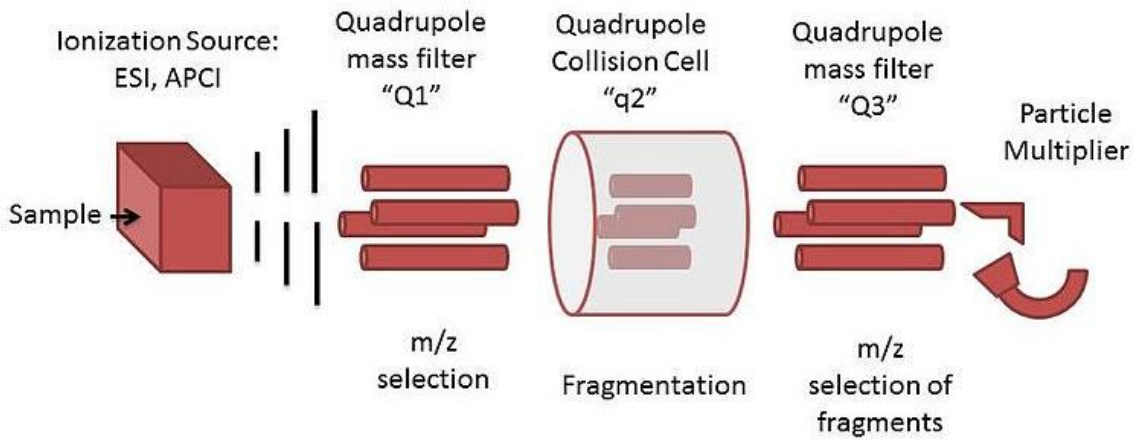


Figure 1.11. Diagram of triple quadrupole mass analyzer

1.5.3.2 Time-of-flight analyzer (TOF)

A time-of-flight (TOF) mass analyzer accelerates ions generated by the same electric potential using an electric field, and then measures the amount of time it takes for each ion to arrive at a detector. Each ion's velocity solely depends on its mass when all of the ions have the same charge, which also means that all of the ions have the same kinetic energy. This implies that lighter ions go faster to the detector than larger ions, which take longer.

Some of the unique benefits of TOF include the following:

- High resolution and high sensitivity studies of the entire mass range can be performed in a single pulse.

- In contrast to unit mass resolving power, greater resolution quadrupole mass filters are better at separating and identifying objects.
- Having quick access to the whole spectrum makes it possible to collect a lot more data in a shorter amount of time. This makes analysis of complex mixtures faster and more accurate.

1.6 Analytical method development approach

Analytical method development and validation are crucial factors to be considered during the drug development process. Analytical methods are typically used to assess quality characteristics such as the quantity of the active ingredient, the amount of impurities, the consistency, and the rate of delivery of dosage units.

The process of developing an analytical method involves steps such as.[43-45]

- Collection and review the literature
- Collection of the structures
- Comparison of the structures
- Mode of choice in chromatography
- Choice of column
- Choice of mobile phase pH
- Choice of the detector
- Choice of organic modifier

The physical and chemical properties of the drug must be studied in order to develop the most effective and appropriate HPLC method. The following is a detailed description of the various processes involved in the method development approach.

1.6.1 Collection and review the literature

One of the key components of how a research problem is formulated is the literature review. Pharmacopeia examines local, national, and worldwide publications for recommendations and information on methods that have already been reported on the drug molecule in order to use it as a starting point for studies. Furthermore, data on the drug's solubility profile, including its solubility in various pH ranges and solvents, analytical reports detailing physico-chemical characteristics like melting point and pKa as well as degradation trails, and stability reviews detailing the drug's sensitivity to moisture, heat, and other environmental factors, will be gathered. Oxidation, reduction, and hydrolysis products are discussed in research papers on metabolic pathways and are important indicators of potential degradation products as well.

1.6.2 Collection of the structures

The starting material, reagents, intermediates, and by-products in the reaction and breakdown products are most likely the molecule's fundamental chemical structure and related impurities.

1.6.3 Comparison of structures

Contamination, intermediates, byproducts, beginning materials, and degradation products with pertinent chemical drug material structures that arrive at polarity, irrespective of the fact that they are more or less polar than the attention composite. By contrasting the structures, we can see how closely linked they are structurally as well as how their functionality and polarity differ.

1.6.4 Selection mode of the chromatography

Because the majority of pharmaceutical medications are polar in nature, reverse phase chromatography is favoured for the majority of compounds. So, they can be efficiently and quickly eluted, however regular phase chromatography should be employed when dealing with enantiomers.

1.6.5 Selection of the columns

The following criteria to be utilized for column selection throughout method development.

←----------Non-polar----------moderately polar----------polar--------→

C18 < C8 < C6/C4 < phenyl < Amino < Cyano < Silica

1.6.6 pH selection in the mobile phase

The analyte's physicochemical properties, or its pKa value, which depends on the molecule's arrangement, are used to determine the buffer's moving phase pH. Analyze the compound's acidity, basicity, and neutrality. It is preferable to utilise an acidic mobile phase if the molecule has acidic functional groups. Employing basic or low pH mobile phases is appropriate if the chemical has a basic character. Neutral mobile phase is the best option if the compound is in a neutral condition.

1.6.7 Selection of detector

The most commonly used detector in HPLC instruments is UV-visible as it detects a wide range of compounds. In addition to this, various detectors used are photodiode array, fluorescence, conductivity, refractive index, electrochemical, mass spectrometer etc.

1.6.8 Selection of organic modifier

The best organic modifier is acetonitrile, which has a low viscosity and good UV transmittance. Use a 5–10% aqueous component in acetonitrile instead of 100% acetonitrile to prevent pumping problems.

1.7 Analytical method validation

Method validation is a continuous, growing process rather than a one-time task that can be neglected after completion. Regulatory agencies might use a well-executed and documented validation as proof that a method is secure and works as intended. In order to succeed, applicants should keep abreast of what regulatory bodies are considering and make an effort to foresee how the rules will evolve. Guidelines from the Food and Drug Administration (FDA), International Conferences on Harmonization (ICH), United States Pharmacopoeia (USP), and others provide a framework for carrying out such validations for pharmaceutical techniques. The objective of the validation of an analytical method is to illustrate that it is suitable for its deliberate use, as determined by means of well-documented experimental investigations.[46-48]

The fundamental purpose of method validation is to establish the reliability and performance of a specific method developed for quantitative measurement of an analyte in a specific biological matrix. Figure 1.12 displays the essential parameters for validation.

The following justifications establish the need for method validation:

- Quality assurance
- Obtaining international agency approval
- Any pharmaceutical product must be registered

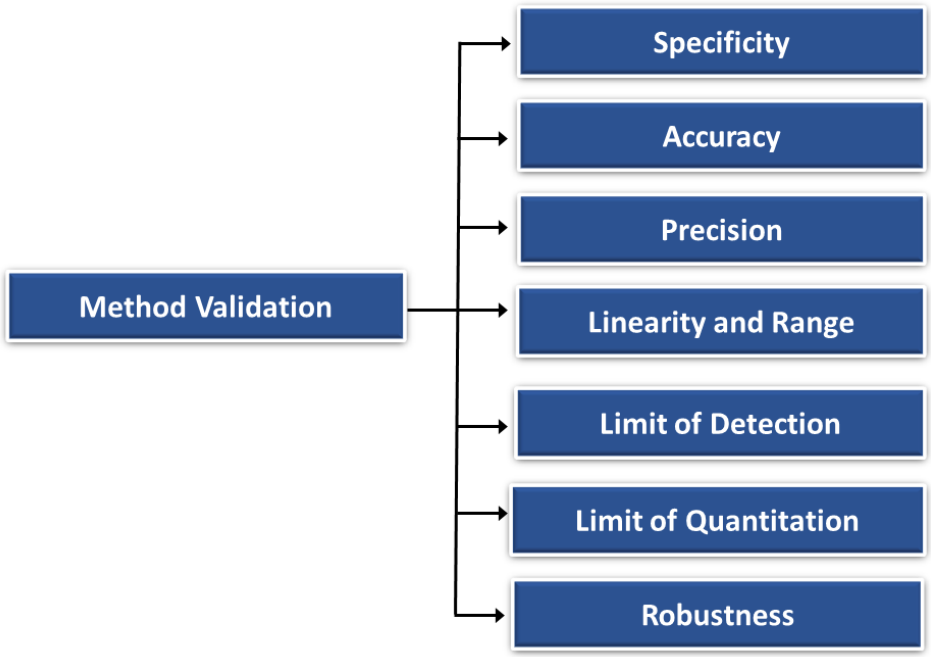


Figure 1.12. Parameters of method validation

1.7.1 Specificity

The ability of an analytical technique to identify and evaluate the analyte in the existence of supplemental constituents in the sample is known as specificity. For precision, at least six different sources of analysis of blank samples of the relevant biological matrix (i.e., plasma,

urine, or another matrix) should be acquired. Every blank sample should be checked for interference, and the lower limit of quantification should be ensured for specificity (LLOQ). Endogenous matrix components, metabolites, byproducts of breakdown, and, in the case of the present investigation, concurrent medications and other exogenous xenobiotics are examples of potential interfering chemicals in a biological matrix. Each analyte should be checked to make sure there is no interference if the method is meant to detect more than one analyte.

1.7.2 Precision

The analytical method's precision specifies how closely successive individual measurements of the analyte match. The coefficient of variation is used to describe precision (CV). For the LLOQ, low QC, medium QC, and high QC samples, precision must be shown. With the exception of the LLOQ, where it should not exceed 20% of the CV, the precision determined at each concentration level should not be more than 15% of the coefficient of variation. Between-run and inter-batch precision, also known as repeatability, is another category of precision that assesses accuracy across time and may involve different analysts, instruments, laboratories, and reagents.

1.7.3 Accuracy

The accuracy of a method is defined as the degree to which mean test results generated using a specific analytical method agree with the true value (concentration) of the analyte. Replicate analyses of samples with known concentrations of the analyte are used to evaluate accuracy. For each concentration, accuracy should be evaluated using a minimum of five determinations. Within the projected concentration range, it is advised to have a minimum of three concentrations. The mean value for bioanalytical procedures should be within 15% of the actual value, with the exception of LLOQ, where it shouldn't vary by more than 20%. The degree to which the mean deviates from the true value is a measure of accuracy. The mean value for non-bioanalytical procedures, on the other hand, should be within 1% of the actual value.

1.7.4 Lower limit of detection and quantitation

The concentration of analyte which gives a signal to noise (S/N) ratio of three is known as the lower limit of detection (LLOD). The concentration of analyte that gives a signal to noise (S/N) ratio of 10 is known as the lower limit of quantitation (LLOQ). With an accuracy of 80–120% and a precision of 20%, an analyte peak (response) at LLOQ should be recognizable, discrete, and repeatable. According to Q2 (R1), the accuracy of the assay of a drug substance can be studied from 80 to 120% of the test concentration. Accuracy solutions of 80%, 100%, and 120% can be prepared in triplicate, analysed according to the test procedure, and the percentage recovery calculated.

1.7.5 Calibration curve and linearity

The correlation between instrument response and known analyte concentrations is known as a calibration curve. Each analyte in the sample requires its own calibration curve to be generated. By injecting the biological matrix with an analyte at a given concentration, a calibration curve should be generated in the same biological matrix as the samples in the intended investigation. How many standards are needed to produce a calibration curve depends on the range of projected results and the type of analyte/response relationship. Based on the concentration range expected in a particular inquiry, standard concentrations should be selected. One blank sample, which is a matrix sample prepared without an internal standard, one blank sample, which was processed with an internal standard, and six to eight non-zero samples covering the anticipated range, as well as the LLOQ, should all be included in a calibration curve.

1.7.6 Robustness

Robustness is a measure of an analytical technique's capacity to remain unaffected by minute but intentional changes in test method parameters, which represents the method's trustworthiness for application in real situations. The robustness study may involve variation in sample extraction time, pH of mobile phase, mobile phase composition, flow rate, temperature, wavelength, different columns, etc. depending on the analytical procedure.

1.7.7 System suitability

Liquid chromatographic techniques always include system suitability tests. They are used to confirm that the chromatographic system's resolution and repeatability are adequate for the desired analysis. The tests are predicated on the notion that the instruments, electronics, analytical procedures, and test samples are all components of one system that can be assessed as a whole.

1.8 Forced degradation studies

Our understanding of the stability of active pharmaceutical ingredients (API) and drug products (DPs) is improved by the use of forced degradation, which also facilitates the development of analytical methodology. Research on forced degradation supports the need for regulations.

- Identification of potential degradation agents
- The therapeutic molecule's intrinsic stability and degradation pathways
- Stability indicating the validation of analytical techniques

The criteria for forced degradation testing are determined by the drug's phase of development. For instance, due to the high rate of compound attrition, phase 2 preclinical requires intensive technique development. As a result, when creating a logical research design, forced degradation

deliverables should be focused more on technique development than product identification and separation. As a drug progresses through phase 2 and registration, method development attempts focus on optimization. The major objectives of stress testing are the characterization and elucidation of degradation products. The necessary solid state and solution state stress conditions are included in forced degradation studies of active pharmaceutical ingredients (API) in accordance with ICH recommendations.[49-54] These are useful for creating a stability indicating technique for a certain active pharmaceutical ingredient (API). These investigations are repeated whenever the procedures, formulations, or methodologies are modified. The API should degrade by roughly 5-20% under the defined stress conditions. The chemical properties of the API will determine the conditions (strength and length) that will be used. The unstressed sample, the appropriate blank, and the stressed sample are compared. In circumstances where a compound does not deteriorate under a specific stress environment, further stressing is not recommended.[50] Forced degradation studies are subjected to hydrolysis, light, thermal/humidity, and oxidative conditions to obtain information about the stability of drug molecules.

1.8.1 Acid degradation

Studies on acid degradation were performed in the solution state. The pH range for acid hydrolysis is 1.0-2.0 using 0.1-1.0 M HCl or H_2SO_4. To ensure dissolution, it is necessary to add a suitable co-solvent or modify the solution's pH to the acidic range for those samples that are barely soluble or insoluble in the acid solution illustrated.[55] A maximum of seven days should be allowed for stress testing. To stop further degradation, the degraded sample is then neutralized with an appropriate acid, base, or buffer.

1.8.2 Base degradation

A base degradation study should also be performed in a solution condition using 0.1-1.0 M NaOH or KOH at a pH range of 12-13.

1.8.3 Oxidation degradation

For oxidation, H_2O_2 and AIBN (2,2'-azobisisobutyronitrile) reagents are usually used. Both H_2O_2 and AIBN act as free radical initiators. As previously mentioned, based on API solubility, it might be necessary to add a suitable co-solvent. Because hydrogen peroxide is an impurity in an excipient, testing for it under stress can be used to determine if a medication is safe.

1.8.4 Thermal degradation

In general, temperatures ranging from 50 to 100 °C are used to assess solid state stability The length of exposure depends on the sensitivity of the API. A phase change can occur under the

thermal conditions. So, it is usually run at temperatures below the critical temperature so that it doesn't change phase.

1.8.5 Photo stability

These tests are carried out in compliance with the ICH photo stability requirements.[56] According to ICH guidelines, the design of forced degradation tests is up to the investigators' judgement, and the degrees of exposure are appropriate. The recommended exposures for confirmatory stability investigations are a total illumination of at least 1.2 million lux hours and an integrated near UV energy of at least 200 Wh/m^2. In a solid state, the solid is exposed to a thickness of 1 mm spread on a watch glass. Acetonitrile is the cosolvent of preference for solution studies. Methoxy radicals generated by exposure to light can cause methanol to develop more artefact degradation products.

1.9 Pharmacokinetic study

A subfield of pharmacology called pharmacokinetics studies the disposition of drugs given externally to living things.[57,58] Theoretically, this field pertains to any compound that is ingested or otherwise provided externally to an organism, such as nutrients, metabolites, hormones, toxins, and so on. But in reality, it is primarily applied to pharmacological compounds. Studies of pharmacokinetics and pharmacodynamics frequently go hand in hand. Pharmacokinetics investigates what the body does to the medication, while pharmacodynamics investigates what a drug does to the body. How a certain medicine interacts with the body after administration is described by pharmacokinetics. Elements like the site of administration and the concentration at which the drug is supplied can have an impact on a drug's pharmacokinetic properties.[59-62]

1.10 *In silico* toxicity study

Drug discovery and development is a very complex and extensive process. To develop a new drug candidate, the average cost reaches approximately 2.6 billion U.S. dollars. The estimated attrition rate of new drug candidates is up to 96%. Drug safety and toxicity are one of the major root causes of this high attrition rate of drugs, which accounts for around 30% of drug failures.[63] In light of these things, making sure that drugs are safe and don't harm people is important for their health.

Toxicity is a measure of any undesirable or adverse effects of chemical substances. It is essential to determine the toxicity of chemicals in order to study their detrimental effects on humans and animals. These adverse effects are generally termed toxicity endpoints, such as carcinogenicity, mutagenicity, and genotoxicity.[64] Earlier, animal models were used for toxicological analysis of chemical compounds, which was time-consuming and costly. But with the advent of *in silico* toxicity assessment tools, which use computational resources to analyze, simulate, or predict

the toxicity of chemical compounds, the use of animals for testing has been reduced.[65] Apart from this, genetic toxicity like carcinogenicity is a very critical end point, for which a safety assessment test is required but cannot be performed on humans. In this context, *in silico* approaches for toxicity evaluation play a major role.

In silico toxicology is based on knowledge acquired from various scientific fields. The toxicity of a chemical substance can be predicted from its molecular structure and inferred from the properties of a similar chemical compound whose toxicological activities are well established.[66] Quantitative structure activity relationships (QSARs) are mathematical equations that are used as a predictive technique to evaluate the toxicity of new chemical substances. It is based on a simulation of a training set of compounds with known activity.[67] Using the knowledge of toxicity gained from the model training set, the QSAR quantifies the features of novel chemical compounds such that the overall poisonous properties of the molecule may be anticipated based on the relationship between the structure and activity. These computational techniques were created to support *in vivo* and *in vitro* toxicity studies, which may eliminate the requirement for animal testing and reduce the expense and time associated with toxicological evaluations.[66] Also, compared to traditional methods, in-silico methods have higher rates of throughput and reproducibility.

1.11 Aims and objectives of research work

The main aim of the current thesis is the development of bioanalytical and stability indicating High Performance Liquid Chromatography (HPLC), and Liquid Chromatography-Mass Spectrometry (LC-MS) based methods for the separation, identification and quantification of selected drugs in biological fluids. The purpose of this study is to reduce spending on analysis time, solvent use, chemical and material costs, and drug sample quantity. The new analytical techniques produce repeatable and trustworthy data to enable accurate interpretation of the investigations they support. The methods were validated as per USFDA as well as ICH guidelines. The objectives of the current work are

I. HPLC-PDA method for the determination of Eluxadoline and Rifaximin in rat plasma and its application to a pharmacokinetics

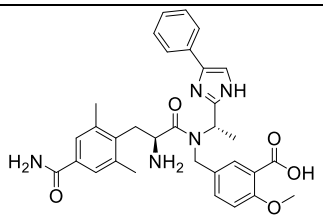
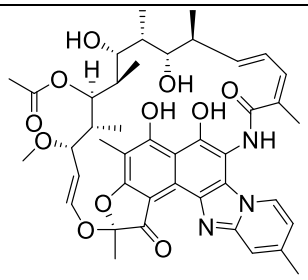
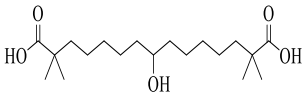
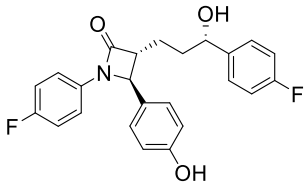
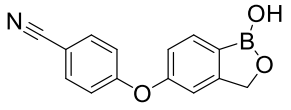
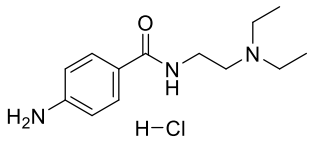
II. Simultaneous determination of Bempedoic acid and Ezetimibe in rat plasma using HPLC-PDA method and its application to a pharmacokinetic study

III. Identification and structural analysis of the stress degradants of Crisaborole using LC-MS/MS and toxicity prediction studies

IV. Degradation behaviour of Procainamide hydrochloride and comparison of its degraded products with known impurities: Development and validation of a stability-indicating HPLC-PDA method

The developed HPLC and LC-MS methods with the aforementioned ideas would offer a substantial opportunity towards the real time analysis of the specified drug molecules from biological fluids and stability studies in the form of active pharmaceutical ingredients. Table.1.1 displays the drugs that were chosen for the research work.

Table 1.1 List of drugs chosen for the current research study

S. No	Name	Chemical structure	Classification
1	**Eluxadoline**		Eluxadoline is a new therapeutic agent that reduced symptoms of IBS with diarrhea in men and women
2	**Rifaximin**		Rifaximin treats traveler's diarrhea and irritable bowel syndrome by stopping the growth of the bacteria that cause diarrhea
3	**Bempedoic acid**		It is used to decrease low-density lipoprotein (LDL) cholesterol ('bad cholesterol') in the blood of adults with familial heterozygous
4	**Ezetimibe**		It is used to treat high blood cholesterol
5	**Crisaborole**		Crisaborole is in a class of medications called phosphodiesterase inhibitors. It is used to treat eczema (atopic dermatitis)
6	**Procainamide hydrochloride**		Procainamide is a medication of the antiarrhythmic class used for the treatment of cardiac arrhythmias

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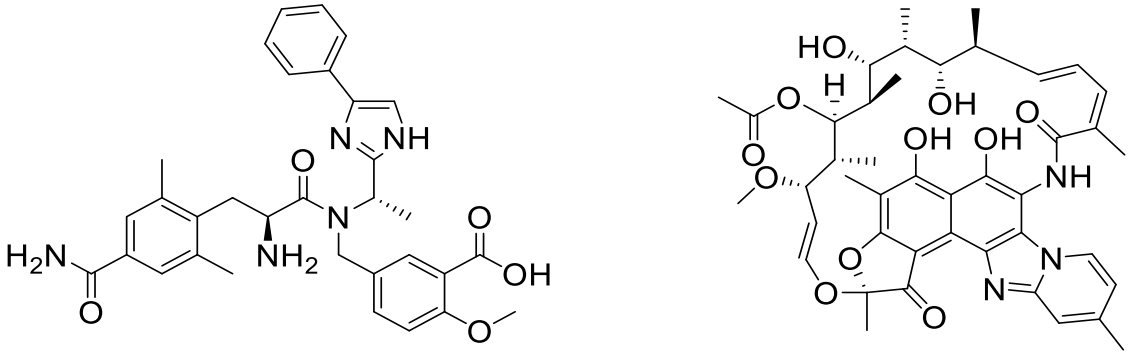
Chapter-II

Validation of the HPLC-PDA Method for the Detection of Eluxadoline and Rifaximin in Rat Plasma and Its Application to a Pharmacokinetic study

2.1 Introduction

Eluxadoline (ELX), is chemically designed as 5-({[(2S)-2-amino-3-(4-carbamoyl-2,6-dimethylphenyl)propanoyl][(1S)-1-(4-phenyl-1*H*-imidazoi-2-yl)ethyl]amino}methyl)-2-methoxybenzoic acid).[1] Eluxadoline is a new oral active medication having opioid effects (μ- and k-opioid receptor agonist and antagonist) and is used to treat IBS with diarrhoea.[2] Due to low gastrointestinal permeability and important presystemic metabolism, eluxadoline has a low oral bioavailability of less than 1%.[3,4] After oral administration of 100 mg of eluxadoline, the area under the plasma concentration-time profile (AUC) was 11-22 ng h mL^{-1} and the peak plasma concentration (C_{max}) was 2-4 ng mL^{-1} at 1.5 to 2.0 h. It binds to plasma proteins 81% of the time and has a half-life of 3.7 to 6.0 hours.[5] In two randomized, double-blind, placebo-controlled phase 3 3 studies (TARGET 1 and 2), in addition to a unique phase 3 retreatment trial (TARGET 3), the safety and efficacy of eluxadoline in patients with irritable bowel syndrome (IBS-D) with diarrhoea were investigated.[6-9] In 2015, FDA approved Rifaximin (RFX) for the treatment of the gastrointestinal disorder (IBS-D) in adults. It is chemically designated as (2*S*,16*Z*,18*E*,20*S*,21*S*,22*R*,23*R*,24*R*,25*S*,26*R*,27*S*,28*E*)-25-(Acetyloxy)-5,6,21,23-tetrahydroxy-27-methoxy-2,4,11,16,20,22,24,26-octamethyl-2,7-(epoxypentadeca[1,11,13]trienimino)benzofuro[4,5-*e*]pyrido[1,2-*a*]benzimidazole-1,15(2*H*)-dione.

Our aim in this present research work is to develop and validate a fast HPLC-PDA method for simultaneously quantifying of ELX and RFX in rat plasma for pharmacokinetic studies. The bioanalytical method employed here has been validated as per the USFDA guidelines. Figure 2.1 depicts the structures of analytes.



(a) **(b)**

Figure 2.1. Chemical structures of (a) Eluxadoline, (b) Rifaximin

2.2 Literature survey

A literature review shown that there are limited reports on the methods to determine these drugs in biological samples. Eluxadoline has been determined in rat plasma using μ-SPE followed by LC-MS/MS.[10] The pharmacokinetics of eluxadoline in healthy South Indian male participants

was investigated using LC-MS/MS technique.[11] RP-HPLC was used to separate and identify eluxadoline and its impurities.[12] It was reported on how the eluxadoline degraded under different stresses, as well as how HR-MS/MS and NMR tests helped to identify the degradation products. [13] Similar to this, RFX in biological matrices and pharmacological dosage forms has been detected using a variety of analytical techniques, including HPLC,[14–17], LC–MS/MS,[18,] LC–ESI–MS,[19,20]. Despite the fact that these techniques have the necessary sensitivity and applicability, some of them demand a significant amount of plasma, many of them involve a time-consuming extraction process, and others are either expensive or unavailable in most laboratories. Furthermore, to our knowledge, no analytical method has yet been reported for the simultaneous measurement of eluxadoline and rifaximin in rat plasma. Whereas, the method used in the present study HPLC-PDA has numerous advantages such as good separation, high sensitivity, outstanding specificity, and low cost as compared to the methods reported earlier.

2.3 Liquid-Liquid extraction (LLE)

LLE is a method that separates compounds in a mixture using water and an immiscible organic solvent. In order to extract an analyte, a suitable organic solvent can be used, LLE of an analyte is based on its partition between an aqueous phase and an immiscible organic phase. The ionic strength, pH, and type of organic solvent used in the aqueous solution, all have an effect on the analyte is distributed between the two phases.[21] In comparison to other methods, the LLE method is simple, rapid, and relatively cost-effective. Through the use of multiple continuous extraction methods, 90% of the drugs can be recovered. In recent days, in place of simple LLE procedures, more complex methods, such as support membrane extraction,[22] liquid phase micro extraction,[23] and single drop liquid phase micro extraction,[24] have been developed.

2.4 Experimental

2.4.1 Materials and reagents

The ELX, RFX, and loperamide hydrochloride were collected from the local pharmaceutical manufacturing industry (Hyderabad, India). Loperamide hydrochloride taken as internal standard (IS). Deionized water for sample processing was obtained using Milli Q ultrapure water purification equipment bought from Millipore (Bangalore, India). Acetonitrile (ACN) was procured from Merck (Mumbai, India). HPLC-grade orthophosphoric acid (OPA) and triethylamine (TEA) were acquired from Sigma Aldrich (Mumbai, India). In the present study, drug-free rat plasma was acquired from Bio Needs Laboratory (Bangalore, India), and it was stored at -20 °C until analysis. The centrifuge type 2-16P (Sigma, Zurich, Switzerland), nylon syringe filters of 0.22 m (Millipore, India), and heparinized tubes were used to collect blood samples.

2.4.2 Apparatus and chromatographic conditions

An Alliance e2695 HPLC system (Waters Corp., Milford, MA, USA) with a quaternary pump, in-line degasser, auto-injector, column compartment, and PDA detector (model 2998) was used to conduct the HPLC analysis. The Empower 2 software was utilized to collect chromatographic data. All the separations were achieved on an Waters Symmetry C8 column (5 µm, 250 mm × 4.6 mm) at room temperature (RT). Isocratic elution with HPLC-grade acetonitrile and 7 mM TEA (pH 2.5) (40:60, v/v) at a flow rate of 1 mL min^{-1}, an injection volume of 10 µL, and detection at 283 nm were used to determine ELX and RFX. A filter paper of 0.45 µm was used to filter all the solutions and solvents (Millipore, India).

2.4.3 Preparation of buffer solution

1 mL of triethyl amine was taken in 1 L of water and using orthophosphoric acid the pH was adjusted to 2.5.

2.4.4 Preparation of standard stock and working solutions

Standard stock solutions were made by accurately weighing 10 mg of ELX and 20 mg of RFX and dissolving them individually in 100 mL of HPLC-grade ACN. Stock solutions were further diluted to prepare the working solutions. The working solution of IS (1.5 ng mL^{-1}) was made by diluting the stock solution of IS with the same diluent. At 4 °C, all the solutions were kept in a volumetric flask and warmed to room temperature just before use.

2.4.5 Preparation of plasma calibration curve (CC) standards and QC samples

The calibration curve standards of the analytes were made by spiking a suitable volume of working solutions into blank plasma, and final concentrations were obtained as 10.0, 25.0, 50.0, 75.0, 100.0, 125.0, 150.0, and 200.0 ng mL^{-1} for ELX and 20.0, 50.0, 100.0, 150.0, 200.0, 250.0, 300.0, and 400.0 ng mL^{-1} for RFX. The quality control samples for both ELX and RFX were prepared at three different concentration levels in blank plasma. In the case of ELX, the lower quality control concentration (LQC) was prepared to be 50.0 ng mL^{-1}, and the medium (MQC) and high quality control concentration (HQC) were prepared to be 100.0 ng mL^{-1} and 150.0 ng mL^{-1}, respectively. Whereas in the case of RFX, the LQC was 100.0 ng mL^{-1}, the MQC was 200.0 ng mL^{-1}, and the HQC was 300 ng mL^{-1}. At -80 °C, all spiked samples were kept in a deep freezer.

2.4.6 Liquid-Liquid extraction (LLE) sample preparation

To extract analytes from the plasma, we have employed the liquid-liquid extraction method.[25-27] A plasma sample of 100 µL spiked with analytes of 600 µL and IS of 500 µL was added to an Eppendorf tube of 5 mL and the mixing process has been carried out for 1 min using a vortex. 800 µL of diluent was added to the samples and vortexed. The mixture was centrifuged at 4500

rpm for 20 min at 10 °C after 1000 µL of ACN was added to the tube and vortexing for 10 minutes. The supernatant solution was taken into another clean vial and dried under a nitrogen atmosphere at 25 °C. The residue was dissolved in 100 µL of mobile phase, of which a 10 µL aliquot was injected into the HPLC instrument. Figure 2.2 schematically extraction of ELX and RFX from rat plasma.

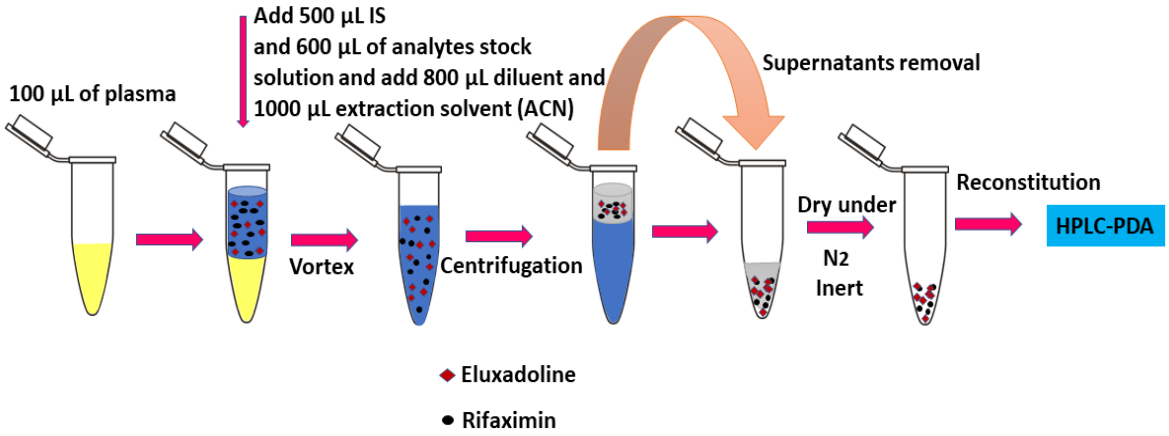


Figure 2.2. Schematic representation of LLE of ELX and RFX from rat plasma

2.4.7 Method validation

The specificity, accuracy, sensitivity, precision, matrix effect, linearity, extraction recovery, and stability of the analytical method were tested using the US FDA bioanalytical method validation rules.[28]

2.4.7.1 Specificity

To determine the specificity, six different samples of rat plasma and blank plasma spiked with target analytes (ELX and RFX) were taken and their retention times were analyzed. By analyzing the retention times of analytes and IS, no evidence of interfering substances was found under the developed chromatographic conditions.

2.4.7.2 Sensitivity

The LLOQC samples were prepared and processed in six replicates to measure sensitivity, precision, and accuracy at the LLOQ level. It was revealed that the precision and accuracy remained within the acceptable ranges of ≤20% and 80-120%, respectively.

2.4.7.3 Carry-over effects

By injecting a plasma blank sample right after the analysis of the upper limit of quantification, carry-over was investigated. The acceptable criteria were 20% of the analyte response at the lower limit of quantification and 5% of the IS peak response.

2.4.7.4 Linearity and LLOQ

To plot calibration curves, the ratio of peak areas of analytes to the IS was plotted against the concentrations of the analytes over the ranges of 5 ng mL^{-1} to 200 ng mL^{-1} for ELX and 10 ng mL^{-1} to 400 ng mL^{-1} for RFX. The internal standard concentration was kept constant at 1.5 ng mL^{-1}. A linear regression analysis was used to evaluate the linearity. The lowest analyte concentration on the calibration curve is defined as the LLOQ. It was confirmed that the precision and accuracy remained within the acceptable ranges of ≤20% and 80–120%, respectively.

2.4.7.5 Accuracy and precision

The determination of the precision and accuracy of this technique was done by analyzing six replicates at four different QC levels: (5.0 ng, LLOQ), (50.0 ng mL^{-1}, LQC), (100.0 ng mL^{-1}, MQC), and (150.0 ng mL^{-1}, HQC) for ELX, and 10.0 (LLOQ), 100.0 (LQC), 200.0 (MQC), and 300.0 ng mL^{-1} (HQC) for RFX. The percentage coefficient of variation (%CV) and percentage recovery were used to determine the precision and accuracy of the method.

2.4.7.6 Extraction recovery

Recovery of analytes was measured by differentiate the peak areas of analytes extracted from six replicates (n = 6) of QC samples at LQC, MQC, and HQC levels to the equivalent areas of un-extracted analytes at the same concentrations.

2.4.7.7 Matrix effect

Matrix effect was evaluated by differentiate the peak area of analytes spiked into blank plasma extracted samples to the peak area of analytes spiked into clean standard solutions. The matrix effect is calculated using six different sources of plasma lots at levels of LQC and HQC. No noticeable matrix effect was found with this method.

2.4.7.8 Stability

The stability was assessed for LQC and HQC concentrations of ELX and RFX in rat plasma, using various storage and handling conditions of samples. The benchtop stability was tested at RT for 6 h. The QC samples were kept in auto-sampler vials at 10 °C for 24 h. For long-term stability, samples were stored in a deep freezer at -80 °C for 28 days. Three freeze-thaw cycles from -80 °C to room temperature were used to investigate freeze-thaw stability.

2.4.7.9 Dilution integrity

The effect of sample dilution on method accuracy and precision was investigated by testing dilution integrity. To investigate the dilution integrity at a 2-fold dilution, spiked samples were

prepared above the ULOQ and tested in six replicates. The precision and accuracy should be within the acceptance criteria of ≤20% and 85–115%, respectively.

2.4.7.10 Application to a pharmacokinetic study

Six healthy rats weighing approximately 200 ± 20 g were used in a pharmacokinetic study. Each rat received an oral dose of ELX at 0.416 mg/kg and RFX at 0.832 mg/kg. The rats were fasted for 12 hours before the medications were administered, and they had access to water during the experiment. The blood samples were composed at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, and 6.0 h after the administration of drugs. Blood samples were taken in heparin-containing tubes and centrifuged at 4500 rpm for 10 min at 8 °C. The plasma samples were then refrigerated at -80 °C until they were analyzed. The WinNonlin programme was used to assess pharmacokinetic parameters.

2.5 Results and discussion

2.5.1 Method development and optimization

The present study aimed to develop and validate an HPLC-PDA method for simultaneously quantifying ELX and RFX in rat plasma samples. A systematic approach was employed to develop and optimize the method during the optimization process of the experimental conditions. To acquire the best separation and resolution from endogenous plasma components and eventually obtain sharp peaks and sufficient peak responsiveness, the chromatographic conditions during the study must be optimized for mobile phase choice, type of column, and flow rate. The separation was carried out using RP-HPLC with a variety of mobile phase combinations, which included organic modifiers such acetonitrile and methanol in various concentrations, as well as buffers including orthophosphoric acid and triethylamine of different strengths on Symmetry RP18, 150 mm x 4.6 mm, 3.5 µm; X-Bridge phenyl, 150 mm x 4.6 mm, 3.5 µm; and Waters Symmetry C8, 250 mm x 4.6 mm, 5 µm columns.

After several trials, the mobile phase containing HPLC-grade acetonitrile and 7 mM TEA in water (pH 2.5) (40:60, v/v) was selected. We have found excellent responses from ELX and RFX with no potential interference from endogenous substances when the flow rate was 1.0 mL min^{-1} and 283 nm was the detection wavelength. Hence, the above-mentioned conditions were selected for analysis. Under the selected conditions, the retention time was found to be 3.06 min for Eluxadoline and 7.82 min for Rifaximin, and the overall runtime was 12 min. Schematic illustration of the LLE of ELX and RFX from rat plasma is depicted in figure 2.3.

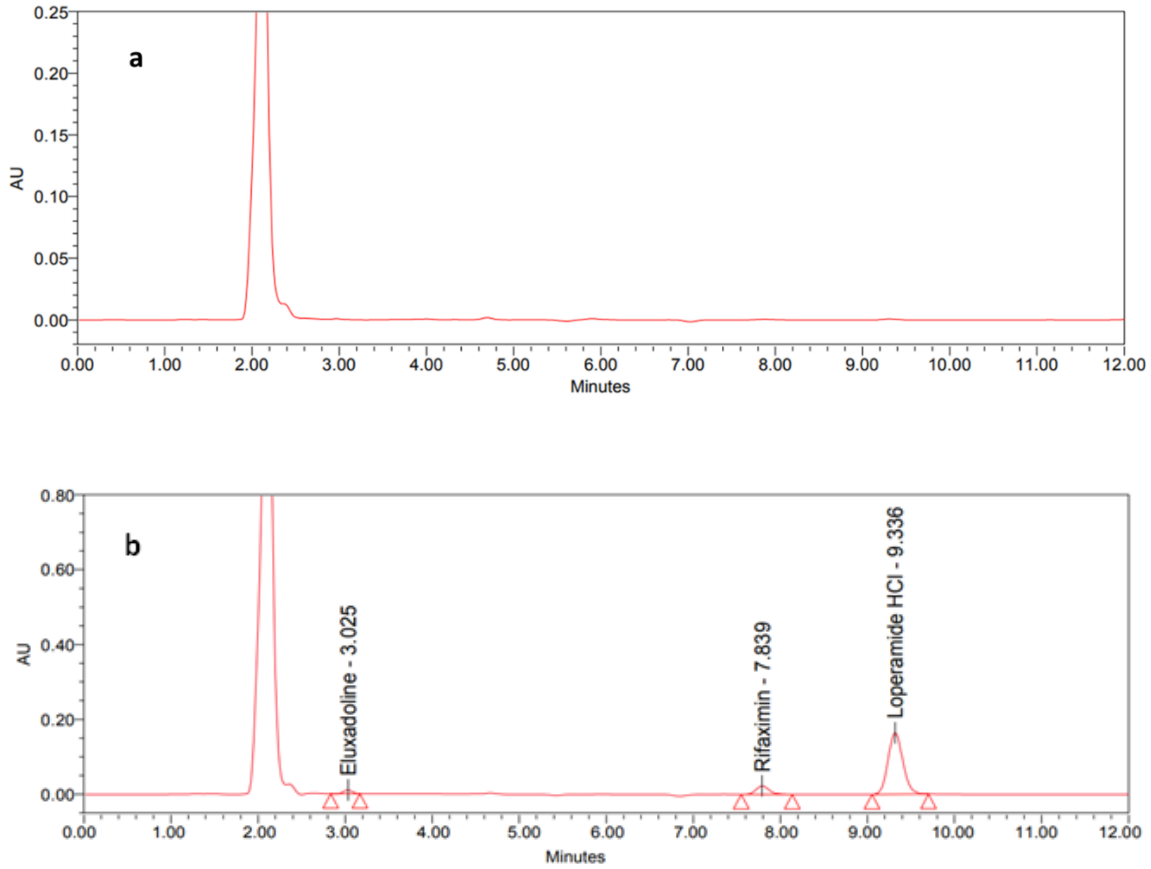


Figure 2.3. Representative chromatograms of a) blank plasma b) two drugs spiked into the plasma

2.5.2 Chromatographic Conditions

Columns	:	Symmetry C8 (4.6 mm x 250 mm, 5µ)
Mobile phase	:	Acetonitrile : 7 µM TEA in water [pH-2.5] (40:60 v/v)
Run Time	:	12 min
Flow rate	:	1.0 mL min^{-1}
Injection volume	:	10 µL
Column temperature	:	Room temperature
Detector	:	Photo diode array (PDA)
Wavelength (λ_{max})	:	283 nm

2.5.3 Method validation

2.5.3.1 Specificity

To evaluate the specificity of the method, six drug-free plasma samples from separate sources were selected. To assess the interferences, the peak responses of the blank plasma and plasma samples spiked with the analytes and internal standard (IS) were compared. As shown in figure

2.4, no interference was seen during the retention of analytes and IS from matrix endogenous components.

2.5.3.2 Sensitivity

The LLOQs for ELX and RFX were determined to be 5.0 ng mL^{-1} and 10.0 ng mL^{-1}, respectively. At this concentration, precision and accuracy results were found to be 0.48 and 100.4% for ELX, respectively, and 1.37 and 101.9%, respectively. The retention times of ELX, RFX, and IS were 3.06, 7.82, and 9.36 min, respectively. The chromatogram of analytes at LLOQ levels is shown in figure 2.4.

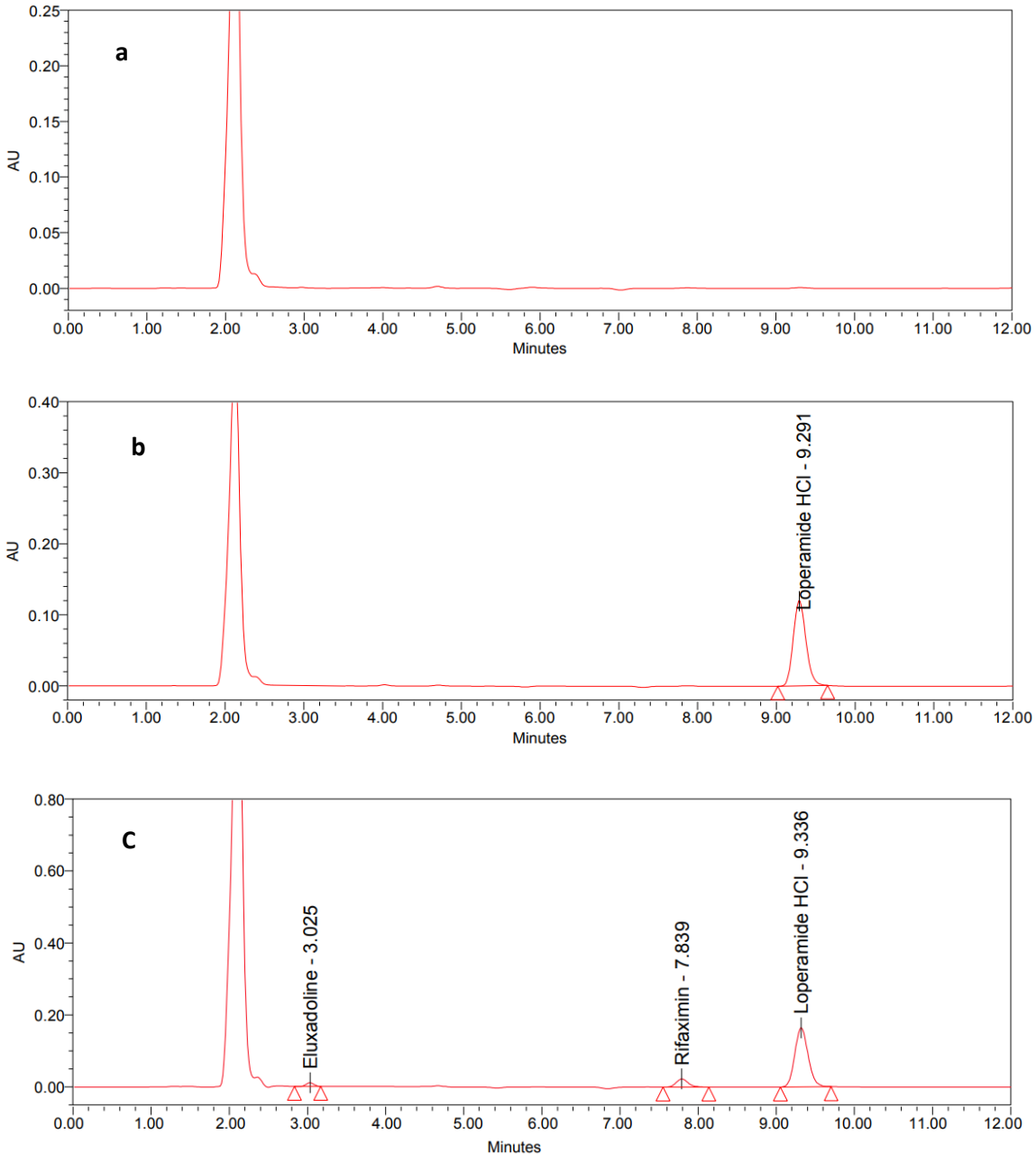


Figure 2.4. Representative chromatograms of (a) Blank plasma, (b) IS and (c) Analytes (5 ng mL^{-1} of ELX and 10 ng mL^{-1} of RFX) spiked at LLOQ levels

2.5.3.3 Carry-over effects

Analysis of blank plasma samples after injection of ULOQ samples revealed no peaks at the retention times of both analytes and IS, representing that the described approach has no carryover effect.

2.5.3.4 Linearity and LLOQ

Linearity was examined by preparing eight calibration standards in rat plasma with concentrations ranging from 5–200 ng mL^{-1} for ELX and 10–400 ng mL^{-1} for RFX. The internal standard concentration remained constant at 1.5 ng mL^{-1}. The ratio of peak areas of analytes to the IS was plotted against concentrations of analytes to plot calibration curves. The linearity of analytes is determined by linear regression analysis. The calibration curves were constructed and found to be linear with good correlation coefficients (r^2) of 0.9997 and 0.9998 for ELX and RFX, respectively (figure 2.5). The LLOQ was determined with tolerable accuracy and precision (≤ 20%). The LLOQs for ELX and RFX were estimated to be 5.0 and 10.0 ng mL^{-1}, respectively. These results are shown in table 2.1. Figure 2.6 shows the represented chromatogram of analytes at LLOQ levels.

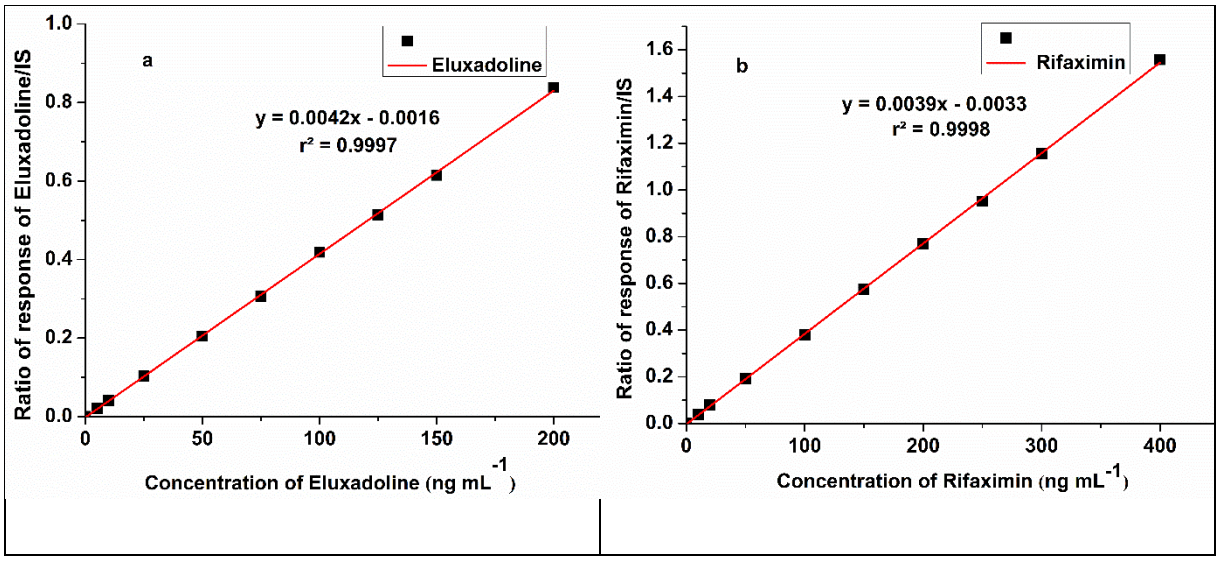


Figure 2.5. Linearity plots for a) ELX and b) RFX

Table 2.1 Linear calibration curve parameters and LLOQ of ELX and RFX

Analytes	Linearity range (ng mL^{-1})	Calibration equation	Coefficient (r^2)	LLOQ (ng mL^{-1})
ELX	5 - 200	y = 0.0042x - 0.0016	0.9997	5.0
RFX	10 - 400	y = 0.0039x - 0.0033	0.9998	10.0

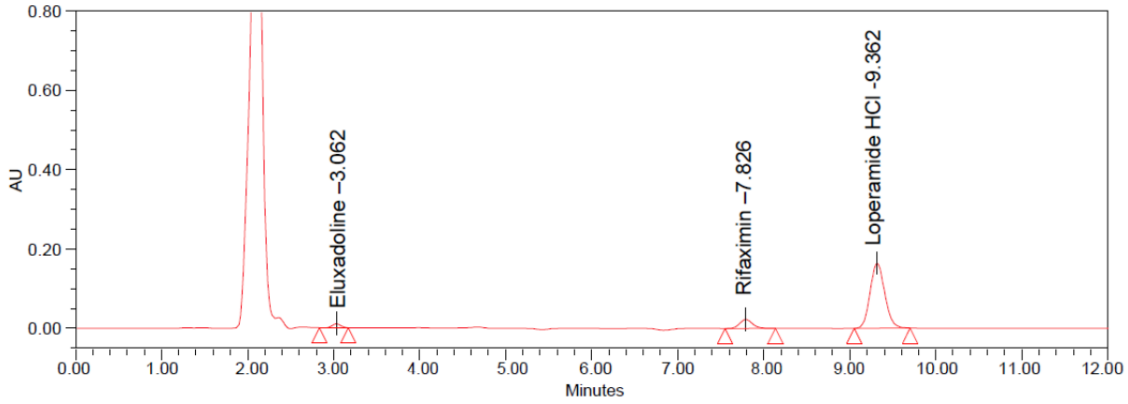


Figure 2.6. Represented chromatogram of ELX and RFX at LLOQ levels

2.5.3.5 Accuracy and precision

By analyzing six replicates of QC samples at four concentrations, the method's intra- and inter-day accuracy and precision were assessed (LLOQ, MQC, HQC and LQC). The precision and accuracy were estimated and depicted as percentages of recovery and percentages of coefficient of variation (%CV), respectively. The acceptance criteria for accuracy are within a range of 85–115% and for precision within 15% of the CV. The precision and accuracy results are shown in table 2.2.

Table 2.2 Intra- and Inter-day accuracy and precision results for ELX and RFX

Analytes	Concentration level (ng mL^{-1})	Intra-day (n = 6)			Inter-day (n =18)		
		Mean found conc. (ng mL^{-1})	CV (%)	Accuracy (%)	Mean found conc. (ng mL^{-1})	CV (%)	Accuracy (%)
ELX	LLOQ (5.0)	5.02 ± 0.023	0.465	99.17	5.04 ± 0.036	0.717	99.6
	LQC (50.0)	50.19 ± 0.207	0.413	99.65	50.43 ± 0.50	1.00	100.13
	MQC (100.0)	99.89 ± 0.134	0.134	99.72	99.78 ± 0.143	0.144	99.6
	HQC (150.0)	149.91 ± 0.311	0.207	99.68	149.94 ± 0.637	0.425	99.71
RFX	LLOQ (10.0)	10.19 ± 0.138	1.358	99.42	10.18 ± 0.12	1.186	99.39
	LQC (100.0)	100.36 ± 0.148	0.147	100.2	100.42 ± 0.131	0.130	100.2
	MQC (200.0)	200.21 ± 0.077	0.038	100.0	200.23 ± 0.143	0.071	100.0
	HQC (300.0)	302.16 ± 2.457	0.813	100.16	300.16 ± 2.157	0.714	100.61

2.5.3.6 Extraction recovery

Analytes recovery was calculated by comparing the peak areas of extracted analytes from six replicate (n = 6) QC samples at three concentration levels (lower, medium, and high QC) with the corresponding areas of unextracted analytes at the equivalent concentrations. The extraction recoveries for the three QC levels (low, medium, and high QC) for ELX were 97.0%, 91.5%, and 93.8%, respectively. The recoveries for RFX were 98.3%, 91.6%, and 95.3%, respectively. The obtained outcomes are tabulated in table 2.3.

Table 2.3 Extraction recovery results for ELX and RFX

Analytes	Spiked conc. level (ng mL^{-1})	Area response (n = 6)			
		Extracted mean response	Post extracted mean response	Extraction recovery (%)	CV (%)
ELX	LQC (50.0)	65540	67563	97.0	0.68
	MQC (100.0)	130424	142522	91.5	0.16
	HQC (150.0)	195784	208552	93.8	0.31
RFX	LQC (100.0)	120553	122549	98.3	0.17
	MQC (200.0)	240481	262371	91.6	0.13
	HQC (300.0)	362942	380569	95.3	0.83

2.5.3.7 Matrix effect

The matrix effect was investigated using six dissimilar plasma lots at two different QC concentrations by comparing the analytes' peak area spiked into extracted blank plasma samples to plain standard solutions at the same concentration (lower and higher QC). The percentage of the coefficient of variation (%CV) is within the acceptable range. No matrix effect was observed in rat plasma. In table 2.4, the results are described.

Table 2.4 Matrix effect results for ELX and RFX

Analytes	Area response (n = 6)				Matrix factor		CV (%)	
	Post extracted mean response		Neat mean response					
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
ELX	65540	195784	67563	208552	0.97	0.93	0.68	0.32
RFX	120553	362942	122549	380569	0.98	0.95	0.18	0.83

2.5.3.8 Stability

Under various storage conditions at LQC and HQC levels, ELX and RFX stability in plasma samples was investigated in six replicates. Analytes were stable at RT for up to 6 h under the benchtop stability conditions. In the auto-sampler, the analytes in the plasma sample were stable at 10 °C for 24 h. In the study of the dry extract, the drugs were found to be stable at 10 °C for 18 h. In the investigation of freeze-thaw stability, frozen samples were found to be stable in three freeze-thaw cycles from -80 °C to RT. The long-term stability test revealed that the analytes retained their stability for up to 28 days when stored at -80 °C. The acceptance criteria for accuracy were found to be within a range of 85–115%, and the precision was ≤15% of the CV. In table 2.5 shows the results of stability study.

Table 2.5 Stability study of ELX and RFX in rat plasma

Storage condition	Conc. level	ELX			RFX		
		Concentration (ng mL^{-1})	(%) CV	(%) Accuracy	Concentration (ng mL^{-1})	(%) CV	(%) Accuracy
Bench top, 6 h at RT	LQC	50.0	0.41	97.0	100.15	0.14	98.3
	HQC	150.0	0.1	93.7	300.3	0.04	94.6
Autosampler, 24 h at 10 °C	LQC	50.0	0.54	96.7	100.15	0.07	98.5
	HQC	150.0	0.09	93.7	300.3	0.04	94.7
Three freeze-thaw cycles, -80 °C to RT	LQC	50.0	0.39	96.6	100.15	0.32	98.4
	HQC	150.0	0.1	93.7	300.3	0.08	94.7
Long term, 28 days at -80 °C	LQC	50.0	0.43	81.7	100.15	1.03	84.3
	HQC	150.0	0.2	74.3	300.3	0.57	74.9
Dry extract, 18 h at 10 °C	LQC	50.0	0.61	97.0	100.15	1.92	100.9
	HQC	150.0	0.98	93.6	300.3	0.62	95.9

2.5.3.9 Dilution integrity

The integrity of dilution was assessed by measuring the concentration of each drug in rat plasma after 2-fold dilution with a blank matrix. The precision expressed as %CV was found to be 1.08 and 0.65 for ELX and RFX, respectively, and the accuracy was found to be 97.5% and 100.8% for ELX and RFX, respectively. By analyzing the results, it can be inferred that the dilution of the samples in the rat plasma with blank plasma has no impact on the precision and accuracy of the procedure.

2.6 A pharmacokinetic study in rats

After giving healthy rats oral doses of 0.416 mg/kg of ELX and 0.832 mg/kg of RFX, respectively, the validated analytical procedure was successfully used to the assay of plasma samples. Figure 2.7 illustrates the mean plasma concentration vs time curves for ELX and RFX. Table 2.6 consists of the pharmacokinetic parameters, namely, maximum plasma concentration (C_{max}), time of maximum plasma concentration (T_{max}), half-life ($t_{1/2}$), the area under the concentration-time curve 0 h to infinity ($AUC_{0-\infty}$), and the area under the concentration-time curve 0 h to time 't'(AUC_{0-t}) which were evaluated by using WinNonlin software.

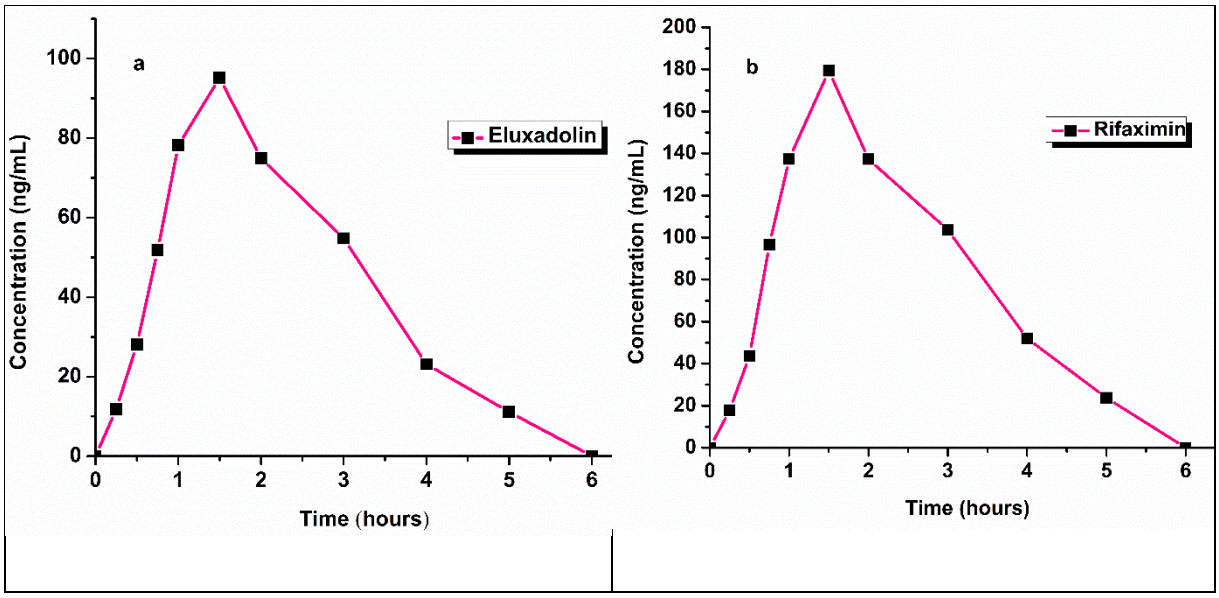


Figure 2.7. The mean plasma concentration-time curve of a) ELX (0.416 mg/kg), b) RFX (0.832 mg/kg) in rats.

Table 2.6 Pharmacokinetic parameters

Parameter	Analytes	
	RFX	ELX
$t_{1/2}$ (h)	5.0	5.0
t_{max} (h)	1.5	1.5
c_{max} (ng/mL)	95.2	179.3
AUC_{0-t} (ng.h/mL)	245	463
$AUC_{0-\infty}$ (ng.h/mL)	245	463
$AUC_{t-\infty}$ (ng.h/mL)	11.9	23.8

2.7 Conclusion

In the current study, an HPLC-PDA technique for ELX and RFX measurement in Wistar rat plasma was successfully developed and validated. This method developed in the present study has the benefit of being simple and cost-effective for liquid-liquid extraction for sample pre-treatment, along with a reduced chromatography acquisition time, and the separation of analytes is possible with an isocratic mode with a mobile phase (HPLC-grade acetonitrile and an aqueous solution of TEA, pH 2.5). The lower limit of quantification for ELX was found to be 5.0 ng mL^{-1}, and for RFX, it was found to be 10.0 ng mL^{-1}. The method was thoroughly validated in terms of accuracy, precision, and stability to meet USFDA recommendations for bioanalytical method validation. The proposed method may be useful for determining ELX and RFX in rat plasma, according to validation conditions. This method was also used to study the pharmacokinetics of ELX and RFX in rats.

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Chapter-III

Simultaneous Determination of Bempedoic Acid and Ezetimibe in Rat Plasma Using HPLC-PDA and Its Application to a Pharmacokinetic study

3.1 Introduction

Hypercholesterolemia is a common risk factor that leads to cardiovascular disease (CVD). One of the most effective ways to lower the risk of cardiovascular disease in hypercholesterolemia patients is to control lipid levels.[1] In multi-pull clinical trials, it has been clearly demonstrated that statins have well-established first-line therapeutic option for low-density lipoprotein cholesterol (LDL-C) lowering, reduce the risk of major cardiovascular (CV) events, and mortality.[2] However, many patients fall short of the LDL-C targets set by recommendations. Furthermore, despite being well-treated, some individuals continue to have a very high or even excessive cardiovascular risk, mainly due to presence of co-morbidities such as diabetes or peripheral artery disease, which significantly increase their global CV risk.[3] On the other hand, many patients are intolerant to statin therapeutic doses which lead to muscle-related adverse effects.[4] According to evidence from randomized controlled trials, statin therapy is linked to lower rates of myalgia, myositis, and rhabdomyolysis.[5,6]

Adenosine triphosphate-citrate lyase (ACL), an enzyme two steps upstream of (3-hydroxy-3-methylglutaryl coenzyme A) HMG-CoA reductase in the cholesterol production pathway, is inhibited by bempedoic acid (BPA), an oral, first-in-class inhibitor. Its chemically named as 8-hydroxy-2,2,14,14-tetramethylpentadecane dioic acid. The very-long-chain acyl-CoA synthetase-1 (ACSVL-1) enzyme, expressed in the liver, but not functional in the skeletal muscle, converts BPA (ETC-1002) to the active form (ETC-1002-CoA).[7] The activated form inhibits ACL by lowering cholesterol production which results in increased expression of the hepatic low-density lipoprotein receptor (LDLR) and, thereby, reduces LDL-C blood levels and increases clearance of circulating LDL-C.[8] According to recent clinical studies, oral BPA effectively suppresses LDL-C in adults with primary hypercholesterolemia or mixed dyslipidemia, regardless of whether such investigations are limited to BPA alone.[9-11]

Ezetimibe (EZE), 1-(4-fluorophenyl)-3(R)-[3(S)-(4-fluorophenyl)-3-hydroxypropyl]-4(S)-(4-hydroxyphenyl)azetidin-2-one is a novel cholesterol-lowering drug that effectively prevents dietary and biliary cholesterol absorption in the intestine and lowers blood cholesterol levels.[12,13] It functions locally in the small intestine's brush barrier, inhibiting cholesterol absorption by enterocytes.[14] By rapidly combining plasma and tissue pools in the faeces, EZE improved reverse cholesterol transport efficiency.[15] Clinical trials have shown that EZE monotherapy is effective in maintaining lipid profiles, but it is even more effective when combined with statins.[16,17] As a result, combining EZE with low-dose stain therapy resulted in a further reduction in cardiovascular events in hypercholesterolemia patients.[18] BPA with EZE (fixed-dose combination) is an adenosine triphosphate-citrate lyase (ACL) inhibitor and a cholesterol absorption inhibitor combination indicated as an adjunct to diet and maximally

tolerated statin therapy for the treatment of heterozygous familial hypercholesterolemia or established (Atherosclerotic cardiovascular disease) ASCVD to reduce LDL-C levels.[19,20] There is a scarcity of data on the efficacy and tolerance of BPA and EZE fixed-dose combination in patients with high cardiovascular disease risk and hypercholesterolemia. Clinical trials show that fixed-dose combination of BPA and EZE, reduced low-density lipoprotein cholesterol in patients with hypercholesterolemia and high cardiovascular disease risk in comparison to EZE monotherapy or placebo and exhibited a favourable safety profile when added to maximally tolerated statin therapy in patients.[21,22]

Chromatographic methods now play an essential role in the development of the pharmaceutical sector. More and more combination dosage forms have been made by the pharmaceutical industry to improve therapeutic effects. A significant challenge is indeed the development of analytical techniques for these combination drug products. As an alternative to developing several reversed-phase (RP) chromatographic methods for evaluating combination pharmaceutical products, the industry currently demands chromatographic methods for each active ingredient. In our present study, the HPLC-PDA method was developed for combined product (BPA and EZE) indicated as an adjunct to diet and maximally tolerated statin therapy for the treatment of heterozygous familial hypercholesterolemia.

In accordance with FDA regulations, the purpose of this research is to develop and validate a quick, accurate, and precise HPLC-PDA approach for the simultaneous detection of BPA and EZE in rat plasma for pharmacokinetic studies. Pitavastatin act as internal standard in this work and figure 3.1 depicts the structural formulae of analytes.

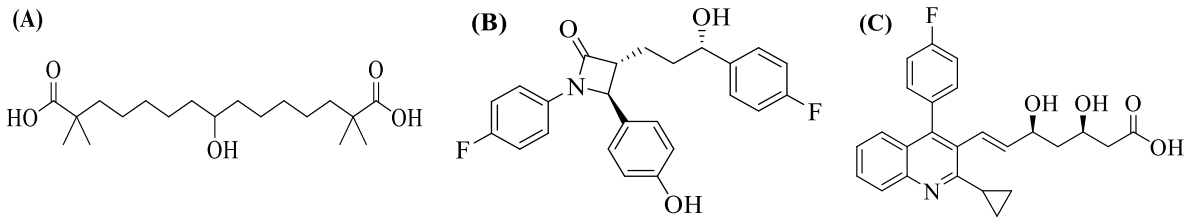


Figure 3.1. Chemical structures of A) bempedoic acid (BPA), B) Ezetimibe (EZE) and C) Pitavastatin (Internal Standard)

3.2 Literature survey

Few spectrophotometric[23-27] and chromatographic methods such as HPLC[28-30] and LC-MS[31-38] techniques, have been published in the literature for the determination and analysis of EZE alone and in combination with other medications. In chromatographic methods, LC-MS/MS approach for the quantification of BPA and its keto metabolite in human plasma and urine has been reported.[39] Also, a current, validated RP-HPLC technique for analysis of Bempedoic acid

and Ezetimibe in bulk drug samples has been reported.[40] A stability-indicating RP-UPLC approach for simultaneous detection of BPA and EZE in bulk products and pharmaceutical formulations[41] as well as a degradation study of BPA and EZE using UPLC-MS/MS[42] have also been reported. A novel HPLC method developed for the estimation of bempedoic acid and ezetimibe in bempedoic acid and ezetimibe pharmaceutical dosage forms of tablets have been reported.[43] To the best our knowledge, no analytical method for the measuring BPA and EZE combination in rat plasma has been published. In comparison to the methods published before, HPLC-PDA method utilized in the current study, offers various advantages including best separation in terms of resolution, high sensitivity, remarkable specificity, and low cost.

3.3 Experimental

3.3.1 Materials and reagents

Biocon Pharmaceutical Industry supplied bempedoic acid, ezetimibe, and also a pitavastatin internal standard (IS) (Bangalore, India). HPLC grade water for sample processing was procured from Millipore's Milli Q purification system, (Bangalore, India). HPLC gradient grade methanol and acetonitrile (ACN) were procured from Merck (Mumbai, India). Analytical grade reagents of formic acid and triethyl amine (TEA) (Mumbai, India) were purchased from Sigma-Aldrich. Drug-free rat plasma used in this study was acquired from the Bioneeds Laboratory (Bangalore, India) and maintained at -20 °C until analysed. The research centrifuge model Tc 4100 F (Electrocoat, Mumbai, India) and 0.22 µm nylon syringe filters (Millipore, India) were used for the study. The mobile phase 0.1% TEA in water (pH 2.5) (formic acid was used to adjust pH) and acetonitrile (40:60, v/v) was used as a diluent.

3.3.2 Apparatus and instrumental conditions

HPLC system is comprised of an alliance e2695 HPLC (Waters Corp., Milford, MA, USA) equipped with a quaternary pump, in-line degasser, auto-injector, column chamber, and PDA detector (model-2998). Empower 2.0 software was employed to acquire chromatographic data. At room temperature, all separations were performed using an X-bridge C18 column (150 × 4.6 mm, 3.5 µm). Isocratic elution mode with 0.1% TEA in water (formic acid was used to adjust pH 2.5) and acetonitrile (40:60, v/v) and the 1 mL min^{-1} flow rate was employed. The injection volume was 10 µL and detection was performed at 236 nm.

3.3.3 Preparation of parent stock solutions and standard stock solutions

Bempedoic acid parent stock solution (7000 ng mL^{-1})

A 100 mL volumetric flask containing 5 mg of bempedoic acid working standard was weighed, transferred, and then diluted to volume using diluent (0.1% TEA in water (pH 2.5) (formic acid

was used to adjust pH) and acetonitrile (40:60, v/v)). Additionally, 1.4 mL of the stock solution was diluted with diluent to obtain 10 mL.

Ezetimibe parent stock solution (4000 ng mL^{-1})

A 100 mL volumetric flask containing 5 mg of ezetimibe working standard was weighed, transferred, and then diluted to volume using diluent. Additionally, 0.8 mL of the stock solution was diluted with diluent to obtain 10 mL.

Standard stock solution (72 ng mL^{-1} of Bempedoic acid and 4 ng mL^{-1} of Ezetimibe)

Transfer 1 mL of bempedoic parent stock and 0.1 mL of ezetimibe parent stock solution into a 100 mL volumetric flask and dilute to volume with diluents to prepare a standard stock solution. The same diluent is used to make a 500 ng mL^{-1} IS stock solution. The IS stock solution was also diluted with the same diluent to create a working IS solution. In a volumetric flask, all the solutions were maintained at 4 °C, and just before use, they were allowed to warm to room temperature.

3.3.4 Preparation of plasma calibration curve standards and quality control (QC) samples

Calibration curve (CC) standards for analytes were produced by spiking appropriate volumes of standard stock solutions in blank plasma, with final concentrations ranging from 1.8 to 36.0 ng mL^{-1} for BPA and 0.1 to 2.0 ng mL^{-1} for EZE. In blank plasma, QC samples were prepared at (low, LQC), (medium, MQC), and (high, HQC) concentrations of 9.0, 18.0, and 27.0 ng mL^{-1} for BPA and 0.5, 1.0, and 1.5 ng mL^{-1} for EZE. All spiked samples were maintained at -20 °C in a deep freezer.

3.3.5 Sample preparation

BPA, EZE, and IS were extracted from plasma samples using a simple liquid-liquid extraction method. The plasma (200 µL) was spiked with 500 µL of stock solution of analytes and 500 µL of IS and 800 µL of ACN to precipitate all the proteins and mixed in vortex cyclo mixture for 1 min followed by centrifuged at 12,000 rpm for 15 min. The upper organic layer was removed and allowed to evaporate at 35 ºC while being gently streamed with nitrogen. The residues were then re-dissolved in 100 µL mobile phase, vortex-mixed for 30 s, and centrifuged for 15 min at 12,000 rpm. Collect 10 µL of the supernatant solution and place in an HPLC vial and injected into the HPLC system. The extraction procedure is displayed schematically in figure 3.2.

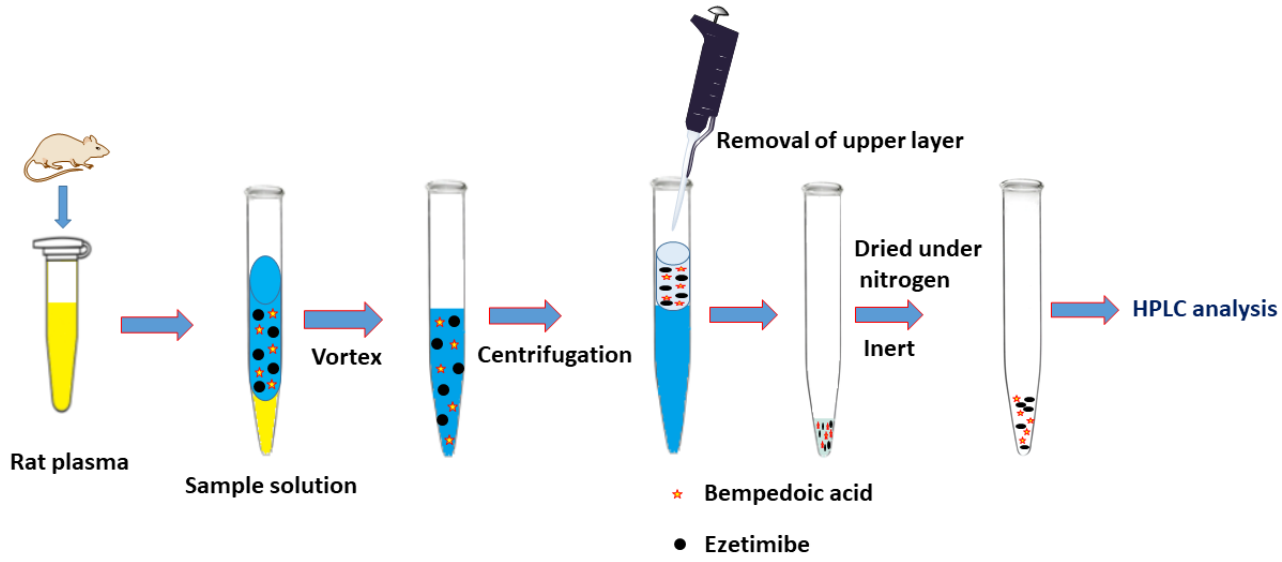


Figure 3.2. Representation of the LLE of BPA and EZE from rat plasma

3.3.6 Method validation

Using the USFDA guidelines for bioanalytical method validation[44], the analytical method was validated for sensitivity, specificity, accuracy, precision, linearity, matrix effect, extraction recovery, and stability studies.

3.3.6.1 Specificity

Six separate specimens of rat blank plasma spiked with target analytes, BPA and EZE were collected, and retention times were evaluated to establish specificity. Under established chromatographic conditions, no evidence of the existence of obvious interfering compounds was detected when the retention times of analytes and IS were examined.

3.3.6.2 Sensitivity

To test sensitivity, precision, and accuracy at the lower limit of quantification (LLOQ) level, six replicate LLOQC samples were prepared and analyzed. The acceptance criteria for measured concentrations showed a precision within ≤20% of CV and an accuracy within 80–120% of the nominal concentration.

3.3.6.3 Carry-over effects

Carryover was confirmed by injecting the samples at the highest concentration level, followed by blank plasma injections. Carryover acceptance criteria is ≤20% of the analyte response at the lower limit of quantification.

3.3.6.4 Linearity and LLOQ

Calibration curves were created by plotting peak area ratios (of the analytes to the internal standard) vs analytes' plasma concentrations over a 1.8 to 36.0 ng mL^{-1} range for BPA and 0.1

to 2.0 ng mL^{-1} range for EZE. The internal standard concentration was held constant at 40.0 ng mL^{-1}. Linear regression analysis was used to examine the linearity. The acceptance criterion for the correlation coefficient should be over 0.99, the precision within ≤20% of the CV, and the accuracy within 80–120%.

3.3.6.5 Precision and accuracy

The accuracy and precision were evaluated by six replicate analyses of quality control samples containing LLOQ, LQC, MQC, and HQC concentrations of 1.8, 9.0, 18.0, and 27.0 ng mL^{-1} for BPA, and 0.1, 0.5, 1.0, and 15 ng mL^{-1} for EZE, respectively. The precision and accuracy of the procedure were evaluated using the percentage coefficient of variation (CV%) and percentage recovery. The acceptance criteria for measured concentrations showed a precision within ≤20% of CV and an accuracy within 80–120% of the nominal concentration.

3.3.6.6 Extraction recovery

Recovery experiments were performed to determine the recovery of the analytes. In these experiments, the corresponding areas of un-extracted analytes at the same concentrations were analyzed to determine the peak areas of analytes extracted from six replicate QC samples at LQC, MQC, and HQC levels.

3.3.6.7 Matrix effect

By comparing the peak regions of each analyte in plasma samples collected prior to spikes with those in clean solution, the matrix effect was determined. The matrix impact was computed using six separate plasma batches from six different sources at the LQC and HQC levels. In this approach, there was no discernible matrix effect.

3.3.6.8 Stability

The stability of BPA and EZE was investigated by analysing LQC, MQC, and HQC plasma samples under various handling and storage conditions. The bench-top stability at room temperature (short-term stability) was evaluated for 6 h. The QC samples were placed in an auto-sampler and analysed at 10 °C for 24 h. For long-term stability, the QC samples were kept at -80 °C in a deep freezer for 28 days. Three freeze-thaw cycles ranging from -80 °C to room temperature were used to investigate the stability of freeze-thawing.

3.4 Application to a pharmacokinetic study

Six healthy rats weighing approximately 250±20 g were used in a pharmacokinetic investigation. Each rat was administered an oral dosage of 0.75 mg/kg BPA and 0.04 mg/kg EZE. Rats were fasted for 12 h before getting the medications and were given water during the experiment. Blood samples were taken at 1.0, 2.0, 4.0, 8.0, 12.0, 16.0, 20.0, and 24.0 h after the

drugs were administered. Blood samples were taken and centrifuged for 15 min at 10 °C at 4500 rpm in K3-EDTA tubes. Afterwards, all the samples were kept at -20 °C prior to examined. Pharmacokinetic parameters are determined using the WinNonlin programme.

3.5 Results and discussion

The current study used with RP-HPLC to simultaneously determine the BPA and EZE in rat plasma, which allowed the study of the pharmacokinetics of these drugs in rat plasma with the RP-HPLC method. Liquid-liquid extraction was used to ensure successful plasma extraction while avoiding matrix impact.

3.5.1 Method development and optimization of chromatographic conditions

Preliminary investigations were used to establish the optimal chromatographic conditions for BPA and EZE in rat plasma. To optimise the chromatographic conditions, several trials were carried out with different buffers, such as 0.1% TFA (trifluoroacetic acid), 0.1% OPA, and 0.1% TEA, and organic solvent acetonitrile in the mobile phase. However, the mobile phase composition was altered in each trial to improve resolution between the peaks, acceptable tailing, and retention times. While using a mobile phase of 0.1% TFA and acetonitrile with different compositions, the resolution between the analytes was low, and the peak shape was not good. The mobile phase with 0.1% OPA and acetonitrile was tried with various portions, but the tailing and resolutions are not within the limits. The better peak shape and improved resolution were found using a mobile phase of 0.1% TEA and acetonitrile.

After several trials, the mobile phase comprised of 0.1% TEA in water (formic acid was used to adjust the pH 2.5), and acetonitrile (40:60, v/v) was chosen with a stationary phase X-bridge C18 (150 mm × 4.6 mm, 3.5 µm) linked to a PDA detector. We observed acceptable responses from BPA and EZE when the flow rate was set to 1.0 mL min^{-1} and the detection wavelength was 236 nm, with no potential interference from endogenous chemicals. As a result, the conditions mentioned above were chosen for analysis. Under the specified conditions, the retention time was detected at 6.83 and 7.96 min, with a total run time of 10 min.

3.5.2 Chromatographic Conditions

Columns	:	X-Bridge C18 column (150 mm x 4.6 mm, 3.5µm)
Mobile phase	:	0.1% TEA in water, pH-2.5 (formic acid was used to adjust the pH): ACN (40:60 v/v)
Run Time	:	10 min
Column temperature	:	Room temperature
Flow rate	:	1.0 mL min^{-1}
Injection volume	:	10 µL

Detector	:	Photodiode array (PDA)
Wavelength (λ_{max})	:	236 nm

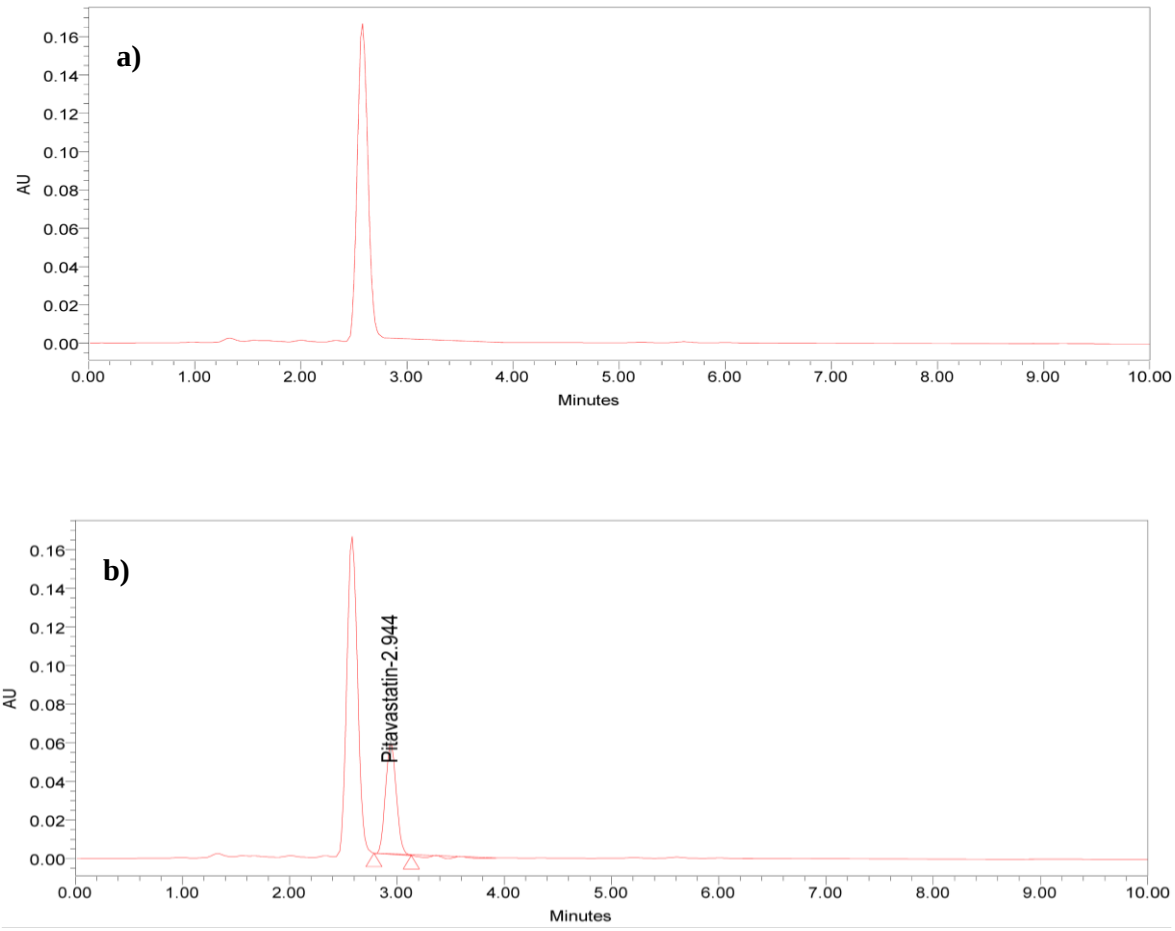
3.5.3 Method validation

3.5.3.1 Specificity

Six drug-free plasma samples from various sources were adopted to examine the method's specificity. To assess the interferences, the peak responses of the blank plasma and plasma samples spiked with the analytes and the internal standard (IS) were compared. As shown in figure 3.3, there was no interference in the retention of analytes and IS from matrix endogenous components.

3.5.3.2 Sensitivity

BPA and EZE were found to have LLOQs of 1.8 ng mL^{-1} and 0.1 ng mL^{-1}, respectively. BPA and EZE precision and accuracy were 0.61, 100.5%, and 3.23, 100.4%, respectively, at this concentration. BPA, EZE, and IS had retention times of 7.96, 6.82, and 2.92 minutes, respectively. Figure 3.3 shows the chromatogram of analytes at LLOQ.



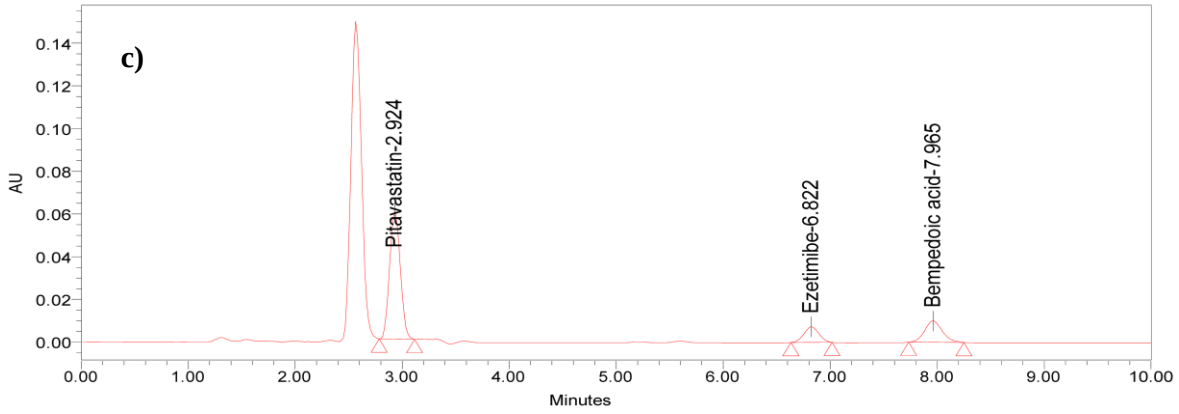


Figure 3.3. Illustrative HPLC chromatograms for a) blank plasma, b) IS and c) plasma sample spiked with 1.8 ng mL^{-1} and 0.1 ng mL^{-1} of BPA and EZE

3.5.3.3 Carry-over effects

After injecting upper limit of quantification (ULOQ) samples, there were no peaks at the retention times of both analytes and IS in blank plasma samples. This shows that the disclosed technique has no carryover effect.

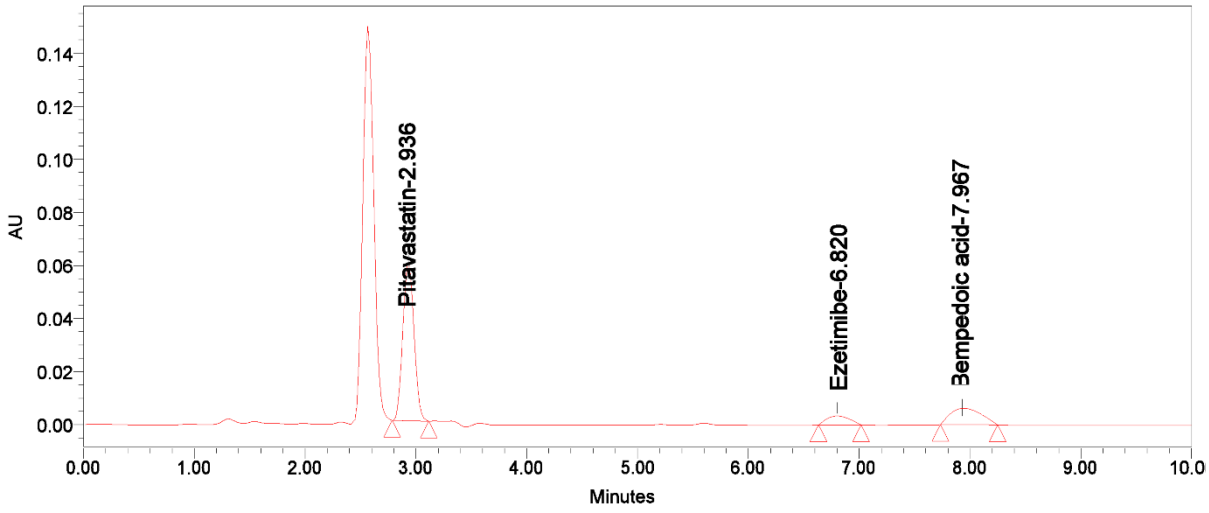
3.5.3.4 Linearity and LLOQ

Linearity was tested in rat plasma employing eight calibration standards with values ranging from 1.8 to 36.0 ng mL^{-1} for BPA and 0.1 to 2.0 ng mL^{-1} for EZE. The internal standard concentration was held constant at 40.0 ng mL^{-1}. To plot calibration curves, the ratio of analyte peak areas to the IS was plotted against analyte concentrations. Linear regression analysis is used to determine the linearity of analytes. The calibration curves for BPA and EZE were constructed and form to be linear, with adequate correlation coefficients (r^2) of 0.9997 and 0.9996, respectively. Limit of quantification (LOQ), at which S/N is 10, and limit of detection (LOD), which indicates the concentration of analyte at a S/N ratio of 3, were determined experimentally for the proposed approach., and the results are displayed in table 3.1. The represented chromatograms of LOD and LOQ are shown in figure 3.4.

Table 3.1 Linear calibration curve parameters of BPA and EZE

Analytes	Linearity range (ng mL^{-1})	Calibration Equation	Coefficient (r^2)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
BPA	1.8 – 36.0	y = 0.0631x + 0.0105	0.9997	0.6	1.8
EZE	0.1 – 2.0	y = 0.2544x + 0.0062	0.9996	0.03	0.1

a)



b)

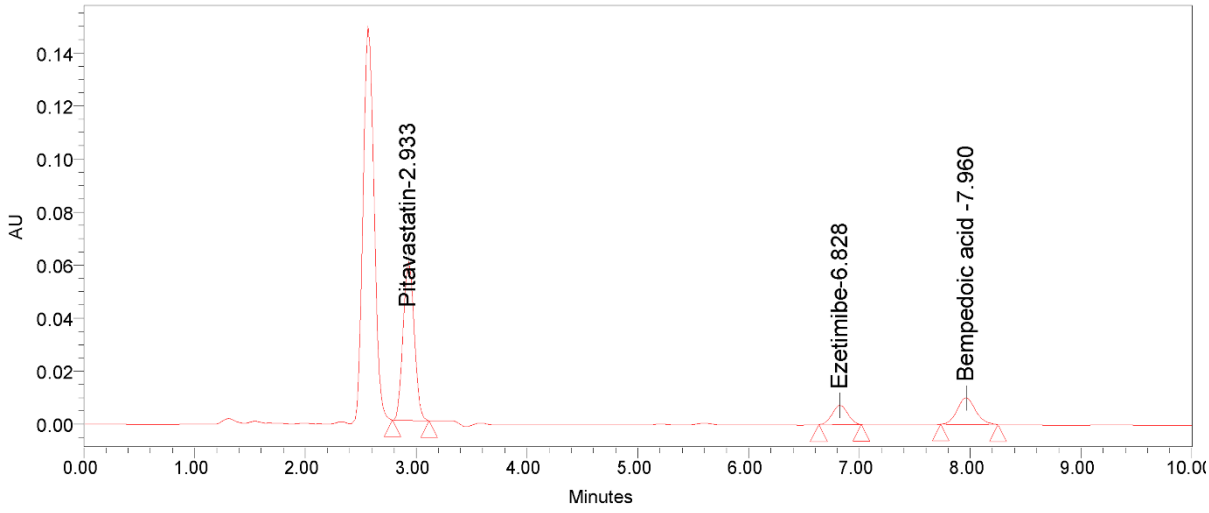


Figure 3.4. Represented chromatograms of BPA and EZE at a) LOD b) LOQ levels

3.5.3.5 Accuracy and precision

Six replicates at four concentrations of QC samples were analysed to establish the method's intra- and inter-day accuracy and, precision (LLOQ, LQC, MQC, and HQC). The percentage of recovery and the percentage of coefficient of variation (%CV) were evaluated and represented as percentages of recovery and %CV, respectively. Precision was found to be within 15% of CV, and accuracy was between 85 and 115%. Table 3.2 shows the results of the accuracy and precision assessments.

Table 3.2 Precision and accuracy of QC samples for BPA and EZE in rat plasma (n = 6)

Analyte	Spiked ($ng\ mL^{-1}$)	Within-run			Between-run		
		Found ($ng\ mL^{-1}$) (mean ± SD)	CV (%)	Accuracy (%)	Found ($ng\ mL^{-1}$) (mean ± SD)	CV (%)	Accuracy (%)
BPA	1.8	1.793 ± 0.0066	0.36	100.54	1.78 ± 0.009	0.52	99.66
	9.0	8.967 ± 0.0525	0.58	100.74	8.94 ± 0.055	0.61	99.76
	18.0	17.934 ± 0.066	0.36	100.51	17.91 ± 0.076	0.42	100.62
	27.0	26.878 ± 0.1623	0.60	99.83	26.90 ± 0.099	0.36	99.78
EZE	0.1	0.09 ± 0.00036	0.36	100.41	0.09 ± 0.0005	0.54	99.59
	0.5	0.49 ± 0.0029	0.58	102.90	0.49 ± 0.0018	0.36	103.0
	1.0	0.99 ± 0.0058	0.58	103.75	0.99 ± 0.0036	0.36	101.73
	1.5	1.49 ± 0.0087	0.58	103.28	1.49 ± 0.0055	0.36	103.24

3.5.3.6 Extraction recovery

The peak areas of post-extracted analytes using six replicated (n = 6) QC samples at three different concentration levels (lower, medium and high QC) were compared to the corresponding areas of pre-extracted analytes at the equivalent concentrations to quantify analyte recovery. Extraction recoveries for BPA were 94.2%, 98.4%, and 97.9%, and recoveries for EZE were 98.8%, 94.4%, and 96.6% for the three QC levels (lower, medium, and high QC), respectively. The results that were observed are displayed in table 3.3.

3.5.3.7 Matrix effect

The matrix effect was investigated using six different plasma lots at two different QC concentrations by comparing analyte peak areas spiked into extracted blank plasma samples to plain standard solutions at the same concentration (lower and higher QC). The CV (coefficient of variation) percentage is within acceptable limits. There were no matrix effects in rat plasma. The findings are displayed in table 3.4.

Table 3.3 Extraction recovery results for BPA and EZE

Analytes	Spiked conc. level (ng mL^{-1})	Area response (n = 6)		Extraction recovery (%) (Mean ± SD)	CV (%)
		Extracted mean response	Post extracted mean response		
BPA	9.0	31453	33376	94.24 ± 0.819	0.86
	18.0	63305	64333	98.4 ± 0.394	0.40
	27.0	94618	96567	97.98 ± 0.245	0.25
EZE	0.5	7844	7944	98.86 ± 0.475	0.48
	1.0	15358	16270	94.39 ± 1.629	1.72
	1.5	23463	24290	96.59 ± 1.449	1.50

Table 3.4 Matrix effect data for BPA and EZE from rat plasma (n = 6)

Analytes	Area response (n = 6)				Matrix factor		CV (%)	
	Post extracted mean response		Neat mean response					
	BPA	EZE	BPA	EZE	BPA	EZE	BPA	EZE
LQC	31668	7848	33376	7955	0.94	0.98	0.86	0.48
HQC	94612	23520	96567	24290	0.97	0.96	0.25	1.50

3.5.3.8 Stability

In six replicates, the stability of BPA, and EZE in plasma samples was interrogate under various storage conditions at LQC, MQC, and HQC levels. Analyses remained stable at room temperature for up to 6 hours under bench-top stability conditions. In an auto-sampler, the analytes in the plasma sample were stable at 8 °C for 26 h. In a study on freeze-thaw stability, frozen samples were found to be stable in three freeze-thaw cycles from -80 °C to room temperature. The analytes were found to be stable for up to 28 days when held at -80 °C in the long-term stability test. The acceptance standards for accuracy were determined to be between 85 and 115%, and the precision was less than 15% of the CV. Table 3.5 shows the results of stability studies.

Table 3.5 Stability results of BPA and EZE in rat plasma (n = 6)

Analyte	Spiked ($ng\ mL^{-1}$)	Bench top (RT, 6 h)		Autosampler (8 °C, 26 h)		Freeze-thaw (-80 °C to RT)		Long term (-80 °C, 28 days)	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
BPA	9.0	0.28	98.66	0.59	98.92	0.58	98.87	0.75	92.28
	18.0	0.25	100.01	0.35	99.84	0.30	99.95	0.34	96.67
	27.0	0.23	99.28	0.29	99.26	0.29	99.37	0.16	96.94
EZE	0.5	0.38	102.51	0.32	102.46	0.31	102.64	0.47	86.94
	1.0	1.19	100.55	1.04	100.34	0.61	100.09	1.47	87.98
	1.5	1.08	102.30	1.07	101.71	1.18	101.65	0.55	90.69

3.5.3.9 Robustness

The robustness study was performed in order to assess the impact of small alterations in the optimized chromatographic conditions. The variables select for this study were mobile phase pH (2.5 ± 0.5), percentage of organic solvent (± 2%), and flow rate (1.0 ± 0.1 mL min^{-1}). The system suitability parameters of analytes and assays were ascertained. The components of the mobile phase were held constant in all of the aforementioned diverse conditions.

In all purposely amended chromatographic conditions (pH, composition of mobile phase, and flow rate) of the robustness study, the resolutions between any two neighboring analytes were >2.0, the tailing factors of each analyte were <1.5, and the assays of BPA and EZE were obtained in the ranges of 99.96–100.05% and 99.95–100.18%, respectively. These results demonstrate the robustness of the suggested approach, which remained unaffected by minor adjustments to the experimental parameters. Table 3.6 shows the results of the robustness study.

Table 3.6 Robustness data for BPA and EZE

Method variables	Changed conditions	Tailing factor		Theoretical plate count		Resolution		% Assay	
		BPA	EZE	BPA	EZE	BPA	EZE	BPA	EZE
Flow rate	1.1 mL min^{-1}	1.05	1.12	12778	10364	3.68	12.75	99.99	99.97
	0.9 mL min^{-1}	1.08	1.36	9896	8994	3.78	13.56	100.03	100.01
Percentage of organic solvent	+2%	1.05	1.18	12654	10549	3.87	12.89	99.96	99.95
	-2%	1.02	1.47	9889	8547	3.53	13.89	100.15	100.18
pH of mobile phase	pH 3.0	1.06	1.15	10992	9154	4.59	13.28	100.01	100.08
	pH 2.5	1.05	0.97	10519	9497	4.75	12.93	99.98	99.97
	pH 2.0	1.02	1.16	11875	9361	4.51	13.33	100.05	99.99

3.6 Pharmacokinetic study in rats

The validated analytical method was effectively applied to determine the plasma concentration of BPA and EZE samples collected from healthy rats. Figure 3.5 depicts the mean plasma concentration vs. time curves for BPA and EZE. WinNonlin software was employed to calculate all of the pharmacokinetic parameters, and the results are summarized in table 3.7. These pharmacokinetic parameters are required for therapeutic drug surveillance investigations along with the study of the relationship between drug dose regimens and concentration-time profiles. The oral administration of BPA (0.75 mg/kg) and EZE (0.04 mg/kg) for each rat was selected for this study. As shown in table 3.7, the results of maximum plasma concentration (C_{max}) for BPA (16.8 ng mL^{-1}), and for EZE (0.9 ng mL^{-1}), time of maximum plasma concentration (T_{max}) for BPA (4 h) and for EZE (12 h), the mean half-life ($t_{1/2}$) of BPA and EZE is 24 h, the area under the concentration-time curve, 't' to infinity ($AUC_{t-\infty}$) is 28 ng.h/mL. For BPA and EZE, the area under the concentration-time curve 0 h to infinity ($AUC_{0-\infty}$) is 244 ng.h/mL and 13 ng.h/mL, respectively and the values of the area under the concentration-time curve 0 h to time 't'(AUC_{0-t}) for BPA and EZE are 244 ng.h/mL and 13 ng.h./mL, respectively.

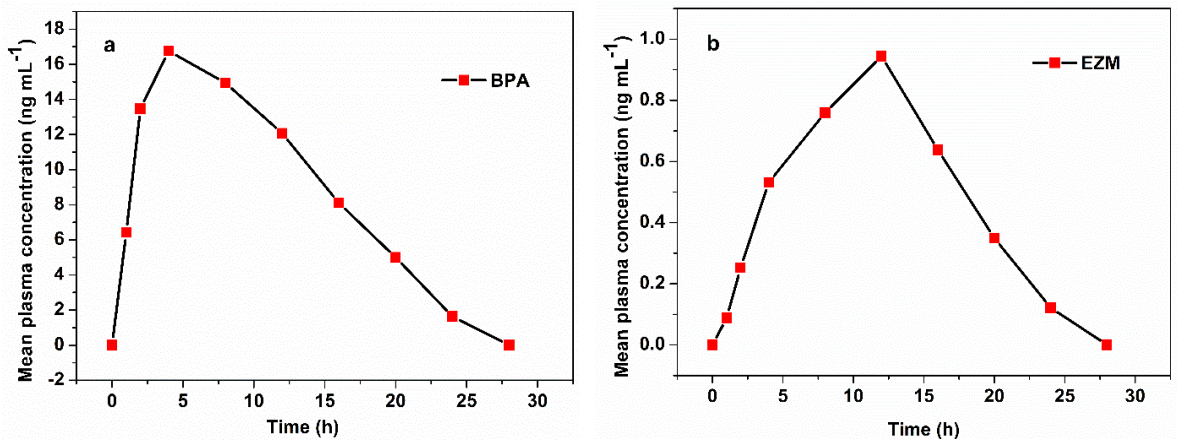


Figure 3.5. Mean plasma concentration versus time curve of a) BPA (0.75 mg/kg) and b) EZE (0.04 mg/kg)

Table 3.7 Pharmacokinetic parameters of BPA and EZE

Parameter	Analytes	
	BPA	EZE
C_{max} (ng/mL)	16.8	0.9
T_{max} (h)	4.0	12.0
$T_{1/2}$ (h)	24.0	24.0
AUC_{0-t} (ng.h/mL)	244.0	13.0
$AUC_{t-\infty}$ (ng.h/mL)	28.0	28.0
$AUC_{0-\infty}$ (ng.h/mL)	244.0	13.0

3.7 Conclusions

In the current study, we firmly established and validated an HPLC-PDA approach for the simultaneous detection of BPA and EZE in rat plasma. Appling this method, sample pre-treatment is fast, plasma concentrations of BPA and EZE are detected in a 10 min run time, and analytes in samples are assured to be stable during sample management, the chromatographic system, and the freezing time. In terms of accuracy, precision, and stability, the method was extensively validated to meet USFDA criteria for bioanalytical method validation. The best results were obtained with a simple extraction approach and rapid chromatographic conditions that were both easy to handle and readily available, allowing for a speedy analytical procedure for future applications of therapeutic drug monitoring and also for clinical analysis.

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Chapter-IV

Identification and Structural Analysis of the Stress Degradants of Crisaborole Using LC-MS/MS and Toxicity Prediction Studies

4.1 Introduction

Atopic dermatitis (AD) is a common chronic skin disorder, and its topical therapies involve administering phosphodiesterase-4 inhibitors (PDE4), calcineurin inhibitors, and corticosteroids for the treatment of patients with mild to moderately severe conditions, in conjugation with systemic treatment.[1-5] Crisaborole (CBE), 4-[(1-hydroxy-1,3-dihydro-2,1-benzoxaborol-5-yl)oxy]benzonitrile, is a boron-based topical non-steroidal PDE4 inhibitor and is used in the treatment of mild to moderate atopic dermatitis in children and adults.[6-9] A 2 % CBE ointment is the first topically applied PDE4 inhibitor, approved by the USFDA in 2016 for the treatment of AD disease. CBE was found to be non-tumorigenic and well-tolerated in mice and female rats.[8] On the other hand, CBE significantly improved the lesional transcriptional profile, epidermal pathomechanisms, and modulated key immune pathways implicated in patients with AD9.[9] In two phase-III studies, CBE had improved disease severity, pruritus, and efficacy with a favourable safety profile.[10] In-vitro studies involving CBE skin permeation and retention were performed with 2% ointment using porcine skin as a barrier.[11] The sensitive skin areas of healthy volunteers were well tolerated on application of CBE ointment (2%) highlighting the potential role of CBE as a topical treatment alternative for patients with AD.[12]

In the process of drug development, stability testing issued by the International Conference on Harmonization (ICH) plays a significant role in determining the quality, safety, and efficacy of the drug product. According to ICH Q1A(R2) guidelines, stress testing provides information about the intrinsic stability of the drug and explore the drug degradation behaviour under various environmental factors.[13,14] To better understand the degradation mechanism under diverse conditions, identification and characterization of degradation products (DPs) are frequently carried out utilizing LC-MS/MS in combination with HRMS.[15-18] Later, the toxicity of the isolated degradation products can be evaluated. These studies play an important role in the drug development process.

4.2 Literature survey

A literature review shown that there are limited analytical methods have been reported for the HPLC analysis of CBE. HPLC-UV method is available for the extraction and quantification of CBE in stripped skin layers of pig ears,[19] and an innovative stability indicating RP-HPLC approach was used for the stress study of crisaborole.[20] If the DP is detected under storage conditions at a level higher than the identification threshold, ICH guidelines Q3A(R2) and Q3B(R2) recommended identifying the degradation impurity.[21,22] Several DPs of drugs were identified under various stress conditions, characterized by LC-MS/MS and accurate mass measurements, and *in silico* toxicity studies were performed.[23-30] However, it should be

mentioned here that, no results on the way CBE degrades under different stress situations according to ICH recommended guidelines have been reported till date. Hence, in the present study, we have investigated the degradation behaviour of CBE under different stress conditions like photolysis, oxidation, hydrolysis, and thermal conditions, and the subsequent identification of the DPs and their structural elucidation of the degradation products (DPs) by using QqLIT LC-MS/MS and accurate mass measurements were performed. Degradation mechanisms for degraded products have also been established. Physico-chemical and absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties, in addition to an *in silico* toxicity evaluation of DPs, were also performed.

4.3 Stability-indicating methods

A stability-indicating approach is an analytical methodology that accurately identifies the active components without the intervention of DPs, process impurities, excipients, or other possible contaminants. Stability-indicating approaches may also effectively quantify important degradants. Forced degradation of the drug during the early stages of research is a proactive strategy for developing a stability-indicating HPLC method, with the essential degradation samples being utilized in method development. The first step before developing a method is forced degradation. Early forced degradation investigations allow for simultaneous method development, identification of primary degradation products, and detection of unknown contaminants. This method concurrently generates information about impurities or degradation, mechanisms of degradation, and verified HPLC methodologies.

4.4 Forced degradation studies

Forced degradation studies aid in the development of analytical methodology, providing insight into the active pharmaceutical ingredients (API), stability of drug products (DPs), and reveal the details DPs and pathways. From a regulatory perspective, the data that can obtained due to forced degradation studies are:

- Identification of possible degradants
- Pathways of drug molecule degradation and its inherent stability
- Validation of stability indicating analytical procedures

The stages of the drug development demand the requirement of force degradation testing. For instance, due to the high rate of compound attrition, phase 2 preclinical requires intensive technique development. As a result, while creating a rational research design, forced degradation deliverables should be zeroed in on method development activities rather than the separation and identification of DPs. The optimization of methods should be the main focus as a compound progresses through registration into later Phase 2. Characterization and elucidation of DPs are the main objectives of stress testing. Forced degradation studies of active

pharmaceutical ingredients (API) include appropriate solid state and solution state stress conditions in accordance with ICH guidelines.[31-36] These are beneficial for the development of a stability-indicating technique for a certain active pharmaceutical ingredient (API). As and when the methodologies, procedures, or formulations alter, these studies are reperformed. The stipulated stress conditions are considered only if there has been an API degradation of at least 5-20%. The particular parameters (intensity and duration) chosen will be based on the chemical characteristics of the API. The unstressed sample, the suitable blank, and the stressed sample are compared. No more stressing is advised if a compound does not degrade under a certain stress condition.[32]

4.4.1 Acid hydrolysis

HCl (0.1-2 M) in the solution form is used for acid hydrolysis experiments. A suitable co-solvent must be added or the solution's pH must be adjusted to the acidic range in order to dissolve some APIs, which are only weakly soluble or insoluble in the specified acidic solution. When selecting the ideal co-solvent, considerable attention is given to the API structure.

4.4.2 Base hydrolysis

NaOH (0.1-2 M) in a solution state is typically used for base hydrolysis. The addition of a suitable co-solvent or adjusting the pH of the solution is necessary to facilitate dissolution for some APIs that are only partially soluble or insoluble in the stated basic solution.

4.4.3 Oxidation

For oxidation, H_2O_2 and AIBN (2,2'-azobisisobutyronitrile) reagents are used. Both H_2O_2 and AIBN act as free radical initiators. As previously indicated, the addition of an appropriate co-solvent may be necessary, depending on API solubility. H_2O_2 stress testing is useful in drug product studies, especially, where hydrogen peroxide is an impurity of an excipient.

4.4.4 Thermal stability

In general, temperatures ranging from 50 to 100 °C are used to assess solid state stability. The length of exposure depends on the sensitivity of the API. Under certain thermal conditions, a phase change can occur. Hence, it is generally run under thermal conditions below the critical temperature to avoid phase change.

4.4.5 Photo stability

The ICH photo stability recommendations are followed in this research.[37] The ICH guideline states that "the design of the forced degradation tests is left to the investigators' discretion." The exposure values are appropriate and take photostability into account. Recommended exposures for confirmatory stability investigations need at least 200 Wh/m2 of integrated near UV

radiation and 1.2 million lux hours of total light. The samples are exposed to at least twice the ICH exposure length in forced degradation investigations to ensure adequate exposure of the sample. With regard to solvents, as methanol produces more artefact degradation products due to the generation of methoxy radicals in the presence of light, acetonitrile is typically chosen as a co-solvent.

In the current work, CBE is taken into consideration for the development and validation of a new analytical method. The chemical structure and name of the CBE are depicted in figure 4.1.

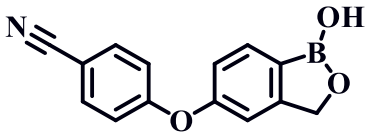


Figure 4.1. Chemical structure of Crisaborole

4.5 Experimental

4.5.1 Chemicals and reagents

From a nearby manufacturing company in Hyderabad, India, pure CBE was obtained. The analytical grade sodium hydroxide, hydrochloric acid, sulphuric acid, hydrogen peroxide, azobisisobutyronitrile (AIBN) and the HPLC grade acetonitrile (ACN), LC-MS grade formic acid were purchased from Merck (Mumbai, India), High purity HPLC grade water was obtained from Milli-Q ultrapure water purification system (Millipore Bedford, MA, USA).

4.5.2 Instrumentation and conditions

An Alliance e2695 HPLC system (Waters Corp., Milford, MA, USA) with a quaternary pump, in-line degasser, auto-injector, column compartment, and PDA detector (model 2998) was used to conduct the HPLC analysis. Chromatographic data was acquired using Empower 2 software. The chromatographic separation was performed using a Symmetry C18 column (150 × 4.6 mm, 3.5 µm) as a stationary phase in isocratic elution mode with a mobile phase constituting of acetonitrile and 0.1% formic acid in aqueous (50:50, v/v) with a flow rate of 1 $mLmin^{-1}$ at an ambient temperature and an injection volume of 10 µL. The UV detection at 254 nm was used to determine the CBE and its DPs. A photodegradation study was performed in a photostability chamber (Newtronic Lifecare Equipment Pvt. Ltd., Maharashtra, India). All the solutions and solvents were filtered through 0.45 µm filter paper (Millipore, India).

An Applied Biosystems/MDS QqLIT 5500 mass spectrometer (SCIEX, Toronto, Canada) was used for the MS/MS analysis in Multiple Reaction Monitoring (MRM) mode. Analyst Software v.1.6.2 was used for data processing and data collection in order to control the mass spectrometer. The ideal conditions for detection included a Turbo Spray electrospray ionization (ESI) source operating in positive ionization mode at 550 °C with the proper setting, a gas

temperature of 250 °C, nitrogen as a drying gas at 55 psi, flow rate of 5 L min^{-1}, nebulizer (nitrogen) gas at 45 psi, ultra-high purity nitrogen as collision cell gas, ionization spray at a voltage of 5500 V, MRM dwell time of 1 s and entrance potential (EP) of 10 V. Under ideal instrumental conditions, the transitions obtained for the investigated compounds were: collision energy (CE), declustering potential (DP), and collision cell exit potential (CXP). (a) CBE: 252 > 234 (CE = 15 V, DP = 40 V, CXP = 7 V); (b) DP-1: 320 > 151 (CE = 14 V, DP = 39 V, CXP = 6 V); (c) DP-2: 226 > 120 (CE = 12 V, DP = 35 V, CXP = 6 V); (d) DP-3: 242 > 104 (CE = 11 V, DP = 38 V, CXP = 7 V); (e) DP-4: 271 > 151 (CE = 10 V, DP = 40 V, CXP = 5 V); (f) DP-5: 256 > 104 (CE = 12 V, DP = 40 V, CXP = 6 V); (g) DP-6: 238 > 104 (CE = 14 V, DP = 42 V, CXP = 6 V).

4.5.3 Forced degradation studies

According to ICH Q1A (R2) recommendations, forced degradation tests of CBE were carried out on the bulk drug. For all of the stress reactions, the drug solutions were prepared at a concentration of 70 µg mL^{-1}. concentration for all the stress reactions. CBE was subjected to acidic hydrolysis (1N HCl and 1N H_2SO_4, reflux at 60 °C, 24 h), basic hydrolysis (1N NaOH, reflux at 60 °C, 24 h), neutral (H_2O, at 60 °C, 24 h) hydrolysis, [26-30] and oxidation (30% H_2O_2 and AIBN, reflux at 60 °C, 18 h) in the solution state. Thermal degradation was performed by placing CBE in an oven at 100 °C for 24 h. Photolytic degradation studies were carried out by irradiating a solid CBE with UV light up to 200 Wh/m^2 and fluorescent light at 1.2 million lux-hour in a photostability chamber for 7 days. Acid and base hydrolyzed samples were neutralized, and all stressed samples were withdrawn at appropriate time intervals and diluted with the mobile phase.

4.5.4 Sample preparation

All the stressed samples (hydrolytic, oxidative, thermal, and photochemical stress) were neutralized and diluted with acetonitrile and 0.1% formic acid in aqueous solution (50:50, v/v). Prior to HPLC and LC-MS analysis, all the solutions were filtered using 0.22 µm membrane filters.

4.5.5 *In silico* physico-chemical and ADMET studies

The QikProp programme of Schrodinger software was used to determine the physical, chemical, and ADMET properties, which aid in predicting the compounds' pharmacokinetically and physicochemically significant descriptors and features.[38] To compare the ranges of molecule or structure attributes with those of known drugs, Schrodinger software's QikProp ADMET programme was utilized. Evaluation of the physico-chemical and ADMET properties of DPs (DP-1 to DP-6) was done using key parameters of the designed molecules.

4.5.6 *In silico* toxicity studies

The software tool admetSAR was used to predict the various degrees of possible toxicity of CBE and its degradation products.[39–41] The toxicological pathways, organ toxicity, and genomic toxicity were identified.

4.6 Results and discussions

4.6.1 Method development and optimization process

Initially, the chromatographic conditions were tested on a X-bridge phenyl column (150 × 4.6 mm id, 3.5 µm) with various acetonitrile (ACN) and water mobile phase compositions. In these different compositions, asymmetrical peak shapes and poor resolutions were observed. Further trials were carried out with different mobile phase compositions consisting of ACN and 0.1% orthophosphoric acid (OPA), in which conditions such as low and high retention times and tailing were observed. As a result, isocratic elution mode with acetonitrile and 0.1% formic acid in aqueous solution (50:50, v/v) and a Symmetry C18 (150 x 4.6 mm, 3.5 µ) column were used to achieve significant isolation of the drug and its DPs. To optimize the chromatographic conditions, the flow rate was adjusted to1.0 mL min^{-1} and the wavelength was set at 254 nm, and a run time of 10 min was used. These optimized chromatographic conditions shown in table 4.1 were used for the isolation of CBE and its DPs, and the same chromatographic conditions were applied for the identification and characterization of DPs and CBE by QqLIT LC-MS/MS, as discussed in the experimental section.

Table 4.1 Optimized chromatographic conditions

S. No	Method Parameters	Optimized Conditions
1	Column	Symmetry C18 (150 mm x 4.6 mm, 3.5 µ)
2	Mobile phase	Acetonitrile: 0.1% formic acid in water (50:50 v/v)
3	Run Time	10 min
4	Flow rate	1.0 mL min^{-1}
5	Column temperature	Room temperature
6	Diluent	Acetonitrile: 0.1% formic acid in water (50:50 v/v)
7	Detector	Photodiode array, 254 nm

4.6.2 Method validation

The proposed HPLC method has been validated in terms of system appropriateness, precision, accuracy, specificity, and linearity, as stated in ICH guidelines Q2(R1).[42]

4.6.2.1 System suitability

System suitability assessments are thought to be an essential aspect of liquid chromatographic methods. They are used to determine whether the chromatographic system has the necessary resolution and reproducibility to carry out the analysis. The tests are founded on the idea that the test samples, equipment, electronics, and analytical techniques together constitute a whole system that can be evaluated as such. Table 4.2 presents the test findings.

Table 4.2 System suitability results of CBE and its DPs

Degradation products	conditions	Retention time (min)	Theoretical plates	Resolution	Tailing factor
CBE	Acid degradation	3.63	5228	2.06	1.01
DP-1		3.35	5100	10.28	0.96
DP-2		1.50	1215	-	1.05
CBE	Base degradation	3.61	5079	12.79	1.03
DP-3		1.33	2969	-	1.03
CBE	Neutral degradation	3.60	5138	12.89	1.07
DP-4		1.16	741	-	1.03
CBE	Thermal degradation	3.56	4957	10.78	1.06
DP-2		1.50	1313	2.03	1.02
DP-6		1.19	763	-	1.05
DP-5		7.44	14643	17.10	0.96

4.6.2.2 Specificity

Specificity of the method was determined by employing CBE to several stress conditions. The obtained DPs were separated from the CBE and the peak purity of CBE was passed in all the degradation stress conditions and the method was found to be specific.

4.6.2.3 Accuracy and precision study

The recovery experiments were performed at three concentration levels ranging from 50-150% (i.e., 35, 70, 105 µg mL^{-1}) in six replicates analysis. Three samples were prepared at three

concentration levels. The solutions were then analysed, and the percentage relative standard deviation (%RSD) along with % recoveries were estimated for each concentration. The %RSD was observed to be <1% and recovery results were observed in the range of 99.1-100.8%.

Precision studies are divided into two categories: intra-day and inter-day (intermediate) precision. Three different concentrations (35, 70 and 105 µg mL^{-1}) were used to determine the inter-day and intra-day precisions, on the same day and on successive days in an analysis with six replications. By estimating %RSD, the method's precision was ascertained to be <1%. The accuracy and precision results were observed to be well within the range. Table 4.3 shows the results of accuracy and precision study.

Table 4.3 Accuracy, precision and recovery data of CBE

Concentration level	Concentration (µg mL^{-1})	Intra-day precision, estimated concentration (µg mL^{-1}) ± SD; (%) RSD	Inter-day precision, estimated concentration (µg mL^{-1}) ± SD; (%) RSD	Recovery (%) ± SD; (%) RSD
50%	35.00	34.67 ± 0.045; 0.13	34.76 ± 0.13; 0.37	99.1 ± 0.13; 0.13
100%	70.00	70.55 ± 0.189; 0.27	70.33 ± 0.259; 0.36	100.8 ± 0.27; 0.27
150%	105.00	105.44 ± 0.23; 0.21	105.43 ± 0.265; 0.25	100.4 ± 0.22; 0.22

4.6.2.4 Linearity

By generating calibration curves for CBE in the concentration range of (0.7-140 µg mL^{-1}), the linearity of the technique was assessed. Excellent linearity was observed in the concentration range 0.7-140.0 µg mL^{-1} of CBE. Statistical analysis of the data was performed using a linear regression model (figure 4.2). Y=25083X+640.88 was found to be the linear regression equation, and the correlation coefficient was found to be 0.9982. Table 4.4 provides an overview of the studies that demonstrate linearity.

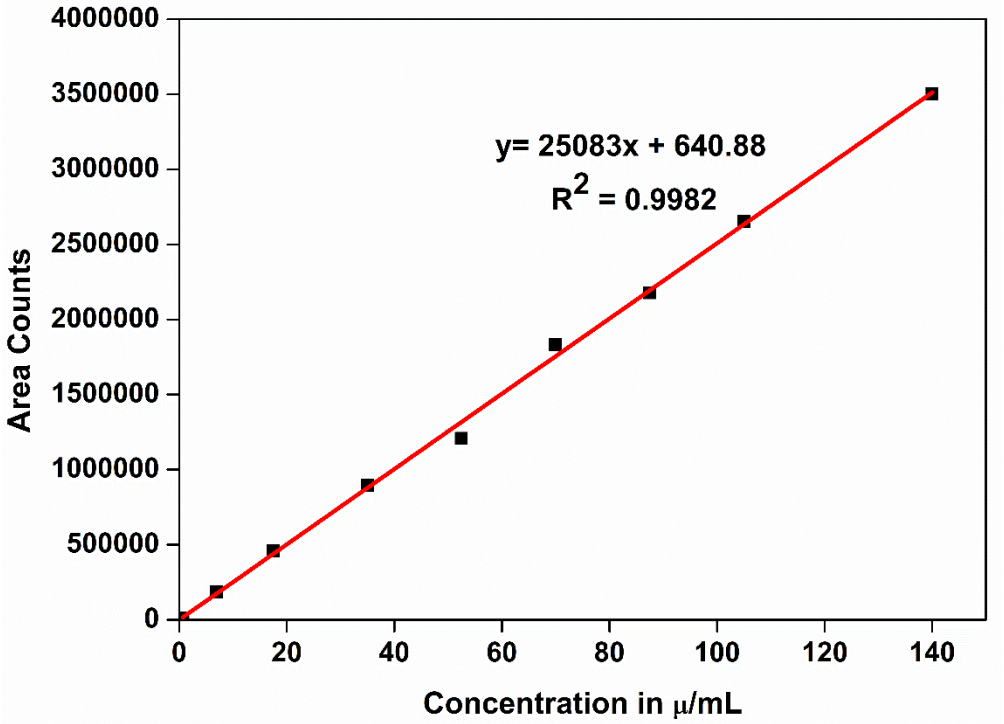


Figure 4.2. Linearity graph of CBE at various concentrations in linear range (0.7–140 µg mL^{-1})

Table 4.4 Shows the linearity of CBE

Parameter	CBE
Linearity range (µg mL^{-1})	0.7 - 140
Regression equation (Y)	Y=25083X+640.88
Slope (m)	25083
Intercept (C)	640.88
Correlation coefficient	0.9982

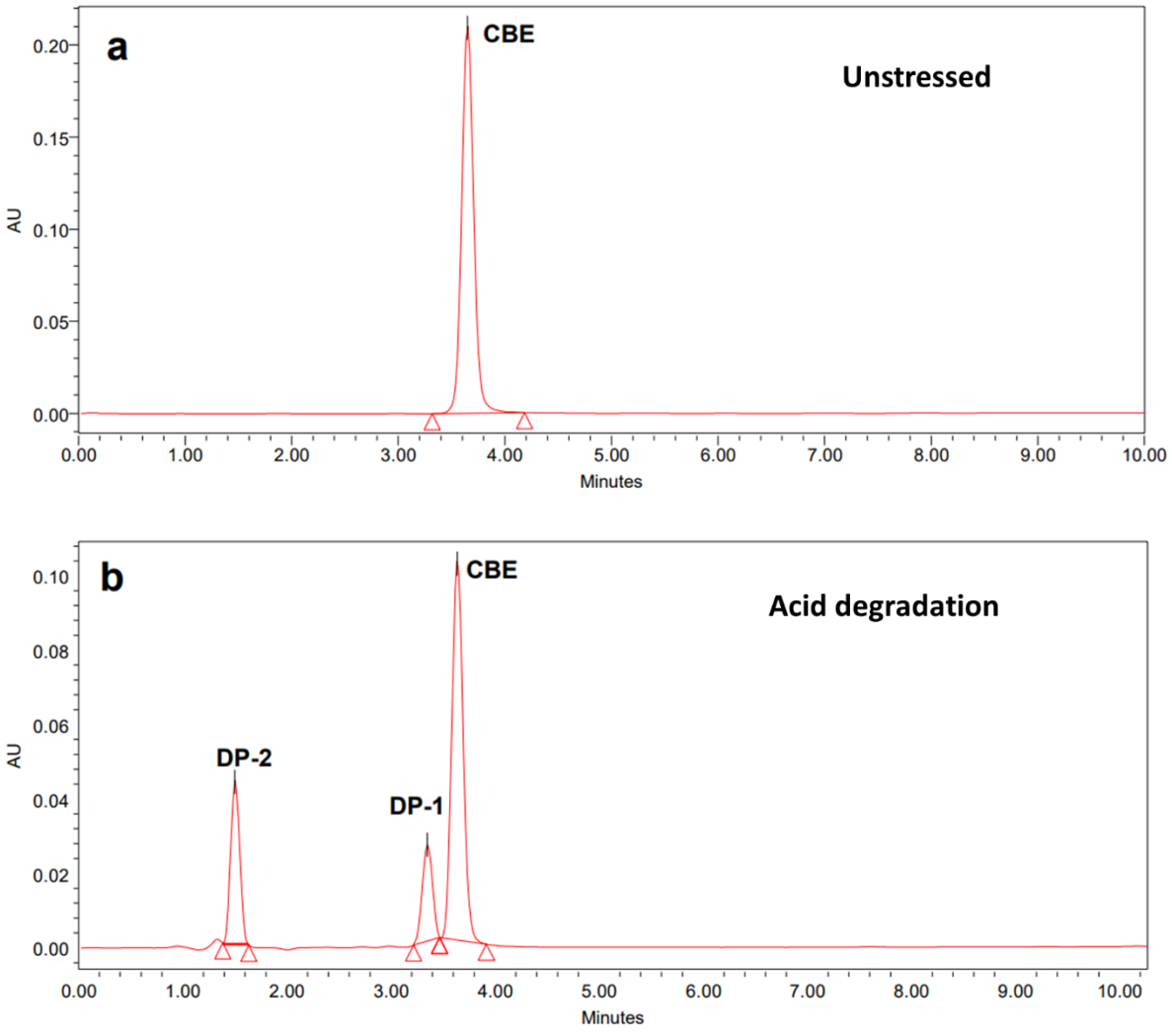
4.7 Degradation behaviour of crisaborole under various stress conditions

The HPLC-PDA (HPLC-Photodiode-Array Detector) method was used to monitor the degradation behaviour of CBE under various stress conditions. Under the conditions of hydrolysis and thermal stress, the drug was highly susceptible and showed significant degradation, while under conditions of oxidation and photolytic stress, it remained stable. The optimized stressed conditions are presented in table 4.5.

Table 4.5 Summary of the forced degradation results of CBE

Stress degradation condition		%Assay of CBE	%Degradation	Mass balance (%assay + %degradation products)	Remarks
Unstressed sample	-	99.58	-	99.58	-
Acid hydrolysis	1 N HCl, 60 °C, 24 h	72.902	26.7	99.58	Significant degradation was observed as DP-1 (R_t 3.35 min) and DP-2 (R_t 1.50 min)
Base hydrolysis	1 N NaOH, 60 °C, 24 h	72.14	27.9	99.58	Significant degradation was observed as DP-3 (R_t 1.33 min)
Neutral	H_2O, 60 °C, 24 h	75.762	24.2	99.58	Significant degradation was observed as DP-4 (R_t 1.66 min)
Thermal	100 °C, 24 h	73.502	26.5	99.58	Significant degradation was observed as DP-6 (R_t 1.19 min), DP-2 (R_t 1.50 min) and DP-5 (R_t 7.44 min)
Oxidation	30 % H_2O_2 and AIBN, 60 °C, 18 h	99.58	0	99.58	No degradation was observed
Photolytic degradation	7 days	99.58	0	99.58	No degradation was observed

Figure 4.3 shows the typical chromatograms of standard CBE (figure 4.3(a)) and DPs formed during the various stress conditions employed. Under acid hydrolysis, two degradation products (DP-1 and DP-2) were observed in 1.0 N HCl at 60 °C for 24 h (figure 4.3(b)). When H_2SO_4 was used for the acid hydrolysis of CBE, no degradation products were observed. DP-3 was observed in 1.0 N NaOH at 60 °C for 24 h (figure 4.3(c)), whereas in the neutral condition, i.e., heating the drug in water at 60 °C for 24 h, only one degradation product (DP-4) was yielded (figure 4.3(d)). In thermal degradation at 100 °C in an oven for 24 h, the drug was significantly degraded to form three degradation products (DP-2, DP-5, and DP-6) (figure 4.3(e)). The drug was found to be stable under oxidation and photolytic conditions.



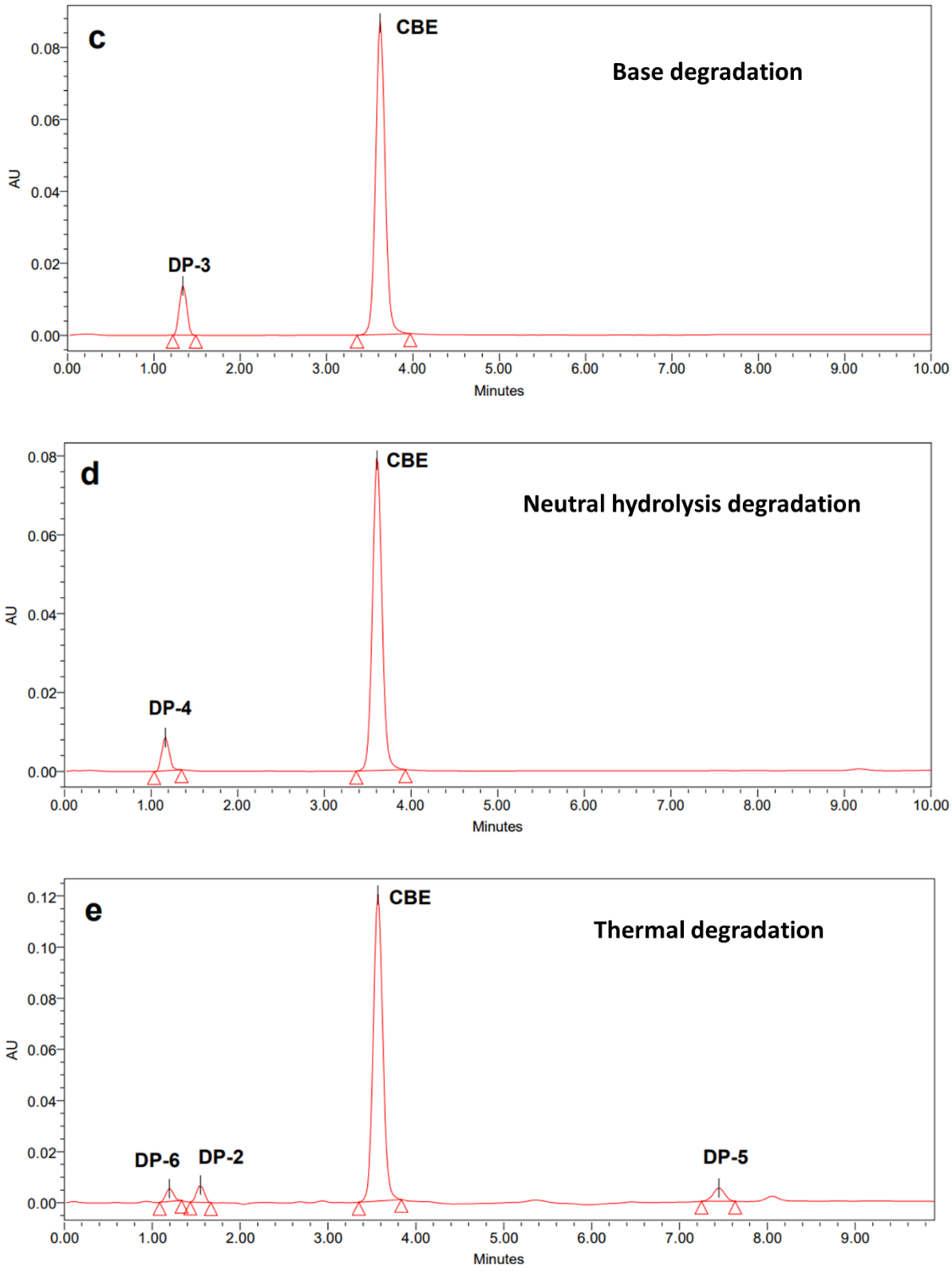


Figure 4.3. Typical HPLC chromatograms of (a) standard CBE and its degradation products under (b) acid hydrolysis, (c) base hydrolysis, (d) neutral hydrolysis, and (e) thermal degradation

Using QqLIT LC-MS/MS, a total of six DPs (DP-1 to DP-6) were recognized and characterized. Accurate mass measurements were used to confirm the elemental composition of DPs and their

product ions, and the MS/MS fragmentation data of the CBE and DPs were used to determine the structure of the DPs. The proposed structures of the DPs of CBE and their respective elemental compositions are illustrated in figure 4.4 and table 4.6, respectively.

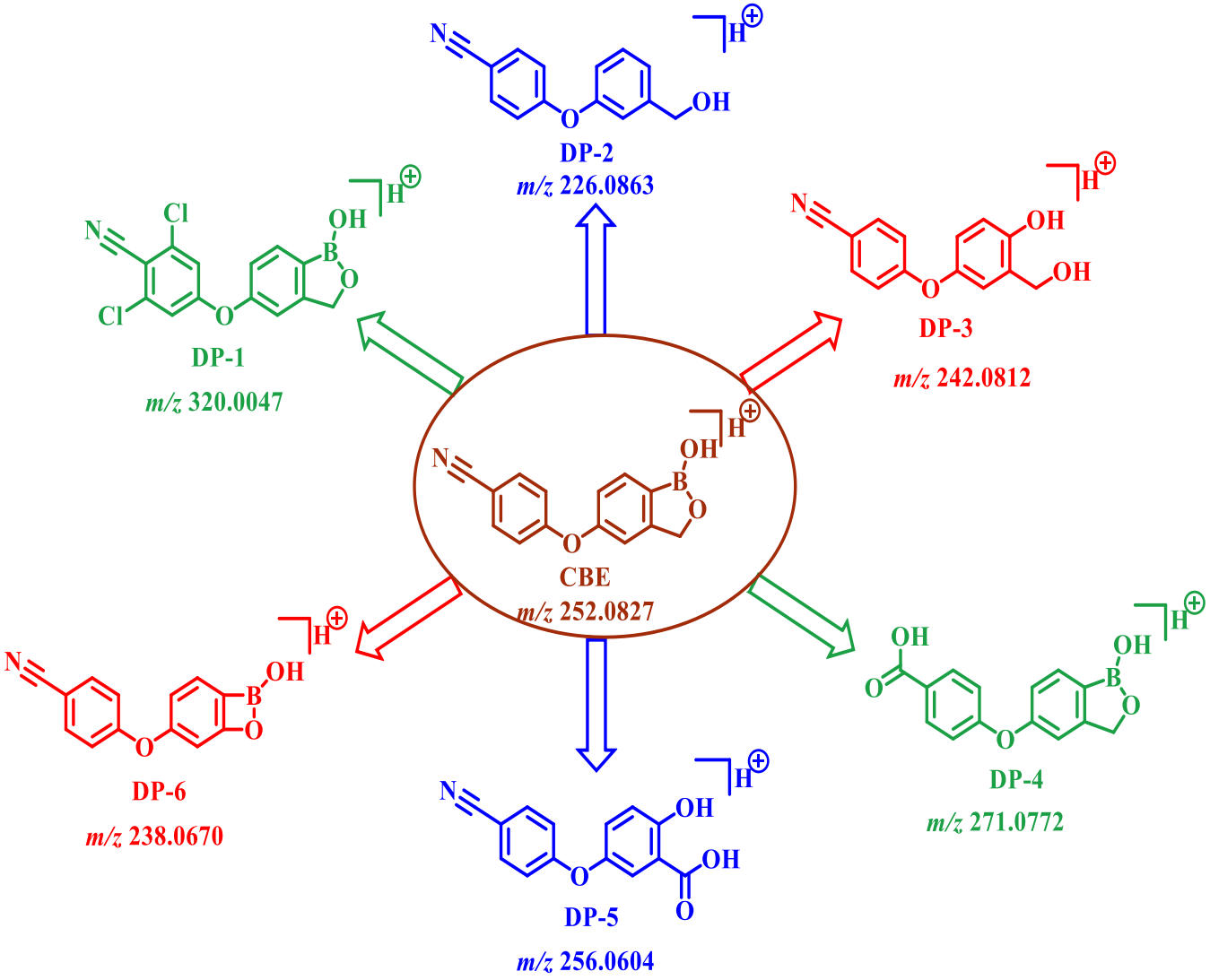


Figure 4.4. Proposed structures of the protonated DPs of crisaborole

Table 4.6 Elemental composition for product ions of CBE and its DPs

CBE and its DPs	Elemental composition	Calculated m/z	Observed m/z	Error ppm
CBE	$C_{14}H_{11}BNO_3^+$	252.0827	252.0832	-1.98
	$C_{14}H_9BNO_2^+$	234.0721	234.0728	-2.99
	$C_{13}H_8NO^+$	194.0600	194.0605	-2.57
	$C_7H_8BO_2^+$	135.0612	135.0619	-5.18
	$C_7H_6NO^+$	120.0444	120.0453	-7.49
	$C_3H_4BO_2^+$	83.0304	83.0312	-9.63

DP-1	$C_{14}H_9BCl_2NO_3^+$	320.0047	320.0049	-0.62
	$C_{13}H_5BCl_2NO^+$	271.9836	271.9843	-2.57
	$C_7H_4Cl_2N^+$	171.9715	171.9724	-5.23
	$C_7H_8BO_3^+$	151.0561	151.0568	-4.63
DP-2	$C_{14}H_{12}NO_2^+$	226.0863	226.0872	-3.98
	$C_{13}H_8NO^+$	194.0600	194.0610	-5.15
	$C_7H_6NO^+$	120.0444	120.0454	-8.33
DP-3	$C_{14}H_{12}NO_3^+$	242.0812	242.0822	-4.13
	$C_{12}H_{10}NO_2^+$	200.0706	200.0714	-3.99
	$C_7H_9O_3^+$	141.0546	141.0555	-6.38
	$C_7H_8O_2^{+\cdot}$	124.0519	124.0519	0.00
	$C_7H_6N^+$	104.0500	104.0504	-3.84
	$C_5H_6O^{+\cdot}$	82.0413	82.0418	-6.09
DP-4	$C_{14}H_{12}BO_5^+$	271.0772	271.0769	1.10
	$C_{13}H_{12}BO_4^+$	243.0823	243.0815	3.29
	$C_7H_8BO_3^+$	151.0561	151.0558	1.98
	$C_7H_7O_2^+$	123.0441	123.0445	-3.25
	$C_6H_7O^+$	95.0491	95.0486	5.26
DP-5	$C_{14}H_{10}NO_4^+$	256.0604	256.0607	-1.17
	$C_{13}H_8NO^+$	194.0600	194.0612	-6.18
	$C_7H_7O_4^+$	155.0339	155.0342	-1.93
	$C_7H_5O_3^+$	137.0233	137.0228	3.64
	$C_7H_6N^+$	104.0500	104.0504	-3.84
	$C_6H_3^+$	75.0229	75.0225	5.33
DP-6	$C_{13}H_9BNO_3^+$	238.0670	238.0667	1.26
	$C_6H_6BO_3^+$	137.0405	137.0411	-4.37
	$C_7H_6NO^+$	120.0444	120.0447	-2.49
	$C_7H_6N^+$	104.0500	104.0504	-3.84

4.8 MS/MS study of the drug and its DPs

4.8.1 MS/MS of CBE ($[M+H]^+$, *m/z* 252.0832)

CBE was eluted at a retention time (R_t) of 3.64 min. The ESI-MS/MS spectrum of $[M+H]^+$ of CBE (*m/z* 252.0832) (figure 4.5) shows product ions at *m/z* 234.0728 (loss of H_2O from *m/z* 252.0832), *m/z* 194.0605 (loss of CH_3BO_2 from *m/z* 252.0832), *m/z* 159.0614 (from *m/z* 252.0832), *m/z* 135.0619 (loss of C_7H_4NO from *m/z* 252.0832) *m/z* 120.0453 (loss of $C_7H_6BO_2$

from *m/z* 252.0832) and *m/z* 83.0312 (loss of C_4H_4 from *m/z* 135.0619) (figure 4.6). Accurate mass measurements have validated the elemental composition of these product ions (table 4.6).

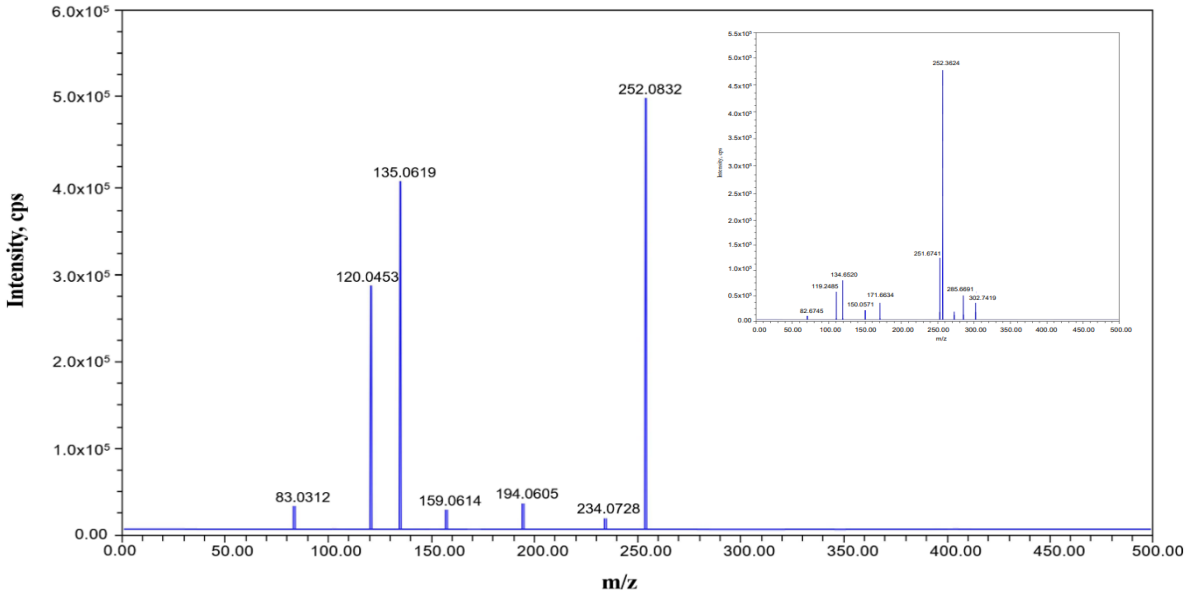


Figure 4.5. Typical LC-MS/MS spectra of protonated CBE (*m/z* 252.0832)

4.8.2 MS/MS of degradation products

DP-1 (pseudo degradation product) ($[M+H]^+$, *m/z* 320.0049)

Pseudo DP-1 was eluted with a retention time (R_t) of 3.35 min under acidic stress conditions. The ESI-MS/MS spectrum of $[M+H]^+$ of DP-1 (*m/z* 320.0049) (figure 4.6) shows the product ions at *m/z* 271.9843 (loss of H_2O and CH_2O from *m/z* 320.0049), *m/z* 213.0271 (from *m/z* 320.0049), *m/z* 171.9724 (loss of $C_7H_5BO_3$ from *m/z* 320.0049), *m/z* 151.0568 (loss of $C_7H_2Cl_2N$ from *m/z* 320.0049) and *m/z* 89.0556 (from *m/z* 213.0271). The product ion at *m/z* 151.0568 suggests the presence of the benzoxaborole moiety in the DP-1 (figure 4.8). Based on the information available, elemental composition of pseudo DP-1 and product ions were evaluated and proposed as shown in the table 4.6. However, it should be mentioned here that, as the expected boron pattern in the mass spectra corresponding to M and (M-1), due to the presence of ^{11}B and ^{10}B isotopes, were observed only in the case of CBE (inset attached in the figure 4.5) and not in case of DP-1 and, in addition, the dichloro pattern was also not observed in the mass spectrum, further studies are needed to authenticate the structure of DP-1.

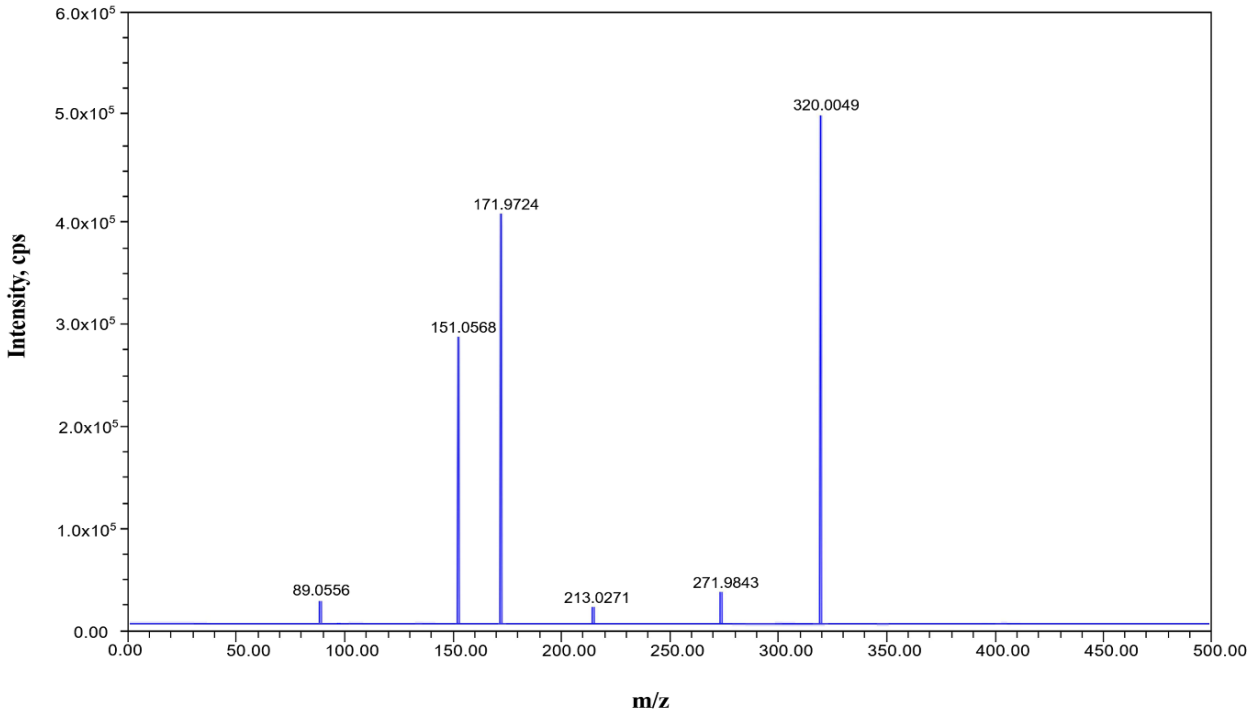


Figure 4.6. LC-MS/MS spectra of protonated DP-1 (*m/z* 320.0049)

DP-2 ($[M+H]^+$, *m/z* 226.0872)

Under acidic and thermal stress conditions, DP-2 was eluted with a retention time (Rt) of 1.50 min. The ESI-MS/MS spectrum of $[M+H]^+$ of DP-2 (*m/z* 226.0872) (figure 4.7) displays the product ions at *m/z* 194.0610 (loss of CH_3OH from *m/z* 226.0872), m/z 120.0454 (loss of C_7H_6O from *m/z* 226.0872), *m/z* 100.0179 (from *m/z* 194.0610), and *m/z* 79.0539 (from *m/z* 194.0610). The neutral loss of methanol authorizes the presence of a hydroxymethyl group in the structure of DP-2 (figure 4.8). Accurate mass measurements were used to confirm the elemental composition of DP-2 and product ions (table 4.6). All of these findings support the proposed structure for DP-2, 4-(3-hydroxymethyl)phenoxy)benzonitrile.

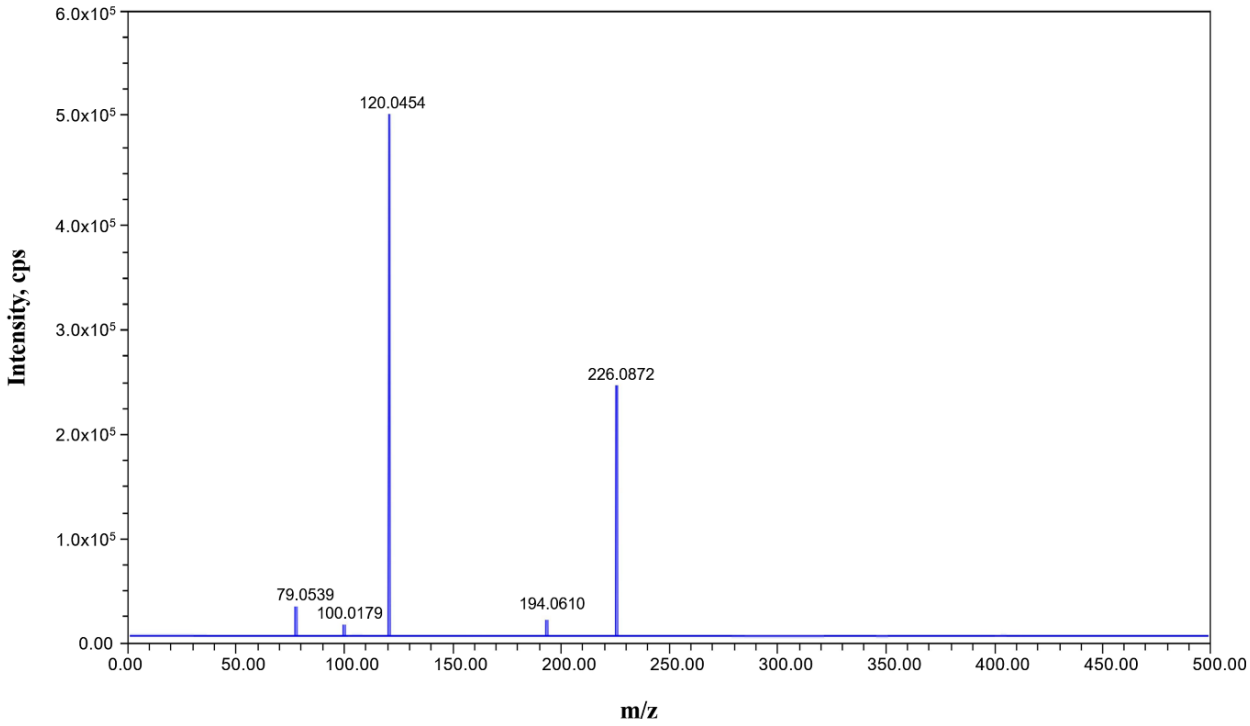


Figure 4.7. LC-MS/MS spectra of protonated DP-2 (*m/z* 226.0872)

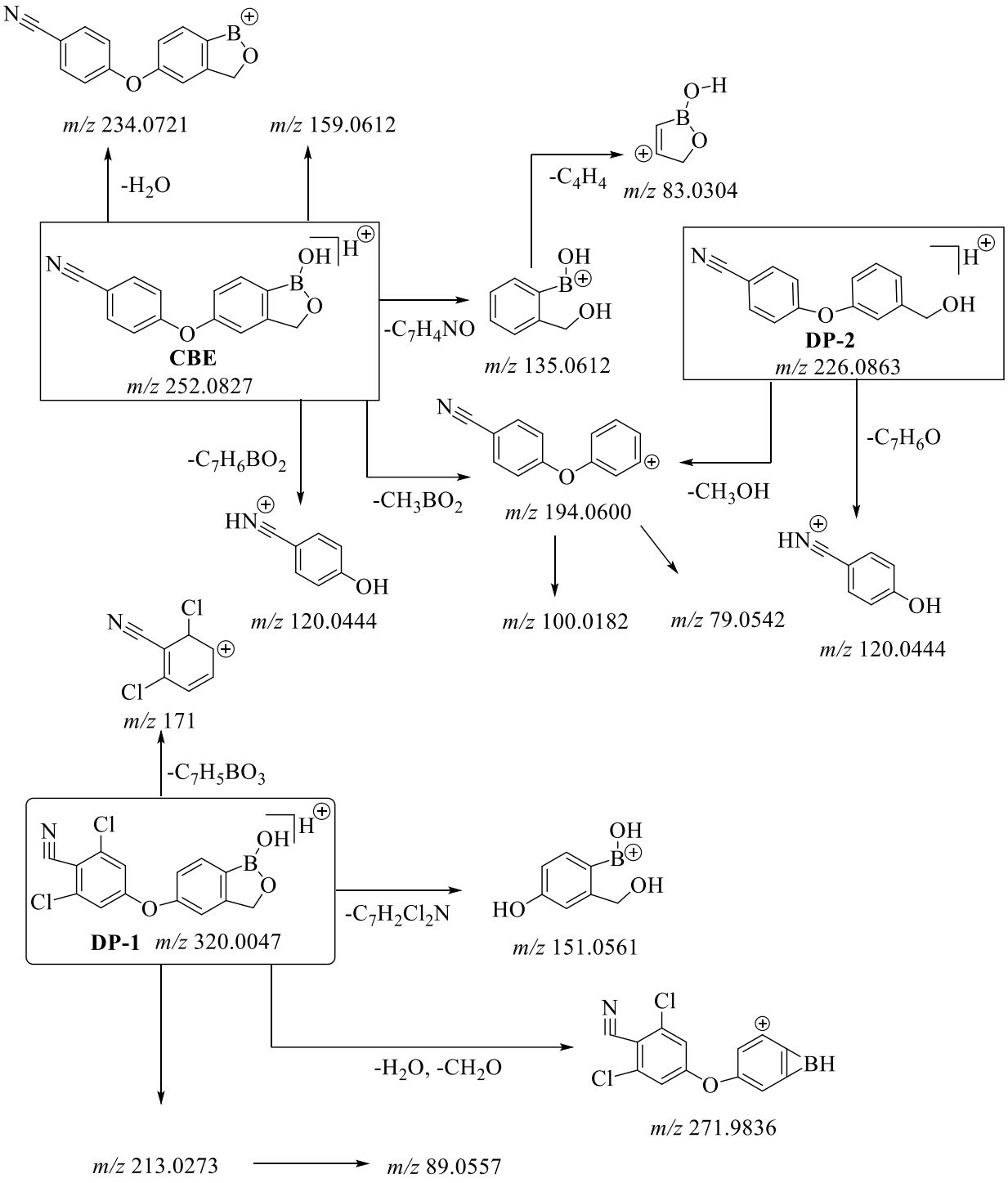


Figure 4.8. Proposed fragmentation pattern for CBE (*m/z* 252.0827), DP-1 (*m/z* 320.0047) and DP-2 (*m/z* 226.0863)

DP-3 ($[M+H]^+$, *m/z* 242.0822)

DP-3 was formed under basic stress conditions and eluted with a retention time (R_t) of 1.33 min. The ESI-MS/MS spectrum of $[M+H]^+$ of DP-3 (*m/z* 242.0822) (figure 4.9) shows the product ions at *m/z* 200.0714 (loss of CH_2=C=O from *m/z* 242.0822), m/z 141.0555 (loss of C_7H_3N from *m/z* 242.0822), m/z 124.0519 (loss of OH radical from *m/z* 141.0555), m/z

104.0504 (loss of $C_5H_4O_2$ from *m/z* 200.0714), and m/z 82.0418 (loss of CH_2=C=O from *m/z* 124.0519). The product ion *m/z* 104.0504 evidently specifies the presence of the nitrile group in DP-3. Whereas the product ions at *m/z* 141.0555 and *m/z* 124.0519 are diagnostic ions for the presence of 2-(hydroxymethyl)phenol skeleton in DP-3 (figure 4.11). Accurate mass measurements confirmed the elemental composition of DP-3 and product ions (table 4.6). All these data are well-matched with the structure of DP-3, which was proposed as 4-(4-hydroxy-3-(hydroxymethyl)phenoxy)benzonitrile.

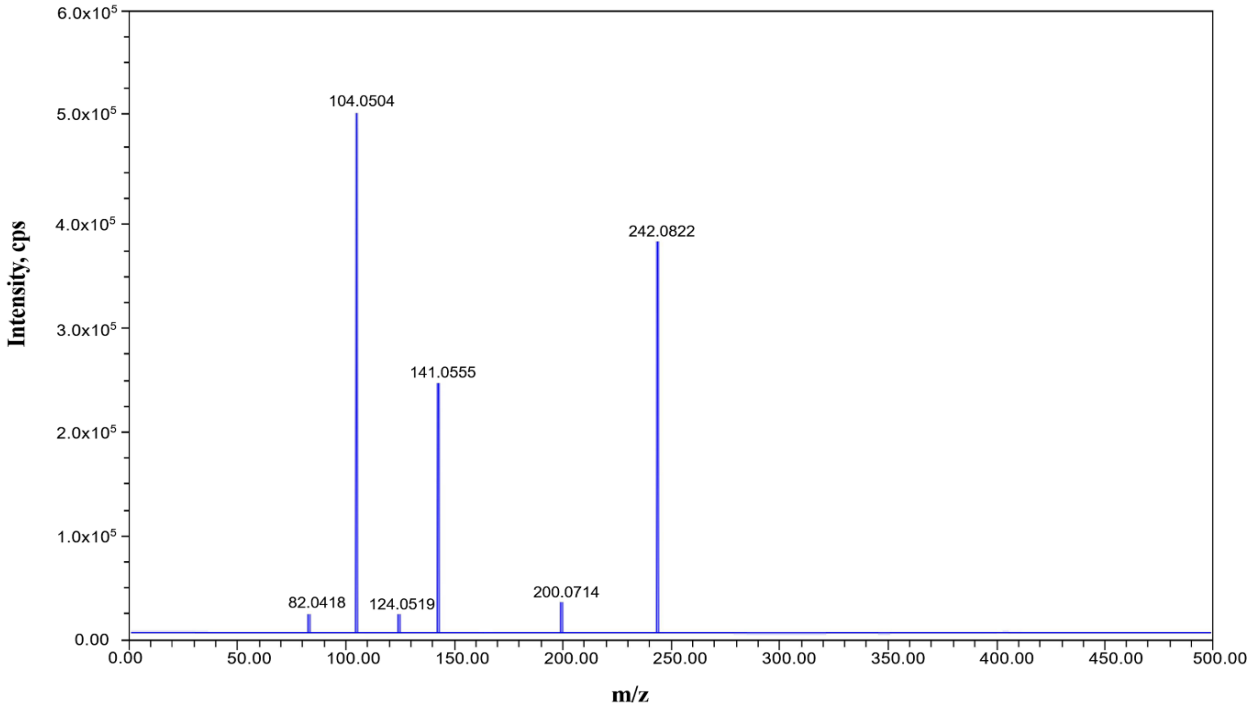


Figure 4.9. Typical LC-MS/MS spectra of protonated DP-3 (*m/z* 242.0822)

DP-4 ($[M+H]^+$, *m/z* 271.0769)

DP-4 was produced under neutral stress conditions and eluted with a retention time (R_t) of 1.16 min. The ESI-MS/MS spectrum of $[M+H]^+$ of DP-4 (*m/z* 271.0769) (figure 4.10) shows the product ions at m/z 243.0815 (loss of CO from *m/z* 271.0769, *m/z* 151.058 (loss of $C_7H_6O_2$ from *m/z* 271.0769), *m/z* 123.0445 (loss of $C_7H_6BO_3$ from *m/z* 271.0769), *m/z* 95.0486 (loss of CO from *m/z* 123.0445). The product ion at *m/z* 123.0445 clearly specifies the existence of an acid group in the structure of DP-4. The product ions at m/z 123.0445 and *m/z* 151.0558 are characteristic ions for DP-4 (figure 4.11). Based on the information available, elemental composition of DP-4 and product ions were evaluated and proposed as shown in the table 4.6. However, it should be mentioned here that, as the expected boron pattern in the mass spectra corresponding to M and (M-1), due to the presence of ^{11}B and ^{10}B isotopes, were observed only in the case of CBE (inset attached in the figure 4.5) and not in case of DP-4, further studies are needed to authenticate the structure of DP-4.

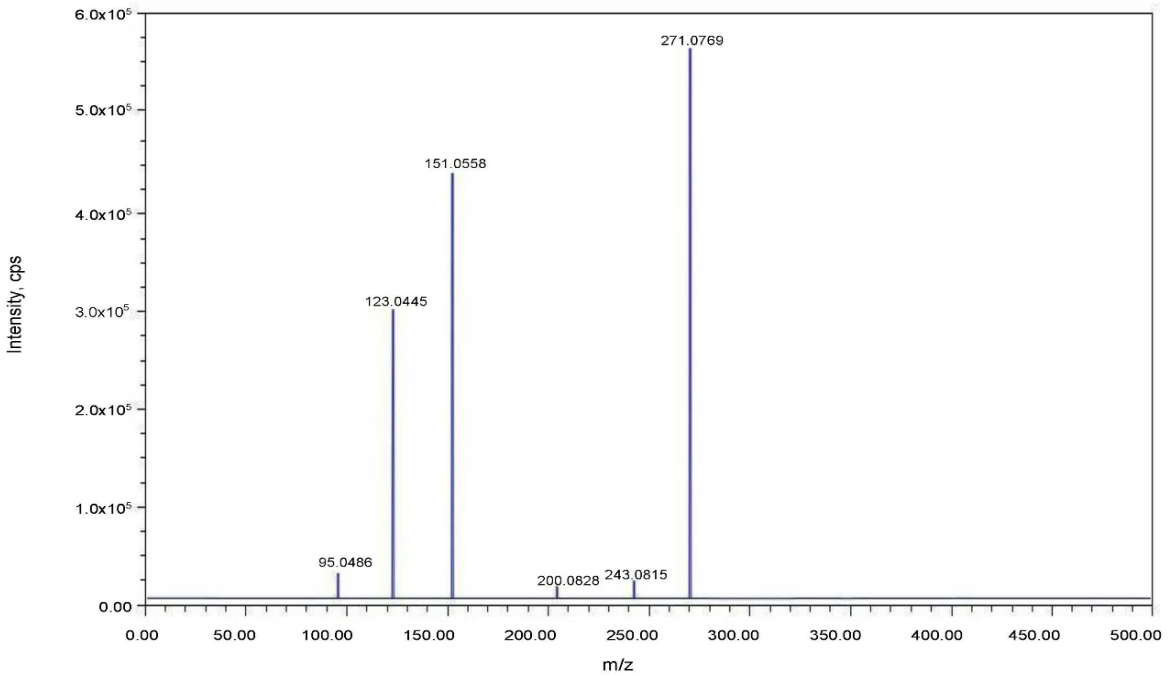


Figure 4.10. LC-MS/MS spectra of protonated DP-4 (*m/z* 271.0769)

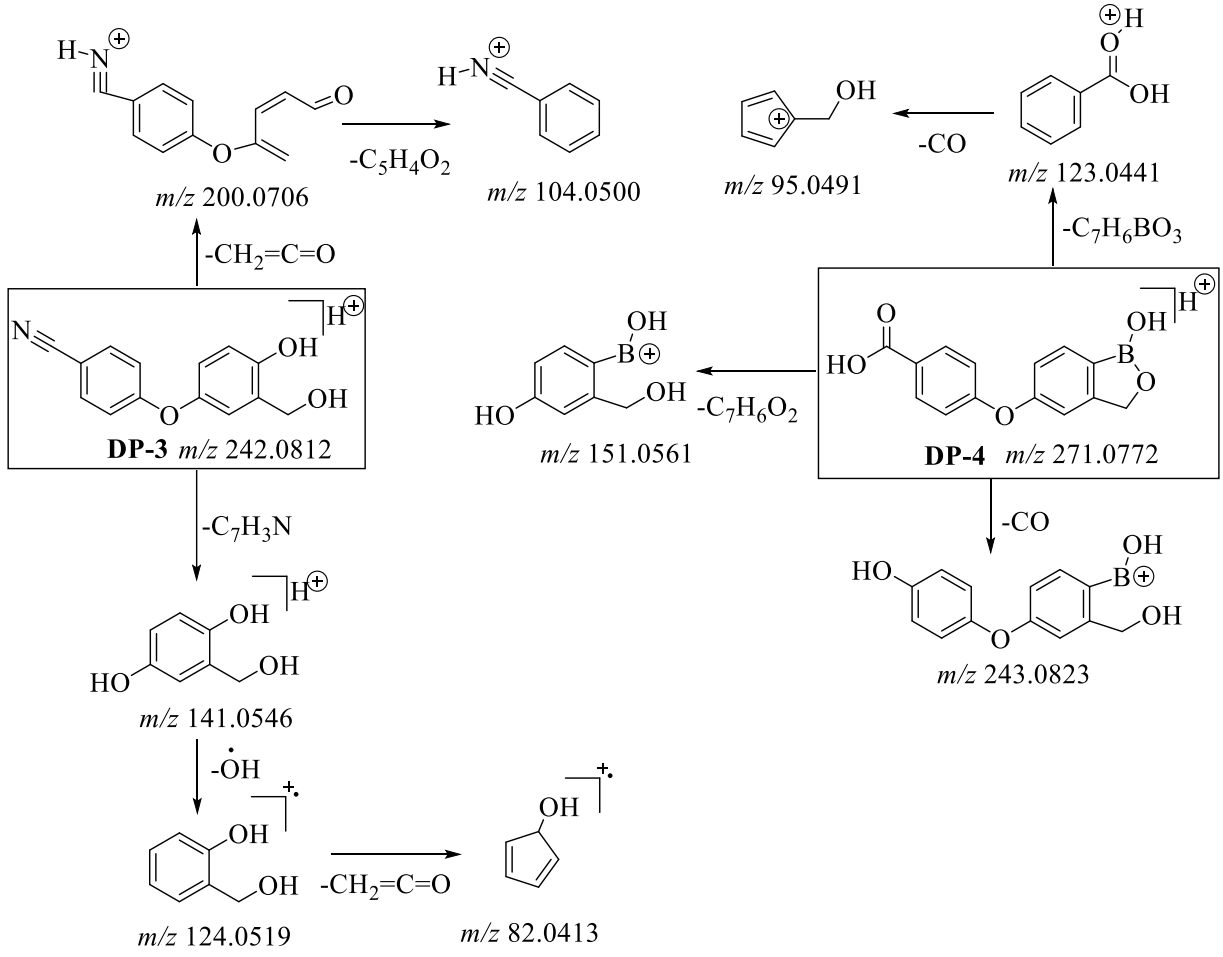


Figure 4.11. Proposed fragmentation pattern for DP-3 (*m/z* 242.0812) and DP-4 (*m/z* 271.0772)

DP-5 ($[M+H]^+$, *m/z* 256.0607)

DP-5 was formed under thermal stress conditions and eluted with a retention time (R_t) of 7.44 min. The ESI-MS/MS spectrum of $[M+H]^+$ of DP-5 (*m/z* 256.0607) (figure 4.12) displays the product ions at *m/z* 194.0612 (loss of CO_2 and H_2O from *m/z* 256.0607), *m/z* 155.0342 (loss of C_7H_4N from *m/z* 256.0607), *m/z* 137.0228 (loss of H_2O from *m/z* 155.0342) *m/z* 104.0504 (loss of $C_7H_5O_4$ from *m/z* 256.0607), and *m/z* 75.0225 (loss of C_7H_5NO from *m/z* 194.0612). The existence of a -COOH and -OH group in the structure of DP-5 is clearly specified by the product ion at *m/z* 194.0612, which is produced by the loss of water molecules and CO2. The product ions at *m/z* 155.0342 and *m/z* 104.0504 are representative ions for DP-5 (figure 4.14). Accurate mass measurements confirmed the elemental composition of DP-5 and product ions (table 4.6). Based on these data, DP-5 was recognized as 5-(4-cyanophenoxy)-2-hydroxybenzoic acid.

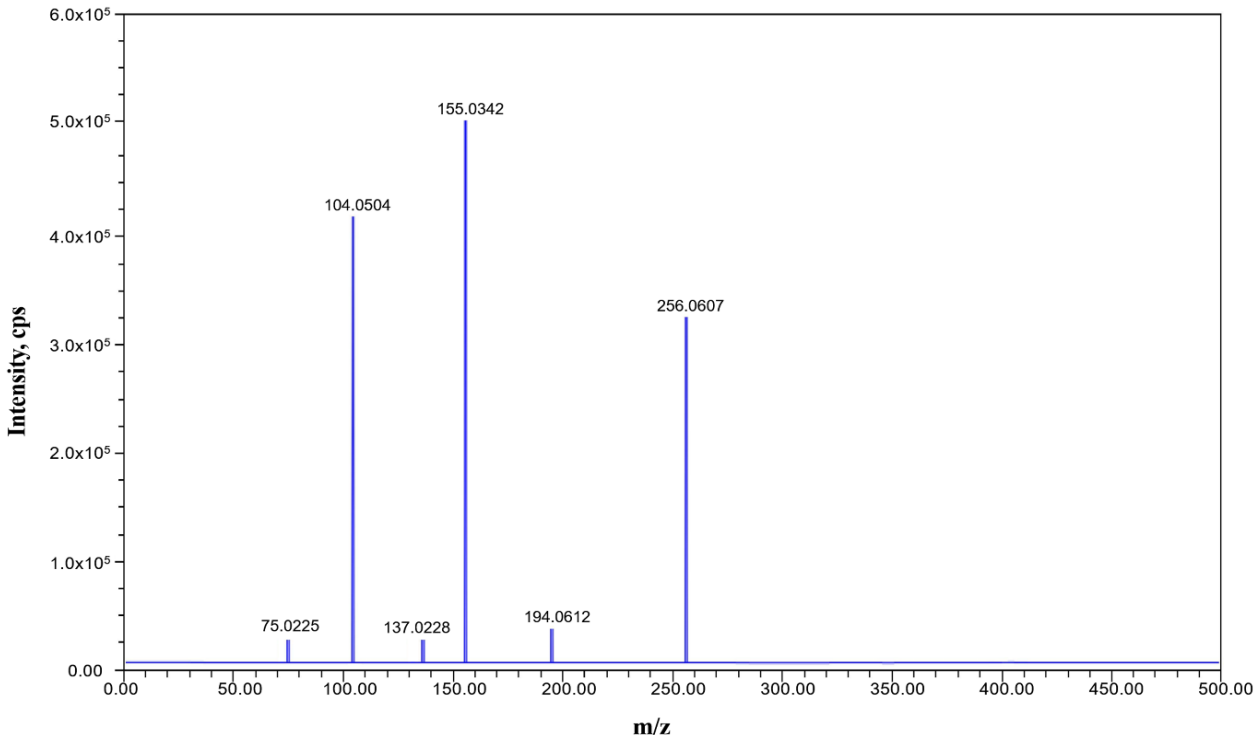


Figure 4.12. Typical LC-MS/MS spectra of protonated DP-5 (*m/z* 256.0607)

DP-6 ($[M+H]^+$, *m/z* 238.0667)

DP-6 was obtained under thermal stress conditions with a retention time (R_t) of 1.19 min. The ESI-MS/MS spectrum of $[M+H]^+$ of DP-6 (*m/z* 238.0667) (figure 4.13) shows the product ions at *m/z* 137.0411 (loss of C_7H_4N from *m/z* 238.0667), *m/z* 120.0447 (loss of $C_6H_4BO_2$ from *m/z* 238.0667), and *m/z* 104.0504 (loss of $C_6H_4BO_3$ from *m/z* 238.0667). The product ions at *m/z* 120.0447 and *m/z* 104.0504 are characteristic ions for DP-6 (figure 4.14). Based on the

information available, elemental composition of DP-6 and product ions were evaluated and proposed as shown in the table 4.6. However, as the expected boron pattern in the mass spectra corresponding to M and (M-1), due to the presence of ^{11}B and ^{10}B isotopes, were observed only in the case of CBE (inset attached in the figure 4.5) and not in case of DP-6, future studies to authenticate the structure of DP-6 are needed.

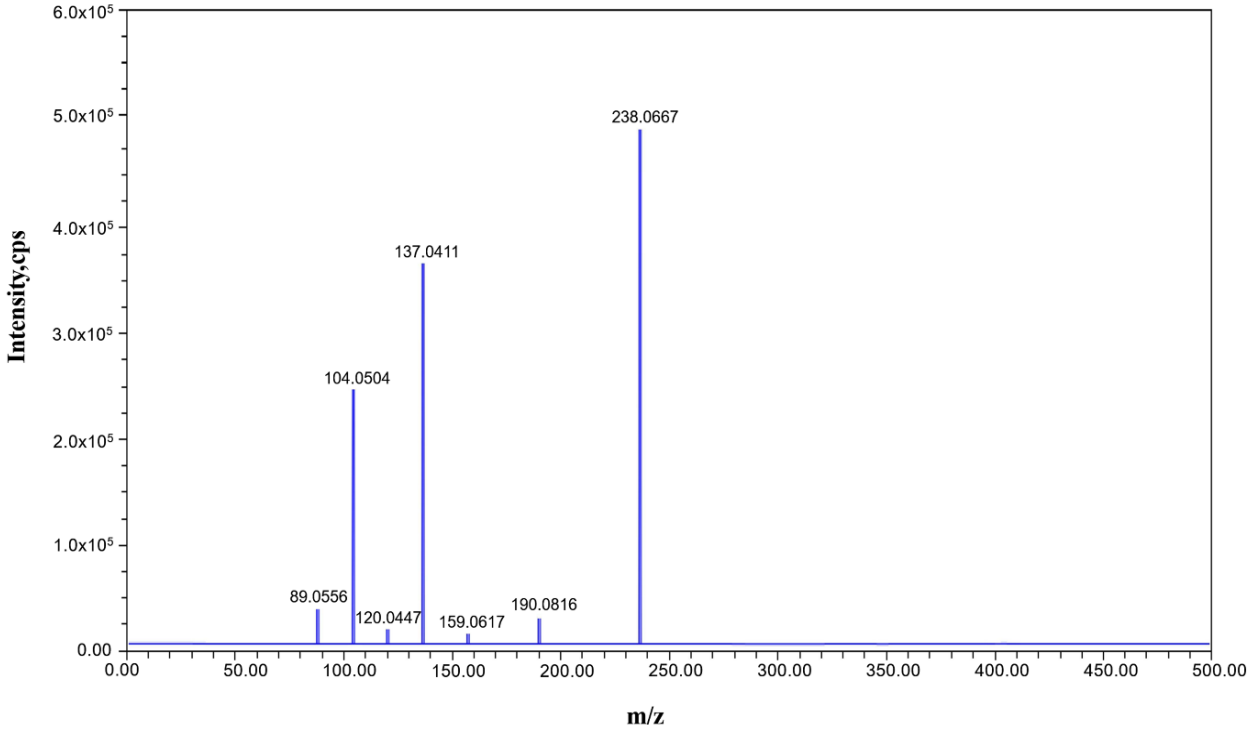


Figure 4.13. LC-MS/MS spectra of protonated DP-6 (*m/z* 238.0667)

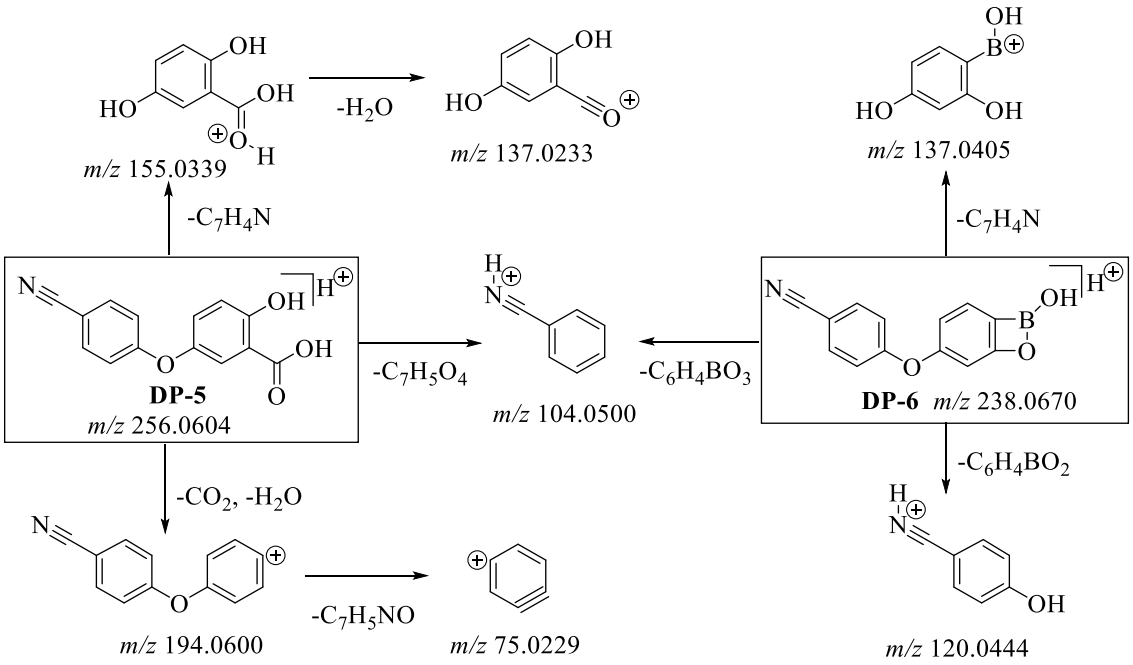


Figure 4.14. Proposed fragmentation pattern for DP-5 (*m/z* 256.0604) and DP-6 (*m/z* 238.0670)

4.9 Mechanistic pathway for the formation of crisaborole DPs

In the presence of an acid hydrolysis condition, CBE was converted to DP-1. Due to electron withdrawing nature of nitrile group in CBE, nucleophilic addition of chloride ion might take place at ortho to the nitrile group on benzene ring. Similarly, for the formation of DP-2, C-B bond cleavage occurred in the oxaborole ring with the attack of H^+ ions and H_2O followed by the elimination of boric acid. A plausible mechanism for the formation of DP-1 and DP-2 are displayed in in figure 4.15.

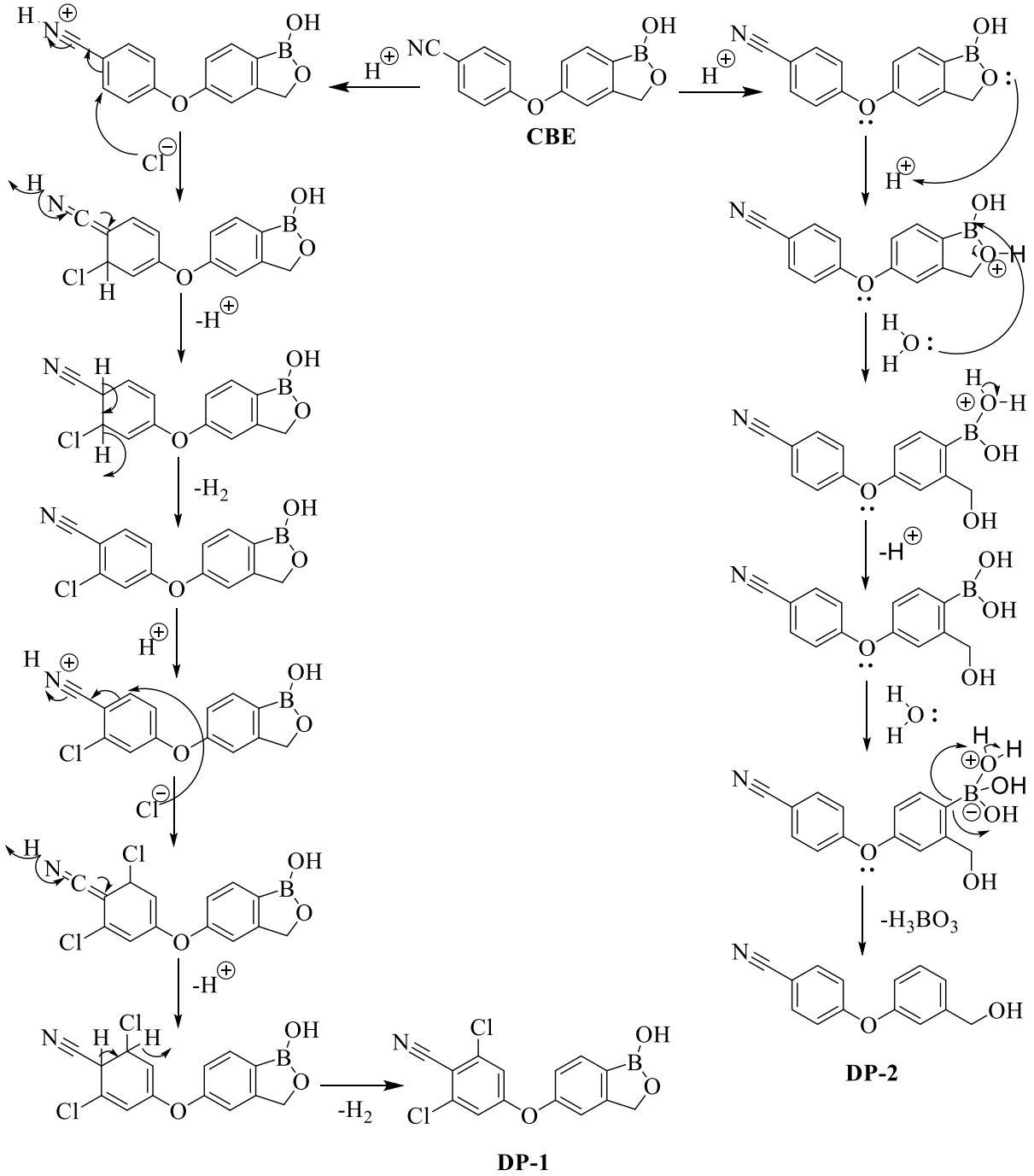


Figure 4.15. Probable mechanism for the formation of DP-1 and DP-2

In the case of base hydrolysis, OH^- ions attack the electrophilic boron atom of the oxaborole ring, which is then hydrolyzed, with the subsequent formation of DP-3. In the case of neutral

hydrolysis, the water molecule may react with the electrophilic carbon of the nitrile group, leading to the formation of amide, followed by the amide hydrolysis to generate DP-4. A possible mechanism for the formation of DP-3 is presented in figure 4.16.

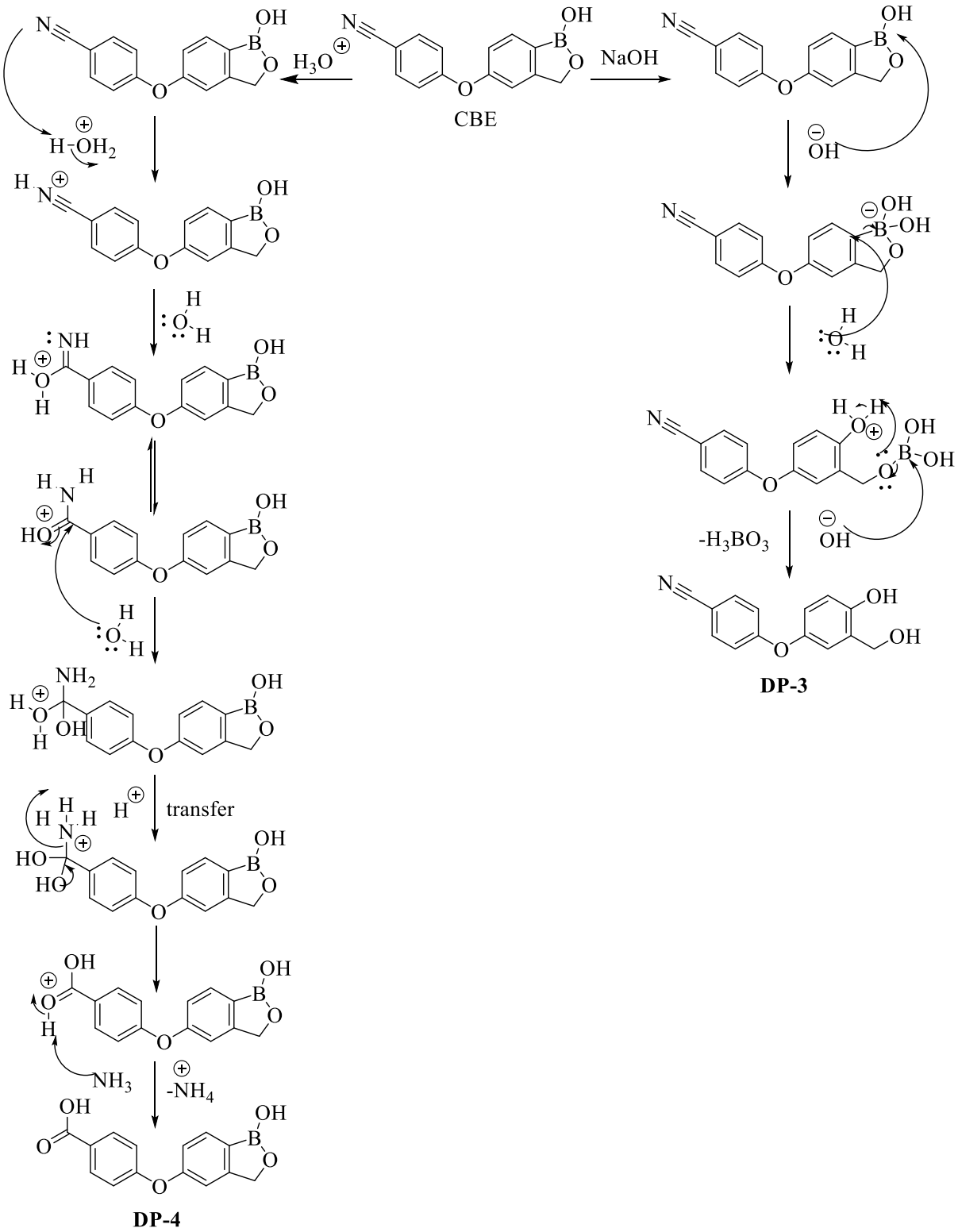


Figure 4.16. Probable mechanism for the formation of DP-3 and DP-4

In thermal conditions, oxidation of CBE generates an oxidized intermediate in which oxaborole ring cleavage occurs in the presence of water. Later, oxidation and the subsequent loss of

boronic acid results in to the formation of DP-5. Similarly, it is proposed that the cleavage of the oxaborole ring in the presence of water with subsequent loss of formaldehyde may lead to the formation of DP-6. In addition, the formation of DP-2 due to water mediated oxaborole ring cleavage followed by the elimination of boric acid was also proposed. A possible mechanism for the formation of DP-3 is presented in figure 4.17.

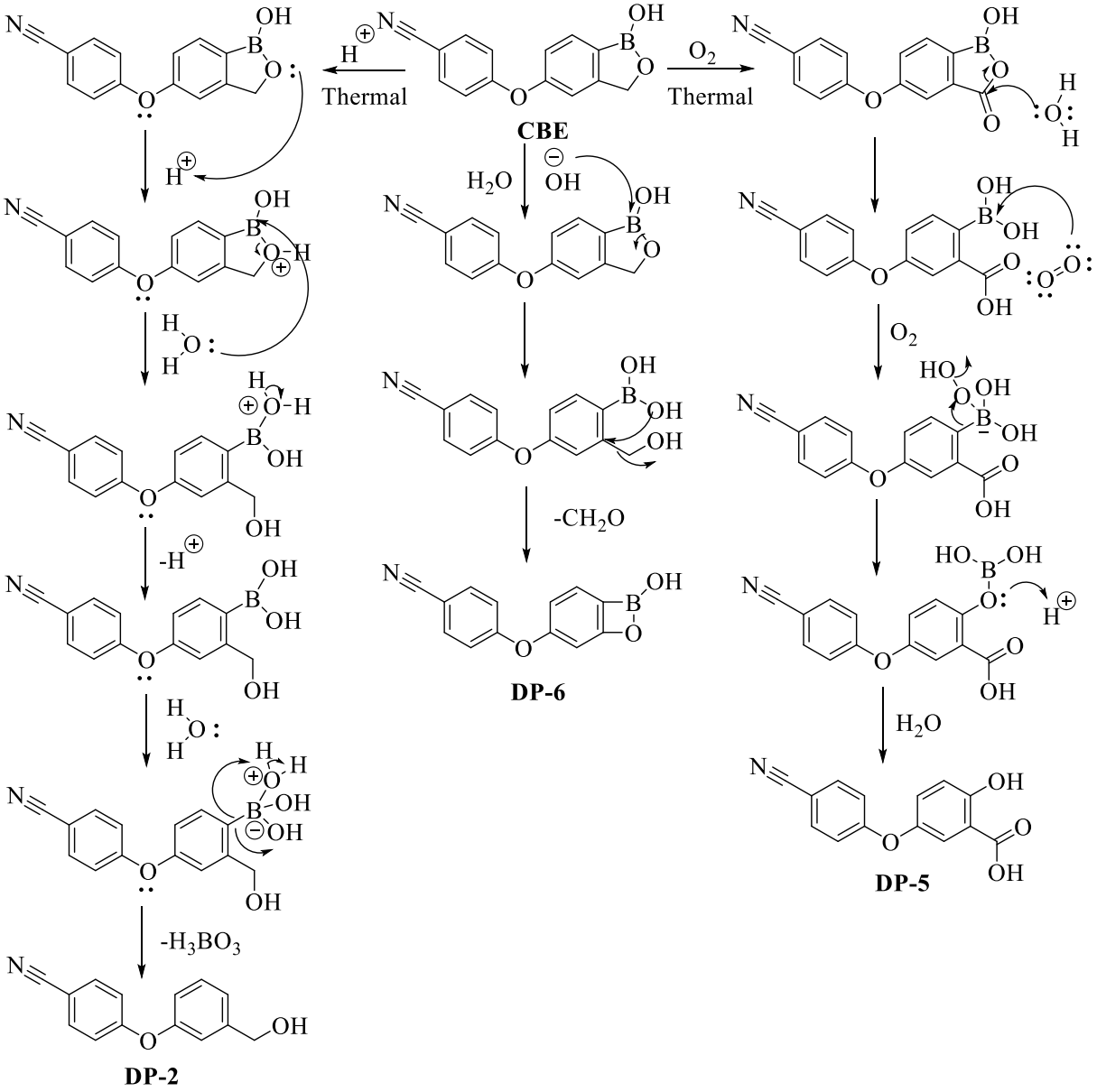


Figure 4.17. Probable mechanism for the formation of DP-2, DP-5 and DP-6

4.10 ADMET prediction studies

The drug CBE and its DPs (DP-1 to DP-6) were evaluated in terms of their key pharmacokinetic properties and physiochemically important descriptors through the QikProp programme of Schrodinger software. The compounds were subjected to QikProp ADMET profiling, and based on the physico-chemical properties of the compounds analysed, they were concluded to exhibit drug-like characteristics. ADMET prediction studies depicted that the CBE and DPs (DP-1 to

DP-6) conformed to Lipinski's rule of +Ve. They exhibited good log P values for desired biological efficacy and exhibited no violations in the prescribed ranges of physicochemical and ADMET parameters. The other related parameters, such as blood-brain permeability and % human oral absorption, are also within the permitted limit. Table 4.7 presents the results.

Table 4.7 Predicted ADMET Properties of CBE and its DPs

Properties	CBE	DP-1	DP-2	DP-3	DP-4	DP-5	DP-6
Physicochemical properties							
No. of Rotatable bonds	2	2	3	3	4	3	3
Polar Surface Area (PSA)	62.48	62.48	53.25	62.48	75.71	64.71	53.25
Log P o/w	1.12	2.1	2.42	2.15	1.82	0.78	1.36
Water Solubility (log mol/L)	-3.11	-4.28	-2.95	-3.07	-2.48	-2.52	-2.95
Pharmacokinetics							
GI absorption	High	High	High	High	High	High	High
BBB permeant	Yes	Yes	Yes	No	Yes	No	Yes
P-gp substrate	Yes	Yes	No	No	Yes	Yes	No
CYP1A2 inhibitor	Yes	Yes	Yes	No	Yes	No	No
CYP2C19 inhibitor	No	No	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	No	No	No
CYP2D6 inhibitor	No	Yes	No	No	No	No	No
CYP3A4 inhibitor	Yes	Yes	No	Yes	Yes	Yes	No
skin permeation (log Kp in cm/s)	-6.28	-5.81	-6.10	-6.15	-6.79	-6.92	-6.23
Drug-Likeness Score	0.55	0.55	0.55	0.55	0.55	0.55	0.55
Medicinal chemistry							
Lead likeness	Yes	Yes	No	No	No	Yes	No
Synthetic accessibility	3.17	3.21	2.04	2.15	1.94	3.11	2.38

4.11 *In silico* toxicity prediction of crisaborole and its degradation products

Drug toxicity that might be harmful to humans is predicted using in silico toxicity studies. It is a supplement to the in vitro or in vivo toxicity studies, minimizing animal testing and lowering the price and duration of toxicity tests in the process. The software tool admetSAR was used to predict the various levels of potential toxicity of CBE and its induced DPs. The toxicological pathways, genetic toxicity, and organ toxicity of CBE were determined. This information revealed the potential underlying mechanisms for toxic responses and the determined outcomes for each target, each of which are assigned a probability value. Table 4.8 lists the expected toxicity of CBE and its DPs.

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Table 4.8 Toxicity prediction of CBE and its degradation products

	Target	CBE and its degradation products						
		CBE	DP-1	DP-2	DP-3	DP-4	DP-5	DP-6
Toxicity class		III	III	III	III	III	III	III
Predicted LD_{50}	Acute oral toxicity (kg/mol)	2.37	1.79	1.67	2.69	2.41	2.15	2.54
Organ toxicity	Hepatotoxicity	+(0.82)	+(0.77)	+(0.70)	+(0.65)	+(0.75)	+(0.75)	+(0.77)
	Human either-a-go-go-ralted gene (hERG) inhibition	-	-	-	-	-	-	-
	Eye irritation	+(0.63)	-	+(0.90)	+(0.86)	+(0.71)	+(0.85)	+(0.92)
	Eye corrosion	-	-	-	-	-	-	-
Genomic toxicity	Ames mutagenesis	+(0.5)	+(0.53)	-	-	-	-	-
	Carcinogenesis	-	-	-	-	-	-	-
	Micronucleus assay	+(0.71)	+(0.75)	-	-	+(0.79)	+(0.74)	+(0.74)
Toxicological pathway	Androgen Receptor (AR)	+(0.61)	+(0.67)	+(0.56)	+(0.73)	+(0.56)	+(0.75)	+(0.60)
	Aromatase	+(0.79)	+(0.89)	+(0.87)	+(0.88)	+(0.84)	+(0.89)	+(0.89)
	Estrogen Receptor (ER)	+(0.88)	+(0.88)	+(0.90)	+(0.91)	+(0.88)	+(0.85)	+(0.80)
	Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	+(0.68)	+(0.60)	+(0.84)	+(0.89)	+(0.76)	+(0.85)	+(0.61)

4.12 Conclusions

The developed HPLC method described in this chapter can isolate all DPs from the drug and also from each other when subjected to various stress conditions. The degradation behaviour of CBE was assessed under different stress conditions like hydrolysis, thermal, photolytic, and oxidation, respectively. The drug was subjected to hydrolytic degradation under acid stress conditions to form two DPs (DP-1 and DP-2), while DP-3 and DP-4 were formed under base and neutral stress conditions, respectively. Three DPs (DP-2, DP-5, and DP-6) were formed under thermal stress conditions, while the drug was stable under photolytic and oxidative stress conditions. The DPs (DP-1 to DP-6) of CBE were identified and well characterized with the aid of QqLIT LC-MS/MS experiments and accurate mass measurements. Further, the *in silico* determined ADMET data and toxicity admetSAR software prediction tool indicated that the probability of CBE and its DPs can be hepatotoxic and genotoxic leading to severe toxicity.

4.13 References

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Chapter-V

Degradation behaviour of Procainamide hydrochloride and their comparison with known impurities: Development and validation of a stability-indicating HPLC-PDA method

5.1 Introduction

Procainamide hydrochloride (PCM; p-amino-N-(2-(diethylaminoethyl)benzamide monochloride)) is a cardiac antiarrhythmic drug that has been used for the treatment of atrial and ventricular arrhythmias for more than 40 years.[1-4] Procainamide is an anti-arrhythmic of class 1A that binds to fast sodium channels and prevents repolarization recovery. It also extends the action potential and slows the conduction of impulses. These effects reduce excitability, raise the effective refractory period, and slow conduction.[5-8] Procainamide is almost completely absorbed in the body, with plasma concentration levels peaking 1 to 2 hours after oral administration. Approximately 50% of the procainamide dose is excreted as an unchanged drug via the kidneys.[9-11] Administration of sustained-release procainamide twice daily results in the desired plasma concentrations in most dogs.[12] N-acetyl procainamide is the active metabolite of procainamide and is generated by polymorphic N-acetyltransferase II.[13,14] It is a pharmacologically active compound that is found in similar amounts to procainamide in the plasma of patients receiving procainamide medication.[15,16] Procainamide causes cardiac depression, which lowers blood pressure, also causes nausea, vomiting, diarrhoea, rashes, granulocytopenia disease, and, on rare occasions, psychosis.[17] Despite its development as a type IA antiarrhythmic drug, procainamide has been demonstrated to have anticancer activity,[18,19] Procainamide hydrochloride was able to protect mice and rats against the toxic effects of cisplatin.[20,21] Procainamide does have the potential to be exploited as an epigenetic demethylating agent to enhance insulin levels in type 2 diabetes.[22]

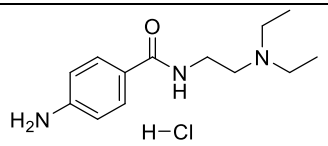
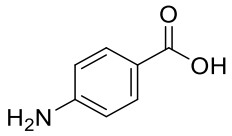
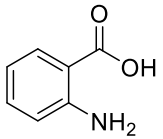
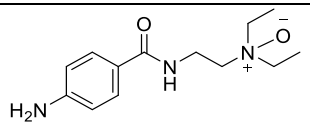
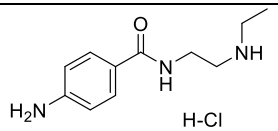
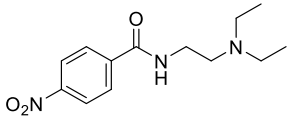
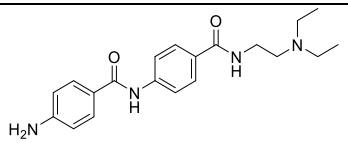
5.2 Literature survey

High-performance liquid chromatography (HPLC) is now considered the most suitable technology for impurity analysis. This is crucial for drug approval and can have a major impact on process development.[23-25] A thorough review of the literature reveals that a few analytical techniques have been published for the determination of PCM, either alone or in with other drugs. These methods include spectrophotometry,[26] spectrofluorometry,[27] capillary zone electrophoresis,[28] multi-spectroscopic techniques,[29,30] HPLC method,[31-39] UHPLC method,[40] and LC-MS method.[41,42] These techniques, however, do not distinguish between and identify the contaminants and degradants generated by the force degradation study. To the best of our knowledge, no stability-indicating method for PCM analysis in the presence of DPs and contaminants has yet been reported. Therefore, the goal of this study was to establish an HPLC-PDA-based stability-indicating analytical method for the separation and determination of potential associated impurities (namely Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, and Imp-F) in PCM drug. The established LC technique was validated with regard to specificity, precision, accuracy, linearity, LOD, LOQ, and robustness. This newly developed method has been

validated and proven to meet the compatibility requirements described in the ICH guidelines. The method proposed here is suitable for routine analysis and stability studies in quality control laboratories.

In the present study, PCM and its related substances were considered for the new analytical method development and validation. The chemical structures and names of the process related impurities and PCM were depicted in table 5.1.

Table 5.1 Chemical structures of Procainamide hydrochloride and its impurities

Name	Chemical structure	Chemical name
Procainamide hydrochloride		4-amino-*N*-(2-(diethylamino)ethyl)benzamide hydrochloride
Imp-A		4-aminobenzoic acid
Imp-B		2-aminobenzoic acid
Imp-C		2-(4-aminobenzamido)-*N*, *N*-diethylethan-1-amine oxide
Imp-D		4-amino-*N*-(2-(ethylamino)ethyl)benzamide hydrochloride
Imp-E		*N*-(2-(diethylamino)ethyl)-4-nitrobenzamide
Imp-F		4-amino-*N*-(4-((2-(diethylamino)ethyl)carbamoyl)phenyl)benzamide

5.3 Experimental

5.3.1 Chemicals and reagents

Pure PCM and impurities were collected from a local manufacturing industry in Hyderabad, India. In addition, HPLC grade acetonitrile (ACN) and LC-MS grade formic acid were purchased from Merck. Hydrochloric acid (HCl), sodium hydroxide (NaOH), sulphuric acid (H2SO4), 30% hydrogen peroxide (H2O2), triethylamine (TEA), hexane sulfonic acid (HSA), and orthophosphoric acid (OPA) of analytical reagent grade were bought from Merck, India. The Milli-Q ultrapure water purifying device (Millipore, Bedford, MA, USA) produced extremely pure water.

5.3.2 Instrumentation

The LC system employed for method development, forced degradation tests, and method validation consisted of a Water Alliance e2695 HPLC system (Waters Corp., Milford, MA, USA) equipped with a quaternary pump, in-line degasser, auto-injector, a column compartment, and a PDA detector (model 2998). Empower 2 software was used to monitor and process the output signal. Photostability studies were performed in a photostability chamber (Newtronic Lifecare Equipment Pvt. Ltd., Maharashtra, India). In a dry air oven, thermal stability investigations were conducted (Kemi, India). A pH meter (Elico LI 120, Mumbai, India) was used to adjust the pH of the mobile phase and all other solutions used for this study. Analytical balance (Shimadzu, ATX224, Kyoto, Japan), and a sonicator (Sonica 2200MH S3, Milano, Italy) were the other instruments used during the study.

5.3.3 Chromatographic conditions

The chromatographic separations were performed on a Waters Symmetry C18 (100 × 4.6 mm, 3.5 µm) column, using a mobile phase composed of acetonitrile and 0.01 M hexane sulfonic acid buffer with 0.1% TEA, pH 2.5 (Orthophosphoric acid was used to bring the buffer's pH down to 2.5) with a gradient elution programme (time (min)/%ACN) of 0/20, 10/50, 20/70, 25/20, and 30/20. The mobile phase flowed at a rate of 1.0 mL min^{-1} at room temperature. The detection wave length was monitored at 258 nm, and an injection volume of 10 µL was used. The diluent consisted of acetonitrile and buffer in a 50:50 v/v ratio.

5.3.4 Preparation of standard stock solution

In the process of preparing the sample, a 50:50 v/v mixture of acetonitrile and buffer was utilized as a diluent. After precisely weighing and transferring 500 mg of PCM into a 100 mL volumetric flask, a stock solution of PCM (5 mg mL^{-1}) was prepared. Then add 70 mL of diluent, and the flask is kept on a sonicator for 15 min, and the volume is made with diluent. The prepared stock solution was maintained at 4 °C until it was time to perform the analysis.

5.3.5 Preparation of impurity stock solution

10 mg each of the impurities Imp-A, Imp-B, Imp-E, and Imp-F, as well as 5 mg of Imp-C and Imp-D, were precisely weighed and then added to a 100 mL volumetric flask to create a stock solution of the impurities. The flask is placed on a sonicator for 15 minutes after adding 70 mL of diluent. The volume is then made with the diluent to obtain 100 µg mL^{-1} for Imp-A, Imp-B, Imp-E, and Imp-F and 50 µg mL^{-1} for Imp-C and Imp-D.

5.3.6 Preparation of spiked solution

In order to achieve a concentration of 500 µg mL^{-1} for PCM; 10 µg mL^{-1} for Imp-A, Imp-B, and Imp-E, and 5 µg mL^{-1} for Imp-C and Imp-D, 5 mL of PCM standard stock solution was transferred into a 50 mL volumetric flask, followed by 20 mL of diluent and 5 mL of impurity stock solution.

5.3.7 Forced degradation studies

The devised LC technique for PCM was tested for its specificity when its contaminants and degradation byproducts were present. In order to demonstrate the method's specificity and stability-indicating nature, forced degradation investigations were also carried out on PCM.[43]. Acidic, basic, and neutral hydrolytic degradations were performed by refluxing the drug in 1N HCl, 1N NaOH, and water at 60 °C for 24 h, respectively. The thermal stress study was conducted for 72 h at 105 °C. In order to perform the oxidative degradation, 30% H_2O_2 was used for 24 hours at 60 °C. In the photostability chamber, the drug was photolytically degraded after being exposed to 1.2 million lux of visible light for 72 hours. The degradation samples were analysed by the HPLC-PDA method as described in the chromatographic conditions. Using a Waters e2695 photo diode array detector (PDA), the peak purity of the PCM stressed samples was examined. All of the stressed samples' purity angles were within the purity threshold limit, confirming the homogeneity of the analyte peak. For each sample, the mass balance (%assay+%of degradation products) was computed. Every stressed sample was collected, neutralized, and diluted with the mobile phase. Before performing HPLC analysis, all of the solutions were filtered using 0.22 µm membrane filters.

5.3.8 Method validation

As prescribed in ICH guidelines Q2(R1),[44] the proposed HPLC method has been validated in terms of system suitability, specificity, accuracy, precision, linearity, and robustness.

5.3.8.1 System suitability test

A system suitability test was conducted to verify the system performance for the proposed method. System suitability parameters, including resolution, number of theoretical plates tailing, and %RSD, were determined.

5.3.8.2 Specificity

Specificity of an analytical method is the capacity to determine the analyte in the presence of other components, such as contaminants and DPs. The specificity of PCM was investigated in the presence of its impurities and DPs. No interference peak was noticed at the retention time of PCM. The approach was proven to be specific because all of the DP and impurity peaks were clearly isolated from one another.

5.3.8.3 Accuracy

To evaluate the accuracy of the method, we performed recovery experiments. Known amounts of the impurities were spiked at three different concentration levels ranging from 50-150% of PCM in six replicate analyses. The accuracy of the PCM assay was evaluated in six replicates at three different concentration levels (50, 100, and 150 µg mL^{-1}). The percentages of recoveries were calculated at each level.

5.3.8.4 Precision

The terms repeatability and intermediate precision were used to describe the method's precision. The repeatability of the suggested method was demonstrated by examining six different test samples of PCM (500 µg mL^{-1}), each spiked with an impurity at the specified level. Using separate analysts and tools on two different days, the same experiment was repeated to assess the intermediate precision of the procedure. The %RSD for each peak's area was computed.

5.3.8.5 Limit of detection (LOD) and Limit of quantification (LOQ)

The limits of detection (LOD) and limits of quantitation (LOQ) for PCM and impurities were calculated with a signal-to-noise ratio of 3:1 and 10:1, respectively. By injecting a series of diluted solutions with known amounts of PCM and its impurities, LOD and LOQ were determined.

5.3.8.6 Linearity

The impurity stock solution was diluted to the necessary concentrations to create the linearity test solutions for the impurities. The solutions were made at six different concentrations: 1.25 to 7.5 g mL-1 for Imp-C and Imp-D, and 2.5 to 15.0 g mL-1 for Imp-A, Imp-B, Imp-E, and Imp-F. Similarly, the linearity of the assay was established by injecting PCM at five different concentration levels ranging from 125 to 750 µg mL^{-1}. Calibration graphs of peak areas against concentrations were plotted for impurities and PCM. The peak area versus concentration data was treated by the least-squares linear regression analysis method. All the samples were analyzed in six replicates.

5.3.8.7 Robustness

By making minimal changes to HPLC technique parameters such as flow rate (1.0 mL min^{-1}), pH of the mobile phase buffer (pH 2.5), and other factors, the robustness of the method was assessed and % of organic modifier in the mobile phase. In each of the adjusted conditions, the system suitability parameters, such as theoretical plates, tailing, and resolution between each neighbouring peak, were assessed.

5.3.8.8 Solution stability

The PCM sample solution and impurities spiked solution were tested for solution stability at RT for 24 h and then cooled at 2-8 ºC for 24 h. The %RSD peak areas and retention times of PCM and impurities were calculated against the initial value for the study period during the solution stability experiments.

5.4 Results and discussions

5.4.1 Method optimization

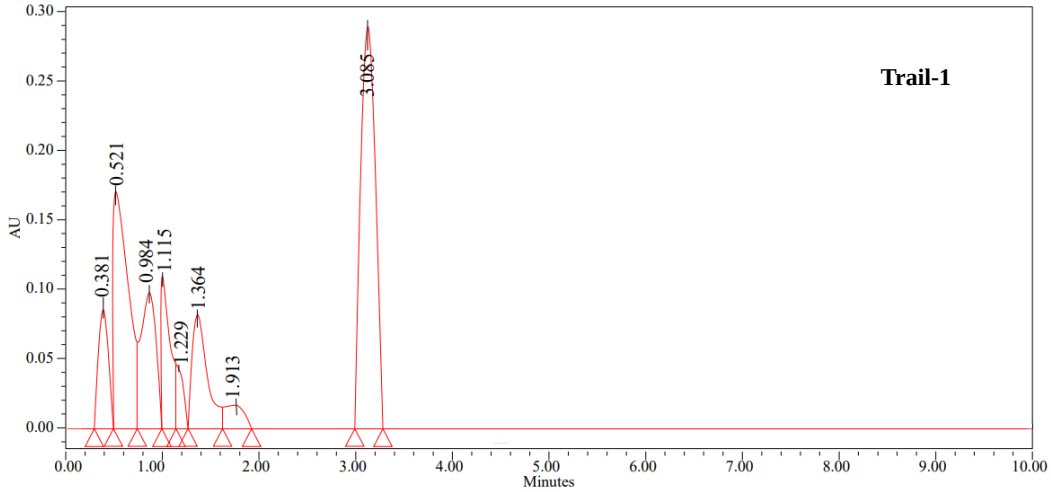
Preliminary studies were carried out to develop a chromatographic method capable of eluting and resolving PCM from its impurities for impurity profiling. Initial trials were carried out to optimise the method development, using an ammonium acetate buffer (pH 3 adjusted with formic acid) and acetonitrile mobile phase with isocratic and gradient elution modes. To improve resolution and achieve acceptable retention times, the composition of the mobile phase in each trial was altered. As a result, the separation of impurities from the PCM was not achieved efficiently, and the resolution was poor.

To optimize the resolution between the impurities and the retention time of the impurities, trails were carried out using a mobile phase of acetonitrile and hexane sulfonic acid buffer with 0.1% TEA (pH 2.5 adjusted with OPA). Various gradient programmes were investigated, and acceptable results were obtained when a gradient programme of (time (min)/%ACN): 0/20, 10/50, 20/70, 25/20, and 30/20. During the optimization process, various stationary phases such as Zorbax SB, C18 250 mm x 4.6 mm, 5 µm, Inertsil ODS column, 150 mm x 4.6 mm, 3.5 µm, and Waters Symmetry C18 column, 100 mm x 4.6 mm, 3.5 µm columns were used for the trials and the results are given in table 5.2. The respective chromatograms of the trial are shown in figure 5.1. Satisfactory results were achieved on a Symmetry C18 column, 100 mm x 4.6 mm, 3.5 µm used as the stationary phase. Finally, after multiple trials, the optimized chromatographic separation of drug and impurities was attained on a Waters Symmetry C18 column, 100 mm x 4.6 mm, 3.5 µm column as the stationary phase. Acetonitrile and 0.01 M hexane sulfonic acid with 0.1% TEA, pH 2.5 (the pH of the buffer was adjusted to 2.5 with orthophosphoric acid), were used as the mobile phase with a gradient elution programme of (time (min)/%ACN): 0/20, 10/50, 20/70, 25/20, and 30/20. At room temperature, the flow rate

of the mobile phase was 1.0 mL min^{-1}. The detection wave length was monitored at 258 nm, and an injection volume of 10 µL was used. The optimized chromatographic separation method is shown in table 5.3. The optimized chromatographic separation of PCM and impurities is shown in figure 5.2.

Table 5.2 Method development trials

Trail	Buffer	Mobile phase	Column	Flow rate	Observation
1	Ammonium acetate pH-3.0 with formic acid	ACN: Buffer; 80:20 v/v	Zorbax SB, C18 250 mm x 4.6 mm, 5 µm	1.0 mL min^{-1}	Peaks are merging and are not eluted properly
2	Ammonium acetate pH-3.0 with formic acid	ACN: Buffer; 70:30 v/v	Zorbax SB, C18 250 mm x 4.6 mm, 5 µm	1.0 mL min^{-1}	Peaks plate counts, tailing and resolution were not within the limits
3	Ammonium acetate pH-3.0 with formic acid	ACN: Buffer; 60:40 v/v	Inertsil ODS column, 150 mm x 4.6 mm, 3.5µm	1.0 mL min^{-1}	Resolution between the impurities not adequate
4	Ammonium acetate pH-3.0 with formic acid	T(min)/ACN: 0/30, 10/70, 20/80, 25/80, 30/80	Inertsil ODS column, 150mm x 4.6mm, 3.5 µm	1.0 mL min^{-1}	Only four peaks were eluted
5	Hexane sulfonic acid and Triethylamine pH-2.5 with OPA	T(min)/ACN: 0/50, 10/50, 20/80, 25/20, 30/20	Inertsil ODS column, 150 mm x 4.6 mm, 3.5 µm	1.0 mL min^{-1}	Peak tailing and resolution were not within the limits
6	Hexane sulfonic acid and Triethylamine pH-2.5 with OPA	T(min)/ACN: 0/20, 10/45, 20/70, 25/10, 30/10	Symmetry C18 column, 100 mm x 4.6 mm, 3.5 µm	1.0 mL min^{-1}	Less resolution between peaks 3 and 4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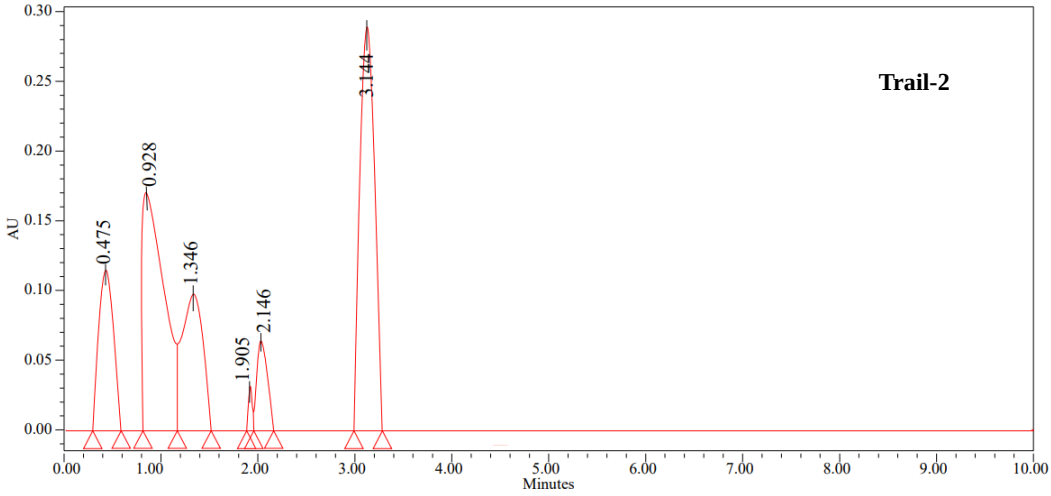
0.521
0.984
1.115
1.229
1.364
1.913
3.085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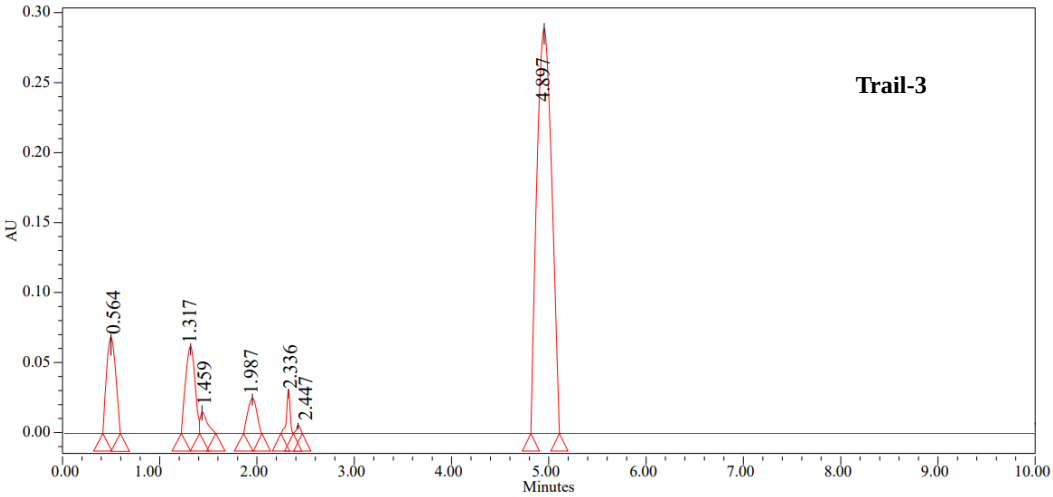
1.905
2.146
3.144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1.987
2.336
2.447
4.897

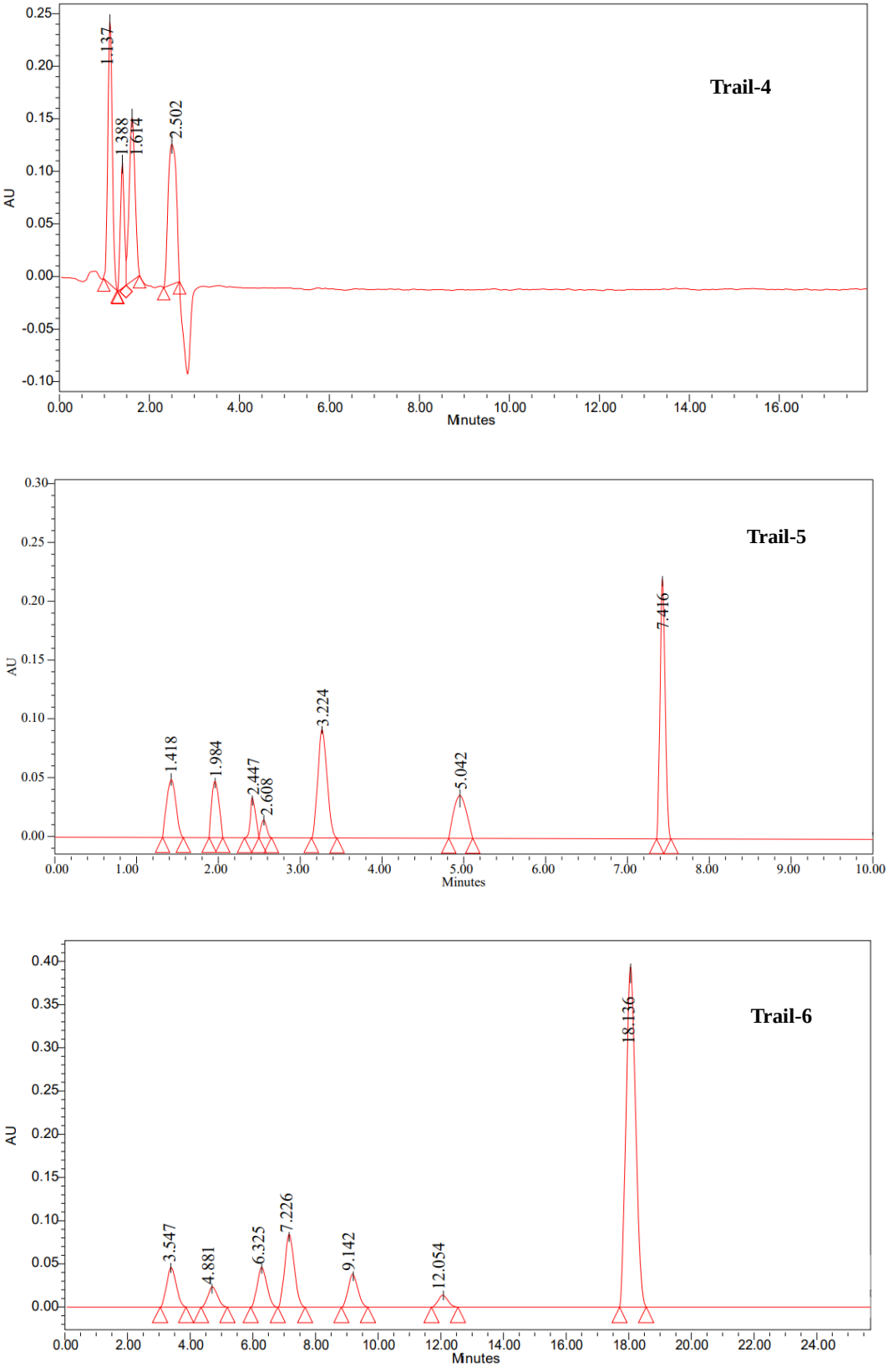


Figure 5.1. Typical LC chromatograms during the method development

Table 5.3 Optimized Chromatographic conditions

S. No	Method Parameters	Optimized Conditions
1	Column	Symmetry C18 (150 mm x 4.6 mm, 3.5 µ)
2	Mobile phase	Acetonitrile: 0.01 M hexane sulfonic acid buffer with 0.1% TEA, pH 2.5
3	Gradient elution	(Time(min)/%ACN): 0/20, 10/50, 20/70, 25/20, and 30/20
4	Run Time	30 min
5	Flow rate	1.0 mL min^{-1}
6	Column temperature	Room temperature
7	Diluent	Acetonitrile: Buffer (50:50 v/v)
8	Detector	Photodiode array, 258 nm

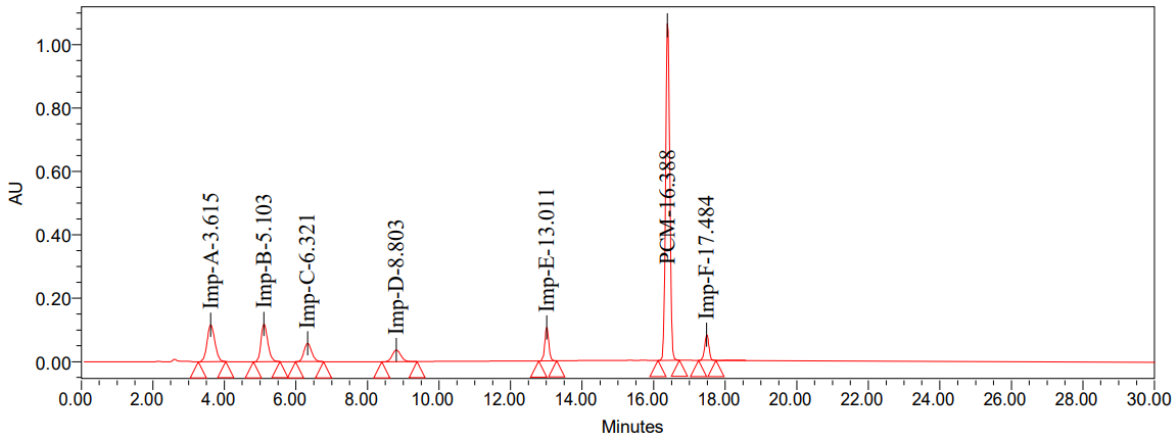
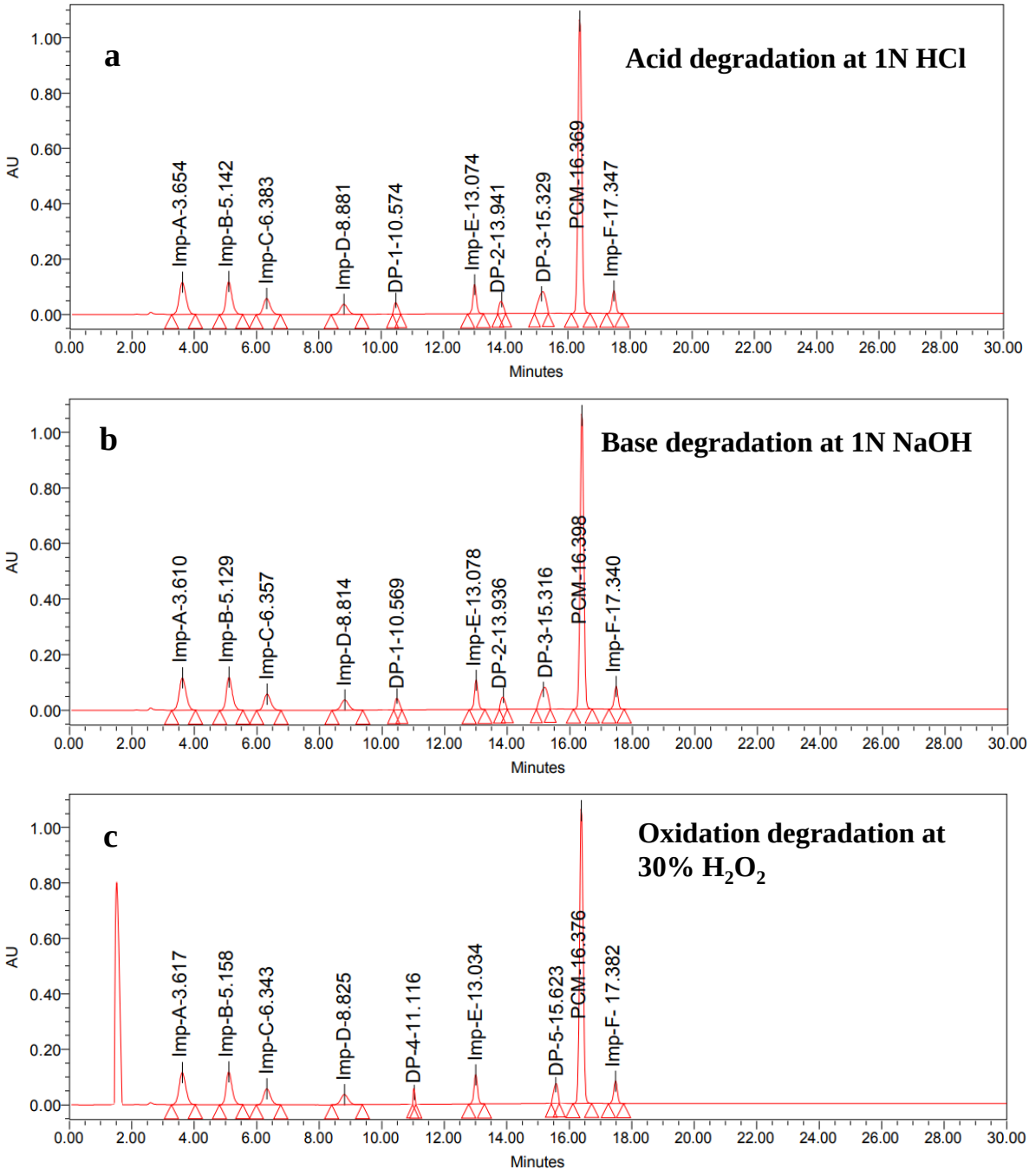


Figure 5.2. Optimized HPLC chromatogram of PCM and its impurities

5.4.2 Degradation behaviour of PCM

The stress degradation behaviour of the PCM was examined using the HPLC-PDA method. The chromatograms in figure 5.3 reveal the degradation behaviour of the PCM under various stress degradation conditions. In neutral hydrolysis and photolytic conditions, degradation of PCM was not observed. However, significant degradation of PCM was seen in (acidic, basic) hydrolytic, oxidation, and thermal stress conditions. In both acidic and basic hydrolysis, three degradation products (DP-1, DP-2, and DP-3) were formed at retention times of 10.57, 13.94, and 15.32 min, respectively. Under oxidation stress conditions, two degradation products (DP-4 and DP-5) were formed at retention times of 11.16 and 15.62 min, respectively. Under thermal stress conditions, the degradation products (DP-6, DP-7, and DP-8) were also observed at

retention times of 14.06, 15.03, and 18.51 min, respectively. Results of peak purity tests attained from the PDA detector indicated that the PCM peak was pure and homogeneous in all of the stress samples analyzed. Stressed samples' mass balance was observed to lie between 99.3 and 99.8%.



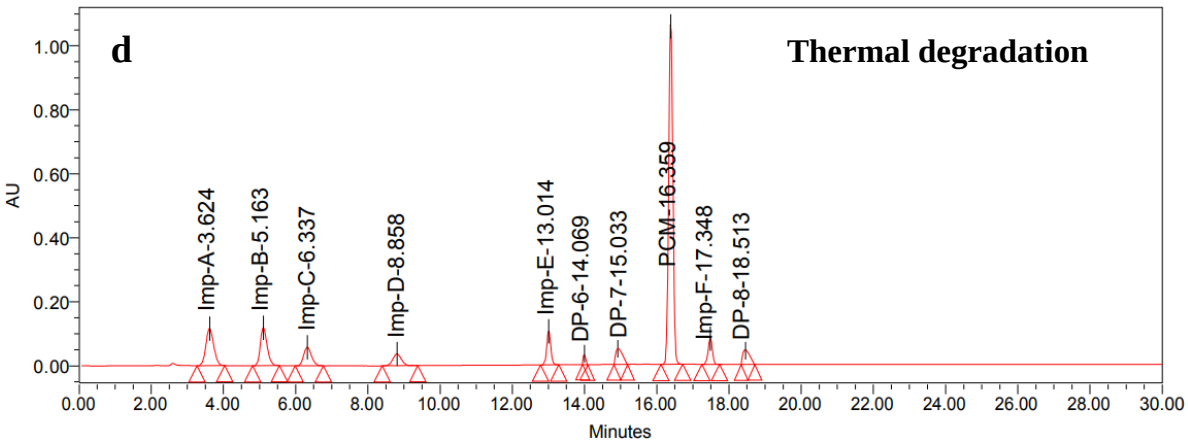


Figure 5.3. Typical HPLC chromatograms of PCM degradation under various conditions at a) 1N HCl, b) 1N NaOH, c) 30% H_2O_2, and d) thermal

Table 5.4 Results of forced degradation study

Degradation condition	Time	%Assay of PCM	%Degradation	Mass balance (%assay + %degradation products)	Remarks
Unstressed	-	99.82	-	99.82	-
Acid hydrolysis (1N HCl, 60 ºC)	24 h	93.72	6.09	99.81	Three degradation products (DP-1, DP-2 and DP-3) were observed
Base hydrolysis (1N NaOH, 60 ºC)	24 h	90.62	9.20	99.82	Three degradation products (DP-1, DP-2 and DP-3) were observed
Neutral hydrolysis (H_2O, 60 ºC)	24 h	99.81	-	99.81	No degradation was observed
Oxidation (30% H_2O_2, 60 ºC)	24 h	86.68	13.52	100.20	Two degradation products (DP-4 and DP-5) were observed
Thermal (105 ºC)	72 h	74.40	25.45	99.85	Two degradation products (DP-6, DP-7 and DP-8) were observed
Photolytic (UV light)	72 h	99.81	-	99.81	No degradation was observed

5.4.3 Method validation

5.4.3.1 System suitability test

In the process of optimizing the conditions of the proposed method, system suitability tests are an important aspect of liquid chromatographic methods. System adequacy trials were performed to ensure that the resolution and repeatability were sufficient for the analysis. Column efficiency (number of theoretical plates), chromatographic peak tailing, peak resolution, and reproducibility of retention as a %RSD. of retention time are the parameters of these tests. Table 5.5 shows the results of system suitability for the recommended method.

Table 5.5 Results of system suitability

Compound name	RT (in min)	RRT	USP resolution	USP tailing	plate count
Imp-A	3.61	0.22	-	1.11	4874
Imp-B	5.10	0.31	3.89	1.25	4578
Imp-C	6.32	0.39	3.18	1.14	3930
Imp-D	8.80	0.54	5.80	1.08	6433
Imp-E	13.01	0.79	12.88	1.04	65729
PCM	16.38	1.00	15.81	1.03	91139
Imp-F	17.48	1.07	5.03	1.03	105428

5.4.3.2 Specificity

The specificity of PCM was investigated in the presence of its impurities and DPs. No interference peak was noticed at the retention time of PCM. The specificity of the method was determined by measuring the peak purity of PCM in a mixture of stressed samples with a photodiode array (PDA) detector. In all stressed samples, the purity angle was within the purity threshold limit, indicating that the analyte peak was homogeneous. All the DPs and impurity peaks were well isolated from each other, and the method was observed to be specific. The peak purity of PCM is presented in figure 5.4.

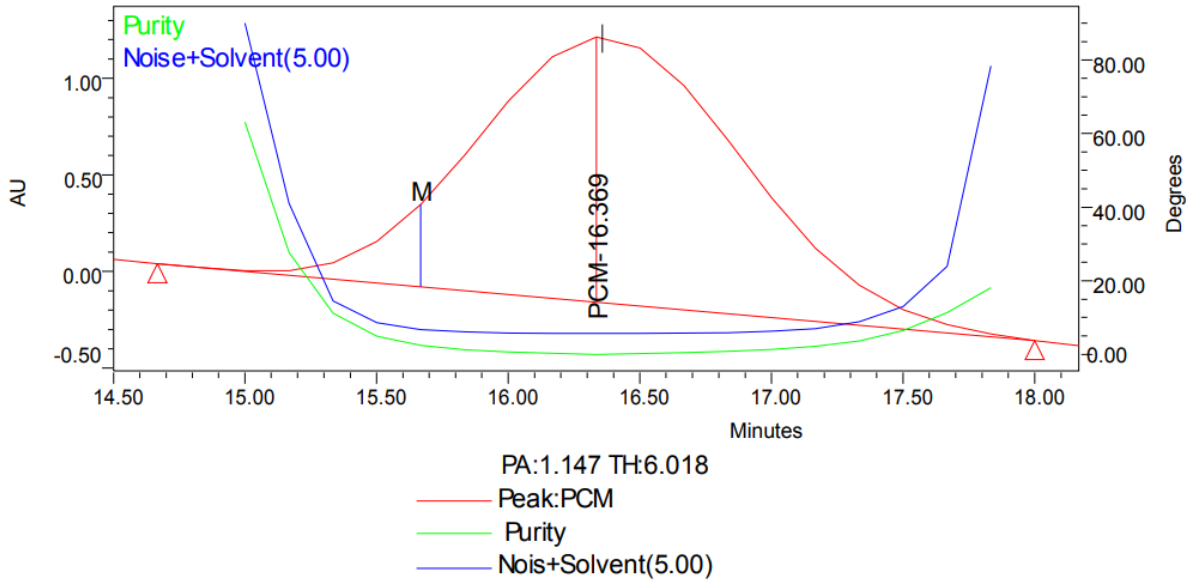


Figure 5.4. Peak purity plot of PCM

5.4.3.3 Accuracy

The accuracy of the analytical technique was assessed by spiking known levels of the impurities into a drug sample of PCM. It was performed in six replicates at three distinct concentration levels ranging from 50 to 150% of the specification level. The observed percent recoveries of the impurities (96.7–104.1%) and PCM (98.5–100.3%) were well within the acceptance criterion of 80–120%. The results are presented in table 5.6, which confirms the accuracy of the method determination.

Table 5.6 Results of accuracy

Amount spiked (%)	Recovery (%) Mean ± SD						
	PCM	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	Imp-F
50%	100.3 ± 0.85	96.7 ± 0.40	102.0 ± 0.40	99.8 ± 0.47	100.5 ± 1.27	100.4 ± 1.15	101.0 ± 1.21
100%	99.5 ± 0.45	97.9 ± 0.15	103.8 ± 0.65	100.5 ± 0.83	100.0 ± 0.60	99.8 ± 0.60	97.8 ± 0.75
150%	98.5 ± 0.96	100.3 ± 0.20	104.1 ± 0.25	102.2 ± 1.82	97.9 ± 1.65	101.0 ± 1.55	100.1 ± 0.75

5.4.3.4 Precision

The precision of the technique was expressed using the terms repeatability and intermediate precision. The repeatability of the proposed technique was illustrated by analyzing six different test samples of PCM (500 µg mL^{-1}) with each impurity spiked at the specification level. The % RSD for the area of each peak was estimated and was observed to be within 2.86%. Using separate analysts and instruments on two different days, the same experiment was repeated to assess the technique's intermediate precision. The %RSD for the area of each peak was estimated and was found to be within 1.17%. The outcomes of the precision study are depicted in table 5.7. These outcomes demonstrate the method's high level of precision.

5.4.3.5 Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ for all the impurities were evaluated by injecting a series of diluted solutions with known concentrations at corresponding signal-to-noise ratios (S/N) of 3:1 and 10:1. The LOD and LOQ values for impurities and PCM are reported in table 5.7. The chromatograms of LOD and LOQ are shown in figure 5.5.

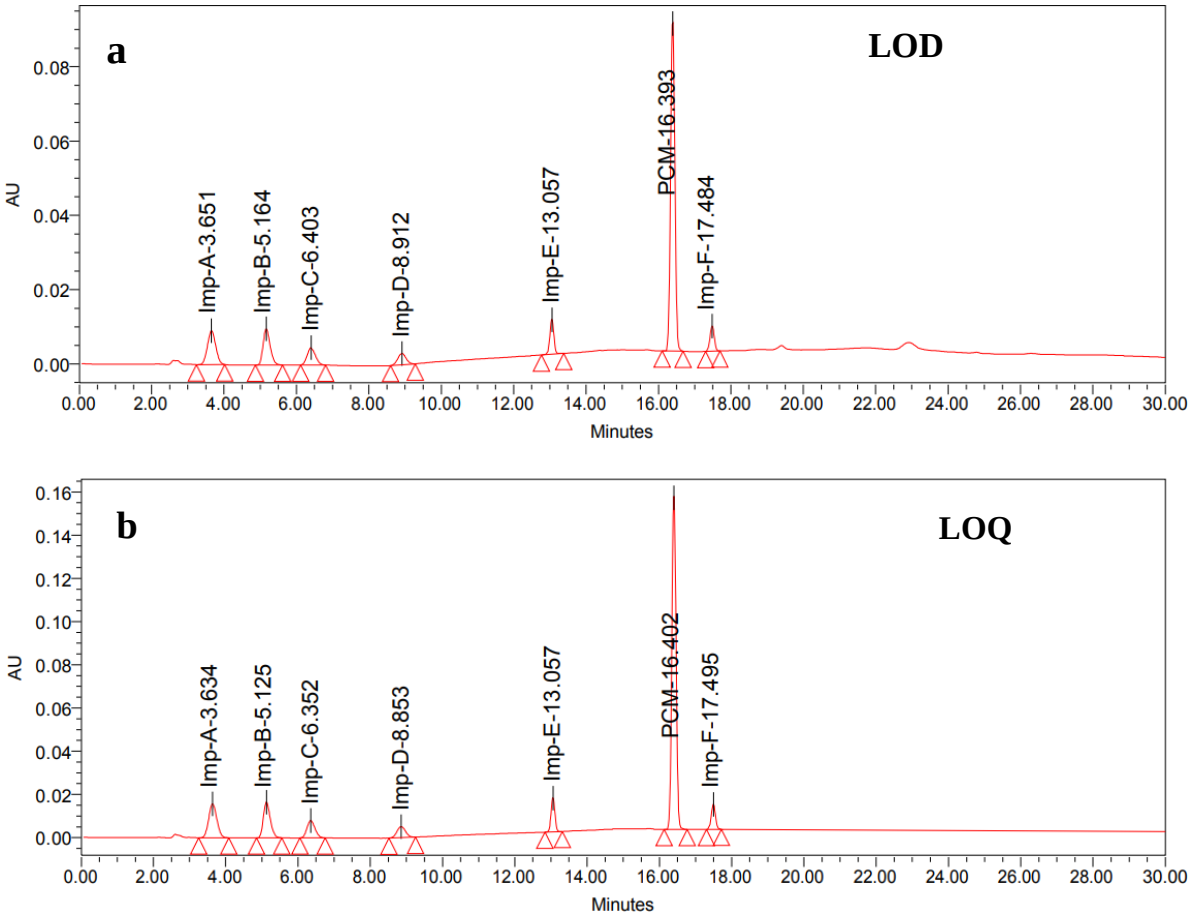


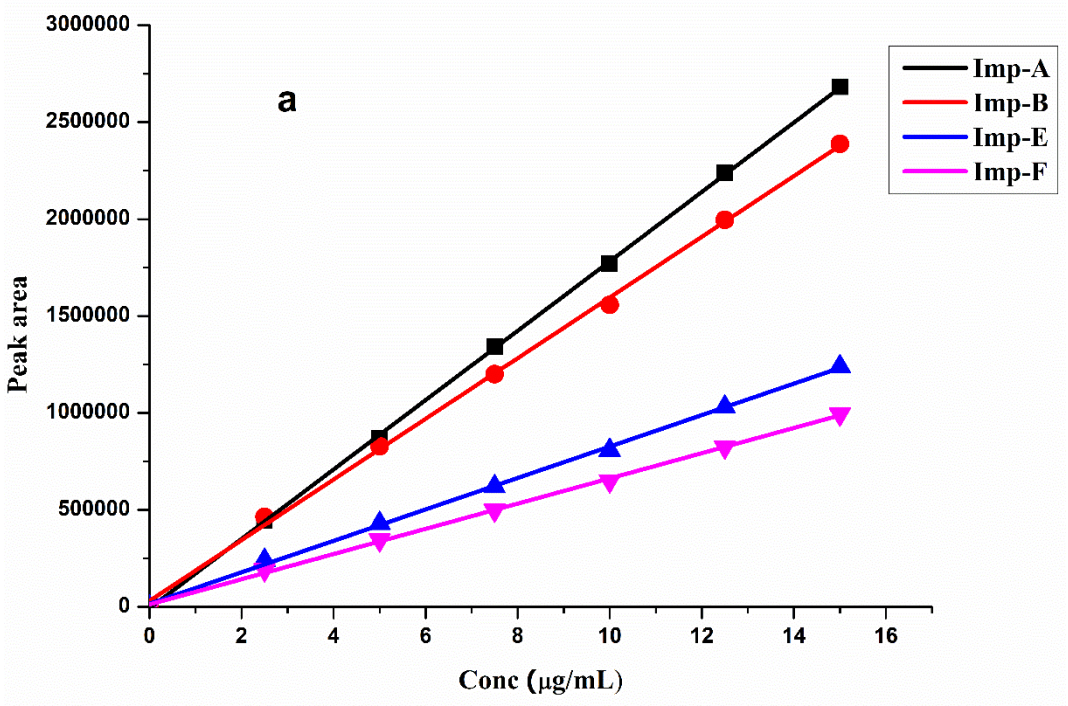
Figure 5.5. Typical HPLC chromatograms of PCM with its impurities at a) LOD and b) LOQ levels

Table 5.7 Results of regression and precision

Parameter	PCM	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	Imp-F
LOD (µg mL^{-1})	1.5	0.01	0.03	0.015	0.015	0.03	0.03
LOQ (µg mL^{-1})	5	0.1	0.1	0.05	0.05	0.1	0.1
Regression equation (*y*)							
Slope (*b*)	16999	178981	156556	175341	121966	81065	65012
Intercept (*a*)	245223.8	-7147.4	30836.6	19361.7	9956.8	16298.4	12531.3
Correlation coefficient	0.9994	0.9999	0.9994	0.9993	0.9995	0.9994	0.9995
Precision (%RSD)	0.81	0.31	0.75	0.46	0.16	0.28	2.86
Intermediate precision (%RSD)	0.81	1.15	1.17	0.24	0.46	0.32	0.17

5.4.3.6 Linearity

From the calibration curve, it was observed that there was good linearity in the concentration range of 125 to 750 µg mL^{-1} for PCM, 2.5 to 15.0 µg mL^{-1} for Imp-A, Imp-B, Imp-E, and Imp-F, and 1.25 to 7.5 µg mL^{-1} for Imp-C and Imp-D. The slope, intercept, and correlation coefficient values for all impurities and PCM were determined by least-squares linear regression analysis. The results of linearity were shown in table 5.7, and the calibration curves of impurities and PCM are presented in figure 5.6.



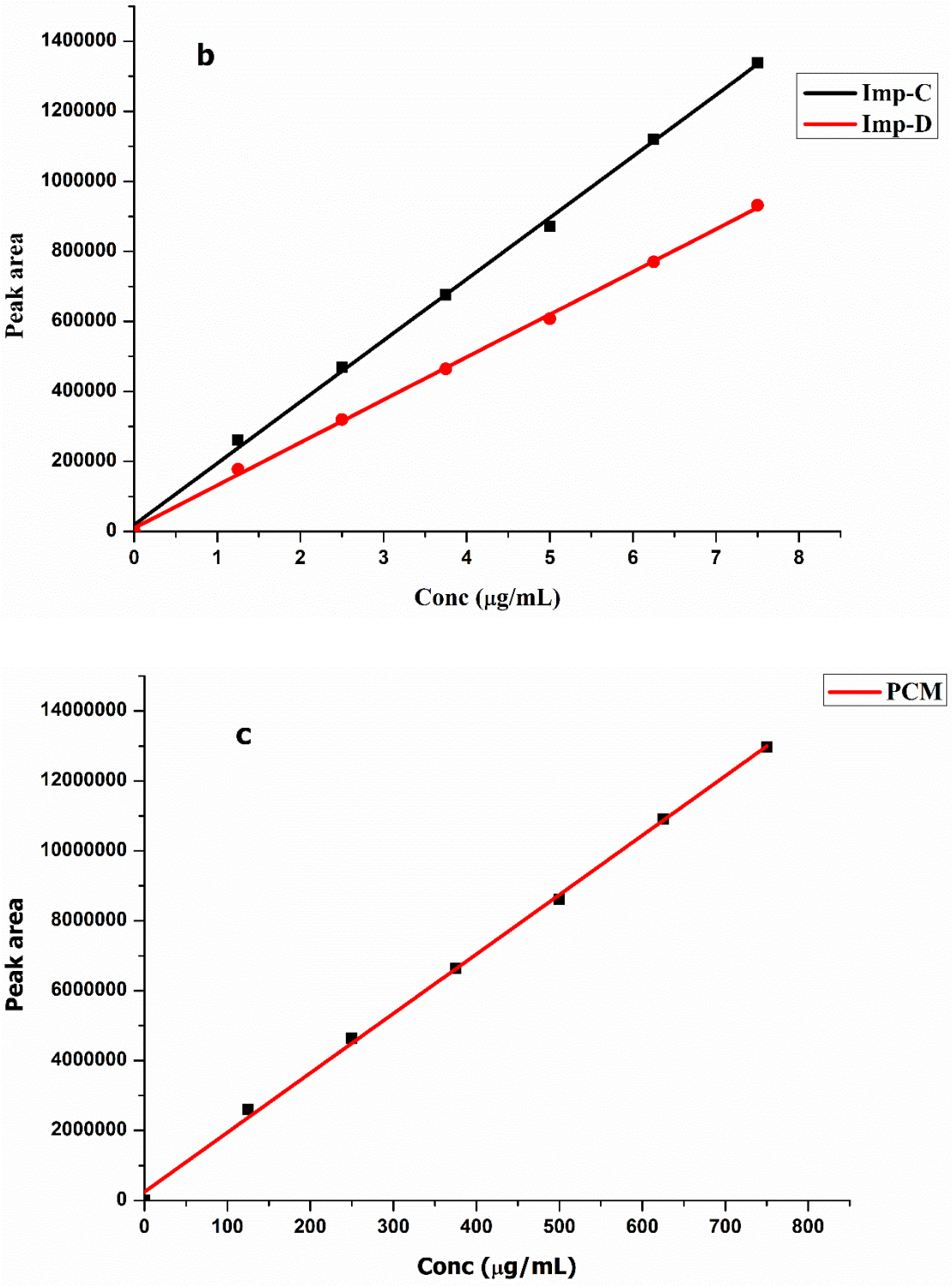


Figure 5.6. Calibration curves of a) Imp-A, Imp-B, Imp-E and Imp-F; b) Imp-C and Imp-D, and c) PCM

5.4.3.7 Robustness

A few deliberate modifications to the original HPLC technique conditions were performed in order to test the robustness of the developed HPLC method. By altering the flow rate to 1.2 and 0.8 mL min^{-1}, the impact of flow rate 1.0 mL min^{-1} on the resolution, tailing, and theoretical plates was studied. By changing the ratio of organic modifier (varying ± 10%) in the mobile phase from its original state, the effect of the ratio of organic modifier was studied. The pH value of the buffer solution was varying to ± 0.5 units, the effect of pH of buffer solution also investigated. For all altered chromatographic conditions, i.e., flow rate, organic modifier, and pH of buffer, the system suitability results, such as theoretical plates and tailing factor, and the resolution between analyte and impurities, were calculated. The robustness of the approach was established within the range of acceptable operational parameters for HPLC. The outcomes are depicted in table 5.8.

Table 5.8 Results of robustness study

Optimum value	Changed value	Plate count	USP tailing	USP resolution b/w Imp-E and PCM	USP resolution b/w PCM and Imp-F
1.0 mL min^{-1}	1.2 mL min^{-1}	91996	1.06	15.10	5.90
	0.8 mL min^{-1}	87840	1.05	15.60	4.53
pH-2.5	pH-3.0	91360	1.09	15.75	5.49
	pH-2.0	87565	1.05	15.39	4.12
0/20, 10/50, 20/70, 25/20, 30/20[a]	0/22, 10/55, 20/77, 25/22, 30/22[a]	78643	1.04	15.41	4.95
	0/18, 10/45, 20/63, 25/18, 30/18[a]	91562	1.03	16.59	6.69

[a](T(min)/% ACN)

5.4.3.8 Solution stability

The solution stability of PCM and its impurities was monitored at RT for 24 h and under cooling conditions of 2–8 ºC for 24 h, and the retention times and %RSD values of the peak areas of the sample solutions were calculated. Throughout the solution stability experiments, there were no appreciable changes in the impurities content. The results from these studies indicate that sample solutions were stable for at least 24 h.

5.5 Conclusion

A simple and rapid HPLC-PDA method was developed for the separation of impurities from PCM drug. All the degradation products and impurities were effectively isolated from the drug. As per the ICH guidelines, the developed approach was validated, and it was found to be specific, exact, accurate, linear, and robust. The developed method can be utilized for routine examination, stability studies, and quality control of bulk production and pharmaceutical formulations because it possesses the stability-indicating power.

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Chapter-VI

Summary and Conclusions

6.1 Summary

Chapter-I: The significance of analytical method development and validation in current pharmaceutical analysis during the drug development process was emphasised in the introduction. A brief description of the importance of identifying and quantifying relative and process impurities in drug molecules using different analytical techniques, including HPLC, LC-MS/MS, and other methods. Discussions were held regarding the requirement of forced degradation studies for newly discovered drug molecules and the evaluation of bioassay analyses using various analytical methods. It was described how to extract drug molecules from biological fluids using various extraction techniques. The identification and quantification of selected pharmaceutical drug molecules from biological fluids using chromatographic techniques were studied. The importance and instrumentation of the HPLC and LC-MS/MS techniques during the drug development process were briefly discussed. Additionally, it was described how the method is influenced by the stationary phase, mobile phase, diluent, flow rate, temperature, etc. The importance of method validation for analytical methods in accordance with USFDA and ICH guidelines was discussed. The objectives of current research on the development of analytical and bioanalytical methods for the detection, identification, and quantification of selected drug molecules and their degradation products were determined.

Chapter-II: In summary, the HPLC-PDA method for the determination of ELX and RFX in rat plasma has been developed and validated. Its application to pharmacokinetics has been demonstrated. The sensitivity and robustness of the approach were improved by optimizing the LLE extraction process and HPLC conditions. The developed method in the present study is a simple and cost-effective for liquid-liquid extraction for sample pre-treatment along with a reduced chromatography acquisition time, the separation of analytes was achieved with an isocratic mode using a mobile phase composition of acetonitrile and an aqueous solution of TEA, pH 2.5 (40:60, v/v). Finally, the achieved lower limit of quantification (5.0 ng mL^{-1} for ELX) and (10.0 ng mL^{-1} for RFX) for plasma determination. The approach was fully validated as per the USFDA guidelines for bioanalytical method validation in terms of accuracy, precision, and stability. According to validation parameters, the mentioned approach could be beneficial for the determination of ELX and RFX in rat plasma. This study demonstrates that LLE is an accurate and reliable method for preconcentration of ELX and RFX in rat plasma for analysis by HPLC-PDA. This method was also used to study the pharmacokinetics of ELX and RFX in rats. The developed method could be applied in bioanalytical and clinical research as well as routine analysis.

Chapter-III: The HPLC-PDA method was developed for the detection of BPA and EZE in rat plasma simultaneously and its application to pharmacokinetics has been demonstrated in this

chapter. The method employed sample preparation by liquid-liquid extraction with adequate recovery as well as chromatographic separation of the analytes with the advantage of speed and sensitivity. Using this method, sample pre-treatment was fast, plasma concentrations of BPA and EZE were detected in a 10 min run time, and analytes in samples were found to be stable during sample management, the chromatographic system, and the freezing time. In terms of accuracy, precision, and stability, the method was extensively validated to meet USFDA criteria for bioanalytical method validation. The validated method has been successfully used to analyze rat plasma samples for the application in pharmacokinetic studies.

Chapter-IV: In this chapter, an HPLC method was developed to monitor degradation products of CBE. The separation of CBE from its degradation products was studied on Symmetry C18 (150 x 4.6 mm, 3.5 µ) column and using a mobile phase combination of acetonitrile and 0.1% formic acid in aqueous solution (50:50, v/v) in isocratic elution mode. The optimal chromatographic conditions were 1.0 mL min^{-1} flow rate, 254 nm wavelength, and 10 min run time. As per the ICH guidelines, the DPs resulted due to stress conditions were separated from the drug and also for each. The stress samples were analyzed using optimized chromatographic conditions. The degradation behaviour of CBE was assessed under different stress conditions like hydrolysis, thermal, photolytic, and oxidation, respectively. The drug was subjected to hydrolytic degradation under acid stress conditions to form two DPs (DP-1 and DP-2), while DP-3 and DP-4 were formed under base and neutral stress conditions, respectively. Three DPs (DP-2, DP-5, and DP-6) were formed under thermal stress conditions, while the drug was stable under photolytic and oxidative stress conditions. The degradation products (DP-1 to DP-6) of CBE were identified and well characterized with the aid of QqLIT LC-MS/MS experiments and accurate mass measurements. Further, the *in silico* determined ADMET data and toxicity admetSAR software prediction tool indicated that the probability of CBE and its DPs can be hepatotoxic and genotoxic leading to severe toxicity. The developed method has stability-indicating power and can be used for routine analysis, stability studies in pharmaceutical quality control labs.

Chapter-V: A simple and rapid HPLC-PDA method was developed for the separation of impurities from PCM drug. Under different stress conditions, the way PCM degradation was studied, following the rules set by the International Conference on Harmonization (ICH). A realistic gradient reversed phase high-performance liquid chromatographic technique has been developed and validated for the determination of a stability-indicating of PCM. The impurities and all of the degradation products were well separated from the PCM drug. According to the ICH guidelines, the developed method was validated, and it was found to be specific, precise, accurate, linear, and robust. As a result, the established method has the potential to indicate

stability and can be utilized for regular analysis, stability studies, and quality control of pharmaceutical formulations and bulk manufacturing.

6.2 Conclusions

Overall, this thesis was about developing different chromatographic methods like HPLC-PDA, and LC-MS/MS to separate, measure, and identify selective drug molecules along with known impurities and degradation products. Also, different liquid-liquid extraction methods were made so that selective drug molecules could be extracted from biological fluids and used to analyze real samples. A summary of the developed analytical and bioanalytical methods for particular drugs and the findings of each method is presented in table 6.1.

The obtained results have clearly proved the efficacy and versatility of the established analytical and bioanalytical methods for the identification, quantification, and determination of drugs in biological samples as well as its degradation products. The developed liquid-liquid extraction approach may serve as a platform for evaluating drug molecules, environmental pollutants, and food sample analysis in the future. These newly established chromatographic techniques can be employed in pharmaceutical industries to analyze drugs on a regular basis.

Table 6.1 The developed analytical and bioanalytical methods for selected drugs and their results of each method

Drug	Extraction and detection method	LOD	LOQ	Linear Range	Sample analysis
Eluxadoline	Liquid-Liquid Extraction HPLC-PDA	1.0 ng min^{-1}	5.0 ng min^{-1}	5 -200 ng min^{-1}	Rat plasma
Rifaximin		2.0 ng min^{-1}	10.0 ng min^{-1}	10 – 400 ng min^{-1}	
Bempedoic acid	Liquid-Liquid extraction HPLC-PDA	0.6 ng min^{-1}	1.8 ng min^{-1}	1.8 – 36 ng min^{-1}	Rat plasma
Ezetimibe		0.03 ng min^{-1}	0.1 ng min^{-1}	0.1 – 2.0 ng min^{-1}	
Crisaborole	LC-MS/MS	0.07 µg min^{-1}	0.7 µg min^{-1}	0.7-140 µg min^{-1}	Forced degradation studies
Procainamide hydrochloride	HPLC-PDA	1.5 µg min^{-1}	5.0 µg min^{-1}	125 – 750 µg min^{-1}	Forced degradation studies

List of publications

Published

1. Validation of the HPLC-PDA method for detection of eluxadoline and rifaximin in rat plasma and application in a pharmacokinetic study.
   Vijendar Reddy Karla, Babji Palakeeti, M. Raghasudha and Raghu Chitta *Future J. Pharm. Sci.* **2022**, *8*.
2. Simultaneous determination of bempedoic acid and ezetimibe in rat plasma using HPLC-PDA and its applications to a pharmacokinetic study.
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