DEVELOPMENT OF HPLC AND LC-MS BASED ANALYTICAL METHODS FOR THE ANALYSIS OF ANTICANCER, ANTIEPILEPTIC AND ANTIHYPERTENSION DRUGS

THESIS SUBMITTED TO THE
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DOCTOR OF PHILOSOPHY
IN CHEMISTRY

BY

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CERTIFICATE

This is to certify that the research work presented in this thesis entitled **"Development of HPLC and LC-MS based analytical methods for the analysis of anticancer, antiepileptic and antihypertension drugs"** submitted by **Mr. Babji Palakeeti** for the award of the degree of **Doctor of Philosophy in Chemistry**, National Institute of Technology, Warangal (Telangana), under my guidance and supervision. This work has not been submitted earlier either in part or in full for any degree or diploma to this or any other university.

Warangal
April 19, 2021

Dr. Jugun Prakash Chinta
Supervisor

Prof. K. V. Gobi
Co-supervisor

DECLARATION

I hereby declare that the research work presented in this thesis entitled **"Development of HPLC and LC-MS based analytical methods for the analysis of anticancer, antiepileptic and antihypertension drugs"** has been carried out by me under the supervision of **Dr. Jugun Prakash Chinta** and Co-supervision of **Prof. K. V. Gobi**, Department of Chemistry, National Institute of Technology-Warangal. I declare that this work is original and has not been submitted in part or full, for any degree or diploma to this or any other university.

P. Babji

(Babji Palakeeti)

Warangal

April 19, 2021

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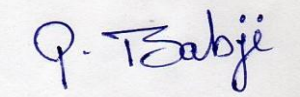


(Babji Palakeeti)

Dedicated to my Family & Teachers

LIST OF ABBREVIATIONS

λ Max	Absorbance maximum
ACN	Acetonitrile
AED	Antiepileptic drug
APCI	Atmospheric pressure chemical ionization
API	Active pharmaceutical ingredients
APPI	Atmospheric pressure photo ionization
BVC	Brivaracetam
CBZ	Carbamazepine
$CHCl_3$	Chloroform
CV	Cumulative variance
DAD	Diode array detector
DMF	Dimethylformamide
DoE	Designing of experiment
ESI	Electrospray ionization
EtOAC	Ethyl acetate
EtOH	Ethanol
FT-IR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GEM	Gemcitabine
GO	Graphene oxide
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
ICH	International conference on harmonization
IP	Indapamide
KOH	Potassium hydroxide
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection

LOQ	Limit of quantification
MeOH	Methanol
MGO	Magnetic graphene oxide
C[6]A	Calix[6]arene
CD	Cyclodextrin
MRM	Multiple/ selected reaction monitoring mode
MSP	Magnetic solid phase
NaOH	Sodium hydroxide
PD	Pharmacodynamics
PK	Pharmacokinetics
PP	Perindopril
QC	Quality control
RCB	Rucaparib
RP-LC	Reverse phase liquid chromatography
RRF	Relative response factor
RRt	Relative retention time
RSD	Related standard deviation
RT	Retention time
SIM	Selected ion monitoring mode
SPE	Solid phase extraction
SPE	Solid phase extraction
TBDE	Tertiary butyl dimethyl ether
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
US FDA	united States food and drug authority
UV	Ultra violet
VSM	Vibrational sample magnetometer
W	Tungsten

Contents

Chapter – I. Introduction of importance of LC and LC-MS method developments in drug discovery and development

Chapter –I
Introduction

1. 1. General Introduction

One of the best quotes of our ancestors is “Health is Wealth”. Everybody may agree with that because in the recent days several new diseases came into the lives of human beings in a variety of ways. In this context, human beings need better drugs to cure ailments. Although naturally available drugs has ability to cure those diseases, the lack of sufficient amounts of these natural drugs to meet the demand make us to move toward synthetic drugs. But the presence of synthetic and process-related impurities in the drugs during the time of the synthesis and purification processes may result in various side effects on human beings. Therefore, there is a need to estimate the impurity profile in active pharmaceutical ingredients (API) in pharmaceutical formulations by developing of stability-indicating methods. It’s very crucial to estimate the purity of the drugs and quantification of the related substances in API’s before going to formulation. Hence there is need to develop a prescribed analytical method for knowing the quantity of process and related impurities in synthesized drugs. The presence of the unwanted chemicals, even in small amount, may affect the efficacy and safety of the pharmaceutical drug products. The typical impurities in pharmaceutical products come through starting materials, critical reagents, intermediates and solvents used in the synthetic procedure that all remains as active pharmaceutical ingredients (API) [1-7]. In addition to these, some more impurities may form through the simultaneously side reactions during the synthesis of drug molecules. The short time presence of these unwanted impurities may decrease the safety and quality of the drug. The pharmaceutical industries needs to control the impurities from raw materials to final formulations [8,9]. In general, the known impurities should be controlled under 0.15% and unknown impurities controlled under 0.10% in the API. The pharmaceuticals labs need to develop good efficient analytical method for knowing the purity of drug and estimate the impurities in drugs. These developed methods is essential to do

systematic validation, because those are efficient to indented use. Thus analytical method development and validation in the modern pharmaceutical analysis is an important task.

1.1.1. Drug discovery

The process of new drug invention is extremely intricate, expensive and hazardous. The development process of a new drug rely on the number of disciplines for the improvement of efficiency and the reduction of led time in the drug discovery procedure. Typically, for the development of new drug and it's ready to use as medication will take at least 10-15 years [10]. For every 5 to 10 thousands of compounds developed in R&D labs for research purpose, only one or two compounds may get approval by regulatory bodies [11]. The idea of new drug invention may come from newly arrived ailments, market value and present necessities in the society. Once the target moieties are finalized for the new development, the pharmaceutical companies, research centers and academic institutions will make efforts on making the substances to the drug development. The schematic representation of drug discovery process was shown in Figure 1.1.

1.1.2. Targets of Drug Discovery and Development

- Perceiving the investigational drug success rates by phases
- Defining Pre-clinical studies
- Defining investigational new drug application - Phase-I, II, III and IV studies.
- Defining new drug application
- Defining phase - IV studies

1.1.3. Investigational Drug success

- Discovery / Screening : 5 - 10k
- Preclinical studies : 250
- Clinical testing : 5
- Approved by regulatory bodies : 1

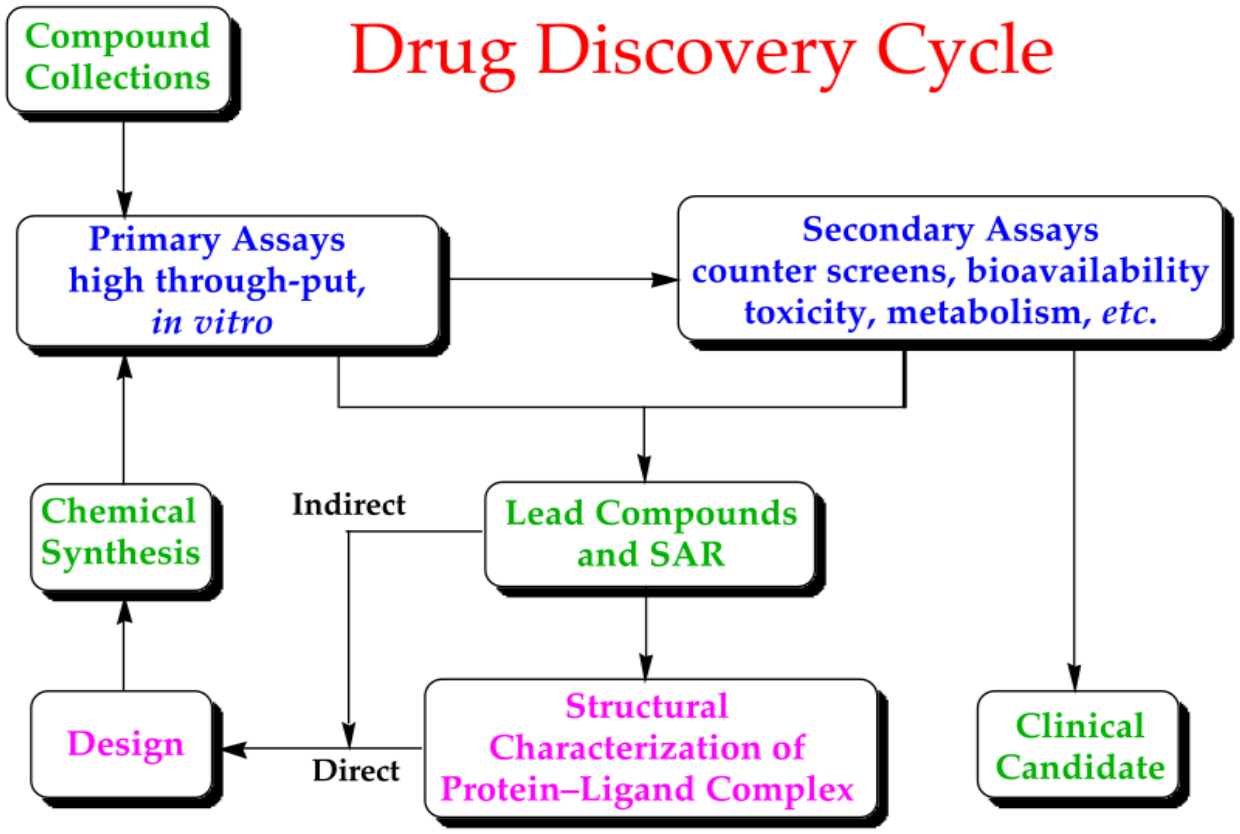


Figure 1.1 The schematic representation of drug discovery process.

During the drug development process, the researchers synthesizes thousands of drug related compounds. Once the promising drug target molecule has been prepared, bioassay analysis will be performed to understand the functioning of the compound such as it's absorption, distribution, metabolization and excretion (ADME) [12,13]. The successful drug must contain the following properties.

- Absorbed easily into the bloodstream
- Distribute the proper action on the particular site in the body
- Metabolize effectively and efficiently
- Excreted successfully from body
- Should be nontoxic

The pharmacokinetic properties (ADME) must be investigated by using different analytical tools like High performance liquid chromatography (HPLC), Gas chromatography (GC), NMR, IR, MS and hyphenated techniques like LC-MS, GC-MS,, LC-NMR, LC-IR etc.

1.2. Significance of ADME and Bio Analysis in new drug development

The absorption, distribution, metabolism and excretion (ADME) has lot of significance in new drug development and discovery (Figure 1.2). These studies are conducted thorough Insilco, in vivo and in vitro models. In vitro and in vivo models will give the ADME parameters, which are useful to know the drug behavior in patients and are most significant for the decision to hold, advance or terminate a drug candidate. However, the misunderstanding of ADME or incomplete ADME analysis may cause failure in drug development. In vitro models generate many ADME parameters, including metabolic stability, apparent permeability reaction, protein binding, phenotyping, blood to plasma partitioning, and drug-drug interaction potentials.

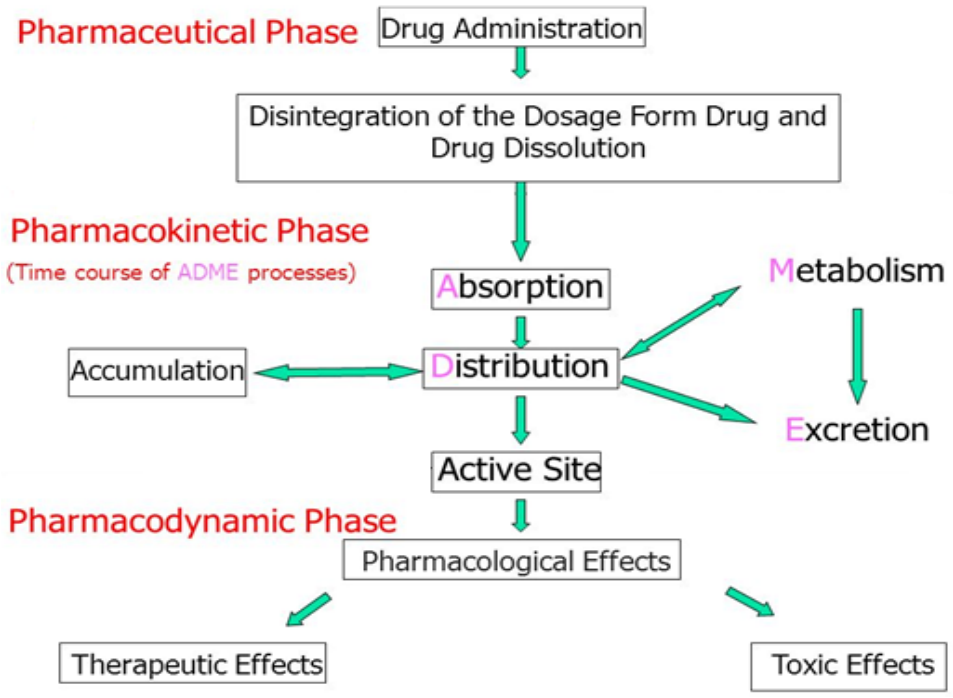


Figure 1.2 Schematic representation of the ADME process.

Bioanalytical methods plays a vital role during the lead optimization conditions. The main goal of the bio analysis is to assess the total parameters of the ADME in the new chemical entities (NCE) [41, 15]. A group of bioanalytical methods are needed to evaluate the total pharmacokinetic parameters in laboratory animals as well as in human beings.

1.2.1. Sample preparation

In bioanalytical methods, sample preparation methods plays a major role in drug discovery and drug development process. These are used for the assessment of drug absorption and disposition in physiological fluids such as urine, blood, plasma, serum and tissue of the drug administered lab test animals. Frequently, the concentration of the NCE levels in biological matrix decreases with time and falls to low level (up to nanogram level), hence the developed bioanalytical methods must be able to quantify at or below nanogram level. In biological sample analysis, stability issue and endogenous material interferences (example, plasma protein, tissues etc.) make the analysis critical to get accurate analytical results. Therefore, developed analytical methods for pharmacokinetic studies should be able to separate the analytes with high resolution from the biological matrix [16]. Typically, some common sample preparation methods needs to develop for removing the interference of the biological matrix from analytes such as Liquid-Liquid extraction, protein precipitation, and solid phase extraction etc.

1.2.2. Sample preparation techniques

Some commonly used sample preparation techniques are [17, 18]

- Liquid-liquid extraction
- Solid phase extraction
- Protein precipitation
- Solid phase micro extraction

- Column - switching
- Ultra filtration
- Drug conjugated hydrolysis
- Direct HPLC injection
- Dried plasma spots
- Dried blood spot technique
- Derivatization

The main sampling techniques are

1.2.3. Liquid-liquid extraction (LLE)

Liquid-liquid extraction (LLE) is one of the best technique for isolating the analytes from the mixture. The Liquid-Liquid extraction method procedure was shown in Figure 1.3.

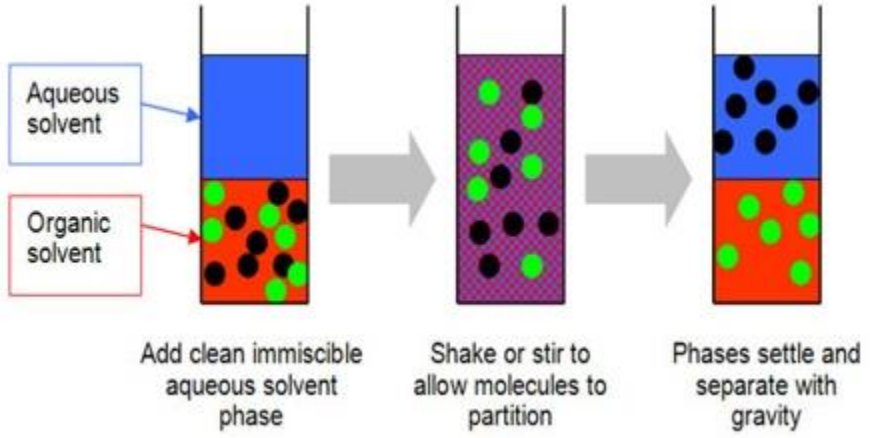


Figure 1.3 Liquid-Liquid extraction method procedure.

LLE operation procedure is very easy and get highly pure analytes from the mixture by using two immiscible solvents. The technique of LLE extract the analytes from the mixture based on partition deference of analytes between the two immiscible solvents. The partitioning of the analytes in the mixture of compounds depends on the selection of proper solvent [19]. In the LLE, the extraction solvents may need to be acidified, basified or low percentage of high polar solvents

are required to extract the metabolites and related compounds. Sometimes there is a need to repeat the extraction procedure for the maximum recovery of analytes. Recent days, several advanced techniques are invented instead of simple LLE methods such as support membrane extraction, liquid phase micro extraction and single drop liquid phase micro extraction.

1.2.4. Solid phase extraction

Solid phase extraction **(**SPE) method is typically used as a versatile technique for the extraction of analyte from the mixture of compounds. Many extraction methods rely on SPE because of lot of advantages such as high pre concentrations of the sample, easy to operate, rapid and selective. The SPE is a powerful technique for the enrichment, isolation, purification of trace level analytes in different type of matrices. The main objects of the SPE is to minimize the interference of matrix in the biological sample, decreasing the final sample volume and maximize the analytes sensitivity. Normally the interferences are rinsed with SPE and then the analytes are eluted by desorption with eluting solvent. The SPEs are available with different formats such as reverse phase, normal phase, restricted access media (RAM), ion exchange and molecular imprinted media [20-22]. The systematic diagram of SPE is shown in Figure 1.4.

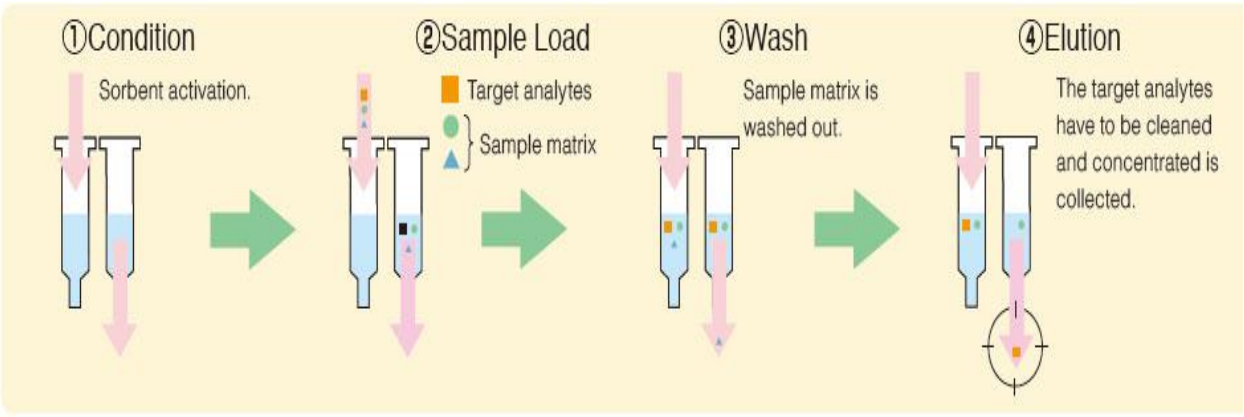


Figure 1.4 Solid phase extraction procedure.

1.3. The role of the chromatography in pharmaceutical analysis

From last two decades, the role of chromatographic techniques in pharmaceuticals have been improved. In chromatography, the analytes are separated from the mixture of compounds based on the deference of partition coefficients between two phases. In these one is mobile phase, it flows continuously through the stationary phase with certain pressure and constant flow rate. The second one is a stationary phase, which is useful for the separation of individual substances from the mixture due to the difference in partition, solubility, adsorption, molecular charge, ionic density and vapour pressure etc.

In the early stage of 19^{th} century, the Russian scientist M. Tswett known as father of chromatography extracted color bands on plants and separated the colors by running through the column, and this process named as 'Chromatography' [23,24].Chromatography is known to be the best analytical technique among other techniques because of high resolving capability of specific analytes. Different types of chromatographic methods can be developed by changing the mobile phase and miscible stationary solid phase. The development of these different chromatographic method needs proper selection of stationary phases, hence many types of stationary phases are available in the market.

1.3.1. Mechanism involved for the separation of analytes in liquid chromatography (LC)

Chromatographic methods are classified based on the following three parameters.

- Classification based on the physical state
- The contact between the stationary phase and mobile phase
- Separation mechanisms

The typical interaction mechanisms in LC are partition, adsorption, size exclusion, chiral interaction, gel permeation and ion exchange [25, 26]. In practice, the mixed mechanisms are

involved in LC separations. A concise explanation for separation mechanism in LC discussed underneath.

1.3.2. Normal-phase chromatography

Normal phase liquid chromatography (NPLC) generally used for the separation of non-polar compounds. In this NPLC method, the stationary phase is made up of polar material and mobile is non polar or moderate polar solutions. In general, the more polar compounds binds with polar stationary phases and non-polar compounds retains long time with non-polar stationary phases because polar to polar and non-polar to non- polar preferable interactions. In the NPLC, during the migration of mixture of compounds through the nonpolar mobile phase, the less polar compounds elute first followed by solutes of increasing polarity which moves slowly.

1.3.3. Reverse phase chromatography

Reverse phase liquid chromatography (RPLC) normally used for the separation of the different molecular weight / water soluble (polar) mixture of compounds. In general, the stationary phase was made up with non-polar material and mobile phase is of polar character in RPLC method. The solution mixture migrate through the non-polar stationary phase with polar mobile phase, in this method the polar compounds elute and detect first due to weak interactions with non-polar stationary phase. Most of the RPLC columns are made with chemically bonded octadecylsilane (ODS) with 18-n-alkyl and 8-n-alkyl carbon chain and sometimes phenyl and cyclohexane groups are used as alternatives.

1.3.4. Chiral chromatography

Chiral chromatography is used for the separation of the enantiomeric compounds. Enantiomers have same identical chemical structures but different chemical, physical properties and optically active. Due to their different chemical properties, the enantiomers must need to separate from the racemic mixture before going to formulations. Chiral chromatography has a chiral stationary

phase, it can be separate the enantiomers due to the formation of transient diastereomers. Some chiral columns are able to separate wide range of compounds, while others are useful for only specific enantiomers in pharmaceutical industries [27-34].

1.4. High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is the most important tool in pharmaceuticals, forensic labs, clinical research labs and environmental study labs etc. HPLC separates individual compounds from the mixture based on the adsorption, partition, ion exchange and chiral separation modes that depends on the nature of stationary phase and mobile phases [35]. The schematic diagram of HPLC is given in Figure 1.5.

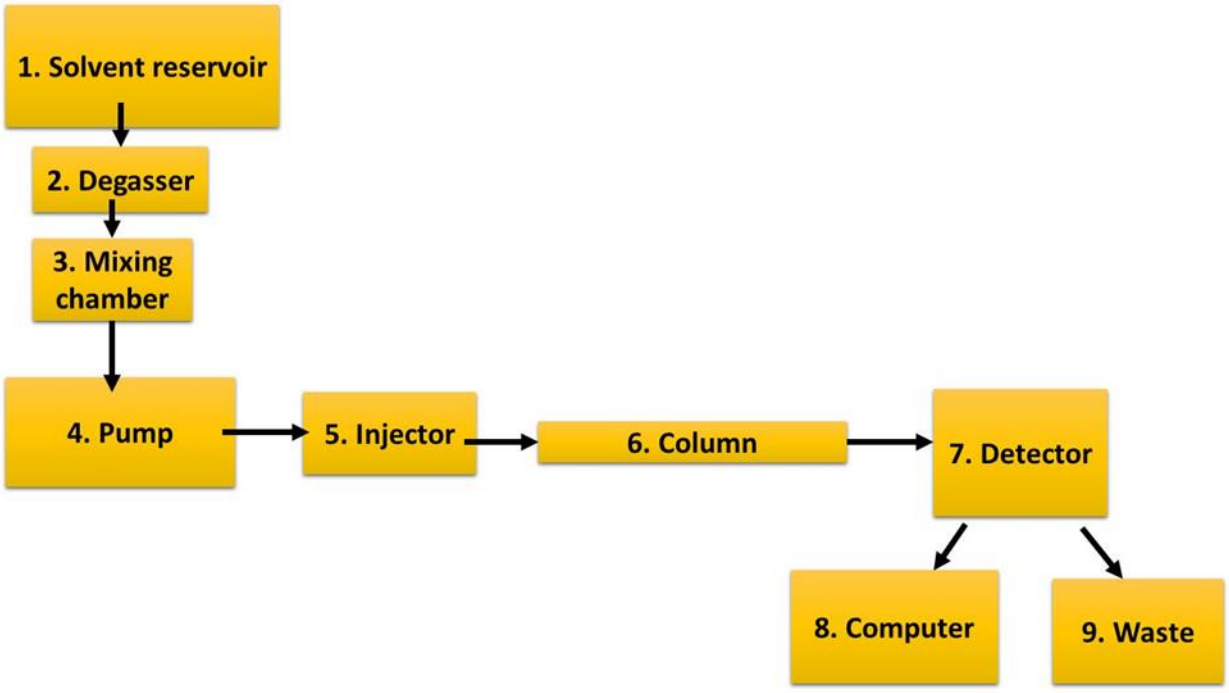


Figure 1.5 Simple schematic diagram of the HPLC components.

HPLC system contains pumps, column, detector, injector, column oven and mobile phase reservoirs. The injector is used for the injection the sample solution in to the HPLC system manually by using syringe or automatic sampler. Different pumps are used in HPLC such as

reciprocating pumps, constant pressure pumps and syringe type pumps which are used for pumping the mobile phase through the column with constant pressure. The column oven is used for maintaining the constant temperature from 5-40^{o}C. Mobile phase reservoirs are used to place the mobile phase solutions. The main part in the HPLC is column, it is made up of round stainless steel tube and filled with stationary phase materials which contains C_{18}, C_8, C_6, C_2, C_4 and C_{22} carbon compounds with functional groups of phenyl, cyano, amino etc. Its length varies from 10-30 cm and diameter from 3-5 mm. Column has retained stainless steel frits on both ends with the mess of ~2 µm and pore size of 60-150 Å. In general, guard columns are used in front of the main column because it helps in protecting the main column from the pressure fluctuations and stop small impurity particle entry in to the column and increases the life time of main columns.

1.4.1. Detectors

In HPLC, the main role of the detector is to identify the deferent types of eluting analytes in the form of peaks (chromatogram). The peak intensities in the chromatogram will depend on the concentration and nature of the samples. The detector position in HPLC is next to the column or stationary phase because it should detect the eluents coming from the column. The detector in the LC must have the ideal properties such as high sensitivity, lower dead volume, low baseline drift, wider linear dynamic range and operational simplicity to detect different compounds, low noise and reliability. Several detectors are available and the selection of detector will depend on the desired detection limits and the nature of the compound to be analyzed.

1.4.2. Ultra Violet Detector (UV detector)

UV detector can be used for the analytes that shows the absorbance maximum (λ_{max}) in the range of 200-400 nm. This would detect the analytes based on the particular absorption maximum of compounds. A number of UV detectors are available for example, fixed wavelength detector, it

measure the absorbance at fixed wavelength and the commonly used wavelength is 254nm. Different wavelength detectors are used to measure the single absorbance value at a time, but the same detector could also use for the wide range of wavelengths.

Diode array detector (DAD) is a widely used detector in LC, because it detects different wavelengths in both UV and Visible (200-800) regions simultaneously. It can detect the number of analyte in the solute with different absorbance wavelengths in between 200-800 nm. This DAD is useful in the estimation of the purity of eluted sample. The commonly used light sources of DAD is deuterium (D_2) and tungsten (W) lamps. The schematic representation of DAD shown in Figure 1.6.

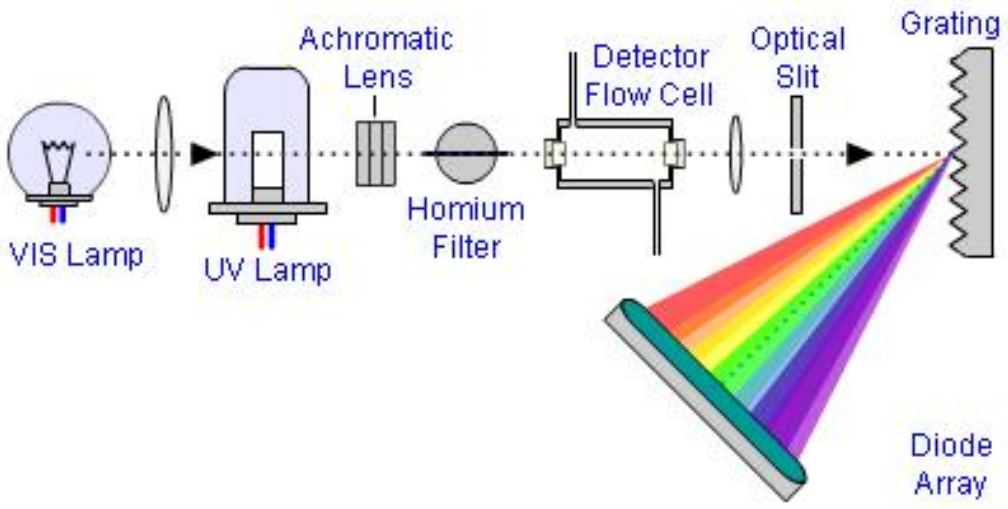


Figure 1.6 Schematic representation of diode array detector.

1.4.3. Refractive Index Detector (RID)

RID detector is useful for the detection of wide variety of analytes such as sugars, polymers, alcohols, fatty acids and carbohydrates. Hence, it is known as universal detector. It is effective and simple for the detection of UV inactive compounds. A limitation of RID is its temperature

dependence, low sensitivity and not useful for gradient programs. In complex mixtures, sample analytes may cover the extensive RI values, but some of those which are the near to the mobile phase RI values will not be visible to the detector.

1.4.4. Fluorescence Detector (FLD)

Fluorescence detector is specific and selective for the detection of analytes in the sample with fluorescence properties. FLD are 10-1000 times more sensitive than UV detector and analytes present in flow cell can be measured with this. FLD is highly sensitive compared with any other LC detectors.

1.4.5. Electrochemical Detector (ECD)

Electrochemical detector coupled with LC is a versatile technique for the detection of phenol compound in food samples, neurotransmitters and environmental assessments. The compounds with redox properties can be detected by using ECD. The main principle involved in this detector is the measurement of electrical signal generated due to the electron flow across the electrodes. The resultant current is directly proportional to the concentration of the analytes.

1.4.6. Evaporative Light Scattering Detector (ELSD)

The ELSD can be used for the detection of compounds which are not done by UV detector. In the ELSD, detection of analytes proceed by evaporating the mobile phase from the sample using a nebulizer, and the nonvolatile analytes are scanned by using the scattered light which is detected by photomultiplier in the detector. The ELSD depends on evaporating mobile phase from the sample by using a nebulizer. Therefore, the mobile phase must be of low boiling point (high volatile nature) compared with analytes. The response peak in the chromatogram depends on the mass of analyte [36, 37]. The schematic diagram of ESLD process is shown in Figure 1.7.

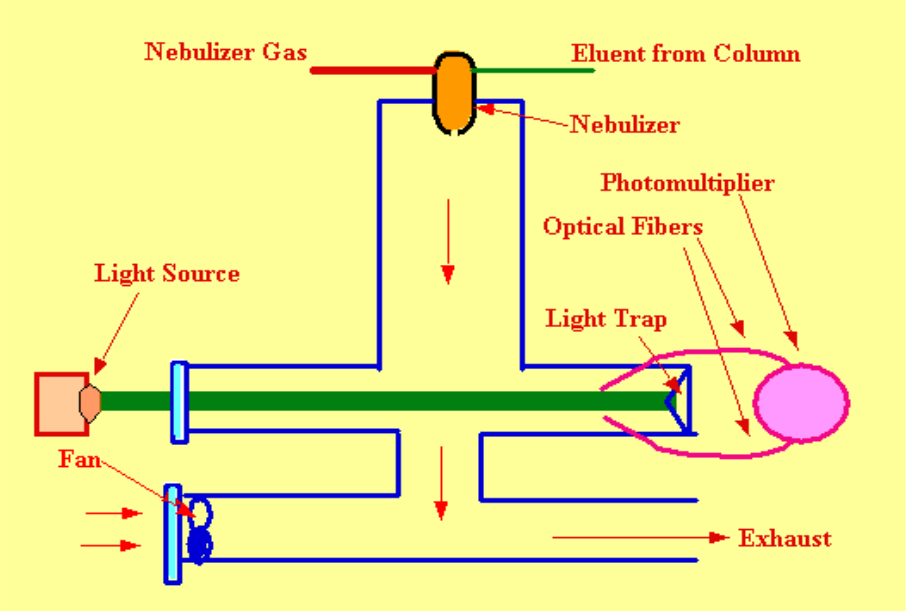


Figure 1.7 Schematic diagram of evaporative light scattering detector.

1.5. Use of hyphenated techniques in Characterizations:

In early days, the characterization of compounds and impurities were done by using conventional methods which are time consuming and insensitive. One such example is use of preparative column for the separation of degradation products which is time consuming, during this process some unstable DPs will be converted to new derivatives. All these problems can be resolved by using hyphenated techniques such as LC-MS, GC-MS, LC-NMR and LC-IR etc. LC-MS is made by connecting liquid chromatography with highly sensitive mass spectrometry. It is a versatile analytical tool for the identification, quantification and purification of compounds, impurities and degradation products at trace level. It is highly used in pharmaceutical industries because of high sensitivity. The major applications of the LC-MS in research and pharmaceutical analysis is the identification and quantification of drug metabolites, degradation products in pharmaceuticals and used in isolation and characterization of drug substances in natural synthetic procedure [38-40]. The major uses of these analytical tools in drug discovery and development are as follows,

- Identification and quantification of degradation products in the drug molecules and for the elucidation of degradation path ways
- For the purification and extraction of metabolisms of drug molecules in biological samples such as blood, urine and tissue by using various extraction methods
- In the assessment of pharmacodynamics / pharmacokinetics (PD/PK) parameters
- For the identification of enantiomeric purity and development of impurity profiling in APIs
- And development of new methods for the chemical substances.

1.5.1. Liquid Chromatography-Mass Spectrometry (LC - MS)

LC-MS is a hyphenated technique in which HPLC is coupled with highly sensitive mass spectrometer. It is highly sensitive and specific when compared with normal LC. In this, the HPLC separates the analytes and introduces in to MS that converts the analytes in to charged particles which are identified and quantified. It is also useful for the structural elucidation and probable molecular formula from the obtained molecular weights of the specific compounds. The schematic diagram of LC-MS detection technique is shown in Figure 1.8.

1.5.2. Instrumentation of Mass Spectrometry

The mass spectrometry is used for analyzing the molecules by the converting them to the charged molecular ions and the formed fragment ions by the using of m/z ratio. Various types of ionization techniques and fragment ion analyzers are available. In practice, LC-MS has different combinations of various ionization sources and mass analyzers in clinical research laboratories. All these combinations are useful for various purposes in clinical laboratories.

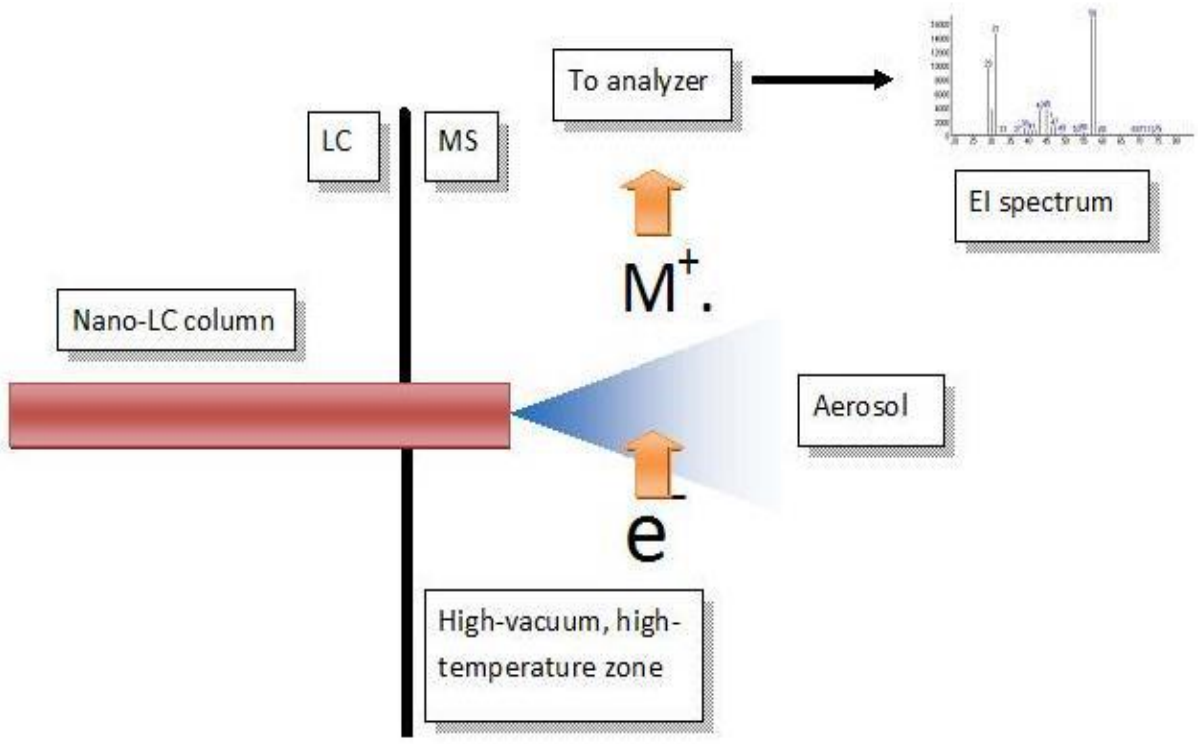


Figure 1.8 Schematic diagram of LC-MS detection technique.

1.5.3. Ion Sources

1.5.3.1 Electrospray Ionization (ESI)

ESI technique is most useful for compounds with moderate polarity and also best suitable for analyzing the metabolites. In this ionization technique, the liquid flow from LC is entered in to a high voltage or heating chamber through a metal capillary. The liquid sample solution is then evaporated to aerosol droplets by the high electrical voltage or heated nitrogen gas flow at atmospheric pressure. The vaporized droplets enters in to a vacuum chamber with the help of small capillary nebulizer. The formed droplets are now de-solvated in this chamber with the help of vacuum and heat, then accelerated in to a gas by applying voltage. In general, the large molecules have one or more charged sites with positive or negative signs, hence the mass of the molecules will be detected based on m/z ratio. The schematic representation of ESI is shown Figure 1.9.

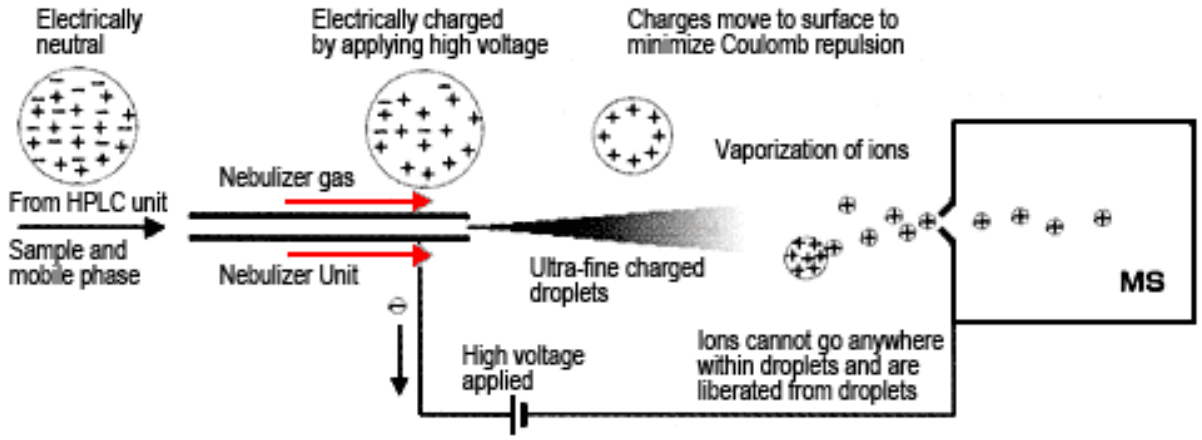


Figure 1.9 Schematic representation of electron spray ionization.

1.5.3.2. Atmospheric Pressure chemical ionization (APCI)

APCI technique is based on two steps, one is the evaporation and de-solvation of analytes and other one is the generation of the charged analyte molecules at vapour phase by using charge transfer reaction. In this APCI technique, solvent containing liquid analytes are nebulized and transferred to large chamber through narrow capillary nebulizer. The schematic representation of APCI shown Figure 1.10.

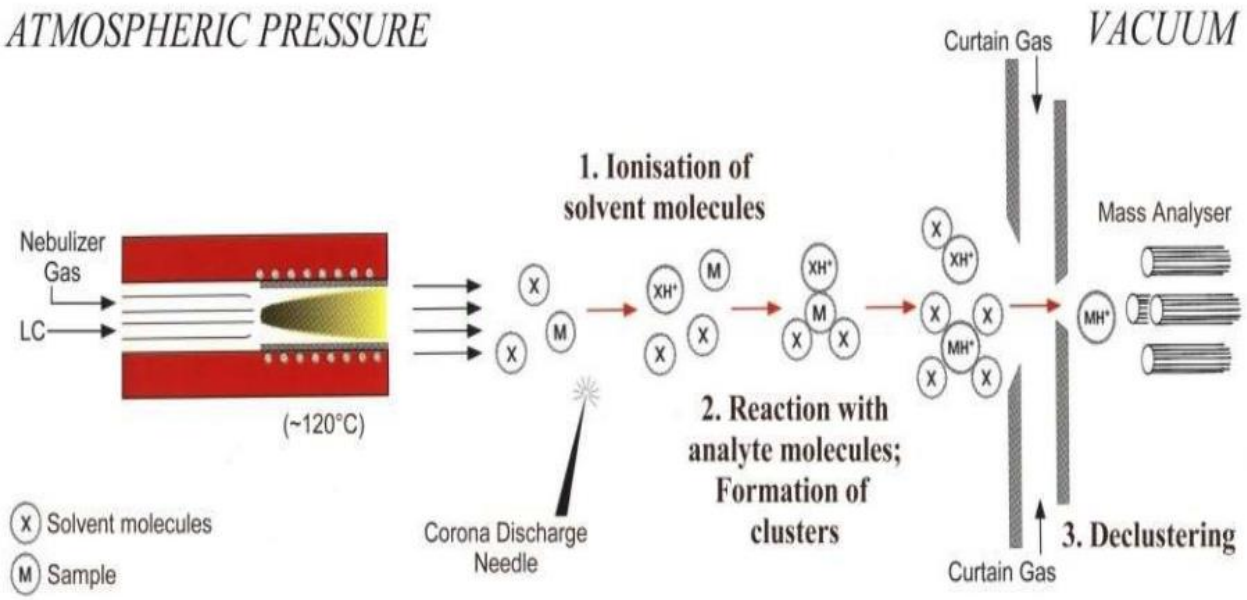


Figure 1.10 Schematic representation of atmospheric pressure chemical ionization.

Then the small droplets will be generated in this heating chamber by evaporating the solvent at the atmospheric pressure. The formed small droplets will be converted in to the ions with the help of ionizing gas at 250 to 400^{o} C. The ions are becoming charged analyte molecules due to chemical ionization reactions and are allowed to enter in to mass analyzer through capillary vent. This technique is widely used for moderate polar, non-polar and small analyte molecules.

1.5.3.3. Atmospheric Pressure Photo Ionization (APPI)

In APPI technique, the analyte molecules are excited and ionized by using the photons. Similar to APCI, APPI also involves vaporization of the liquid eluents to gaseous phase by using heated nitrogen gas. In this technique, Krypton (Kr) lamp is used for generating high energy photons which converts gaseous analyte molecule to excited and ionized molecules. The energy of photons should be controlled to decrease the ionization of analytes. The resulting ionized molecules will be transferred to the mass analyzer by the capillary orifice. APPI is suitable for analyzing of non-polar compound and is the one of the complicated ionization technique compared with ESI and APCI.

1.5.4. Mass Analyzers

After the ionization of molecular ions, the ions are entered into mass analyzer and are separated based on the m/z ratios. The commonly used mass analyzer will depends on its time, speed, rate and its reaction.

1.5.4.1. Quadrupole Mass Analyzer

It is one of the most commonly used technique due to its high sensitivity. It contains two plain rods arranged in parallel to one another in between the detector and ion source. In this, the ions are separated according to the m/z ratio with respect to space or time. The linear quadrupole mass analyzer contains the four cylindrical or hyperbolic rods which are placed in radial manner. The

positive (+ve) or negative (-ve) charges are imposed on opposite rods by the using of direct current (DC) at which oscillating radio frequency (RF) and alternate current voltage is superimposed. By using these AC and RF currents, the rods can generate the electromagnetic field. The generated electromagnetic field filters the ions based on m/z ratio. The stable ions are transported to detector and remaining unstable ions are discharged on the rods.

LC-MS triple quadrupole system has three quadrupole units (Q_1, Q_2 and Q_3) arranged in linear manner. Among these three, the first one (Q_1) is scans parent ions selectively, then second quadrupole (Q_2) filters the ions and allows active ions to Q_3 and the remaining ions are discharged. The third quadrupole (Q_3) transports the highly intense daughter ions to the detector. The schematic diagram of triple quadrupole mass analyzer are shown in Figure 1.11.

Quadrupole mass analyzer can detect the analyte molecules in different modes

- **Scanning mode (Scan):** The mass analyzer scans the analyte molecules in a range of m/z ratio.
- **Selected Ion Monitoring Mode (SIM):** It is highly sensitive when compared with scan mode, because SIM mode avoids the effect of impurities and unwanted analytes. In SIM mode, only selected ions are monitored and send through the quadrupole and detected.
- **Multiple/ Selected Reaction Monitoring mode (MRM):** The mass analyzers that consists of more than one quadruples is only applicable to MRM mode. In this mode, the user can selectively transport particular ions from the first mass spectrometer to second mass spectrometer. In the second mass spectrometer, the selected analyte ions will be fragmented like precursor ions and selected for detection.

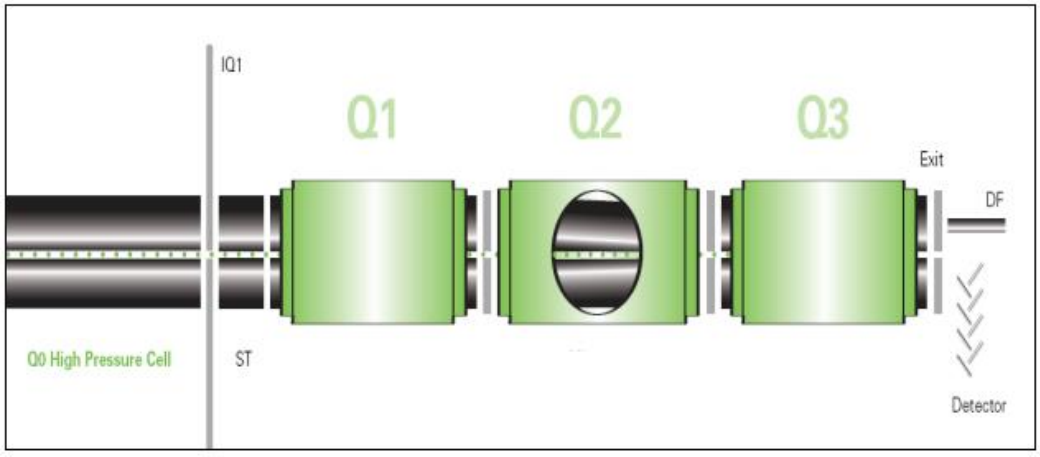


Figure 1.11 Schematic diagram of triple quadrupole mass analyzer.

1.5.4.2. Time of Flight Analyzer (TOF)

The TOF is a most robust analyzer used for different inlet systems and sources. This is a simple electrostatic and straight forward technique when compared with quadruple, where there is no magnetic fields maintenance is required. The ions coming from various ion sources will be entered in to a vacuum chamber with an electrostatic accelerations. The ions are then moving different distances with time in the TOF based on their mass to charge ratio. The travelling time taken by the single charged ion to reach the detector is directly proportional to the mass of ion. Therefore, lighter ions reach the detector first and detected. In this analyzer, scanning and detection of ions can be done simultaneously and it is useful for the detection of the large m/z ratios also.

1.6. Applications of LC-MS / MS

- Identification of degradation products
- Identification of metabolites
- The analysis of biological samples
- Quantification of analytes in biological matrix
- Selective identification of compounds in complex mixture
- Peptide mapping etc.

1.6.1. Use of LC-MS in Forced degradation studies

1.6.2. Forced Degradation studies

The pharmaceutical industry needs to submit the stability studies of the related API to the regulatory agencies if they want to register a new drug in the form of active pharmaceutical ingredient (API). The stability report in general contains structures and their quantification details of degradation products formed during storage as per the International Conference on Harmonization (ICH) guidelines. The stability studies are critical due to the formation of tiny quantities of degradation products, intermingling with API and its related substances. The forced degradation studies also called stress studies has been used to know the stability of drug products. These studies were done by using the external physical and chemical stress conditions on the APIs. The formed degradation products will be assessed and compared with the obtained degradants in standard storage conditions. The large quantities of degradation products will be isolated and characterized, these will be useful for the elucidation of degradation path ways and better understanding the chemistry of degradation of API's. Forced degradation studies are most useful in formulations for understanding the drug excipient interactions, and in the manufacturing and packing. Hence, the degradation studies play an important role in pharmaceutical industry. The stress studies are done by the following below mentioned ICH guidelines.

- ICH Q_1 A: The testing of stability studies of new drug substances and its related products.
- ICH Q_1 B: The photo stability studies of new drug substances and its related products.
- ICH Q_2 B: The validation parameters of developed analytical method.

1.6.3. Various Stress Conditions for forced degradation studies

1.6.3.1. Hydrolysis:

In hydrolysis, the drug product subjected to reaction with water at different pH and it ionizes the susceptible groups that are present in drug substances. For maintain acidic conditions, normally

hydrochloric acid (HCl) or sulfuric acid (H_2SO_4) and for basic medium sodium hydroxide (NaOH) or potassium hydroxide (KOH) with concentration range of 0.01 to 1N are used. This reaction cannot be more than seven days and after completion of the reaction time intervals the solutions are to be neutralized then the samples should be analyzed as soon as possible.

1.6.3.2. Oxidation:

In general for oxidation degradation studies, hydrogen peroxide (H_2O_2) or Azobisisobutyronitrile (AIBN) (0.1 to 10%) are used at room temperature. The concentration of oxidizing agent will chose based on the drug structure and the mechanism involved in this degradation studies is either electron transfer or oxidation of drug molecules.

1.6.3.3. Thermal Degradation:

In this thermal degradation studies, the solid drug substance will be exposed to the heat with temperatures ranging from 40-80^oC at dry or wet conditions. But in the case of liquid sample only dry conditions are used.

1.6.3.4. Humidity:

Humidity also plays a major role in the degradation of the drug substance and finished products. In this study, the drug product will be exposed to minimum seven days with 90% humidity.

1.6.3.5. Photolytic Degradation:

Photolytic degradation studies will produce the primary degradation products of the drugs. As per ICH guidelines, most commonly used wavelength is 1.2 million lux hours and 2 Wh/m^2 and should not be more than 6 million lux hours. In photolytic degradation, the photo sensitive groups such as n-oxide, O-H, weak C-H bonds, chlorides and sulfides etc. will results in radical formation.

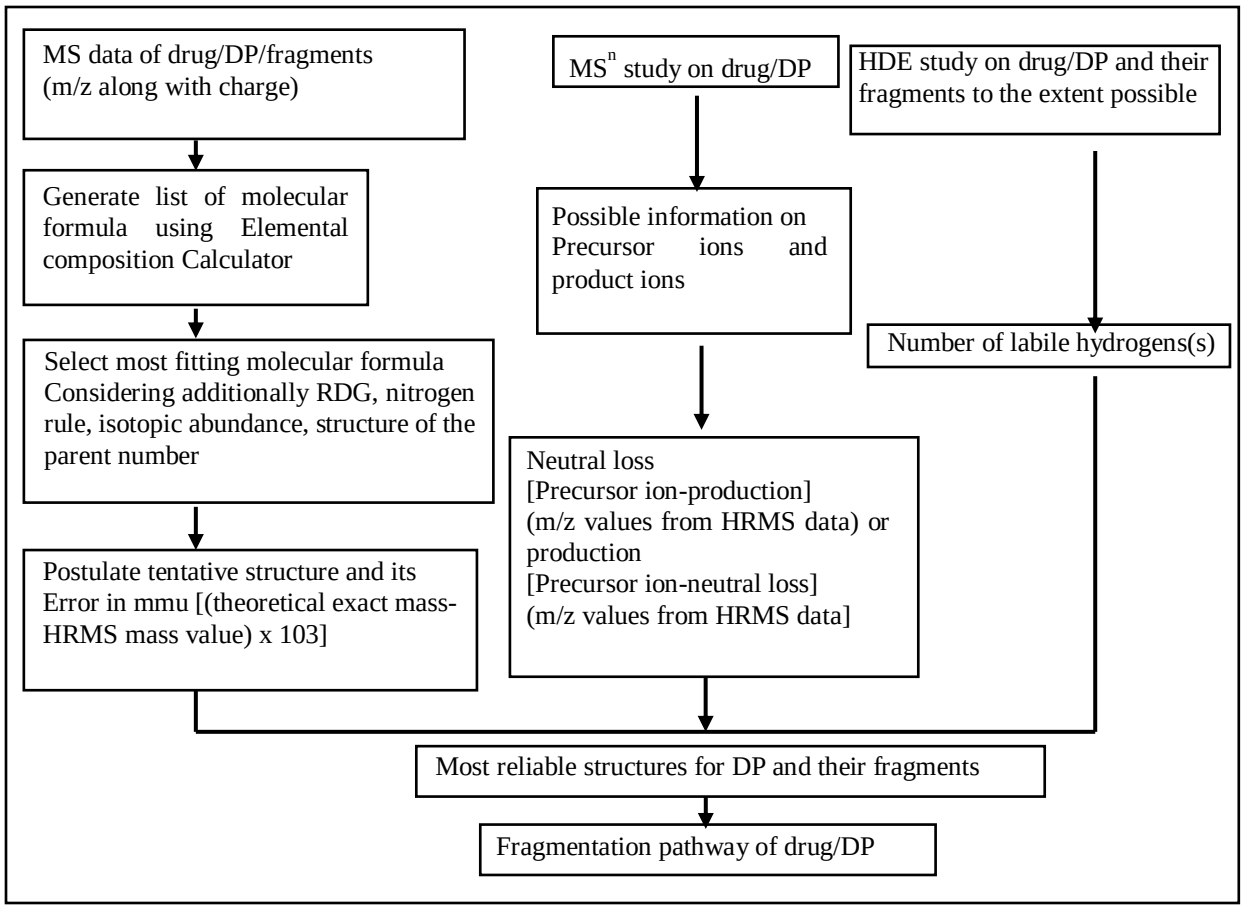


Figure 1.12 Use of MS data in in the forced degradation studies.

1.7. Process of LC Method Development

Generally the following steps are involved in the conventional new HPLC method development procedure [41, 42].

- Literature survey of drug molecules
- Chemical structure
- Selection of diluent
- Stationary phase (column) selection
- Mobile phase selection
- Selection of detector
- HPLC instrument parameters

1.7.1. Literature survey of drug molecules

For the development of new HPLC method for newly developed drug molecules, one need to know the physio chemical properties, reported degradation pathways, reported impurities and extraction method from biological samples etc. of the drug. All these information mostly available from chromatography journals and different pharmacopeias.

1.7.2. Chemical structure

The finalized structures of drug molecules, related and process impurities and degradation products will be known from synthetic schemes and degradation studies. These structure will give functional group information that are present in the drug molecules. It helps in finalizing suitable HPLC method for the elution of analytes.

1.7.3. Diluent selection

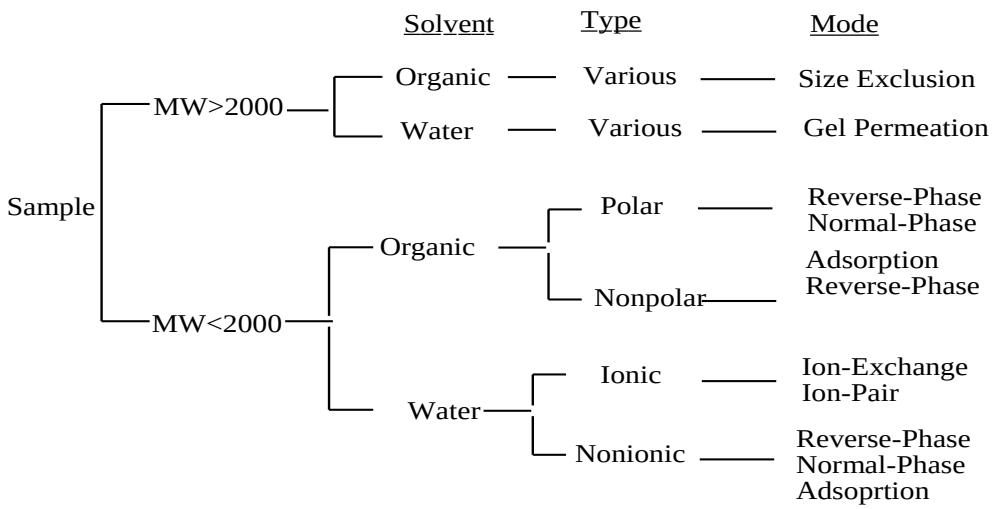


Figure 1.13 Selection of chromatography mode using the solubility.

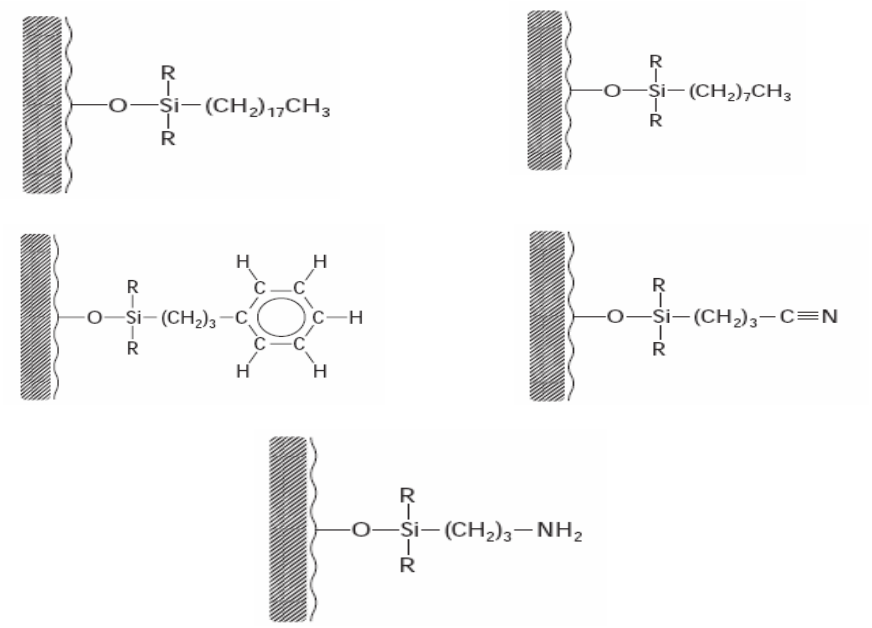
The selected diluent solution must have the ability to dissolve the drug molecules and its related components. The selected diluent should be able to dissolve completely the drug substances without undiluted components and precipitation. It must be companionable with obtaining Gaussian peak shapes of the analytes. If mobile phase is used as diluent it aids in controlling the abating unwanted peaks at blank solution in gradient eluting program.

1.7.4. Stationary phase selection

The column selection depends on the polarity of the analytes. If the analytes is nonpolar the stationary phase must be a polar and if analytes is polar the stationary phase must be a nonpolar. These phenomenon are called normal and reverse phase methods respectively.

1.7. 5. Reverse phase columns

Depending on the polarity of analytes the stationary phase is selected. Various types of reverse phase columns are available with different functional groups (cyano (CN), amino ($-NH_2$) and phenyl (C_6H_6)), with different carbon chains (C_8, C_{18}) and cross linked Si-OH groups [43]. The following Figure 1.14 shows variation of polarity in carbon chains that are linked with Si-OH.



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

$C_{18} < C_8 < C_6 / C_4 <$ Phenyl < Amino < Cyano < Silica

Figure 1.14 Variation of polarity in carbon chains that are linked with Si-OH.

The column stability depends on pH of the mobile phase and is limited to the pH range from 2-8. But now a days highly stable columns are available which are stable in the range from 1.0 to 11 pH.

1.7.6. Normal phase columns

Normal phase columns are made with polar stationary phase that contains cyano and silanol functional groups. These are used for the separation of the positional isomers in the anlytes. For normal phase columns mobile phase must be a non polar.

1.7.7. Chiral columns

Typically, chiral columns are used in pharmaceuticals to seperate the recemic mixtures of drug molecules [44]. The enentiomers are seperated in chiraral columns due to the formation of reversible tranisient diasteriomers with chiral satationary phase (CPS). The formed diastereiomers bonds with CPS show different free energy interactions and stability. There is no single CPS is available for the separation of enantiomers, hence column selection is critical in the enantiomers separations.

1.7.8. Detector selection

In pharmaceuticals, drug molecules and its related compound mostly UV active due to the presence of chromophores. Therefore, for method development in pharmaceuticals UV and PDA detectors are preferred in the initial stages.

1.7.9. Mobile phase selection

In reverse phase method, mobile phase is polar and stationary phase is in non-polar. The polar nature of the mobile make less hydrophobic analytes elute first with low retention time on stationary phase and highly hydrophilic analytes are eluted next with high retention time. If mobile phase pH is increased, the acidic analytes gets ionized due to the loss of proton, and if the mobile phase pH is decreased, basic compounds are ionized due to the gaining of proton from mobile phase. Hence, for the separation of acidic or basic analytes change in the pH of mobile phase is required and this will be done by using the appropriate buffer solution. In

reverse phase method, mobile phase is prepared along with buffer and organic modifier. Commonly used organic modifiers are methanol (MeOH) and acetonitrile (ACN). The organic modifier acetonitrile helps in lowering of mobile phase viscosity and it helps in lowering the UV cut off than other organic modifiers such as Methanol, isopropyl alcohol, tetrahydrofuran etc. The commonly used solvents in normal phase is n-heptane, n- hexane, 1,4 dioxane and isopropanol etc.

1.8. Method validation

For all the developed analytical methods, method validation needs to be performed. It is a procedure to ascertain the suitability and intended use of method by generating a systematic documental confirmations. For the effective validation for analytical methods, the ICH laid down some specific guidelines [45, 46]. Some published reports are also useful in ensuring the validation parameters effectively and in logical manner by using available laboratory resources. The following parameters should be evaluated for the validation of the analytical process as guided by regulatory authorities [47-49].

1.8.1. Specificity

In method validation process, the parameter specificity is the ability to quantify with selectively and accurately the target analyte existing in the sample mixture. It considers the level of interference from other excipients, degradation products and impurities etc. And also specificity ensures that a peak response is due to a single analyte and it shows overlap with another. In general, specificity is measured as tailing factor, resolution and plate number of the particular analyte. Specificity measures the quantity and identity of the main analyte in the assay or related impurity methods in HPLC. If method is specific, it gives the adequate resolution and peak purity of analyte in-between the other components.

1.8.2. Precision

In analytical method, precision is well-defined intimacy of agreement between the test results from repetitive analysis of the homogeneous sample solution. In general precision is determined in three different ways repeatability, reproducibility and inter day precision.

1.8.3. Repeatability

Repeatability also called intraday precision and it determined the capability of method for generating the similar results with small intervals of time in the same day and same conditions same laboratory. Typically, for the assessment of precision at least six to nine determinations at 100% test concentrations are to be made. After completion of the analysis, the results are summarized and related standard deviation (RSD) are calculated.

1.8.4. Reproducibility

Reproducibility determines the precision among the measurement results of method in different laboratories. It is useful for method standardization and for usage in various laboratories, and it is not required when used in the single laboratory.

1.8.5. Intermediate precision

Generally, intermediate precision is useful to determine the small variations in agreements of the results when performed with different analysts, different times and equipments. This examines the individual variables of the results and the obtained large variations are shown in standard deviation (SD) results.

1.8.6. Accuracy

Accuracy determine the closeness or exactness of method value from the sample with the accepted reference value or true value. Accuracy can be measured in assay analysis of drugs through the percentage of recovery. In general, the accuracy of drug assessed by the assay

results of drug compared with the reference standard value. For the assay of drug or impurities, the accuracy is established by spiking the known amounts of components. According to regulatory methods, for evaluation of accuracy at least three level concentration solutions must be prepared in triplicates and analyzed. The percentage of recovery will depends on the quantity of sample added and recovered.

1.8.7. Sensitivity

The method sensitivity can be assessed by the demonstration of limit of detection (LOD) and limit of quantification (LOQ).

1.8.8. Limit of detection (LOD)

The method LOD defines the lowest concentration of analyte that can be detected by the developing method and it is not necessary to quantify at that level. Typically, it can be measured by the visual examination or signal to noise (S/N) ratio or by using the standard deviation value of the response with slope of the liner plot.

Visual examination can be done by either instrumental or non-instrumental analysis, which means that peak elution in chromatogram and the colour change in titrations. It is not a commonly used method. In S/N ratio type of LOD determination, low known concentrations of sample solutions are to be prepared and run by using developed analytical method, and then compared with the peak height with the blank sample noise. The common detectible ratio of LOD is 3:1 or 2:1. Statistical calculation of LOD is done by the using the following equation of standard deviation of results and the slope of the linear curve.

$$LOD = 3.3*\sigma / S$$

Where ‘S’ is slope of the calibration curve and ‘σ’ is Standard deviation.

1.8.9. Limit of quantification (LOQ)

LOQ is determines the ability of proposed method to quantify at lower concentration of analyte in sample with acceptable accuracy and precision. LOQ is assessed by using the same method for detection of LOD, S/N ratio method or statistical calculation method. In S/N ratio method, the acceptable ratio is 10:1. Statistical calculation of LOQ is done by the using the following equation of standard deviation of results and the slope of the linear curve.

$$LOQ = 10*\sigma / S$$

Where 'S' is slope of the calibration curve and 'σ' is Standard deviation.

1.8.10. Linearity and Range

Linearity determines the ability of proposed method to show the results are directly proportional to the concentration of the analyte in the sample in one particular concentration range. Linearity report contains the correlation coefficient (R^2) value by producing linear curve, slope and intercept. According to ICH guidelines, the linearity determination needs at least five different concentrations of solutions with gradual increase in order.

Range is a interval of high and low concentrations of sample solutions, it is used to determine the linearity with acceptable accuracy and precisions by using the proposed method. The units of range is expressed as in test results obtained in the proposed method. Minimum five concentrations should be required for range as that in linearity and some methods may need specific range depends on method conditions.

1.8.11. Robustness

Robustness can be defined as the ability of proposed method to give acceptable results by changing the small but deliberate variations in method parameters. It gives the reliability and suitability of proposed method for general use. During the robustness study, the method

parameters are changed intentionally to know the effect of change in parameters on results. In general, change in method parameters such as pH of buffer in mobile phase, column oven temperature, flow rate, organic modifier in mobile phase and detector wavelength are considered. The effect of change in those parameters on results can be verified by the changes that are occurred in resolution between adjusting peaks, tailing factor, plate count and RSD of the results. The ICH guidelines presented the importance of robustness. If any minute susceptible results are observed during the robustness study, those parameters should be controlled and must be mentioned in method documentation.

1.9. Development of Quality-by-Design (QbD) approach to analytical methods

QbD has gained a lot of importance in pharmaceutical industries since its inception by United States food and drug authority (US FDA). The QbD main goal is to improve the product quality and patient safety. The ICH defines the QbD as a systematic process for the development of drugs, which starts with pre-defined objectives. And also risk management approaches uses science and procedure understanding, and finally process control [50, 51]. During a one factor at a time (OFAT) approach where one parameter is changed consecutively until an appropriate method is produced. This kind of development may produce a sufficient method but provides an inadequate understanding of method abilities and robustness. Instead of OFAT, the systematic screening approach assess a method at different pH ranges, variable stationary phases and organic modifiers hence it would provide highly thorough going approach to analytical method development [52]. The QbD approach for analytical method development uses statistical calculations for the designing of experiment (DoE) for developing a robust (design space) method. The design space define the stability of method,

which means that the change in parameters of the method will not show more effect on results. The following steps demonstrates the development of analytical method by the QbD approach.

1.9.1. 1st Step: Define method goal

In accordance with the QbD principles of pre-defined goals, the goal and purpose of the method to be developed must be clearly defined [53]. The goal of the HPLC method is the separation and quantification of the main compound and impurities those are generally affect the quality of the formulated product [54].

1.9.2. 2nd Step: Method scouting and Evaluation

This systematic experimental design involves the better understanding of the effects on the developing method with individual factor changes and its mutual interactions with method that eventually will lead to method optimization. Automatic method scouting can be hired with effective and comprehensive experimental design to assess the key components like column, pH and organic modifier of the RP-LC. The QbD can be generated from the chromatographic database and it will help in better understanding of the method selection and optimization.

1.9.3. 3rd Step: Method selection and risk assessment

Depends on the method scouting and evaluation results, the method that best reaches the target can be selected for risk assessment. Prioritizing and identifying in a structural manner followed by robustness and ruggedness tests are parts of the risk assessment. For the risk assessment some of the tools such as failure mode effects analysis (FMEA), fishbone diagrams and polarization matrix are used [55]. These tools will be helpful for identifying the unintentional changes to method parameters such as analysts, laboratories, period of time and instrument reagents and prioritize potential risks to the method.

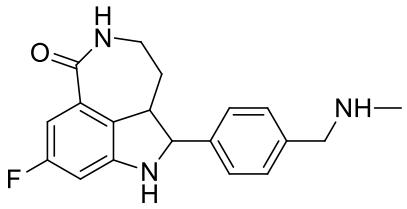
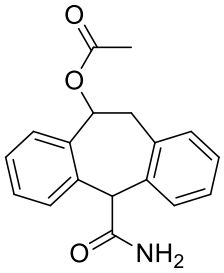
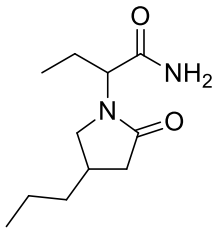
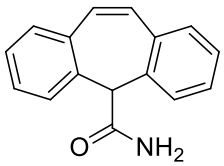
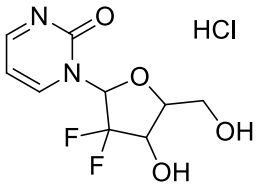
1.9.4. 4th Step: Define analytical method performance control strategy

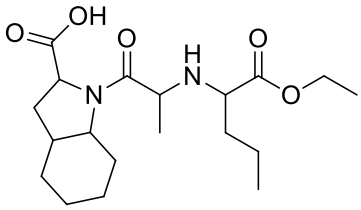
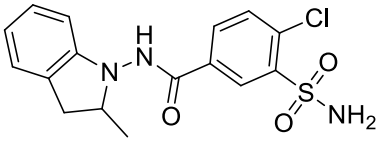
Control strategy ensures that whether the method maintains its intended purpose on a general basis. Analytical method performance control strategy as well as suitable system compatibility criteria can be defined to ensure the risk management and the method that provides the desired method characteristics. If method is getting to high risk and not possible to manage, the analyst has to go back to described database in the second step and can select the more appropriate method.

1.10. Aims and objectives

The integral aims of this study is to develop a new validated HPLC and mass spectrometry methods for identification and quantification of different drug molecules and its related substances, degradation products as per ICH guide lines. These play major role in decreasing the analyzing time and in getting the results with high accuracy and precision. This thesis also aims at developing new extraction methods like liquid-liquid extraction and solid phase extraction methods for extraction of drug molecules and it related compounds from the biological fluids. These extractions methods will help in the extraction of the drugs and its metabolites from biological fluids with low extraction time, low consumption of solvents, quick and in easy way. These developed analytical methods showed reliable and reproducible data for the applications to future work.

Table 1.1 List of selected drugs for the present research work.

S. No	Name of the Drug	Chemical Structure	Therapeutic activity
1.	**Rucaparib**		Rucaparib is a PARP inhibitors. It workings by the killing of cancer cells.
2.	**Eslicarbazepine Acetate**		Eslicarbazepine is used for the treat certain type of seizures.
3	**Brivaracetam**		Brivaracetam is us as medication for control the partial onset seizures.
4	**Carbamazepine**		Carbamazepine is used to treat the epilepsy.
5	**Gemcitabine**		Gemcitabine is used to treat the lung cancer.

6	**Perindopril**		Perindopril is used to treat the decrease of hyper tension.
7	**Indapamide**		Indapamide is used to treat the decrease of high blood pressure.

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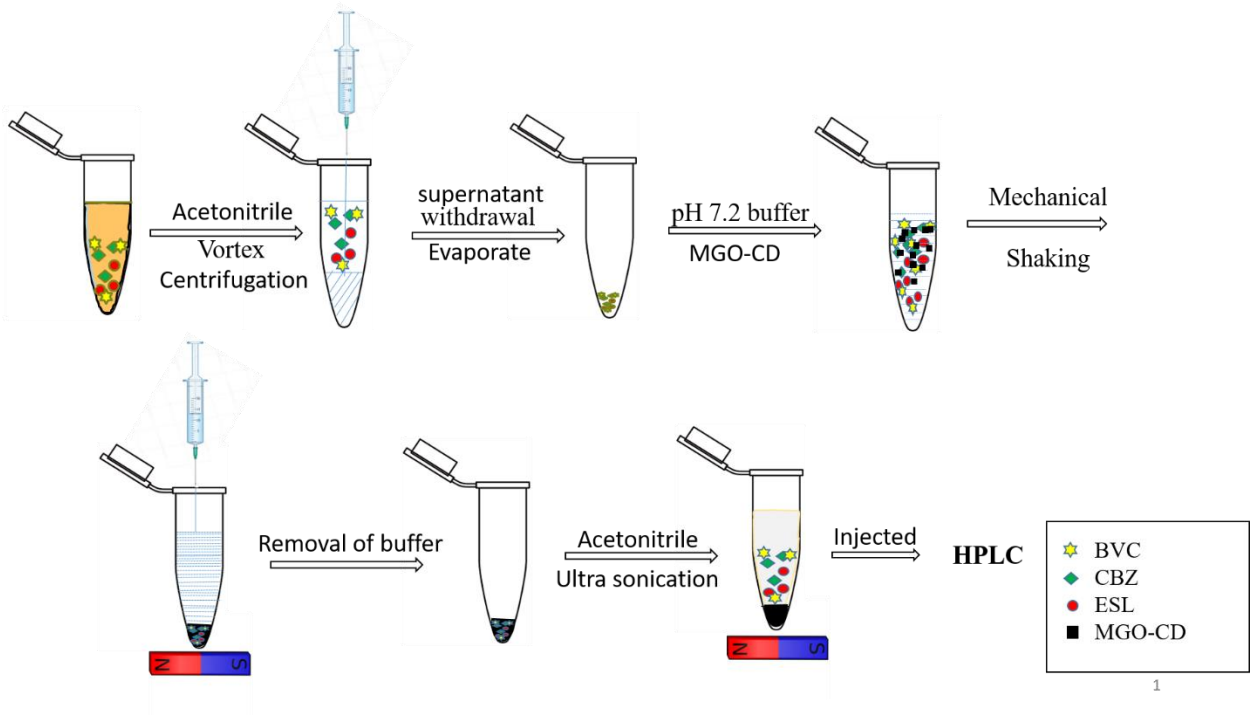
Chapter –II

Development and validation of HPLC method for the analysis of three antiepileptic drugs in human plasma by using solid phase extraction

Abstract

A new solid phase (MGO-CD) was developed for the extraction of three anti-epileptic drugs (BVC, ESL and CBZ) from human plasma and analyzed by using a HPLC-UV method. Morphology, magnetic properties and structure of the synthesized MGO-CD were characterized by using FT-IR, SEM, XRD and VSM. Solid phase extraction (SPE) methods was used to extract the analytes from human plasma. Among the different extraction solvents used, acetonitrile (ACN) showed better extraction ability towards drug molecules from MGO-CD. The linearity analysis showed good correlation coefficient values (R^2) of 0.9989, 0.9995 and 0.9982 for BVC, ESL and CBZ respectively. The LOD and LOQ ranges were found to be 6.14-28.32 ng mL^{-1} and 20.45- 94.31 ng mL^{-1}.



Schematic diagram for extraction of all three antiepileptic drugs from human plasma

2.1. Introduction

Epilepsy is a chronic brain disease described by sudden and transient brain dysfunction caused by recurrent episodes of neurons in the brain [1-3]. It is identified with symptoms like seizures and should be treated immediately with a quick-action antiepileptic drug (AED). The treatment results in lowering the potential sequelae, predominantly excitotoxic and ischemic neuronal cell loss, which initiates within minutes of uninterrupted seizure activity [4-8]. Brivaracetam (BVC), Eslicarbazepine acetate (ESL) and Carbamazepine (CBZ) drugs are widely used drugs for the treatment of this condition [9]. The IUPAC name of BVC is ((2S)-2-[(4R)-2-oxo-4-propylpyrrolidin-1-yl]), which is a 4-n-propyl equivalent of racetum and levetiracetam derivative and is primarily employed for partial onset of seizures in adults and adolescents [10-11]. The mode of action of BVC is through binding to the pervasive synaptic vesicle glycoprotein 2A (SV2A), like levetiracetam but has a 20-fold greater affinity [12-15]. CBZ is a mood stabilizing and anticonvulsant tricyclic lipophilic drug. This drug is the first one to treat epilepsy for psychomotor, partial onset of seizures and also used for variability of indications, including schizophrenia, attention-deficit hyperactivity disorder (ADHD), paroxysmal dangerous pain disorder, phantom limb disease and post-traumatic stress syndrome [16-18].

ESL is a modern and third-generation single enantiomer drug belongs to the dibenzoazepine family. ESL is an anticonvulsant medication, effectively administrated in the adjunctive therapy for partial-onset seizures [19, 20]. ESL is a prodrug that is intensively transformed to eslicarbazepine, which is an important metabolite in the human body [21-23]. ESL was known to wield anticonvulsant activity by inhibiting the repetitive neuronal firing and also by stabilizing the inactive sodium channels [24-26].

Considering the importance of these molecules as antiepileptic drugs, it is important to develop an analytical method for the extraction these drugs from biological fluids. The available literature revealed that the different analytical methods such as fluorescence-based immunoassays, enzyme linked immunosorbent assays, electro chemical, and spectrophotometric methods are known for detection of these drugs. These methods gave results with high accuracy, sensitivity and also rapid by using small amount of blood sample. The main drawback with these methods is their high operating costs. Solid Phase Extraction (SPE) method is the commonly used extraction method compared with Liquid-Liquid Extraction (LLE) due to its advantages such as high preconcentration value, low solvent consumption and easy handling. Adsorbent plays a key role in SPE method, various adsorbents such as simple graphene oxide, silica gel, activated carbon, ionic liquids, calixarenes and chelating resins were reported. But these materials normally showed low absorbance values and made the elution procedure critical. Therefore, the development of method which overcomes all the limitations mentioned here is of utmost importance for the analysis of drugs in biological samples.

In the current method, iron oxide-Graphene oxide-β-Cyclodextrin composite was used as an adsorbent. β-cyclodextrin, is a cyclic oligosaccharide consisting of seven D-glucopyranose units bound by β-1, 4-glycosidic bonds [27]. The usage of β-cyclodextrin in the composite further increases the adsorption capacity due to the presence of hydrophilic outer shell due to the presence of more hydroxyl groups and hydrophobic cavity due to its carbon chain conformation. Owing to this dual character, it acts as the host guest moiety by capturing of compounds with suitable dimensions into its cavity [28-31]. Due to its enrichment capability and high supra molecular recognitions make them widely used in different analytical aspects especially in separation methods [32, 33]. Graphene oxide (GO), the other component of the composite is made through

the oxidation of graphene. It contains hydroxyl, carboxyl, and epoxide derivative as functional groups, which increases the dispersibility in solution. GO is known for its exceptional electrical, optical and mechanical properties due to the high surface area. Thus, the combination of these three components makes this composite an ideal adsorbent for the extraction of drugs from plasma. Iron oxide induces magnetic property to adsorbent, this made the extraction process easy and quick with low matrix effect and high recovery percentage of analytes. There is no need to go for high speed centrifugation instead a simple magnet can be used for the separation. The MGO-CD was prepared through the encapsulation of β-cyclodextrin on MGO with the help of linker tetrafluoterthalonitrile. The liker tetrafluoterthalonitrile, which helped for building a porous structure of MGO and β-cyclodextrin via nucleophilic aromatic substitution reactions [34]. The MGO-CD morphology, structure and magnetic nature were confirmed by using of SEM, VSM, IR and XRD studies. Simultaneous, identification and quantification synergy of BVC, ESL and CBZ in human blood plasma by using MGO-CD as solid phase has been accomplished with HPLC.

2.2. Literature survey

Few stability indicating and extraction methods were reported for BVC, ESL and CBZ in the form of bulk drugs and formulations. Stability indicating methods were reported for the identification, quantification and characterization of drug molecules, relative impurities and degradation products. Several methods were reported for the extraction, identification and quantification of the drug molecules and relative substances from biological fluids such as blood, urine, plasma and serum.

Shouzhu Liao et al., developed a systematic impurity profile research for anti-epileptic drug of brivaracetam and the drugs were identified and quantified by using HPLC, mass spectrometry and NMR techniques [35]. Noura. M. Mansour et al., developed and validated a

novel green and accurate HPLC method for the simultaneous determination and quantification of three anti-epileptic drugs brivaracetam, carbamazepine and piracetam from human plasma [36]. Emmanuel Bourgogne et al., were developed and validated a new liquid chromatography-mass spectrometric (LC-MS/MS) method for the identification and quantification of antiepileptic drug brivaracetam acid metabolites in human kidney tissue, liver and blood by using a solid phase extraction method [37]. Muzaffar Iqbal et al., developed and validated ultra-performance liquid chromatography-mass spectrometric (UPLC-MS/MS) method for identification and quantification of anti-epileptic drug brivaracetam from plasma by using a liquid-liquid extraction method [38]. Vijayakumar Baksam et al., successfully separated brivaracetam stereo isomeric impurities with high resolution by using a newly developed and validated high performance liquid chromatography (HPLC) method [39]. Pankaj Bhamre et al., developed a stability indicating LC-PDA method for brivaracetam [40].

Shereen M. Azab et al., developed a electrochemical method for the determination of eslicarbazepine and its base metabolite S-licarbazepine in urine sample [41]. Mahesh Kumar Mone et al., developed and validated a chiral liquid chromatographic method for the separation of R-enantiomer from eslicarbazepine acetate [42]. Anne-Catherine Servais et al., developed and validated a chiral liquid chromatographic method for the separation of eslicarbazepine and its metabolites (R-licarbazepine, oxcarbazepine, and optical antipodes of ESL) [43]. Saji Thomas et al., identified and characterized eslicarbazepine and its synthesized process related impurities by using LC-ESI-IT-MS, ^{1}H-^{1}H COSY and ^{1}H, ^{13}C NMR [44]. A.I. Loureiro et al., identified and quantified the eslicarbazepine acetate, oxcarbazepine, R-licarbazepine in human plasma by developing a enantioselective validated LC-MS/MS method [45].

Zahra Rezaei et al., developed a HPLC and partial least square (PLS) calibration methods for the simultaneous determination of two anti-epileptic drugs (carbamazepine and phenytoin) in human plasma [46]. Ana Fortuna et al., developed a HPLC-UV method for the simultaneous determination and quantification of carbamazepine, eslicarbazepine acetate, oxcarbazepine and their metabolites in human plasma [47]. Ana Serralheiro et al., quantified simultaneously six anti-epileptic drug (primidone, carbamazepine, lamotrigine, phenobarbital, phenytoin and phenobarbital) in human plasma by using an efficient HPLC-UV method [48]. Bahram Hemmateenejad et al., determined the carbamazepine and it metabolite carbamazepine-10, 11-epoxide by using the PLS method in plasma and compared with HPLC-UV [49]. K.M. Patil et al., have developed a validated HPLC method for the simultaneous quantification of carbamazepine, phenytoin and lamotrigine in human serum [50]. Mohammad Behbahani et al, extracted carbamazepine and lamotrigine anti-epileptic drugs at trace level in biological fluids by developing mesoporous silica based a dispersive micro solid phase extraction method [51]. Elizabeth Greiner-Sosanko et al, have developed a HPLC method for simultaneous detection of three anti-epileptic drugs carbamazepine, lamotrigine and zonisamide in human plasma sample [52]. R D. Scheyjir et al., have developed a simple, precise HPLC method for the determination of Carbamazepine and their metabolite carbamazepine epoxide in human brain micro dialysis [53]. A. Kumps developed a HPLC method for simultaneous detection of oxcarbazepine, carbamazepine and their three metabolites in serum samples [54].

2.3 Sample preparation methods

Bioanalytical sample preparation methods plays a major role in the drug discovery and drug development processes. These are used for the assessment of drug absorption and disposition in physiological fluids such as urine, blood, plasma, serum and tissue of the drug administered lab

test animals. Frequently, the concentration of the NCE levels in biological matrix decreases with time and fall to low level (nanogram level), hence the developed bioanalytical methods must be able to quantify the analytes at or below nanogram level. In the analysis of biological sample, stability issue and endogenous materials (example, plasma protein) interferences make the analysis critical to get accurate analytical results. Therefore, developed analytical methods for pharmacokinetic studies should be able to separate the analytes with high resolution from the biological matrix. Typically, some common sample preparation methods are used such as Liquid-Liquid extraction, protein precipitation, and solid phase extraction etc., methods for removing the interference of the biological matrix from analytes.

2.3.1. Protein precipitation method (PPM)

Protein precipitation method (PPM) is a simple method for the removal of biological macro molecules when compared with any another protein separation methods. In general, different organic solvents (acetonitrile, methanol, and ethanol etc.,) in which analytes exhibit good solubility are used in PPM method [55]. Since all these organic solvents have less capability of removing total protein content from biological sample, a number of endogenous interference peaks will appear along with main analytes. In some cases acidic solutions (orthophosphoric acid, hydrochloric acid etc.,) may also be used for removing of protein through the precipitation. In such cases the acids may induce the external stress on analytes thus the percentage recovery will decrease and new degradation peaks may come. The PPM is a time taking process when we do many samples at a time. Extensive efforts such as automation make this process more efficient and give higher-output in Biological analysis. However, it is not efficient method in drug discovery, clinical research and bioanalytical methods because some analytes may bind with proteins and will be removed from the sample solution.

2.3.2. Liquid-Liquid extraction (LLE)

Liquid-liquid extraction (LLE) is one of the best technique for isolating the analytes from the mixture. LLE operation procedure is very easy and get highly pure analytes from the mixture by using two immiscible solvents. The technique of LLE extract the analytes from the mixture based on partition deference of analytes between the two immiscible solvents. The partitioning of the analytes in the mixture of compounds depends on the selection of proper solvent [56]. In the LLE, the extraction solvents may need to be acidified, basified or low percentage of high polar solvents are required to extract the metabolites and related compounds. Sometimes there is a need to repeat the extraction procedure for the maximum recovery of analytes. Recent days, several advanced techniques are invented instead of simple LLE methods such as support membrane extraction, liquid phase micro extraction and single drop liquid phase micro extraction.

2.3.3. Solid phase extraction (SPE)

Solid phase extraction **(**SPE) method is typically used as a versatile technique for the extraction of analyte from the mixture of compounds. Many extraction methods rely on SPE because of lot of advantages such as high pre concentrations of the sample, easy to operate, rapid and selective. The SPE is a powerful technique for the enrichment, isolation, purification of trace level analytes in different type of matrices. The main objects of the SPE is to minimize the interference of matrix in the biological sample, decreasing the final sample volume and maximize the analytes sensitivity. Normally the interferences are rinsed with SPE and then the analytes are eluted by desorption with eluting solvent. The SPEs are available with different formats such as reverse phase, normal phase, restricted access media (RAM), ion exchange and molecular imprinted media [57-59].

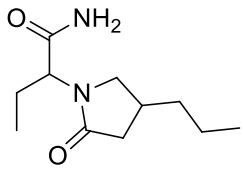


Figure 2.1 Chemical structure of Brivaracetam.

2.4. BVC drug information

IUPAC name	:	(2S)-2-[(4R)-2-oxo-4-propyltetrahydro-1H-pyrrol-1-yl] butanamide
Molecular formula	:	$C_{11}H_{20}N_2O_2$
Molecular weight	:	212.29 g/mol
Brand name	:	Briviact
Maximum daily dosage	:	200 mg
Dosage strength	:	10, 25, 50, 75 and 100 mg
Dosage form	:	Tablet

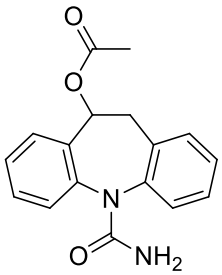


Figure 2.2 Chemical structure of Eslicarbazepine acetate.

2.5. ESL drug information

IUPAC name	:	[(5S)-11-carbamoyl-5, 6-dihydrobenzo[b] [1] benzazepin-5-yl] acetate
Molecular formula	:	C17H16N2O3
Molecular weight	:	296.32g/mol
Brand name	:	Eslistar, Eslizen

Maximum daily dosage	:	1200 mg
Dosage strength	:	200, 400, 600 and 800 mg
Dosage form	:	Tablet

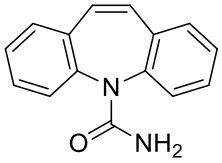


Figure 2.3 Chemical structure of Carbamazepine.

2.6. CBZ drug information

IUPAC name	:	5H-dibenzo [b, f] azepine-5-carboxamide
Molecular formula	:	$C_{15}H_{12}N_2O$
Molecular weight	:	236.27g/mol
Brand name	:	Mazetol, Zeptol
Maximum daily dosage	:	1600 mg
Dosage strength	:	100, 200, 300 and 400 mg
Dosage form	:	Tablet

2.7 Experimental

2.7.1 Chemicals and Materials

All the chemicals and solvents used in the current studies are of Analytical grade. Graphene powder, $FeCl_3$. $6H_2O$, Ammonium hydroxide solution (28.0-30% NH_3 basis) were procured from Sigma-Aldrich. Acetonitrile, Methanol, Chloroform, TBDE, Acetone, Ethanol, Ethyl acetate and DMF purchased from (Merck) Mumbai India; Double distilled deionized water is used for entire analysis; Tetrafluoroterepthalonitrile, β-Cyclodextrin (>98%), sodium acetate, mono-, di- and poly

ethylene glycol were procured from Sigma Aldrich. CBZ, BVC and ESL were obtained from Mylan & Hetero laboratories as gifted samples.

2.7.2 Instruments and analytical conditions

HPLC having binary pump system with diode array detector and LC-Solutions software (Shimadzu, Japan) were used for developing the method. Luna RP C18 (4.6x150mm, 5 µ) column was used, column oven temperature maintained at ambient conditions. Acetonitrile and 0.1% Formic acid (65:35) mixture was used as a mobile phase. FT-IR spectra were recorded on Perkin Elmer, USA using KBr pellets at ambient temperature. Powder X-Ray diffractograms of GO, MGO, and synthesized MGO-CD were recorded by using Bruker AXS D8 diffractometer with Cu K_α radiation (1.5406 A°), step size 2 mdeg and 0.5 s per step scan speed. The morphology of MGO-CD was analyzed by FEI Apreo LoVac equipped with an Aztec Standard EDX System. The magnetic properties of samples were measured at room temperature by using vibrational sample magnetometer (VSM, Lakeshore 7400, West-erville Ohio, USA) in magnetic fields up to 15 kOe. Raman spectra were taken on a SENTERRA Dispersive Raman microscope (Bruker, Germany) with a wavelength 785.0 nm incident laser light.

2.7.3 Synthetic procedure for MGO-CD

2.7.3.1 Synthesis of Graphene Oxide

The modified Hummer's method was used for the synthesis of graphene oxide (GO) from graphite nanopowder with a set of modifications (Figure 2.4) [60]. In brief, graphite powder (2 g) was added slowly in to 50 mL of concentrated sulfuric acid, and for this black mixture 2 g of sodium nitrate was added a for the initiating the reaction. For the above reaction mixture, potassium permanganate was added by maintaining the temperature below 20^{o}C. The resultant mixture was stirred at 60^{o}C for four hours. At this stage, the temperature was raised up to 90^{o}C after adding the 100 mL of

deionized water and stirred for 15.0-20.0 min. The addition of 20 mL of 30% H_2O_2 and 200 mL of warm water resulted in colour change to bright yellow indicates the formation of GO. The solid was collected and washed several times with 5% hydrochloric acid and water and then dried for 12 h.

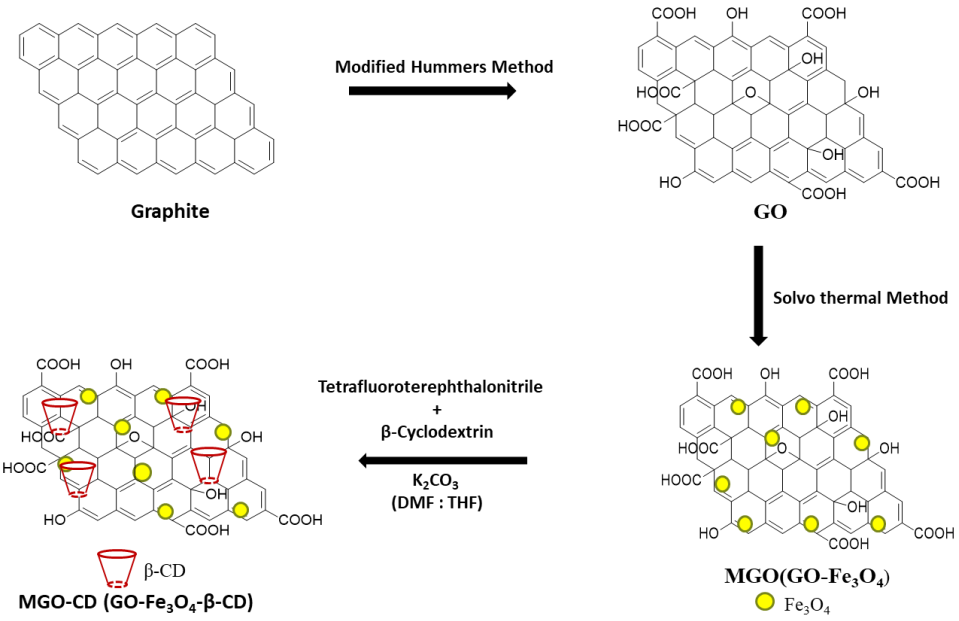


Figure 2.4 Schematic diagram for synthetic procedure of MGO-CD.

2.7.3.2 Synthesis of M-GO

M-GO was prepared with the help of simple and ecofriendly solvothermal route [61]. The GO (0.2 g) was dispersed in to 250 mL round bottom flask containing the mixture of mono- (20 mL) and di-ethylene glycol (60 mL), and sonicated for about 2 h. To this, 0.68 g of $FeCl_3.6H_2O$ was added and the dispersion was stirred for 25 min. Thereafter, poly ethylene glycol (2.25 g) and sodium acetate (8.10 g) were added slowly and sonicated for 30 min. The reaction mixture was transferred in to a stainless-steel autoclave and the reaction was carried out at 180 °C for 12 h. Finally, the formed product was collected by using the centrifugation and washed several times with water and ethanol and then dried for 12 h at 50 °C in vacuum oven.

2.7.3.3 Preparation of MGO-CD composite

MGO (0.0615 g), tetrafluoroterphthalonitrile (0.4 g), cyclodextrin (0.615 g) and potassium carbonate (0.96 g) were taken in a three-neck round bottom flask containing the solvent mixture of tetrahydrofuran (THF, 4 mL) and dimethylformamide (DMF, 26 mL). The reaction mixture was deoxygenated by purging nitrogen gas for about 30 min. After that, the reaction mixture was stirred mechanically at 85°C with constant speed for about 24 h. After the completion of reaction time, the formed product was collected by using centrifugation for 5.0 min at 5000 RPM. Then the product was washed with deionized water and HCl until the supernatant is neutralized. Further, the product was washed with THF and dichloromethane for the removal of unbound cyclodextrin and dried at 50^{o}C in the oven for 10 h [59].

2.7.4 Preparation of standard and spiked human plasma sample solutions

The standard stock solutions of BVC, ESL, and CBZ (1000 µg mL^{-1}) were prepared by dissolving in acetonitrile and stored at 4°C in the refrigerator. The drug free human plasma was collected from our institute dispensary and stored at -20°C. The protein content from the plasma was removed by adding 1 mL acetonitrile 1:4 (v/v) ratio to 250 µL of plasma 2 mL eppendorf tube. The sample was vortexed for 5 min and then centrifuged for 4 min at 4000 rpm and repeated this procedure till 4 mL of supernant was acquired. The 4 mL of supernant was taken in 5 mL glass vial and dried with N_2 gas at 40°C in vacuum oven. The reconstitution of the solution was done by adding phosphate buffer (pH 7.2) to the residue. In order to construct the calibration curve, eight spiked plasma sample solutions containing abstained the final concentrations of three anti-epileptic drugs of BVC, ESL, and CBZ (0.5-50.0,0.1-40.0 and 0.25- 60.0 µg mL^{-1} respectively) were prepared. For QC analysis three level spiked sample solutions (Low: BVC, ESL and CBZ is 1.0, 0.5, 0.75 µg mL-1; Middle: 10.0, 5.0, and 7.5 µg mL^{-1}; High: 20.0, 10.0 and 15.0 µg mL^{-1}) were

prepared. Eighteen milligrams of MGO-CD was added and then the solutions were mixed up to 25 min. The MGO-CD was separated from the solution by using a strong magnet. The analytes were extracted with 1.0 mL acetonitrile through ultrasound and the solution was evaporated and reconstituted with acetonitrile. From this 20 µL solution was directly injected into HPLC for the analysis (Figure 2.5).

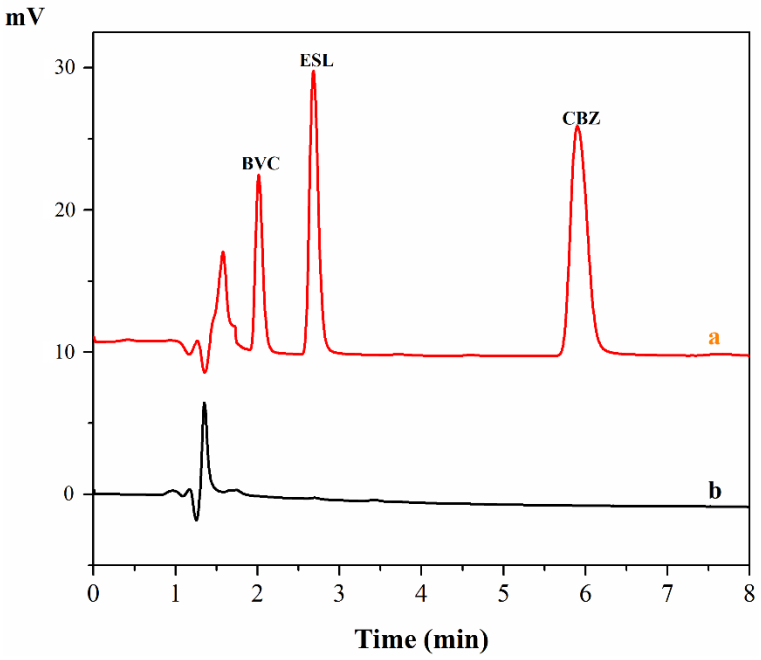


Figure 2.5 Typical HPLC chromatograms (a) human blank plasma sample after the extraction (b) Spiked sample solution after extraction with SPE at low level concentration.

2.7.5 The study of MGO-CD properties

2.7.5.1 Adsorption capacity of MGO-CD

Different amounts of MGO-CD (1.0-12.0 mg) in different centrifuge tubes were taken and then three antiepileptic drugs at a concentration of each 100.0 µg/mL were added. After continuous shaking of this mixture up to 120 min, the supernatant was collected by a strong external magnet and then directly injected into HPLC (Figure 2.6 (a)).

2.7.5.2 The study of adsorption kinetics

Ten milliliters of three antiepileptic drug solution containing concentrations of each BVC, ESL, and CBZ (100.0 µg/mL) was taken and added 18.0 mg of MGO-CD for the study of adsorption kinetics. The mixture was shaken continuously with different time ranges from 5 to 180 min. The unbounded solution was separated by using a strong external magnet and directly injected into HPLC analysis (Figure 2.6 (b)).

2.7.5.3 Study of adsorption isotherm

For the study of adsorption isotherm, 2 mg of MGO-CD in 1 mL drug solutions with different concentrations (5.0-700.0 µg mL^{-1}) was taken. The solutions are kept for 30.0 min and then supernatants were collected from MGO-CD by using a strong magnet. 20.0 µL of each supernatant was directly injected into HPLC-UV and the concentrations are determined (Figure 2.6 (c)).

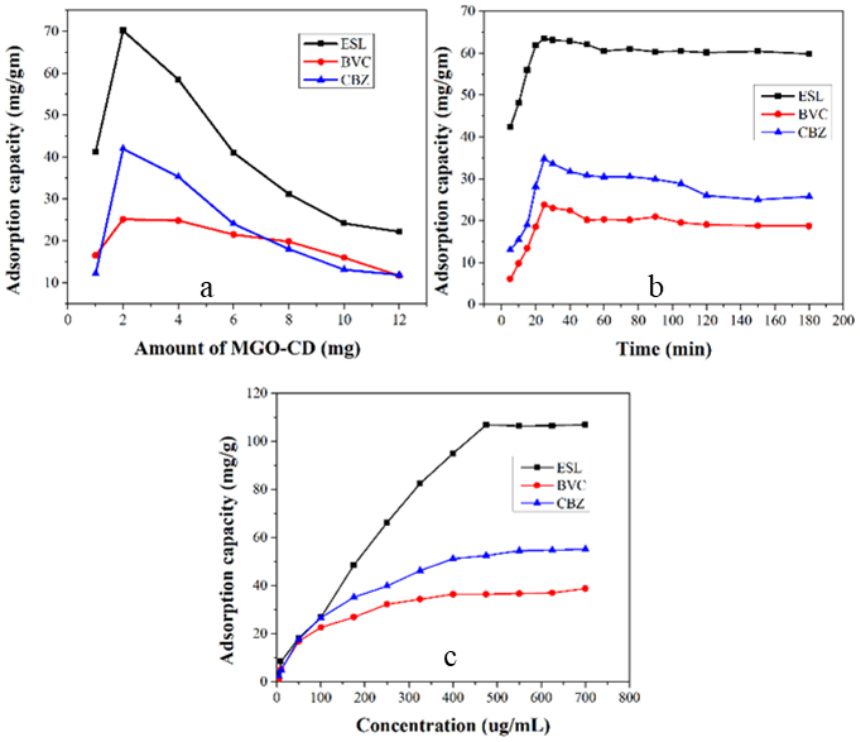


Figure 2.6 (a) Adsorption capacity of MGO-CD (b) The study of adsorption kinetics (c) Study of adsorption isotherm.

2.8 Results

2.8.1 Characterization of synthesized MGO-CD

The magnetic graphene oxide and cyclodextrin (MGO-CD) composite was synthesized as depicted in the experimental details. The MGO-CD composite was characterized by FT-IR, powder XRD, SEM and VSM. The MGO showed one specific band at 591 cm^{-1} in the Infra-red spectrum which corresponds to the Fe-O vibration in Fe_3O_4. The MGO-CD exhibited an additional band at 2255 cm^{-1} attributed to the stretching frequency of -C≡N in the linker (Figure 2.7). The IR spectra also exhibited a weak band at 1156 cm^{-1} and strong peaks at 1422 and 1580 cm^{-1} corresponds to C-F and aromatic C=C stretching frequencies respectively. The stretching frequencies of O-H, C-H and C-O were also found at 3453, 2945 and 1032 cm^{-1}. These results suggested the formation of magnetic graphene oxide cyclodextrin composite (MGO-CD).

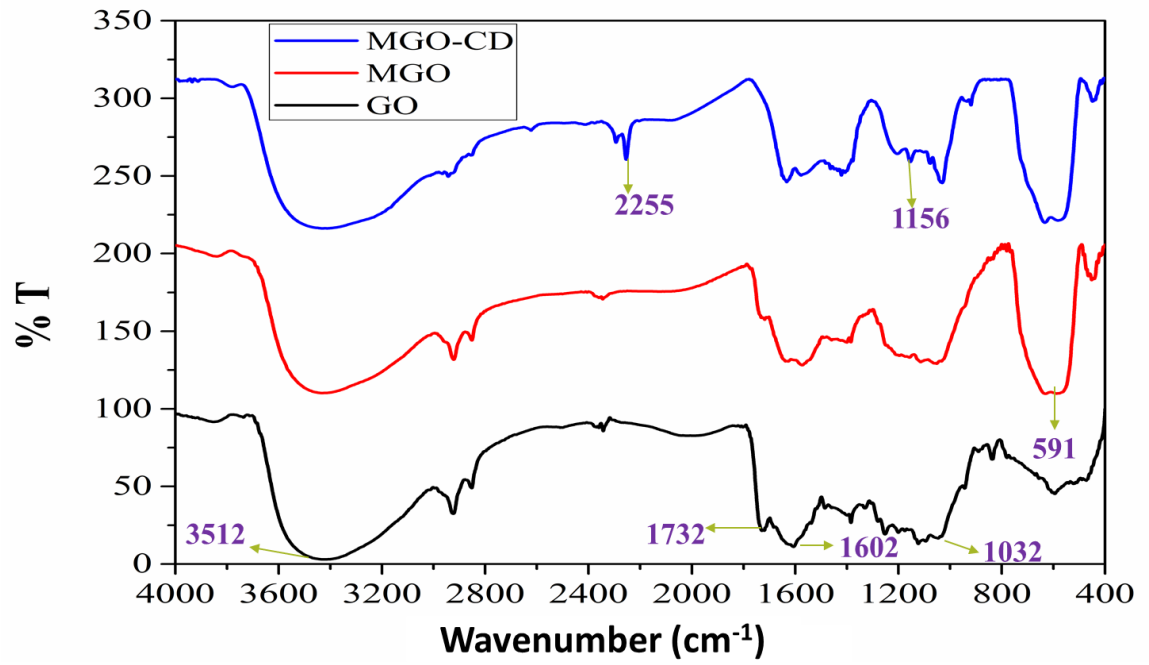


Figure 2.7 FTIR spectra's of GO, MGO, and MGO-CD.

Powder X-ray diffractogram of GO exhibited a peak at 10.56° which correspond to the characteristic diffraction from graphitic carbon in exfoliated GO. The peak was disappeared in

both MGO and MGO-CD due to the disturbance in stacking of GO sheets after loading with iron oxide (Fe_3O_4) and CD. The diffraction peaks observed at 30.0°, 35.9°, 43.0°, 57.3° and 62.4° were assigned to (220), (311), (400), (422), (511), and (440) planes, which corresponds to the cubic phase of Fe_3O_4 with a face-centered cubic structure as found in the literature reports. The intensity of XRD peaks were found to be slightly decreased on loading with cyclodextrin. These results supported the incorporation of CD on to MGO (Figure 2.8).

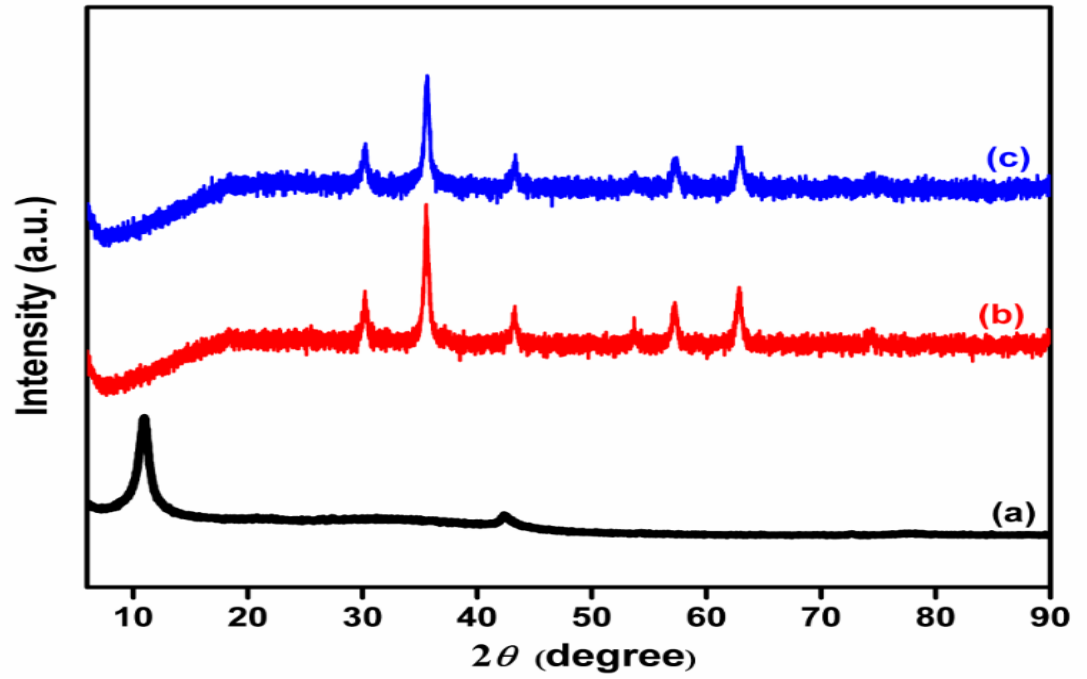


Figure 2.8 XRD patterns of (a) GO (b) MGO (c) MGO-CD.

There will be irregularity occurred in sp2 carbon network in GO, due to incorporation of CD and Fe_3O_4. Hence, the ordered and disordered crystal structures of fabricated MGO-CD needs to be investigated using Raman spectroscopy. The obtained Raman spectra of GO and MGO-CD are plotted in Figure 2.9. D-band and G-band are the parameters corresponding to the structural defects and imperfections, and first order scattering of the E2g phonon of the sp^2 carbon domains, respectively. From the spectrum of GO, it can be observed that D-band and G-band are assigned at 1345 and 1585 cm^{-1}, respectively. The disordering in the structure of GO is measured using the

intensity ratio of ID/IG. It can also be observed that there is an increase in the intensity ratio of MGO-CD than that of GO due to the incorporation of CD and Fe_3O_4, which indicates significant amount of structural defects of sp^2 carbon network in GO.

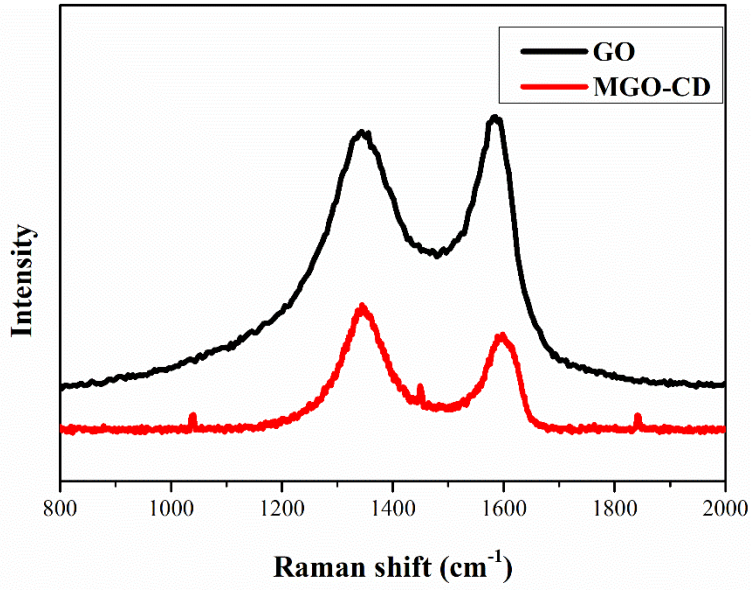


Figure 2.9 Raman spectra of GO and MGO-CD.

The morphology of the synthesized composite was examined by SEM. Figure 2.10(A) showed sheets like structure of the exfoliated GO. This was further employed for the synthesis of MGO-CD composites. Figure 2.10(B) exhibited the shining dots of Fe_3O_4 that are appeared on thin layers of GO sheets. The thin layered GO sheets has a large matrix which helps to anchor the Fe_3O_4 particles on it. The loading of Fe_3O_4 particles also increased due to the presence of a large surface area on the wrinkled GO. The Fe_3O_4 particles were deposited heterogeneously and distributed randomly in clusters on thin layers of GO sheets. There was clear diffraction observed from Figure 2.10(C) shows the formation of Fe_3O_4 composites on the GO sheets. The particles have been observed clearly along with the stacking of the GO sheets because of the adherence of Fe_3O_4 and CD.

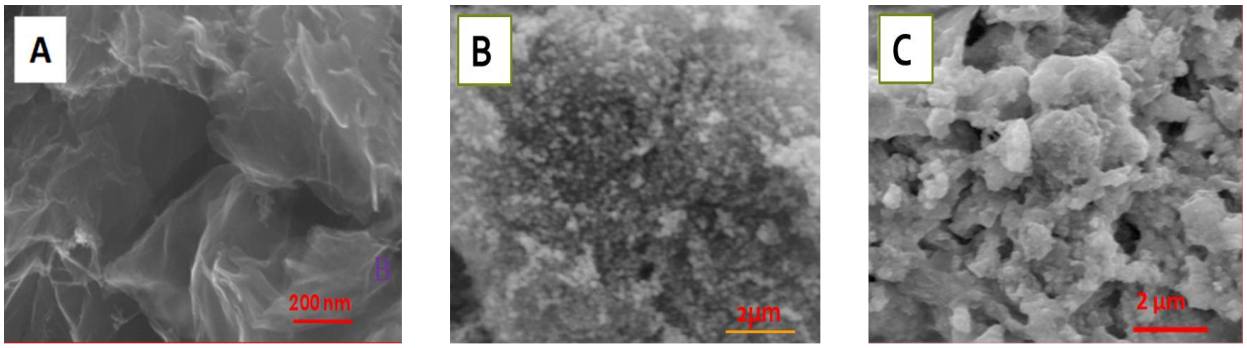


Figure 2.10 SEM images of (A) GO and (B) MGO and (C) MGO-CD.

The vibrational sample magnetometer (VSM) data was collected to understand the magnetic properties of MGO-CD, the hysteresis loops of MGO-CD were shown in Figure 2.11. S-like curves of magnetic hysteresis loops observed indicates the paramagnetic nature of MGO-CD because of no remnant magnetization or coercivity at room temperature. The specific saturation magnetization (Ms) of the composite was observed to be 43.96 emu g^{-1}, which is appropriate for the separation of composites with a magnet. The MGO-CD could be easily separated from the mixture of composites in the solution by placing a permanent magnet.

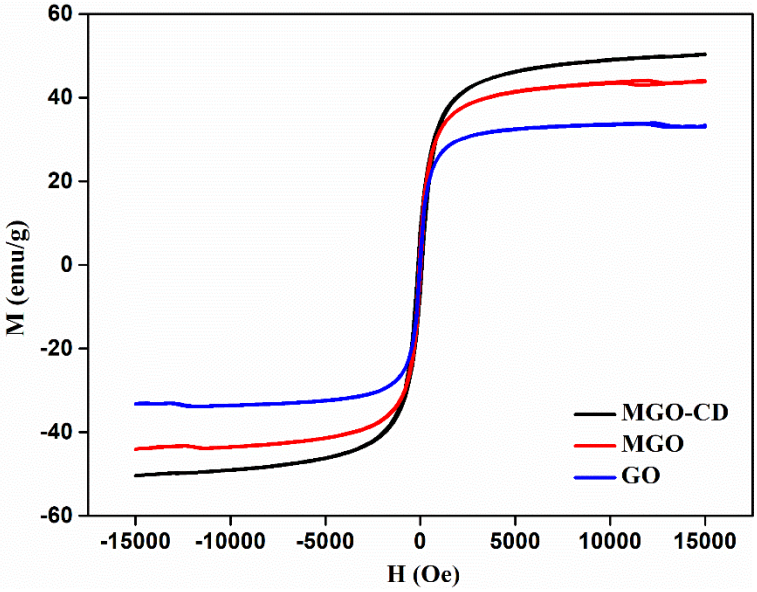


Figure 2.11 Magnetization hysteresis loops of GO, MGO and MGO-CD.

2.8.2 Optimization of extraction conditions

In this method, parameters such as extraction solvent (eluent) (ACN, MeOH, acetone, $CHCl_3$, TBDE, and EtOAC), sorbent amount (2.0-22.0 mg), extraction time (intermissions from 5.0-30.0 min) and eluent amount (0.5 to 2.5 mL) were optimized. The mixture of three antiepileptic drug solution contains the concentrations of BVC (10.0 µg mL^{-1}), ESL (10.0 µg mL^{-1}) and CBZ (20.0 µg mL^{-1}) respectively.

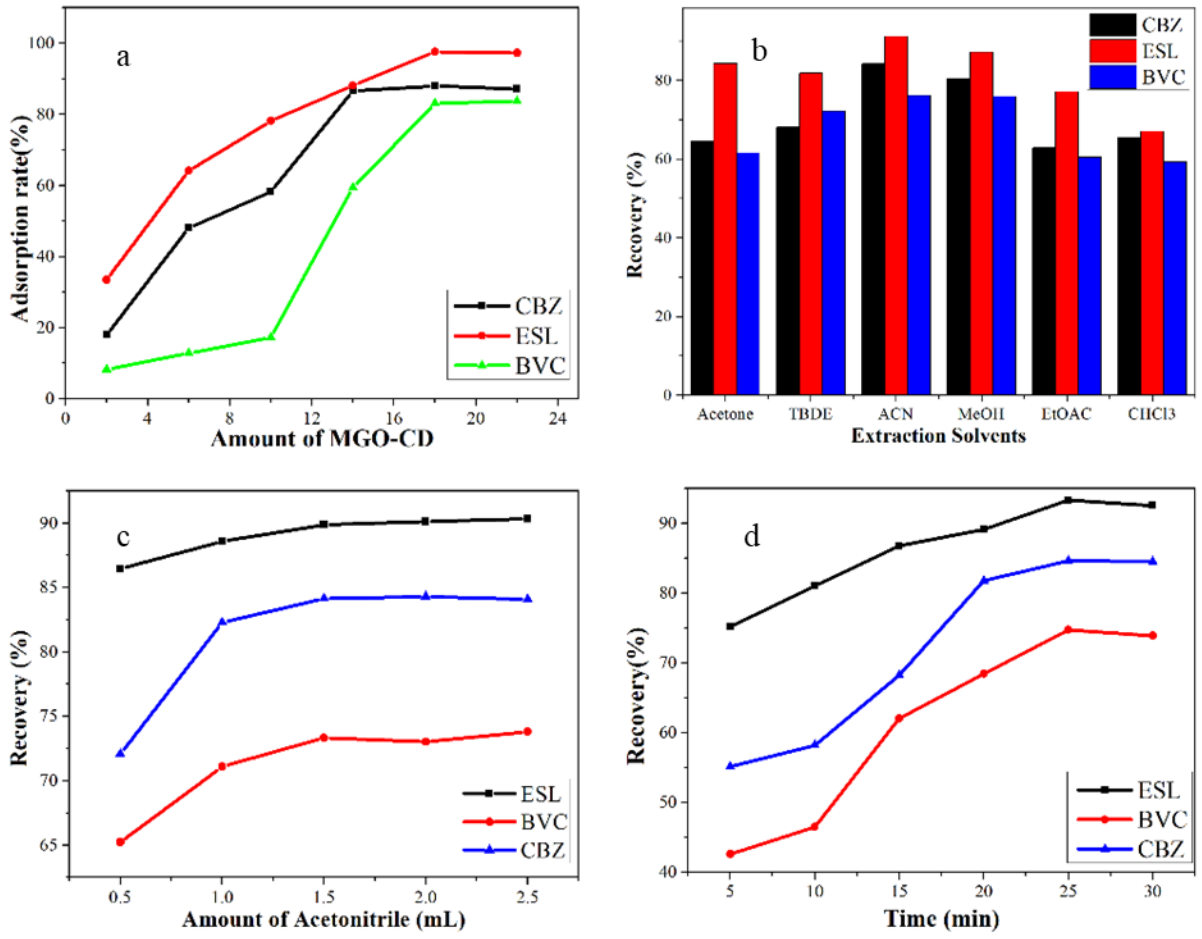


Figure 2.12 (a) The effect of MGO-CD amount (b) The effect of deferent solvents (c) The effect of solvent amount (d) The effect of time on extraction.

2.8.2.1 Effect of adsorbent amount

In order to get the amount of adsorbent required for better extraction, the effect of adsorbent quantity on the extraction of these drugs was studied. These studies revealed that at 2.0 mg of

absorbent, the adsorption rates were found to be 33.50% (ESL), 17.95% (CBZ), and 8.19% (BVC). Increasing in the four-unit volume of the adsorbent amount, the adsorption rate for ESL gradually increased and it reaches equilibrium (97.62%) at 18.0 mg. In the case of BVC, the rate of adsorption was slow up to 10.0 mg and it reaches maximum (83.08%) at 18 mg whereas the adsorption rate for CBZ was gradually increased (87.98%) and reaches maximum at 18.0 mg (Figure 2.12.(a)). These studies suggest that the maximum rate of adsorption was found to be at 18.0 mg in all the three drugs.

2.8.2.2 Effect of eluent solvent

The choice of desorption solvent plays a significant role in the extraction of analytes from the adsorbent. In the previous methods of extraction of these three drugs, acetonitrile, methanol, acetone, and a mixture of solvents under acidic conditions were used. In the present method, methanol, TBDE, acetone, acetonitrile, chloroform, and ethyl acetates were used as desorption solvents. Based on the polarity scale, acetonitrile is expected to give high desorption efficiency for these three antiepileptic drugs among all solvent used and chloroform is of low eluting efficiency. The same pattern has been found in our experimental results (Figure 2.12(b)). The percentage recovery of these three drugs was found to be higher in the case of acetonitrile and lower with chloroform as eluent. From the HPLC chromatograms, the polarity sequence of the three antiepileptic drugs is BVC > ESL > CBZ respectively (Figure 2.5).

2.8.2.3 Effect of eluent amount

The rate of desorption of analytes from any adsorbent is known to depend not only on the nature of the eluent but also on the amount. To study the effect of eluent amount on desorption rate, different volumes of eluent were used from 0.5 to 2.5 mL. The studies clearly suggested that the 1.5 mL of eluent gives maximum desorption of drugs (Figure 2.12(c)). The variation was found to

be in the range of 10% between 0.5 and 2.5 mL. Based on these studies, 1.5-mL eluent volume was found to be the optimal condition to get the best desorption efficiency**.**

2.8.2.4 Effect of time on the extraction

Time also showed a significant effect on desorption of three antiepileptic drugs from MGO-CD. The effect of time on extraction has been studied between 5.0 and 30.0 min. From Figure 2.12(d), it is clear that the extraction increases with the increase in time from 5.0 to 20.0 min (up to 30%) and becomes saturated after 25.0 min. Based on this study, the optimized desorption was found to be 20.0 min for effective desorption of these drugs from the MGO-CD.

2.8.3 Adsorption capacity

For the evolution and determination of adsorption capacity of MGO-CD, different initial concentrations of drug solutions (5.0-700.0 $\mu g\, mL^{-1}$) were prepared. The equilibrium adsorption capacity Qe (mg/g) of MGO-CD was measured by changing the concentrations of sample solutions and the values were calculated by using the subsequent equation

$$\mathbf{Q_e = (C_0 - C_e)\ V/m}$$

where Qe (mg/g) is the amount of three antiepileptic drugs adsorbed per unit weight of adsorbent at equilibrium, C_0 ($\mu g\, mL^{-1}$) is the initial concentration, C_e ($\mu g\, mL^{-1}$) is the equilibrium concentrations of drugs in the solution, m (g) is the mass of MGO-CD, and V (L) is the volume of the sample solution.

The apparent binding amount of the MGO-CD was calculated by using following Langmuir isotherm model.

$$\mathbf{C_e / Q_e = C_e\ (1 / Q_m) + (1 / Q_m\ K)}$$

Where, K is constant coefficient, Q_m is maximum sorption capacity of the MGO-CD, Q_e is equilibrium sorption capacity and C_e is equilibrium concentration. This transformation (Ce / Qe

versus Ce) gives information about the binding characteristics of the equilibrium adsorption. The Q_m values can be obtained from the plot of C_e/Q_e versus C_e. By using this transformation, the maximum sorption values for BVC, ESL and CBZ were found to be 36.38, 106.86 and 54.49 mg g^{-1} respectively.

2.9 Method validation

2.9.1 Linearity

For the determination of linearity, six different concentrated solutions of three antiepileptic drugs were prepared in the range of 0.5-50.0, 0.1-40.0 and 0.25-60.0 µg mL^{-1} respectively for BVC, ESL and CBZ. The calibration curves give good correlation coefficient (R^2) values ≥0.9982 with acceptable linearity (see Table 2.1).

Table 2.1 Analytical parameters of the three anti-epileptic drugs quantitative analysis.

Drug	Linear range (µg mL^{-1})	Calibration curve equation	R^2	LOD (ng mL^{-1})	LOQ (ng ml^{-1})
Brivaracetam	0.5 – 50	y = 36563x + 22801	0.9989	28.32	94.31
Eslicarbazepine acetate	0.1 – 40	y = 77215x + 15516	0.9995	6.14	20.45
Carbamazepine	0.25 – 60	y = 310443 - 47234	0.9982	14.86	49.48

2.9.2 LOD & LOQ

The sensitivity of the method was assessed by measuring the limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ of the developed method was evaluated by using signal to noise ratio (S/N) method. The LOD was set as lowest concentration that can be distinguished with signal to noise ratio over 3, whereas the lower limit of quantification of the analytes was assessed by using signal to noise ratio is 10. The LOD for BVC, ESL and CBZ were 28.32, 6.14 and 14.86 ng mL^{-1} and LOQ values for BVC, ESL and CBZ were 94.31, 20.45 and 49.48 ng mL^{-1} respectively.

2.9.3 Precision & Accuracy

Precision and accuracy of the method were analyzed by preparing three different QC level samples as described in Table 2.2. (Low, middle and high) of three antiepileptic drugs BVC, ESL and CBZ. Each level of QC samples repeatedly injected in triplicate and evaluated the recovery percentage. Inter and intraday recoveries of analytes results showed in Table 2.2. The results clearly showed the low interference effect on target analyte peaks and this demonstrates the good specificity of the method. From the results, it is clear that the recovery ranges in both intra and inter day are 80.25 - 101.11% and CV ranges is 1.86 - 5.50%. From these tests results, it is clear that the developed HPLC method will be useful for analysis of these three drugs in human plasma samples.

Table 2.2 Intra-day and inter-day precision and accuracy values of analytes in plasma sample.

	Intra – day (%)			Inter – day (%)		
	Low	Middle	High	Low	Middle	High
Brivaracetam	81.23 ± 3.41	84.65 ± 4.24	86.06 ± 3.66	80.25 ± 3.72	86.08 ± 3.97	87.61 ± 4.11
Eslicarbazepine acetate	96.62 ± 2.48	98.68 ± 1.84	101.11 ± 3.68	95.02 ± 4.63	97.55 ± 3.87	99.13 ± 3.22
Carbamazepine	91.27 ± 4.43	94.83 ± 4.10	98.36 ± 3.45	92.97 ± 5.12	95.63 ± 3.88	99.07 ± 3.72

2.9.4 Robustness

The robustness of current method was studied by preparing standard solutions of drugs (10.0 µg mL^{-1}) and by varying conditions such as flow rate, column oven temperature and mobile phase ratio. From the Table 2.3, it is clear that no substantial difference in the results were observed at different chromatographic condition and the RSD were found to vary between 5.84-1.42%. These results indicate the reliability and the good performance of developed HPLC method.

Table 2.3 Robustness Study.

Chromatographic changes	Level	Brivaracetam				Eslicarbazepine acetate				Carbamazepine			
		RT	Area	TF	N	RT	Area	TF	N	RT	Area	TF	N
Mobile phase ratio	38 : 62	1.98	301592	0.915	5846	2.78	741964	1.057	6715	6.41	4201875	1.345	14025
	40 : 60	2.05	334200	0.926	6264	2.80	750839	1.015	6806	6.45	4322257	0.99	15248
	42 : 58	2.16	350124	0.925	6584	2.93	780485	1.037	7142	6.49	4436481	1.025	16055
Mean ± SD			328639 ± 24739				757763±20172				4320204±117316	1.02	
RSD (%)			3.40				2.66				2.72		
Flow rate (mL/min)	0.7	2.34	300188	0.930	6845	3.21	708355	1.12	7038	6.93	4158410	1.10	16655
	0.8	2.05	334200	0.926	6264	2.80	750839	1.015	6806	6.45	4322257	0.99	15248
	0.9	1.82	305486	0.921	6081	2.03	735995	1.014	6682	5.91	4025014	1.00	14081
Mean ± SD			313291±18300				758396±26985				4201894±156473		
RSD (%)			5.84				3.56				3.72		
Column temperature (°C)	45°C	1.87	301542	0.926	6522	2.78	762844	0.99	6325	6.34	4211326	0.97	16051
	40°C	2.05	334200	0.926	6264	2.80	750839	1.015	6806	6.45	4322257	0.99	15248
	35°C	2.23	318921	0.932	6125	2.83	777202	1.01	6412	6.49	4225953	0.99	14583
Mean ± SD			318221±16340				763628±13199				4253179±60629		
RSD (%)			2.28				1.73				1.42		

2. 10 Discussion

LC-MS method has been quite widely employed for the analysis of drugs in plasma due to its high sensitivity and selectivity. But the method is expensive in terms of solvents usage and maintenance that increase the burden for institutions and patients which in-turn limit the applicability of the method. In the case of liquid-liquid extractions, the method needs high amount of organic solvents and it causes pollution. Therefore solid phase extraction method is considered as an alternative method for the analysis of drugs in plasma due to its good extraction ability, convenient operation and consumption of low amount of organic solvents and it needs low amount of adsorbent.

Table 2.4 Comparison of previous published method for Brivaracetam, eslicarbazepine acetate and carbamazepine.

Drug	Extraction method	Determination method	LR (µg mL^{-1})	LOD (ng mL^{-1})	RSD (%)	Recovery (%)	Ref
Brivaracetam	LLE	UPLC-MS/MS	0.001 - 2.00	0.80	5.64 - 9.69	91.5-108.7	[62]
Brivaracetam	SLE	LC-MS/MS	0.001-0.20	-	1 - 8.7	91.6-101	[63]
Brivaracetam	LLE	LC-MS/MS	0.16-8.0	39.0	0.59-1.96	95.7-106.5	[64]
Eslicarbazepine acetate	LLE	LC-MS/MS	50.08-15020	-	0.72-4.11		[65]
Eslicarbazepine acetate	SPE-Bond-Elut C_{18} cartridges	LC-MS/MS	0.050-1.000	-	0.9-6.1	93.2-106.3	[66]
Eslicarbazepine acetate	µSPE -Oasis® HLB	HPLC-DAD	25-100	7.6	1.1-5.6	≥ 93%	[67]
Cabamazepine	LLE	HPLC-UV	0.5-40	250.0	0.53-3.7	97.53-103.58	[68]
Cabamazepine	SPE-sulfur nano particles		0.0005-0.2	0.16	2.2-3.7	97.5-101.3	[69]
Cabamazepine	SPE -Oasis® HLB	HPLC-UV	0.10-50	10.0	2.97-10.54	86.37-88.89	[70]
Brivaracetam	SPE-MGO/CD	HPLC-UV	0.5-50	28.32	4.63-6.74	81.23-87.61	Present
Eslicarbazepine acetate	SPE-MGO/CD	HPLC-UV	0.1-40	6.14	1.91-2.85	96.62-99.13	Present
Cabamazepine	SPE-MGO/CD	HPLC-UV	0.25-60	14.86	2.48-3.93	91.27-99.07	Present

Compared with earlier reported methods by using different equipment's, our method showed high precision, wider linear range and comparable detection limit (see Table 2.4) [62-70].

These results demonstrate the advantages of the current method over the available methods and in addition to this there were no studies were reported on the simultaneous extraction of these three antiepileptic drugs using the solid phase extraction method.

2.11. Conclusions

Magnetic graphene oxide composite of β-Cyclodextrin with good water dispersibility was synthesized and characterized by FT-IR, SEM, powder XRD, and the magnetic property of the material was established by VSM. The material was found to be paramagnetic with sufficient magnetization for the separation of composite with a conventional magnet. The developed material was used as MSPE sorbent for the extraction of three anti-epileptic drugs from the human plasma. The method showed high precision with wider linear range and good detection limits. This developed HPLC-UV method has good efficiency for recoveries, good linearity and simple to handle. And also it gave low retention time for three antiepileptic drugs within 8 minutes. It provides high efficiency for extraction of trace level substances from human plasma.

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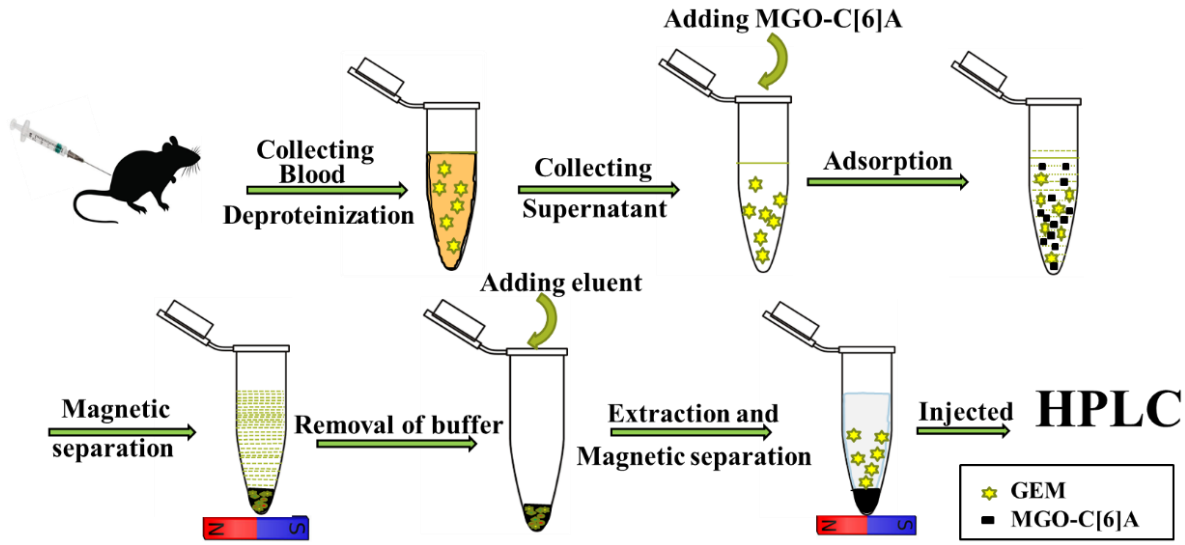
Chapter –III

Development and validation of HPLC method for the analysis of gemcitabine in human plasma and applications to pharmacokinetic analysis

Abstract

A highly accurate, precise and sensitive HPLC-UV method was developed for identification and quantification of Gemcitabine anti-cancer drug in rat plasma. A new magnetic graphene oxide based calixarene composite was developed and used as a solid phase for the extraction of gemcitabine from rat plasma. Extraction efficiency has been studied by varying different experimental variables (eluent type, sorbent amount, extraction time and eluent volume etc.) and these were evaluated and optimized. The composition, morphology and magnetic nature of the synthesized hybrid material was characterized by using XRD, FT-IR, SEM and VSM. Under the optimized conditions, linearity was evaluated with good correlation coefficient value R^2 (0.9993) by taking the different concentration solutions ranging from 0.5 to 25.0 µg/mL. Limit of detection (2.0 ng/mL) and Limit of quantification (13.0 ng/mL) was assessed by using signal to noise ratio method. The recovery percentages of gemcitabine at three QC level concentrations were obtained in the range from 97.6 to 100.2%.



Schematic diagram for solid phase extraction of GEM from rat plasma.

3.1. Introduction

Pancreatic cancer is a dangerous adenocarcinoma, it occupies fourth place for causing cancer related deaths globally [1]. Despite the progress in combination chemotherapy for the pancreatic cancer, it has less than 5% of survival rate [2]. Gemcitabine (GEM) is one of the USFDA approved drug for the pancreatic cancer [3–8]. The chemical name of Gemcitabine is (2', 2'-difluorodeoxycitidine (dFdC)) and it is active for various carcinogenic solid tumors such as bladder, breast and lung cancer [9–12]. As anti-carcinogenic agent, GEM has low toxicity profile and well tolerate. The main catabolic pathway is the metabolization of the GEM by cytidine deaminase in to the inactive metabolite 2', 2'-difluoro-2'-deoxyuridine (dFdU). GEM enters the concourses of the nucleoside transporters by penetrating through the cells [13, 14] and then it is phosphorylated in presence of deoxycytidine kinase to mononucleotide. Subsequently it converts in to active metabolites such as GEM diphosphate (dFdCDP) and triphosphate (dFdCTP) in presence of nucleotide kinases [15]. Predominantly, the GEM triphosphate is assimilated in to DNA and its leads to covered chain termination, at the same time GEM diphosphate prevents the activity of ribonucleotide reductase. It leads to DNA repair and synthesis by the reduction of deoxynucleotides concentration [16].

In general, sample preparation for bioanalytical methods is a critical task due to its time consuming nature, low sample sensitivity, matrix effect and labor intensive. Therefore, an appropriate sample preparation method must be developed before the evaluation of the sample. Various sample pretreatment such as liquid -liquid extraction (LLE), solid phase extraction (SPE), and matrix solid phase extraction (m-SPE), dispersive micro solid phase extraction (d-µ-SPE), and liquid phase micro extraction (LPME) etc. techniques have been reported. Among these, SPE sample pretreatment technique is most commonly used one due to its operational simplicity, high

sensitivity, economical, low extraction time, low consumption of organic solvents and high enrichment factor. However, traditional SPE sample preparation method have some disadvantages of time consumption, large secondary waste, expensive complex equipment and large amount of solvent loss etc. To overcome the limitations of classical SPE, magnetic nature is introduced to the solid phase which has the capability to address abovementioned disadvantages.

Selection of suitable magnetic solid phase (MSP) play a key role to acquire the adequate recovery of target molecules from biological samples. Iron oxide induces magnetic property to the adsorbent and this makes the extraction process easy and quick with low matrix effect and high recovery percentage of analytes. There is no need to go for high speed centrifugation instead a simple magnet can be used for the separation. In this work, a new magnetic solid phase material constitutes of magnetic graphene oxide and calixarene (MGO-C[6]A) was developed.

Graphene oxide (GO), the other component of the composite is made through the oxidation of graphene. It contains hydroxyl, carboxyl, and epoxide derivative as functional groups, which increases the dispersibility in solution. GO is known for its exceptional electrical, optical and mechanical properties due to the high surface area. Thus, the combination of these three components makes this composite an ideal adsorbent for the extraction of drugs from plasma.

Calixarenes are cavitands and macrocyclic host compounds which consists of hydoxyalkylation products of aldehyde and phenol [17–19]. Depending on their structural properties, calixarenes have a remarkable capacity to serve as receptors for a number of guests such as ions, metal ions and amino acids [20–22]. The properties of calixarenes can be changed by adding functional groups or by increasing the size of the substituents as part of the scaffold. Calixarenes are possibly the most promising of the macrocyclic compounds for application in the field of host-guest, identification of pharmaceutical and toxicological molecules. Due to the

presence of hydrophilic outer surface and hydrophobic inner cavity surface, low toxicity, presence of reactive sites and synthetic availability makes them very relevant for supramolecular chemistry. They are widely used in variety of applications such as drug design, sensing, catalysis, extraction and chromatography due to their stability, conformation flexibility, and easy makeover functionalization of calixarenes [23–28]. Hence, calixarenes can used as trapping agents to encapsulate the target molecules through host-guest interactions. Herein, magnetic graphene oxide (MGO) and calixarene were conjugated with the help of the linker tetrafluoroterephthalonitrile and used for the extraction of GEM from plasma.

3.2. Literature Survey

Stability indicating and extraction methods were reported for GEM in the form of bulk drugs and formulations. Stability indicating methods were reported for the identification, quantification and characterization of drug molecules, relative impurities and degradation products. Extraction methods were reported for the extraction, identification and quantification of the drug molecules and relative substances from biological fluids such as blood, urine, plasma and serum. Several chromatographic methods were developed for the analysis of anticancer drugs. For example, V. Rajesh, et al., estimated the gemcitabine and capecitabine anti-cancer drugs simultaneously from combined tablet dosage form by using reverse phase HPLC method [29]. S. Kurbanoglu et al., developed modern analytical assay techniques such as liquid chromatography and electroanalytical methods for anticancer drugs [30]. M. A. Vaudreuil et al., developed a reverse phase LC-MS/MS method for the analysis of polar anti-cancer drugs from waste water [31]. Quinmei Zhou et al., developed a new HPLC method for the analysis of gemcitabine liposome injection by using light scattering detector [31]. Tashinga E et al., have developed a new LC-MS/MS method for the quantification of gemcitabine and its two metabolites gemcitabine triphosphate and 2,2-

diXuorodeoxyuridine in tumour tissue [31]. R. Losa et al., have developed a HPLC-UV method for detection of gemcitabine metabolites di and tri phosphates in cancer cells and human blood [32]. Chester Bowen et al., developed a selective and sensitive LC-MS/MS method for the identification of gemcitabine and its metabolite 2,2-difluoro-2-deoxyuridine in human plasma [33]. Enaksha R et al., have developed a new LC-MS/MS method for the simultaneous estimation of gemcitabine, gemcitabine prodrug and its metabolite dFdU in human plasma [33]. Tina Kamceva et al, have developed a new ion pair LC-MS/MS method for the simultaneous determination of gemcitabine metabolite and eight endogenous nucleotides in human blood [33].

Richard J et al., have developed a new LC-MS/MS method for the determination of gemcitabine and its phosphorylated metabolites in tumour cell lines [34]. N. A. Parshina et al., have developed a isocratic HPLC method for the quantification of anticancer drug gemcitabine in plasma [35]. Brian R et al., have developed a new HPLC method for the estimation of gemcitabine and its prodrug amino acid ester and application to pharmacokinetic analysis [36]. S. G. Hiriyanna et al., have separated, isolated and characterized the gemcitabine impurities formed during the anomerization by using HPLC, HPTLC, FT-NMR and LC-MS/MS [37]. Yilin Sun et al., have developed a new UFLC-MS/MS method for the determination of gemcitabine, its prodrug and its main metabolite dFdU simultaneously [38]. L. Malatesta et al., have developed a high performance liquid chromatography-photo diode array method for the simultaneous determination and quantification of irinotecan and gemcitabine in rat plasma [39]. Ch. S. N. Malleswararao et al., have developed a new stability indicating ultra-performance liquid chromatography method for the determination and separation of gemcitabine related components [40]. Maryam Gomar et al., have developed a new solid phase molecular imprinted polymer for the extraction of gemcitabine in human urine and plasma and determined by using HPLC method [41].

In view of pharmaceutical importance of GEM, numerous analytical techniques have been developed for the determination and quantifications of GEM in biological fluids and individually. The reported methods are based on high performance liquid chromatography (HPLC) [42], gas chromatography (GC) [43], liquid chromatography with mass spectrometry (LC-MS) [44], fluorescence sensing [45] and electrochemical determination [46]. The main aim of this study is to develop a simple, precise, accurate, fast and sensitive analytical method for the determination and quantification of GEM from plasma. The synthesized calixarene based MGO-C[6]A composite was successfully applied as magnetic solid phase(MSP) for the extraction of GEM from the plasma, followed by the reverse phase high performance liquid chromatography (RP-HPLC). As far as we know, the extraction of GEM from plasma using MGO-C[6]A have not been reported till now.

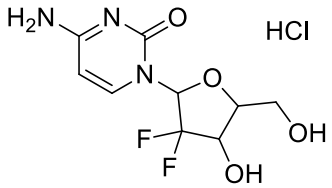


Figure 3.1 Chemical structure of Gemcitabine.

3.3. GEM drug information

IUPAC name	:	4-amino-1-[(2R,4R,5R)-3,3-difluoro-4-hydroxy-5 -(hydroxymethyl) oxolan-2-yl] pyrimidin-2-one;hydrochloride
Molecular formula	:	$C_9H_{12}ClF_2N_3O_4$
Molecular weight	:	299.66 g/mol
Brand name	:	Gemcite, Celgem and Abingem etc.
Maximum daily dosage	:	1000 mg
Dosage strength	:	200-1000 mg
Dosage form	:	Tablet and Injection

3.4 Experimental

3.4.1 Chemicals and Reagents

The analytical grade chemicals and solvents were used for the analysis. The compound calix[4/6]arene was gifted by Prof. C. P. Rao (IIT Tirupati). The water was made Ultra-pure by using Milli Q system (Millipore, USA) and used for entire analysis. HPLC grade or analytical grade solvents (Ethyl acetate, methanol, acetonitrile, chloroform, dichloromethane and tertiary butyl diethyl ether) are purchased from sigma Aldrich (Mumbai, India). Graphene powder, $FeCl_3$. $6H_2O$, Ammonium hydroxide solution (28.0-30% NH_3 basis) were procured from Sigma-Aldrich. Tetrafluoroterepthalonitrile, sodium acetate, mono-, di- and poly ethylene glycol were procured from Sigma Aldrich. The drug Gemcitabine was procured from sigma Aldrich (Mumbai, India).

3.4.2 Instrumentation and Analytical Conditions

HPLC having binary pump system with diode array detector and LC-Solutions software (Shimadzu, Japan) were used for developing the method. Phenomenex Synergi Fusion-RP (50×2 mm, 4 μm) column was used and column oven temperature maintained at ambient conditions. Methanol: 0.1% acetic acid in water: ACN (60:30:10) mixture was used as a mobile phase. FT-IR spectra were recorded on Perkin Elmer, USA using KBr pellets at ambient temperature. Powder X-Ray diffractograms of GO, MGO, and synthesized MGO-C[6]A were recorded by using Bruker AXS D8 diffractometer with Cu K_α radiation (1.5406 A°), step size 2mdeg and 0.5 s per step scan speed. The morphology of MGO-C[6]A was analyzed by FEI Apreo LoVac equipped with an Aztec Standard EDX System. The magnetic properties of samples were measured at room temperature by using vibrational sample magnetometer (VSM, Lakeshore 7400, West-erville Ohio, USA) in magnetic fields up to 15kOe.

3.4.3 Synthesis of MGO-C[6]A composite

Modified hummers method with a set of variations has been used for the synthesis of graphene oxide (GO) from graphite nanopowder. In brief, 50 mL of concentrated sulfuric acid was taken in to a 250 mL round bottom flask and 2 g of graphite powder was added slowly to this solution. Subsequently, 2 g of sodium nitrate was added to this black colored reaction mixture for initiating the reaction. To this reaction mixture, potassium permanganate was added slowly by maintaining the temperature less than 20^{o}C. The resultant mixture was stirred on magnetic stirrer for four hours at 60^{o}C. At this stage, 100 mL of water was added to reaction mixture and raised the temperature to 90^{o}C and then stirred for 20 to 25 min. The color of the reaction mixture was changed to bright yellow color at that time 200 mL of warm water and 20 mL of 30% H_2O_2 solution were added. The colour change indicates that the formation of GO and the formed solid GO was collected by using centrifugation and washed several times with 5% hydrochloric acid followed by water. The washed compound was dried for 15 hours in oven.

Solvothermal route is an ecofriendly and simple way to synthesize the magnetic graphene oxide (MGO). For the synthesis of MGO, 120 mL of diethylene glycol and 40 mL of mono ethylene glycol mixture was taken in 500 mL round bottom flask and 0.4 g of the synthesized GO was added to this and sonicated for 2 h for the better dispersibility. To this mixture, $FeCl_3$ (1.36g) was added and stirred for 25 minutes at room temperature. After that, 4.50 g of poly ethylene glycol and 16.20 g of sodium acetate were added and sonicated for about 30 min. Then the reaction mixture was transferred to a stainless steel autoclaves and the reaction was carried out 12 h at 180^{o} C. After completion of the reaction time, the formed solid product was collected by using centrifugation and washed many times with water and ethanol. Then the compound was dried at 50^{o}C for 12 h in oven.

The MGO-C[6]A composite was synthesized successfully by using in situ reaction (Figure 3.2). In this reaction, MGO, organic linker tetrafluoroterphthalonitrile and calix[6]arene (1:8:10) were taken in a round bottom flask containing dimethylformamide (DMF) solvent. The reaction mixture was deoxygenated by using nitrogen purging for 20 min. Then, the reaction was carried out in basic condition by using potassium carbonate at 120°C for 12 h. After the completion, the reaction mixture was cooled to room temperature. The formed solid compound was separated by using centrifugation at 4000 RPM for five minutes and was washed several times with HCl and water. Then the solid was washed with dichloromethane and kept in oven for 12 h for drying.

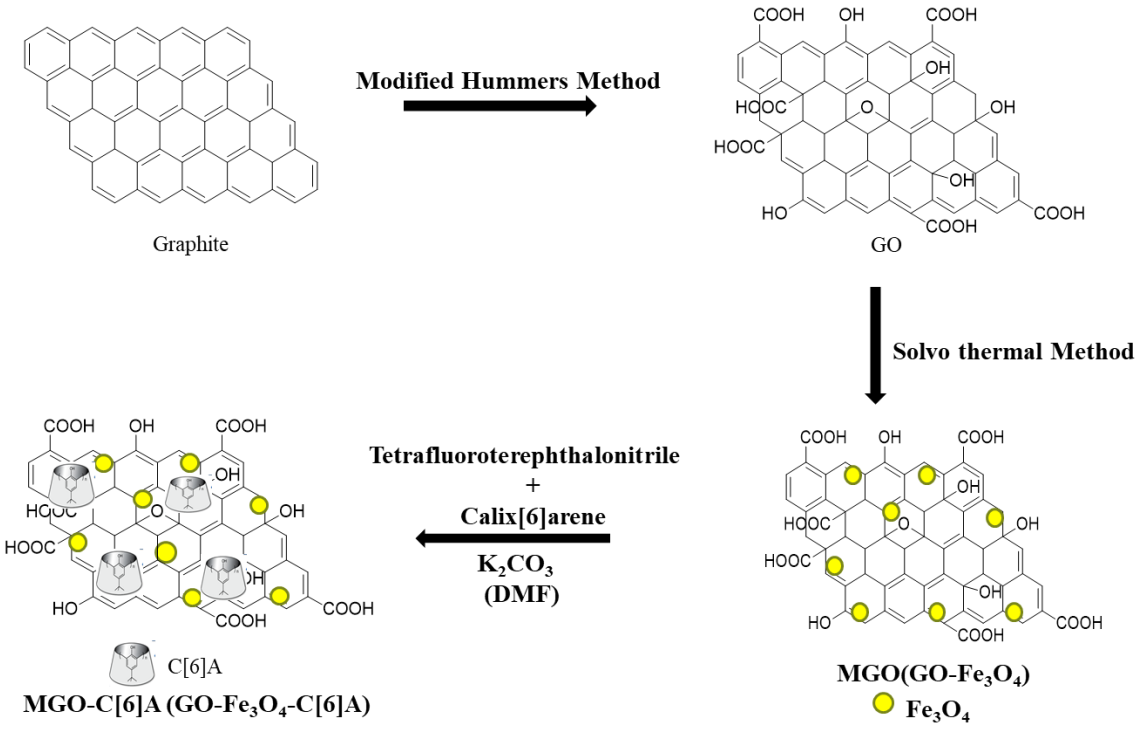


Figure 3.2 Schematic diagram for synthetic procedure of MGO-C[6]A.

3.4.4 Preparation of stock and standard solutions

Stock solution of gemcitabine (1 mg/mL) prepared by taking 100 mg in 100 mL volumetric flask and dissolved by using diluent (MeOH:H_2O:ACN (60:30:10)) and stored at -20^0 C. The appropriate working standard solutions were prepared daily by diluting the stock solution in diluent and stored

at room temperature. For the constructing a calibration graph, six different concentrations of GEM (0.5-25.0 µg/mL) were prepared by spiking the appropriate amount of working standard solutions with aliquots of plasma. For the preparations of three QC (low (0.5 µg/mL), middle (5.0 µg/mL) and high (25.0 µg/mL) level sample were spiked the appropriate amounts of working standard solution with aliquots of plasma.

3.4.5 Sample preparation

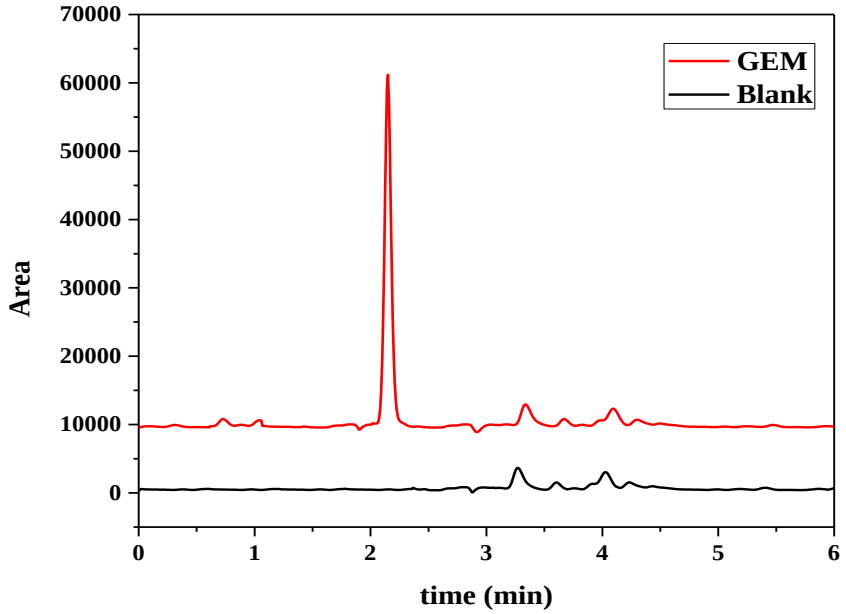


Figure 3. 3 HPLC chromatograms of blank and gemcitabine after SPE.

The sample solutions were prepared by adding 100 µL of working standard solution to 250 µL of plasma. Protein precipitation method was used for removal of protein from the plasma. In this method, 1 mL of ACN was added to 250 µL of human plasm sample, then vortexed for 5 min and centrifuged for 10 min with 12000 rpm at 4^{o}C. Then the supernant was collected in glass vial and evaporated the solution by passing N_2 gas at 40^{o}C in vacuum oven. For the reconstruction of sample, 1 mL of phosphate buffer (pH 7.0) was added to residue and vortexed for 2 min. 5 mg of adsorbent (MGO-C[6]A) was added to this solution and vortexed for 5 min then mechanically shaken for 30 min. The external magnet was used to separate the MGO-C[6]A and buffer solution.

For the extraction of analytes, 1 mL eluent solvent (ethyl acetate) was added to MGO-C[6]A, vortexed and sonicated for 1 min. The supernant was collected in a glass vial by using external magnet and evaporated by using N_2 gas. To this residue, 1 mL diluent was added for reconstruction of sample solution. The analytes containing sample solution was filtered with 0.45 µ nylon filter and 10 µL of this solution was directly injected into HPLC for the analysis (Figure 3.3).

3.5 Results

3.5.1 Characterization of MGO-C[6]A

The magnetic graphene oxide calixarene (MGO-C[6]A) composite was successfully synthesized as mentioned in experimental section and the formed GO, MGO and MGO-C[6]A was confirmed by taking the SEM, VSM, powder XRD, and FT-IR instrumental characterizations. The resulting FT-IR spectra were depicted in Figure 3.4.

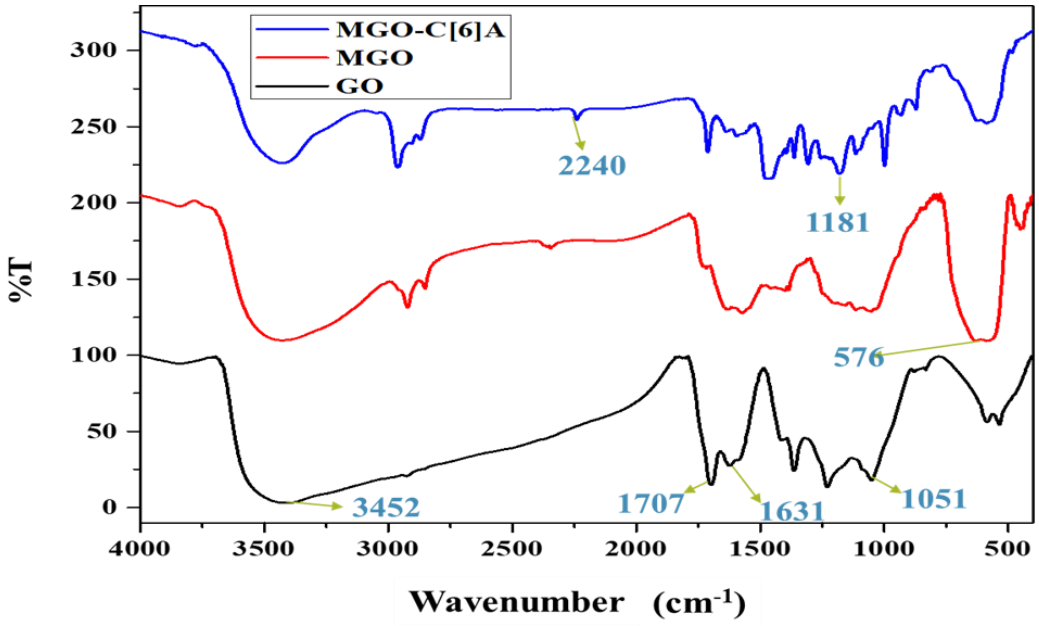


Figure 3.4 IR spectra of GO, MGO and MGO-C[6]A.

The peaks obtained in the FT-IR spectra of GO at 1707, 1631, and 1051 cm^{-1} corresponds to stretching frequency of -C = O in carboxylic group, stretching frequency of C = C in graphitic carbon, stretching frequency C-O that are presented on GO surface. The FT-IR spectrum of MGO

and MGO-CR showed a characteristic peak at 576 cm^{-1} which corresponds to Fe-O functional group vibration. The IR spectrum of MGO-C[6]A exhibited two peaks, one at 2240 cm^{-1} and other weak band at 1181 cm^{-1} which corresponds to stretching frequency of C ≡ N and C-F groups in the linker. These FT-IR results suggested the formation of MGO-C[6]A composite.

The morphology of formed GO, MGO and MGO-C[6]A were analyzed by SEM. The SEM images of GO revealed the flakes and layers like structures with folded and rippled wavy shapes. (Figure 3.5(a). Due to the oxidation process, the exfoliated GO sheet edges are crumpled. All these confirms the formation of exfoliated GO sheets from the graphite powder with stacked graphene layers.

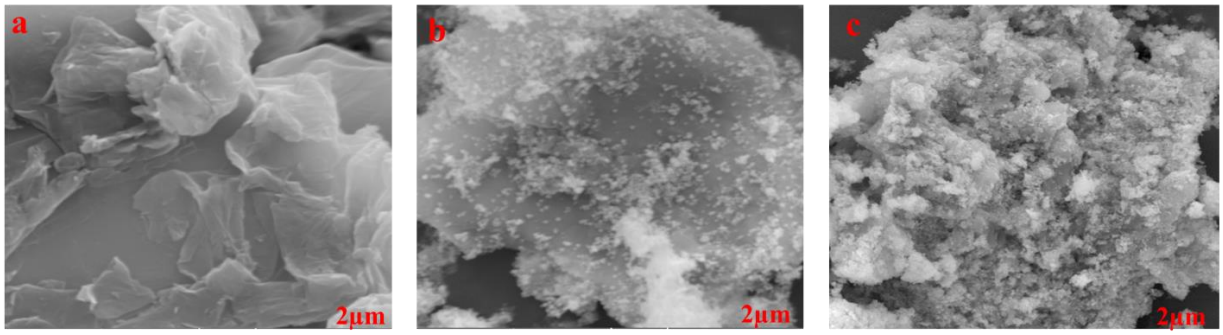


Figure 3.5 SEM images of (a) GO, (b) MGO and (c) MGO-C[6]A.

The SEM image given in (Figure 3.5(b) exhibited the shining dots of Fe_3O_4 that are appeared on thin layers of GO sheets. The thin layered GO sheets has a large matrix which helps in anchoring the Fe_3O_4 particles on it. The loading of Fe_3O_4 particles also increased due to the presence of a large surface area on the wrinkled GO. The Fe_3O_4 particles were deposited heterogeneously and distributed randomly in clusters on thin layers of GO sheets [47, 48]. MGO-C[6]A showed lamellar structure which is due to the distribution of Fe_3O_4 particles with calix[6]arene on GO sheets (Figure 3.5(c)).

The XRD diffractograms of GO, MGO and MGO-C[6]A were depicted in Figure 3.6. The obtained experimental results were compared with reported experimental results [49]. In general, the normal graphene show a diffraction peak at 2θ=26.6 with the basic plane (002) and d-spacing of 35 nm. The above mentioned graphite peak was disappeared in the XRD spectrogram (Figure 3.6(a) of synthesized GO and a new diffraction peak at 2 θ =11.06^{o} with crystal pane is (001) and d-spacing (0.85nm) was appeared. The main reason of increasing the d-spacing in GO is due to the addition of oxygen containing functional groups by the strong oxidization process and also intercalation of H_2O molecules on the graphene. The XRD diffractograms (Figure 3.6(b) and (c)) of MGO and MGO-C[6]A showed a six new diffraction peaks at (2θ = 30.1^{o}, 35.4^{o}, 43.3^{o}, 53.6^{o}, 57.1^{o} and 62.7^{o}) corresponding to the planes of (220), (311), (400), (422), (511) and (440) respectively and these are the facets of the face centered cubic spinel crystal planes of the Fe_3O_4 (JCPDS No. 19-0629). These results indicates the aggregation of Fe_3O_4 particles on GO surface and formation of MGO.

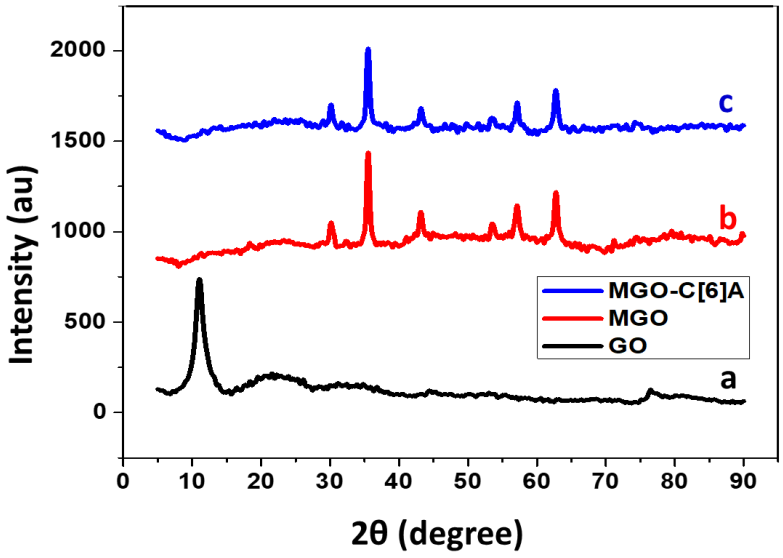


Figure 3.6 XRD diffractograms of (a) GO, (b) MGO and (c) MGO-C[6]A.

The magnetic properties of synthesized MGO and MGO-C[6]A were assessed by doing the VSM analysis at room temperature. The obtained magnetic hysteresis loops of MGO and MGO-C[6]A

showed S - shaped curves which passes through the zero point magnetization (Figure 3.7). These curves indicates the super magnetic nature of MGO and MGO-C[6]A. The saturation magnetization point of MGO-C[6]A was found to be at 38.42 emu/g and this value is sufficient for the separation of material by using external magnet.

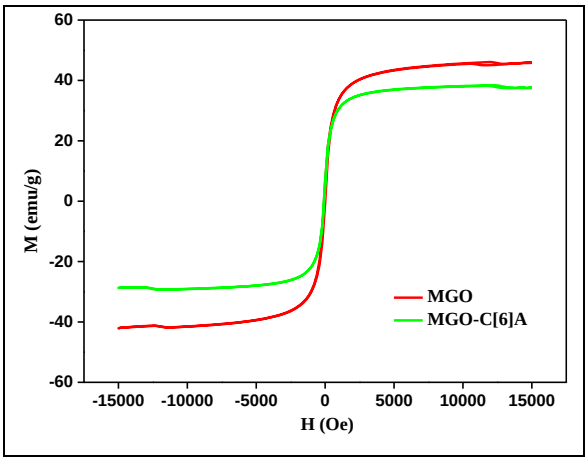


Figure 3.7 Magnetization hysteresis loops of MGO and MGO-C[6]A.

3.5.2 Optimization of MSPE conditions

In order to acquire better recoveries of target anlytes from plasma, various extraction conditions such as adsorbent amount (2-12 mg), eluent solvent (ethyl acetate, methanol, acetonitrile, chloroform, dichloromethane and tertiary butyl methyl ether), eluent amount (0.4-1.6 mL), extraction time (5-30 min) and desorption time (1-7 min) were optimized (Figure 3.8). The concentration of GEM in sample solution was taken as 10 µg/mL.

3.5.2.1 Effect of amount of magnetic solid phase

Amount of adsorbent plays a significant influence on the extraction of analyte from sample solution. In order to attain good adsorption efficiency towords the target analyte, different amounts (2-12 mg) of adsorbent were taken. Figure 3.8(a) shows that the results of adsorption rates at various adsorbent amounts. The amount of analytes adsorbed by the adsorbent was increased

sharply from 2-8 mg and then saturated. Based on these results, the optimized amount of adsorbent found as 8 mg which is sufficient to get good adsorption of target analytes.

3.5.2.2 Effect of the type of desorption solvent

The selection of desorption solvent plays a key role in the recovery percentages of analyte in the MSPE method. To ensure the better recoveries of analyte from the sorbent, six different organic solvents such as ethyl acetate, methanol, acetonitrile, chloroform, dichloromethane and tertiary butyl methyl ether were used in this analysis. The results are depicted in a histogram (Figure 3.8(b)). Among all, the organic desorption solvents ethyl acetate given a better recovery percentages of analytes from the sorbent. These results are might be related with the polarity of desorption solvents and analytes. The polarity sequence of desorption solvents is TBME < $CHCl_3$ < CH_2Cl_2 < Ethyl Acetate < ACN < MeOH. We have observed the slight change in recovery of gemcitabine in ACN and ethyl acetate when used as extraction solvents.

3.5.2.3 Effect of eluent amount

For attaining the good recovery percentages of analyte, the volume of desorption solvent amount (from 0.4 to 1.6 mL) were optimized and shown in Figure 3.8(c). The eluent solvent amount of 1.2 mL showed better recovery of analyte and slight decrease in recovery was observed above this limit. Therefore, 1.2 mL of ethyl acetate was optimized for better recovery and used same amount for the analysis.

3.5.2.4 Effect of adsorption and desorption time

The extraction and desorption time also influences the recovery percentage of analyte. For evaluating the recovery percentages of analytes, the extraction time between 5-30 min was optimized. At 20 min time interval, the high adsorption rate of analyte was observed. Hence, the adsorption time is 20 min was taken as optimized one and the results are shown in Figure 3.8(d).

And desorption time was also optimized by taking between 1- 7 min at different intervals. At 5 min of desorption time, the maximum recovery of analyte was obtained (Figure 3.8(e)). After this the recoveries were slightly increased and saturated. From these results, the optimized desorption time is fixed at 5 min.

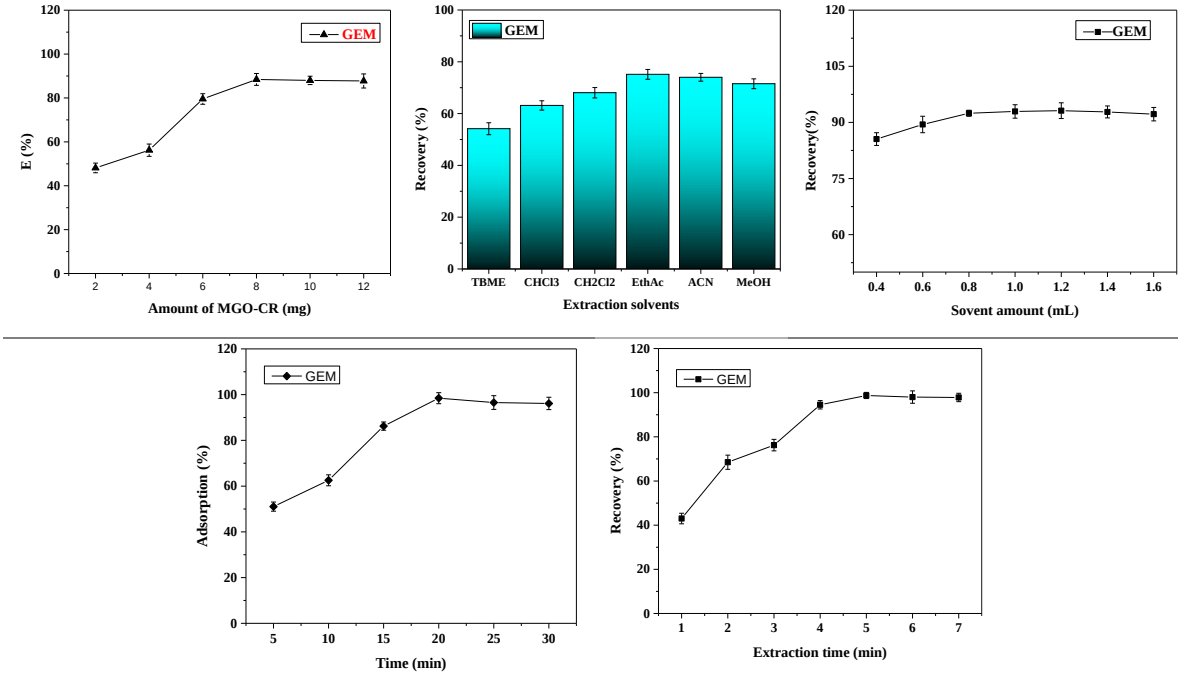


Figure 3.8 optimized extraction conditions (a) effect of amount of solid phase (b) effect of desorption solvent (c) effect extraction solvent amount (d) effect of adsorption time (e) effect of desorption time.

3.6 Preparation for real sample analysis

The developed magnetic solid-phase extraction method was successfully applied to the quantification of GEM from rat plasma after oral administration. Six different Wistar rats (250 ±20g) are allowed to adapt to the standard housing environment for three days prior to analysis. Prior to the experiment, the rats were fasted overnight and allowed to drink only water throughout the experiment. The rats were administered with 15 mg/kg of GEM individually and the blood was collected at different time points (0 to 6 hour) then centrifuged for 4 min at 12000 rpm. The

extraction of analyte from rat plasma was carried out by following the developed extraction procedure. The resultant plasma samples were stored at -20°C for the analysis. The mean plasma concentration vs time curves of GEM were shown in Figure 3.10. The pharmacokinetic parameters such as the peak plasma concentration (C_{max}), the time to C_{max} (t_{max}), elimination half-life ($t_{1/2}$), the AUC from 0 to infinity ($AUC_{0-\infty}$) the AUC from 0 to time (AUC_{0-t}), were calculated for each subject by the 'Ramkin' software. The pharmacokinetic parameters of GEM are shown in Table 3.1.

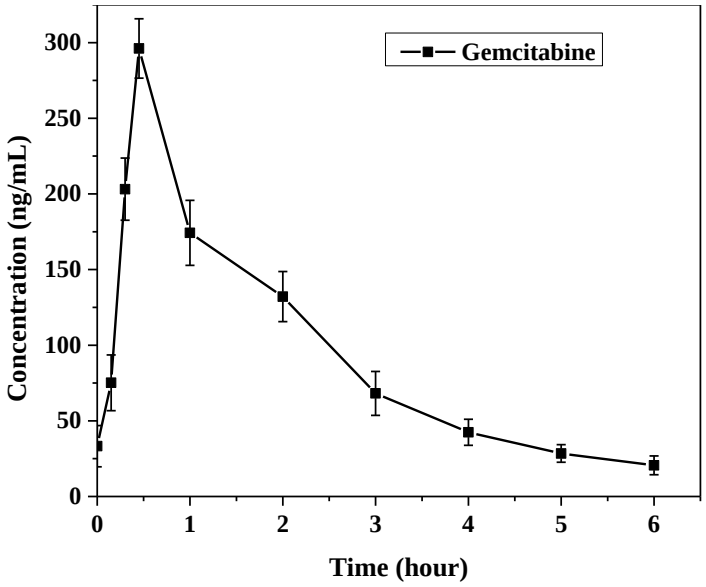


Figure 3.10 Concentration vs. Time profiles of GEM in plasma of rat (n=6).

Table 3.1 The pharmacokinetic parameters of GEM in rats after oral administration.

Parameter	Mean value
T_{max} (h)	0.45
C_{max} (ng/mL)	296
$AUC_{0\rightarrow t}$ (ng/mL/h)	564
$AUC_{0\rightarrow\infty}$ (ng/mL/h)	636
$t_{1/2}$ (h)	2.43

C_{max} (ng/mL): maximum plasma concentration
T_{max} : time to C_{max}
AUC: area under

3.7 Analytical method validation

3.7.1 Linearity

Calibration curve was obtained for GEM via plotting the concentrations against the peak areas of analyte. For the evaluation of linearity, six various concentrated (0.5-25 µg/mL) calibration solutions were prepared and the each sample ran parallelly for thee times. The acquired correlation co-efficient value of GEM is (0.9993) which is in the acceptable linear range (Table 3.2) and the results indicates the satisfactory linearity.

Table 3.2 Pharmacokinetic parameters of GEM in rats after oral administration.

Validation parameters	Results
Linearity range (µg/mL)	0.5-25.0
Calibration curve equation	Y = 18523 x + 9251
Correlation coefficient (R^2)	0.9993
LOD (ng/mL)	2.0
LOQ (ng/mL)	13.0

3.7.2 LOD and LOQ

Limit of detection (LOD) and limit of quantification (LOQ) of GEM was determined by using signal to noise ratio method. In this method, LOD's for the GEM was assessed by taking lower concentration of samples that can be distinguished with the signal to noise ratio of 3 and also LOQ was evaluated by using the signal to noise ratio of 10. The determined LOD and LOQ concentrations were 2 ng/mL and 13 ng/mL respectively (Table 3.2).

3.7.3 Precision and Accuracy

The overall performance of the method was evaluated in terms accuracy, interday and intraday precisions. The precision and accuracy has been assessed by using the optimized magnetic solid

phase extraction method to three replicate spiked human plasm samples at three different QC (low, middle and high) level samples of target analyte. The obtained relative standard deviation (RSD) values of accuracy, interday and intraday precisions for the spiked QC samples were summarized in Table 3.3. Intraday precision RSD values of GEM were found to be less than 4.6% and interday precision values are less than 6.8%. The above results confirms that the developed MSPE method has good precision and accuracy. The results also showed that the recovery percentage of GEM is in the range of 97.6-100.2%. Thus, this method is suitable for analysis of GEM in plasma sample.

Table 3.3 The resulting validation parameters of accuracy and precision.

Analyte	**Spiked value (µg/mL)**	**Accuracy**		**Precision (%RSD)**	
		Recovery (%)	RSD%	Inter - day	Intra - day
GEM	0.5	97.6	4.8	6.8	4.1
	5.0	100.2	5.1	5.9	4.6
	25.0	98.8	4.4	5.2	3.8

3.7.4 Robustness

The robustness of the method was analyzed by changing the HPLC method parameters. Flow rate (±0.2 mL), methanol percentage in mobile phase (±5), the detector wavelength (±5 nm) and column temrature (±5^{o}C) were changed for analyzing the robustness of method. The obtained RSD values are in between 1.2-4.6%, which means that no significant changes in results were observed. These results indicates that the developed method has efficient performance and reliability.

3.8 Discussion

The control analysis for the extraction of GEM were also performed by taking MGO, MGO-C[4]A and MGO-C[6]A separately at room temperature and compared. The obtained results showed that MGO- C[6]A exhibited significantly high recoveries of GEM when compared with remaining two adsorbents. Reusability analysis of developed magnetic adsorbent is the most important factor in

environmental and economic aspects. The factor of adsorbent reusability will help to reduce the need for new sorbent amount and decreases the waste of adsorbent which would make the developed SPE method more environmental and economically friendly. In order to evaluate the reusability of prepared adsorbent, they were washed with methanol (2.0 mL) and water (2 mL) and dried at ambient temperature after the extraction of analytes. Then, the material was reused for the extraction of analyte according to the developed sample preparation method. The obtained results showed that the adsorbent can be used up to six times without significant loss of extraction recoveries (<5%) of analytes. These results indicate that the developed magnetic adsorbent has great potential to reuse in the sample preparation.

In general, LC-MS/MS method show high sensitivity and selectivity in the analysis of drug molecules in comparison with HPLC method. But the problem with LC-MS/MS method is expensive equipment and solvents, therefore maintenance is burden to the institutions. Hence, the development of HPLC method is always economically viable and have several advantages. Solid phase extraction (SPE) method is widely used versatile method for the extraction of drug molecules from the biological fluids. In case of liquid-liquid extraction (LLE) methods, the extraction of drug molecules from biological samples needs high amount of organic solvents, show high interference from biological matrix and there is problem of co-elution along with main analytes. In comparison with LLE, SPE has high enrichment factor, high recovery percentages and easy to handle. There are several LLE based extraction methods were reported for Gemcitabine. Very few SPE based methods were reported for Gemcitabine and the comparison of our method with the reported methods were given in Table 3.4. The present method shown good recovery percentages with high precision, comparable detection limit and extensive linear range (see Table 3.4) [37–44].

Table 3.4 Comparison of previous reported method for gemcitabine.

Drug	Extraction method	Determination method	LR (μg mL^{-1})	LOD (ng mL^{-1})	RSD (%)	Recovery (%)	Ref
Gemcitabine	LLE	HPLC-DAD	0.1-18.0	0.3	≤7.0	81.2-87.6	[50]
Gemcitabine	LLE	LC-MS/MS	0.125-40.0	-	1.30-4.70	92.-111.0	[51]
Gemcitabine	LLE	HPLC-UV	20.0-150.0	-	<2	97.0	[52]
Gemcitabine	LLE	HPLC-UV	250.0-1000	220	<2	-	[53]
Gemcitabine	LLE	HPLC-UV	0.5-50.0	0.15	<2	100.2-100.4	[54]
Gemcitabine	SPE	LC-UV	0.01-50.0	-	3.8-6.4	95.6-103.6	[55]
Gemcitabine	SPE	UPLC-MS/MS	0.005-4.0	-	0.9-10.4	81.4-100.8	[56]
Gemcitabine	Oasis® HLB	LC-MS	0.005-1.0	-	0.3-7.0	86.8-89.4	[57]
Gemcitabine	MSPE	HPLC-UV	0.5-25	0.002	3.8-6.8	97.6-100.2	Present

3.9 Conclusions

The proposed method is convenient, quick, reusable and eco-friendly due to less amount of organic solvents used. All these advantages make the method suitable for real plasma sample analysis. In conclusion, a simple, rapid and sensitive magnetic solid phase extraction method was developed for analyzing the anticancer drug GEM in plasma sample. The developed magnetic graphene oxide calix[6]arene composite showed efficient extraction and high recovery percentage from plasma solution due to hydrophobic and π-π interactions. The proposed method is convenient, quick, reusable and eco-friendly due to less amount of organic solvents used. All these advantages make the method suitable for real plasma sample analysis.

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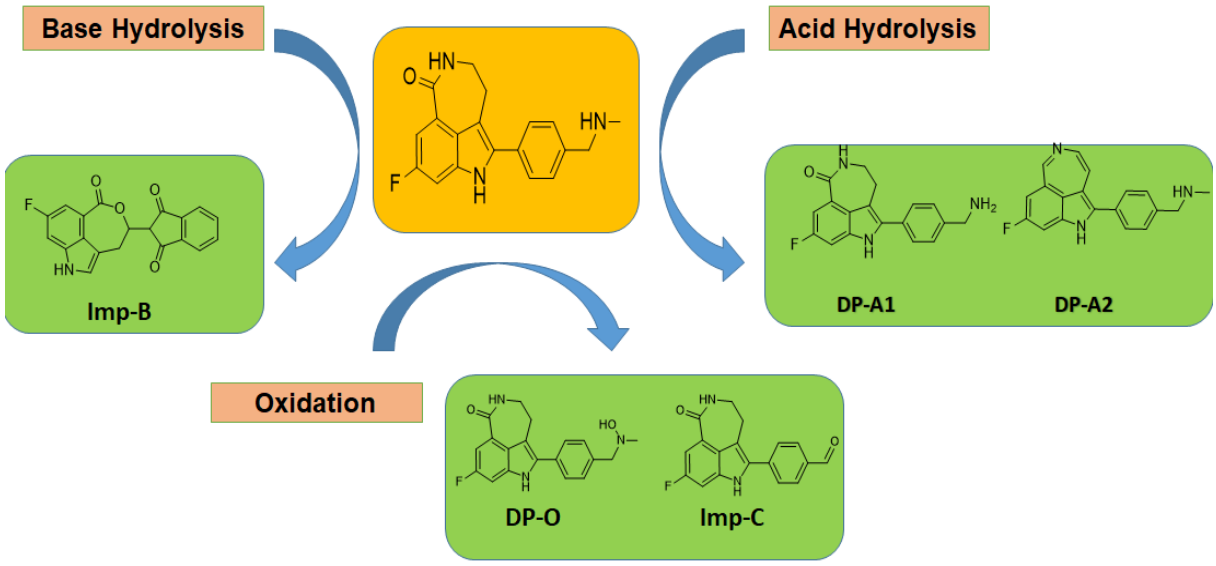
Chapter –IV

Development and validation of LC-MS method for the identification and quantification of forced degradation products of rucaparib

Abstract

A reversed-phase HPLC (high performance liquid chromatographic) method is employed to identify and quantify the degradation impurities and related substances of Rucaparib (RCB), API bulk drug. The present study focused on understanding the degradation phenomenon of RCB. The chromatographic separation is achieved with in 25 min run time, where Zorbax Bonus RP column is used with a gradient elution of trifluoroacetic acid (TFA) – ACN –water as mobile phase. Oxidative, basic and acidic stress conditions showed significant impact on degradation compared to thermal and photolysis. The LC-MS technique is used for the characterization of the degradation products and the plausible pathways of fragmentation were proposed. Qualified reference standards were used to quantify the stressed samples. Results of this investigation confirmed the efficacy of the proposed new method to determine the RCB drug stability ICH guidelines were also considered to authenticate our results.



Schematic diagram for stability studies of Rucaparib.

4.1 Introduction

Cancer is the second leading cause of death in the world, followed by heart disease and heart stokes. In every year 10million peoples at worldwide were diagnosed with various type of cancers, in all of them half of the peoples has been dying. Due to the significant development of treatment and prevention of heart related diseases cancer will become number one cause to death in various places at worldwide. Cancer is the name assumed to a group of related ailments. Cancer is nothing but the unstoppable growing of body cell in any pars of the body and these are cause damages to adjacent tissue and damage of DNA. In general, compared with younger peoples elders have more chances to susceptible to cancer, and also many countries have been facing the problem from population aging around the worldwide hence, it will make some critical. The main five behavioral reasons are responsible for people getting cancer. Those are 1. Tobacco use 2. Alcohol use 3. High body mass index 4. Low vegetable and fruit intake 5. Lack of physical activities. And also the following three external factors causes to fall ill with cancer

1. Physical carcinogens (ionizing radiation and UV radiations)
2. Chemical carcinogens (a food contaminant of aflatoxin, and arsenic drinking water contaminant)
3. Biological carcinogens (infections from bacteria, virus, and parasites)

Different type of cancers are known, for example, Sarcomas is the type of cancer that arises from the supporting tissues of the body such as cartilage, connective tissue, bone, fat and muscle. Lymphomas arises from the tissue of the body immune system and lymph nodes. Leukemia is the other type of cancer that arises from growing of blood cells in bones marrow and tend to accumulate in great numbers in the bloodstream of the body. The most common type of cancer is Carcinoma**,** it cover the external or internal body surface cells such as Prostate, lungs, colon and breast cancers.

Most of the cancers can be prevented (30-50%) by avoiding risk factors such as alcohol consumption, smoke etc., and implementing the available evidence based prevention strategies. The diagnosis of cancer play a major role to prevent the cancer by using chemotherapy, surgery or radiotherapy. The ovarian cancer is one type of carcinoma. This is highly noticed in the women at the age of 40 years in the United States and UK [1, 2]. Ovarian cancer (OC) is the fifth leading cause of cancer-related deaths in women. In general, the treatment procedures depend on type of ovarian cancer and its stages in specific conditions. The advanced stage of ovarian cancer can be treated by the RCB [3]. RCB is an US FDA (Food and Drug Administration) approved orphan drug for the landmark treatment of female patients with certain types of ovarian cancer [4]. RCB is a type of PARP (Poly (ADP-Ribose) Polymerase) inhibitor [5, 6], which is an enzyme that normally helps in the repair of damaged DNA cell through the activation of base excision repair and alternative end-joining pathways and inhibition of the non-homologous end-joining pathway [7–9]. The BRCA gene is responsible for the repair of damaged DNA and usually serves to prevent tumor growth. However, its genetic mutations can lead to specific cancers such as ovarian cancer [10, 11]. The patients in the advanced stage of ovarian cancer, i.e., if mutation in BRCA is detected even after one platinum-based chemotherapy, may be suitable for treatment with RCB [12, 13].

In pharmaceutical industries, stress studies of API drugs play an important role during the storage and formulations. The forced degradation studies of drug product/related substances will give the impurity profile, which is useful to know the stability of drug under the influence of light, temperature and humidity. During the forced degradation studies, the drug loses its efficiency and forms some new drug related impurities. Based on these studies, the adverse effects of drug due to the formation of toxic degradation compounds can be understood. During the synthesis of RCB, the process related substances A, B, and C (Table 4.1) were observed. Hence, there is a great need

to develop a new HPLC method for determination of RCB, related substances along with its degradation products.

4.2 Literature Survey

Few stability indicating and extraction methods were reported for RCB in the form of bulk drugs and formulations. Stability indicating methods were reported for the identification, quantification and characterization of drug molecules, relative impurities and degradation products. Extraction methods were reported for the extraction, identification and quantification of the drug molecules and relative substances from biological fluids such as blood, urine, plasma and serum.

Very few HPLC and LC-MS methods were reported for the identification and quantification of RCB in biological fluids. For example, M.A.C. Bruin et al., developed LC-MS/MS method for the RCB with another four PARP inhibitors olaparib, talazoparib, niraparib and veliparib. It is a gradient reverse phase method, 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) were used as mobile phase with Acquity UPLC BEH C18 column (particle size 1.7µm, 100 × 2.1mm) [14]. Rolf W. Sparidans et al. developed a LC-MS bio analytical method for the extraction and determination of RCB from plasma sample. In this reverse phase LC-MS method, Polaris 3 C18 column (50×2mm, 3 µm) and mobile phase of 0.02% formic acid (A) and methanol were used and the method showed good recovery, accuracy and precise values [15]. Vamseekrishna Gorijavolu et al., have developed a LC-MS method for the analysis of RCB from human plasma. In this isocratic reverse phase method, mobile phase of10Mm ammonium formate, methanol in the ratio of (20:80) and X-bridge C18 (50 x 4.6, 5 µm) column were used. Rucaparib-d3 was used as internal standard in this analysis [16]. Diane D et al., have introduced pharmacokinetic and pharmacodynamics models for rucaparib PARP inhibitor drug and assessed the PARP activity in tumor tissue and peripheral blood lymphocytes [17]. Bernard T. Golding was

wrote the entire history about rucaparib [18]. Mitchell S et al., have summarized the FDA approval of rucaparib anti-cancer drug for the treatment of BRCA mutated prostate cancer in patients [19]. Gilberto S et al., have developed ^{1}H-MRS (proton magnetic resonance Spectroscopy) method for the detection and identification of the changes in liver tissue and NAD (nicotinamide adenosine diphosphate) levels in cells due to the usage of rucaparib [20]. Zhen Chen et al., have examined the efficiency of the rucaparib to overcome ATP binding cassette mediated multidrug resistance in cervical cancer cells [21]. Nicoletta Colombo et al., have investigated the safety and efficiency of rucaparib and their effect with respect to age in phase-III [22].

Stability indicating method is a method that detects and quantify the active ingredients without any interference from the relative substances, process impurities, degradation products and other excipients. The method that can detect and quantify the degradation products accurately is considered as a stability indicating method. Initial stages of drug development process must need to study the stability of drugs in different conditions. At the early stages of a new drug development, an effective approach to develop a new stability indicating HPLC method is forced degradation study, it is the first step before method development. If forced degradation studies are performed early, the unknown degradation impurities and relative substances are run parallel in the developed method. These stability indicating methods can be useful to identify the degradation path way and developing a validated HPLC methods simultaneously.

4.3 Forced degradation studies

Forced degradation studies provide the information of drug products and API's degradation path ways, it also facilitate the analytical methodology for API and drug product. The forced degradation studies will produce the following supporting data in the prospective of regulatory affairs.

- The intrinsic stability of drug molecules and degradation path ways
- Identification and quantification of degradants
- Validation of developed stability indicating analytical method.

The requirement of forced degradation study depends on the stage of drug development. For example, the drug in phase-II clinical trial needs a high penetrating method development because the rate of compound attrition is high at this stage. Hence, a rational design of forced degradation studies needs no focus on isolation and identification of degradation products. After phase-II, the compound progresses for registration, at this stage the method development activities should be optimized. The focus of stress testing is directed to elucidation and characterization of degradation products. The forced degradation studies of API, in solid or liquid state will be held in accordance with the ICH guidelines [23–25]. These are useful in developing a new stability indicating method for appropriate active pharmaceutical ingredient (API). The stated stress conditions should result in approximately 5 - 20% degradation of the API. Depends on chemical characteristics of API, the specified conditions such as time duration and intensity are used. The percentage of degradation of sample will be evaluated by comparing with unstressed sample and blank.

4.3.1 Acid hydrolysis

In general, acid hydrolysis was performed by using HCl (0.1-2 N) in sample solution. The insoluble and partially soluble API will be dissolved by using co-solvents or by changing the pH of the acidic solution. The selection of appropriate co-solvent will depend on the chemical structure of the API.

4.3.2 Base hydrolysis

The base hydrolysis stress study was performed by using NaOH (0.1-2 N) in sample solution and insoluble or partially soluble API will be dissolved by using co-solvents or by changing the pH of

the basic solution. The selection of appropriate co-solvent will depend on the chemical structure of the API.

4.3.3 Oxidation

In general, oxidation degradation study of drug was carried out by using the free radical initiators such as 2, 2'-azobisisobutyronitrile (AIBN) and hydrogen peroxide (H_2O_2). As mentioned earlier, insoluble or partially soluble APIs may need co-solvents.

4.3.4 Thermal stability

Thermal stability test for solid API was performed under the 50-100^oC temperature. Some APIs may change the phase at high temperatures, in such cases thermal stability will be tested below the critical temperature to avoid any phase changes. The time duration for this study depends on sensitivity of API.

4.3.5 Photo stability

The photo stability studies of API was conducted based on ICH guidelines [26]. Based on ICH guidelines, the sample should be exposed to the light not less than 1.2 million lux hours and the intensity of integrated UV wavelength should be no less than Wh/m^2. In general, the sample will be exposed to the length of 2x ICH exposer length for adequate exposure. In general, acetonitrile is preferred as a co-solvent over methanol because methanol forms more artefact degradation products due the formation of methoxy radicals in the presence of light.

In the present study, RCB and its related substances were considered for the new analytical method development and validation. The chemical structures and names of the process related impurities of RCB is depicted in Table 4.1.

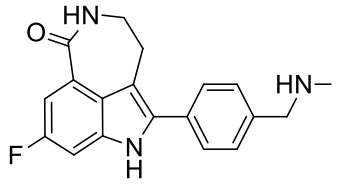
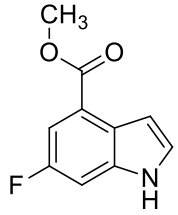
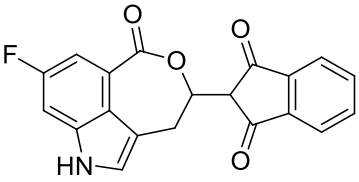


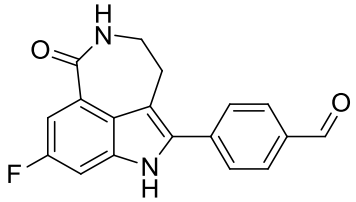
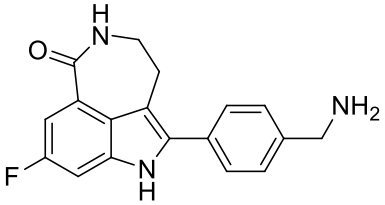
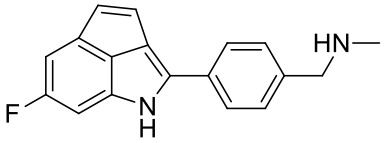
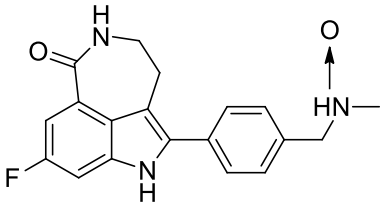
Figure 4.1 Chemical structure of Rucaparib.

4.4 RCB drug information

IUPAC name	:	6-fluoro-2-[4-(methylaminomethyl) phenyl]-3, 10-diazatricyclo [6.4.1.04, 13] trideca-1, 4, 6, 8(13)- tetraen-9-one
Molecular formula	:	$C_{19}H_{18}FN_3O$
Molecular weight	:	323.4 g/mol
Brand name	:	Rubraca
Maximum daily dosage	:	600 mg
Dosage strength	:	200, 250, 300 mg
Dosage form	:	Tablet

Table 4.1 chemical structures and names of the process related impurities of RCB.

Impurity	**Chemical name**	**Structure**	**Source of impurity**
Imp - A	Methyl 6-fluoro-1*H*-indole-4-corboxylate		Process related impurity
Imp - B	2-(8-fluro-6-oxo-1,3,4,6-tetrahydooxepino[5,4,3-*cd*]indol-4yl)-1*H*-indene-1,3(2*H*)-dione		Process related impurity

Imp - C	4-(-8-fluoro-6-oxo-1,4,5,6-tetrahydo-1*H*-azepino-[5,4,3-*cd*]indole-2-yl)benzaldehyde		Process related impurity
Imp - A1	2-(4-(aminomethyl)phenyl)-8-fluoro-4,5-dihydro-1*H*-azepino[5,4,3-*cd*]indol-69(3*H*)-one		Degradation impurity in acidic condition
Imp - A2	1-(4-(6-fluoro-1*H*-cyclopenta[*cd*]indol-2-yl)phenyl)-*N*--methylmethanamine		Degradation impurity in acidic condition
Imp - O	8-fluoro-2-(4-((methylamino)methyl)phenyl)-4,5-dihydro-1*H*-azepino[5,4,3-*cd]*indol-6(3*H)*-one-n-oxide		Degradation impurity in oxidation

LC-MS (Liquid chromatography-tandem mass spectrometry) has been used for bio-analytical assay method development of RCB in plasma [15]. In stress studies, LC-MS is becoming an emerging and adaptable tool for the determination of drug impurities and degradation products [27]. To date, no systematic characterization and mechanistic pathway approach have been developed for the degradation of RCB under the stress conditions as specified by ICH Q1A (R2) [28]. The main purpose of this study is to examine the degradation behavior in several conditions and to define degradation product of drugs. Herein, drug fragmentation patterns and their degradation products were established by exposing the drug to ICH suggested oxidation, thermal,

photolytic and hydrolysis stress conditions and analyzed the resultant solution through LC-MS, MS / MS, MS^n and precise mass measurements.

4.5 Experimental

4.5.1 Chemicals and Reagents

RCB and three impurities (A, B and C) were received from Mylan laboratories Pvt. Ltd, Hyderabad, India,). HPLC grade ACN was purchased from Merck (Mumbai, India) and AR grade H_2O_2 (Hydrogen Peroxide), HCl (Hydrochloric acid), NaOH (Sodium Hydroxide) and CF_3CO_2H (TFA) were purchased from S.D. Fine Chemicals (Mumbai, India). The Millipore synergy apparatus (Millipore, France) was used for the water purification.

4.5.2 Instrumentation

The method development, validation and simulated degradation studies were carried out on HPLC (Alliance Waters 2695) system along with DAD (Diode Array Detector). Zorbax Bonus Reverse Phase (Agilent, USA) column with particle size 3.5 µ & 150 X 4.6mm was used for the separation of the compounds. HP-Vectra (Hewlett Packed, Waldron, Germany) computer system along with Millennium data software was used for chromatographic data recording. The Agilent1100 online ion trap MSD mass spectrometer along with an auto-sampler (G1329A), APCI source in +ve mode and Diode Array Detector G1315B (Waldbronn, Germany, Agilent Technologies) was used to perform the LC-MS/MS. The mass data was obtained using a Q-TOF- HRMS (High-Resolution Mass Spectrometer) fortified with an ESI source (QSTAR XL, MDS Sciex /Biosystems, USA) and QS software.

4.5.3 Stress studies

Hydrolysis, Oxidation, Thermal and Photolytic methods were employed as stress conditions as per ICH recommendation for degradation studies. The photolytic study was carried by using 1.2

million lux hours of visible light on thin layer of RCB for 11 days [29]. Thermal stress study was performed for 10 days at 105°C in vacuum oven. The base and acid stress studies were done at ambient temperature (25 ± 2°C) with 1N NaOH for 42 hours and 0.1N HCl for 24 hours. The oxidation stress study was carried out for 24 hours at room temperature with 1% H_2O_2 solution. RCB reference standards were used to quantify all the stressed samples. Photo Diode Array (PDA) was used for the examination of the spiked samples of RCB and the peak purity of RCB in stressed samples with its known related impurities. The LC-MS was used to check the mass numbers of the unknown degradation impurities.

4.5.4 Chromatographic conditions

The water: TFA and ACN: TFA in the ratio of 100:0.10 (v/v) were used as a mobile phase A and mobile phase B in the gradient LC method respectively. Zorbax Bonus RP column was used to achieve the chromatographic separation. A flow rate of 1.0 mL/min was used and the LC gradient program is set as (T/%B) = 0/40, 10/50 and 20/90 and 25/90 which changes every 5 minutes of its 25 minutes run time. A constant column temperature of 40°C is maintained throughout the study. The injection volume and the detection wavelength are set at 10 µL and 245 nm respectively. The diluent is a mixture of ACN and water in the ratio of 50:50.

4.5.5 Mass spectrometric conditions

The same conditions used in LC were applied to LC-MS method (4.0 kV capillary voltage, 300°C source temperature, 600 L/h gas flow rate). The characteristic source conditions were: 5.00 kV capillary voltage, (4 kV for +ve mode); 60 V de-clustering potential; 220V focusing potential; 10V second de-clustering potential; 10,000 resolution (full-width half-maximum). Ultra-high pure Nitrogen gas was used as a collision and carrier gas, zero air was used for nebulizer. The precursor ion was chosen for the experiments of CID (Collision Induced Dissociation), Quadruple analyzers

was used for analyzing the ions of product. Photostability study was performed in a photostability UV chamber (Leicestershire, Sanyo, UK). Stress studies under hydrolysis conditions were carried out in Cintex Digital Water Bath and Cintex Dry Air Oven was used for thermal stress studies.

4.6 Preparation of standard sample solutions

For the development of an assay for RCB and its related impurities, the stock solutions of (500 µg/ml) were prepared by dissolving appropriate amounts of impurities (A, B and C) and RCB in the diluent. According to ICH Q3A guidelines, the impurities should not exceed more than 0.15% in a drug substance. Hence, the standard solutions were prepared by spiking the appropriate amounts of impurity solutions to drug solution. All three known impurity solutions were spiked at the level of 0.15% w/w to target analyte concentration (RCB-500 µg/mL) for the analysis.

4.6.1 Preparation of RCB test solution

The RCB drug solution was prepared by taking 50 mg of RCB in a 50 mL standard flask with diluent ACN:Water (50:50). For the estimation of impurities, 500 µg/ml concentrated RCB solution was taken.

4.6.2 Preparation of sample solution for stress studies

The stress study was carried out by taking single batch RCB sample as it helps in the uniform degradation of material. Forced degradation studies (oxidation, hydrolysis, thermal and photo degradation) were carried out by taking single batch RCB drug substance. After the degradation, the samples were neutralized and diluted by using diluent. The samples were filtered by using 0.45 µ nylon membrane filters before introducing in to the HPLC.

4.6.3 Preparation of acid hydrolysis sample solution

5mL of 0.1 N HCl was added to a sample solution containing 5 mL of RCB (1 mg/mL) in 10mL round bottom flask and the resultant solution was continuously stirred for 24 h at 25±2°C. After

completion of reaction time, the solution was neutralized by using 0.1 N NaOH and diluted. The diluted solution was directly injected in to HPLC for Identification of degradation products.

4.6.4 Preparation of base hydrolysis sample solution

5 mL of 1.0 N NaOH solution was added to a sample solution containing 5 mL of RCB (1 mg/mL) in 10 mL round bottom flask and the solution was stirred for 42 h at 25± 2°C. After the completion of 42 h, the solution was neutralized by using 1.0 N HCl solution and diluted. The diluted solution was filtered by using 0.45 µ nylon membrane filter and injected in to HPLC for the identification of degradation products.

4.6.5 Preparation of thermal sample solution

10 mg of solid RCB was taken on a petri dish and spread the sample uniformly as a thin layer. The petri dish with the sample was placed in vacuum oven at 105ºC for 10 days. After completion of 10 days, the sample was taken from oven and dissolved by using diluent. The solution was filtered by using 0.45 µ nylon membrane filter and injected in to HPLC for the analysis of degradation products.

4.6.6 Preparation of oxidation sample solution

The sample solution for oxidation was prepared by adding 5 mL of 1% H_2O_2 to 5 mL of RCB (1 mg/mL) sample solution taken in 10 mL round bottom flask. The obtained mixture was kept on magnet stirrer and continuously stirred for 24 h at room temperature. After completion of 24 h, the sample solution was diluted and filtered by using 0.45 µ nylon membrane filter, and injected in to HPLC for the identification of degradation products.

4.6.7 Preparation of photolytic sample solution

10 mg of solid RCB drug substance was taken on a petri dish and spread the sample uniformly as a thin layer. The petri dish with the sample was placed in UV chamber and maintained 1.2 million

lux hours of visible light for 11 days. After completion of 11 days, the sample was taken from UV chamber and dissolved in diluent. The resultant solution was injected in to HPLC for further analysis of degradation products.

4.7 Method development and optimization

The chromatographic method development for the separation of any compounds depends on their physicochemical properties such as polarity, molecular weight, hydrophilic or lipophilic nature, acidic or basic nature, etc. RCB is slightly basic and polar compound due to the presence of amino groups. The usage of normal phase columns for the RCB make the elution critical due to strong interactions between the amino groups of RCB and the hydroxyl groups on silica. Therefore, the reverse phase elution method was chosen for the current studies. Most of the pharmaceutical drug molecules were analyzed by using reverse phase method and the samples with higher molecular weights (~2000) are separated by using size exclusion chromatography. Since the molecular weight of RCB is less than 2000, the adsorption chromatographic method was adapted for the analysis of RCB.

Selection of stationary phase is a most important influenced factor for the separation of compounds which depends on its particle size, column diameters, pore size, and volume. The main objective of our method is the determination and identification of all degradation products and potential impurities from the analyte (B & C are critical impurities) and also to obtain the better peak shape for RCB. In the initial attempts of method development, numerous diverse stationary phases and mobile phases were used and the results were given in Table 4.2 and the corresponding LC chromatograms has been showed in Figure 4.2. Zorbax Bonus RP column is suitable in the separation of basic compounds [30]. By using Zorbax Bonus RP column (150 X 4.6 mm and 3.5 µm) as a stationary phase and mobile phase solutions A and B, satisfactory the resolution of closely

eluting impurities and peak shapes were achieved and the resulting LC chromatogram were shown in Figure 4.3. The stationary phase played a major role in achieving the resolution between RCB, Imp-B and Imp-C. Highly polar amide groups embedded in the stationary phase and end-capping procedures are the key in the deactivation of unwanted silanol interactions and in achieving the good resolution. TFA is one of the best acidic additive found to show the ability to adjust the selectivity with regard to the strength in the mobile phase [31–33]. Kailin Guan et.al reported that zwitterionic and weak basic compounds could be separated by modulating TFA concentration in mobile phase [34]. Therefore, TFA is used in mobile phase in the current study as RCB is a weakly basic compound. The results depicted that the best resolution in peaks and good peak shape were achieved. The gradient program also played a significant role for the separation of Imp-B & C from the RCB. The optimized gradient program was found to be suitable for achieving low retention time of the RCB. The RCB tailing factor was observed as ~1.0 at column temperature 40°C and its three potential impurities were well isolated with a resolution greater than 2. The characteristic chromatogram of RCB and its associated impurities is shown in Figure 4.3.

Table 4.2. Results of LC method development during the optimization.

Trial	Column	Dimensions	Mobile phase	Conclusion
1	Zorbax SB-C18	150 X 4.6 mm 5.0 µ	Acetate buffer with pH 2.5/Acetonitrile	Imp-B and Imp-C were co-eluted with analyte
2	Zorbax XDB-C18	150 X 4.6 mm 3.5 µ	Water/Acetonitrile/ Trifluoroacetic acid	Imp-C was co-eluted with analyte
3	Zorbax XDB-C8	150 X 4.6 mm 3.5 µ	Water/Acetonitrile/ Trifluoroacetic acid	Imp-C was co-eluted with analyte
4	Zorbax SB-Cyano	150 X 4.6 mm 3.5 µ	Water/Acetonitrile/ Trifluoroacetic acid	Imp-B and Imp-C were co-eluted with analyte
5	Zorbax SB-Phenyl	150 X 4.6 mm 3.5 µ	Water/Acetonitrile/ Trifluoroacetic acid	Imp-C was co-eluted with analyte

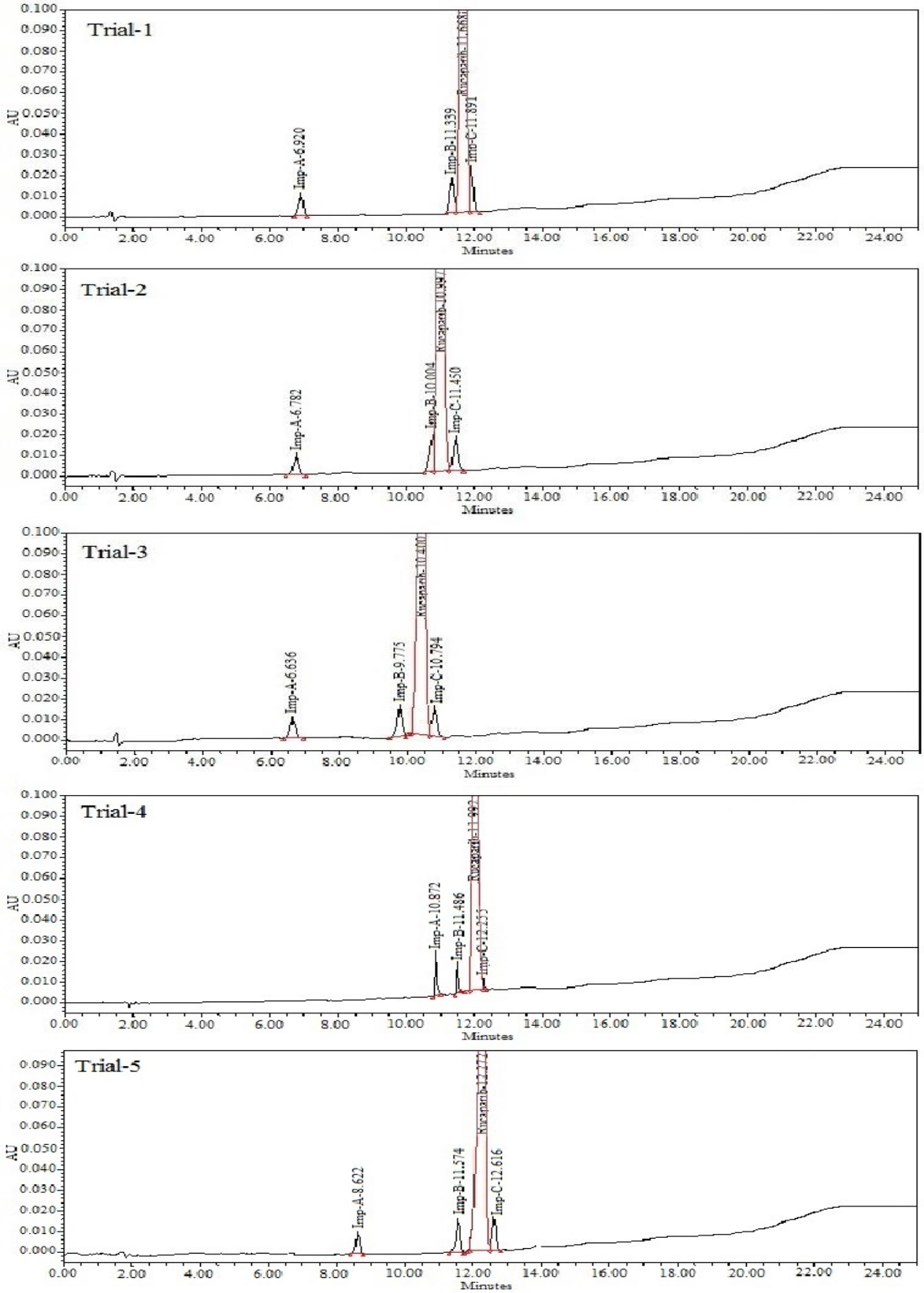


Figure 4.2 LC Chromatograms during the method development.

Table 4.3 Optimized chromatographic conditions.

Method Parameters	Optimized conditions
Column	Zorbax Bonus Reverse Phase column with particle size 3.5 µ & 150 x 4.6mm
Column oven temperature	40°C
Mobile phase	Mobile phase (A): Water: TFA (100:0.10) Mobile phase (B): ACN: TFA (100:0.10)
Mode of elution	Gradient
Gradient program	Time (min) / % Mobile phase (B): 0.01/40, 10/50, 20/90, 25/90.
Wavelength	245nm
Diluent	ACN: Water (50:50)
Flow rate	1.0mL/min
Injection volume	10 µL
Run time	25 minutes

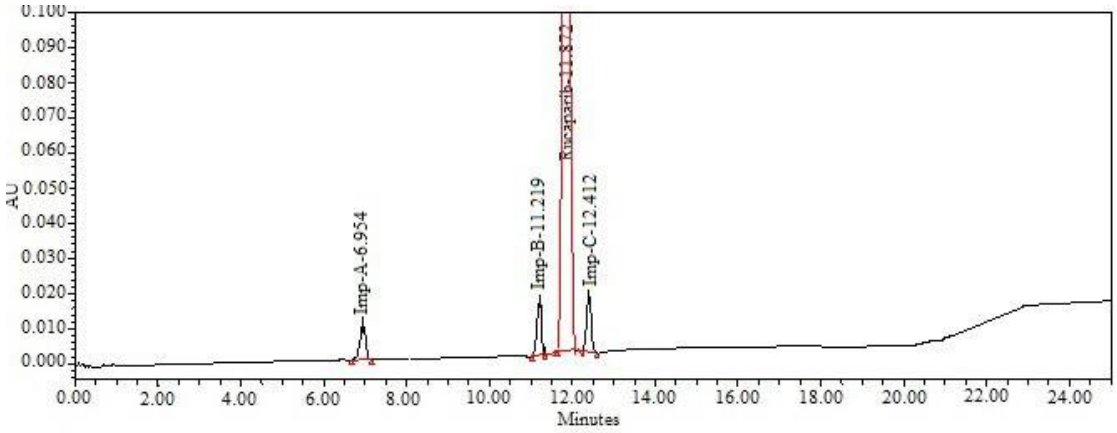


Figure 4.3 LC- Chromatogram for RCB and its related impurities after optimization.

4.8 Validation

As per ICH guidelines, the developed analytical LC method was validated for determination of RCB and its related substances[35].

4.8.1 System suitability test

In chromatographic methods, system suitability test plays a vital role to corroborate the adequacy of resolution, reproducibility, and column efficiency of the chromatographic system for a particular analysis. The studies were conducted using Zorbax Bonus RP column and the test results are tabulated in Table 4.4.

Table 4.4 Results of system suitability test.

Compound	RT (min)	RRT[a] (n=3)[c]	USP resolution R_s[b] (n=3)[c]	USP tailing factor T (n=3)[c]	No. of theoretical plates (*N* tangent method)
Imp-A	6.9	0.58±0.01	--	1.0±0.05	46,308
Imp-B	11.2	0.94±0.01	3.03±0.32	1.0±0.02	59,078
Imp-C	12.4	0.96±0.01	4.01±0.41	1.0±0.03	65,751
RCB	11.9	1.0	3.15±0.57	1.0±0.02	71,124

[a] Relative retention times (RRT) were calculated against the retention time (RT) of RCB.
[b] Resolution calculated between two adjacent peaks.
[c] Mean ± SD.

4.8.2 Specificity

The specificity of RCB was checked in the presence of its degradation products and its potential impurities. The products and impurities were separated from the peak of RCB with a specified resolution as shown in Figure 4.3 and the peak purity was shown in Figure 4.4.

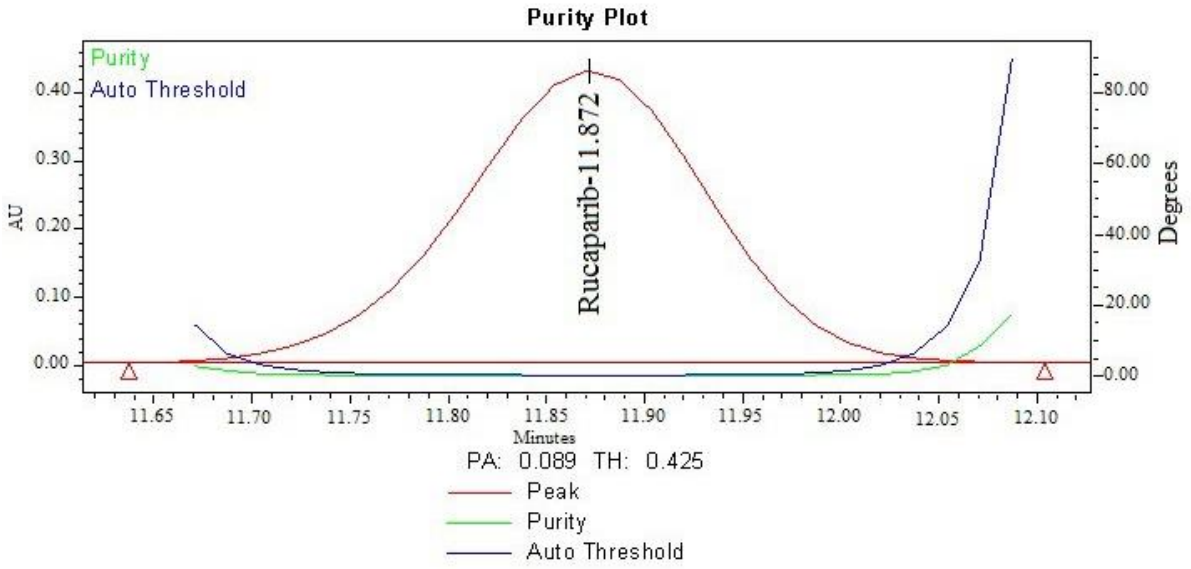


Figure 4.4 Peak purity plot for RCB.

4.8.3 Precision

During the precision studies, RSD's within 0.2% to 2.0 % were obtained in the assay results of RCB and all three impurities respectively. Six individual measures of RCB with three potential impurities (0.15 % w/w) with the reference of TAC were used for the analysis. In the intermediate precision study, the assay results of RCB and all three impurities RSD's were achieved at less than 0.4 % and 1.5 % respectively. The reduction of individual values confirms that the method has good precision. %RSD values of RCB and related impurities are given in Table 4.5.

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

LOD and LOQ values for RCB and its three related impurities (A, B and C) were assessed by using signal to noise ratio (S/N) method. The LOD and LOQs were established by the diluting the standard solutions of RCB and its three potential impurities, the studies showed that the S/N of 3: 1 and 10:1 for LOD and LOQ respectively. The precision of the developed method was assessed by using six different solutions of RCB and its three related impurities. Accuracy was determined by preparing three recovery solutions for the impurities associated with the RCB at LOQ level,

then the recovery percentages of all relevant impurity were calculated. The quantitative limit of these impurities helps in the process optimization to control the level of impurities. The recovery values were observed from 98.9 to 101.0 with impurity content close to LOQ level with RSD lower than 2.0 %. The results of the LOD and LOQ level accuracy are shown in Table 4.5 and Figure 4.4.

Table 4.5 Results of validation parameters for related impurities.

Parameter	RCB	Imp-A	Imp-B	Imp-C
LOD (mg/mL)	0.011	0.013	0.012	0.009
LOQ (mg/mL)	0.035	0.044	0.041	0.031
Correlation coefficient	0.9999	0.9997	0.9999	0.9992
Precision at LOQ level (%RSD)[a]	0.98	0.72	1.01	1.24
Precision (%RSD)[b]	0.12	1.10	1.23	1.35
Relative response factor	1.0	1.0	1.0	1.0

[a]Linearity range was from LOQ to 0.30 %w/w of RCB and its related impurities with respect to analyte concentration.
[b] (n=6)

4.8.5 Linearity

Linearity at a higher level is established by comparing five different RCB solutions of 80, 90, 100, 110 and 120% w/w. Linearity at a lower level was determined by adding six various solutions starting from LOQ, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30% w/w of three impurities (A, B, C) and RCB. The linear regression analysis was carried out by plotting the peak area versus concentration. The correlation coefficients of regression (R^2), y-intercept, and the slope of the calibration curves were calculated. The correlation coefficients of not less than 0.999 were achieved for all impurities. The relative response factor (RRF) from the plot of each impurity was determined by using the slope of RCB and slope of each impurity. At the level 0.15% w/w of all impurities, the response

of y-intercept of each plot was found to be within the 2.0%. RRF values and linearity results were tabulated in Table 4.5.

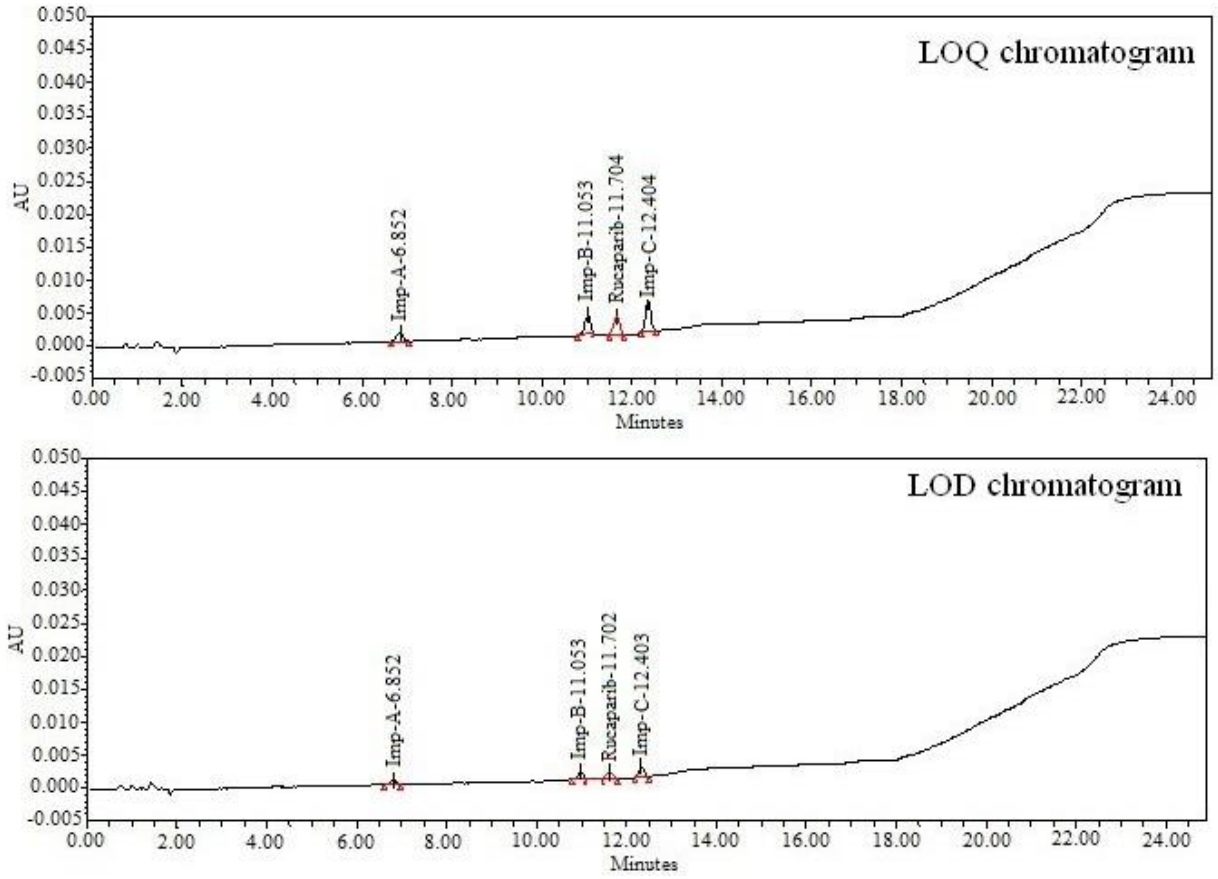


Figure 4.5 LOD and LOQ chromatograms of Rucaparib and its known impurities.

4.8.6 Accuracy

The assay accuracy was evaluated at three times with three different concentrations of RCB (400, 500 and 600 µg/mL) and the recovery percentages were calculated at each level. Studies were performed at TAC of 0.075, 0.15 and 0.225% w/w. The recovery percentages for all three impurities were calculated by taking into consideration of the number of spiked impurities, the amount of impurities present in the un-spiked samples, and the amount of impurities recovered after RRF correction. The recovery percentage of all three impurities was in the range 98.9 to 102.1

in the bulk drug sample (as indicated in Table 4.6). All the impurities values were within confidence intervals of the mean value.

Table 4.6 Evaluation of accuracy for related impurities.

Amount spiked[a]	% Recovery[b]		
	Imp-A	Imp-B	Imp-C
LOQ	101.0 ± 0.32	101.0 ± 0.25	98.9 ± 0.12
80%	99.7 ± 0.69	101.4 ± 0.56	101.3 ± 0.07
100%	102.1 ± 0.82	100.8 ± 0.32	101.1 ± 0.17
120%	100.6 ± 0.24	100.1 ± 0.82	100.6 ± 0.48

[a]Amount of impurities spiked with respect to specification level
[b] (n=3)

4.8.7 Robustness

To determine the robustness of the established LC method, certain deliberate variations were made to the original LC method conditions. The effect of flow rate on the resolution, tailing and theoretical plates was studied by changing from 1 mL/min to 1.2 and 0.8 mL/min. The wavelengths of 243 nm and 247 nm were used instead of usual 245 nm. The temperature impact on the column has been studied at 45 °C and 35 °C in the place of usual standard temperature of 40 °C. The impact of the change in the ratio of organic modifier was studied by changing the ratio (varying ±2% absolute) in both mobile phase-A as well as B from its original state. The system suitability parameters (theoretical plates, tailing factor of the analyte and the resolution) were evaluated. The tailing factor of RCB was found to be less than 1.2, theoretical plates were more than 61,000 and the resolution between RCB and impurities (Imp-B and Imp-C) was greater than 1.5 under the modified chromatographic conditions. The small changes observed in the theoretical plate, tailing

factor and resolution illustrate the robustness of the method. The results were tabulated in Table 4.7.

Table 4.7. Results of robustness.

Actual value	Changed value	No. of theoretical plates (*N* tangent method)	USP tailing factor (*T*)	USP resolution (R_s) between Imp-B and RCB	USP resolution (R_s) between RCB and Imp-C
1.0 mL/min	0.8 mL/min	62,154	1.1	2.2	2.9
	1.2 mL/min	82,263	1.0	1.6	2.4
245 nm	243 nm	68,968	1.0	2.1	2.8
	247 nm	69,794	1.0	2.1	2.8
40°C	35°C	61,459	1.0	2.0	2.5
	45°C	75,936	1.0	2.4	3.1
[a]0/40, 10/50, 15/70, 20/90, 25/90	[a]0/36, 10/45, 15/63, 20/81, 25/81	65,265	1.0	2.2	2.9
	[a]0/44, 10/55, 15/77, 20/99, 25/99	84,789	1.0	1.6	2.5

[a](T/%B)

4.9 Solution stability and mobile phase stability

The stability of RCB solution with its related impurities was studied at room temperature (25±2°C) and at cooling conditions (5±2°C) for 48 h. The assay of the analyte and all potential impurities was performed at time intervals of 4 h during the course of 48 h. The mobile phase stability was determined by evaluating the assay of RCB solution and its spiked solution with known impurities. The assay was performed by injecting the freshly prepared spiked solution at regular intervals of time i.e., every 4 h for 48 h with the same mobile phase. The solution was

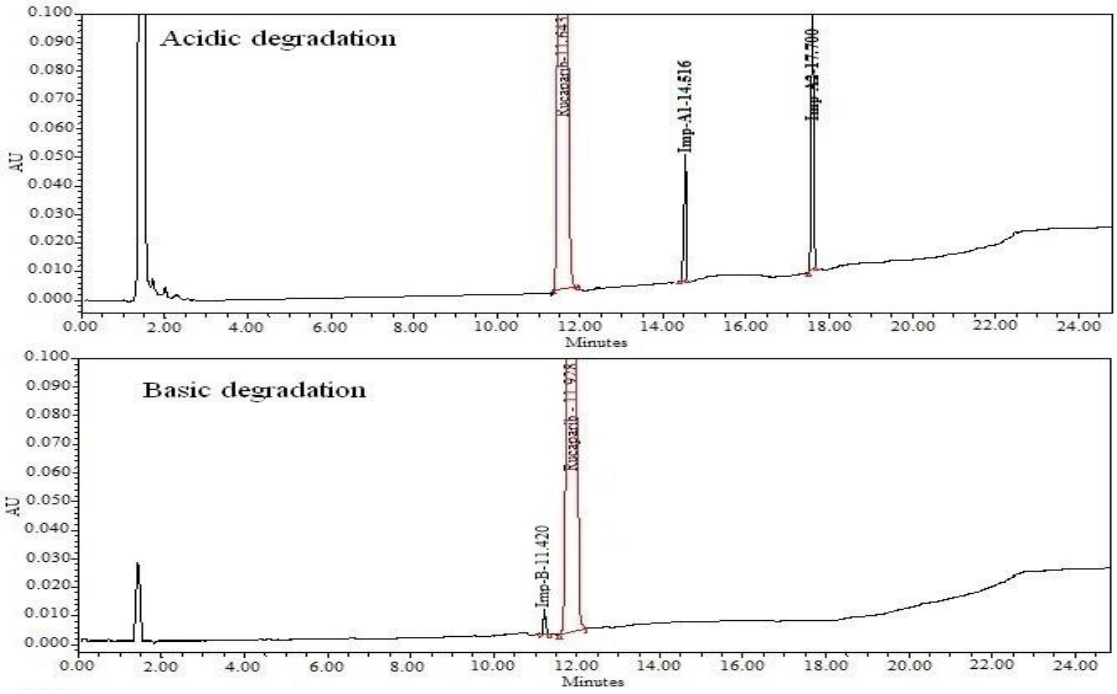
found to be stable for 4 h at room temperature and 24 h at cooling conditions. The RSD was within 0.60% and no major changes in the impurities were observed during the stability experiments.

4.10 Identification of degradation products

The LC-MS studies were used for the determination of the m/z values of the degradation products formed during acidic hydrolysis and oxidation processes. The m/z values at 309.1, 260.0 and 339.2 were assigned to the unknown degradation impurities A_1, A_2 and O. List of the proposed structures of the degradation impurities were depicted in Table 4.1.

4.11 Degradation behavior

The purity and assay of RCB before and after the degradation under various stress conditions (acid and base hydrolysis, thermal, oxidation and photolytic) were evaluated and the chromatographs of stressed samples were depicted in Figure 4.6.



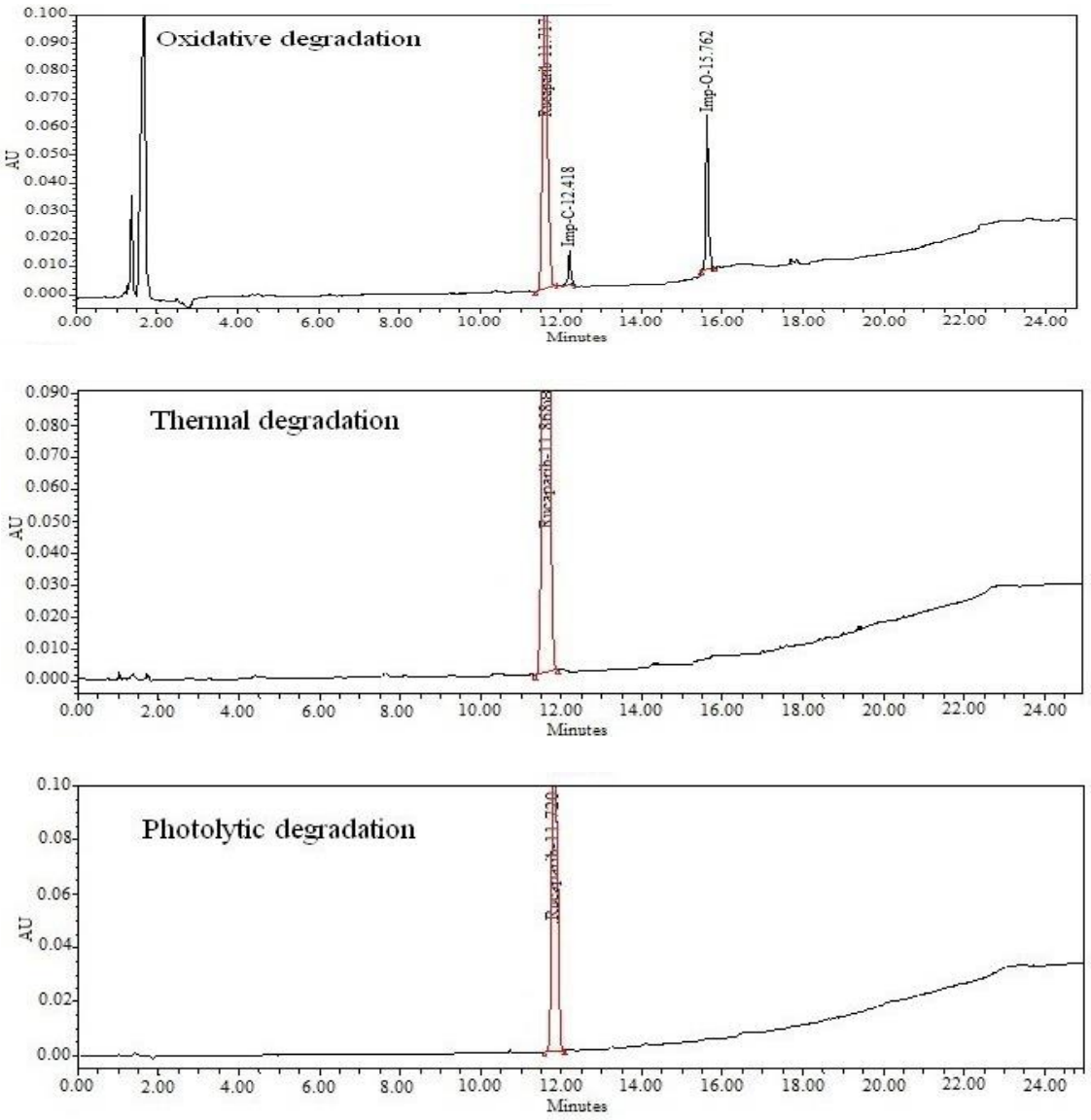


Figure 4.6 LC chromatographs of stressed samples.

Thermal and photolytic conditions resulted in no degradation of the RCB. However, the drug degraded and formed two unknown impurities during the acidic hydrolysis. The basic hydrolysis stress condition also lead to the formation of known impurity B. One unknown impurity along with the known impurity C was formed during the oxidative degradation of the drug. The different notations used for the impurities and degradation products are indicated in Table 4.8. The mass spectra of known impurities and unknown degradation products are depicted in Figure 4.7.

Table 4.8 Summary of forced degradation results.

Stress condition	Time	Purity of Analyte after degradation	Assay of Analyte after degradation	Remarks
Unstressed sample	--	99.8	99.7	--
Acid hydrolysis (0.1N HCl)	24 h	86.3	86.9	Significant degradation was observed. Unknown degradation impurity-A_1 and A_2 were formed.
Base hydrolysis (1N NaOH)	42 h	89.1	89.2	Significant degradation was observed. Known impurity-B was formed.
Oxidation (1% H_2O_2)	24 h	89.1	89.9	Significant degradation was observed. Unknown degradation impurity-O along with known impurity-C were formed.
Thermal (105° C)	10 days	99.8	99.6	Significant degradation was not observed.
Photolytic Degradation	11 days	99.8	99.5	Significant degradation was not observed.

The determined retention time, resolution factor, tailing factor, peak purity index, and relative retention time which are commonly termed as chromatographic parameters for the degradation products were summarized in Table 4.9. The peak purity index obtained in all the stressed samples was used for the calculation of the specificity for the analyte. The peak homogeneity study established that the RCB peak is homogeneous in all the analysed stress samples.

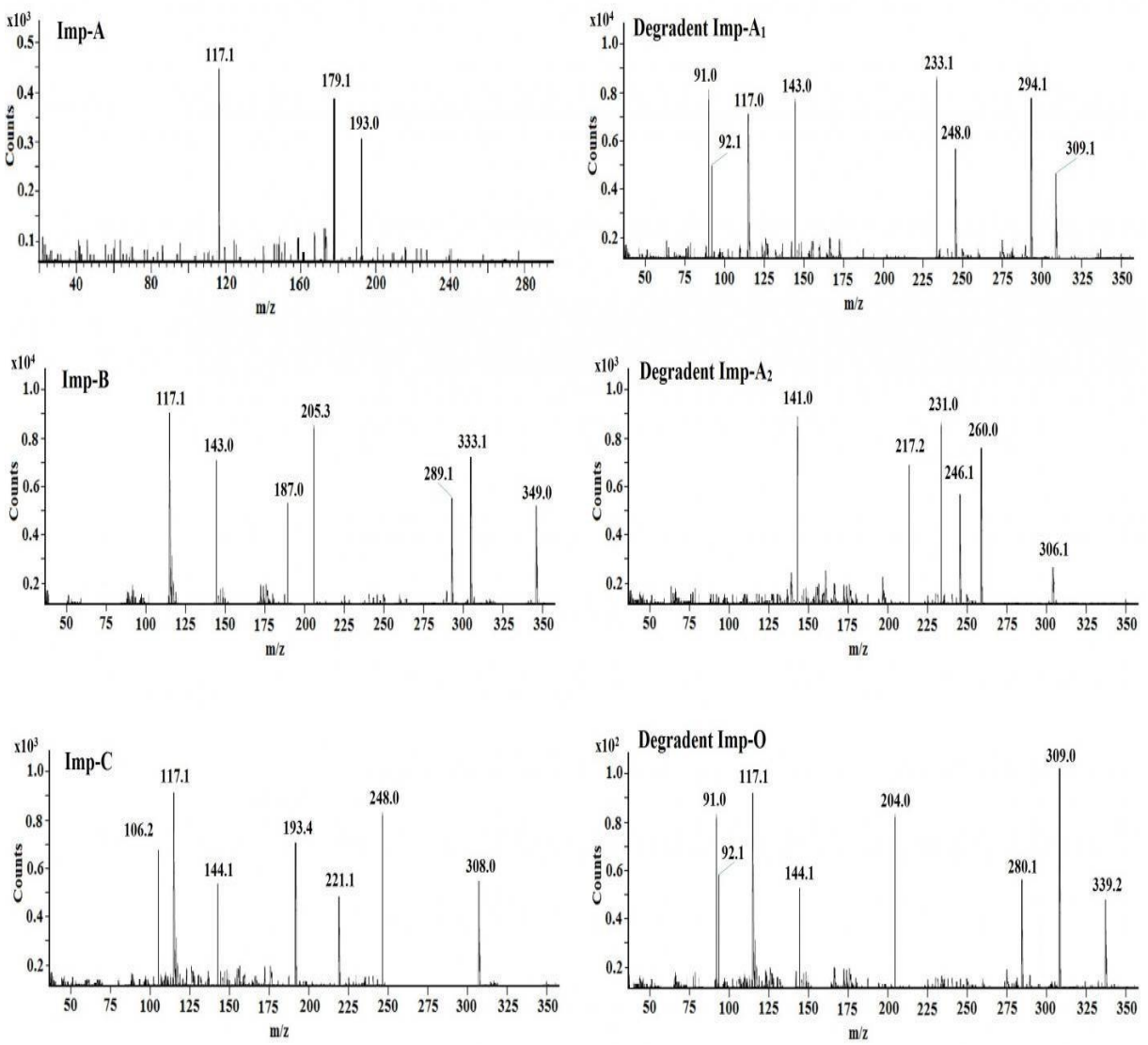


Figure 4.7 Mass spectra's of known impurities and unknown degradation products.

Table 4.9 Peak, purity and chromatographic data of the products formed after degradation.

Degradation product	R_t (min)	P.P.I	R_s	T_f	Relative retention time (RR_t) min
A1	14.51	0.9982	4.37	1.02	1.52
A2	17.70	0.9966	6.53	1.07	1.43
O	15.76	0.9993	5.66	1.05	1.45

R_t, Retention time; R_s, Resolution factor; T_f, Tailing factor; P.P.I, peak purity index; RR_t, Relative retention time.

4.12 Characterization of degradation products

4.12.1 Acidic Hydrolysis Process

The acidic hydrolysis of RCB resulted in the formation of two degradation products A1 and A2. The product A1 was formed due to the elimination of the methyl group from RCB which is evidenced by the appearance of molecular ion at m/z 309.1 Da. The retention of the peaks A1 and A2 detected by LC-MS were at 14.5 and 17.7 min respectively. Figure 4.6 shows the spectra of the acidic degradation products of LC-ESI-MS/MS.

Fragmentation of the A1 leads to the fragment ions of m/z 294, 248, 233 Da. The detailed fragmentation is shown schematically in the Figure 4.8. The fragmentation patterns were established by their elemental composition $C_{18}H_{16}FN_3O$ and calculation from the accurate masses of elements. The A1 forms the fragment ions by the elimination of amine and addition of CO_2 with HF having m/z values 294 and 248 respectively. A stable fragment from the degraded product A1 is the 2*H*-cyclopenta [cd] indole ion. The formation of indole ion can be explained by the nucleophilic attack of hydroxide ion of benzylamine moiety of the drug followed by the degradation of fragment ion with m/z 91.

Another degraded product A2 with m/z 306.1 Da was also formed due to the loss of water molecule from RCB during acidic hydrolysis. Fragmentation of A2 leads to the formation of fragment ions with m/z 260, 246, 231, 217 and 141 Da. The detailed fragmentation is shown schematically in the Figure 4.8. The list of m/z values of fragmentation ions for degradation products and all the mass values with molecular formulas of each LC-MS/MS results were tabulated in Table 4.10.

4.12.2 Oxidation Process

The degradation products O and C were formed during the oxidative degradation process in 1% H_2O_2 at room temperature in 24 h. The product O was formed by the oxidation of amine to N-oxide which was evidenced by its m/z 339 corresponding to molecular ion. The mass fragment

ions of O were obtained at m/z 309, 280, 204, 144, 117 and 91. The elimination of tropylium cation from the fragment ion with m/z 309 yielded the fragment with m/z 117. The probable chemical formula given by LC-MS/MS is $C_{19}H_{18}FN_3O_2$ for the degradation product O.

The second oxidative degradation product C was formed with m/z 308 by eliminating the cyanide group. Further, MS/MS fragments were observed at m/z 248, 221, 193, 144, 117, 106. Illustration of the oxidation degradation process for the formation of products and its fragmentation is shown schematically in Figure 4.9. The mass spectra results support the proposed structures as shown in Table 4.1.

Table 4.10. m/z values of degradation products and its fragment ions of RCB.

Degradation product	Molecular ion m/z (amu)	Most probable molecular formula	Fragment ions m/z (amu)
A_1	309	$C_{18}H_{16}FN_3O$	294, 248, 233, 143, 117, 92, 91
A_2	306	$C_{19}H_{16}FN_3$	260, 246, 231, 217, 141
O	339	$C_{19}H_{18}FN_3O_2$	309, 280, 204, 144, 117, 92, 91

4.13 Conclusions

A precise, linear and rapid LC method was developed and validated for the assay of RCB and its related substances. The stability of bulk samples of RCB shelf life was also checked by using the developed LC method. The result of the RCB being subjected to different stress conditions led to the fact that it was found to be stable during thermal, photolytic stress conditions. It degraded extensively under acidic hydrolysis as well as oxidative stress conditions. The LC-MS/MS data results were used in characterization of the three resultant degradation products and valid fragmentation pathways. The obtained stability studies, degradation pattern and proposed fragmentation pathway could potentially be useful for better understanding of RCB during storage and stable formulations and mend pharmaceutical safety.

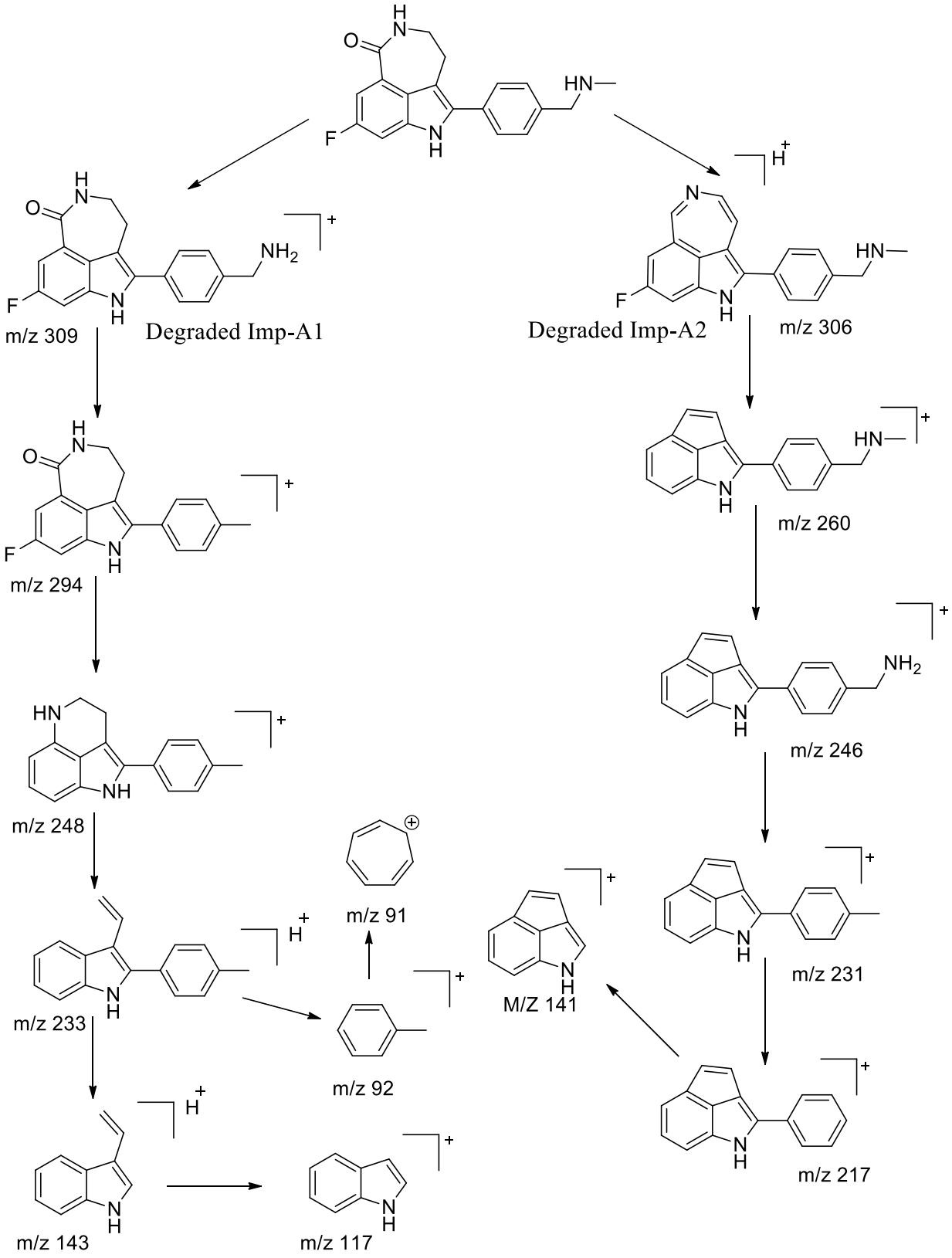


Figure 4.8 The probable degradation path way for the acidic degradation products

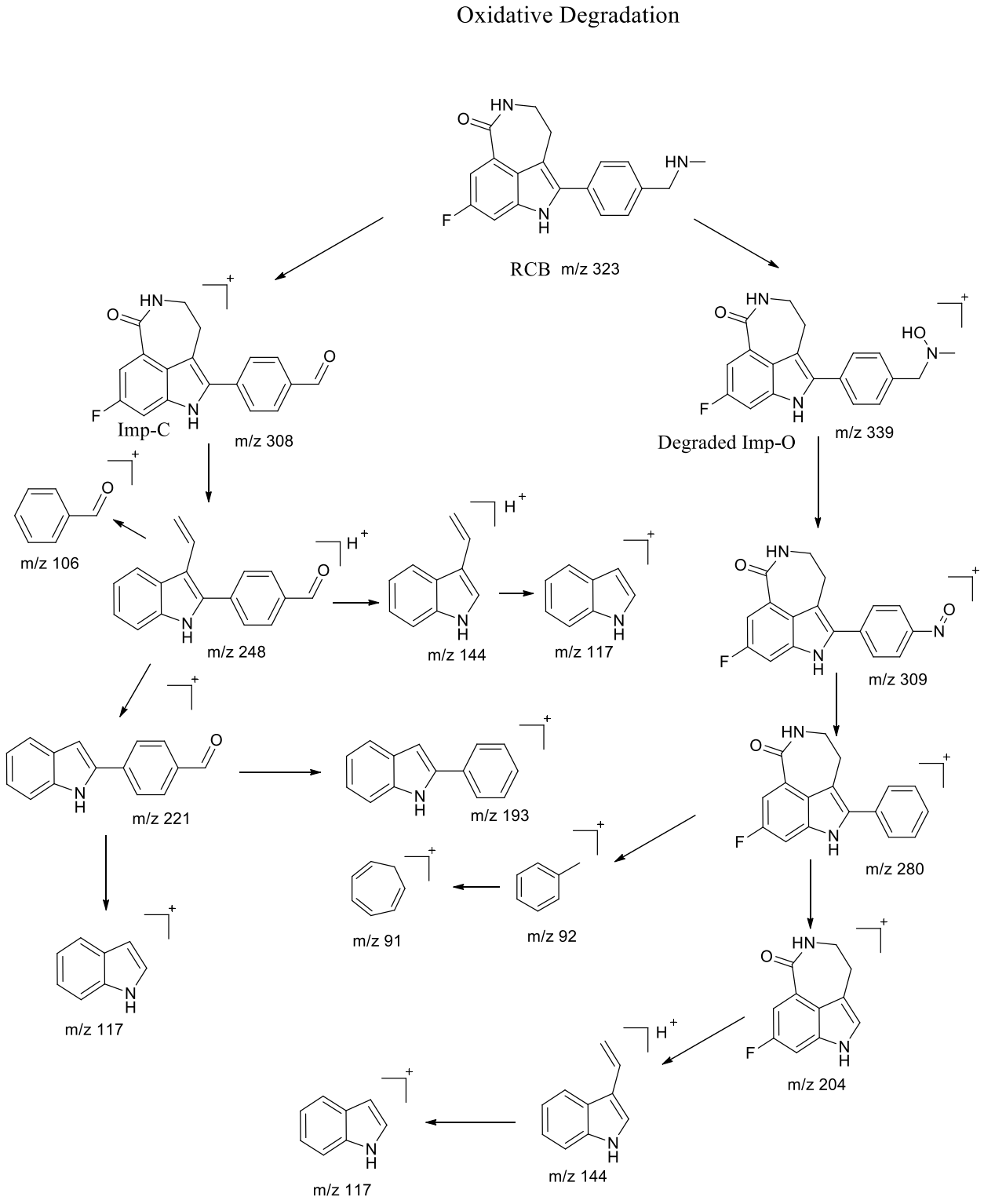


Figure 4.9 The probable degradation path way for the oxidation degradation products

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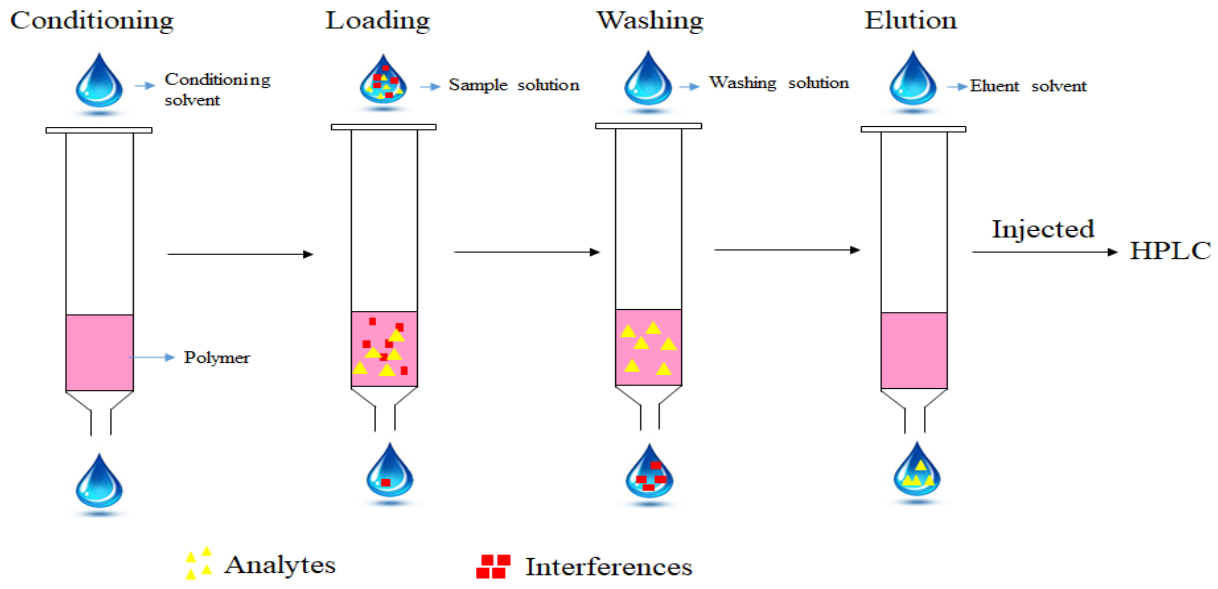
Chapter –V

Development and validation of UPLC method for the analysis of two antihypertension drugs from human plasma by using solid phase extraction

Abstract

A new, simple, highly accurate and precise stability indicating ultra-performance liquid chromatography (UPLC) method was developed for the identification and quantification of PP and IP from human plasma. In this UPLC method, HSS C_{18} column (100x2.1 mm, 1.8 µm) and mobile phase ACN, phosphate buffer solution (pH 3.0) mixture was used in the ratio of 65:35. SPE method was used for the extraction of PP and IP from human plasma. Different solvents were used to extract the analyte from SPE such as, ACN, methanol, acetone, tertiary butyl diethyl ether (TBDE), chloroform ($CHCl_3$), and ethanol (EtOH). Among these, ACN gave good recovery percentages (94.56 to 101.58%). From the linearity graph, good correlation coefficient values of 0.9996 for PP and 0.9997 for IP were achieved. The %RSD values for intra and inter day precision is in between 1.08 to 12.5%. The LOD and LOQ values were determined by the signal to noise ratio method. LOD and LOQ values for IP and PP were found to be 8.6 and 33.5 ng/mL and 28.33 and 110.5 ng/mL respectively. The developed method was statistically validated as per ICH guidelines.



Schematic diagram for solid phase extraction.

5.1. Introduction

Cardiovascular ailments such as hypertension and stokes are the most leading diseases to cause death worldwide. In general, the monotherapy was used to reduce the blood pressure in patients, but it works in few patients only. Therefore, the combination therapy was introduced to effectively reduce the cardiovascular illnesses [1, 2]. Combination therapy has been found to decrease the hypertension, due to their numerous mechanisms of actions and showed reduced side effects due to the lower dosage of medications [3-6].

Perindopril is commonly used to treat coronary artery ailments such as heart failure and hypertensions [7-10]. The chemical name of perindopril is (2S,3aS,7aS)-1-[(2S)-2-{[(2S)-1-ethoxy-1-oxopentan-2-yl] amino}propanoyl]-octahydro-1H-indole-2-carboxylic acid. It is an ethyl ester of non-sulphahydryl derivative and angiotensin converting enzyme (ACE) inhibitor, it selectively reduces the level of angiotensin I by converting it to angiotensin II due to the hydroxylation of perindopril to produce its active metabolite perindoprilat. Consequently, inhibits the angiotensin II activities like stimulation of aldosterone secretion in adrenal cortex and vasoconstrictions [11, 12].

Indapamide (4-chloro-N-(2-methyl-2,3-dihydroindol-1-yl)-3-sulfamoylbenzamide) is a thiazide or sulphonamide derivative. It is used to treat the mild to moderate hypertensions due to the activities of calcium antagonist and diuretic effects [13-15]. Different studies revealed the antihypertension activity of indapamide due to the peripheral vascular resistance and decreasing the vascular reactivity.

The indapamide and perindopril combination therapy has shown to be effective for reducing high blood pressure [16-19]. This combination therapy has proved to show major effects on microcirculatory alterations, systolic blood pressure and arterial stiffness. Thus, many

pharmaceutical industries competing to produce the various combinations of indapamide hemihydrate and perindopril arginine to prevent the hypertension and maintain the normal blood pressure in patients. The British and U.S pharmacopeias officially approved Indapamide, and perindopril [13, 20].

The number of methods were reported for the identification and quantification of indapamide and perindopril individually. To the best of our knowledge, only few methods has been reported for the simultaneous estimation of indapamide and perindopril drugs in biological fluids [21-26]. Till now, no one reported the stability indicating reverse phase UPLC method for the simultaneously identification and quantification of Indapamide and perindopril from human plasma. Therefore, the current study demonstrated the stability of perindopril and indapamide by doing the forced degradation studies, successfully extracted and quantified these two drug molecules from human plasma.

5.2. Literature survey

Few stability indicating and extraction methods were reported for PP and IP in the form of bulk drugs and formulations. Stability indicating methods were reported for the identification, quantification and characterization of drug molecules, relative impurities and degradation products. Extraction methods were reported to extraction, identification and quantification of the drug molecules and relative substances from biological fluids such as blood, urine, plasma and serum.

Sarah S. Saleh et al., have developed and validated a HPLC method for the identification of drug combinations of indapamide, amlodipine, and perindopril by using an ecofriendly eluents [27]. Alina Porfire et al., have developed a validated chemometry and near infrared spectroscopy methods for characterization of sustainable indapamide tablets [28]. S. Udrescu et al., have

developed a liquid-liquid extraction method for the extraction of indapamide in whole blood and determined by using a LC-MS/MS method [29]. Bin Du et al., have developed an accurate and precise enantioselective HPLC method for the separation of enantiomers of indapamide in the whole blood sample [30]. Alina Porfire et al., have developed a chemometric-NIR method for pharmaceutical characterization and assay of indapamide in tablet power blend [31]. Yi Tao et al., were separated and determined of perindopril, indapamide and its active metabolite perindoprilat in whole human blood by developing a new UPLC-MS method [32]. Ioan Tomuta et al., were evaluated the pharmaceutical properties and drug content of indapamide (API) by developing a new high throughput chemometric-NIR method [33]. Suresh Kumar Kailasa et al., have selectively quantified the indapamide drug from biological fluids by developing a new synthesized fluorescent gold clusters with introducing the cellulose cysteine Schiff base as a ligand [34]. Eyad Rashed Dawud et al., have developed a simple, accurate validated HPLC method for the simultaneous identification of indapamide and hydrochlorothiazide (inhibitors of angiotensin converting enzyme) in formulations [35].

M. Medenica et al., have developed a new reverse phase HPLC method for the detection of perindopril and its impurity level in tablet dosage form [36]. Andelija Malenovic et al., have developed a micro emulsion liquid chromatography system for the selective analysis of perindopril and its four related impurities by using desirability based optimization conditions [37]. Ramzia I et al have developed a simultaneous detection method for the three hypertension drugs (indapamide, perindopril and amlodipine) in bulk and formulations by using HPLC method [38]. Kalaiyarasi Duraisamy et al have developed a new UHPLC-MS/MS method for the identification and quantification of amlodipine and perindopril in the form of bulk and formulations [39]. Nevin Erk was developed a reverse phase HPLC method for the detection of indapamide and perindopril

in the form of formulations and compared with derivative spectrometric method [40]. Hari Hara Theja Dugga et al., have studied degradation analysis by using HPLC method for the perindopril by applying the external stress conditions [41]. P.S. Jain et al have developed a simple validated HPLC method for dissolution analysis of indapamide and perindopril combination tablets [42]. S. Bouabdallah et al., have introduced a unified equation for kinetic studies of isomerization of perindopril and analyzed by using a dynamic reverse phase HPLC method [43]. G. M. Khomushku et al., have developed a simple unified reverse phase HPLC method for analysis of anti-hyper tension drugs perindopril, quinapril and Lisinopril [44]. Kirtan P et al., have developed a simple and efficient reverse phase HPLC method for the detection of anti-hypertension drugs (amlodipine, perindopril and indapamide) in the form of bulk and formulations. Ion Valentin et al., have developed HPLC and capillary zone electrophoresis (CBZ) two analytical methods for the analysis of inhibitors of hypertension drugs indapamide and perindopril [45]. Said A et al., have separated, identified and quantified simultaneously perindopril and bisoprolol drugs and its degradation products by developing a HPLC, capillary electrophoresis methods and IR, mass characterization techniques [46]. Anna Gumieniczek et al., have developed a new reverse phase HPLC method for the in vitro degradation analysis for amlodipine and perindopril hypertension drugs in the form of formulations [47]. Deepak S et al., developed a solid phase extraction method for the extraction of perindopril and its metabolite from human plasma and analyzed by developing a new validated LC-MS/MS method [48].

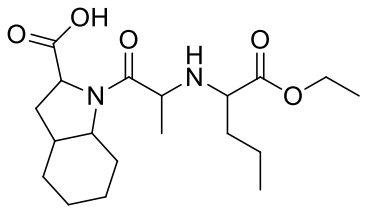


Figure 5.1 Chemical structure of Perindopril.

5.3 PP drug information

IUPAC name : (2S, 3aS, 7aS)-1-[(2S)-2-[[(2S)-1-ethoxy-1-oxopentan-2- yl] amino] propanoyl]-2,3,3a,4,5,6,7,7a-octahydroindole -2-carboxylic acid;2-methylpropan-2-amine

Molecular formula : C19H32N2O5

Molecular weight : 368.5 g/mol

Brand name : Coversyl

Maximum daily dosage : 16 mg

Dosage strength : 2, 4, 8 mg

Dosage form : Tablet

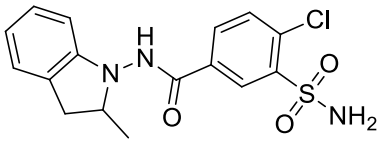


Figure 5.2 Chemical structure of Indapamide.

5.4 IP drug information

IUPAC name : 4-chloro-N-(2-methyl-2, 3-dihydroindol-1-yl)-3- sulfamoyl benzamide

Molecular formula : C16H16ClN3O3S

Molecular weight : 365.83 g/mol

Brand name	:	Lorvas
Maximum daily dosage	:	5 mg
Dosage strength	:	1.25, 2.5 mg
Dosage form	:	Tablet

5.5 Experimental

5.5.1 Chemical and reagents

For the UPLC analysis and extraction, analytical grade methanol, ACN, TBDE, chloroform ($CHCl_3$) and ethanol (EtOH) were procured from Sigma Aldrich, U.S. Hydrogen peroxide (H_2O_2), hydrochloric acid (HCl), KH_2PO_4, orthophosphoric acid and sodium hydroxide (NaOH) were purchased from sigma Aldrich, U.S. Milli-Q system Millipore, U.S, used for the purification of water. The reference standards perindopril (potency (99.8)), Indapamide (potency (99.8)) and active pharmaceutical ingredients (API) were obtained from Mylan laboratories and Glenmark pharmaceutical as gifted samples.

5.5.2 Instrumentation

Stability indicating method development and validation was established on UPLC (Acquity, waters, USA) by using the Empower II software. Acquity HSS C18 column (waters) was used for the eluting the peaks with high resolution. Milli-Q Millipore system (USA) was used for the purification of water. Sonicator (sonica, spincotech-Italy), vortex mixture (India), Elico pH meters, and 0.45-micron nylon filters (Merck, Millipore) were are used in the sample preparations.

5.5.3 Analytical conditions

Isocratic method was used for the simultaneous determination and quantification of indapamide and perindopril in human plasma. Mobile phase acetonitrile ACN and 10 mM KH_2PO_4 buffer solution (pH 3.0) was used in the ratio of 65:35. Column oven temperature was maintained at 30^oC,

flow rate of 1 mL per minute and injection volume of 10 µL, UV wavelength (λ max) of 254 nm and run time is 5 min were used. Stock solutions were prepared in ACN and the mobile phase was used as a diluent for the further dilutions.

5.5.4 Preparations of linearity and quality control (QC) sample solutions

Stock solutions of perindopril (1 mg/mL) and indapamide (1 mg/mL) were prepared in ACN and stored at -4^{o}C. The working standard solutions were prepared by diluting the stock solutions by using mobile phase. Six different concentrations of perindopril (0.75, 1.5, 3, 7, 15 and 25 µg/mL) and indapamide (0.15, 0.45, 1.25, 2.5, 7.5 and 12 µg/mL) spiked solutions with human blank plasma were prepared for the construction of calibration curve. Three different quality control samples low (LQC), middle (MQC) and high (HQC) were prepared individually by spiking with 500 µL aliquots of human blank plasma. The solutions of indapamide at the LQC 0.45, MQC 2.5 and HQC 12 µg/mL and perindopril at the LQC 1.5, MQC 7, and HQC 25 µg/mL solutions were prepared. All spiked solutions are stored in refrigerator at -20^{o}C.

5.5.5 Extraction procedure

Human plasma (250 µL) was taken in 2 mL eppendorf tube containing 500 µL of potassium phosphate buffer (pH 4). The samples were mixed with vortex for few minutes. Then the samples were loaded into the Oasis HLB (1 mL, 30 mg) cartridges. Before loading the sample in to solid phase (cartridges), these were preconditioned with 1 mL of acetonitrile, 1 mL of methanol and 1 mL of water: acetonitrile (95:05 v/v). The sample loaded cartridges are washed five times with 1 mL of methanol: water (10: 90, v/v) at -30 kPa [49]. Then the sorbents were dried for 10 min under the air flow. After drying, the analytes were eluted from the sorbent by using 1mL of acetonitrile under gentle vacuum. The solution was evaporated by using N_2 gas at 40^{o}C in vacuum oven. The sample residues were reconstituted by adding 100 µL of mobile phase solution, vortexed for 2 min

and sonicated for 1 min. Then, the solutions were filtered by using 0.2-micron nylon filters and 10 µL of sample solution was directly injected in to UPLC for analysis.

5.6 Forced degradation studies of drug substances

The stability of the developed UPLC method was examined by doing the forced degradation of drug substances. The stability studies of drugs was carried out in presence of acidic, basic, neutral, oxidative, photolytic and thermal conditions.

5.6.1 Acidic degradation

During the acidic degradation of drug substances, 1.0 N hydrochloric acid (HCl) was used. One milli liter of indapamide (1 mg/mL) and perindopril (1 mg/mL) mixed solution was taken in 10 mL volumetric flask stirred with 1 mL of 1.0 N HCl for 1 h at 60 ± 2^{o}C. The acid treated solution was neutralized with 1 mL of 1.0 N sodium hydroxide for preventing further degradations and make up to the volume with diluent.

5.6.2 Basic degradation

Alkaline degradation study of drug molecules was carried out by using 1 N sodium hydroxide solution (NaOH). 1.0 mL stock solution was refluxed with 1.0 mL 1 N NaOH in 10 mL volumetric flask at 60 ± 2^{o}C for one hour. The solution was neutralized by adding 1.0 mL of 1.0 N HCl solution and make up to the mark with diluent.

5.6.3 Thermal degradation

Thermal degradation studies were carried out at 80 ± 5^{o}C for 8 h. One milli litere of sample solution was taken in 10 mL volumetric flask and kept under the above mentioned conditions and then solution was diluted with diluent.

5.6.4 Oxidative degradation

Oxidative stress study of drugs was carried out by using 10% hydrogen peroxide (H_2O_2). Suspension of 1 mL of drugs solution into 1 mL of 10% H_2O_2 and stirred at 30 ± 2°C for one hour then the solution was diluted up to 10 mL with diluent.

5.6.5 Photolytic degradation

Photolytic degradation was carried out by using UV light. 1 mL of sample solution was exposed to UV light for 24 h in UV chamber and the solution was diluted up to 10 mL by using diluent.

5.6.6 Neutral degradation

HPLC grade water was used in the neutral degradation. One milli liter HPLC grade water was added into 1 mL sample solution and refluxed at 60 ± 2°C for 5h and then solution was diluted up to 10 mL with diluent.

All degradation samples are filtered with 0.45 µ filters and each degradation sample (10 µL) was injected individually in to UPLC and recorded the chromatogram.

5.7 Results

5.7.1 Method development and optimization

The main objectives of this work is the isolation, quantification of IP and PP drug molecules form human plasma by developing a novel stability indicating UPLC method . In the process of method development, different compositions of mobile phases with different pH were used. In general, the pH of the buffer will retain the ionization of compounds and provide low tailing factor values. The pKa values of indapamide and perindopril are 8.8 (nearly neutral) and 5.7 (weak acidic) respectively. The selected mobile phase acetonitrile and KH_2PO_4 buffer (pH=3) (65:35) would give good retention and tailing factor peaks. The optimized chromatograms were shown in Figure 5.3. Among all the columns used in the current method, HSS C_{18} column (100x2.1 mm, 1.8 µm)

was found to be suitable for getting adequate resolution, plate count and tailing factors (indapamide (1.7) and perindopril (1.6)) for the drug molecules simultaneously. This column gave high theoretical plate count values (indapamide (8254) and perindopril (6411)), which indicates the efficiency of column for the separation with high resolution, narrow and sharp peaks. The system parameters such as flow rate (1 mL/min) and injection volume (10 µL) were optimized based on the minimal consumption of mobile phase and peak resolution.

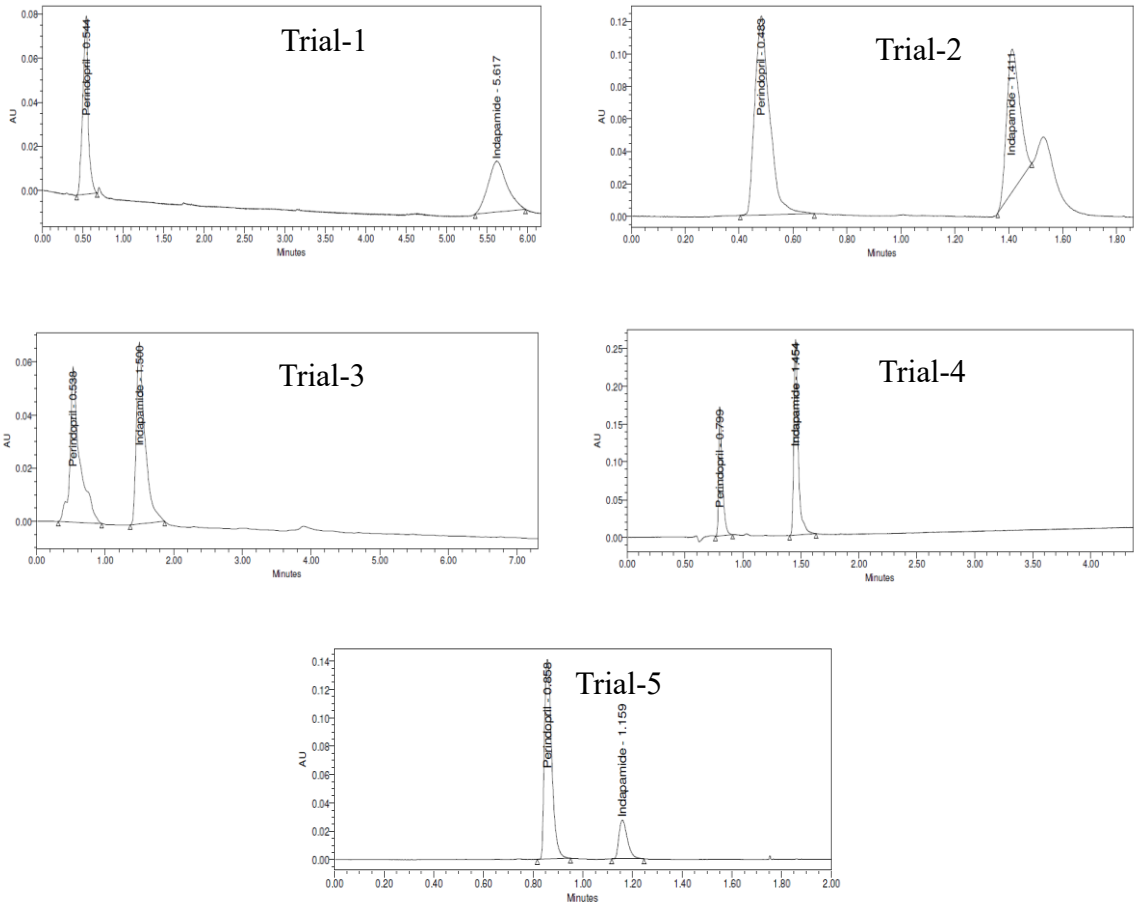


Figure 5.3 Optimized resulting UPLC chromatograms with different columns.

In the forced degradation studies, the drug degradation between 5 to 20% is acceptable and that was considered as stability indicating method and is reasonable for the validation of chromatographic method. In the present method, stability studies were carried out in different conditions such as acidic, basic, neutral, thermal, oxidation and photolysis. In those, the drugs

were degraded only in acidic and basic conditions and were stable in remaining conditions. In acidic condition, 1.56% of Indapamide and 8.65% of perindopril and in basic condition, 6.42% of indapamide and 3.59% of perindopril were degraded (Figure 5.4(a), (b) and Table 5.1).

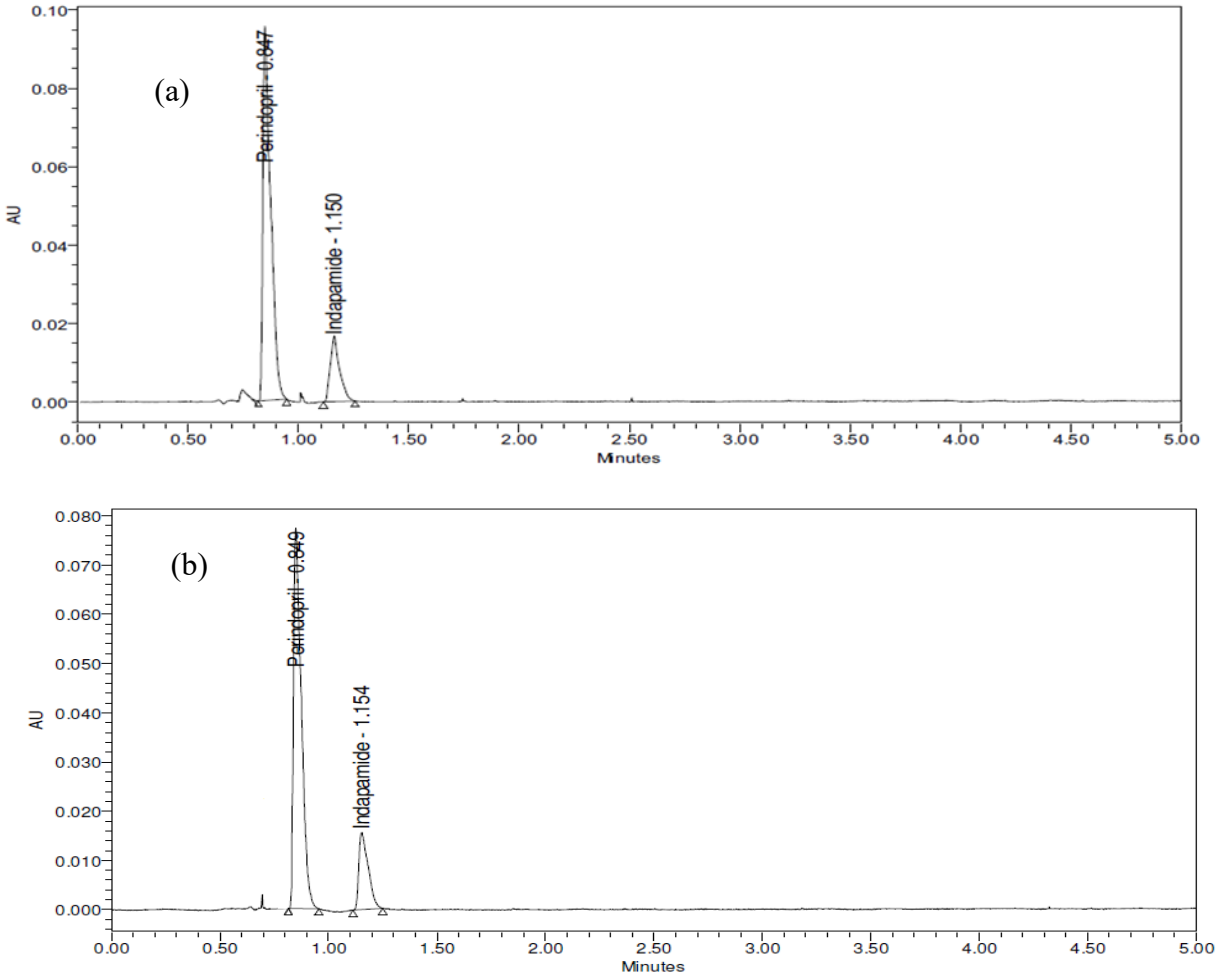


Figure 5.4 (a) acidic degradation (b) Basic degradation HPLC chromatograms.

The assay % of drugs were calculated by using the following formula:

$$\% \text{ Assay} = \frac{\text{Sample area}}{\text{Standard area}} \text{ X } \frac{\text{dilution of Standard}}{\text{dilution of sample}} \text{ X } \frac{\text{P}}{100} \text{ X } 100$$

Solid phase extraction (SPE) method was used for the extraction of drug molecules from human plasma. Various organic solvents (MeOH, ACN, $CHCl_3$, EtOH, acetone and TBDE) were used for

the extraction of drug molecules from SPE, when compared with all of those ACN was given good recovery percentages of Indapamide (96.64-98.64%) and perindopril (98.51-101.25%).

Table 5.1 Summary of forced degradation study results.

Stress condition	Time	Assay% of PP after degradation	Assay% of IP after degradation	Remarks
Unstressed sample	---	99.63	100.2	--
Acid hydrolysis (1.0N HCl)	60 min	91.15	98.64	The Significant degradation was observed.
Base hydrolysis (1.0N NaOH)	60 min	96.01	93.78	The slight degradation of analytes was observed.
Oxidation (10% H_2O_2)	60 min	98.65	99.12	Significant degradation was not observed.
Thermal (80° C)	8 hr	99.32	99.81	Significant degradation was not observed.
Photolytic degradation	24hrs	99.53	99.98	Significant degradation was not observed.

5.8 Method Validation

The developed stability indicating UPLC-UV method was validated for the following parameters statistically as per the ICH guidelines [50].

5.8.1 Selectivity

Simultaneous extraction of perindopril and indapamide was carried out from human plasma by using SPE method. Perindopril and indapamide peaks are arrived (0.87 and 1.16) within 2 min of retention time (Figure 5.5). The chromatograms revealed that the peaks are pure, symmetric, well separated and no other endogenous peaks are eluted. The results indicated that the SPE method was sufficient to isolate the perindopril and indapamide from human plasma.

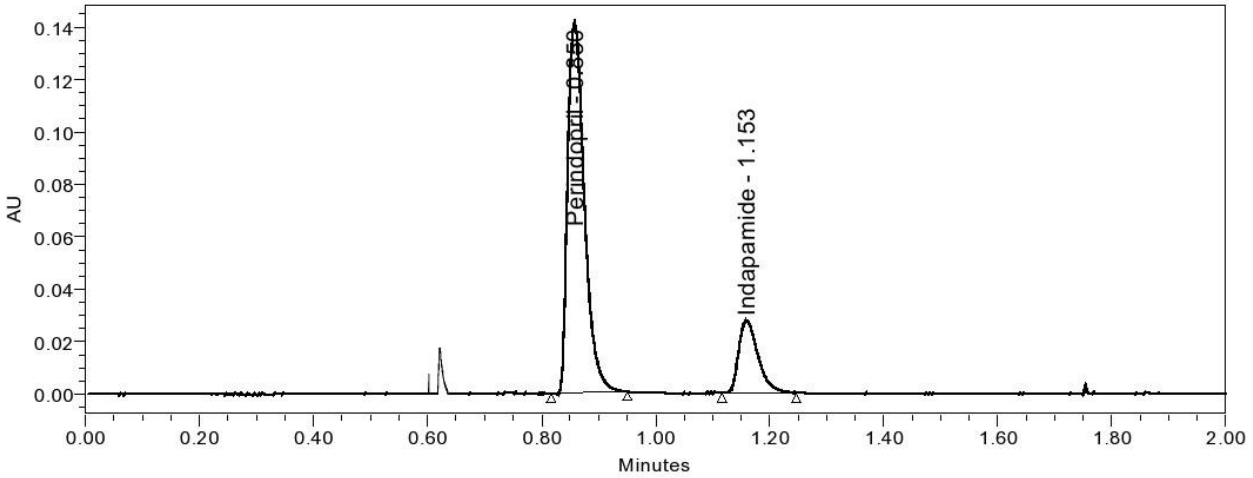


Figure 5.5 The final UPLC chromatogram of Indapamide and Perindopril after the SPE.

5.8.2 Specificity

The specificity and selectivity was determined by running six different blank plasma sample solutions using the above optimized UPLC method. No interference peaks were observed at respective retentions times of perindopril and indapamide. Moreover, the specified drugs also did not give any interference peaks during the analysis.

5.8.3 Calibration curves

The linearity of the developed method was evaluated by using standard solutions of six different concentrations of indapamide (0.15, 0.45, 1.25, 2.5, 7.5 and 12 µg/mL) and perindopril (0.75, 1.5, 3, 7, 15 and 25 µg/mL) plasma sample solutions.

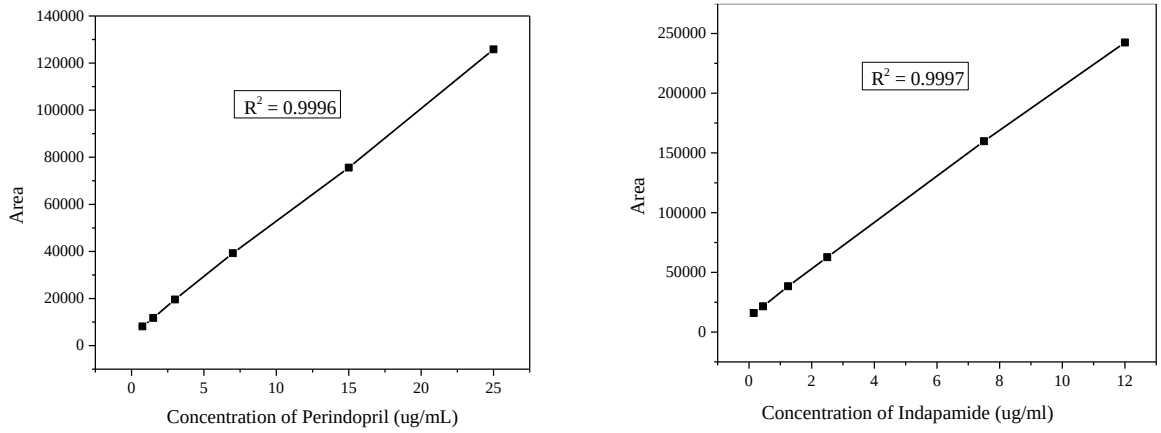


Figure 5.6 The linearity plots of Perindopril and Indapamide.

The linearity graphs were constructed by plotting the peak area against the concentrations of plasma sample solutions (Figure 5.6) and the results are shown in Table 5. 2. The linearity plots gave acceptable correlation coefficient (R^2) values for indapamide (0.9997) and perindopril (0.9996).

Table 5.2 Linearity of IP and PP

IP (µg/mL) [X]	Mean peak area [Y]	PP (µg/mL) [X]	Mean peak area [Y]
0.15	15954	0.75	8139
0.45	21585	1.50	11751
1.25	38384	3.00	19584
2.50	62645	7.00	39258
7.50	159785	15.0	75581
12.0	242445	25.0	125842
Linear regression equation	Y=19163 x + 13957	Linear regression equation	Y=4820 x + 4710
Slop	19163	Slop	4820
Intercept	13957	Intercept	4710
Correlation coefficient (R^2)	0.9997	Correlation coefficient (R^2)	0.9996

Average (n) =3

5.8.4 Precision and accuracy

The precision was determined by using three different concentrations (LQC, MQC and HQC) of QC samples. For determining the intra-day precision, the experiments were carried out five times within same day. The analysis was also done in different days for the inter-day precision measurements and both precision values are expressed in percentage (1.08-12.5%) of cumulative variance (CV) of the peak area of three different QC sample solutions. The accuracy was determined in terms of mean percentage of analytes (94.56-101.2%) recovered from the plasma (Table 5.3).

Table 5.3 Validation parameters results for determination of perindopril and indapamide.

Analyte	Nominal value (µg/mL)	Intra day			Inter day		
		Found concentration (µg/mL)	Precision (RSD %)	Accuracy (%)	Found concentration (ng/mL	Precision (RSD %)	Accuracy (%)
Perindopril	1.5	1.46 ± 0.28	8.34	97.45	1.48 ± 0.65	6.48	97.12
	7	7.16 ± 0.83	4.66	101.58	7.24 ± 2.51	3.51	101.2
	25	25.12 ± 2.73	2.4	98.6	24.68 ± 1.54	1.08	98.05
Indapamide	0.45	0.39 ± 0.16	12.5	95.16	0.41 ± 1.23	11.12	94.56
	2.5	2.48 ± 0.79	5.36	100.4	2.49 ± 0.64	3.05	99.2
	12	11.22 ± 1.68	4.8	98.7	11.56 ± 3.5	3.61	97.92

5.8.5 LOD and LOQ

Limit of detection (LOD) and limit of quantification (LOQ) of the developed method was assessed by using signal to noise (S/N) method. The LOD and LOQ are useful for the assessment of sensitivity of developed method. In this method, the LOD value of perindopril (33.5 ng/mL) and indapamide (8.6 ng/mL) was determined by using S/N ratio of baseline over 3 times signal level of sample. LOQ values of perindopril (110.5 ng/mL) and indapamide (28.33 ng/mL) was assessed by using S/N ratio of baseline over 10 times signal level of sample.

5.8.6 Robustness

The robustness of the developed method was evaluated by changing the conditions such as flow rate, column temperature, mobile phase ratio and UV wavelength. The flow rate ±0.2 mL per minute, organic solvent of mobile phase ±5%, column oven temperature ±5^{o}C and UV wavelength of detector ±4 nm were changed for the evaluation of robustness of the developed method. The analyte solution containing 10 µg/mL concentration of each drug indapamide and perindopril were

prepared for these studies. There is no substantial difference were observed with the change in the above mentioned conditions and RSD values vary in between 1.04 - 5.77 (Table 5.4). These results indicates that the developed method have good performance and reliability even with small variation in method conditions.

Table 5.4 Robustness study.

Chromatographic changes	Level	Indapamide				Perindopril			
		RT	Area	TF	N	RT	Area	TF	N
Mobile phase ratio	70 : 30	0.74	246854	1.64	8254	1.05	125654	1.65	5957
	65 : 35	0.87	256481	1.74	8102	1.16	121842	1.65	6264
	60 : 40	1.02	241506	1.72	7650	1.82	118156	1.61	6441
Mean ± SD			248280± 7589				121884±3749		
RSD (%)			3.06				3.07		
Flow rate (mL/min)	0.8	1.14	265894	1.65	6845	1.71	144245	1.62	6038
	1.0	0.87	248456	1.65	8125	1.16	138824	1.71	6506
	1.2	0.72	265894	1.61	7845	1.03	145489	1.65	6382
Mean ± SD			260081±10068				142852±3543		
RSD (%)			3.87				2.48		
Column temperature (°C)	35°C	0.85	264584	1.70	8201	1.12	148425	1.64	6218
	30°C	0.87	248454	1.71	8054	1.16	145699	1.64	6151
	25°C	0.89	235844	1.71	7654	1.19	145894	1.62	6424
Mean ± SD			249627±14406				146673±1521		
RSD (%)			5.77				1.04		
UV wavelength	258	0.87	246895	1.71	7951	1.14	145299	1.63	5984
	254	0.87	248155	1.72	8156	1.16	140258	1.61	6068
	250	0.85	240184	1.71	7899	1.17	139521	1.61	6248
Mean ± SD			245078±4285				141693±3145		
RSD (%)			1.75				2.22		

5.9 Stability of solutions

The study of solution stability also play a key role for determining the effects on long time storage of solutions. For knowing stability of sample solutions, three different concentrations QC (LQC, MQC and HQC) samples are kept under the RT for 4 h, 4^{o}C for 48 h and 7days at -20^{o}C. No significant changes were observed during the stability studies and hence the sample solutions are stable under these conditions and the results are summarized in Table 5.5.

Table 5.5 Stability studies of analytes in Human plasma and mobile phase.

Analyte	Nominal value (µg/mL)	Stability Conditions			
		Human plasm (Analyte concentration (%))			Mobile phase
		RT (4 hour)	4^{o}C (48 hour)	-20^{o}C (15 days)	4^{o}C (24 hour)
Perindopril	1.5	97.25	102.4	96.15	98.45
	7	100.06	98.2	95.29	99.1
	25	98.33	99.65	96.72	99.58
Indapamide	0.45	101.6	103.08	97.26	102.5
	2.5	97.05	98.15	93	100.25
	12	98.3	100.2	95.52	97

5.10 Discussion

A new stability indicating UPLC method was developed to detect at low concentration (nano-gram) level of perindopril and indapamide in human plasma. The established UPLC method was optimized adequately by using various parameters, different UPLC columns, mobile phases for satisfactory peak shape, high sensitivity and good resolutions. The results showed that the UPLC-UV method is suitable for simultaneous quantification and identification of perindopril and indapamide drugs in human plasma. Very few LLE and SPE methods were reported for the

quantification of perindopril and indapamide in human plasma [51-54] which are time consuming and cost effective for the separation of drugs from biological samples. However, there is no SPE stability indicating UPLC method was reported for the identification of these drugs. The method reported in the present paper is very simple, easy to operate, quick and less cumbersome and showed adequate recovery percentage (93.00-101.58) of drugs. The established method was able to give low LOD and LOQ values and able to give high sensitivity, accuracy and precision to determine indapamide and perindopril in human plasma.

5.11 Conclusions

In summary, a stability indicating UPLC-UV method was developed and validated for the simultaneous identification and quantification of perindopril and indapamide drugs in human plasma and tested the stability studies as per ICH guidelines. It is a simple, accurate and specific method for the extraction of these two drugs from human plasma with quick elution time of two minutes. The method showed high recovery rate as well as low detection and quantification limits of two drugs. The developed method could be used for routine analysis as well as in bioanalytical and clinical studies.

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Chapter –VI
Summary and Conclusions

6.1 Summary

Chapter-I: Introduction focused on the importance of analytical method development and validation in the modern pharmaceutical analysis during the drug development procedure. Brief discussion on the importance of identification and quantification of process and relative impurities of drug molecules by using different types of analytical tools such as HPLC, UPLC, LC-MS etc. Necessity of forced degradation studies for newly developed drug molecules and evaluation of bioassay studies by using different analytical tools were discussed. The usage of different extraction method for the extraction of drug molecules from biological fluids were discussed. Several chromatographic methods for the identification and quantification of various pharmaceutical drug molecules (anti-cancer, anti-hypertension, anti-bacterial, anti-viral, and anti-epileptic agents etc.,) from biological fluids were reviewed. A brief discussion on the importance and instrumentation of HPLC and LC-MS techniques during the drug development procedure was given. And also the influence of stationary phase, mobile phase, diluent, flow rate column oven temperature etc. on the method was discussed. The importance of method validation according to ICH and USFDA guidelines, and also development of quality by design approach to analytical method were discussed. The objectives of current studies on the development of analytical and bio-analytical method for detection, identification and quantification of the anticancer, antihypertension and antiepileptic drug molecules and its degradation products were defined.

Chapter-II: The development of LC and LC-MS methods for the detection, quantification and identification of RCB along with its known impurities and degradation products were studied. The forced degradation sample preparation and method validations were carried out as per ICH guidelines. A precise, linear and rapid LC method was developed and validated for the assay of RCB and its related substances. The stability of bulk samples of RCB shelf-life was also checked

by using this developed LC method. The result of the RCB being subjected to different stress conditions led to the fact that it is stable during thermal, photolytic stress conditions. It degraded extensively under acidic hydrolysis as well as oxidative stress conditions. The LC-MS/MS data results were used in characterization of the three resultant degradation products and valid fragmentation pathways. The obtained stability studies, degradation pattern and proposed fragmentation pathway could potentially be useful for better understanding of RCB during storage and stable formulations and mend pharmaceutical safety.

Chapter-III: The development of HPLC-UV method for the detection and quantification of GEM from rat plasma was discussed. The solid phase extraction (sample preparation technique) for GEM was developed and method validations were done as per ICH guidelines. The proposed method is convenient, quick, reusable and eco-friendly due to less amount of organic solvents used. All these advantages make the method suitable for real plasma sample analysis. In conclusion, a simple, rapid and sensitive magnetic solid phase extraction method was developed for analyzing the anticancer drug GEM in plasma sample. The developed magnetic graphene oxide calix[6]arene composite showed efficient extraction and high recovery percentage from plasma solution due to hydrophobic and π-π interactions. The proposed method is convenient, quick, reusable and eco-friendly due to less amount of organic solvents used. All these advantages make the method suitable for real plasma sample analysis.

Chapter-IV: The development of HPLC-UV method for the simultaneous detection and quantification of BVC, ESL and CBZ from human plasma was discussed. Magnetic graphene oxide composite of β-Cyclodextrin with good water dispersibility was synthesized and characterized by FT-IR, SEM, powder XRD, and the magnetic property of the material was established by VSM. The material was found to be paramagnetic with sufficient magnetization for

the separation of composite with a conventional magnet. The developed material was used as MSPE sorbent for the extraction of three anti-epileptic drugs from the human plasma. The method showed high precision with wider linear range and good detection limits. This developed HPLC-UV method has good efficiency for recoveries, good linearity and simple to handle. And also it gave low retention time for thee antiepileptic drugs within 8 minutes. It provides high efficiency for extraction of trace level substances from human plasma.

Chapter-V: The development of UPLC-UV method for the simultaneous detection and quantification of IP and PP from human plasma was discussed in this chapter. A stability indicating UPLC-UV method was developed and validated for the simultaneous identification and quantification of perindopril and indapamide drugs in human plasma and tested the stability studies as per ICH guidelines. It is a simple, accurate and specific method for the extraction of these two drugs from human plasma with quick elution time of two minutes. The method showed high recovery rate as well as low detection and quantification limits of two drugs. The developed method could be used for routine analysis as well as in bioanalytical and clinical studies.

6.2 Conclusions

Overall the current thesis focused on the development of different type of chromatographic methods such as HPLC, UPLC and LC-MS for the separation, quantification and identification of selective drug molecules along with known impurities and degradation products (Table 6.1). Further, various magnetic solid phase materials such as MGO-CD and MGO-C[6]A were developed for extraction of selective drug molecules from biological fluids and applied to real sample analysis.

Table 6.1 Summary of the developed analytical and bio-analytical methods for selected drugs and their respective findings.

Drug	Extraction and detection method	LOD (ng/mL)	LOQ (ng/mL)	Linear Range (µg/mL)	Sample Analysis
RCB	LC-MS	11.0	35.0	0.03-300	Degradation studies
GEM	MSPE HPLC-UV	2.0	13.0	0.5-25.0	Rat plasma
BVC	MSPE HPLC-UV	28.3	94.31	0.5-50.0	Human plasma
ESL		6.1	20.4	0.1-40.0	
CBZ		14.8	49.5	0.25-60.0	
PP	SPE UPLC-UV	33.5	110.5	0.75-25.0	Human plasma
IP		8.6	28.3	0.15-12.0	

The obtained results has clearly showed the efficacy, versatility of the developed stability indicating analytical and bioanalytical methods for the determination, quantification and identification of drugs in biological samples and its degradation products. The developed solid phase extraction method may provide a platform for the future prospects in assessing the drug molecules, environmental pollutants and food sample analysis. These developed chromatographic methods can be used in routine analysis of drugs in pharmaceutical industries.

List of Publications

1. **Babji, P**., Ramesh, T., Reddy, K. V., Konakanchi, R., Rao, P. N., & Gobi, K. V. (2019). Identification and Characterization of Rucaparib Degradation Products and Their Comparison with Known Impurities. Chromatographia, 82(2), 591-604.

2. **Babji, P**., Rao, P. N., Jugun, P. Ch. (2021) Development of new stability indicating UPLC-UV method for the extraction and quantification of perindopril and indapamide from human plasma. *Future Journal of Pharmaceutical Sciences*, 7:71

3. **Babji, P**., Reddy, K. V., Rao, P. N., & Gobi, K. V., Jugun, P. Ch. (2021) Simple and Efficient Method for the Quantification of Antiepileptic Drugs in Human Plasma by using magnetic Graphene oxide-β-Cyclodextrin Composite as a sorbent. *Future Journal of Pharmaceutical Sciences. (Accepted)*

4. **Babji, P**., Amar, B. N. L., Gobi, K. V., Jugun, P. Ch. (2021) Development of a magnetic solid phase extraction method for gemcitabine from rat plasma by using magnetic graphene oxide calix[6]arene composite and its application to pharmacokinetics. *Journal of Pharmaceutical and Biomedical Analysis (Under review).*

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ORIGINAL

Identification and Characterisation of Rucaparib Degradation Products and Their Comparison with Known Impurities

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Abstract

A reversed-phase HPLC (high-performance liquid chromatographic) method is employed to identify and quantify the degradation impurities and related substances of Rucaparib (RCB), API bulk drug. There is no literature available that elucidates degradation phenomenon of RCB. Thus, the present study focuses on understanding the degradation phenomenon of RCB. The chromatographic separation is achieved within 25 min run time, where Zorbax Bonus RP column is used with a gradient elution of trifluoroacetic acid–ACN–water as the mobile phase. In this study, various forced degradation conditions such as photolysis, hydrolysis, oxidation, and thermal degradation were employed on RCB. Oxidative, basic, and acidic stress conditions showed a significant impact on degradation compared to thermal and photolysis. LC–MS technique was used for the characterization of degradation products and the plausible pathways of fragmentation were proposed. Qualified reference standards were used to quantify the stressed samples and the mass balance was found close to 99.5% (w/w) considering the response of the degradants to be equivalent to RCB. Results of this investigation confirmed the efficacy of the proposed novel method to determine drug stability of RCB, and ICH guidelines were also considered to authenticate our results.

Keywords Rucaparib · Liquid chromatography · Stability-indicating method · Forced degradation · LC–MS · Validation

Introduction

Ovarian cancer was frequently noticed in the women over the age of 40 years in the US and UK [1, 2]. Ovarian cancer (OC) is the fifth leading cause of cancer-related deaths in women. In general, treatment procedures depend on ovarian cancer type and its stages in specific conditions. Advanced stage of ovarian cancer can be treated by RCB [3]. RCB is a US FDA (Food and Drug Administration) approved orphan drug for the treatment of female patients with certain types of ovarian cancer [4]. RCB is a type of PARP [poly(ADP-ribose) polymerase] inhibitor [5, 6], which is an enzyme, that normally helps in the repair of damaged DNA cell through the activation of base excision repair and alternative end-joining pathways and inhibition of the non-homologous end-joining pathway [7–9]. The BRCA gene is responsible for the repair of damaged DNA and usually serves to prevent tumor growth. However, its genetic mutations can lead to specific cancers such as ovarian cancer [10, 11]. Patients in advanced stages of ovarian cancer, i.e., if mutation in BRCA is detected even after more than one platinum-based chemotherapy, are eligible for treatment with RCB [12, 13].

In pharmaceutical industries, stress studies of API drugs play an important role during the storage and formulations. The forced degradation studies of drug product and related substances give the impurity profile, which is useful to examine the stability of drug under the influence of factors like light, temperature, and humidity. In general, during the forced degradation studies, the drug loses its efficiency and forms some new drug related impurities. Based on these studies, the adverse effects of drug due to the formation of toxic degradation compounds can be understood.

LC–MS (liquid chromatography–tandem mass spectrometry) is used to study the bio-analytical assay method for RCB in plasma by Sparidans et al. [14]. In stress studies, LC–MS is emerging an adaptable tool for the determination of drug impurities and degradation products [15].

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To date, no systematic characterization and mechanistic pathway approach have been developed for the degradation of RCB under the stress circumstances specified by ICH Q1A (R2) [16]. The major purpose of this study is to examine the degradation behavior in several conditions and to define the degradation product of the drugs. Drug fragmentation patterns and their degradation products were established by exposing the drug to ICH suggested oxidation, thermal, photolytic, hydrolysis stress conditions, and analyzed the resultant solution to optimize through LC–MS, MS/MS, MS^n, and precise mass measurements.

Experimental

Chemicals and Reagents

RCB and three impurities (A, B, and C) (gift samples (Fig. 1) were obtained from Mylan laboratories Pvt. Ltd., Hyderabad, India). HPLC grade ACN was purchased from Rankem (Mumbai, India), and AR grade H_2O_2 (hydrogen peroxide), HCl (hydrochloric acid), NaOH (sodium hydroxide), and CF_3CO_2H (TFA) were purchased from S D Fine Chemicals (Mumbai, India). The Millipore Synergy apparatus (Millipore, France) was used for water purification of water.

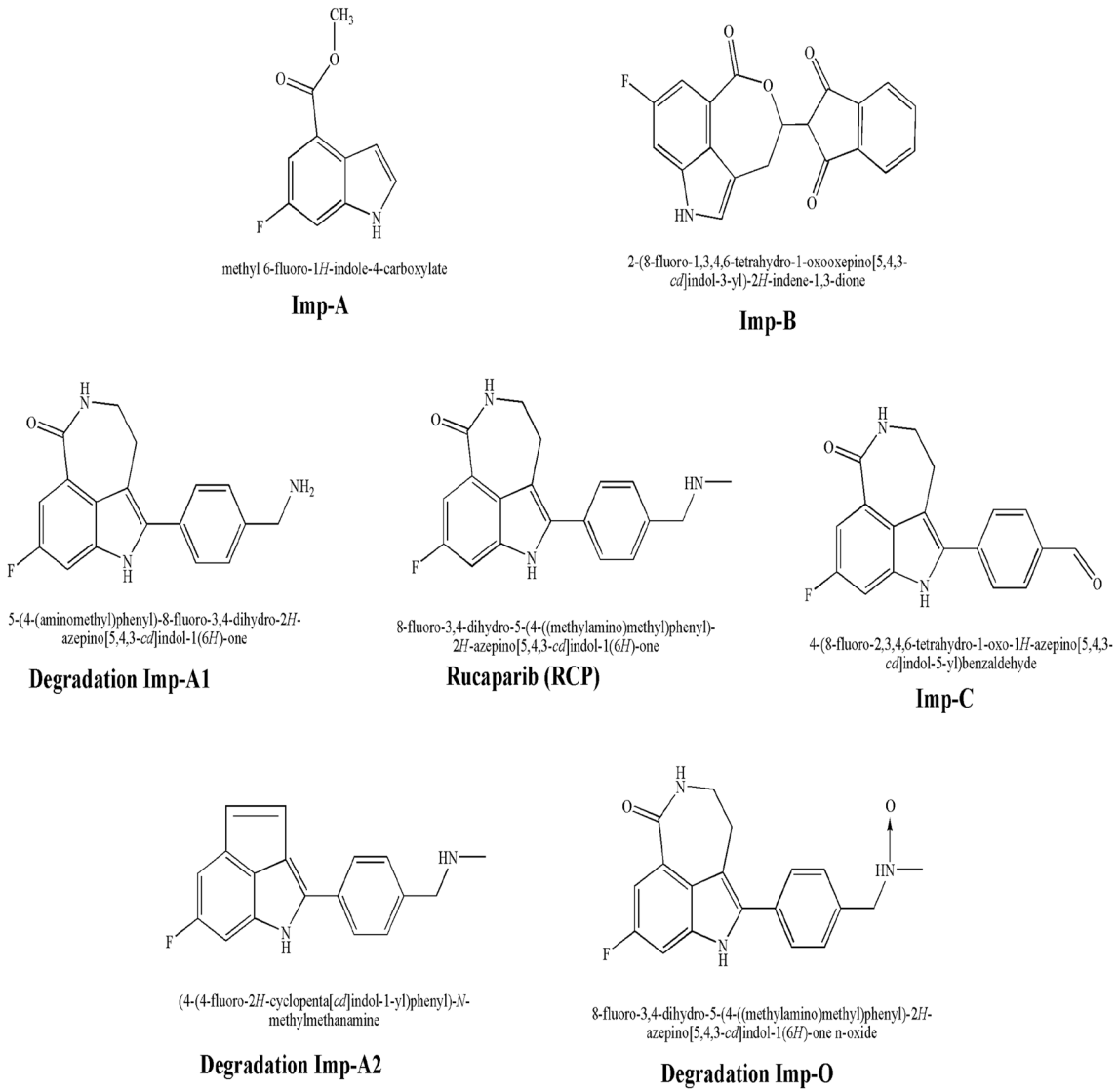

Fig. 1 Chemical structure of RCB, its related impurities, and forced degradation impurities

Instrumentation

For the method development, validation and simulated degradation studies' HPLC (Alliance Waters 2695) system along with DAD (Diode Array Detector) was used. To separate the compounds, Zorbax Bonus Reverse Phase (Agilent, USA) column with particle size 3.5 µm and 150 × 4.6 mm was used. HP-Vectra (Hewlett Packed, Waldron, Germany) computer system along with Millennium data software was used for chromatographic data recording. Agilent1100 online ion trap MSD mass spectrometer along with an auto-sampler (G1329A), APCI source in + ve mode, and Diode Array Detector G1315B (Waldbronn, Germany, Agilent Technologies) was used to perform the LC–MS/MS. The mass data were obtained using a Q-TOF-HRMS (High-Resolution Mass Spectrometer) fortified with an ESI source (QSTAR XL, MDS Sciex/Biosystems, USA) and QS software. The same LC circumstances were used for LC–MS (4.0 kV capillary voltage, 300 °C source temperature, and 600 L h^{-1} gas flow rate). The characteristic source circumstances were: 5.00 kV capillary voltage, (4 kV for + ve mode); 60 V declustering potential; 220 V focusing potential; 10 V second de-clustering potential; 10,000 resolution (full-width half-maximum). Ultra-high pure nitrogen gas was used as a collision and carrier gas, and zero air was used for nebulizer. The precursor ion was chosen for the experiments on CID (collision-induced dissociation); using the TOF and quadruple analyzers, the ions of the product were analyzed. Photostability study was performed in a photostability chamber (Leicestershire, Sanyo, UK). For hydrolysis stress studies, Cintex Digital Water Bath was used and Cintex Dry Air Oven was used for thermal stress studies.

Chromatographic Conditions

The gradient LC method uses water:TFA and ACN:TFA in the ratio of 100:0.10 (v/v) as a mobile phase-A and mobile phase-B, respectively. Zorbax Bonus RP column was used to achieve the chromatographic separation for the method. A flow rate of 1.0 mL min^{-1} is set for the mobile phase. The LC gradient program was set as (*T*/%B) = 0/40, 10/50, 20/90, and 25/90 which changes every 5 min of its 25 min run time. A constant column temperature of 40 °C was maintained throughout the study. The injection volume and the detection wavelength were set at 10 µL and 245 nm, respectively. The diluent was a mixture of ACN and water in the ratio of 50:50.

Preparation of Standard and Sample Solutions

The RCB solution and each of the impurity stock solutions of the concentration 500 µg mL^{-1} in the diluent were prepared for the determination of assay and related substances. The stock solutions were diluted to get the required concentration of standard solutions containing impurities and drug. All three potential impurities were mixed to prepare a stock solution and the RCB was spiked at a level of 0.15% w/w resulting in a target analyte concentration (TAC) of 500 µg mL^{-1}.

Stress Studies

Hydrolysis, oxidation, and thermal and photolytic methods were employed as stress conditions as per ICH recommendation for degradation studies. Using 1.2 million lux hours of visible light on the thin layer of RCB, the photolytic study was carried out for 11 days [17]. The thermal stress study was performed for 10 days at 105 °C. The base and acid stress studies were done at the ambient temperature (25 ± 2 °C) with 1N NaOH for 42 h and 0.1 N HCl for 24 h. The oxidation stress study was carried out for 24 h at room temperature with 1% H_2O_2 solution. RCB reference standards were used to quantify all the stressed samples. Photodiode array (PDA) was used to examine the spiked solution of RCB and the peak purity of RCB in stressed samples with its known related impurities. The LC–MS was used to check the mass numbers of the unknown degradation impurities. The structures of RCB, its related impurities, and degradation products are presented in Fig. 1.

Results and Discussion

Method Development and Optimization

The main objective of our method is to isolate all degradation products and potential impurities from the analyte, in particular B & C which are critical impurities, and also, to obtain the better peak shape of RCB. In the initial attempts at method development, numerous diverse stationary phases were used and the results are enumerated in Table 1 and the corresponding LC chromatograms are shown in Fig. 2. Zorbax Bonus RP column (150 × 4.6 mm and 3.5 µm) as stationary phase and using mobile phase solutions A and B, the resolution of closely eluting impurities and peak shapes achieved was satisfactory and the resulting LC chromatogram is shown in Fig. 3. In achieving the resolution between RCB, Imp-B, and Imp-C, stationary phase played a major role. Highly polar amide groups embedded in the stationary phase and end-capping procedures are the key in deactivation unwanted silanol interactions and achieving the good resolution. Zorbax Bonus RP column is suitable for the separation of basic compounds [18]. It was reported that TFA is one of the best acidic additives that has the ability to adjust the selectivity with regard to the strength in the mobile phase [19–21]. Kailin Guan et al. reported that zwitterionic and

Table 1 Results of method development

Trial	Column	Dimensions	Mobile phase	Conclusion
1	Zorbax SB-C18	150×4.6 mm 5.0 µm	Acetate buffer with pH 2.5/acetonitrile	Imp-B and Imp-C were co-eluted with analyte
2	Zorbax XDB-C18	150×4.6 mm 3.5 µm	Water/acetonitrile/trifluoroacetic acid	Imp-C was co-eluted with analyte
3	Zorbax XDB-C8	150×4.6 mm 3.5 µm	Water/acetonitrile/trifluoroacetic acid	Imp-C was co-eluted with analyte
4	Zorbax SB-Cyano	150×4.6 mm 3.5 µm	Water/acetonitrile/trifluoroacetic acid	Imp-B and Imp-C were co-eluted with analyte
5	Zorbax SB-Phenyl	150×4.6 mm 3.5 µm	Water/acetonitrile/trifluoroacetic acid	Imp-C was co-eluted with analyte

weak basic compounds could be separated by modulating TFA concentration in the mobile phase [22]. In the current study, as RCB is a weak basic compound; we have employed TFA in the mobile phase has been employed. The results reported the best resolution in peaks and good peak shape. For the separation of Imp-B & C from the RCB peak, the gradient program also plays a significant role. The optimized gradient program is suitable for the retention time of the RCB at column temperature 40 °C, and found that RCB tailing factor was about 1.0. RCB and its three potential impurities were well isolated with a resolution greater than 2, under optimized conditions. The characteristic chromatogram of RCB and its associated impurities is shown in Fig. 3.

Validation

As per the ICH guidelines, the developed analytical LC method was validated for the determination of RCB and its related substances [23].

System Suitability Test

In chromatographic methods, system suitability test plays a vital role to corroborate the adequacy of resolution, reproducibility, and column efficiency of the chromatographic system for particular analysis. The studies were conducted using Zorbax Bonus RP column and the test results are tabulated in Table 2.

Specificity

The specificity of RCB was checked in the presence of its degradation products and its potential impurities. The products and impurities were separated from the peak of RCB with a specified resolution, as shown in Fig. 3.

Precision

During the precision studies, RSDs were obtained within 0.2% and 2.0%, respectively, in the assay results of RCB and all three impurities. To obtain the results, six individual measures of RCB with three potential impurities (0.15% w/w) with the reference of TAC were used. In the intermediate precision study, the assay results of RCB and all three impurities' RSDs were achieved at less than 0.4% and 1.5%, respectively. The reduction in individual values confirms that the method has good precision. RCB and related impurities' %RSD values are reported in Tables 3 and 4, respectively.

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

The dilution of the standard stock solutions of RCB and its three potential impurities to solutions of known concentrations, the LOD and LOQs, were established which resulted in SNR (signal-to-noise ratio) of 3:1 and 10:1, respectively. Six separate RCB formulations with relevant impurities at LOQ level were prepared and the percentage of RSD calculated. The precision for the RCB and the impurities in the region of RCB accuracy were also achieved by preparing three recovery solutions for the impurities associated with the RCB at the LOQ level and then calculating the percent recovery of all relevant impurity areas. The LOD of RCB and its three potential impurities at the injection volume 10 µL was observed to be lower than or equal to 0.02% w/w (TAC) (Fig. 4). The LOQ of RCB and its three potential impurities were found to be lower than or equal to 0.05% w/w (Fig. 4; Table 4). The dose of RCB which is between 10 and 80 mg per day is advantageous for the method to know the LOQ of the reported threshold for impurities and APIs. The quantitative limit of these impurities helps, during process optimization, to achieve the acceptable levels of impurities. The recovery values were from 98.9 to 101.0 at impurity content close to LOQ level and the RSD was lower than 2.0%. The results of the LOQ level accuracy are shown in Table 5.

Linearity

Linearity at a higher level is established by comparing five different RCB solutions of 80, 90, 100, 110, and 120% w/w, respectively, in terms of TAC. Linearity at a lower level was determined by adding six various solutions with LOQ 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30% w/w in terms of TAC of three impurities (A, B, C) and RCB. The linear

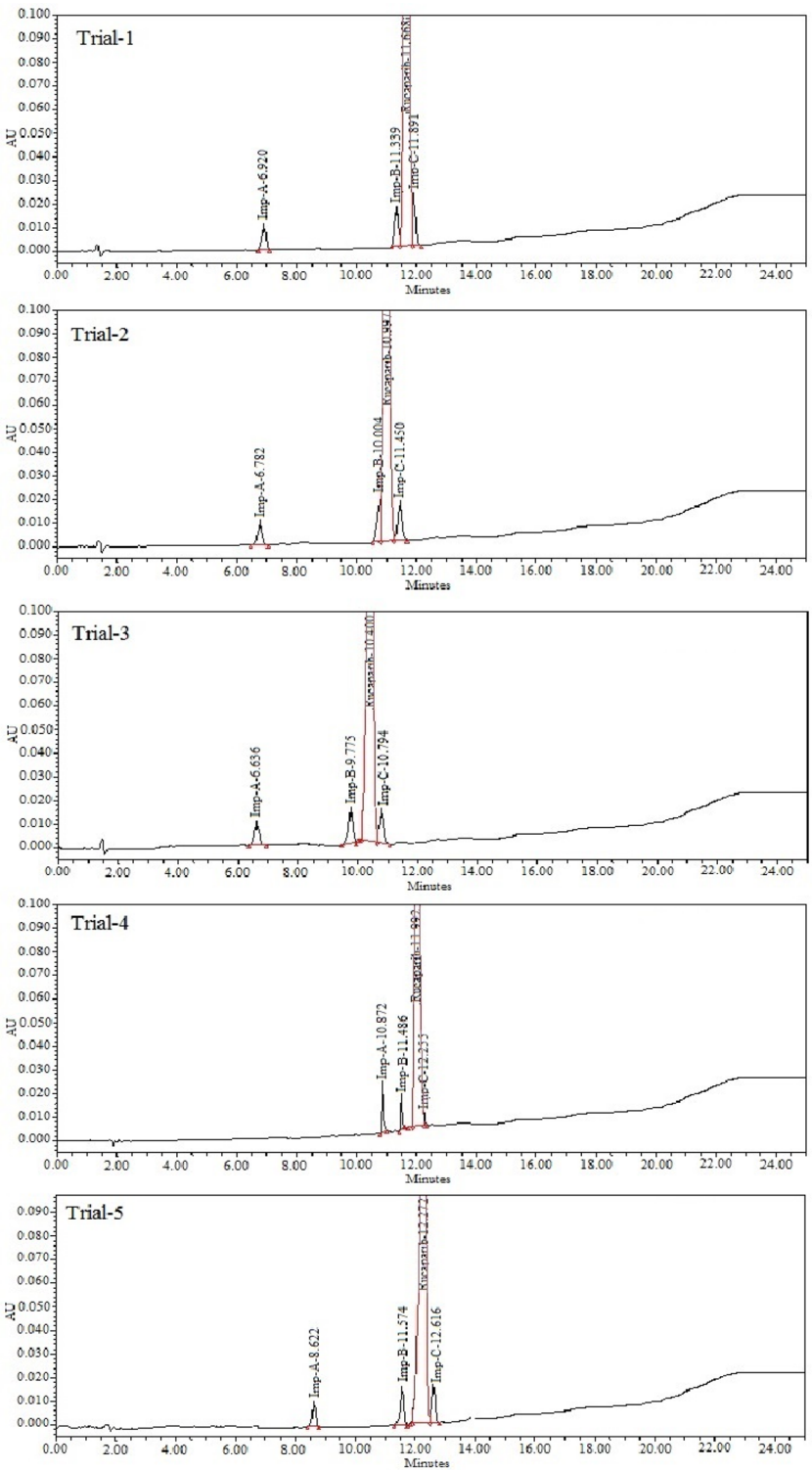

Fig. 2 Typical LC chromatograms during method development

regression analysis was plotted with the peak area versus concentration data. The correlation coefficients of regression, percent, *y*-intercept, and the slope of the calibration curves were calculated. The correlation coefficients achieved greater than 0.999 for all impurities. The plot of RRF of each impurity was determined using the slope of RCB plot versus each impurity. At the level of 0.15% w/w of all impurities, the response of *y*-intercept of each plot was within the 2.0%, which is running almost through the origin, making it possible to obtain an accurate value of RRF. RRF values and linearity results are tabulated in Table 4.

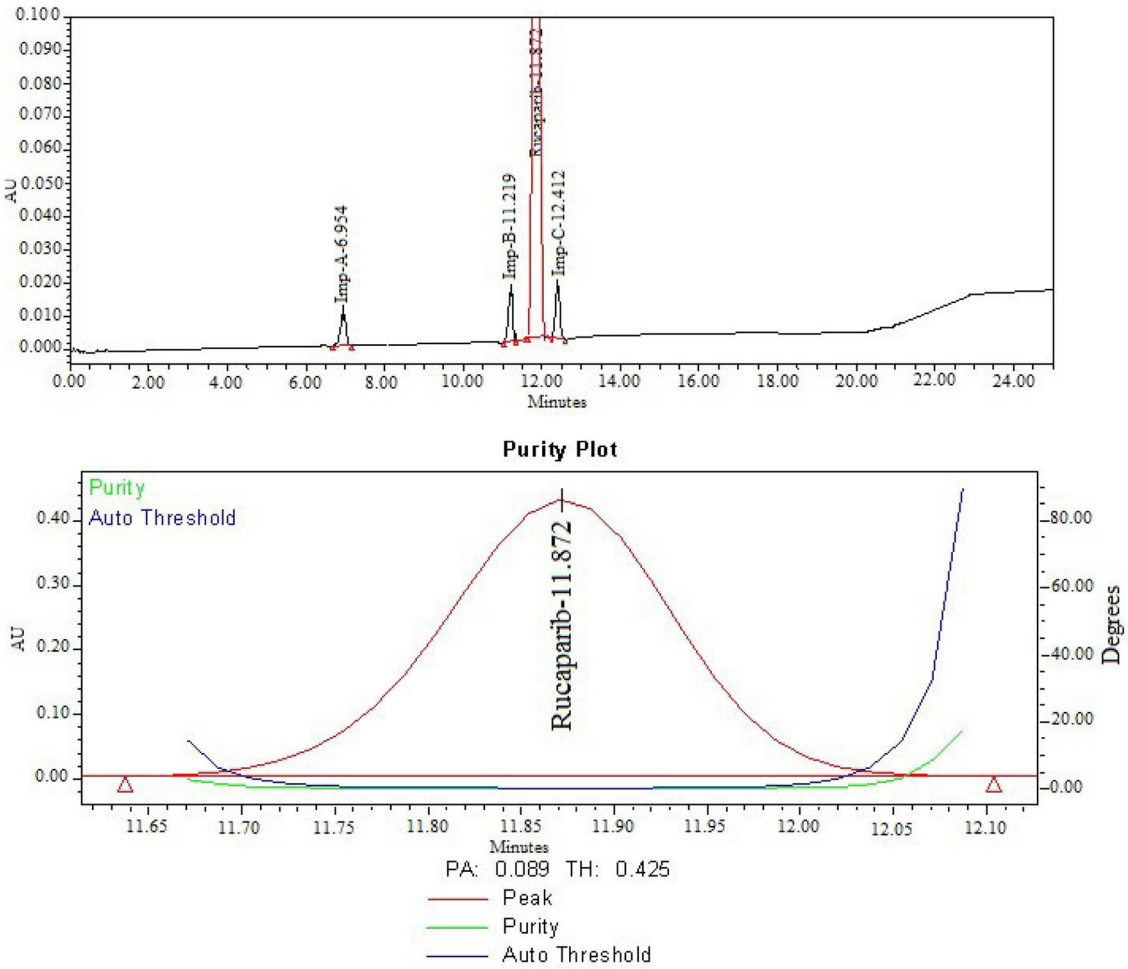

Fig. 3 Typical LC chromatogram and peak purity spectrum of RCB spiked with its related impurities at specification level

Table 2 Results of system suitability test

Compound	RT (min)	RRT[a] ($n=3$)[c]	USP resolution R_s[b] ($n=3$)[c]	USP tailing factor T ($n=3$)[c]	No. of theoretical plates (N tangent method)
Imp-A	6.9	0.58 ± 0.01	–	1.0 ± 0.05	46,308
Imp-B	11.2	0.94 ± 0.01	3.03 ± 0.32	1.0 ± 0.02	59,078
Imp-C	12.4	0.96 ± 0.01	4.01 ± 0.41	1.0 ± 0.03	65,751
RCB	11.9	1.0	3.15 ± 0.57	1.0 ± 0.02	71,124

[a]Relative retention times (RRT) were calculated against the retention time (RT) of RCB

[b]Resolution calculated between two adjacent peaks

[c]Mean ± SD

Accuracy

The assay accuracy was evaluated at three times with three different concentrations of RCB of concentrations 400, 500, and 600 µg mL^{-1}, and the recovery percentages were calculated at each level. To verify the accuracy of the relevant substances in the same way and to quantify all three impurities of RCB, the standard adding and recovery experiments were carried out. Studies were performed at TAC of 0.075, 0.15, and 0.225% w/w. The recovery percentages of all three impurities were calculated by taking into consideration the number of spiked impurities, the amount of impurities present in the unspiked samples, and the amount of impurities recovered after RRF correction.

Table 3 Results of validation parameters for RCB at assay level

Parameter	RCB
Regression equation (*y*)	
Slope (*m*)	234.1
Intercept (*C*)	− 4.2
% *Y*-intercept	− 0.56
Correlation coefficient	0.9999
Precision (%RSD)[a]	0.12
Ruggedness(%RSD)[a]	0.36
% Recovery[b] at 50% level	99.9 ± 0.13
% Recovery[b] at 100% level	99.5 ± 0.69
% Recovery[b] at 150% level	99.7 ± 0.78

[a]($n = 6$)

[b]($n = 3$)

Table 4 Results of validation parameters for related impurities

Parameter	RCB	Imp-A	Imp-B	Imp-C
LOD (mg mL^{-1})	0.011	0.013	0.012	0.009
LOQ (mg mL^{-1})	0.035	0.044	0.041	0.031
Regression equation (*y*)				
Slope (*m*)	451.21	472.12	455.91	436.54
Intercept (*C*)	− 2.50	− 3.20	0.98	2.31
% *Y*-intercept	− 0.21	− 0.32	0.12	1.10
Correlation coefficient	0.9999	0.9997	0.9999	0.9992
Precision at LOQ level (%RSD)[a]	0.98	0.72	1.01	1.24
Precision (%RSD)[b]	–	1.10	1.23	1.35
Ruggedness (%RSD)[b]	–	0.98	0.93	1.10
Relative response factor	1.0	1.0	1.0	1.0

[a]Linearity range was from LOQ to 0.30% w/w of RCB and its related impurities with respect to analyte concentration

[b]($n = 6$)

The recovery percentage of all three impurities was in the range 98.9–102.1 in the bulk drug sample (as indicated in Table 5). The recovery percentage of RCB in the bulk drug sample was in the range of 99.5–99.9 (tabulated in Table 3). All the impurities values were within confidence intervals of the mean value.

Robustness

To determine the robustness of the established LC method, certain deliberate variations were made to the original LC method conditions. The effect of flow rate 1.0 mL min^{-1} was studied on the resolution, tailing, and theoretical plates by changing it to 1.2 and 0.8 mL min^{-1}. The impact of the wavelengths at 243 nm and 247 nm was studied instead of the usual 245 nm. The temperature impact on the column was also investigated at 45 °C and 35 °C in its place of the usual standard temperature of 40 °C. By changing the ratio of organic modifier (varying ± 2% absolute) in both mobile phase-A as well as B from its original state, the impact of the ratio of organic modifier was studied. For all altered circumstances, i.e., wavelength, flow rate, organic modifier, and temperature, the system suitability results such as theoretical plates and tailing factor of the analyte and the resolution between analyte and Imp-B and Imp-C were calculated. The varied chromatographic conditions mentioned above were achieved, when the tailing factor of RCB was less than 1.2, theoretical plates were more than 61,000, the resolution between RCB, Imp-B, and Imp-C was greater than 1.5. The very small changes in the theoretical plate, tailing factor, and resolution tailing factor results observed in all robustness conditions illustrate the robustness of the method. When considering the ability of the column, higher column temperature results in a better resolution than the nominal temperature. The results are tabulated in Table 6.

Solution Stability and Mobile Phase Stability

The stability of RCB solution with its related impurities was studied at room temperature (25 ± 2 °C) and at cooling conditions (5 ± 2 °C) for 48 h. The assay of the analyte and all potential impurities were identified during the course of 48 h with a time interval of 4 h. The mobile phase stability was determined by evaluating the assay of RCB solution and its spiked solution with known impurities at a specific level. For this purpose, freshly prepared spiked solution was injected at regular intervals of time, i.e., every 4 h for 48 h with the same mobile phase. The solution was found to be stable for 4 h at room temperature and 24 h under cooling conditions. The RSD was within 0.60% and no major changes in the impurities were observed during stability experiments of mobile phase solutions.

Identification of Degradation Products

The LC–MS studies were carried out using Agilent 1100 series LC system, which was coupled with a triple quadrupole mass spectrometer, is used to determine the *m/z* values of the degradation products formed during acidic hydrolysis and oxidation process. The *m/z* values at 309.1, 260.0, and 339.2 were assigned to the unknown degradation product impurities A_1, A_2, and O. Figure 1 lists the proposed structures of the degradation impurities.

Degradation Behavior

The purity and assay of RCB before and after the degradation under various stress conditions like acidic and basic hydrolysis, thermal, oxidation, and photolytic are tabulated

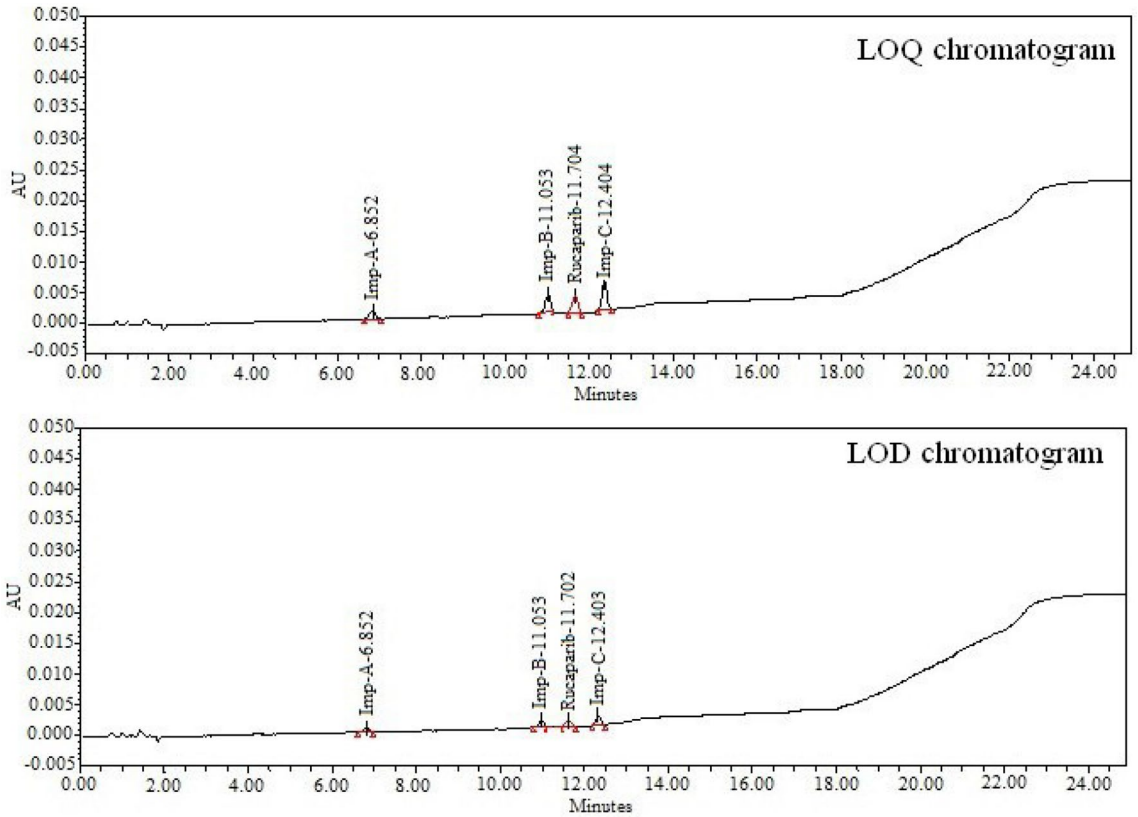

Fig. 4 Typical LOQ and LOD chromatograms of RCB with its related impurities

Table 5 Evaluation of accuracy for related impurities

Amount spiked[a]	% Recovery[b]		
	Imp-A	Imp-B	Imp-C
LOQ	101.0 ± 0.32	101.0 ± 0.25	98.9 ± 0.12
80%	99.7 ± 0.69	101.4 ± 0.56	101.3 ± 0.07
100%	102.1 ± 0.82	100.8 ± 0.32	101.1 ± 0.17
120%	100.6 ± 0.24	100.1 ± 0.82	100.6 ± 0.48

[a]Amount of impurities spiked with respect to specification level

[b]($n = 3$)

in Table 7, and the chromatographs of stressed samples are depicted in Fig. 5.

There is no degradation noticed when the RCB was subjected to thermal and photolytic conditions. However, the drug degraded and formed two unknown impurities during the acidic hydrolysis. In addition, the basic hydrolysis stress condition led to the formation of known impurity-B. One unknown impurity along with the known impurity-C was formed during the oxidative degradation of the drug. The different notations used for the impurities and degradation products are indicated in Table 7. The total ion chromatograms under different stress conditions are depicted in Fig. 6.

The retention time that was determined, resolution factor, tailing factor, peak purity index, and relative retention time, which are commonly termed as chromatographic parameters, for the degradation products are summarized in Table 8. For the analyte, the peak purity index obtained in all stressed samples was used to calculate the specificity. The peak homogeneity study established that the RCB peak is homogeneous besides being pure in all the analyzed stress samples. When the RRF of the degradants was considered to be one, the mass balance was 99.5%. The summary of the forced degradation study is shown in Table 7.

Characterization of Degradation Products

Acidic Hydrolysis Process

The acidic hydrolysis of RCB resulted in the formation of two degradation products, A1 and A2. Product A1 was formed due to the elimination of the methyl group from RCB which is evidenced by the appearance of molecular ion at *m/z* 309.1350 Da. The retention of the peaks A1 and A2 detected by LC–MS was at 14.5 and 17.7 min, respectively. Figure 6 shows the spectra of the acidic degradation products of LC–ESI–MS/MS.

Table 6 Results of robustness

Actual value	Changed value	No. of theoretical plates (*N* tangent method)	USP tailing factor (*T*)	USP resolution (R_s) between Imp-B and RCB	USP resolution (R_s) between RCB and Imp-C
1.0 mL min^{-1}	0.8 mL min^{-1}	62,154	1.1	2.2	2.9
	1.2 mL min^{-1}	82,263	1.0	1.6	2.4
245 nm	243 nm	68,968	1.0	2.1	2.8
	247 nm	69,794	1.0	2.1	2.8
40 °C	35 °C	61,459	1.0	2.0	2.5
	45 °C	75,936	1.0	2.4	3.1
0/40, 10/50, 15/70, 20/90, 25/90[a]	0/36, 10/45, 15/63, 20/81, 25/81[a]	65,265	1.0	2.2	2.9
	0/44, 10/55, 15/77, 20/99, 25/99[a]	84,789	1.0	1.6	2.5

[a](*T*/%B)

Table 7 Summary of forced degradation results

Stress condition	Time	Purity of analyte after degradation	Assay of analyte after degradation	Remarks
Unstressed sample	–	99.8	99.7	–
Acid hydrolysis (0.1 N HCl)	24 h	86.3	86.9	Significant degradation was observed. Unknown degradation impurity-A_1 and impurity-A_2 were formed
Base hydrolysis (1 N NaOH)	42 h	89.1	89.2	Significant degradation was observed. Known impurity-B was formed
Oxidation (1% H_2O_2)	24 h	89.1	89.9	Significant degradation was observed. Unknown degradation impurity-O along with known impurity-C was formed
Thermal (105 °C)	10 days	99.8	99.6	Significant degradation was not observed
Photolytic degradation	11 days	99.8	99.5	Significant degradation was not observed

Fragmentation of A1 leads to the fragment ions having *m/z* 294 Da, 248, 233, by losing NH and COF from successive ions. The detailed fragmentation is shown schematically in Fig. 7. This fragmentation was established by the elemental composition of $C_{18}H_{16}FN_3O$ and the calculation of the masses of elements accurately. The CID MS^2 of A1 formed the fragment ions by the elimination of amine and the addition of CO_2 with HF having *m/z* values of 294 and 248, respectively. A stable fragment from the degraded product A1 is the ion of 2*H*-cyclopenta[cd]indole. The formation of indole ion can be explained by the nucleophilic attack of hydroxide ion of benzylamine moiety of the drug, followed by the degradation of fragment ion with *m/z* 91.

Another degraded product A2 with *m/z* 306.1 Da was also formed due to the loss of a water molecule from RCB during acidic hydrolysis. Fragmentation of A2 led to the formation of fragment ions with *m/z* 260, 246, 231, 217, and 141 Da. The detailed fragmentation is shown schematically in Fig. 7. Table 9 lists the *m/z* values of fragmentation ions and degradation products. For degradation products, all the mass values along with an error (in ppm) were determined. Molecular formulas of each HRMS result have been tabulated in Table 10.

Oxidation Process

The degradation products O and C were formed during the oxidative degradation process in 1% H_2O_2 at room temperature in 24 h. The product O was formed by the oxidation of amine to N oxide which was evidenced by its *m/z* 339 corresponding to the molecular ion. The MS^2 fragment ions of O were obtained at *m/z* 309, 280, 204, 144, 117, and 91. The elimination of tropylium cation from the fragment ion with *m/z* 309 yielded the fragment with *m/z* 117. The probable chemical formula given by HRMS is $C_{19}H_{18}FN_3O_2$ for the degradation product O.

The second oxidative degradation product C was formed with *m/z* 308 by eliminating the cyanide group. In addition, MS/MS fragments were observed at *m/z* 248, 221, 193, 144, 117, and 106. The oxidation degradation process for the formation of products and its fragmentation is shown schematically in Fig. 8 from the knowledge of LC–ESI–MS/MS spectra. The HRMS results support the proposed structure, as shown in Table 10.

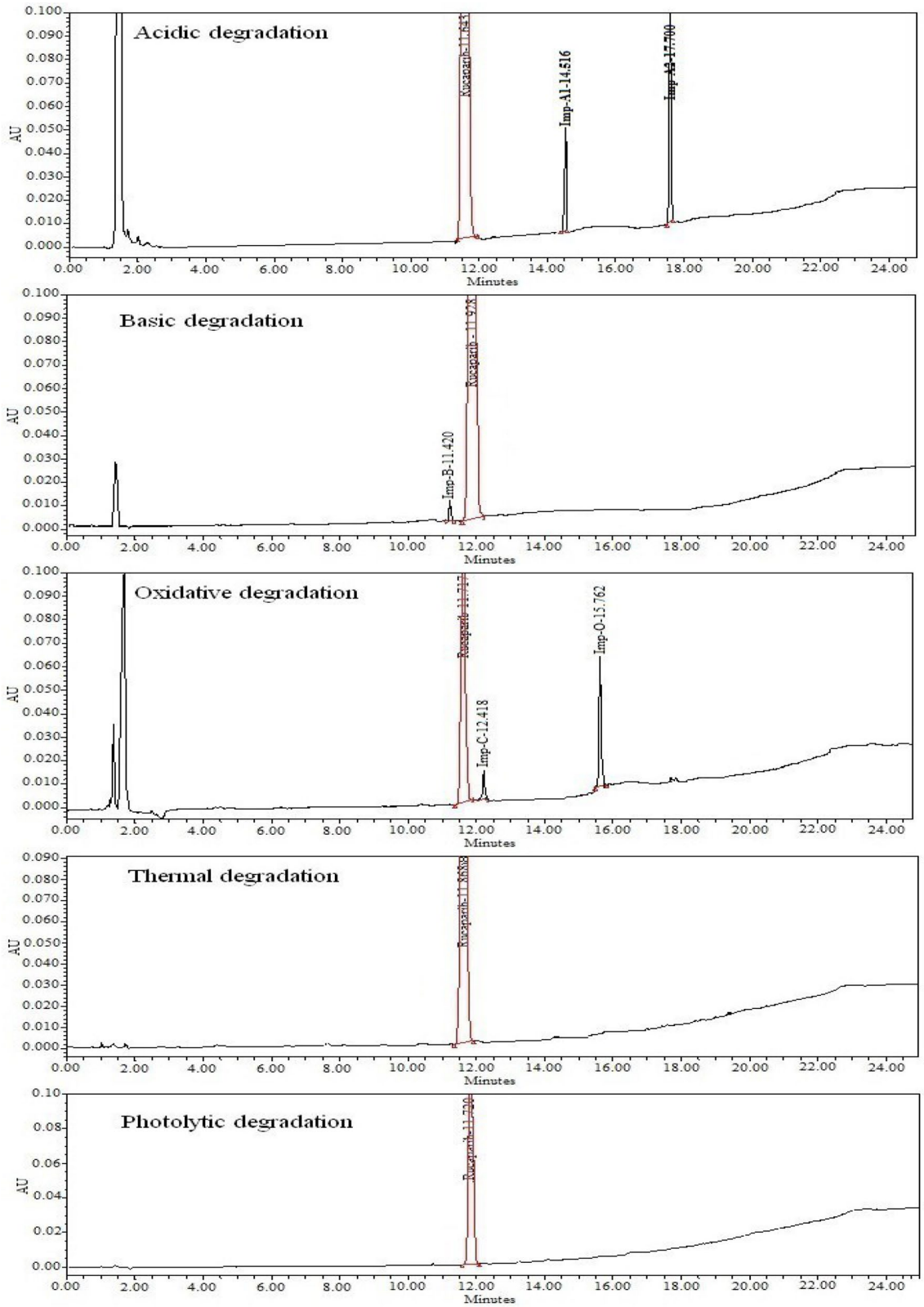

Fig. 5 Typical LC chromatograms of all stress conditions

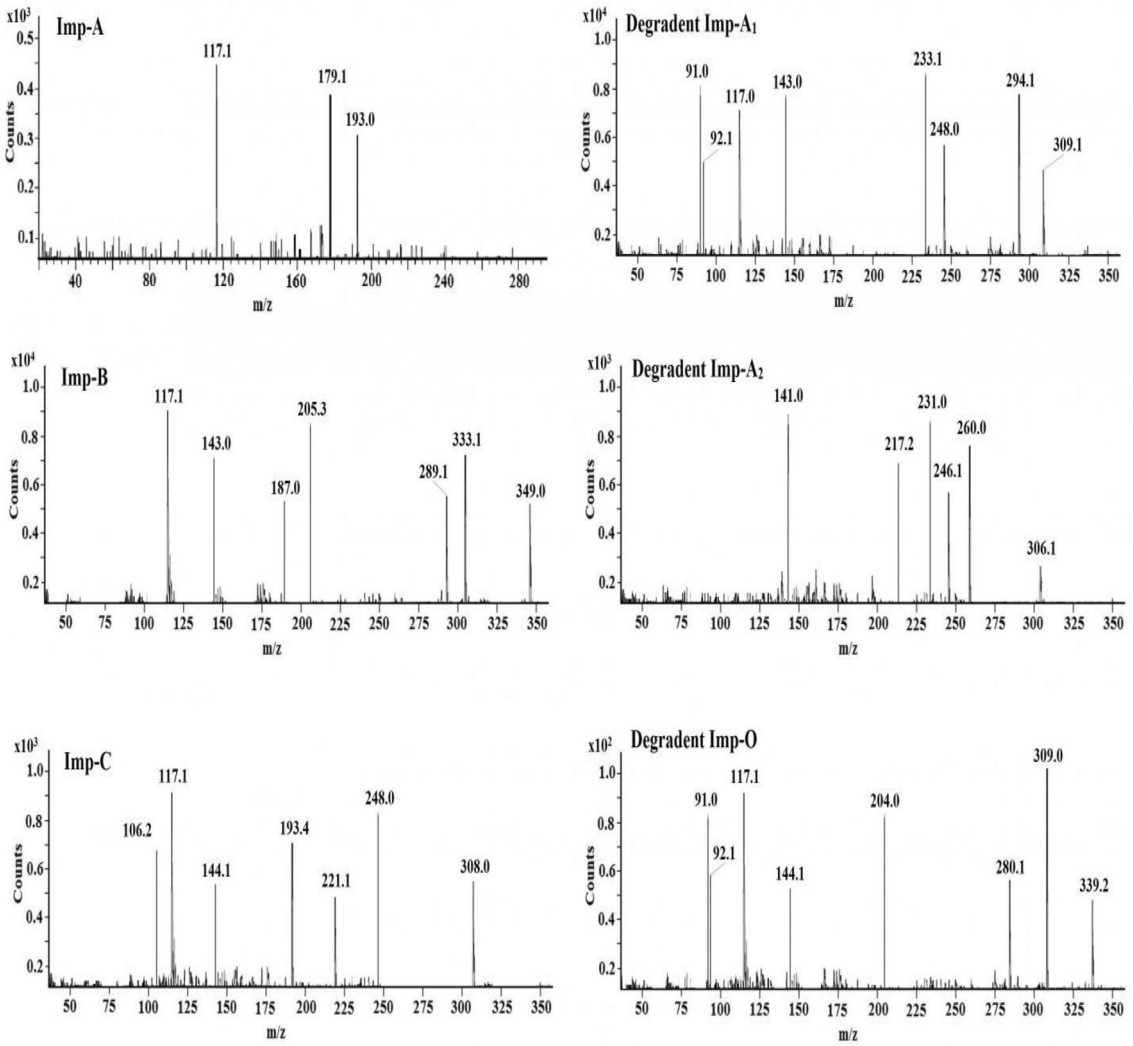

Fig. 6 LC–ESI–MS/MS spectrum of $[M+H]^+$ ions of Imp-A (*m/z* 193)at 18 eV; Imp-B (*m/z* 349) at 20 eV; Imp-C (*m/z* 308) at 16 eV; Imp-A_1 (*m/z* 309) at 15 eV; Imp-A_2 (*m/z* 260) at 19 eV; Imp-O (*m/z* 339) at 16 eV

Table 8 Peak, purity, and chromatographic data of the products formed after degradation

Degradation product	R_t (min)	P.P.I	R_s	T_f	Relative retention time (RR_t) min
A1	14.51	0.9982	4.37	1.02	1.52
A2	17.70	0.9966	6.53	1.07	1.43
O	15.76	0.9993	5.66	1.05	1.45

R_t, retention time; R_s, resolution factor; T_f, tailing factor; P.P.I, peak purity index; RR_t, relative retention time

Conclusion

A precise, linear, and rapid LC method was developed and validated for the assay of RCB and its related substances. The stability of bulk samples of RCB during its storage was also checked using the developed LC method. The result of the RCB being subjected to different stress conditions led to the fact that it was found to be stable during thermal, photolytic stress conditions. It degraded extensively under acidic hydrolysis as well as oxidative stress conditions. The LC–MS/MS data and HRMS results were used in characterization of three resultant degradation products and valid fragmentation pathways. Obtained stability studies, degradation pattern, and proposed fragmentation pathway, thus, obtained could potentially be useful for a better understanding of RCB during storage and stable formulations and contribute to

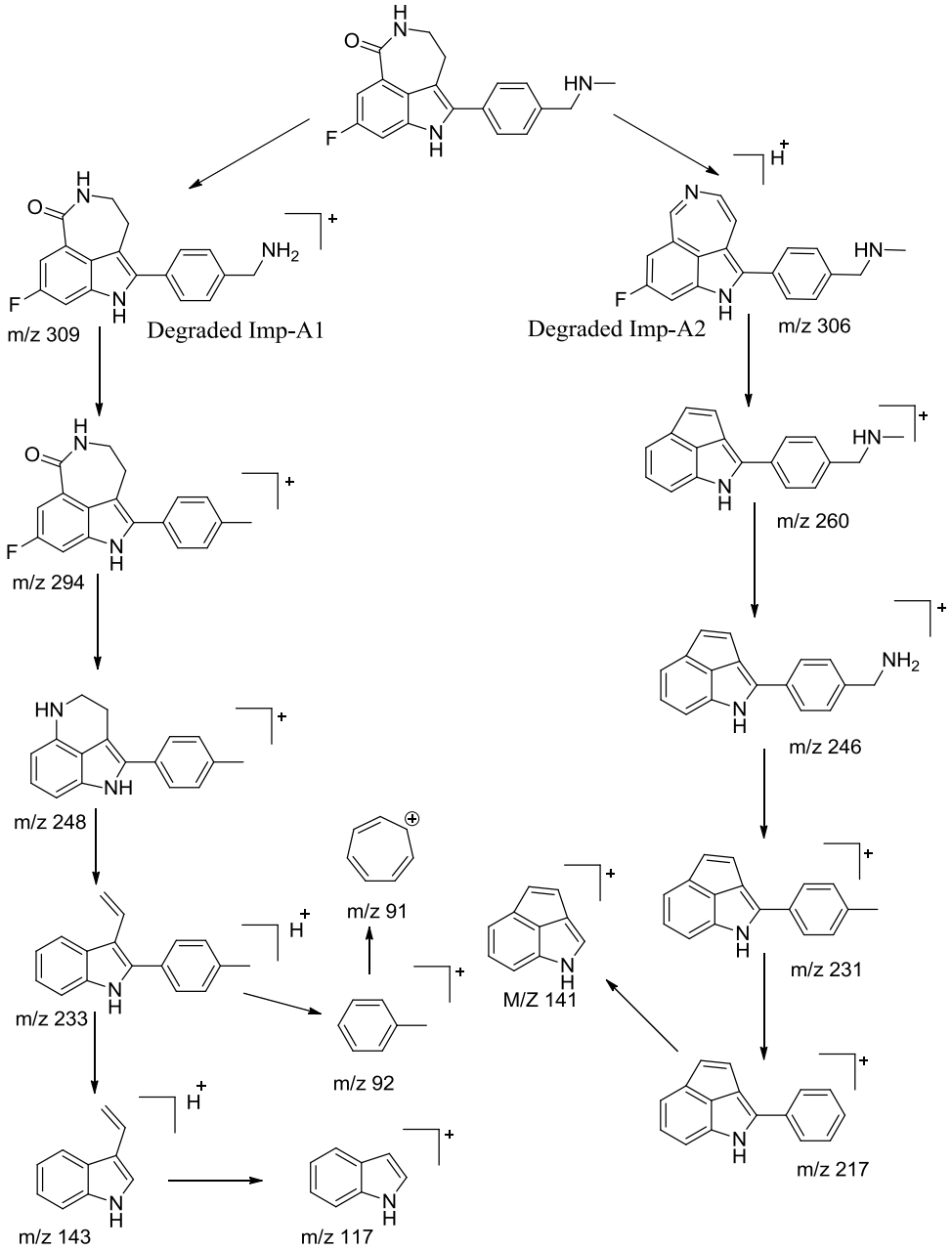

Fig. 7 Proposed fragmentation pathway of degradation products of RCB under acidic hydrolysis

Table 9 *m/z* values of degradation products and its fragment ions of RCB

Degradation product	Molecular ion *m/z* (amu)	Fragment ions *m/z* (amu)
A_1	309	294, 248, 233, 143, 117, 92, 91
A_2	306	260, 246, 231, 217, 141
O	339	309, 280, 204, 144, 117, 92, 91

Table 10 HRMS data of degradation products of RCB

Degradation product	Theoretical mass *m/z* (amu)	Most probable molecular formula	Observed mass *m/z* (amu)	Error (ppm)
A_1	309.1350	$C_{18}H_{16}FN_3O$	309.1345	− 1.326
A_2	306.0045	$C_{19}H_{16}FN_3$	306.0031	0.008
O	339.2037	$C_{19}H_{18}FN_3O_2$	339.2042	0.013

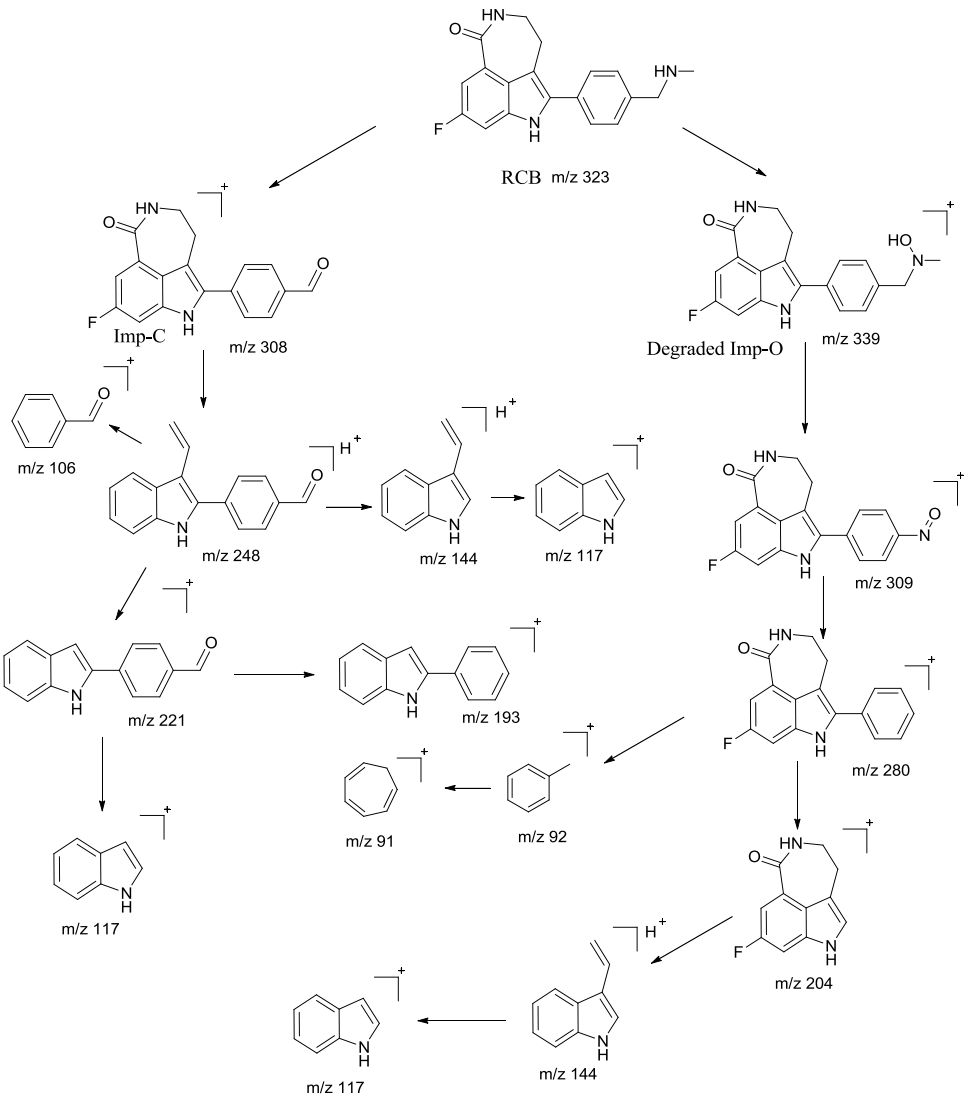

Fig. 8 Proposed fragmentation pathway of degradation products of RCB under oxidation

the safety of RCB being manufactured in pharmaceutical laboratories.

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Compliance with Ethical Standards

Conflict of interest Author Babji palakeeti declares that he has no conflict of interest. Author Dr. Tippani Ramesh declares that he has no conflict of interest. Author Vijender Reddy K declares that he has no conflict of interest. Author Ramaiah Konakanchi declares that he has no conflict of interest. Dr. K. Vengatajalabathy Gobi declares that he has no conflict of interest. The corresponding author Prof. Pothuraju Nageswara Rao declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors. This article does not contain any studies with human participants or animals performed by any of the authors.

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Development of new stability indicating UPLC-UV method for the extraction and quantification of perindopril and indapamide from human plasma

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Abstract

Background: The hypertension and cardiovascular ailments are the leading cause of deaths worldwide. The combination therapy was found to be effective on the cardiovascular illness by reducing the blood pressure. The indapamide and perindopril combination therapy showed excellent results on reducing high blood pressure. With this in mind, the stability indicating reverse phase UPLC method was developed for the simultaneous identification and quantification of indapamide and perindopril from human plasma. In this work, we developed a new solid phase extraction method for the extraction of indapamide and perindopril in human plasma. It is a simple, accurate, and selective method for the extraction of these two drugs from human plasma with elution time of 2 min. The extracted drugs were identified and quantified by using stability indicating UPLC method. The method showed high recovery rate as well as low detection and quantification limits of two drugs.

Results: A novel, simple, highly accurate, and precise stability indicating ultra-performance liquid chromatography (UPLC) method was developed for the identification and quantification of perindopril (PP) (brand name Coversyl) and indapamide (IP) (brand name Lorvas) from human plasma. In this UPLC method, HSS C_{18} column (100 × 2.1 mm, 1.8 μm) and mobile phase acetonitrile (ACN), 10 mM KH_2PO_4 buffer solution (pH 3.0) mixture was used in the ratio of 65:35. Colum temperature of 30 °C, flow rate of 1.0 mL per minute and UV wave length of 254 nm were used. PP and IP were eluted below 2 min runtime with high resolution. Solid phase extraction (SPE) method was used for the extraction of PP and IP from human plasma. Different solvents were used to extract the analyte from SPE such as ACN, methanol, acetone, tertiary butyl diethyl ether (TBDE), chloroform ($CHCl_3$), and ethanol (EtOH). Among these, ACN gave good recovery percentages (94.56 to 101.58%). From the linearity graph, good correlation coefficient values of 0.9996 for PP and 0.9997 for IP were achieved. The coefficient variance values for intra and inter day precision is in between 1.08 and 12.5%. The LOD and LOQ values were determined by the signal to noise ratio method. LOD and LOQ values for IP and PP were found to be 8.6 and 33.5 ng/mL and 28.33 and 110.5 ng/mL respectively. The developed method was statistically validated as per ICH guidelines.

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Conclusion: In summary, a novel stability indicating UPLC-UV method was developed and validated for the simultaneous identification and quantification of perindopril and indapamide drugs in human plasma and tested the stability as per ICH guidelines. It is a simple, accurate, and specific method for the extraction of these two drugs from human plasma and eluted within 2 min runtime. The method showed high recovery rate as well as low detection and quantification limits of two drugs. The developed method is suitable for routine analysis as well as in bioanalytical and clinical studies.

Keywords: Perindopril, Indapamide, UPLC, Solid-phase extraction, Hypertension

Background

Cardiovascular ailments such as hypertension and stokes are the most leading diseases to cause death worldwide. In general, the monotherapy was used to reduce the blood pressure in patients, but it works in few patients only. Therefore, the combination therapy was introduced to effectively reduce the cardiovascular illnesses [1, 2]. Combination therapy has been found to decrease the hypertension, due to their numerous mechanisms of actions and showed reduced side effects due to the lower dosage of medications [3–6].

Perindopril is commonly used to treat coronary artery ailments such as heart failure and hypertensions [7–10]. The chemical name of perindopril is (2S,3aS,7aS)-1-[(2S)-2-{[(2S)-1-ethoxy-1-oxopentan-2-yl] amino}propanoyl]-octahydro-1H-indole-2-carboxylic acid. It is an ethyl ester of non-sulphahydryl derivative and angiotensin converting enzyme (ACE) inhibitor, it selectively reduces the level of angiotensin I by converting it to angiotensin II due to the hydroxylation of perindopril to produce its active metabolite perindoprilat. Consequently, it inhibits the angiotensin II activities like stimulation of aldosterone secretion in adrenal cortex and vasoconstrictions [11, 12].

Indapamide (4-chloro-N-(2-methyl-2,3-dihydroindol-1-yl)-3-sulfamoylbenzamide) is a thiazide or sulphonamide derivative. It is used to treat the mild to moderate hypertensions due to the activities of calcium antagonist and diuretic effects [13–15]. Different studies revealed the antihypertension activity of indapamide due to the peripheral vascular resistance and decreasing the vascular reactivity.

The indapamide and perindopril combination therapy has shown to be effective for reducing high blood pressure [16–19]. The structures of indapamide and perindopril showed in Fig. 1. This combination therapy has proved to show major effects on microcirculatory alterations, systolic blood pressure and arterial stiffness. Thus, many pharmaceutical industries competing to produce the various combinations of indapamide hemihydrate and perindopril arginine to prevent the hypertension and maintain the normal blood pressure in patients. The British and US pharmacopeias officially approved indapamide, and perindopril was approved by British pharmacopeia [13, 20].

The number of methods was reported for the identification and quantification of indapamide and perindopril individually. To the best of our knowledge, only few methods have been reported for the simultaneous estimation of indapamide and perindopril drugs in biological fluids [21–26]. Till now, no one reported the stability indicating reverse phase UPLC method for the simultaneously identification and quantification of indapamide and perindopril from human plasma. Therefore, the current study demonstrated the stability of perindopril and indapamide by doing the forced degradation studies, successfully extracted and quantified these two drug molecules from human plasma.

Methods

Pure samples

The reference standards perindopril (potency (99.8)), indapamide (potency (99.8)), and active pharmaceutical ingredients (API) were obtained from Mylan laboratories and Glenmark pharmaceutical as gifted samples.

Chemical and reagents

For the UPLC analysis and extraction, analytical grade methanol, ACN, TBDE, chloroform ($CHCl_3$), and ethanol

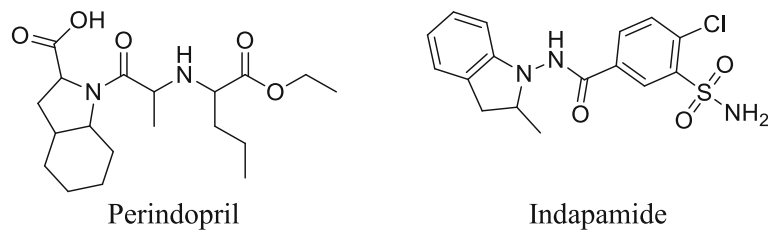

Fig. 1 Structures of perindopril and indapamide

(EtOH) were procured from Sigma Aldrich, USA, hydrogen peroxide (H_2O_2), hydrochloric acid (HCl), KH_2PO_4, orthophosphoric acid, and sodium hydroxide (NaOH) were purchased from sigma Aldrich, USA, Milli-Q system Millipore, USA, used for the purification of water.

Instrumentation

Stability indicating method development and validation was established on UPLC (Acquity, waters, USA) by using the empower II software. Acquity HSS C_{18} column (waters) was used for the eluting the peaks with high resolution. Milli-Q Millipore system (USA) was used for the purification of water. Sonicator (sonica, spincotech-Italy), vortex mixture (India), Elico pH meters, and 0.45-micron nylon filters (Merck, Millipore) were are used in the sample preparations.

Analytical conditions

Isocratic method was used for simultaneous determination and quantification of indapamide and perindopril in human plasma. Mobile phase acetonitrile ACN and 10 mM KH_2PO_4 buffer solution (pH 3.0) was used in the ratio of 65:35. Column oven temperature was maintained at 30 °C, flow rate of 1 mL per minute and injection volume of 10 μL, UV wavelength (λ max) of 254 nm and run time is 5 min were used. Stock solutions were prepared in ACN, and the mobile phase was used as a diluent for the further dilutions.

Preparations of linearity and quality control (QC) sample solutions

Stock solutions of perindopril (1 mg/mL) and indapamide (1 mg/mL) were prepared in ACN and stored at −4 °C. The working standard solutions were prepared by diluting the stock solutions by using mobile phase. Six different concentrations of perindopril (0.75, 1.5, 3.0, 7.0, 15.0, and 25.0 μg/mL) and indapamide (0.15, 0.45, 1.25, 2.5, 7.5, and 12.0 μg/mL) spiked solutions with human blank plasma were prepared for the construction of calibration curve. Three different quality control samples: low (LQC), middle (MQC), and high (HQC) were prepared individually by spiking with 500 μL aliquots of human blank plasma. The solutions of indapamide at the LQC 0.45, MQC 2.5, and HQC 12.0 μg/mL and perindopril at the LQC 1.5, MQC 7.0, and HQC 25.0 μg/mL solutions were prepared. All spiked solutions are stored in refrigerator at −20 °C.

Extraction procedure

Human plasma (250 μL) was taken in 2 mL Eppendorf tube containing 500 μL of potassium phosphate buffer (pH 4). The samples were mixed with vortex for few minutes. Then the samples were loaded into the Oasis HLB (1 mL, 30 mg) cartridges. Before loading the sample in to solid phase (cartridges), these were preconditioned with 1 mL of acetonitrile, 1 mL of methanol, and 1 mL of water: acetonitrile (95:05 v/v). The sample loaded cartridges are washed five times with 1 mL of methanol: water (10: 90, v/v) at −30 kPa [27]. Then, the sorbents were dried for 10 min under the air flow. After drying, the analytes were eluted from the sorbent by using 1 mL of acetonitrile under gentle vacuum. The solution was evaporated by using N_2 gas at 40 °C in vacuum oven. The sample residues were reconstituted by adding 100 μL of mobile phase solution, vortexed for 2 min, and sonicated for 1 min. Then, the solutions were filtered by using 0.2-micron nylon filters, and 10 μL of sample solution was directly injected in to UPLC for analysis.

Forced degradation studies of drug substances

The stability of the developed UPLC method was examined by doing the forced degradation of drug substances. The stability studies of drugs were carried out in presence of acidic, basic, neutral, oxidative, photolytic, and thermal conditions.

Acidic degradation

During the acidic degradation of drug substances, 1.0 N hydrochloric acid (HCl) was used. One milliliter of indapamide (1 mg/mL) and perindopril (1 mg/mL) mixed solution was taken in 10 mL volumetric flask stirred with 1 mL of 1.0 N HCl for 1 h at 60 ± 2 °C. The acid treated solution was neutralized with 1 mL of 1.0 N sodium hydroxide for preventing further degradations and make up to the volume with diluent.

Basic degradation

Alkaline degradation study of drug molecules was carried out by using 1.0 N sodium hydroxide solution (NaOH). In total, 1.0 mL stock solution was refluxed with 1.0 mL 1.0 N NaOH in 10.0 mL volumetric flask at 60 ± 2 °C for 1 h. The solution was neutralized by adding 1.0 mL of 1.0 N HCl solution and make up to the mark with diluent.

Thermal degradation

Thermal degradation studies were carried out at 80 ± 5 °C for 8 h. One milliliter of sample solution was taken in 10.0 mL volumetric flask and kept under the abovementioned conditions and then solution was diluted with diluent.

Oxidative degradation

Oxidative stress study of drugs was carried out by using 10% hydrogen peroxide (H_2O_2). Suspension of 1.0 mL of drugs solution into 1.0 mL of 30% H_2O_2 and stirred at

30 ± 2 °C for 1 h then the solution was diluted up to 10.0 mL with diluent.

Photolytic degradation

Photolytic degradation was carried out by using UV light. One milliliter of sample solution was exposed to UV light for 24 h in UV chamber, and the solution was diluted up to 10.0 mL by using diluent.

Neutral degradation

HPLC grade water was used in the neutral degradation. One milliliter HPLC grade water was added into 1.0 mL sample solution and refluxed at 60 ± 2 °C for 5 h and then solution was diluted up to 10 mL with diluent.

All degradation samples are filtered with 0.45-micron nylon filters and each degradation sample (10.0 μL) was injected individually in to UPLC and recorded the chromatogram.

Results

Method development and optimization

The main objectives of this work are the isolation, quantification of IP, and PP drug molecules form human plasma by developing a novel stability indicating UPLC method. In the process of method development, different compositions of mobile phases with different pH were used. In general, the pH of the buffer will retain the ionization of compounds and provide low tailing factor values. The pKa values of indapamide and perindopril are 8.8 (nearly neutral) and 5.7 (weak acidic) respectively. The selected mobile phase acetonitrile and KH_2PO_4 buffer (pH=3) (65:35) would give good retention and tailing factor peaks. The optimized chromatograms were shown in Fig. 2. Among all the columns used in the current method, HSS C_{18} column (100×2.1 mm, 1.8 μm) was found to be suitable for getting adequate resolution, plate count, and tailing factors (indapamide (1.7) and perindopril (1.6)) for the drug molecules simultaneously. This column gave high theoretical plate count values (indapamide (8254) and perindopril (6411)), which indicates the efficiency of column for the separation with high resolution, narrow, and sharp peaks. The system parameters such as flow rate (1.0 mL/min) and injection volume (10.0 μL) were optimized based on the minimal consumption of mobile phase and peak resolution.

In the forced degradation studies, the drug degradation between 5.0 to 20% is acceptable and that was considered

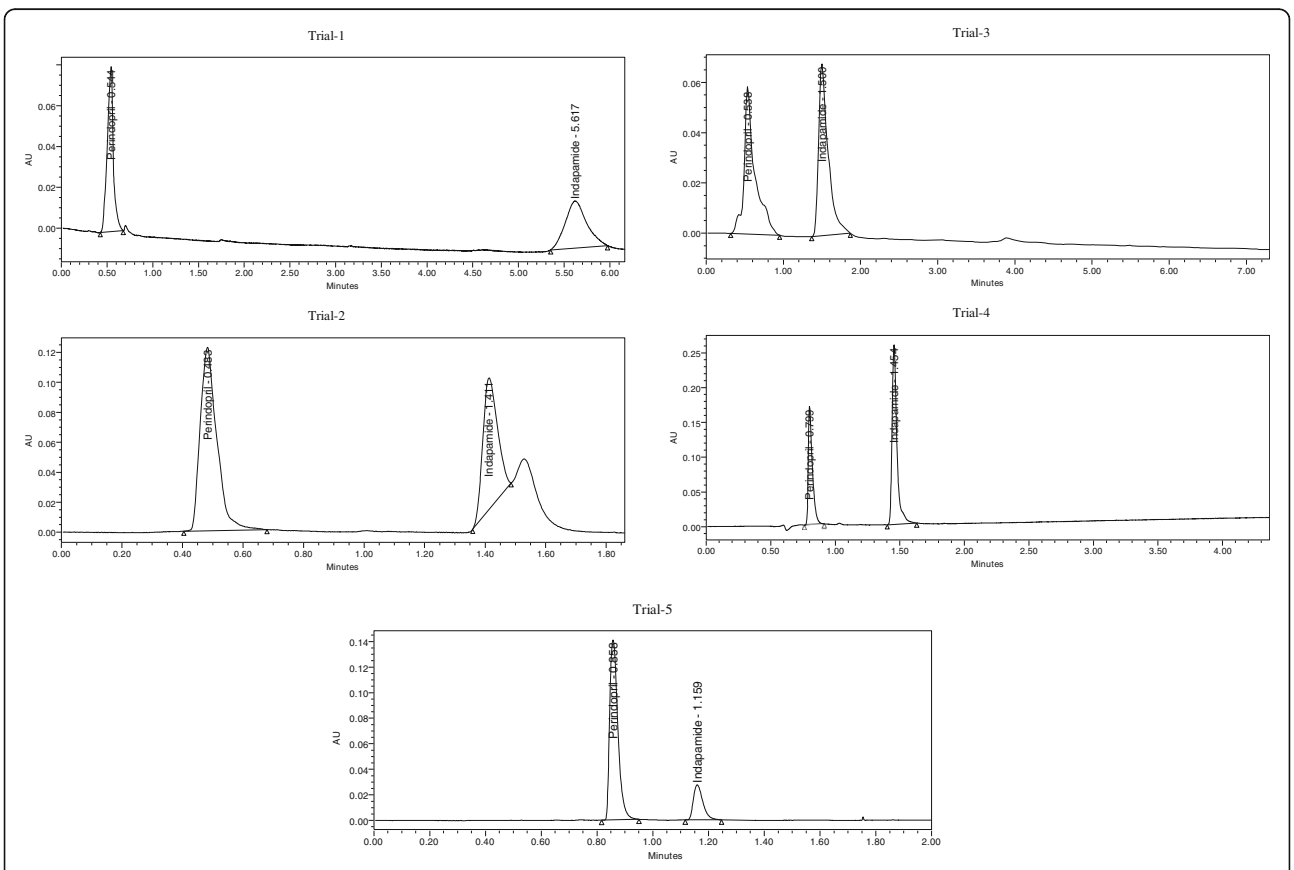

Fig. 2 Optimized resulting UPLC chromatograms with different columns

Table 1 Summary of forced degradation study results

Stress condition	Time	Assay% of PP after degradation	Assay% of IP after degradation	Remarks
Unstressed sample	–	99.63	100.2	–
Acid hydrolysis (1.0N HCl)	60 min	91.15	98.64	The significant degradation was observed.
Base hydrolysis (1.0N NaOH)	60 min	96.01	93.78	The slight degradation of analytes was observed.
Oxidation (10% H_2O_2)	60 min	98.65	99.12	Significant degradation was not observed.
Thermal (80 ° C)	8 h	99.32	99.81	Significant degradation was not observed.
Photolytic degradation	24 h	99.53	99.98	Significant degradation was not observed.

as stability indicating method and is reasonable for the validation of chromatographic method. In the present method, stability studies were carried out in different conditions such as acidic, basic, neutral, thermal, oxidation, and photolysis. In those, the drugs were degraded only in acidic and basic conditions and were stable in remaining conditions. In acidic condition, 1.56% of indapamide and 8.65% of perindopril and in basic condition, 6.42% of indapamide, and 3.59% of perindopril were degraded (Fig. S2 and Table 1). The assay % of drugs were calculated by using the following formula:

$$\%\text{Assay} = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Dilution of standard}}{\text{Dilution of sample}} \times \frac{\text{P}}{100} \times 100$$

Solid phase extraction (SPE) method was used for the extraction of drug molecules from human plasma. Various organic solvents (MeOH, ACN, $CHCl_3$, EtOH, acetone, and TBDE) were used for the extraction of drug molecules from SPE, comparing with all of those ACN was given good recovery percentages of indapamide (96.64-98.64%) and perindopril (98.51-101.25%).

Method validation

The developed stability indicating UPLC-UV method was validated for the following parameters statistically as per the ICH guidelines [28].

Selectivity

Simultaneous extraction of perindopril and indapamide was carried out from human plasma by using SPE method. Perindopril and indapamide peaks are arrived (0.87 and 1.16) within 2 min of retention time (Fig. 3). The chromatograms revealed that the peaks are pure, symmetric, well separated, and no other endogenous peaks are eluted. The results indicated that the SPE method was sufficient to isolate the perindopril and indapamide from human plasma.

Specificity

The specificity and selectivity was determined by running six different blank plasma sample solutions using the above optimized UPLC method. No interference peaks were observed at respective retention times of perindopril and indapamide. Moreover, the specified drugs also did not give any interference peaks during the analysis.

Calibration curves

The linearity of the developed method was evaluated by using standard solutions of six different concentrations of indapamide (0.15, 0.45, 1.25, 2.5, 7.5, and 12.0 μg/mL) and perindopril (0.75, 1.5, 3.0, 7.0, 15.0, and 25.0 μg/mL) plasma sample solutions. The linearity graphs were constructed by plotting the peak area against the concentrations of plasma sample solutions (Fig. 4), and the results are shown in Table 2. The linearity plots

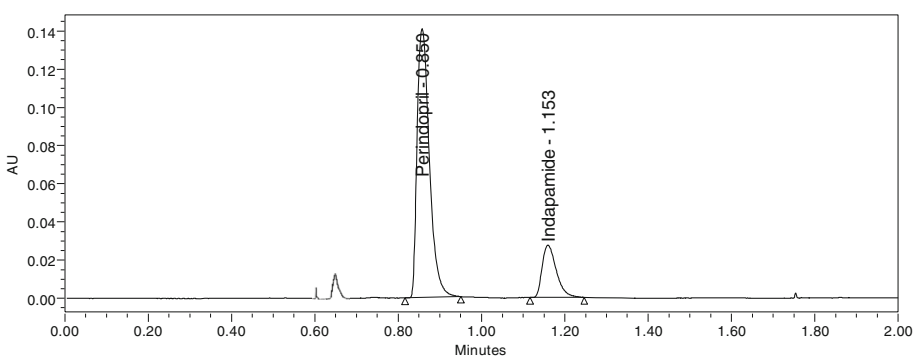

Fig. 3 The final UPLC chromatogram of indapamide and perindopril after the SPE

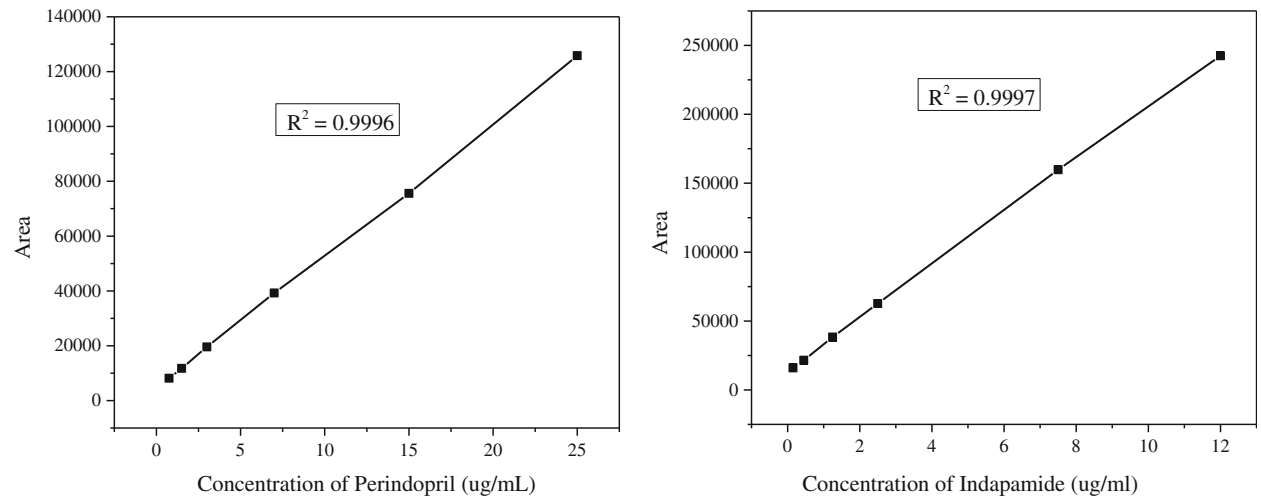

Fig. 4 The linearity plots of perindopril and indapamide

gave acceptable correlation coefficient (R^2) values for indapamide (0.9997) and perindopril (0.9996).

Precision and accuracy

The precision was determined by using the different concentrations (LQC, MQC, and HQC) of QC samples. For determining the intra-day precision, the experiments were carried out five times within same day. The analysis was also done in different days for the inter-day precision measurements and both precision values are expressed in percentage (1.08-12.5%) of cumulative variance (CV) of the peak area of three different QC sample solutions. The accuracy was determined in terms of mean percentage of analytes (94.56-101.2%) recovered from the plasma (Table 3).

LOD and LOQ

Limit of detection (LOD) and limit of quantification (LOQ) of the developed method were assessed by using signal to noise (S/N) method. The LOD and LOQ are useful for the assessment of sensitivity of developed method. In this method, the LOD value of perindopril (33.5 ng/mL) and indapamide (8.6 ng/mL) was determined by using S/N ratio of baseline over 3 times signal level of sample. LOQ values of perindopril (110.5 ng/mL) and indapamide (28.33 ng/mL) was assessed by using S/N ratio of baseline over 10 times signal level of sample.

Stability of solutions

The study of solution stability also plays a key role for determining the effects on long time storage of solutions. For knowing stability of sample solutions, three different concentrations QC (LQC, MQC, and

Table 2 Linearity of IP and PP

IP (μg/mL) [*X*]	Mean peak area [*Y*]	PP (μg/mL) [*X*]	Mean peak area [*Y*]
0.15	15954	0.75	8139
0.45	21585	1.50	11751
1.25	38384	3.00	19584
2.50	62645	7.00	39258
7.50	159785	15.0	75581
12.0	242445	25.0	125842
Linear regression equation	Y=19163x + 13957	Linear regression equation	Y=4820x + 4710
Slope	19163	Slope	4820
Intercept	13957	Intercept	4710
Correlation coefficient (R^2)	0.9997	Correlation coefficient (R^2)	0.9996

Average (n) =3

Table 3 Validation parameters results for determination of perindopril and indapamide

Analyte	Nominal value (μg/mL)	Intra day			Inter day		
		Found concentration (μg/mL)	Precision (RSD %)	Accuracy (%)	Found concentration (ng/mL)	Precision (RSD %)	Accuracy (%)
Perindopril	1.5	1.46 ± 0.28	8.34	97.45	1.48 ± 0.65	6.48	97.12
	7	7.16 ± 0.83	4.66	101.58	7.24 ± 2.51	3.51	101.2
	25	25.12 ± 2.73	2.4	98.6	24.68 ± 1.54	1.08	98.05
Indapamide	0.45	0.39 ± 0.16	12.5	95.16	0.41 ± 1.23	11.12	94.56
	2.5	2.48 ± 0.79	5.36	100.4	2.49 ± 0.64	3.05	99.2
	12	11.22 ± 1.68	4.8	98.7	11.56 ± 3.5	3.61	97.92

HQC) samples are kept under the RT for 4 h, 4 °C for 48 h, and 7 days at −20 °C. No significant changes were observed during the stability studies and hence the sample solutions are stable under these conditions and the results are summarized in Table 4.

Robustness

The robustness of the developed method was evaluated by changing the conditions such as flow rate, column temperature, mobile phase ratio, and UV wavelength. The flow rate ±0.2 mL per minute, organic solvent of mobile phase ±5%, column oven temperature ±5 °C and UV wavelength of detector ±4 nm was changed for the evaluation of robustness of the developed method. The analyte solution containing 10 μg/mL concentration of each drug indapamide and perindopril were prepared for these studies. There is no substantial difference observed with the change in the abovementioned conditions and RSD values vary in between 1.04 and 5.77 (Table 5). These results indicate that the developed method have good performance and reliability even with small variation in method conditions.

Discussion

A new stability indicating UPLC method was developed to detect at low concentration (nanogram) level of perindopril and indapamide in human plasma. The established UPLC method was optimized adequately by using various parameters, different UPLC columns, mobile phases for satisfactory peak shape, high sensitivity, and good resolutions. The results showed that the UPLC-UV method is suitable for simultaneous quantification and identification of perindopril and indapamide drugs in human plasma. Very few LLE and SPE methods were reported for the quantification of perindopril and indapamide in human plasma [29–33] which are time consuming and cost effective for the separation of drugs from biological samples. However, there is no SPE stability indicating UPLC method was reported for the identification of these drugs. The method reported in the present paper is very simple, easy to operate, quick and less cumbersome, and showed adequate recovery percentage (93.00-101.58) of drugs. The established method was able to give low LOD and LOQ values and able to give high sensitivity, accuracy, and precision to determine indapamide and perindopril in human plasma.

Conclusion

In summary, a novel stability indicating UPLC-UV method was developed and validated for the simultaneous

Table 4 Stability studies of analytes in human plasma and mobile phase

Analyte	Nominal value (μg/mL)	Stability conditions			
		Human plasma (analyte concentration (%))			Mobile phase
		RT (4 h)	4 °C (48 h)	−20 °C (15 days)	4 °C (24 h)
Perindopril	1.5	97.25	102.4	96.15	98.45
	7	100.06	98.2	95.29	99.1
	25	98.33	99.65	96.72	99.58
Indapamide	0.45	101.6	103.08	97.26	102.5
	2.5	97.05	98.15	93	100.25
	12	98.3	100.2	95.52	97

Table 5 Robustness study

Chromatographic changes	Level	Indapamide				Perindopril			
		RT	Recovery (%)	TF	*N*	RT	Recovery (%)	TF	*N*
Mobile phase ratio	70:30	0.74	97.89	1.64	8254	1.05	98.38	1.65	5957
	65:35	0.87	98.06	1.74	8102	1.16	99.35	1.65	6264
	60:40	1.02	96.54	1.72	7650	1.82	97.64	1.61	6441
Mean ± SD			97.49±0.83				98.45±0.85		
RSD (%)			0.85				0.87		
Flow rate (mL/min)	0.8	1.14	97.49	1.65	6845	1.71	98.14	1.62	6038
	1.0	0.87	98.26	1.65	8125	1.16	99.52	1.71	6506
	1.2	0.72	96.07	1.61	7845	1.03	97.84	1.65	6382
Mean ± SD			97.27±1.11				98.5±0.89		
RSD (%)			1.14				0.9		
Column temperature (°C)	35 °C	0.85	99.04	1.70	8201	1.12	98.47	1.64	6218
	30 °C	0.87	98.63	1.71	8054	1.16	99.04	1.64	6151
	25 °C	0.89	97.82	1.71	7654	1.19	97.22	1.62	6424
Mean ± SD			98.49±0.62				98.24±0.93		
RSD (%)			0.63				0.94		
UV wavelength	258	0.87	96.14	1.71	7951	1.14	99.81	1.63	5984
	254	0.87	98.36	1.72	8156	1.16	98.74	1.61	6068
	250	0.85	97.03	1.71	7899	1.17	96.28	1.61	6248
Mean ± SD			97.17±1.11				98.27±1.81		
RSD (%)			1.14				1.84		

identification and quantification of perindopril and indapamide drugs in human plasma and tested the stability studies as per ICH guidelines. It is a simple, accurate, and specific method for the extraction of these two drugs from human plasma with quick elution time of 2 min. The method showed high recovery rate as well as low detection and quantification limits of two drugs. The developed method could be used for routine analysis as well as in bioanalytical and clinical studies.

Abbreviations

PP: Perindopril; IP: Indapamide; SPE: Solid-phase extraction; ACN: Acetonitrile; MeOH: Methanol; $CHCl_3$: Chloroform; TBDE: Tertiary butyl diethyl ether; EtOH: Ethanol; LOD: Limit of determination; LOQ: Limit of quantification; RP: Reverse phase; UPLC: Ultra performance liquid chromatography; RSD: Relative standard deviation

Supplementary Information

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Additional file 1: Figure S2. Acidic and basic degradation HPLC chromatograms.

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Authors' contributions

All the authors have read and approved the manuscript. BP has framed the methodology of the work, and investigated, validated by performing the formal analysis. BP and JC have drafted the original paper. PN and JC have helped in conceptualization of the work.

Availability of data and materials

All data and materials are available upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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