DEVELOPMENT AND VALIDATION OF CHROMATOGRAPHIC METHODS FOR SELECTED DRUGS AND INTERMEDIATES BY HPLC AND UPLC

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CERTIFICATE

This is to certify that the thesis entitled “***DEVELOPMENT AND VALIDATION OF CHROMATOGRAPHIC METHODS FOR SELECTED DRUGS AND INTERMEDIATES BY HPLC AND UPLC*** ” is submitted by **I. Ugandar Reddy,** for the award of the degree of ***Doctor of Philosophy in Chemistry,*** National Institute of Technology, Warangal is based on the results of the investigations carried out by him under my guidance and supervision. This work has not been submitted earlier either in part or in full for any degree or diploma to any other institution.

Place: Warangal
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DECLARATION

I hereby declare that the matter embodied in the thesis entitled "***DEVELOPMENT AND VALIDATION OF CHROMATOGRAPHIC METHODS FOR SELECTED DRUGS AND INTERMEDIATES BY HPLC AND UPLC***" is based entirely on the results of the investigations and research work carried out by me under the supervision of **Prof. P. Nageswara Rao**, Department of Chemistry, National Institute of Technology, Warangal and co-supervisor of **Dr. V. Ranga Reddy**, AR&D Department, Dr. Reddy's Laboratories, Hyderabad. I declare that this work is original and has not been submitted in part or in full, for any degree or diploma to this or any other institution.

(I. Ugandar Reddy)

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I. Ugandar Reddy

DEDICATED TO
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LIST OF ABBREVIATIONS

API	Active Pharmaceutical Ingredient
ICH	International Conference on Harmonization
LC	Liquid Chromatography
HPLC	High-Performance Liquid Chromatography
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultra-Violet
NPLC	Normal Phase Liquid Chromatography
RPLC	Reversed-Phase Liquid Chromatography
ECD	Electrochemical detector
ELSD	Evaporative Light Scattering Detector
mm	Millimeter
mL/min	Milliliter per minute
μm	Micrometer
μL/min	Microliter per minute
MW	Molecular Weight
Å	Angstrom
CSP	Chiral Stationary Phase
Inc.	Incorporated
imp	Impurity
nm	Nanometre
pKa	Acid dissociation constant
QbD	Quality by Design
USP	United States Pharmacopoeia
h	Hour
g/mol	Grams per mole
LC-MS	Liquid Chromatography-Mass Spectrometry
HPTLC	High-Performance Thin-Layer Chromatography
TLC	Thin-Layer Chromatography
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
EP	European Pharmacopoeia
RH	Relative Humidity
°C	Degree Celsius

µg/mL	Microgram/ milliliter
µL	Microliter
mg	Milligram
mL	Milliliter
MP	Mobile Phase
min	minute
RRT	Relative Retention Time
LOD	Limit of Detection
LOQ	Limit of Quantitation
SD	Standard deviation
t_R	Retention Time (minutes)
$\mathrm{T_f}$	Tailing factor
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
R_s	Resolution
α	Alpha
β	Beta
γ	Gamma
w/w	Weight by Weight
v/v	Volume by Volume

LIST OF FIGURES

LIST OF TABLES

CONTENTS

P.No

Chapter 1: Brief introduction of impurity profiling in APIs by Liquid chromatography

Chapter 2: Development and Validation of HPLC Method for the Separation and simultaneous determination of process related substances of Irbesartan in bulk drugs

Chapter 3: Quality by Design approach for the separation of Clopidogrel bisulfate and its related substances by Ultra Performance Liquid Chromatography

Chapter 4: Stability indicating UPLC method for the determination of Ramelteon and their degradation products in Active pharmaceutical ingredients

Chapter 5: Quality by Design approach for the separation of Naproxcinod and its related substances by fused core particle technology column

Chapter 6: Development and Validation of Chiral Liquid Chromatographic Method for the Enantiomeric Separation of CPE, an Intermediate of Ticagrelor

CHAPTER – 1

Brief introduction of impurity profiling in APIs by Liquid chromatography

1. Introduction

Active pharmaceutical ingredients, widely known as bulk drugs, are used for therapeutic effects in pharmaceutical formulations. It is essential to assess the purity and safety of the APIs thoroughly before using them in different formulations. To establish the quality of synthesized pharmaceutical compounds and to quantify the impurities present in the desired product, suitable analytical methods are required. Development of suitable analytical methods is a critical issue in the domain of Pharmaceutical industries. Hence, analytical method development is equally important to synthetic process development of pharmaceutical compounds. An impurity as defined by the ICH guidelines is “Any component of the drug substance that is not the chemical entity defined as the active substance or an excipient in the product”. The definition of impurity profile is “A description of the identified and unidentified impurities present in the medicinal product”. The possible impurities in pharmaceuticals are like starting materials, intermediates, critical reagents and solvents used in the manufacturing process that remain with the active pharmaceutical ingredients (APIs). Impurities may form upon aging of API/drug product and also impurities may appear in main component due to some side reactions during synthetic process. The presence of these undesired components even in minute levels may influence the quality and safety of drug products. Hence, it is required to control the impurities from raw material to finished dosage form [1,2]. In general each known impurity should be controlled <0.15% and unknown impurity should be controlled <0.10% in APIs whose daily dose is less than 2.0g/day, to provide acceptable safety for the therapeutic use of the drugs. To ensure the quality of drug product or to control and monitor the impurities, analytical methods should be more efficient. To prove the effectiveness of developed analytical method for its indented use, systematic validation of analytical procedure is highly essential. Thus, the analytical method development and its validation became highly essential task in modern pharmaceutical analysis.

1.1. Importance of impurity profiling in APIs

The quality of the APIs is determined either by establishing its purity or by determining its content/assay with the help of a stability indicating method. During the early stages of development of pharmaceutical industry, chromatographic techniques were not available, hence the drug purity was evaluated based on the non-specific photometric and titrimetric methods supported by physical constants determination and for known compounds limit tests by color reactions. The deficiencies of the above approaches are well known. In early editions of pharmacopoeias, due to the presence of non-specific methods for determination of impurities, highly contaminated drug substances could meet set acceptance criteria.

With advancements in technology from the last two decades, completely new possibilities have been established for the purity determination of drug materials. In fact, it is possible to replace all non-specific purity/assay methods with highly precise and specific

Chromatographic (mainly LC) methods thus significantly improving the value of the quantitation of the active ingredient content of bulk drugs. Almost all organic impurities are quantified by chromatographic or related methods of which HPLC/UPLC has been the most important for over a decade and half.

Available literature is an evidence for the widespread usage of LC, during analysis of impurities in drugs. Most of the pharmaceutical compounds are UV active organic compounds, hence LC with UV detection is a versatile technique used across the pharmaceutical industries for analysis. LC-UV is most reliable, sensitive, accurate and precise to estimate UV active analytes. In some cases, this technique can be used for quantification of UV inactive compounds by introducing chromophore into that analyte. In general, to introduce chromophore, derivatization techniques can be used. In fact, this analytical tool/technique has set the standard against which others can compare.

1.2. Significance of impurity estimations in pharmaceutical industry

Safety and efficacy of the Pharmaceuticals are the two issues of fundamental importance in drug therapy. Pharmacological and Toxicological profile as well as the impurities present in the APIs and dosage forms influence the safety of the drug. The impurities in drugs often possess unwanted pharmacological or toxicological effects by which any benefit from their administration may be out weighted [3].

The complete characterization of the APIs upto the optimal extent is an indispensable requirement. The quality and safety of a drug is generally assured by monitoring and controlling the impurities effectively. Thus, the analytical activities concerning impurities in drugs are among the most important issues in modern pharmaceutical analysis [4-7].

To purify a material and to remove or control the undesired components one should understand the nature and source of the impurity. Various regulatory bodies laid down the guidelines to control the impurities in drug substances/drug products based on toxicity or nature of impurities and daily dosage of drug product [8-13]. To market the developed drug, regulatory bodies are insisting the pharmaceutical industries to control the impurities as per guidelines.

1.3. Classification of possible impurities

Possible impurities in Pharmaceutical compounds have been classified based on the nature of impurity [14].

1.3.1. Organic impurities

Organic impurities may arise during synthetic process or storage of the drug product/drug substance [15,16]. Impurities related to manufacturing process can be obtained from starting materials, intermediate stages of the process, ligands, reagents and catalysts used in the chemical synthesis, in addition to that due to side-reactions caused during a chemical processes by-products may be formed as impurities. Degradation products are a part of possible organic impurities; they may form due to chemical degradation of drug substances or drug products upon storage or in stress conditions. They may be identified or un-identified. However, they should be quantified and controlled in drug substance/drug product. The following are possible organic impurities drawn or taken from different sources in different Active Pharmaceutical Ingredients.

1.3.1.1 Starting materials or intermediates

The most common impurities that are found in every drug substance are the starting materials and intermediates that may appear if appropriate care is not taken to wash away the un-reacted materials throughout the multi-step synthesis. There are always probabilities of having the un-reacted starting materials that may remain, even though the end products are always purified with solvents, unless the manufacturers are extremely cautious about the impurities [17].

1.3.1.2 By-products

Achieving an end product with 100% yield and purity is very uncommon in synthetic organic chemistry. The possibility of formation of by-products is always there. For example, Diacetylated paracetamol may form as a by-product during the synthesis of paracetamol drug.

1.3.1.3 Degradation products

Degradation of the final product during manufacturing of APIs can also result in impurities. However, common impurities in the drug products are the degradation products resulting from storage or formulation to different dosage forms or aging. A famous instance of degradation products is the degradation of penicillin and cephalosporin, which is caused by the presence of a ß-lactam ring along with that of an α-amino group in the C6/C7 side chain [18].

1.3.1.4 Reagents, catalysts and ligands

These are less commonly found chemicals in drug substances; they may create a problem as impurities in some cases. Usually, an active pharmaceutical ingredient may contain all of the aforementioned types of organic impurities at levels differing from negligible to considerable. A review paper describes methods of isolation, detection and quantification of degradation products and antigenic polymeric by-products in cephalosporin and penicillin. Studies show the existence of traces of ampicillin polymers and hydrolyzed products in the API [19]. The presence of certain chemical substances like triethylamine causes degradation in some medicines. Presence of more than 7000ppm of triethylamine in Ampicillin trihydrate samples showed substantial degradation, where as samples with triethylamine less than 4000ppm were found to be stable under accelerated stability testing. The limit tests for the traces of impurities present in amoxycillin and ampicillin bulk raw materials were incorporated recently in Pharmacopoeia [20].

1.3.1.5 Enantiomeric impurities

Now, a single enantiomeric form of a chiral drug is considered as a superior chemical entity that may offer an enhanced pharmacological profile and an improved therapeutic index with a more favourable adverse reaction profile [21]. The unwanted stereoisomers in drug control are considered in the same manner as other organic impurities by the manufacturers of single enantiomeric drug (eutomer). Levalbuterol (R-Albuterol), Esomeprazole (S-Omeprazole), Levofloxacin (S-Ofloxacin) are some of the well-known single isomer drugs, which are being marketed.

1.3.2 Inorganic impurities

Inorganic impurities can result from the manufacturing process. They are normally known and identified and include,

- ✓ Reagents, ligands and catalysts
- ✓ Heavy metals or other residual metals
- ✓ Inorganic salts
- ✓ Other materials used as filter aids and charcoal

1.3.3 Residual solvents

Solvents are commonly used chemicals in synthetic processes of pharmaceutical compounds as these chemicals can be used to dissolve solids or to make suspension and to extract some chemical substances. The residual solvents in drug substances have to be controlled and monitored as per ICH guidelines [10]. Based on the known toxicity of the solvents, limits for most of solvents were clearly defined.

1.4. The role of Chromatography in estimation of impurities

Role of chromatography in Pharmaceutical analysis has been extensively enhanced from the last two decades. Chromatography is a process by which solutes are resolved due to a dynamic differential migration process in a system having two migration phases. One is mobile phase which moves continuously with specified speed in mL per min, other one is stationary phase in which the individual substances exhibit different mobilities due to difference in adsorption, partition, solubility, vapour pressure, molecular size and ionic charge density etc.

In the initial stages of 19th century, a Russian scientist Tswett came across colour bands on plant extracts that moved down the column and he named that process as chromatography. As Tswett had created the name, which is popular and prevalent till date, he is considered as the father of chromatography. In fact, the greatest advantage of this technique over any other analytical techniques is the capability of resolving specific analytes, a feature that appeals to different branches of science, which enables to invent and analyze unknown elements or chemical compounds. Chromatography includes a group of different methods that allow the separation of complex chemical mixtures. As discussed, all chromatographic techniques consist of two phases i.e. mobile phase and immiscible stationary phase. Based on the requirement of analytical scientists, different types of stationary phases are available in market.

1.4.1 Separation Mechanisms involved in Liquid Chromatography

A valuable classification of the various LC techniques is based on the type of distribution which is responsible for the separation. The common interaction mechanisms in LC are adsorption, partition, ion-exchange, gel permeation or size exclusion and chiral interaction. In practice, most of the LC separations are the result of mixed mechanisms. A brief description of the separation mechanisms is discussed below.

1.4.1.1 Normal-phase chromatography

Normal-phase liquid chromatography (NPLC) is a technique that uses columns packed with polar stationary phases combined with nonpolar or moderately-polar mobile phases to separate the components of mixtures. The rate at which individual solutes migrate through NPLC columns is primarily a function of their polarity. Less polar solutes move the fastest and therefore exit the column and are detected first, followed by solutes of increasing polarity which move more slowly.

1.4.1.2 Reverse phase (RP) - Chromatography

RPLC utilizes nonpolar stationary phases and aqueous-based polar mobile phases, and the elution order of solutes in a mixture is related to their hydrophobicity, not polarity; more polar solutes move the fastest and appear first, followed by solutes of decreasing polarity. RPLC is useful for separating mixtures in which components differ in molecular weight and/or water solubility. Chemically bonded octadecylsilane (ODS), an n-alkane with 18 carbon atoms, is the most frequently used stationary phase. C8 and shorter alkyl chains and also cyclohexyl and phenyl groups provide other alternatives. Phenyl groups are more polar than alkyl groups.

1.4.1.3 Ion-Exchange Chromatography

Separation is generally achieved by attraction and repelling of ions to each other. Stationary phases for ion-exchange separations are characterized by the nature and strength of the acidic or basic functions on their surfaces and the types of ions that they attract and retain. Cation exchange is used to retain and separate positively charged ions on a negative surface. Conversely, anion exchange is used to retain and separate negatively charged ions on a positive surface. The mode of separation is suitable for the separation of amino acids, ionic metabolic products and organic ions [22]. The ion exchange mechanism is shown in Fig. 1.4.F1.

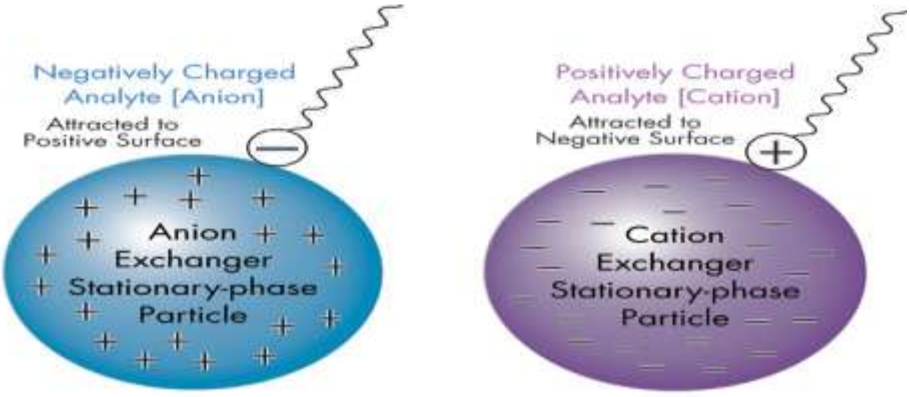


Fig.1.4.F1: The schematic diagram of ion exchange mechanism.

1.4.1.4 Ion Chromatography

Ion chromatography was developed as a means of separating the ions of strong acids and bases (e.g. Cl^-, NO_3^-, Na^+, K^+). It is a special case of ion-exchange chromatography but the equipment used is different.

1.4.1.5 Ion-Pair Chromatography

Ion-pair chromatography may also be used for the separation of ionic compounds and overcomes certain problems inherent in the ion-exchange method. Ionic sample molecules are ‘masked’ by a suitable counter ion. The main advantages are, firstly, that the widely available RP system can be used, so no ion exchanger is needed, and, secondly, acids, bases and neutral products can be analyzed simultaneously.

1.4.1.6 Size-Exclusion Chromatography

This mode of chromatography separates the molecules by size and is typically done on stationary phases that have been synthesized with a pore-size distribution over a range that permits the analytes of interest to enter, or to be excluded from, more or less of the pore volume of the packing. Smaller molecules penetrate more of the pores on their passage through the bed. Larger molecules may only penetrate pores above a certain size so they spend less time in the bed. The biggest molecules may be totally excluded from pores and pass only between the particles, eluting very quickly in a small volume. This is divided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solutions).

1.4.1.7 Affinity Chromatography

Affinity chromatography is one of the most diverse and powerful chromatographic methods for purification of a specific molecule or a group of molecules from complex mixtures. It is based on highly specific biological interactions between two molecules, such as interactions between enzyme and substrate, receptor and ligand, or antibody and antigen. These interactions, which are typically reversible, are used for purification by placing one of the interacting molecules, referred to as affinity ligand, onto a solid matrix to create a stationary phase while the target molecule is in the mobile phase [23]. Affinity chromatography can be used to isolate proteins (enzymes as well as structural proteins), lipids, etc., from complex mixtures without involving any great expenditure.

1.4.1.8. Chiral chromatography

Enantiomers are stereoisomers which are non-super imposable mirror images of each other. They have identical chemical properties but behave differently in a chiral environment due to the presence of chiral centers. In chiral chromatography, mobile phase or stationary phase should be chiral in nature. The common principle of separation in all these techniques relies on formation of reversible transient diastereomers between enantiomer and chiral selector.

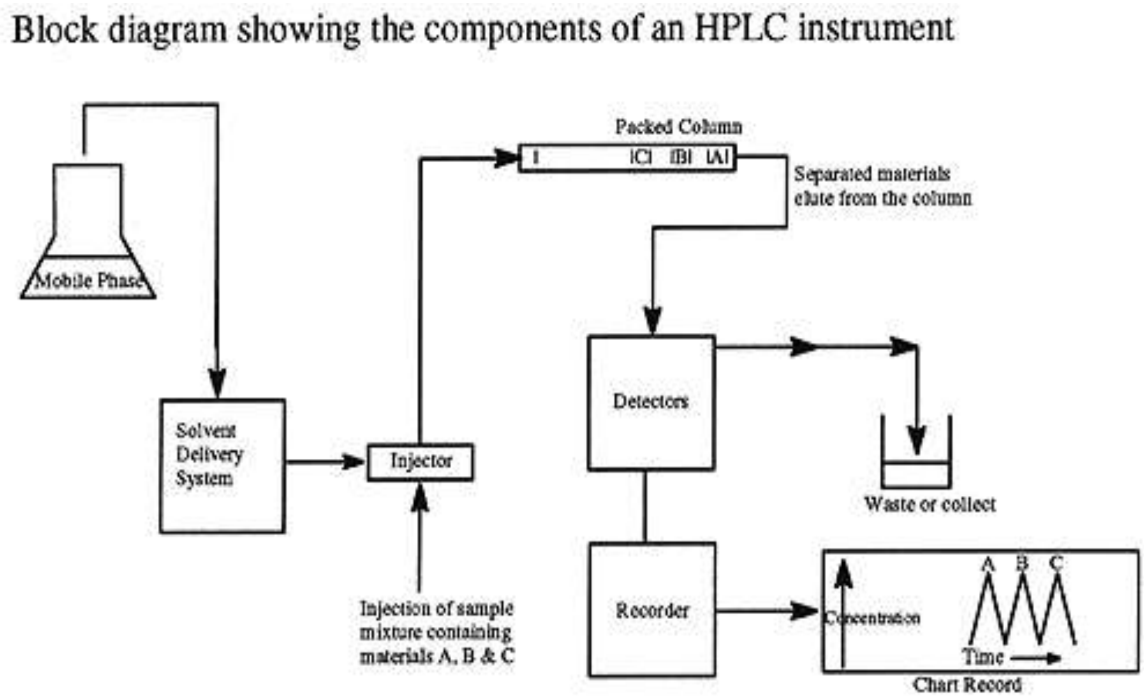
As mentioned above, due to several separation mechanisms and availability of different types of stationary phases, Liquid Chromatography is a versatile technique used across the pharmaceutical industries [24-29].

1.5. Liquid chromatography

Two types of Liquid Chromatographs are available in the market. In HPLC, separation of various compounds is based on partition, adsorption, chiral separation or ion exchange depending on the nature of stationary phase. The schematic diagram of HPLC is presented in Fig.1.5.F1.

The HPLC consists of pumps, an injector, column, mobile phase reservoir, column oven, and detector. The injector introduces the sample into the HPLC system. This is either done by hand with a syringe, or automated with an auto-sampler. There are several different types of pumps available for use with HPLC. They include reciprocating pumps, which are the most common, syringe type pumps, and constant pressure pumps. Column oven is used to maintain constant temperature throughout the analysis.

1.5.1 High performance liquid chromatography (HPLC)



Block diagram showing the components of an HPLC instrument

Fig.1.5.F1: Simplified schematic of an HPLC system

Column is typically a stainless tube filled with stationary phase, having a length of 10-30 cm and inner diamter of 3-5mm. The stationary phase is retained at each end by thin stainless steel frits with a mesh of 2μm or less. The most widely used stationary phase material is Octadecyl silica, which contains C_{18} chains. The other stationary phases include C_2, C_4, C_6, C_8 and C_{22} Chains.

Guard columns are often used in front of the column. This short column helps to protect the analytical column and increase its lifespan by removing larger particles and impurities before they can enter the column. The composition is similar to that of the analytical column.

1.5.1.1 Detectors

Detector in HPLC shows responses in the form of peak based on the eluting sample nature and concentration. All the peaks in the sample detected by the detector will appear as a chromatogram. Detection in HPLC system positions immediately posterior to the stationary phase or column in order to detect the compounds as they elute from the column. Regardless of the principle of operation, an ideal LC detector should have properties like low baseline drift and noise, high sensitivity, wider linear dynamic range, lower dead volume, operational simplicity to detect different compounds and reliability.

Based on the nature of compound to be analyzed and desired detection limit of that particular analyte, different types of detectors are available.

1.5.1.1.1 Refractive Index Detector (RID)

Refractive index detector contains two channels in the flow cell. This can detect the change in refractive index of the column eluent and mobile phase. It is useful for analysis of all types of analytes, called as universal detector. Output signal response is based on the difference of refractive index between mobile phase and column eluent which contains analytes.

1.5.1.1.2 Ultra Violet detector (UV detector)

The compounds which show absorbance at UV region (200-400 nm) can be analyzed by using this detector. Detection process involves absorbance of UV light at particular wavelength by analyte. This can be established at one or several wavelengths.

Several types of UV detectors are available like Fixed wavelength detector, which measures the absorbance of analyte at single wavelength, 254 nm. Variable wavelength detectors are used by measuring the absorbance at one wavelength at a time, but we can use same detector for wide range of wavelengths.

Diode array detector is most useful UV detector for analytical method development. It scans the solute at multiple wavelengths, it covers total UV range. It gives UV spectrum of each analyte simultaneously. To ensure the purity of eluted peak, this detector can be used, that mean analyte peak contain single compound or it may be co-eluted with any other compounds that can be detected based on UV spectra of compound(s). Diode array detector contains Deuterium or Xenon lamp. Schematic diagram of Diode array detector is shown in Fig.1.5.F2.

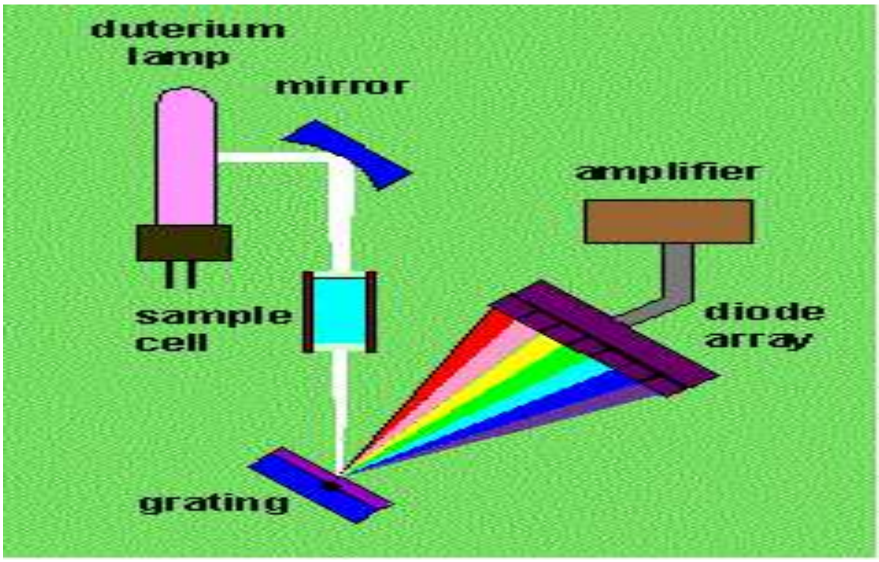


Fig. 1.5.F2: Schematic diagram of Diode array detector

1.5.1.1.3 Fluorescence detector (FLD)

Compounds having Fluorescence properties can be analyzed by using Fluorescence detector. This detector measures fluorescent light of a particular analyte at specified wavelengths.

1.5.1.1.4 Electrochemical detector (ECD)

The compounds which undergo oxidation or reduction can be analyzed by using ECD. Electron flow generated by the reaction across the electrodes detects as an electrical signal.

1.5.1.1.5 Evaporative Light Scattering Detector (ELSD)

This detector is independent on compound properties like electro activity, absorbance and fluorescence. The principle involved in detection is analyte should be less volatile than the mobile phase. Analyte forms as a particulate matter, scattered light through the solute can be detected by photomultiplier in the detector. Response of peak depends on the particulate mass of solute. The schematic diagram of ELSD is shown in Fig.1.5.F3.

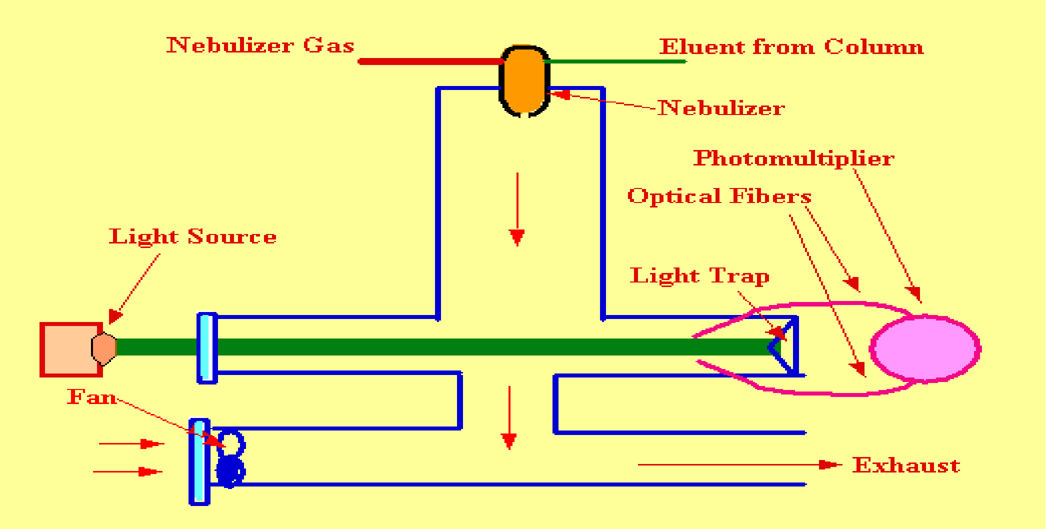


Fig: 1.5.F3. Schematic diagram of ELSD

HPLC can be performed on three scales, preparative, analytical, or micro scale. Preparative HPLC is concerned with the isolation and/or purification of a target analyte, while analytical and micro-HPLC involve the qualitative and/or quantitative analysis of a mixture of analytes.

Analytical HPLC generally uses column inner diameters (i.d.) of 2.1 to 4.6 mm and flow rate from 0.5 to 3 ml/min [30]. Micro-HPLC columns, on the other hand, are typically fashioned from fused silica capillaries (10 to 320 µm i.d) and use much lower flow rate (0.1 to 100 µl/min) [31].

1.5.2 Ultra Performance Liquid Chromatography (UPLC)

Ultra Pressure Liquid Chromatography (UPLC) is a relatively advanced technique which offers similar separation capabilities of HPLC with additional benefits of lower run times and less solvent consumption. This latest class of separation science has been developed based on new sub 2-µm particles used as column packing material and instruments designed to take advantage of these small particles. These particles allow operating at higher pressures and increased flow rates while still providing high resolution. The obvious results from this new class are improved separation, speed and enhanced sensitivity. Although this UPLC technology has been available since early 2000, few laboratories have embraced the new technology as an alternative to HPLC. Besides the resistance to investing in new capital, another major obstruction is converting existing HPLC methodology to UPLC without disruption. Various HPLC methods can be converted to UPLC to get faster results with higher resolutions resulting in more informative, robust methods. This will give us better situational response time in research and more samples analyzed per system and scientist with less solvent consumption in the industry. As discussed earlier a new class of chromatographic columns i.e. Ethylene Bridged Hybrid (BEH) are introduced for UPLC.

Another vital factor in UPLC is sample introduction. The manual or automatic injection valves that are used in conventional HPLC would not be able to withstand the pressure of UPLC. Further there is also a scope for a fast pulse free injection cycle in order to protect the column from pressure fluctuations and to maintain the speed that UPLC technology offers [32]. In order to have minimal carryover and low volume injections on the columns and, to maintain sensitivity, a static split injection technique is used in UPLC [33]. However this technique has some drawbacks which include large sample volumes and irreproducible long injections. As a result a pressure balance injection technique is being used. This is more efficient, reproducible and uses much less sample as compared to the split injection technique. This injection process is only 1-3 seconds long. The detectors that are coupled with UPLC have a very fast sampling rate to obtain sufficient number of data points of the analyte peak. In UPLC detectors, the analyte peak is accurately integrated and uses large number of data points thus taking complete advantage of the small particle chemistry.

1.6. Sequential steps involved in LC method development

The conventional approach for an HPLC method development is presented here consists of the following steps [34,35].

A) Literature collection

B) Chemical structure

C) Diluent selection

D) Selection of stationary phase

E) Detector selection

F) Mobile phase selection

G) Flow rate and Column temperature

H) Degradation studies

1.6.1. Literature collection

Literature search is performed to collect the solubility data, physico chemical properties, degradation path ways, reported impurities etc. It is generally performed through the Pharmacopoeia and Chromatography journals.

1.6.2. Chemical structure

Based on the synthetic scheme and reported degradation pathways chemical structure of main component and its related compounds, like starting materials, intermediates and process related impurities if available has to be verified for the functional groups present in them. Presence of acidic, basic or neutral functional groups helps in predicting the order of elution based on the selected chromatography mode.

1.6.3. Selection of Mode of chromatography

Solubility of analyte plays an important role in the selection of chromatographic mode. For the analytes which are soluble in polar solvents like water, methanol and acetonitrile the reversed phase chromatography is the appropriate mode. Whereas analytes insoluble in polar solvents, and showing solubility in non-polar solvents like 1,4-doxane, Ethyl acetate and n-hexane etc., normal phase is the best mode of chromatography. Generally for separation of chiral mixtures and analysis of non-polar compounds normal phase chromatography is preferable. For analysis of ionic compounds, ion-pair chromatography is a useful technique. Whereas for analysis of high molecular weight compounds such as protein, size exclusion chromatography can be useful. Flow chart for selection of chromatography mode has been provided in Fig.1.6.F1.

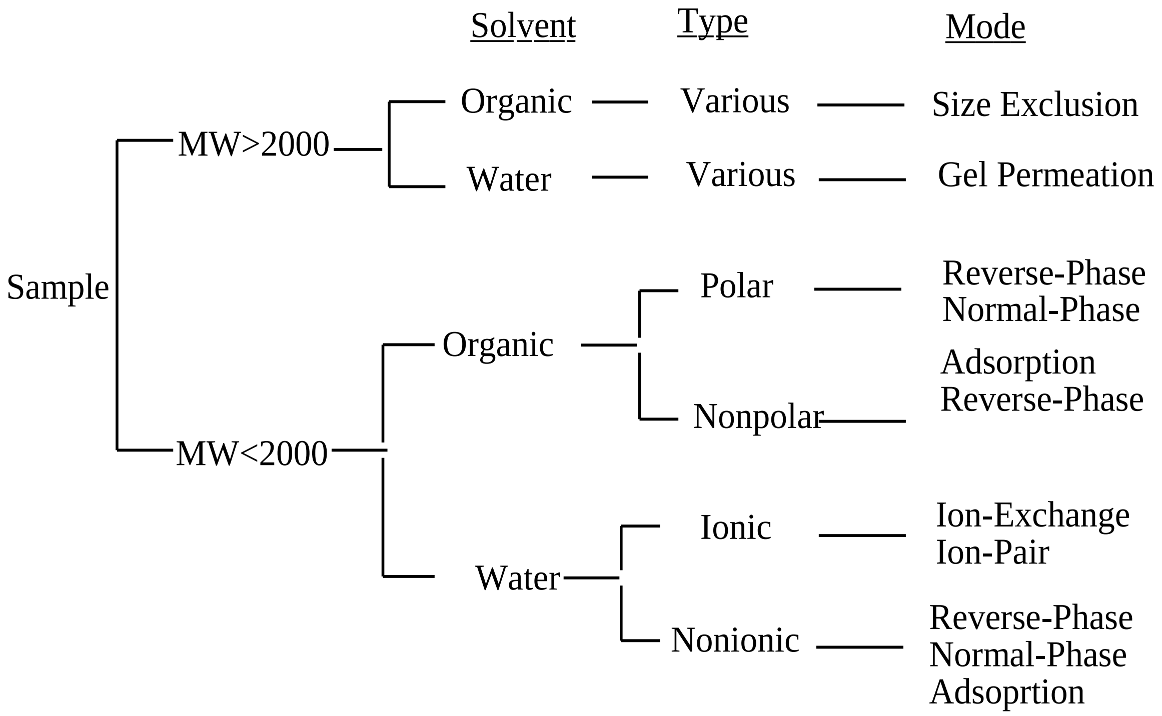


Fig: 1.6.F1: Flow chart for selection of chromatography mode

1.6.4. Solubility and diluent selection

Diluent should be selected in such a way that all the components of the mixture should be soluble. It should be compatible to get Gaussian peak shapes of analytes. It is more advisable to have mobile phase or the initial composition of the gradient elution mode as diluent for reduction of blank interference, if all the analytes get dissolved in that particular mobile phase. The selected diluent should produce a clear solution of analyte.

1.6.5. Selection of Column

1.6.5.1Reverse phase columns

Bonding phase can be selected based on the polarity of the molecule. For Reverse phase chromatography, a wide variety of columns are available (covering a wide range of polarity) by cross-linking the Si-OH groups with alkyl chains like C8, C18 and nitrile groups (CN), phenyl groups ($-C_6H_6$) and amino groups ($-NH_2$)[36] etc [Fig.1.6.F2].

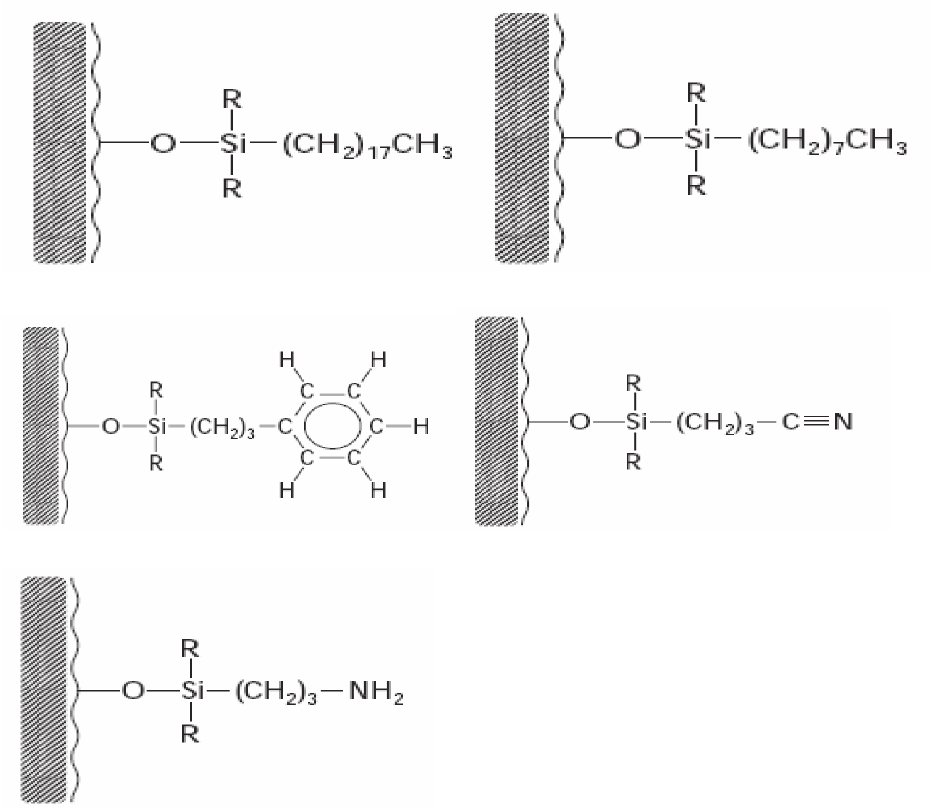


Fig.1.6.F2: Different alkyl chains attached to Si-OH

Silica based columns, with different cross linking, in the increasing order of polarity are as follows:

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

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

1.6.5.1.1 Particle shape:

Particles are either spherical or irregular in shape. Irregular particles have higher surface areas and higher carbon loads. Spherical particles provide higher efficiency, better column stability and lower back-pressures compared to irregularly shaped particles.

1.6.5.1.2 Particle size:

Particle size for HPLC column packing refers to the average diameter of the packing particles. Particle size affects the back-pressure of the column and the separation efficiency. Column back-pressure and column efficiency are inversely proportional to the square of the particle diameter. As the particle size decreases, the column back-pressure and efficiency increase. The particle diameter range is about 2–20 µm. Smaller particles offer higher efficiency.

1.6.5.1.3 Surface Area:

The surface area is the sum of particle outer surface and interior pore surface in square meters per gram. High surface areas generally provide greater retention, capacity, and resolution for separating complex, multi component samples. The physical structure of the particle substrate determines the surface area of the packing material. Surface area is determined by pore size. Pore size and surface area are inversely related. A packing material with a small pore size will have a large surface area, and vice versa. High surface area materials offer greater capacity and longer analyte retention times. Low surface area packing offer faster equilibration time and are often used for large molecular weight molecules.

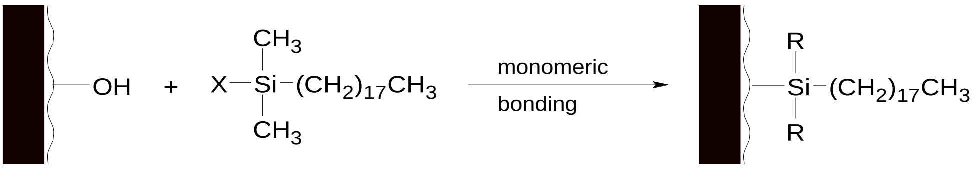
1.6.5.1.4 Pore size:

The pore size of a packing material represents the average size of the pores within each particle. Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics especially for larger analytes. Pore size defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface. This is especially important because the ratio of the outer particle surface to its inner one is about 1:1000. The surface molecular interaction mainly occurs on the inner particle surface. Larger pores allow larger solute molecules to be retained longer through maximum exposure to the surface area of the particles. Choose a pore size of 150Å or less for sample MW ≤ 2000. Choose a pore size of 300Å or greater for sample MW > 2000.

1.6.5.1.5 Bonding Type:

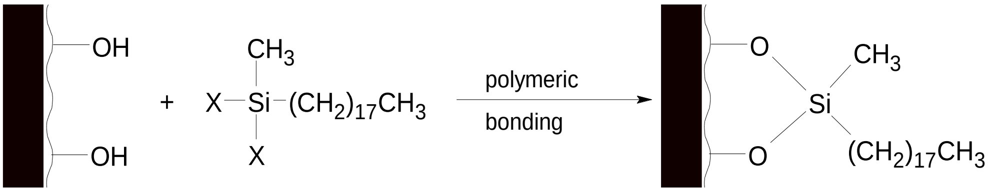
Monomeric bonding offers increased mass transfer rates, higher column efficiency, and faster column equilibration.

Monomeric bonding



Polymeric bonding offers increased column stability, particularly when highly aqueous mobile phases are used. Polymeric bonding also enables the column to accept higher sample loading.

Polymeric bonding



1.6.5.1.6 Carbon load:

The carbon load is the amount of bonded phase attached to the base material, expressed as the percentage of carbon. High carbon loads generally offer greater resolution and longer run times for hydrophobic samples. Low carbon loads shorten run times and often show different selectivity.

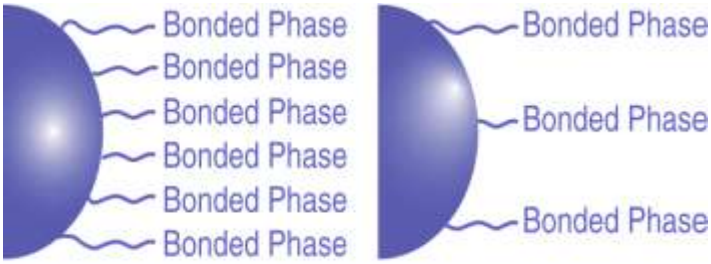


Fig.1.6.F3: A representative diagram for carbon load.

1.6.5.1.7 pH limitations of HPLC column:

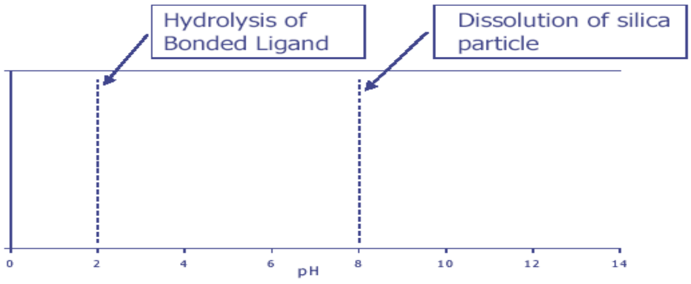


Fig.1.6.F4: Diagram for understanding the pH limitations

Most of the columns are limited between pH 2 and 8. Recently many of column manufactures are offering a wide range of pH scale i.e around 1.0 to 11.

1.6.5.2 Normal phase Columns

Columns containing polar stationary phases when compared to alkyl chain stationary phases like cyano and silica stationary phases can be used for normal phase category. Mobile phase should be non-polar in normal phase chromatography. The impact of the bonded phase, internal diameter, particle size, surface area and pore volume on theoretical plates, peak symmetry, selectivity and resolution should be evaluated. Smaller particle size provides more surface area and better separation.

1.6.5.3 Chiral columns

Chiral columns are used for separation of stereo isomeric pharmaceutical molecules [37]. Resolution depends on the formation of reversible transient diasteromers on the chiral stationary phases that have different free energies of interaction and therefore stability. The stereoisomer forming the most stable diastereomer with the CSP will be the most retained and vice versa. There is no single CSP that can be considered as universal, i.e., having the ability to separate all classes of racemic compounds. Selection of the right CSP for the enantiomeric separation of a chiral compound is a critical issue. The decision depends mostly on empirical data. Most of the chiral separations achieved on CSPs, however, were based upon the accumulated trial and error knowledge of the analyst’s, intuition and often simply by chance.

1.6.5.3.1 Derivatized polysaccharide CSPs

These phases are derived from either amylase or cellulose, derivatized with different chemical groups through carbamate or ester linkages. The below listed polysaccharide CSPs are extremely successful for enantiomeric separation of wide ranges of chiral molecules.

i. Amylose based polysaccharide CSPs

a. Chiralpak-AD (Amylose tris(3,5-dimethyl phenylcarbamate))

b. Chiralpak-AS (Amylose tris(4-methylbenzoate))

ii. Cellulose based polysaccharide CSPs

a. Chiralcel-OD (Cellulose tris(3,5-dimethyl phenylcarbamate))

b. Chiralcel-OJ (Cellulose tris(4-methylbenzoate))

1.6.5.3.2 Macrocylic antibiotic CSPs

The most successful macrocyclic antibiotic CSPs are based on the glycopeptides antibiotics supplied by Advanced Separation Technologies Inc.

i. Chirobiotic V (Vacomycin)

ii. Chirobiotic T (Teicoplanin)

iii. Chirobiotic R (Ristocetin A)

1.6.5.3.3 Pirkle type CSPs

These CSPs consist of low molecular weight amino acid derivatives. The phases are covalently bonded to silica supports which are chemically stable with universal mobile phase compatibility. Whelk-O1 and Whelk –O2 are the commercially available Pirkle type CSPs, supplied by Regis Technologies Inc.

1.6.5.3.4. Cyclodextrin CSPs

These CSPs are natural macrocyclic polymers of D-glucose units attached by α-(1,4) glycosidic linkages. Below are the different types of Cyclodextrin phases commercially available and supplied by Astec Inc.,

i. α-Cyclodextrin (Six glucose units)
ii. β-Cyclodextrin (Seven glucose units)
iii. γ -Cyclodextrin (Eight glucose units)

Inclusion complexation is responsible for retentions. Cyclodextrin CSPs used in reversed phase mode, hence buffer ionic strength and pH of mobile phase are the two important parameters to alter the strength of interactions.

1.6.5.3.5. Protein CSPs

Proteins are polymers of L-amino acid containing several chiral centres. Protein based CSPs are used exclusively in reversed phase chromatographic mode. The retentions can be influenced by the variations in type of buffers, strength of buffer, pH of the buffer, temperature and organic modifier. The below listed are some of the examples for protein based CSPs.

i. Chiral AGP (α- glycoprotein)
ii. Chiral CBH (cellobiohydrolase)
iii. Chiral HSA (human serum albumin)

1.6.6. Detector selection

Detector selection depends on nature of the analytes to be estimated. Most of the pharmaceutical compounds and its intermediates contain chromophore and are UV active. Hence, UV detector is most widely used in pharmaceutical analysis. For initial development trials of analytical method, Photodiode array (PDA) detector is very essential to verify the wavelength of maximum of each analyte and to check purity of analyte peaks. Wavelength maxima of all the analytes should be considered while selecting UV detector wavelength for method development and also at the selected wavelength (nm), all the analytes should give optimum response.

PDA detector is a powerful tool for evaluating the specificity of method. Diode array detectors will extract the spectra at different wavelength ranges from 200-400 nm at each data point across the analyte peak. Collected data will be processed through software for spectral comparison and provide information on homogeneity or non-homogeneity of that particular analyte peak. If any difference observed in the spectral comparison at any data point of the analyte peak, it will appear as purity flag. In this way PDA detector is very useful for development of specific chromatographic techniques.

1.6.7. Mobile phase selection

In reversed phase chromatographic mode, stationary phase is non-polar and mobile phase is polar in nature. Due to high polar mobile phases, based on the hydrophobicity of the analytes, retention times will be varied. If, the analyte is more hydrophobic it will retain in the stationary phase for long time when compared with less hydrophobic analyte. When an analyte gets ionized at the mobile phase pH it becomes less hydrophobic and therefore it will elute from the stationary phase with less retention. Acidic analytes lose a proton and become ionized when mobile phase pH increases and basic analytes gain a proton and become ionized when mobile phase pH decreases. Therefore, while separating mixture of acidic and basic analytes in reversed phase mode, it is required to maintain the pH of mobile phase by using appropriate buffer. Based on the pKa or dissociation constant of the buffers, different buffers will have different buffering capacities. Types of buffer and its buffering ranges were listed in below Table 1.6.T1.

Table: 1.6.T1. Commonly used Buffers for Reversed Phase HPLC.

Buffer	pKa	Buffer Range	UV Cutoff (nm)
Phosphate	2.1 7.2 12.3	1.1 – 3.1 6.2 – 8.2 11.3 – 13.3	200
Formic Acid*	3.8	2.8 – 4.8	210
Acetic Acid*	4.8	3.8 – 5.8	210
Citric Acid	3.1 4.7 5.4	2.1 – 4.1 3.7 – 5.7 4.4 – 6.4	230
Tris	8.3	7.3 – 9.3	205
Triethylamine*	11.0	10.0 – 12.0	200
Pyrrolidine	11.3	10.3 – 12.3	200

*Volatile buffers

For acidic analytes, it is preferable to use acidic mobile phases and for basic analytes, basic mobile phases. For analysis of neutral molecule, neutral pH is useful to get high retention times and there by more resolutions. To develop robust methods, mobile phase pH should not be within the range of ±1.0 pH units to the pKa value of the analyte. pKa values for different functional groups were listed in below Table 1.6.T2.

Table-1.6.T2: pKa values for different functional groups.

	pKa			
	Acid		Base	
	Aliph[a]	**Arom**[b]	**Aliph**[a]	**Arom**[b]
Sulfonic acid	1	1	---	---
Amino acid	2 – 4	---	9 – 12	---
Carboxylic acid	4 – 5	4 – 5	---	---
Thiol	10 – 11	6 – 7	---	---
Purine	---	2 – 4	---	9
Phenol	---	10 – 12	---	---
Pyrazine	---	---	1	---
Sulfoxide	---	---	1 – 2	---
Thiazole	---	---	1 – 3	---
Amine	---	---	8 – 11	---
Imidazole	---	---	---	7
Piperazine	---	---	10	---

[a]Aliphatic substituent, [b]Aromatic substituent

In reversed phase chromatographic mode, along with the buffer and its pH, selection of organic modifier is also important for mobile phase preparation. Methanol and Acetonitrile are the most commonly used organic modifiers in mobile phases. Due to lower UV cut-off and low viscosity, Acetonitrile, is the best organic modifier when compared to any other solvents like methanol, Isopropanol and Tetrahydrofuran etc., But, to avoid the pumping problems of 100 % Acetonitrile as mobile phase, it is preferable to use Acetonitrile with 5-10% of aqueous phase.

In normal phase chromatographic mode, n-Hexane, n-Heptane, Isopropanol, Ethanol, Methanol and 1,4-Dixane are the suitable solvents based on the requirement.

1.6.8. Method optimization by conducting stress studies

Once separation is achieved for all the known analytes, to optimize or finalize the chromatographic conditions, forced degradation samples of the main analyte (oxidation, acid hydrolysis, base hydrolysis, water hydrolysis, thermal and photolysis) will be analyzed by using PDA detector to verify the peak purity of the known components and formed degradation products. The main objective of the stability indicating method is to resolve the degradation products from the known components.

1.7 A Quality by Design approach to analytical methods

The application of QbD principles to analytical method development is focused on the concept of building quality into the method during development, as opposed to testing methods after development, for quality. ICH defines QbD as a systematic approach to drug development, which begins with predefined objectives and uses science and risk management approaches to gain product and process understanding and ultimately process control [38,39]. During a one-factor-at-a time (OFAT) approach where one variable is changed sequentially until a suitable method is produced. This type of development may create an adequate method but provides a limited understanding of method capabilities and method robustness. Rather, a systematic screening approach that evaluates a number of stationary phases, pH ranges and organic modifiers provides a more thorough approach to method development. A quality by Design (QbD) approach to method development uses statistical design of experiments (DoE) to develop a robust method ‘design space’. The design space defines the experimental region in which changes to method parameters will not significantly affect the results. The structured method development strategy using QbD approach to analytical methods can be exemplified as follows:

Step 1: Define method goal

The goal and intent of the method to be developed must be clearly defined, to comply with the QbD principles of predefined objectives [40]. The method goal for an HPLC method is generally to separate and quantify main compound and the critical quality attribute (CQA) impurities that may hamper the quality of the formulated product [41].

Step 2: Method scouting and Evaluation

This involves the systemic experimental design to understand the influence of each individual factor and their mutual interactions on the method performance which will eventually lead to the method optimization. Automated method scouting can be employed with efficient and comprehensive experimental design to evaluate the key components of the RP-LC (column, pH and organic modifier). The QbD forms a chromatographic database that will assist with method understanding, optimization and selection. In addition to this, it can be used to evaluate and implement change of the method, as needed in the future.

Steps 3: Method selection and Risk assessment

Based on the method scouting and evaluation results, the method that best meet the method goal can be selected for risk assessment. Identification and prioritization of risks in a structured fashion, followed by ruggedness and robustness testing is part of risk assessment. Fishbone diagrams, failure mode effects analysis (FMEA) and the prioritization matrix [42] are some of the tools used for the risk assessment. These tools help identify and prioritize potential risks to the method caused by small unintentional changes to method parameters, changes that might occur between the laboratories, analysts, instruments, reagents and period of time in which the method is performed.

Step 4: Define analytical method performance control strategy

Control strategy assures that the method is performing its intended purpose on a routine basis. The analytical method performance control strategy along with appropriate system suitability criteria can be defined to manage risk and ensure a method that delivers a desirable method attributes. If the risk is high and is hard to manage, it is an opportunity for the analyst to go back to the database described in step 2 to find a more appropriate method.

1.8. Analytical method validation

Method validation is a process to demonstrate the developed analytical procedure with systematic documental evidence to prove the suitability of the procedure for its indented use. ICH and USP laid down the guidelines for effective validation of analytical methods [43,44].

Several literatures are available to utilize the laboratory resources in a logical and efficient manner for validation of analytical methods to fulfill the regulatory guidelines [45-47].

As guided by regulatory authorities, below parameters are to be evaluated for validation of analytical procedure.

1.8.1. Specificity

Specificity is the first and foremost parameter to be evaluated during analytical method validation. The terms specificity and selectivity are often used interchangeably. Specificity indicates that the proposed analytical procedure is specific for estimation of that particular analyte in the presence of other possible substances to be present in the analyte; the word selectivity indicates a method specificity for number of analytes to be estimated by the proposed analytical procedure.

Specificity can be evaluated by analyzing main component spiked with all the known impurities, available degradation products and excipients. Ensure adequate separation between the components and peak purity of the main components in the presence of all the chemical entities expected to be present in the main component which indicates that the method is specific.

Forced degradation study is also a part of specificity evaluation, as discussed in section 1.6.8. The stress samples shall be analyzed in proposed analytical procedure. To perform the specificity of the HPLC-UV methods, PDA detector should be used to ensure the homogeneity of the analyte peaks.

1.8.2. Precision

Precision is the measure of the degree of repeatability of an analytical procedure under defined chromatographic conditions. Precision of the method can be expressed in terms of relative standard deviation for the content of analytes in different measurements of same analyte solution.

To demonstrate the precision of the method, repeatability, reproducibility and intermediate precision or ruggedness of the analytical procedure is to be evaluated. Repeatability experiment can be conducted by analyzing six preparations of spiked solution by using same set of analytical conditions. To perform the intermediate precision, repeatability experiment to be conducted by a different analyst, using different system, different lots of reagents and different column. Reproducibility refers the collaborative laboratory precision studies.

1.8.3. Accuracy

Accuracy is a measure of exactness of an analytical method, or the closeness of agreement between the measured value and the value that is accepted either as a conventional, true value or an accepted reference value. Accuracy can be demonstrated by performing recovery study hence, it can be expressed in terms of percentage recovery. As per regulatory guidelines, accuracy should be estimated by using minimum nine determinations at three minimum concentration levels, which should cover the specified range indicating that each concentration should be analyzed in triplicate.

Accuracy solution should be prepared by spiking known amount of all impurities at three different concentration levels to sample solution (preferably, 50%, 100% and 150% to the specification limits of impurities). Each concentration should be prepared in triplicate and analyzed. Percentage recovery of each solution to be estimated based on the amount obtained and added.

1.8.4. Sensitivity

Sensitivity of the method can be demonstrated by determining Limit of Detection (LOD) and Limit of Quantitation (LOQ).

1.8.4.1. Limit of detection

LOD can be defined as the minimum amount of analyte that can be detected by using the proposed analytical procedure, it may not be quantifiable. During estimation of LOD for particular analyte peak, signal to noise ratio should be considered as about 2 or 3.

1.8.4.2. Limit of quantitation

LOQ can be defined as the minimum amount of analyte that can be quantified by using the proposed analytical procedure. At that concentration of LOQ analyte should be determined with acceptable accuracy and precision. During estimation of LOQ of particular analyte peak, signal to noise ratio should be considered as about 10.

1.8.5. Linearity and Range

Linearity is the ability of the proposed analytical procedure to elicit test results that are directly proportional to concentration of analyte within a given range. Linearity results can be derived from linear regression analysis, it can be reported in terms of correlation coefficient, slope, intercept and y-bias. As per ICH guidelines, minimum of five concentration levels should be considered for linearity demonstration. It should start from LOQ level and shall cover 150% of specification level.

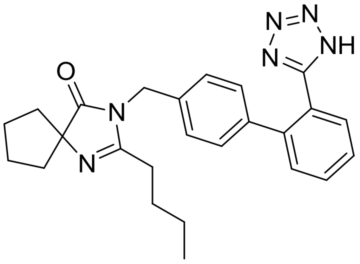
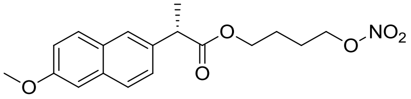
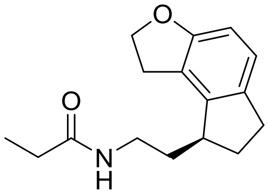
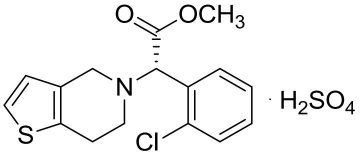
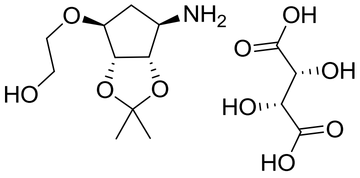
Range is an interval between the upper and lower levels of analyte concentration that have been demonstrated to be estimated with acceptable precision, accuracy and linearity using defined set of operational conditions. The range can be expressed in the same units as the test results of analytes expressed by the method.

1.8.6. Robustness

Robustness is the ability of the analytical method to remain unaffected by small, but deliberate variation of the defined analytical conditions. To study the robustness method parameters like flow rate, pH of buffer, organic strength of mobile phase and column temperature are to be considered. Defined system suitability parameters like resolutions between critical pair of analytes, theoretical plates and Tailing factor of the main component can be verified during the study. ICH guidelines recommend that robustness of the method should be studied during analytical method development itself. In addition, if the results of a method or other measurements are susceptible to minute variations in method parameters, these parameters should be effectively controlled and a precautionary statement should be included in the method documentation.

1.9 Aims and objectives of research work

Table 1.9.T1: List of APIs and Intermediate taken for research.

S.No.	Chemical name of API/Intermediate	Structure	Therapeutic activity
1.0	3-((2'-(1H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-2-butyl-1,3-diazaspiro[4.4]non-1-en-4-one		Irbesartan is used for the treatment of hypertension
2.0	(4-(nitrooxy) butyl-(2s)-2-(6-methoxy-2-naphthyl) propanoate		Naproxcinod is a nitric oxide-donating cyclo oxygenase inhibitor in development for Duchenne Muscular Dystrophy
3.0	(S)-N-(2-(1,6,7,8-tetrahydro-2*H*-indeno[5,4-b]furan-8-yl)ethyl)propionamide		Ramelteon is highly selective melatonin MT_1 and MT_2 receptor agonist used for sleep disorders
4.0	(+)-(S)-α-(2-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4H)acetate sulfate (1:1)		Clopidogrel bisulphate is a platelet aggregation inhibitor
5.0	-{[(3aR,4S,6R,6aS)-6-amino-2,2-dimethyl tetrahydro-3aH-cyclopenta [d][1,3]-dioxol-4-yl]oxy}-1-ethanol,L-tartrate		CPE, an intermediate of Ticagrelor which is an anti coagulant drug.

As per the regulatory agencies, development of specific and sensitive liquid chromatographic methods is essential for the quantification of potential impurities present in the Active pharmaceutical ingredients and their intermediates. No literature deals with the impurity profiling of the APIs selected for the research study using the HPLC and UPLC techniques. In the light of the above, the present research involves the description of the specific reverse phase liquid chromatographic methods for the accurate quantification of impurities present in Irbesartan, Clopidogrel bisulphate, Ramelteon, Naproxcinod, and reverses phase chiral method for the quantification of enantiomer of CPE, an intermediate of Ticagrelor. The work also takes into account the validation parameters of the established analytical methods as per the requirement of ICH and proves the suitability of developed methods to check the stability of API. The author’s present research objective is to develop liquid chromatographic methods for the APIs and Intermediate listed in Table 1.9.T1 using HPLC and UPLC techniques.

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CHAPTER – 2

Development and Validation of HPLC Method for the Separation and simultaneous determination of process related substances of Irbesartan in bulk drugs

2.1 Introduction to Irbesartan and survey of analytical methods

Irbesartan, 3-((2'-(1H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-2-butyl-1,3-diazaspiro[4.4]non-1-en-4-one (IRB), is an angiotensin II receptor (AT_1 subtype) antagonist mainly used for the treatment of hypertension. As shown in seven placebo controlled clinical trials, Irbesartan provides clinically significant dose related reductions in blood pressure in patients with mild-to-moderate hypertension [1-3]. Once daily dosing provides full 24 h blood pressure control with blood pressure reductions equivalent to those of twice daily dosing, and long-term control with monotherapy in a high percentage of patients. The anti-hypertensive effect of Irbesartan is comparable to or exceeds that of leading antihypertensive agents [4]. The empirical formula for Irbesartan is $C_{25}H_{28}N_6O$, and the molecular weight is 428.53 g/mol

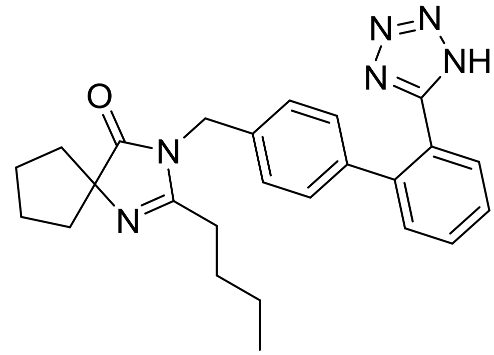


Fig. 2.1.F1: Chemical structure of IRB

Analytical literature survey revealed that some methods were available in the literature for the determination of Irbesartan in biological fluids. The techniques used include High-performance liquid chromatography (HPLC) with fluorescence detector [5,6]. UV [7,8], Diode-array [9]. LC-MS method was described for determining the levels of 4-methyl-2-cyano biphenyl and 4-bromomethyl-2-cyano biphenyl, which are key starting materials of Irbesartan [10].

Table: 2.1.T1 IRB Drug information

Molecular weight	:	428.53 g/mol
Molecular formula	:	$C_{25}H_{28}N_6O$
Brand name	:	Avapro
Dosage strength	:	75mg, 150mg, 300mg
Dosage form	:	Tablets
Maximum daily dose	:	300mg

HPLC method was mentioned in United States pharmacopeia [11] and European pharmacopoeia [12-14] for the estimation of impurity-C. While studying the synthetic process (Fig. 2.1.F2) we observed A, B, D, E, F, G and H as process-related impurities apart from impurity-C in the crude samples of IRB. Thus, there is a great need for the development of analytical methods for the simultaneous determination of IRB, including its degradation products along with the above mentioned process related impurities.

A thorough literature search has revealed that only a few analytical methods for determination of Irbesartan in bulk drug preparations and formulation dosage forms by HPLC method were reported. V. Zorica et all., have developed Simultaneous Analysis of Irbesartan and Hydrochlorothiazide: An Improved HPLC Method with the Aid of a Chemometric Protocol [15].

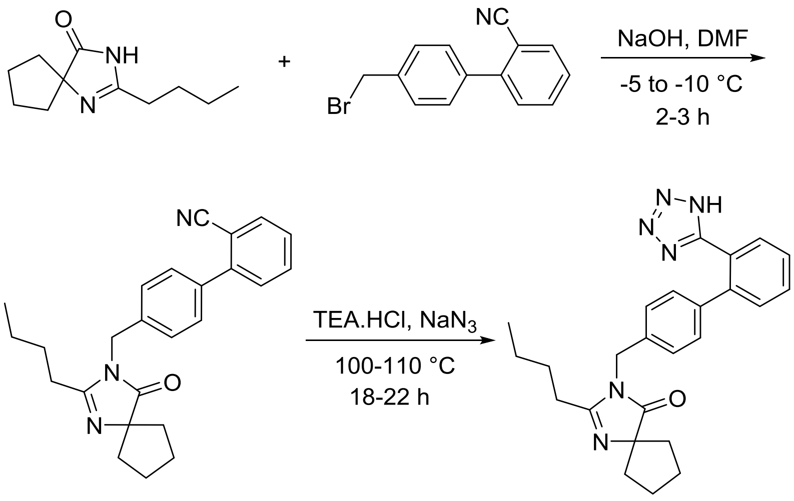


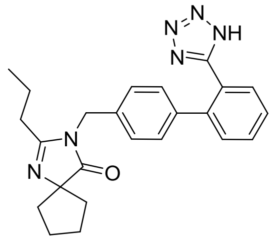
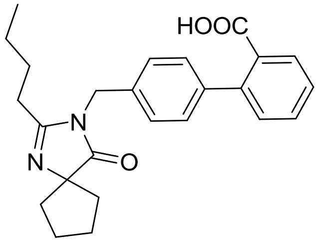
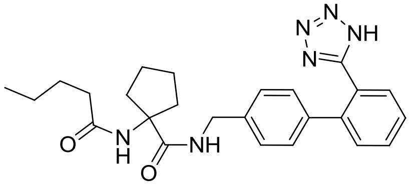
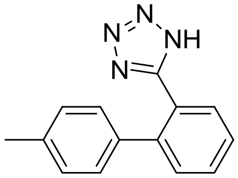
Fig. 2.1.F2: Synthetic scheme of IRB

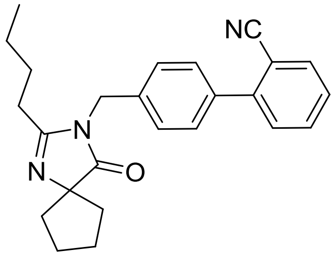
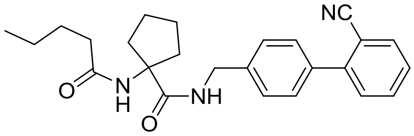
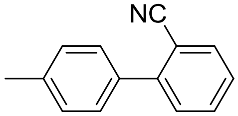
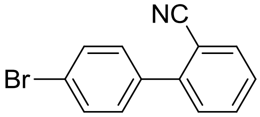
S. Hemamrutha et all., have developed RP-HPLC method for simultaneous estimation of irbesartan and hydrochlorothiazide in bulk and pharmaceutical dosage forms [16]. R. Ramesh Raju and N. Bujji Babu were developed and validated HPLC method for the estimation of irbesartan in pharmaceutical dosage form [17]. G. Kumaraswamy et all., have developed RP-HPLC method for simultaneous estimation of irbesartan and simvastatin in tablet dosage form [18]. D. M. Reddy et all., have developed simultanius method for the estimation of hydrochlorothiazide and irbesartan in formulation forms by HPLC [19]. R. Prashanthi et all., have developed sensitive RP-HPLC-PDA method for assay of irbesartan in pure and pharmaceutical dosage forms [20]. N. Anitha et all., have developed and validation for the estimation of irbesartan in tablet dosage form by HPLC [21].1. R. Youssef et all., have developed RP-HPLC method for the estimation and separation of valsartan, losartan and irbesartan in bulk and pharmaceutical formulation [22]. B. Raja et all., have developed RP-HPLC method for the simultaneous estimation of irbesartan and hydrochlorthiazide in pharmaceutical dosage form [23]. N. J. Shah et all., have developed HPTLC method for the simultaneous estimation of irbesartan and hydrochlorothiazide in tablet dosage form [24]. R. A. Mhaske et all., have developed RP-HPLC method for simultataneous determination of irbesartan, losartan, hydrochlorothiazide and chlorthalidone–application to commercially available drug products [25]. V. P. Rane et all., have developed stability indicating LC method for simultaneous determination of irbesartan and hydrochlorothiazide in pharmaceutical preparations [26]. A. S. Khodke et all., have studied a validated stability indicating HPTLC method for simultaneous estimation of irbesartan and hydrochlorothiazide [27].

To the best of our knowledge there is no reported HPLC method for the determination of IRB along with the above mentioned process related impurities.

In the present study, in addition to the reported impurity –C other process related impurities were considered for the analytical method development. Chemical structures of possible impurities and degradation products are provided in Table 2.1.T2.

Table: 2.1.T2 Details of related substances of IRB drug substance

Imp	**Chemical structure**	**Chemical name**	**Source of impurity**
Imp-A		3-((2'-(1H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-2-propyl-1,3-diazaspiro[4.4]non-1-en-4-one	Process related impurity
Imp-B		4'-((2-butyl-4-oxo-1,3-diazaspiro[4.4]non-1-en-3-yl)methyl)-[1,1'-biphenyl]-2-carboxylic acid	Process related impurity
Imp-C		N-((2'-(1H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-1-pentanamidocyclopentanecarboxamide	Basic degradation product
Imp-D		5-(4'-methyl-[1,1'-biphenyl]-2-yl)-1H-tetrazole	Process related impurity

Imp-E		4'-((2-butyl-4-oxo-1,3-diazaspiro[4.4]non-1-en-3-yl)methyl)-[1,1'-biphenyl]-2-carbonitrile	N-1 stage of IRB
Imp-F		N-((2'-cyano-[1,1'-biphenyl]-4-yl)methyl)-1-pentanamidocyclopentanecarboxamide	Process related impurity
Imp-G		4'-methyl-[1,1'-biphenyl]-2-carbonitrile	Process related impurity in KSM of IRB
Imp-H		4'-bromo-[1,1'-biphenyl]-2-carbonitrile	KSM of IRB

During the evaluation of chromatographic method mentioned in USP and EP for the estimation of related substances of Irbesartan it was observed that the impurity- E elutes at about 157 minutes by virtue of its hydrophobic nature and due to the isocratic mode of this method.

As per the ICH guidelines, all the process related and degradation products must be controlled to a appropriate level in the drug substance to ensure the safety and efficacy of the drug. Attempts were made to develop a single HPLC method for the separation and quantification of process related and degradation products of Irbesartan. To the best of our knowledge, there is no stability indicating HPLC method for the quantitative determination of the impurities mentioned in table: 2.1.T2. The current international conference on harmonization (ICH) guidelines emphasize that the analysis of stability should be done by using stability-indicating methods, developed and validated after stress testing on drug under ICH recommended conditions such as hydrolysis (acidic, basic and aqueous), oxidation (agent: hydrogen peroxide), photolysis and thermal degradation. This chapter deals with the development of a stability-indicating analytical method for determination of process related impurities and degradation products by using samples generated from forced degradation studies. All the impurities were separated with good resolution and the developed method was validated as per the ICH guidelines [26-29].

2.2 Development of stability indicating method for Irbesartan

2.2.1 Materials

Materials or chemicals used for this study were listed in the Table 2.2.T1.

2.2.2 Apparatus

The LC system used for the method development and forced degradation studies was Waters made LC system with 2996 PDA detector (Waters Corporation, Milford, USA). The output signal was monitored and processed by using empower software. Method validations performed on

Agilent made 1100 series HPLC system. The output signal was monitored and processed by Chemstation software. The stability studies were carried out in humidity chamber (75%RH- 40°C, 65%RH-25°C. Thermo lab humidity chamber, India), photo stability chamber (Sanyo photo stability chamber, Leicestershire, UK), Thermal stability studies were performed in a hot air oven (Mackpharmatech, Hyderabad, India).

Table: 2.2.T1. Details materials and chemicals

S.No.	**Name of the compound**	**Grade**	**Procured from../synthesized by..**
1.	IRB drug substance samples	--	Dr.Reddy's Laboratories, INDIA
2.	Acetonitrile	Chromatography grade	Merck, India
3.	KH_2PO_4	AR grade	Merck, India
4.	Ortho phosphoric acid	AR grade	Merck, India
5.	Water	High pure	Purified by Milli-Q plus system, USA
6.	IRB impurities	--	Dr.Reddy's Laboratories, INDIA

2.2.3 Preparation of solutions

2.2.3.1 Preparation of impurity standard solution

Impurity stock solution were prepared by dissolving appropriate amount of all the known impurities i.e. Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H in diluent. (Acetonitrile and mobile phase-A in the ration of 1:1) to get the final concentration of each impurity in stock solution as 100 µg mL^{-1}.

As per ICH Q3A guidelines, specification limit of each known impurity in drug substance should not be more than 0.15 %, where the maximum daily dosage of drug is less than or equal to 2.0 g [30]. As described in Table 2.1.T1, maximum daily dosage of IRB is less than 2.0 g. Hence, considered specification limit for all the known impurities as 0.15 % and prepared impurity blend solution of 0.15 % by spiking appropriate volume of impurity stock solution in µL to 1000 µg mL^{-1} IRB drug substance test solution.

2.2.3.2. Preparation of IRB test solution

IRB drug substance test solution was prepared by weighing 10 mg of drug substance in 10 mL volumetric flask dissolved and diluted in 5mL of acetonitrile and made to volume with mobile phase-A. The concentration of IRB drug substance used for the estimation of related substances was 1000 µg mL^{-1}.

2.2.4 Preparation of forced degradation samples

As per the ICH stability guidelines (Q1AR2): “stress testing is likely to be carried out on single batch material (through out the study uniform material).

One lot of IRB drug substance was selected and subjected to different stress conditions like acid hydrolysis, base hydrolysis, water hydrolysis, oxidation, photo degradation and Thermal degradation.

2.2.4.1. Preparation of Photo degradation sample

For photo degradation study, IRB drug substance was taken in Petri dish and exposed to ultraviolet radiation providing an overall energy of not less 200 Watt hours/m^2 and exposed to visible radiation providing an overall energy of not less than 1.2 million lux hours in a photo stability chamber, sample was taken-off from the chamber and prepared test solutions of 1000 μg mL^{-1}.

2.2.4.2. Preparation of Thermal degradation sample

IRB drug substance was placed in Petri dish and spread uniformly. Later the Petri dish was kept in the oven and maintained the temperature of oven at 105 °C for 7 days. After 7 days, IRB samples were taken-off from the oven and test solutions were prepared to get the final concentration as 1000 μg mL^{-1}.

2.2.4.3. Preparation of oxidative degradation sample

100 mg of IRB drug substance was transferred to 100 mL volumetric flask, dissolved in 50 mL of Acetonitrile and made upto the mark with 12 % peroxide solution. Placed magnetic stirrer in the solution and kept solution at room temperature under continuous stirring for 48

h. Used the solution directly for the estimation of Oxidative degradation products.

2.2.4.4. Preparation of Aqueous hydrolysis sample

100 mg of IRB drug substance was transferred to 100 mL volumetric flask, dissolved in 50 mL of Acetonitrile and made upto mark with water. Placed magnetic stirrer in the solution and kept solution at 70 °C temperature under continuous stirring for 48 h.

2.2.4.5. Preparation of Acid hydrolysis sample

100 mg of IRB drug substance was transferred to 100 mL volumetric flask dissolved in 50 mL of Acetonitrile and made upto mark with 1.0N hydrochloric acid. Placed magnetic stirrer in the solution and kept solution at 70 °C temperature under continuous stirring for 48 h.

2.2.4.6. Preparation of Base hydrolysis sample

100 mg of IRB drug substance was transferred to 100 mL volumetric flask dissolved in 50 mL of Acetonitrile and made upto mark with 0.1N Sodium hydroxide solution. Placed magnetic stirrer in the solution and kept solution at room temperature under continuous stirring for 48 h.

2.3 Method development and optimization of chromatographic conditions

Impurity blend solution spiked to IRB at the specification level i.e 0.15% has been used for method development trials.

2.3.1 Selection of wavelength

10 ppm solution of each known impurity and IRB Drug substance were prepared in Acetonitrile diluent. By using UV Visible spectrophotometer, scanned the solution in the UV range of 200-400 nm against Acetonitrile as blank. Overlaid UV spectra of all the related components and IRB were given as Fig. 2.3.F1. IRB and its related compounds showing wavelength maximum around 220 nm, hence it has been selected as UV detector wavelength for LC method development

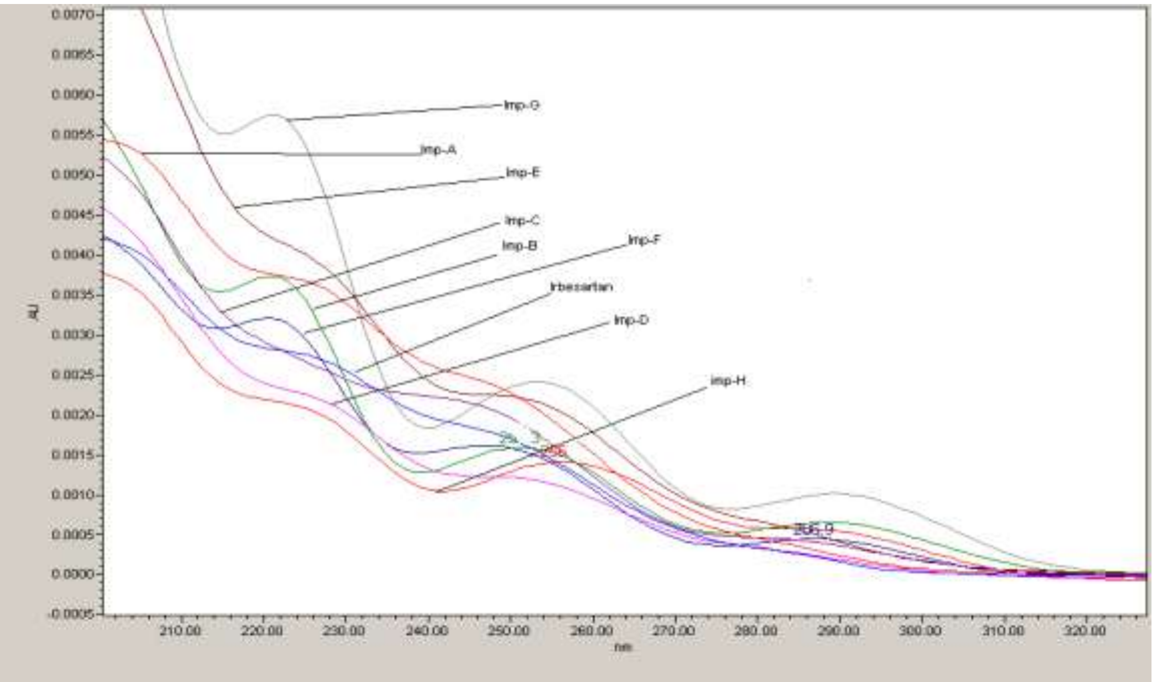


Fig. 2.3.F1: Overlay of UV spectra of IRB and its impurities

2.3.2. Important method development Trials in sequence

2.3.2.1. Trial-1

Method was reported in USP and EP for the estimation of related substances by using a Nucleosil C18 column having dimentions of 250mm length and 4.0 mm internal diameter packed with 7µm particles. pH 3.2 buffer was prepared by dissolving 5.5ml of phosphoric acid in 950ml of water and adjusting the pH 3.2 with Triethyl amine. The mobile phase was prepared by mixing 670ml of buffer and 330ml of acetonitrile. The flow rate was set as 1.0ml/min. The eluants were monitored at 220nm. The composition of the mobile phase was kept constant during the course of analysis (Isocratic mode). The chromatogram was presented in Fig.2.3.F2.

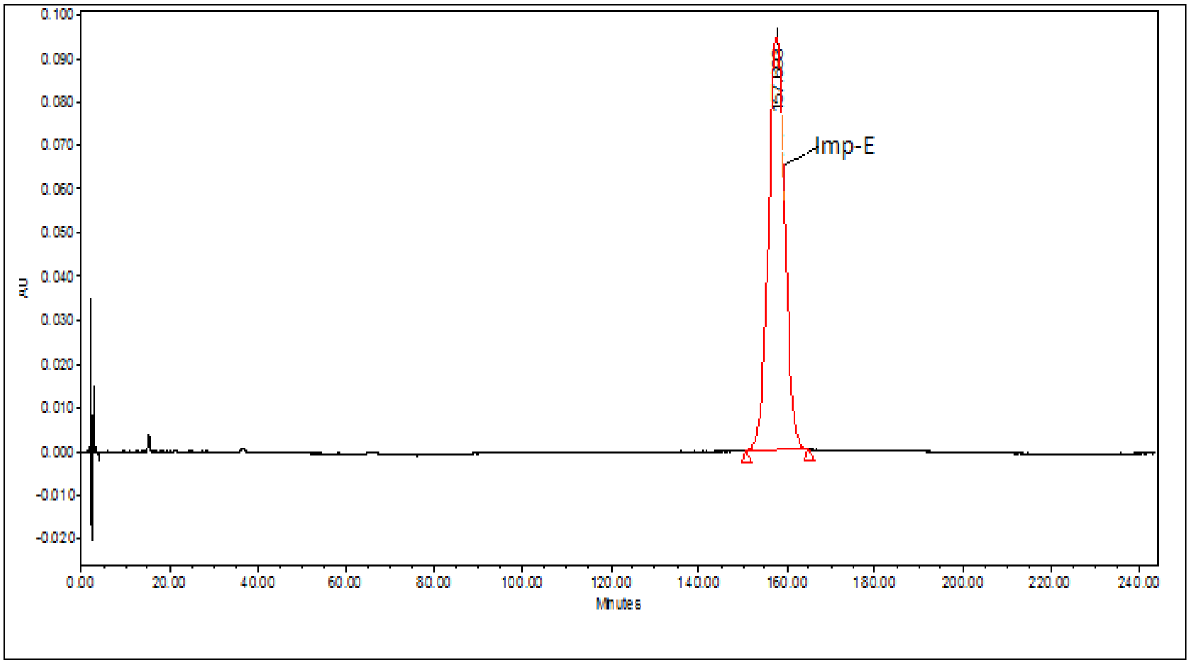


Fig. 2.3.F2: Elution of Imp-E in USP method

The late elution of Imp-E was due to its high hydrophobicity and due to the isocratic nature of the method. Efforts were made to develop a method by employing gradient elution mode so that Imp-E retention time will be reduced.

Before starting the method development trials, the information about the pKa value of IRB was collected to arrive at the correct buffer pH. The pKa of IRB are 4.12 and 7.4. Hence, pH of buffer was chosen as 2.6 without overlapping the ionization pH of IRB [31]. The retention of Imp-A, Imp-B, Imp-C and Imp-D depends on the pH of the mobile phase due to the presence of the polar functional groups. To maintain the pH of mobile phase at 2.6 potassium dihydrogen orthophosphate buffer was selected. To ensure elution of all the non-polar compounds in the test sample, development initiated with gradient mode of elution. Mixture of 90% Buffer and 10% acetonitrile used as Mobile phase-A. To have high elution strength in mobile phase-B, acetonitrile was selected as organic solvent. Due to low viscosity of Acetonitrile, 100% solvent usage as mobile phase- B may lead to pumping problem. To avoid this, Acetonitrile with 20 % water has been used as Mobile phase-B.

2.3.2.2. Trial-2

The method development started with a conventional C18 stationary phase like Kromasil C18 having carbon load of 19%, with dimensions of 250mm length and internal diameter of 4.6mm. Injected impurities blend solution into the chromatographic system.

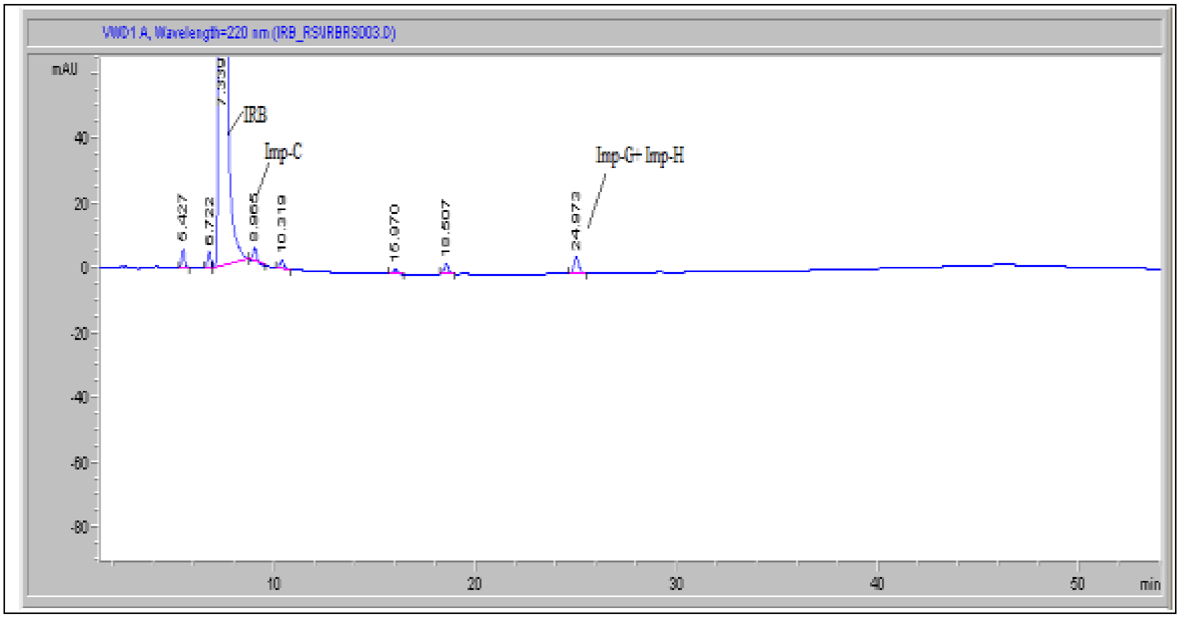


Fig: 2.3.F3. Chromatogram of IRB impurity blend in Trial-2

In this trial Imp-C was closely eluting with the IRB peak and there is no resolution between the Imp-G and Imp-H. The chromatogram of this trial is presented in Fig.2.3.F3.

2.3.2.3. Trial-3

To bring the Selectivity between the Imp-G and Imp-H, column chemistry was changed from conventional end capped C-18 to polar embedded column like Symmetry shield RP18. Due to the presence of polar embedded carbamate groups, this column brings selectivity difference between the structurally similar moieties.

The mobile phase-A was prepared by mixing water, acetonitrile and phosphoric acid in the ratio of 85:15:0.1(v/v/v). Mobile phase –B was prepared by mixing water and acetitrile in the ratio of 20:80 (v/v). Flow rate was set as 1.0ml/min. The gradient programe for this trial was shown in Table: 2.3.T1.

Table: 2.3.T1. Gradient program for Trial-3

Time	0.01	5	40	50	52	60
% of B	20	20	90	90	20	20

Mixture of Imp-G and Imp-H was injected into the chromatographic system and then the responses of the peaks were recorded. Imp-G and Imp-H peaks were separated in this trial. The chromatogram of this trial is presented in Fig.2.3.F4.

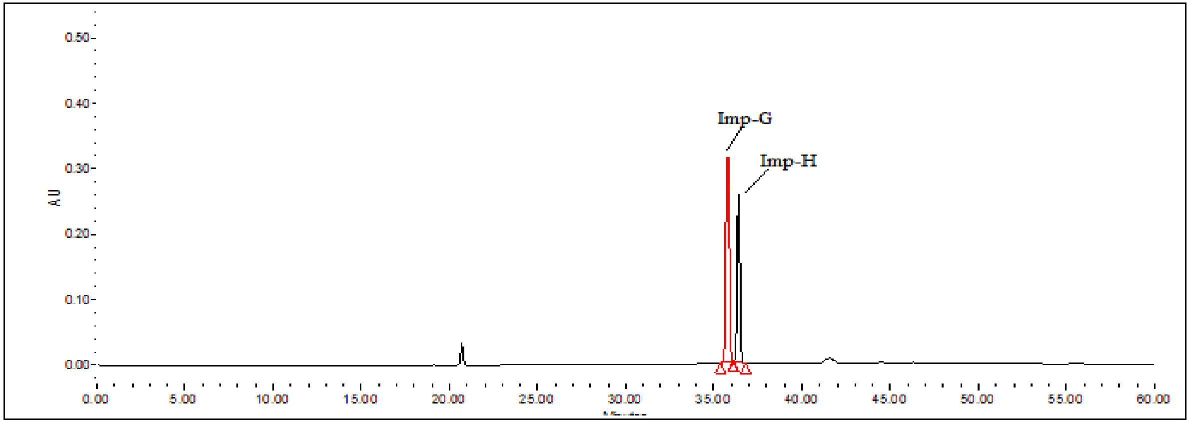


Fig: 2.3.F4. Chromatogram of Imp-G and Imp-H in Trial-3

2.3.2.4. Trial-4

Preliminary degradation studies were conducted to ensure the separation of potential degradants from the IRB peak. One of the Oxidation (with 3.0% Peroxide for 17H at 60°C) degraant was closely eluting with the IRB peak (Fig.2.3.F5.)

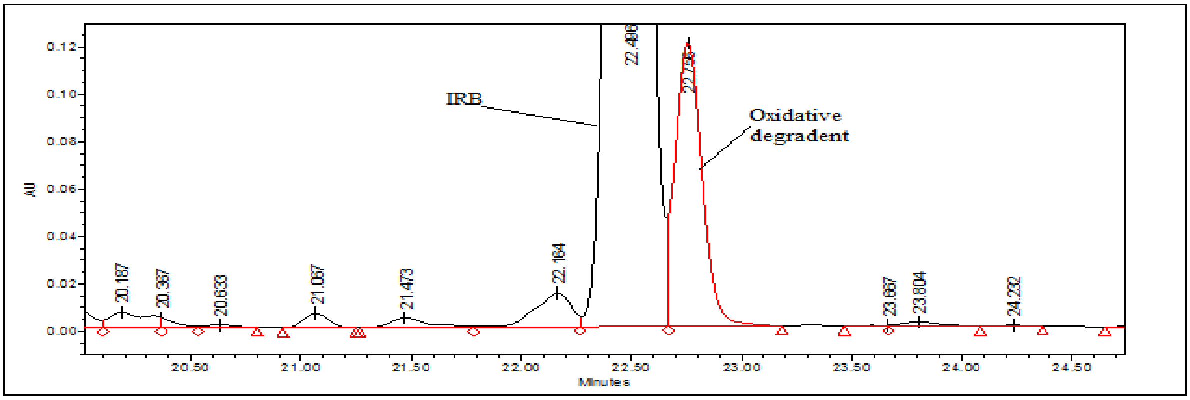


Fig: 2.3.F5. Chromatogram of Oxidative degradation.

To separate this impurity from IRB the pH of the mobile phase was increased to 2.6. Mobile phase-A was prepapred by mixing 850mL of 0.01M KH_2PO_4 and 150mL of acetonitrile. The composition of mobile phase-B was kept constant during the trials. Column oven was maintained at 30°C.The gradient programme remained same as above trial. The resolution between the oxidative degradant and IRB peak was more than 2.0 (Fig.2.3.F6.)

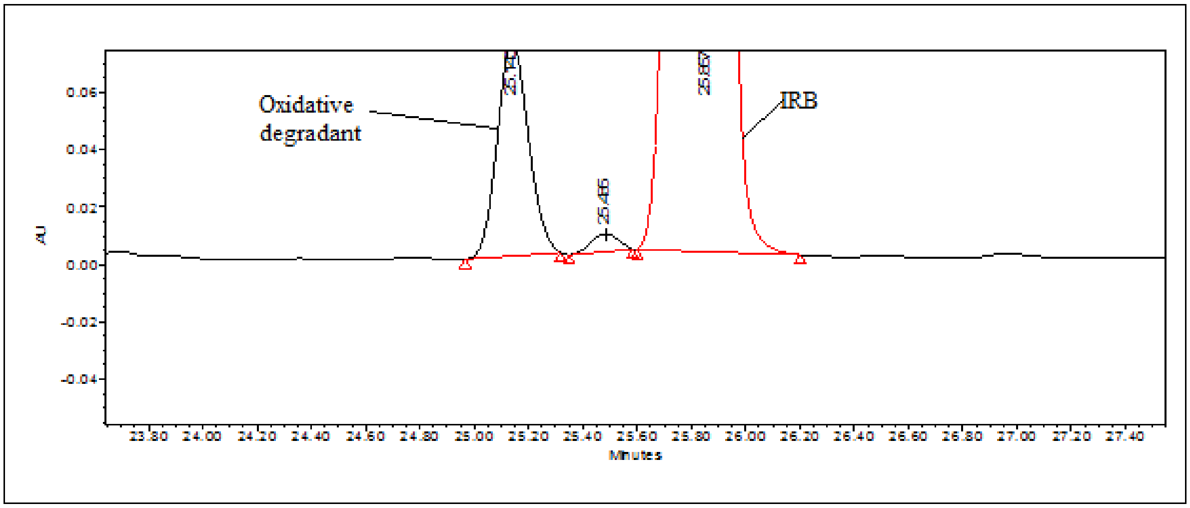


Fig: 2.3.F6. The resolution between the IRB and Oxidative degradant

2.3.2.5. Trial-5

The gradient programme is further optimized to reduce the retention time of IRB from 25 minutes. Injected blend solution and forced degradation samples and found that the method is capable to separate all the related compounds including degradants. Detailed discussion on forced degradation study is provided in separate section, 2.4. Blank chromatogram of Trial-5 put on view as Fig.2.3.F7 and gradient program as shown in Table: 2.3.T2.

Table: 2.3.T2. Gradient program for Trial-5

Time	0.01	5	43	53	55	60
% of MP-B	30	30	80	80	30	30

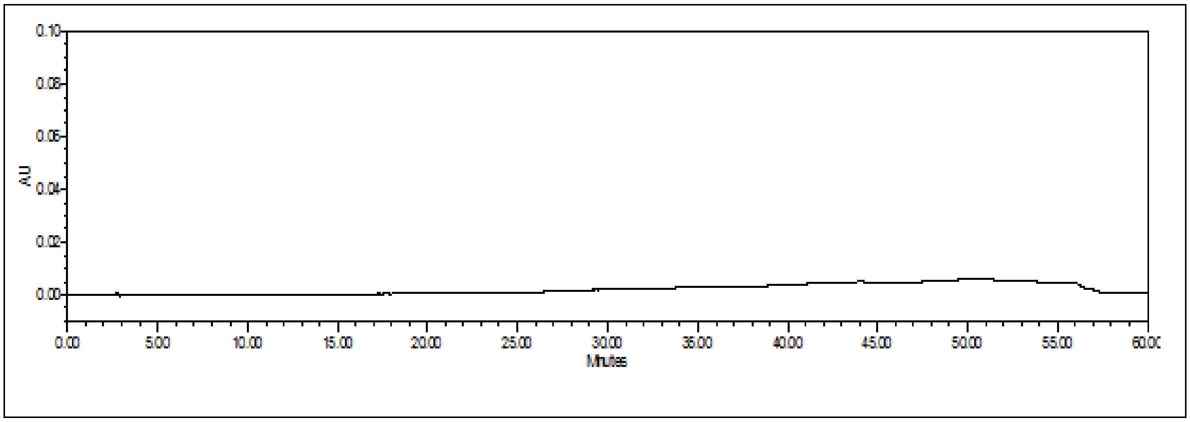


Fig: 2.3.F7. Typical blank chromatogram

Blend chromatogram of IRB with all related impurities presented as Fig.2.3.F8. As the above method is showing Gaussian peak shape of IRB, well resolution between all the related compounds and neat blank, this Trial was finalized for estimation of related substances of IRB.

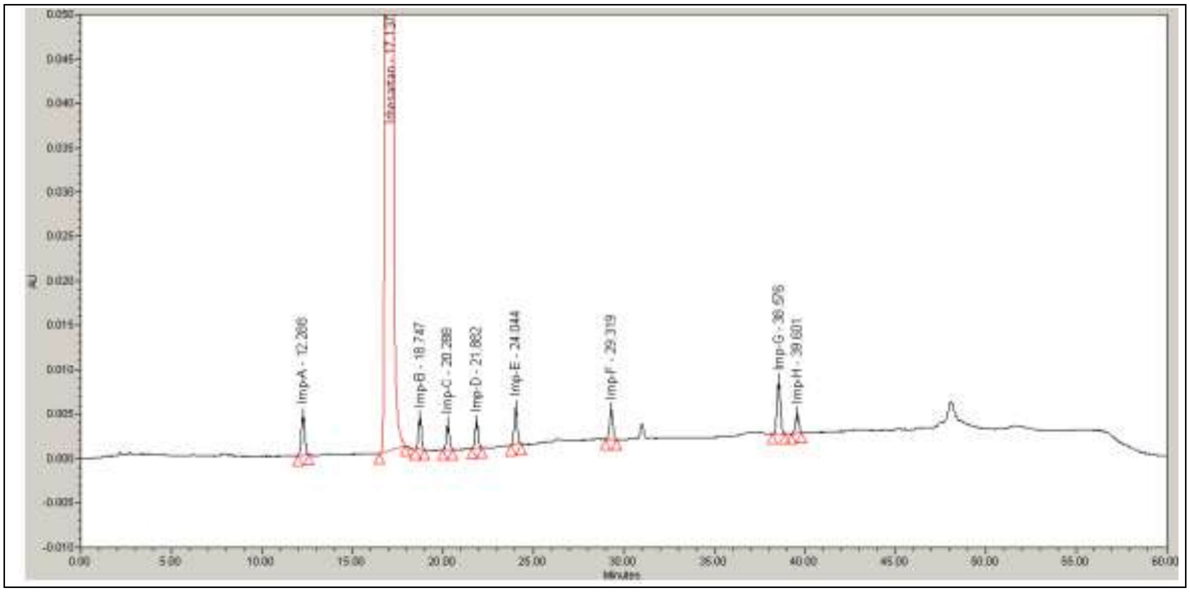


Fig: 2.3.F8. IRB Blend chromatogram of Trial-5

Table: 2.3.T3. Final chromatographic conditions of IRB method

Column	Waters Symmetry shield RP18, 250mm x 4.6mm, 5µ particle size.
Buffer	0.01M KH_2PO_4 in Milli-Q water, pH adjusted to 2.6 with dilute ortho phosphoric acid solution
Mobile phase	Mobile phase-A : Buffer : Acetonitrile : 90: 10 (v/v)
	Mobile phase-B : Water : Acetonitrile : 20: 80 (v/v)
Mode of elution	Gradient
Flow rate	1.0 mL min^{-1}
Column temperature	30 °C
Wavelength of detection	220 nm
Injection volume	10 µL
Run time	60 min
Diluent	Acetonitrile and Mobile phase-A in the ratio 1:1
Gradient program	Time (min) / % Mobile phase- B: 0.01/30, 5/30, 43/80, 53/80, 55/30 and 60/30.
Concentration	For related substances estimation : 1000 µg mL^{-1}

2.3.3. Optimized chromatographic conditions

Optimized chromatographic conditions for related substances estimation in IRB was given in Table 2.3.T3. The retention times (RTs) and relative retention times (RRTs) of all the known compounds are presented in Table 2.3.T4.

2.4 Discussion on Forced degradation studies

Analyzed IRB drug substances spiked with all the known impurities, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H at 0.15% level with respect to test concentration. The spectral homogeneity of main component was ensured in spiked solution. Chromatogram of unstressed sample and peak purity plot for IRB peak are presented in Fig.2.4.F1.–F2.

Table: 2.3.T4. RTs and RRTs of known components in finalized method

S.No.	Name of the Analyte	Retention time (min)	Relative retention time w.r.t. IRB peak
1	Imp-A	12.194	0.72
2	Imp-B	18.829	1.12
3	Imp-C	20.142	1.19
4	Imp-D	21.880	1.30
5	Imp-E	24.139	1.43
6	Imp-F	29.138	1.73
7	Imp-G	38.682	2.29
8	Imp-H	39.712	2.36
9	IRB	16.860	---

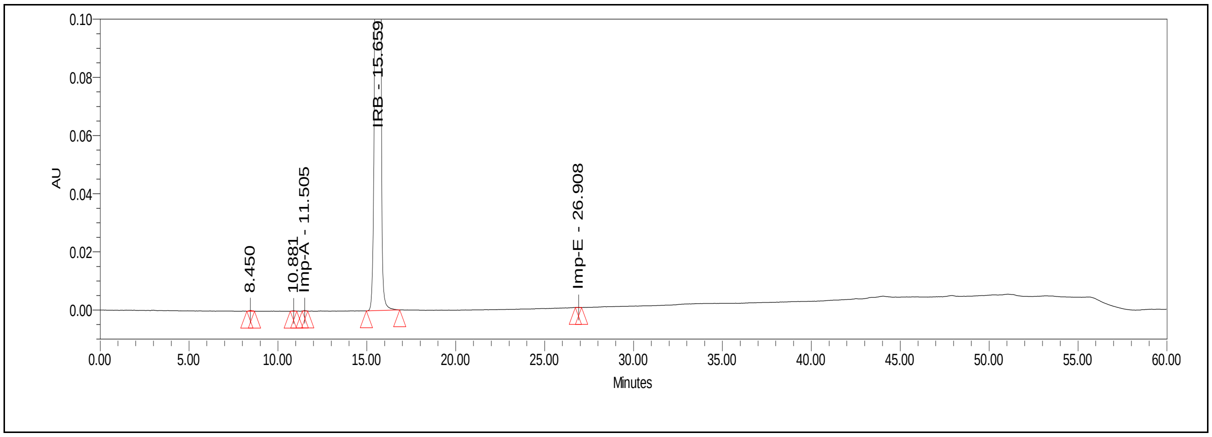


Fig: 2.4.F1. Chromatogram of unstressed IRB drug substance

All the forced degradation samples were analyzed in HPLC method by using PDA detector. Homogeneity of IRB peak in all the degradation samples was evaluated by means of peak purity evaluation.

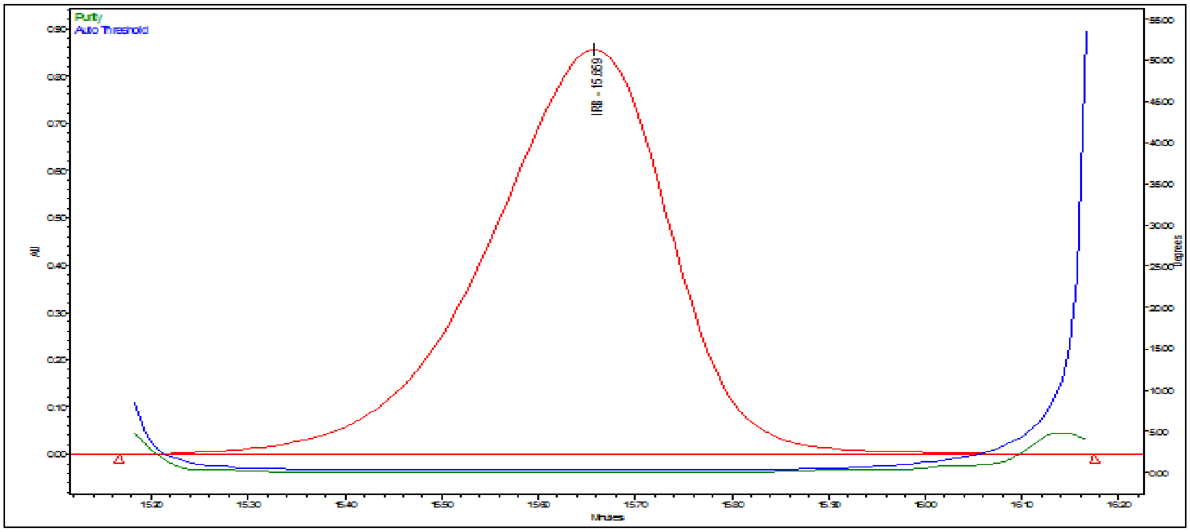


Fig: 2.4.F2. Peak purity of IRB peak

2.4.1. Degradation of IRB during oxidation

IRB drug substance was sensitive towards the oxidation, it underwent degradation and formed impurity at RRT 0.96 around 1.8 %.

Peak purity of IRB peak in oxidized drug substances was confirmed as homogeneous. Chromatogram of degraded IRB sample and peak purity plot of IRB peak is presented in Fig. 2.4.F3 and 2.4.F4.

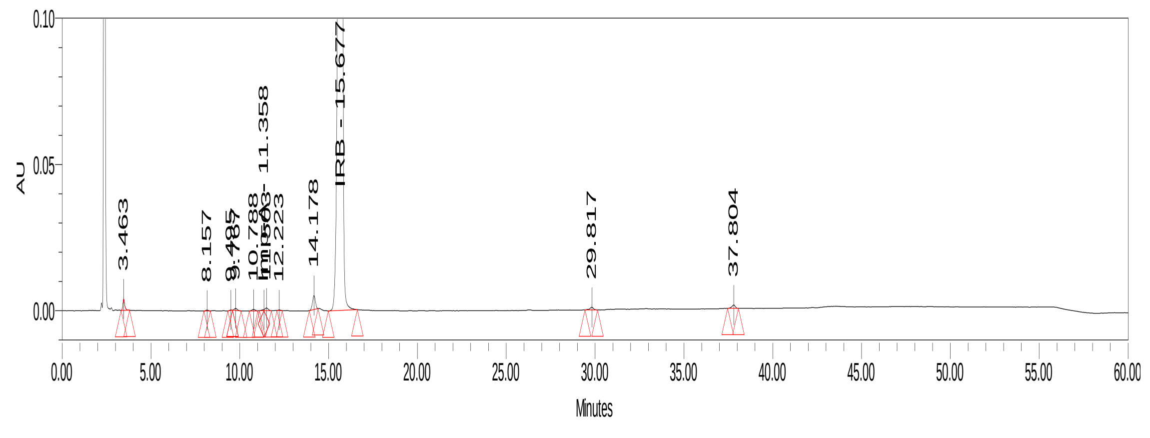


Fig: 2.4.F3. Chromatogram of oxidized IRB sample

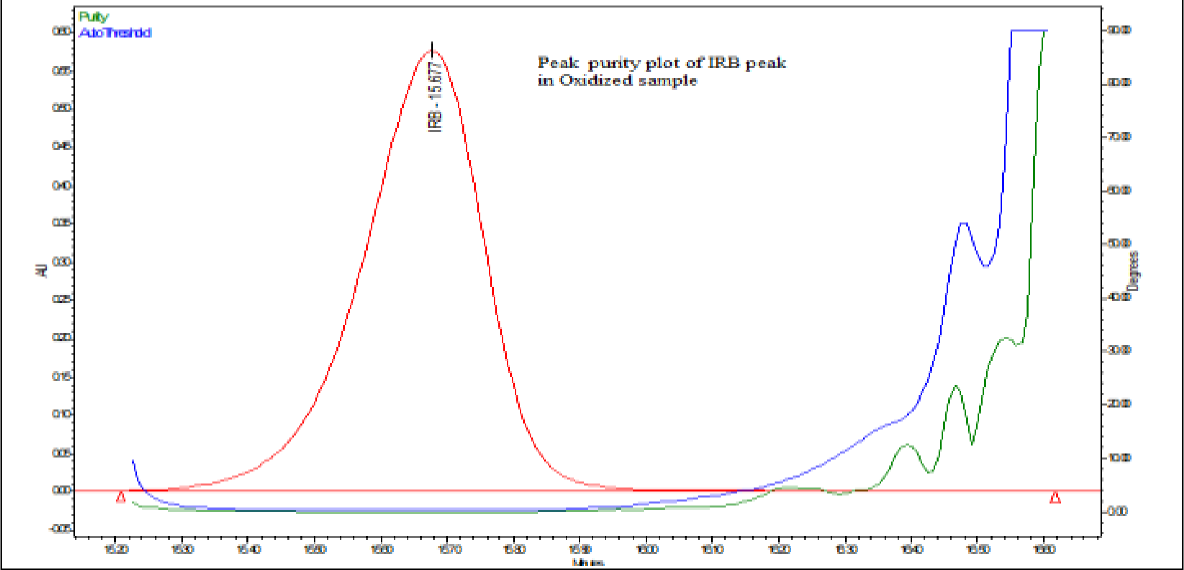


Fig: 2.4.F4. Peak purity plot for IRB peak in oxidized sample

2.4.2. Degradation during base hydrolysis

IRB drug substance was subjected to base hydrolysis by using 0.05N sodium hydroxide at room temperature for 48 hours. Significant degradation was observed in basic conditions resulting in the formation of Imp-C. Based on the peak purity data it was confirmed that the main component in base hydrolyzed sample is homogeneous. Chromatogram of base hydrolyzed IRB sample and purity plots are presented in Fig.2.4.F5 and Fig.2.4.F6 respectively.

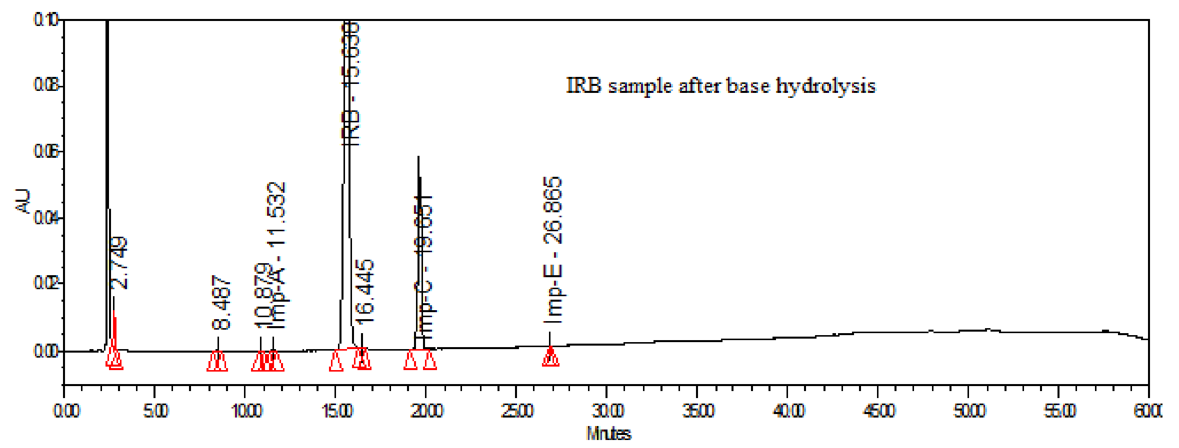


Fig: 2.4.F5. Chromatogram of base hydrolysis IRB sample

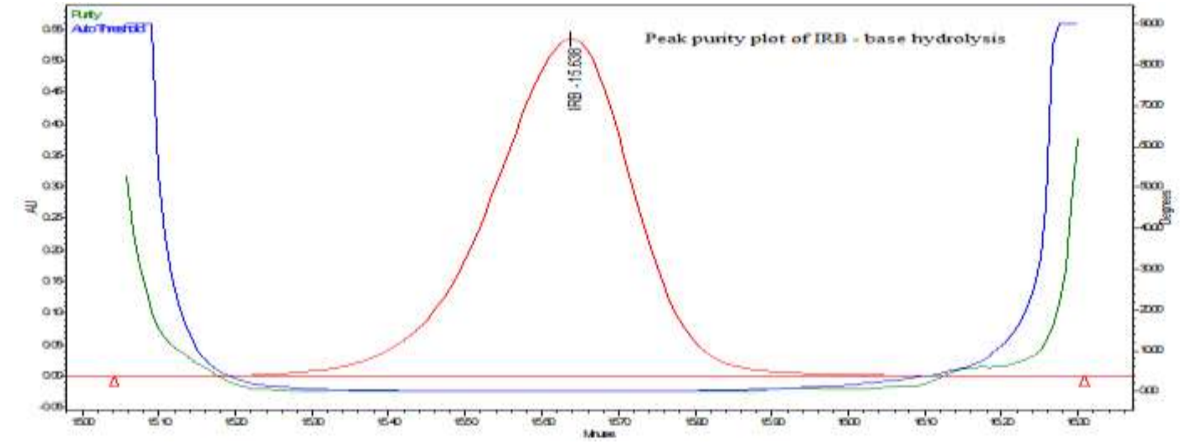


Fig: 2.4.F6. Peak purity plot for IRB peak in base hydrolysis

2.4.3. Degradation during Acid hydrolysis

IRB drug substance was subjected to acid hydrolysis by using 0.5N Hydrochloric acid at 70 °C for 12 h; around 5.5% of IRB was getting degraded. Peak purity of IRB peak in acid hydrolyzed sample was found to be homogeneous. LC chromatogram of acid hydrolyzed sample and peak purity plots are shown in Fig. 2.4.F7 and Fig.2.4.F8 respectively.

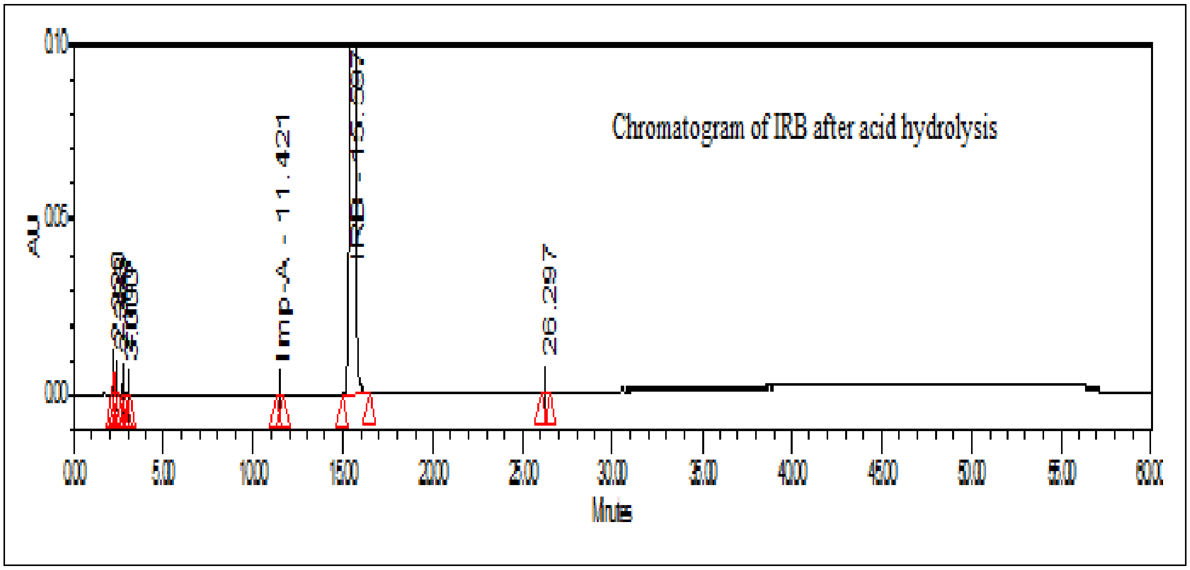


Fig: 2.4.F7. Chromatogram of Acid hydrolysis IRB sample

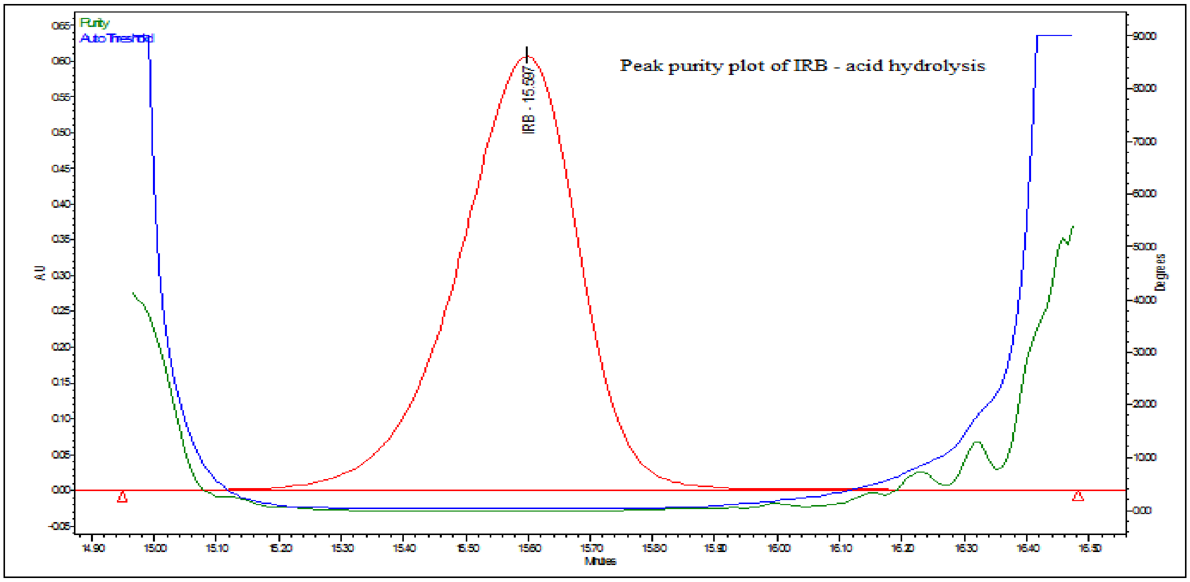


Fig: 2.4.F8. Peak purity plot for IRB peak in acid hydrolysis

2.4.4. Degradation during water hydrolysis

IRB drug substance is more stable towards water hydrolysis; IRB was not degraded even after 48 h of water hydrolysis at 70 °C. Peak purity data of IRB peak in final sample indicates that peak is homogeneous. Chromatograms of stressed sample and peak purity plot of IRB are shown in Fig.2.4.F9 and Fig. 2.4.F10 respectively.

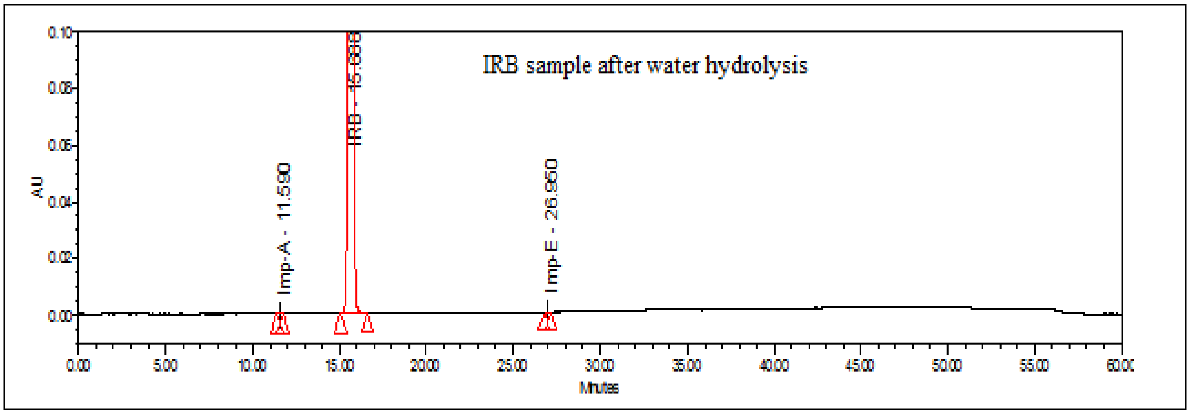


Fig: 2.4.F9. Chromatogram of water hydrolysis IRB sample

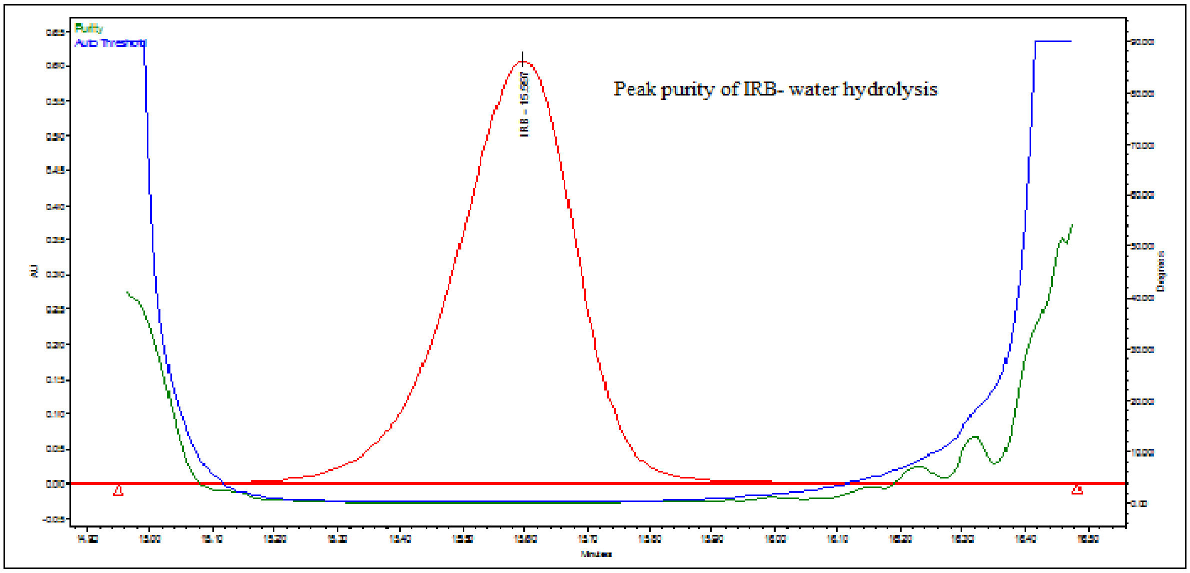


Fig: 2.4.F10. Peak purity plot for IRB peak in water hydrolysis

2.4.5. Thermal degradation

IRB drug substance was thermally stable. The drug was exposed to 105 °C temperature for 7 days, practically it was not degraded. IRB peak in thermally degraded sample was found to be spectrally pure. Chromatogram of stressed sample and peak purity plot was put on view as Fig.2.4.F11 and Fig.2.4.F12.

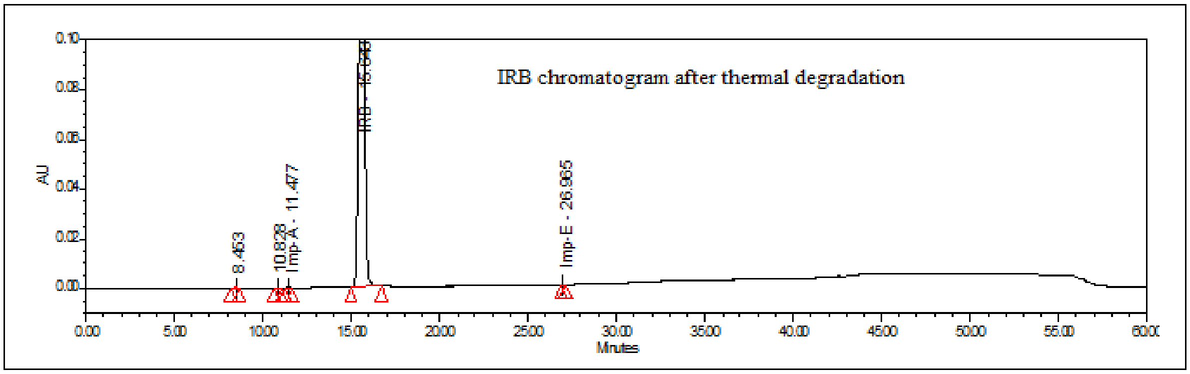


Fig: 2.4.F11. Chromatogram of Thermal degradation IRB sample

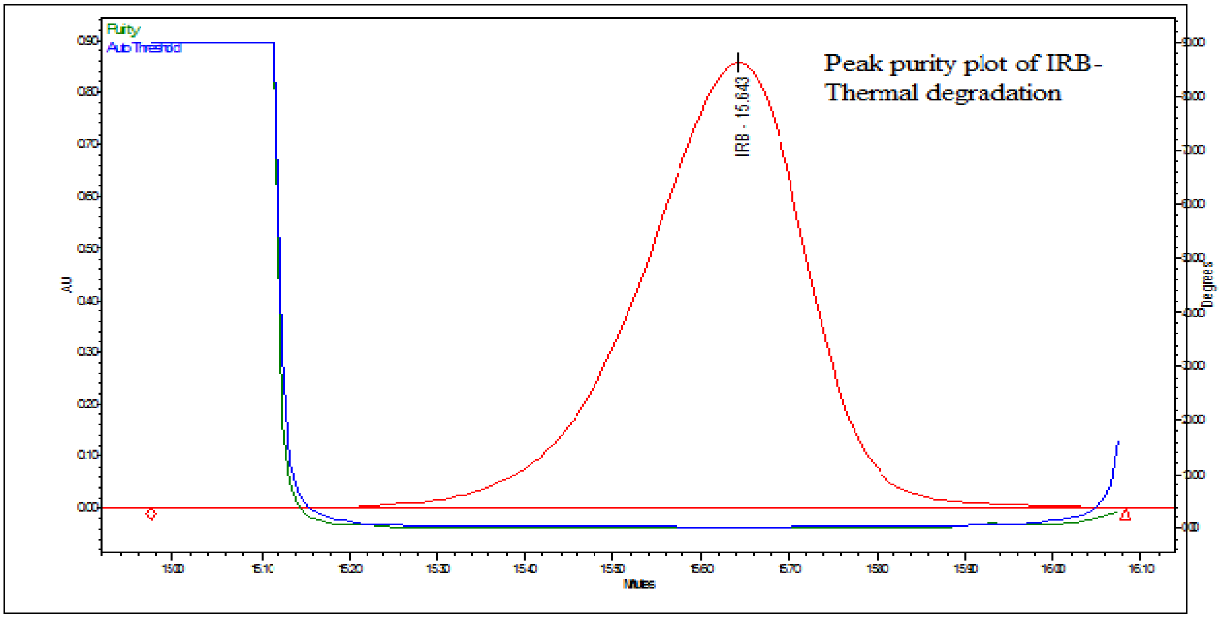


Fig: 2.4.F12. Peak purity plot for IRB peak in Thermal degradation Sample

2.4.6. Photolytic degradation

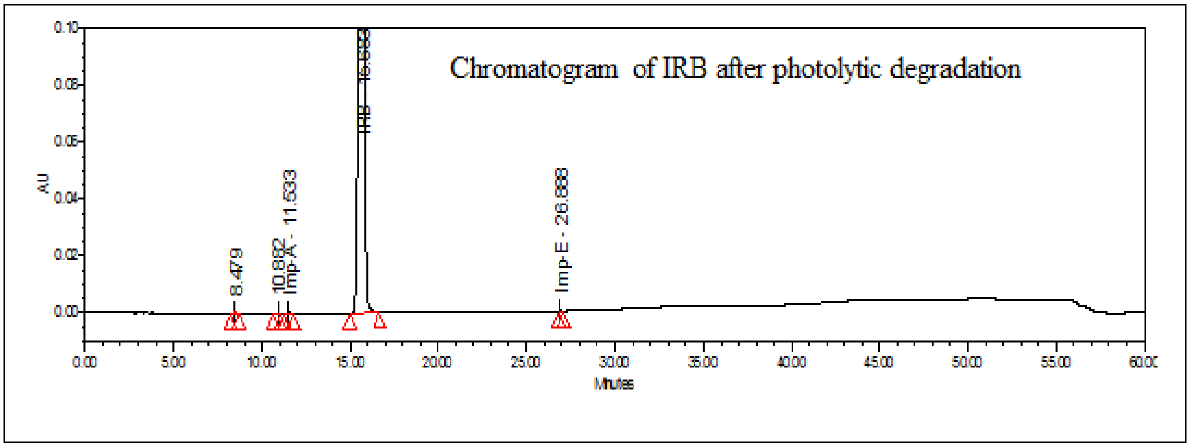


Fig: 2.4.F13. Chromatogram of Photolytic degradation IRB sample

IRB drug substance was exposed to UV and visible radiations for 7 days. No considerable degradation was observed. To confirm the stability of IRB drug substance towards photolytic condition, peak purity of IRB peak was verified in degraded sample and found that it was homogeneous. Chromatogram of photolytic degradation sample and peak purity plot was put on display as Fig. 2.4.F13 and 2.4.F14 respectively.

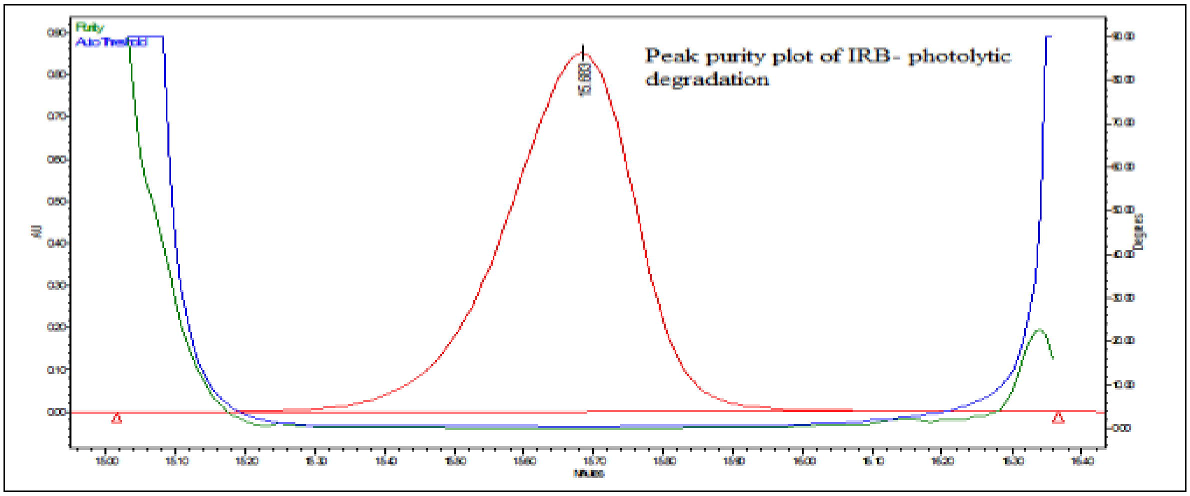


Fig: 2.4.F14. Peak purity plot for IRB peak in photolytic degradation sample

2.4.7. Results of Forced degradation studies

To ensure the mass balance of stressed samples, an assay of final degraded samples were estimated against qualified IRB drug substance as a reference standard. The sum of % of degradation products, all other impurities and % assay of corresponding stressed sample was considered as the mass balance of each stressed sample. The mass balance of stressed samples was found to be in the range of 99.5 to 101.6 %. Mass balance results are evident that the developed LC method is stability indicating and specific for estimation of assay in presence of its related components, Imp-1, Imp-2, Imp-3 and Imp-4. Also this stability indicating method is suitable for determination of all the related substances and degradation products in IRB drug substance during stability testing. % of all specified related compounds, % assay and % mass balance in all the stressed samples are presented in Table 2.4.T1 and 2.4.T2 respectively.

Table: 2.4.T1. Results of forced degradation studies

Stress Condition	Imp-C	Imp-E	Unk-1 ~0.17 RRT	Unk-2 ~0.20 RRT	Unk-3 ~0.52RRT	Unk-4 ~0.96 RRT	% Deg
As such sample	ND	0.002	-	-	-	-	-
Photo Deg.(U.V&Visible) With "Al" Foil	ND	0.007	-	-	0.02	-	-
Base Deg. 0.05N NaOH (RT) 48th Hour	4.24	0.006	0.20	-	0.03	-	4.6
Water Hydrolysis 48th Hour under reflux	0.04	0.02	0.02	0.004	0.02	-	0.4
Acid Deg. 0.5N HCl 12th Hour under reflux	ND	ND	2.25	2.72	0.04	-	5.5
6%H_2O_2 Deg. (RT) 48th Hour	0.05	ND	-	-	0.36	1.78	5.2
Thermal Deg. (105°C) 7th Day	0.005	0.004	-	-	-	-	-

2.5 Analytical method validation

The developed and optimized LC method was fully validated as per ICH and USP guidelines.

Table: 2.4.T2. % Mass balance in each stress condition

Degradation condition	**Assay (% w/w)**	**% Total impurities***	**%Mass balance**
As such sample	99.5	-	--
Photolyticl degration	99.5	-	99.5
Thermal degradation	99.5	-	99.5
Base Deg. 0.05N NaOH (RT) 48thHour	94.7	4.6	99.8
Water Hydrolysis 48th Hour under reflux	99.7	0.4	100.6
Acid Deg. 0.5N HCl 12th Hour under reflux	95.6	5.5	101.6
6%H_2O_2 Deg. (RT) 48th Hour	94.0	5.2	99.7

*Total impurities = sum of degradation products and all other impurities

2.5.1. System suitability test (SST)

Prepared six different solutions of IRB drug substance standard in test concentration, spiked with all specified known impurities, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H at 0.15 % level with respect to test concentration. These solutions were injected into LC system and ensured that the USP resolution (Rs) between any known pair of peaks should not be less than 2.0 and USP Tailing factor for any known compound should be less than 1.5. System suitability results were tabulated in Table 2.5.T1.

2.5.2. Method Precision

To verify the repeatability of the developed method, prepared six individual preparation of IRB drug substance spiked with Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H at 0.15 % level with respect to test concentration. Calculated the %RSD for each individual known impurity content. Results were tabulated in below Table 2.5.T2.

Table: 2.5.T1. SST results for IRB related substances

Name of the Compound	Retention time (min)	RRT w.r.t. IRB peak	USP resolution [b]	USP tailing factor
Imp-A	11.14	0.73	_	0.96
IRB	15.68	1.00	11.2	1.43
Imp-B	18.99	1.21	8.20	0.94
Imp-C	19.79	1.26	2.10	0.95
Imp-D	21.16	1.35	3.60	0.95
Imp-E	27.76	1.77	16.9	1.04
Imp-F	28.84	1.84	2.80	1.14
Imp-G	36.01	2.30	18.3	0.97
Imp-H	37.10	2.37	2.80	1.16

[b] Resolutions were calculated between two adjacent known peaks.

Table: 2.5.T2. Results of IRB related substances method precision

Sample solution	% of Related substances							
	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	Imp-F	Imp-G	Imp-H
Prep-1	0.22	0.14	0.17	0.22	0.18	0.08	0.34	0.23
Prep-2	0.22	0.14	0.17	0.22	0.18	0.08	0.34	0.23
Prep-3	0.22	0.14	0.17	0.22	0.18	0.08	0.34	0.23
Prep-4	0.22	0.14	0.17	0.22	0.18	0.08	0.34	0.23
Prep-5	0.21	0.14	0.17	0.22	0.19	0.08	0.34	0.23
Prep-6	0.22	0.14	0.17	0.22	0.18	0.08	0.34	0.23
Mean	0.22	0.14	0.17	0.22	0.18	0.08	0.34	0.23
%RSD	1.87	0.0	0.0	0.0	2.25	0.0	0.0	0.0

The above results are evident that the method is repeatable within acceptable limits of % RSD for six preparations of related substances are 0.0-2.3.

2.5.3. Sensitivity

Sensitivity of the method was demonstrated in terms of Limit of Quantitation (LOQ) and Limit of Detection (LOD) values of specified analytes. LOQ, LOD values were established for Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H and IRB based on signal to noise ratio of each peak.

2.5.3.1 Limit of quantification (LOQ)

Table: 2.5.T3. LOQ values of IRB and its related compounds

S.No.	Name of the analyte	S/N Ratio	LOQ in µg/mL	% w.r.t. test concentration
1	Imp-A	10.1	0.06	0.006%
2	Imp-B	9.7	0.05	0.005%
3	Imp-C	10.0	0.10	0.010%
4	Imp-D	10.0	0.10	0.010%
5	Imp-E	10.2	0.10	0.010%
6	Imp-F	9.7	0.08	0.008%
7	Imp-G	10.1	0.05	0.005%
8	Imp-H	9.9	0.13	0.013%

Prepared a series of dilutions of IRB, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H in different concentrations and injected them into the liquid chromatography until to get the signal to noise ratio 10 (i.e. 9.5 to 10.0). Limit of quantitation values of all the analytes are presented in Table 2.5.T3.

2.5.3.2. Limit of detection (LOD)

Table: 2.5.T4. LOD values of the impurities and IRB peak

S.No.	Name of the analyte	S/N Ratio	LOD in μg/mL	% w.r.t. test concentration
1	Imp-A	2.3	0.020	0.0020%
2	Imp-B	2.6	0.020	0.0020%
3	Imp-C	2.7	0.030	0.0030%
4	Imp-D	2.5	0.020	0.0024%
5	Imp-E	2.9	0.020	0.0020%
6	Imp-F	2.4	0.030	0.0030%
7	Imp-G	2.1	0.016	0.0016%
8	Imp-H	2.0	0.026	0.0026%

Prepared a series of dilutions of IRB, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H in different concentrations and injected them into the liquid chromatography until to get the signal to noise ratio 2 to 3. Limit of detection values of all the analytes are presented in Table 2.5.T4.

2.5.3.3. Precision at Limit of quantification level

Six preparations of IRB, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H at LOQ level were injected individually and the % RSD for the areas of each analyte was calculated. No significant variation observed in the area of each analyte for six consecutive injections, RSD of all the components ranges from 2.9 to 7.9 %. Results are summarized in 2.5.T5.

Table: 2.5.T5. LOQ precision results of IRB and its related compounds

Sample solution	Area of impurities							
	Imp-A	**Imp-B**	**Imp-C**	**Imp-D**	**Imp-E**	**Imp-F**	**Imp-G**	**Imp-H**
Prep-1	2.46	1.52	3.18	4.87	3.78	1.43	6.31	7.84
Prep-2	2.29	1.53	3.41	4.96	3.95	1.50	6.07	7.48
Prep-3	2.41	1.60	3.37	5.03	3.99	1.44	5.95	7.67
Prep-4	2.20	1.41	3.33	5.10	4.13	1.57	6.23	7.11
Prep-5	2.37	1.49	3.59	4.76	4.31	1.60	5.56	7.97
Prep-6	2.57	1.62	3.67	5.15	3.41	1.48	6.14	7.57
Mean	2.38	1.53	3.43	4.98	3.93	1.50	6.04	7.61
Stdev.	0.13	0.08	0.18	0.15	0.31	0.07	0.27	0.30
%RSD	5.44	4.99	5.22	2.93	7.90	4.58	4.43	3.96

Above results indicate that IRB RS method is precise at LOQ level.

2.5.3.4. Accuracy at LOQ level

IRB sample was injected in test concentration i.e. 1000 μg mL^{-1} to estimate the content of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H. Three different sample solutions (1000 μg mL^{-1}) of IRB containing Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H at LOQ level were prepared and injected each solution once. From the corrected area of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H, % recovery of each impurity was calculated. Results are summarized in Table 2.5.T6.

2.5.4. Linearity

2.5.4.1 Linearity of the related substances method

Table: 2.5.T6. Results of Accuracy at LOQ level

Name	Workup	Amount Added (µg/mL)	Amount obtained (µg/mL)	% Recovery	% Mean Recovery
Imp-A	1	0.06	0.0633	105.5	105.3
	2		0.0628	104.6	
	3		0.0635	105.9	
Imp-B	1	0.05	0.0514	102.9	105.1
	2		0.0544	108.9	
	3		0.0518	103.5	
Imp-C	1	0.10	0.0110	110.3	107.8
	2		0.0103	102.7	
	3		0.0110	110.3	
Imp-D	1	0.10	0.0100	99.7	98.3
	2		0.0097	97.3	
	3		0.0098	97.9	
Imp-E	1	0.10	0.0104	103.8	105.3
	2		0.0106	106.3	
	3		0.0106	105.8	
Imp-F	1	0.08	0.0723	90.3	91.6
	2		0.0769	96.1	
	3		0.0707	88.4	
Imp-G	1	0.05	0.0512	102.4	102.2
	2		0.0484	96.7	
	3		0.0534	107.6	
Imp-H	1	0.13	0.1245	90.7	92.4
	2		0.1278	93.1	
	3		0.1268	92.4	

A series of linearity solutions were prepared containing IRB, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H standard solution at different concentrations i.e. LOQ, 0.038 %, 0.075 %, 0.15 %, 0.15 %, 0.19 % and 0.225 % of working concentration (1000 µg mL^{-1}) by performing appropriate dilutions to achieve the targeted concentrations. The above prepared solutions of IRB and its impurities are LOQ, 25 %, 50 %, 100 %, 125 % and 150 % to known impurity specification limit i.e. 0.15 %. Each solution was injected once and calibration plots drawn for concentration of each component versus peak area of corresponding known component. Linearity plot of each analyte with best fit linear equation is put on view in Fig.2.5.F1-2.5.F8. Linear regression analysis has been performed for each analyte and data is presented in Table 2.5.T7- 2.5.T14.

Table: 2.5.T7. Linearity of Imp-A

S.No.	**Conc. (µg mL^{-1})**	**Imp-A peak area**	**Calculated response through trend line equation**	**Residual**	**Residual square**	**Sensitivity**
1	0.06	2.65	2.65	0.00	0.00	44.2
2	0.375	18.35	17.96	-0.39	0.16	48.9
3	0.75	35.71	36.18	0.47	0.22	47.6
4	1.125	54.22	54.40	0.18	0.03	48.2
5	1.5	72.72	72.63	-0.09	0.01	48.5
6	1.875	91.1	90.85	-0.25	0.06	48.6
7	2.25	108.9	109.07	0.09	0.01	48.4
Regression coefficient		0.99997	Residual sum of squares		0.49	
Slope		48.59				
Intercept		-0.27				
% y-Intercept		-0.37	Linearity equation		$y = 48.59x-0.27$	

Fig: 2.5.F1. Linearity plot for Imp-A

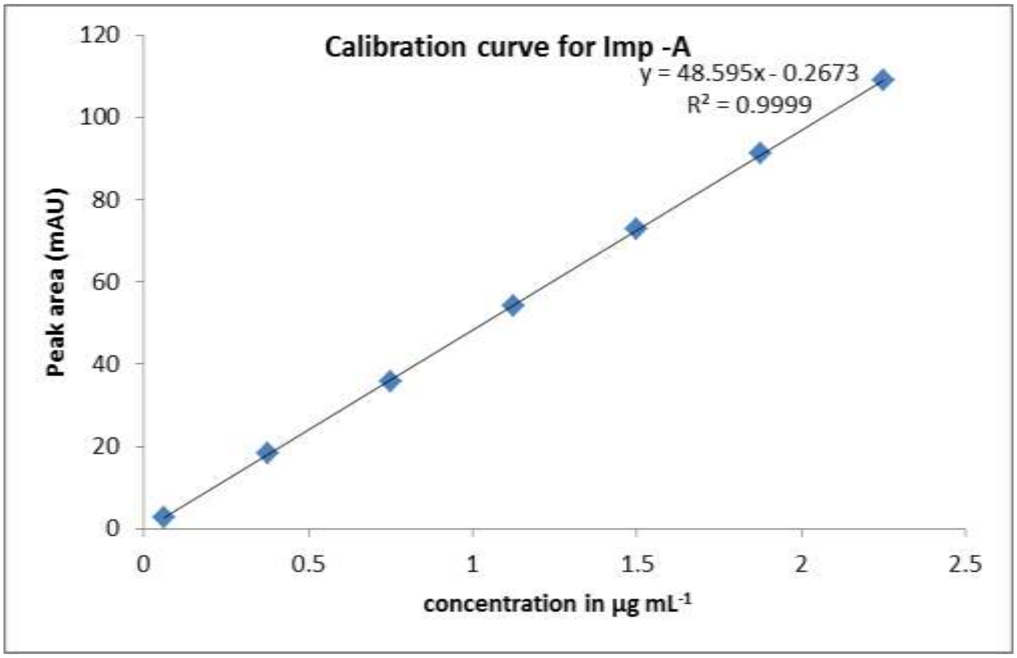


Table: 2.5.T8. Linearity of Imp-B

S.No.	Conc. (µg mL^{-}1)	Imp-B peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.05	1.63	1.72	0.09	0.01	32.6
2	0.375	13.45	13.18	-0.27	0.07	35.9
3	0.75	26.04	26.40	0.36	0.13	34.7
4	1.125	39.49	39.62	0.13	0.02	35.1
5	1.5	53.38	52.84	-0.54	0.29	35.6
6	1.875	66.02	66.07	0.05	0.00	35.2
7	2.25	79.11	79.29	0.18	0.03	35.2
Regression coefficient		0.99994	Residual sum of squares		0.55	
Slope		35.26				
Intercept		-0.04				
% y-Intercept		-0.08	Linearity equation		*y* = 35.26*x -0.04*	

Fig: 2.5.F2. Linearity plot for Imp-B

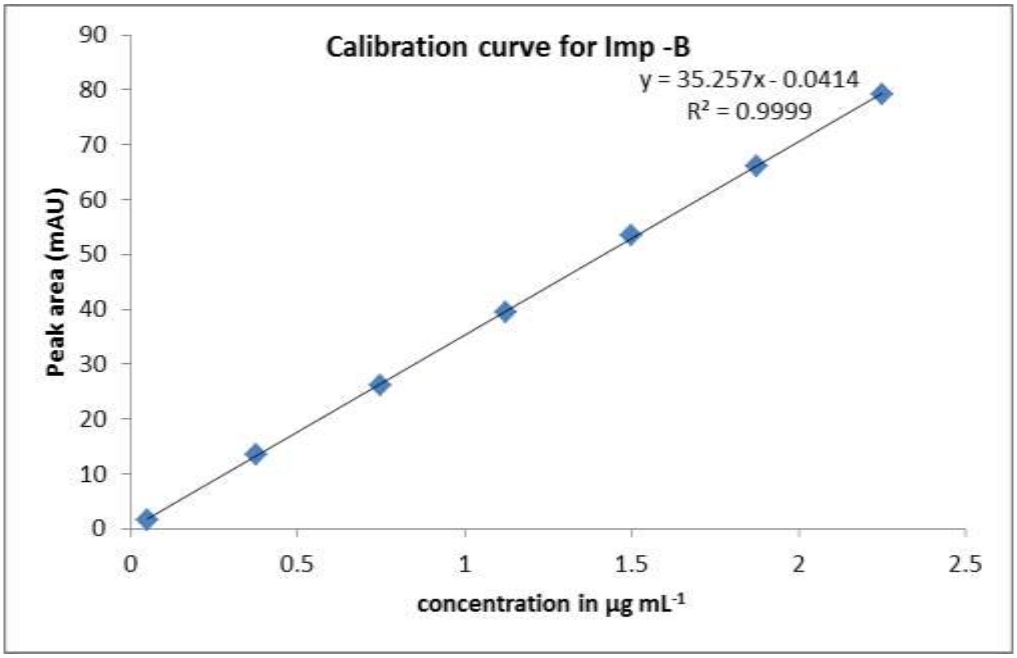


Table: 2.5.T9. Linearity of Imp-C

S.No.	Conc. (µg mL^{-1})	Imp-C peak area	Calculated response through trend line	Residual	Residual square	Sensitivity
1	0.1	3.41	3.28	-0.13	0.02	34.1
2	0.375	13.35	13.20	-0.15	0.02	35.6
3	0.75	26.35	26.73	0.38	0.15	35.1
4	1.125	40.15	40.26	0.11	0.01	35.7
5	1.5	53.78	53.79	0.01	0.00	35.9
6	1.875	67.76	67.32	-0.44	0.19	36.1
7	2.25	80.65	80.85	0.20	0.04	35.8
Regression coefficient		0.99996	Residual sum of squares		0.43	
Slope		36.08				
Intercept		-0.33				
% y-Intercept		-0.61	Linearity equation		*y* = 36.1*x-0.33*	

Fig: 2.5.F3. Linearity plot for Imp-C

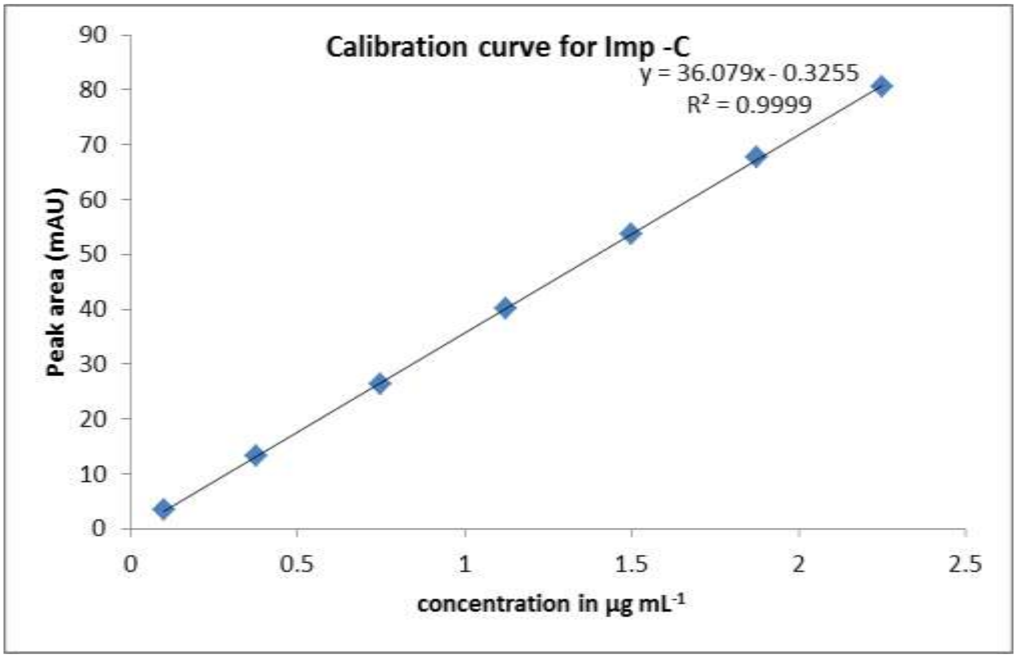


Table: 2.5.T10. Linearity of Imp-D

S.No.	Conc. (µg mL^{-}1)	Imp-D peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.1	5.04	4.63	-0.41	0.17	50.4
2	0.375	20.22	19.96	-0.26	0.07	53.9
3	0.75	40.15	40.87	0.72	0.52	53.5
4	1.125	61.76	61.77	0.01	0.00	54.9
5	1.5	82.32	82.68	0.36	0.13	54.9
6	1.875	103.5	103.58	-0.01	0.00	55.2
7	2.25	124.9	124.49	-0.41	0.17	55.5
Regression coefficient		0.99995	Residual sum of squares		1.05	
Slope		55.74				
Intercept		-0.94				
% y-Intercept		-1.14	Linearity equation		y = 55.74x -0.94	

Fig: 2.5.F4. Linearity plot for Imp-D

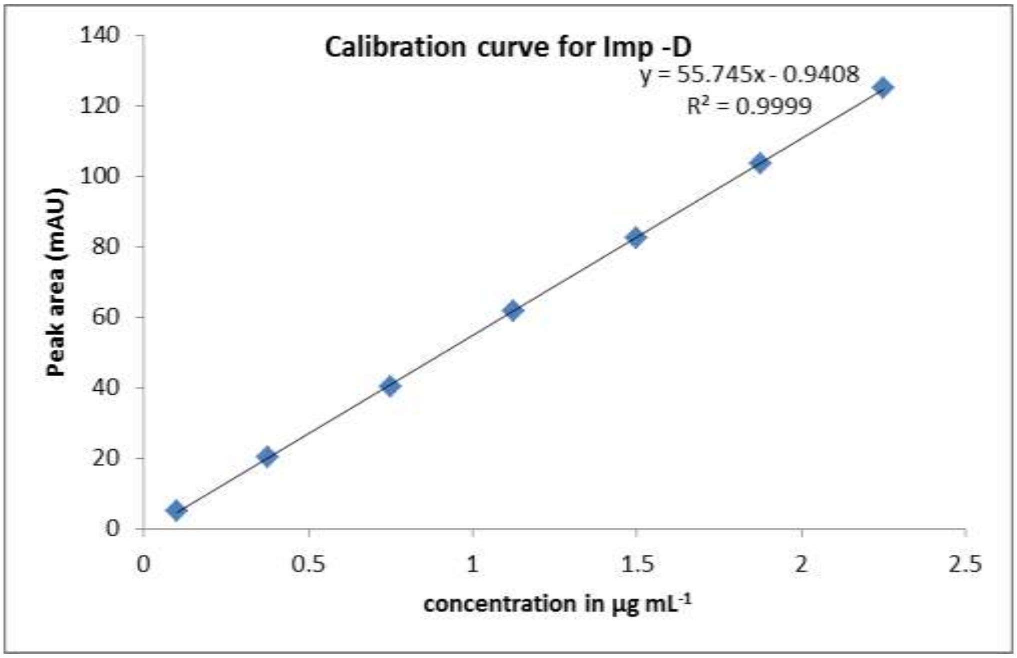


Table: 2.5.T11. Linearity of Imp-E

S.No.	Conc. (µg mL-1)	Imp-E peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.1	3.91	3.49	-0.42	0.18	39.1
2	0.375	15.83	15.54	-0.29	0.08	42.2
3	0.75	31.85	31.99	0.14	0.02	42.5
4	1.125	49.14	48.43	-0.71	0.50	43.7
5	1.5	64.74	64.87	0.13	0.02	43.2
6	1.875	76.14	81.32	5.18	26.81	40.6
7	2.25	101.7	97.76	-4.03	16.23	45.2
Regression coefficient		0.99695	Residual sum of squares		43.84	
Slope		43.85				
Intercept		-0.90				
% y-Intercept		-1.39	Linearity equation		y = 43.85*x* -0.90	

Fig: 2.5.F5. Linearity plot for Imp-E

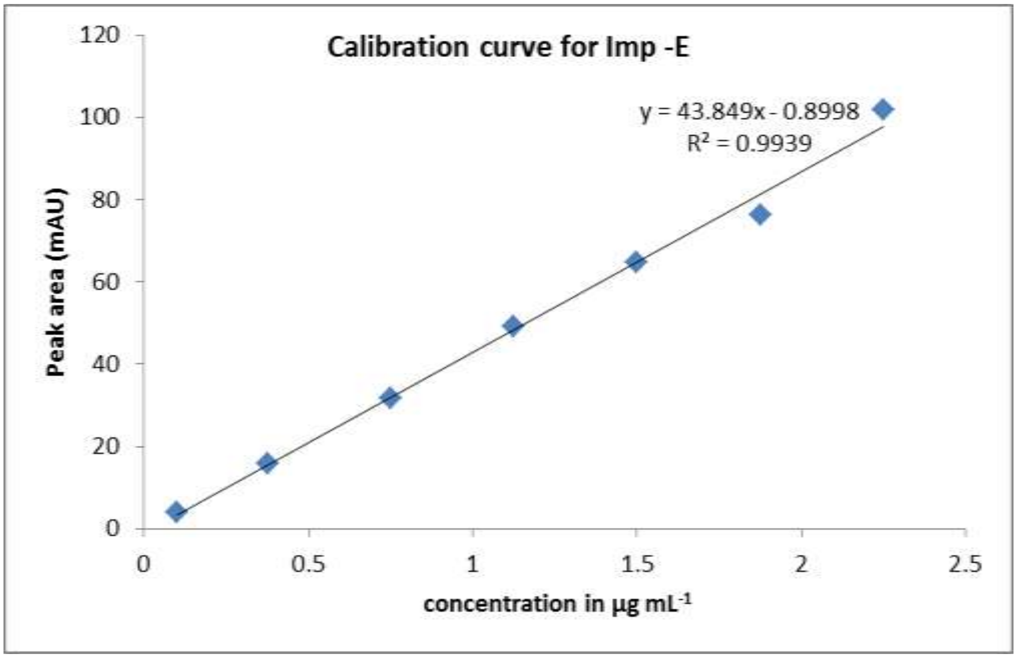


Table: 2.5.T12. Linearity of Imp-F

S.No.	Conc. (µg mL-1)	Imp-F peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.08	1.58	1.63	0.05	0.00	19.8
2	0.375	7.62	7.45	-0.17	0.03	20.3
3	0.75	14.66	14.85	0.19	0.04	19.5
4	1.125	22.2	22.26	0.06	0.00	19.7
5	1.5	30.04	29.66	-0.38	0.14	20.0
6	1.875	36.68	37.06	0.38	0.15	19.6
7	2.25	44.6	44.47	-0.13	0.02	19.8
Regression coefficient		0.99987	Residual sum of squares		0.38	
Slope		19.74				
Intercept		0.05				
% y-Intercept		0.16			y = 19.74x+0.05	

Fig: 2.5.F6. Linearity plot for Imp-F

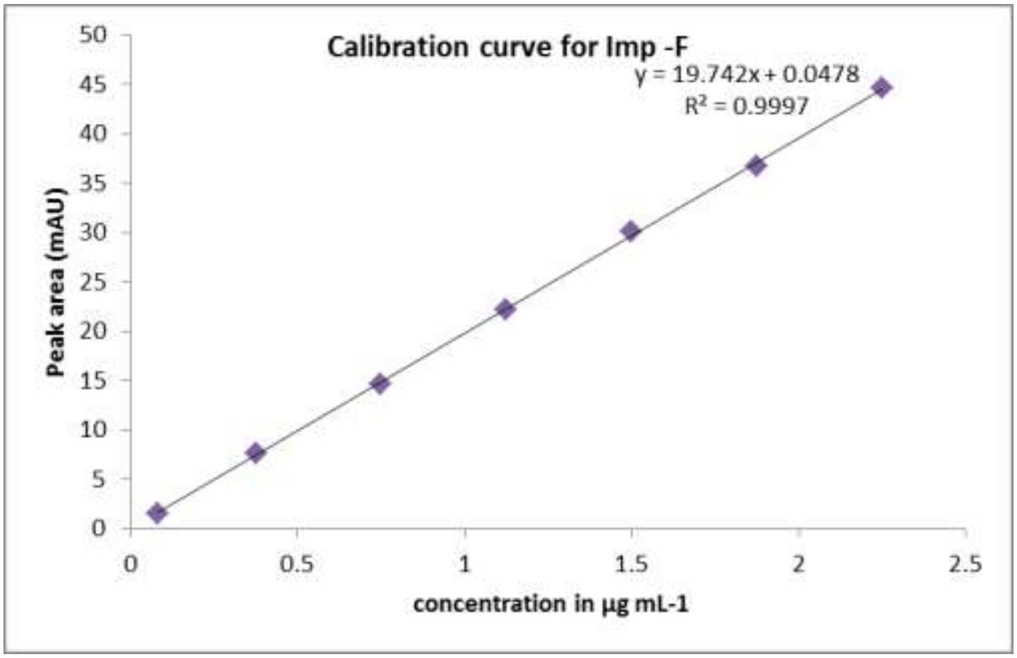


Table: 2.5.T13. Linearity of Imp-G

S.No.	Conc. (µg mL-1)	Imp-G peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.05	4.6	3.75	0.05	0.00	19.8
2	0.375	30.85	31.28	-0.17	0.03	20.3
3	0.75	62.94	63.05	0.19	0.04	19.5
4	1.125	94.59	94.82	0.06	0.00	19.7
5	1.5	126.56	126.58	-0.38	0.14	20.0
6	1.875	156.68	158.35	0.38	0.15	19.6
7	2.25	191.73	190.12	-0.13	0.02	19.8
Regression coefficient		0.99988	Residual sum of squares		6.36	
Slope		84.71				
Intercept		-0.49				
% y-Intercept		0.38			y = 84.71x-0.49	

Fig: 2.5.F7. Linearity plot for Imp-G

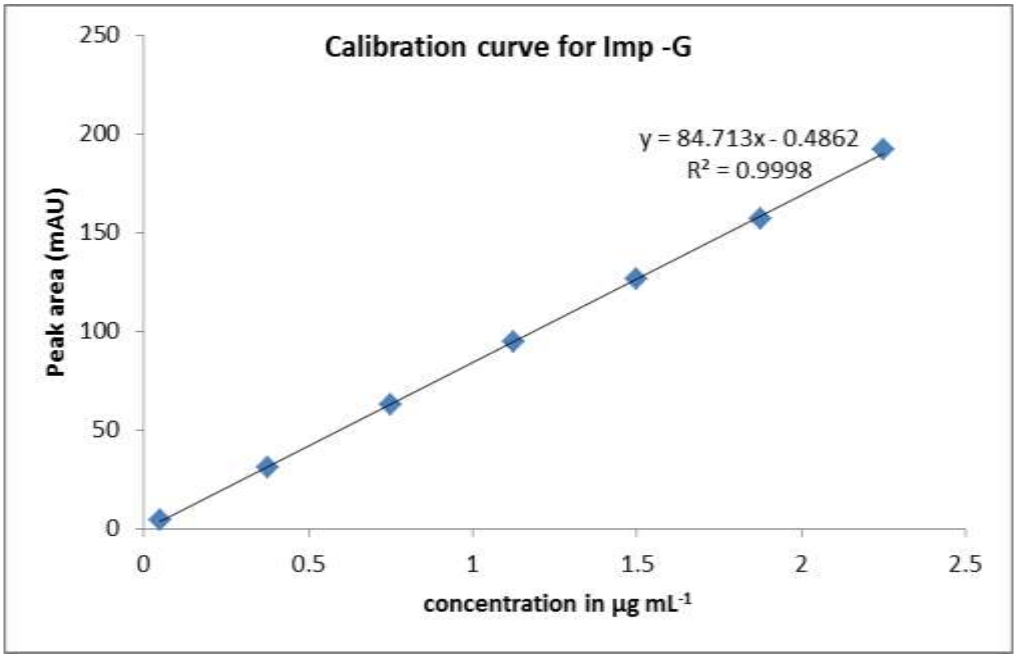
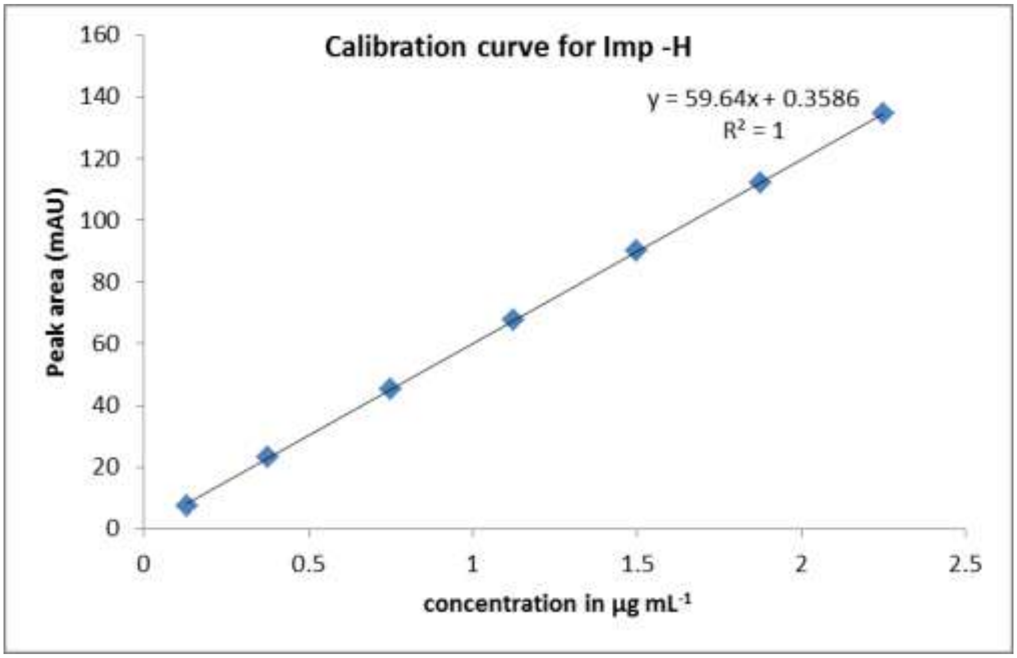


Table: 2.5.T14. Linearity of Imp-H

S.No.	Conc. (µg mL^{-1})	Imp-H peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.13	7.6	8.11	0.51	0.26	58.5
2	0.375	23.29	22.72	-0.57	0.32	62.1
3	0.75	45.05	45.09	0.04	0.00	60.1
4	1.125	67.49	67.45	-0.04	0.00	60.0
5	1.5	89.94	89.82	-0.12	0.01	60.0
6	1.875	112.05	112.18	0.13	0.02	59.8
7	2.25	134.51	134.55	0.04	0.00	59.8
Regression coefficient		0.99998	Residual sum of squares		0.62	
Slope		59.64				
Intercept		0.36				
% y-Intercept		0.40			y = 59.64x+0.36	

Fig: 2.5.F8. Linearity plot for Imp-H



Regression coefficients for concentration against peak area of all the related compounds of IRB from LOQ to 150 % level to the specification limit were more than 0.99. And also % y-bias with respect to 100 % specification of known compounds is within the limit of ± 2.0 indicates that developed related substances by LC method for IRB is linear.

2.5.5. Accuracy

Solutions of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H at three different concentration levels i.e. 50 %, 100 % and 150 % w.r.t. the specification limit (0.15%) of working concentration was spiked with IRB sample solution (conc. 1000 µg mL^{-1}), each level was prepared in triplicate and each of nine solutions were injected once.

Amount of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H obtained in each solution was calculated as % recovery. Accuracy results at three levels were summarized in Table 2.5.T15. % Recoveries obtained for three different levels were ranges from 96.2 to 108.0. Standard deviation for the average of % recoveries of each individual analyte is less than 3.0. Above accuracy results reveal that the method is highly accurate.

2.5.6. Range

As evident from Linearity, accuracy and precision study of related substances method, range has been established for all the analytes i.e. IRB, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F and Imp-H from LOQ to 150 % of specification limit.

2.5.7. Stability of analyte in solution form

Analyzed freshly prepared spiked solution of IRB with all the specified analytes, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H. Spiked solution was stored at room temperature and injected into liquid chromatography on day1 and day-2. Results are presented in Table 2.5.T16. The above data reveals that spiked solution of IRB with all the related components was stable upto 48 h study period at room temperature. No significant variation observed in % impurity content after 2 days.

Table: 2.5.T15. Accuracy results

Name of the analyte	Concentration of analyte w.r.t. specification limit	Amount of impurity spiked to IRB* (µg mL-1)	Amount of impurity recovered (µg mL-1)	%Mean recovery ± SD
Imp-A	50%	0.7515	0.8119	108.0±0.7
	100%	1.5030	1.4902	99.2±1.0
	150%	2.2545	2.1685	96.2±0.2
Imp-B	50%	0.7508	0.7355	98.0±0.6
	100%	1.5015	1.4965	99.7±0.3
	150%	2.2523	2.2458	99.7±0.2
Imp-C	50%	0.7500	0.7535	100.5±0.6
	100%	1.5000	1.4840	98.9±1.8
	150%	2.2500	2.2515	100.1±0.8
Imp-D	50%	0.7523	0.7546	100.3±0.6
	100%	1.5045	1.5072	100.2±0.2
	150%	2.2568	2.2697	100.6±0.3
Imp-E	50%	0.7508	0.7945	105.8±0.8
	100%	1.5015	1.5105	100.6±3.0
	150%	2.2523	2.2343	99.2±0.3
Imp-F	50%	0.7515	0.7534	100.3±0.5
	100%	1.5030	1.5108	100.5±0.1
	150%	2.2545	2.2622	100.3±0.3
Imp-G	50%	0.7515	0.7480	99.5±0.6
	100%	1.5030	1.5105	100.5±0.9
	150%	2.2545	2.2889	101.5±0.3
Imp-H	50%	0.7508	0.7131	95.0±0.9
	100%	1.5015	1.4819	98.7±0.5
	150%	2.2523	2.2163	98.4±0.3

Table: 2.5.T16. Solution stability data of related substances

Study interval	% of Related substances							
	Imp-A	**Imp-B**	**Imp-C**	**Imp-D**	**Imp-E**	**Imp-F**	**Imp-G**	**Imp-H**
Initial	0.22	0.14	0.17	0.22	0.18	0.08	0.34	0.23
Day-1	0.21	0.14	0.16	0.22	0.18	0.08	0.34	0.23
% Variation	4.5	0.0	5.9	0.0	0.0	0.0	0.0	0.0
Day-2	0.22	0.14	0.16	0.22	0.18	0.08	0.34	0.23
% Variation	0.0	0.0	5.9	0.0	0.0	0.0	0.0	0.0

2.5.8 Robustness

To evaluate the influence of minute changes in finalized method parameters on separation of known components, robustness study was performed. Study was conducted by deliberately altering the method conditions from the original method parameters and verified RRTs of impurities and system suitability parameters of standard solution.

Method parameters selected for the study were, flow rate (±0.2 mL/min), and column temperature (±5°C). Robustness study data of IRB related substances method is provided in Table 2.5.T17.

Above results of IRB RS method robustness study reveal that no significant variation was found in the SST results and RRTs of IRB related substances. Hence, the developed method has been considered as robust.

Table: 2.5.T17. Robustness study data

Parameter and Variation	RRTs of impurities							
	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	Imp-F	Imp-G	Imp-H
As such conditions	0.73	1.21	1.26	1.35	1.77	1.84	2.30	2.37
Flow rate (mL min^{-1})								
a. 0.8	0.74	1.18	1.50	1.55	1.69	1.91	2.36	2.42
b. 1.2	0.68	1.22	1.47	1.73	1.85	2.14	2.66	2.74
Column Temperature (°C)								
a. 25	0.72	1.20	1.39	1.44	1.79	1.91	2.33	2.41
b. 35	0.70	1.20	1.38	1.35	1.72	1.86	2.37	2.43

2.5.9. Conclusion from analytical method validations

The developed RP-LC method developed for quantitative determination of related substances of IRB in drug substance is precise, accurate, selective and linear as per the ICH recommended guidelines.

The Robustness and ruggedness or intermediate precision study reveals that the method is highly rugged and robust for its intended use.

2.6. Conclusion

The proposed RP-HPLC method is sensitive, linear, precise and accurate for quantitation of related substances of IRB and its degradation product. As the method was fully validated as per ICH and proved the stability indicating power, can be used for estimation of impurities of IRB for routine analysis, stability testing in Pharmaceutical quality control labs.

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CHAPTER – 3

Quality by Design approach for the separation of Clopidogrel bisulfate and its related subtances by Ultra Performance Liquid Chromatography

3.1 Introduction to Clopidogrel bisulphate and details of available analytical literature

Clopidogrel bisulfate (CLP) is a thienopyridine class inhibitor of P2Y12 ADP platelet receptors. Chemically it is methyl (+)-(S)-α-(2-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4H)acetate sulfate (1:1) (Fig.3.1.F1). The molecular formula is $C_{16}H_{16}ClNO_2S \bullet H_2SO_4$ and molecular weight is 419.9 g/mol [1-2]. Complete drug information of Clopidogrel (CLP) is given in Table 3.1.T1.

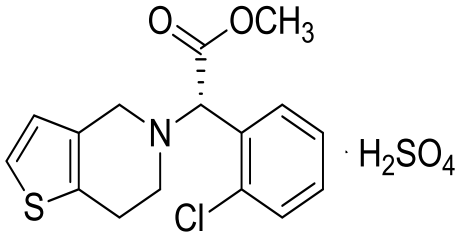


Fig: 3.1.F1. Chemical structure of CLP

Table: 3.1.T1. CLP Drug information

Chemical name	:	(+)-(S)-α-(2-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4H)acetate sulfate (1:1)
Molecular weight	:	419.9
Molecular formulae	:	$C_{16}H_{16}ClNO_2S \bullet H_2SO_4$
Brand name	:	Plavix
Dosage strengths	:	75 and 300mg
Dosage form	:	Tablet
Maximum daily dosage	:	300 mg

Analytical literature survey revealed that CLP is listed in both USP [3] and EP [4]. As per the literature this method is not capable of separation and quantification of impurities generated during stability studies [5] and covers only two impurities. The method reported in European pharmacopeia employs ion pair reagent with a runtime of 68 minutes. There were many reported analytical methods for the determination of CLP by using HPTLC in bulk drugs, formulation dosage forms and combined dosage forms of CLP and aspirin [6-7], by TLC method for the determination of CLP and its impurity SR26334 in pharmaceutical products[8], by Capillary electrophoresis for the quantification of CLP with and three impurities, [9], for determination of (+)-S Clopidogrel carboxylic acid metabolite in human plasma and urine designed for biopharmaceutic studies[10], Normal phase LC method for the quantification of CLP and five of its impurities[11], by RP-HPLC method for the determination of CLP in pharmaceutical preparations[12], for the High –Throughput analysis of CLP in pharmaceutical formulations using a monolithic silica column[13], for the determination of CLP in coated tablets[14], for the assay of bulk CLP and for some tablet forms[15], stability indicating method for the determination of CLP in tablets and its application to content uniformity testing[16], By RP-HPLC for the quantification of CLP in combined dosage forms with aspirin[17-20],for the purity control of CLP and acetyl salicyclic acid in combined dosage form[21], for the determination of aspirin and CLP in combined tablets in the presence of degradation products formed under ICH recommended stress conditions. [22], ion pairing method for the simultaneous determination of aspirin and CLP in tablet and capsule dosage forms [23], for the simultaneous determination of aspirin, atorvastatin calcium and CLP in capsules[24] in Chemometric method for the estimation and aspirin and CLP [25] HPLC and HPTLC method for the simultaneous determination of acetyl salysilic acid and CLP [26] Characterization of degradation product in amorphous and polymorphic forms under solid state stress conditions described [27], Spectrophotometric and spectrodensitometric determination of CLP with kinetic study of its alkaline degradation described [28], for simultaneous estimation of atorvastatin, CLP and aspirin in capsule dosage forms using UV-Spectroscopy[29], Identification and characterization of oxidation impurity in drug substance and drug product [30], HPLC-UV methods for the determination of CLP in pharmaceutical dosage forms and human plasma[31-32], HPLC method for the determination of carboxylic acid metabolite of CLP in human plasma and its application to a pharmacokinetic study[33] and in human serum and its application to bioequivalence study[34], HPLC-MS/MS methods for the determination of CLP and its metabolite in human plasma [35-41], Quantitative analysis of CLP and Aspirin by first derivative Spectrophotometric method in Tablets[42], determination of CLP by differential pulse differential pulse voltammetry in bulk form and pharmaceutical preparations with a glassy carbon[43].

In the present study, in addition to reported related impurities of CLP and degradation product, total twelve possible impurities were identified by considering CLP route of synthesis (Fig.3.1.F2) and from forced degradation pathways. Chemical structures of possible impurities are provided in Table 3.1.T2.

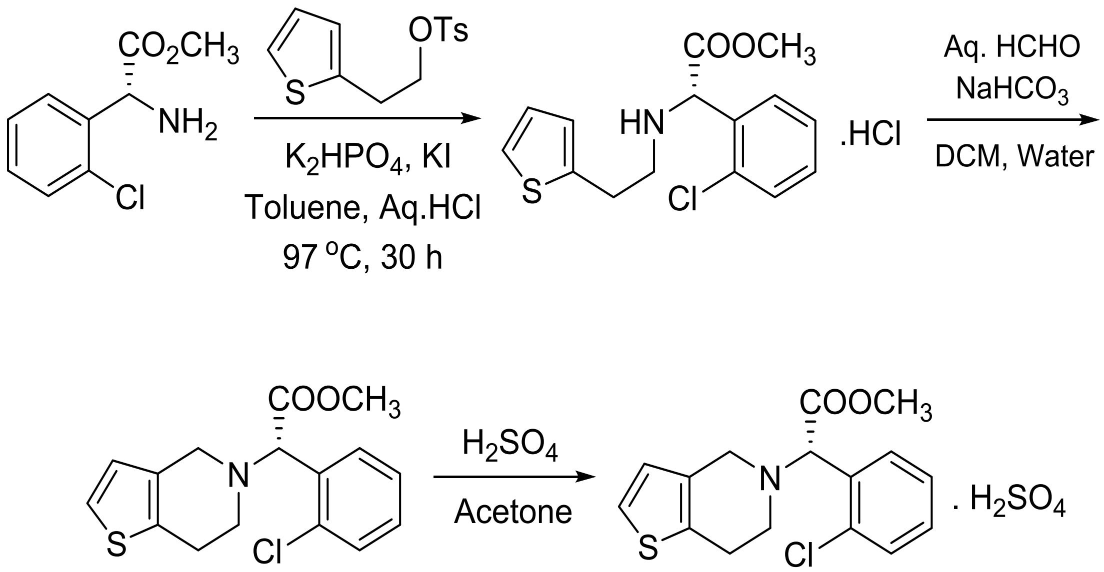
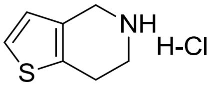
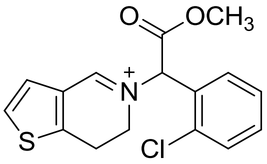
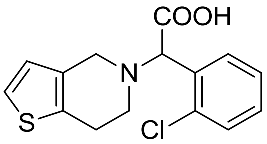
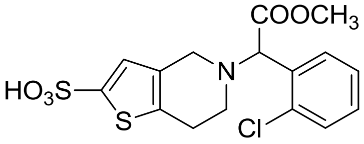
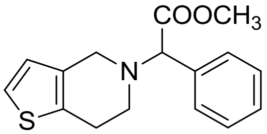
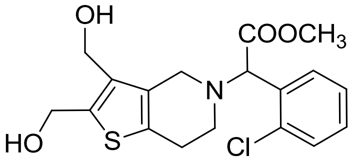
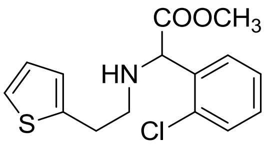
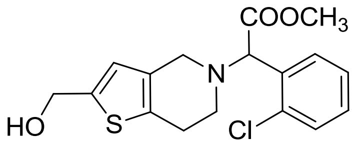
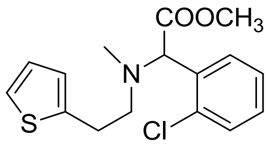
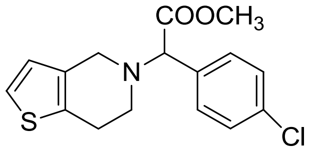
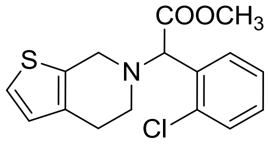
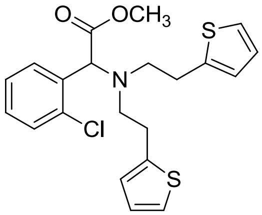


Fig: 3.1.F2. Synthetic scheme of CLP

Table: 3.1.T2. Details of related substances of CLP drug substance

Chemical structure of impurity	**Chemical name**
Imp-1 	4,5,6,7-tetrahydrothieno[3,2-c]pyridine hydrochloride
Imp-2 	5-(1-(2-chlorophenyl)-2-methoxy-2-oxoethyl)-6,7-dihydrothieno[3,2-c]pyridin-5-ium
Imp-3 	2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)acetic acid
Imp-4 	5-(1-(2-chlorophenyl)-2-methoxy-2-oxoethyl)-4,5,6,7-tetrahydrothieno[3,2-c]pyridine-2-sulfonic acid
Imp-5 	methyl 2-(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)-2-phenylacetate
Imp-6 	methyl 2-(2,3-bis(hydroxymethyl)-6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)-2-(2-chlorophenyl)acetate
Imp-7 	methyl 2-(2-chlorophenyl)-2-((2-(thiophen-2-yl)ethyl)amino)acetate

Chemical structure of impurity	Chemical name
Imp-8 	methyl 2-(2-chlorophenyl)-2-(2-(hydroxymethyl)-6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)acetate
Imp-9 	methyl 2-(2-chlorophenyl)-2-(methyl(2-(thiophen-2-yl)ethyl)amino)acetate
Imp-10 	methyl 2-(4-chlorophenyl)-2-(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)acetate
Imp-11 	methyl 2-(2-chlorophenyl)-2-(4,5-dihydrothieno[2,3-c]pyridin-6(7H)-yl)acetate
Imp-12 	methyl 2-(bis(2-(thiophen-2-yl)ethyl)amino)-2-(2-chlorophenyl)acetate

To establish retest periods, storage conditions and shelf life of CLP drug substance, stability studies to be performed. As per guidelines of United State Pharmacopoeia (USP) and International Conference on Harmonization (ICH) to perform stability testing, stability indicating analytical method is required [44, 45]. This work presents development of stability indicating UPLC method for quantitation of CLP and its twelve related compounds in CLP drug substance within shorter runt time of 18 minutes. The developed analytical method was validated as guided by ICH [46, 47].

3.2. Experimental

3.2.1. Materials

Materials or chemicals used for this study were listed in below Table 3.2.T1.

Table: 3.2.T1. Details materials and chemicals

S.No.	Name of the compound	Grade	Procured from../synthesized by..
1.	CLP drug substance samples	--	Dr.Reddy's Laboratories, INDIA
2.	Acetonitrile	HPLC grade	Merck, India
3.	Methanol	HPLC grade	Merck, India
4.	Ortho phosphoric acid	AR grade	Merck, India
5.	Water	High pure	Purified by Milli-Q plus system, USA
6.	Imp-1 to Imp-12	--	Dr.Reddy's Laboratories, INDIA

3.2.2 Equipments

LC analysis was carried out on a Waters Acquity UPLC with a 2998 photodiode array detector. The output signal was monitored and processed using Empower Software. All experimental design work was carried out using 'Design Expert 8.0.6' software by Stat-Ease (Minneapolis, USA). The chromatographic column used was Waters Acquity UPLC BEH SHIELD RP_{18} 100 mm, 2.1 mm, and 1.7μm particle size.

3.2.3. Preparation of solutions

3.2.3.1. Preparation of impurity standard solution

Impurity stock solution was prepared by dissolving appropriate amount of all the known impurities i.e. Imp-1 to Imp-12 in diluent (acetonitrile and buffer-A in the ratio of 2:8) to get the final concentration of each impurity in stock solution as 15 μg mL^{-1}. Specification limit for all the known impurities was considered as 0.15 % and prepared impurity blend solution of 0.15 % by spiking appropriate volume of impurity stock solution in μL to 150 μg mL^{-1} CLP drug substance test solution.

3.2.3.2. Preparation of CLP test solution

CLP drug substance stock solution was prepared by weighing 15 mg of drug substance in 100 mL volumetric flask dissolved and diluted to volume with diluent. Final concentration of the solution was 150 μg mL^{-1} of CLP drug substance, which was used for related substances estimation. A solution containing 0.15 μg mL^{-1} was prepared from this stock solution for quantification of related substances.

3.2.4 Preparation of Forced degradation samples

As per ICH guidelines to generate degradation samples, one lot of CLP drug substance was selected and subjected to different stress conditions like acid hydrolysis, base hydrolysis, water hydrolysis, oxidation, photo degradation and Thermal degradation.

3.2.4.1. Preparation of Photo degradation sample

For photo degradation study, CLP drug substance was taken in Petri dish and kept in UV cabinet. The sample was exposed to 254 nm and 365nm. After 10 days, sample was taken-off from UV cabinet and prepared test solution of 150 µg mL^{-1} for related substances analysis.

3.2.4.2. Preparation of Thermal degradation sample

CLP drug substance was placed in Petri dish and spread uniformly. Kept the Petri dish in oven and maintained the temperature of oven at 90°C for 10 days. After 10 days, CLP samples was taken-off from oven and prepared the test solution to get the final concentration as 150 µg mL^{-1} for related substances by HPLC analysis.

3.2.4.3. Preparation of Oxidative degradation sample

15 mg of CLP drug substance was transferred to 100 mL volumetric flask, dissolved in 25 mL of diluent and made upto mark with 3 % peroxide solution. Placed magnetic stirrer in the solution and kept solution at room temperature under continuous stirring for 24 h. The resultant solution used for related substances testing.

3.2.4.4. Preparation of Water hydrolysis sample

15 mg of CLP drug substance was transferred to 100 mL volumetric flask, dissolved in 50 mL of diluent and made upto mark with water. Placed magnetic stirrer in the solution and kept solution at 60 °C under continuous stirring for 24 h. The resultant solution used for related substances testing.

3.2.4.5. Preparation of Acid hydrolysis sample

15 mg of CLP drug substance was transferred to 100 mL volumetric flask, dissolved in 25 mL of diluent and made upto mark with 0.1N Hydrochloric acid. Placed magnetic stirrer in the solution and kept solution at 60 °C under continuous stirring for 24 h. Considered this solution for related substances testing.

3.2.4.6. Preparation of Base hydrolysis sample

15 mg of CLP drug substance was transferred to 100 mL volumetric flask, dissolved in 25 mL of diluent, and made up to mark 0.01N Sodium hydroxide solution. Placed magnetic stirrer in the solution and kept solution at room temperature under continuous stirring for 1 h. The resultant solution used for related substances testing.

3.3. Sequential steps in method development and optimization

During initial development, impurity blend solution containing potential impurities i.e., Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and Imp-12 at specification level in CLP drug substance was used. And to confirm stability indicating power of the method, forced degradation samples were also considered for development. Analytical method was developed by following Quality by Design approach.

3.3.1. Understanding the Source of impurities:

The crude samples of CLP were subjected to LC-MS study and based on the molecular mass number (m/z) values of the unknown peaks, based on the process knowledge, tentative structures were proposed. The impurities were synthesized and injected into the HPLC system to confirm retention time of these impurities. Impurity 1 to Impurity 3 were degradation products, impurity 4 was due to the sulfonation of the thiophene ring, impurity 5 was the analogue due to the presence of its precursor in the starting material, impurity 6 was a by product due to the di hydroxy methylation , impurity 7 was the n-2 stage of CLP, impurity 8 was due to the hydroxy methylation, impurity 9 was due to the N-methylation of n-1 stage, impurity 10 was the positional isomer of CLP due to the presence of its corresponding precursor in the starting material, impurity 11 was the regio isomer of CLP and impurity 12 was the byproduct due to the addition of one more molecule of thiophene 2-ethanol to the stage 1.

The objective of the present work is to separate all the above process related impurities of CLP with a resolution greater than 1.5 using the quality by design approach. Quality by design approach involves the following steps. The first step is to identify the target measurement; in the present study it was to separate the 12 process related impurities with a resolution greater than 1.5 and with a run time less than 20 minutes.

The second step in qbd approach was to identify the technique. As the objective was to separate the impurities with shorter run time, a UPLC was used for the trials.

3.3.2 Simultaneous optimization of Column and buffer pH

The selection of column was crucial as the target impurities selected for the separation were a blend of highly polar and non polar moieties. To find out the best stationary phase for this set of mixture, column screening was performed (Table.3.3.T1)

Table: 3.3.T1. Detais of column screening

S. No	**Column Name**	**Observation**
1	BEH C 8 100 * 2.1, 1.7 um	Imp 3, 4; Imp 9,10; CLP and Imp 11 co-elution
2	BEH C18 100*2.1, 1.7um	Co-elution of Imp 5 and 6
3	BEH Phenyl 100*2.1, 1.7um	Co-elution of Imp 5,6 ; CLP and Regio isomer
4	BEH HSS T3 100*2.1, 1.8um	Co-elution of Imp-3 and 4
5	BEH Shield RP 18 100*2.1, 1.7um	Baseline separation of all impurities with Resolution > 1.5

From the results, the BEH Shield column chemistry was found to be giving baseline resolution of all the impurities due to the presence of hydrophilic carbamate group with in a C18 chain [48] which facilitates the retention of polar compounds and reduces the retention of highly hydrophobic impurities (Table.3.3.T1).

The effect of pH on the retention of impurities was performed at pH 2.0, 3.0 and 6.0. The pH range of 3.0 to 6.0 was not considered due to the fact that the pKa of clopidogrel bisulphate was ~4.5. The retention of clopidogrel increased with the increase of pH. At pH 2.0 the resolution between imp 4, 5, 6 was less than 1.0 and resolution between imp. 9 and 10 was also less than 1.0.

Table: 3.3.T2. Detais of pH screening

S. No	**pH of the mobile phase A**	**Observation**
1	pH 2.0	Co-elution of imp 4,5 and 6; Res. greater than 3.0 for CLP and imp 11
2	pH 3.0	Co-elution of CLP and impurity 11
3	pH 6.0	Co-elution of CLP and impurity 11; Change of elution pattern imp 10 after the CLP peak.

The resolution between CLP and imp 11 was more than 6.0. Increase in pH leading to the increase in resolution between the impurities 1 to 6 where as impurity 10 and 11 were co-eluting with CLP. Efforts were made to find the optimum pH where all the peaks can be resolved with a resolution greater than 2.0. pH was screened from 2.0 to 3.0 with an increments of 0.2 units but no pH has served the purpose. Then it was decided to go for a pH gradient. The pH of buffer in solvent A was maintained at 2.5 and pH of the buffer in solvent B was maintained at 2.0 (Table.3.3.T2).

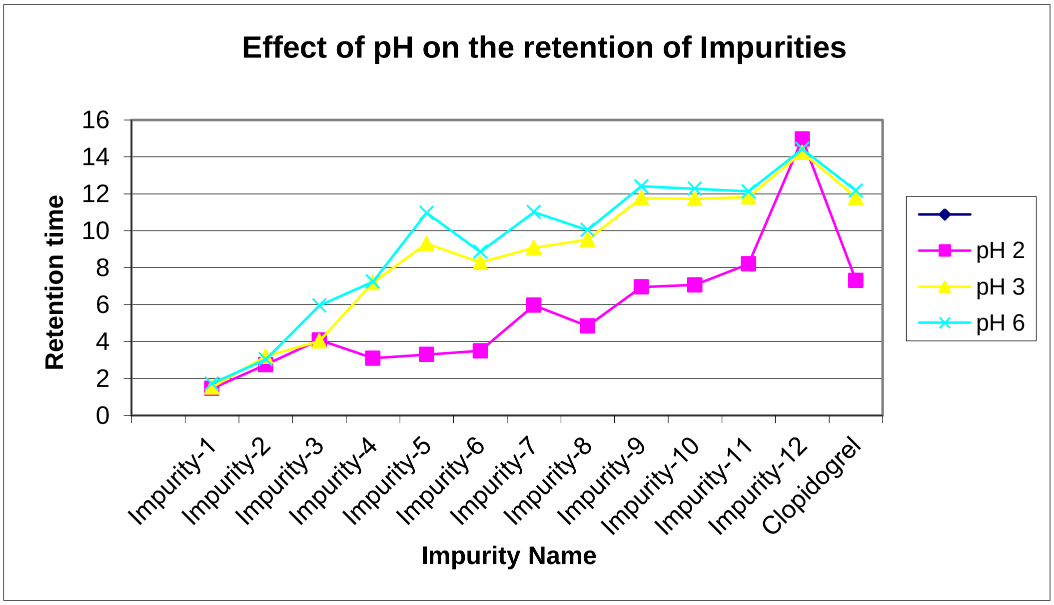


Fig: 3.3.F1. Effect of pH on retention of impurities

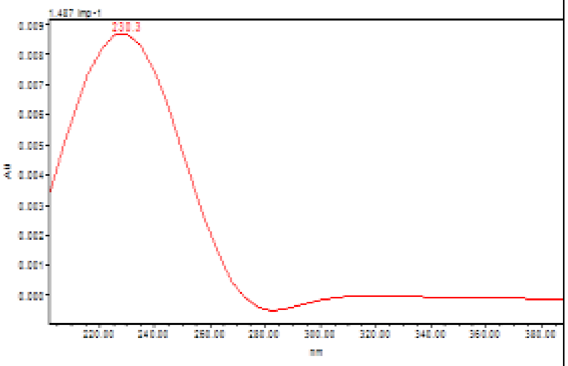
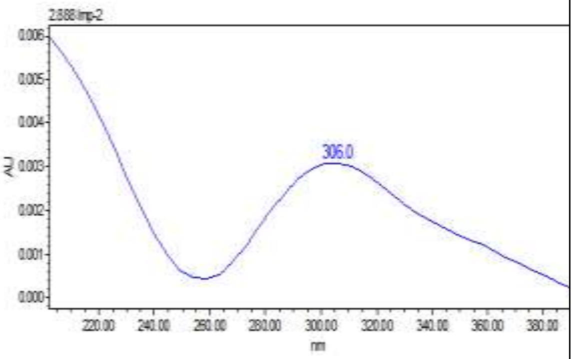
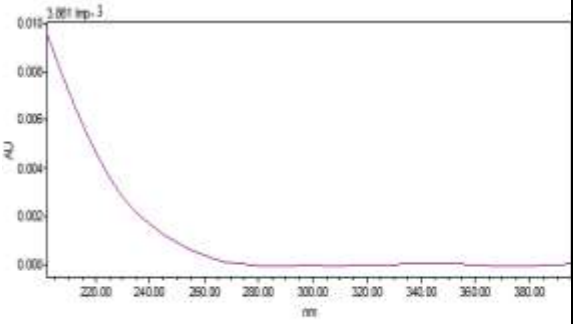
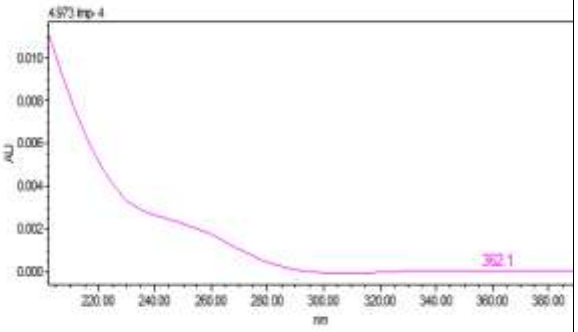
The final optimized gradient programme was (T/%B) 0.01/5, 2.5/5, 6.0/40, 11.0/90, 11.50/100,17.0/100,17.01/5.0 and 18.0/5.

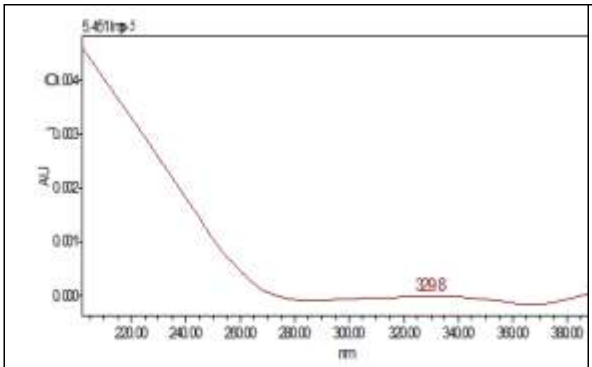
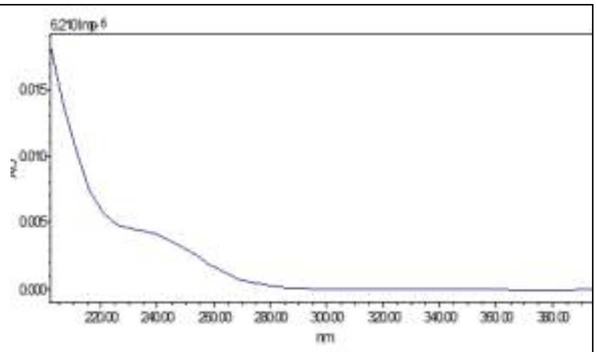
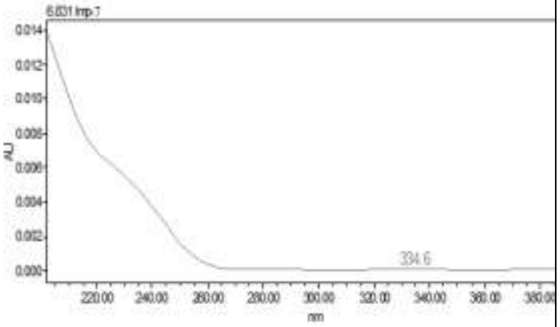
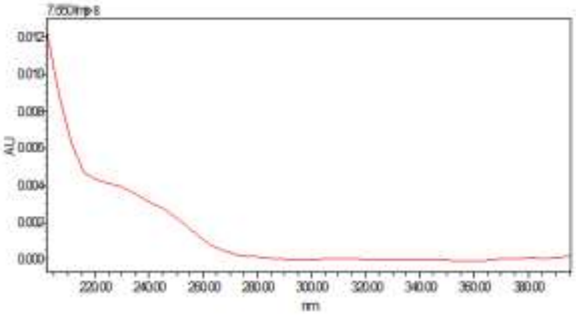
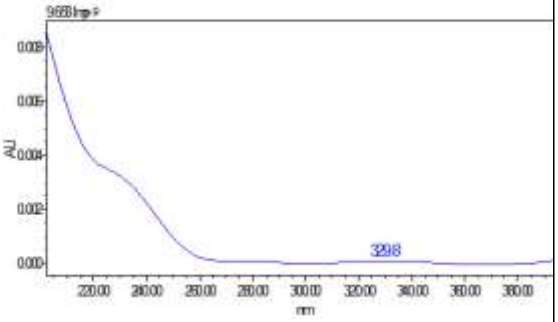
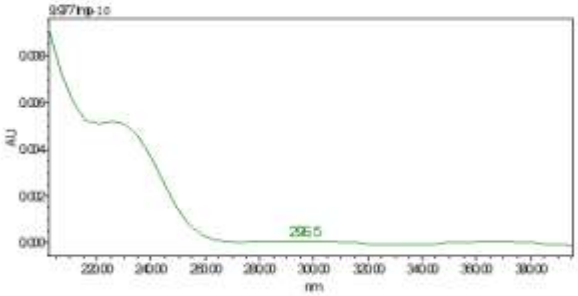
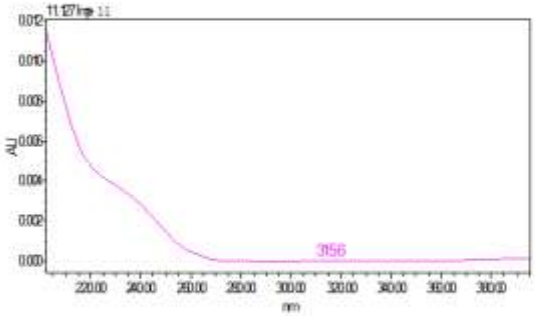
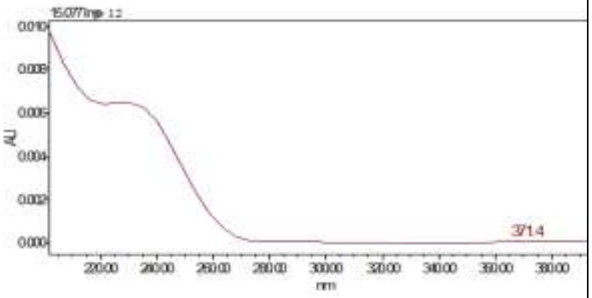
3.3.3. Selection of Diluent

Based on solubility of CLP and all the known impurities, Acetonitrile and mobilephase-A in the ratio of 3:7 was selected as diluent.

3.3.4. Selection of Wavelength

10 ppm solution of each known impurity and CLP Drug substance were prepared in Acetonitrile diluent. By using UV Visible spectrophotometer, the solution was scanned in the UV range of 200-400 nm against Acetonitrile as blank. UV spectra of all the related components and CLP were given as Fig. 3.3.F2. CLP is showing wavelength maxima at 220 nm. Most of the related compounds of CLP have wavelength maximum at around 220 nm; hence it has been selected as UV detector wavelength for LC method development.

UV spectra of Imp-1	UV spectra of Imp-2
	
UV spectra of Imp-3	UV spectra of Imp-4
	
UV spectra of Imp-5	UV spectra of Imp-6

	
UV spectra of Imp-7	UV spectra of Imp-8
	
UV spectra of Imp-9	UV spectra of Imp-10
	
UV spectra of Imp-11	UV spectra of Imp-12
	

UV spectra of CLP

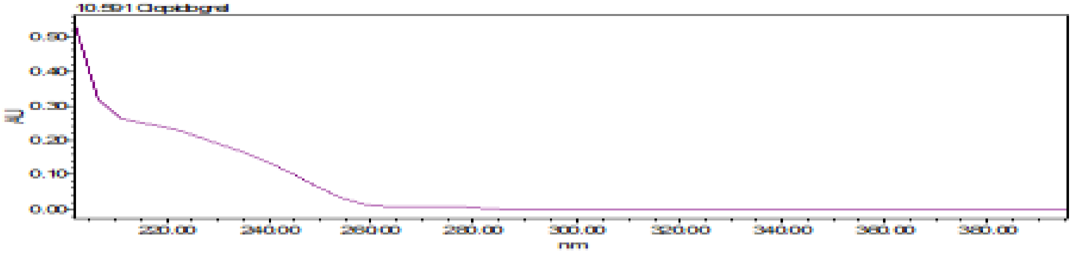


Fig: 3.3.F2. Typical UV spectra of CLP and its related impurities

3.3.5. Design of Experiments

3.3.5.1. Identification of critical analytical method factors:

To find out the significant few factors among the selected factors for the study, a fractional factorial design was selected, which will study the main effects and their two level interactions (Resolution V method) in sixteen runs. Factors and their "low" (−) and "high" (+) levels are presented in the 3.3.T3.

Table: 3.3.T3. Factors and levels

	Levels	
Factors	**-1**	**+1**
Factor-1 (Flow rate)	0.16mL/min	0.2mL/min
Factor-2 (pH of Buffer in solvent A)	2.0	3.0
Factor-2 (Temperature)	27°C	35°C
Factor-4 (Initial gradient time t_G in minutes)	2.5	4.5
Factor-5 (% of Methanol in solvent B)	80	120

Table: 3.3.T4. Design of Experiments and responses

Run No	Factor-1 (Flow rate)	Factor-2 (pH)	Factor-3 (Temperature)	Factor-4 (Initial gradient time)	Factor-5 (% of Methanol in Mobile phase-B)	R_s-1	R_s-2
1	-1	-1	-1	-1	+1	1.31	7.0
2	+1	-1	+1	+1	-1	1.17	7.0
3	+1	-1	-1	+1	+1	1.15	6.7
4	+1	-1	-1	-1	-1	1.14	5.9
5	+1	+1	+1	-1	-1	1.57	2.5
6	+1	+1	+1	+1	+1	1.60	2.5
7	+1	+1	-1	+1	-1	2.00	2.9
8	+1	-1	+1	-1	+1	1.23	6.2
9	-1	+1	+1	+1	-1	1.59	2.2
10	-1	-1	+1	-1	-1	1.34	5.9
11	-1	-1	+1	+1	+1	1.39	7.3
12	-1	+1	-1	+1	+1	1.93	2.5
13	-1	-1	-1	+1	-1	1.30	6.9
14	-1	+1	-1	-1	-1	2.18	2.8
15	+1	+1	-1	-1	+1	1.96	2.7
16	-1	+1	+1	-1	+1	1.56	2.3

3.3.5.2. DoE – Results & Discussion:

ANOVA table for response Rs-1 is represented in Table.3.3.T5. According to the ANOVA for response Rs-1, the model F-value was 80.42, implying that the model is significant. Values of "Prob > F" for the terms A, B, C and BC were 'less than 0.0500' indicating that these model terms are significant. The fraction of variation by means of coefficient of determination R2 was also evaluated. The "Pred R-Squared" of 0.9300 is in reasonable agreement with the "Adj R-Squared" of 0.9549.

Table: 3.3.T5. ANOVA Table for response Rs-1

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model *significant*	1.62	4	0.40	80.42	< 0.0001
A-Flow rate	0.039	1	0.039	7.76	0.0177
B-pH	1.19	1	1.19	237.34	< 0.0001
C-Temp	0.14	1	0.14	28.34	0.0002
BC	0.24	1	0.24	48.23	<0.0001
Residual	0.055	11	0.005		
Cor Total	1.67	15			
Std. Dev.	0.071			R-Squared	0.9669
Mean	1.53			Adj R-Squared	0.9549
C.V. %	4.64			Pred R-Squared	0.9300
PRESS	0.12			Adeq Precision	22.482

ANOVA table for response Rs-2 is represented in Table 3.3.T5. According to the ANOVA for response Rs-2, the model F-value was 204.85, implying that the model is significant. Values of "Prob > F" for the terms A, B, C, AC, C2 were ‘less than 0.0500’ indicated that these model terms are significant. The fraction of variation by means of coefficient of determination R2 was also evaluated. The "Pred R-Squared" of 0.9611 is in reasonable agreement with the "Adj R-Squared" of 0.9817.

Table: 3.3.T6. ANOVA Table for response Rs-2

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model *significant*	66.02	1	66.02	377.42	< 0.0001
B-pH	66.02	1	66.02	377.42	< 0.0001
Residual	2.45	14	0.17		
Cor Total	68.46	15			
Std. Dev.	0.42			R-Squared	0.9642
Mean	4.58			Adj R-Squared	0.9617
C.V. %	9.13			Pre R-Squared	0.9533
PRESS	0.056			Adeq Precision	27.475

From the above data it can be concluded that out of the 5 variables selected, pH has significant impact on the selected response. The method is further optimized by using the full factorial design. Factors and their “low” (−) and “high” (+) levels are presented in the 3.3.T7.

Table: 3.3.T7. Factors and levels

Factors	Levels		
	-1	**0**	**+1**
Factor-A (Flow rate)	0.16mL/minute	0.18mL/minute	0.20mL/minute
Factor-B (Temperature)	25°C	30°C	35°C
Factor-C (pH)	2.3	2.5	2.7

The design and the responses obtained against the experiments are presented in 3.3.T8. The information gathered from these experiments was used to evalutate the robustness of the method as well.

Table: 3.3.T8. Design of Experiments and responses

Run No	Factor-A (Flow rate)	Factor-B (Temperature)	Factor-C (pH)	R_s-1
1	1	1	-1	5.1
2	-1	1	1	3.6
3	0	0	0	4.9
4	0	0	0	4.7
5	-1	-1	1	3.5
6	0	0	0	4.5
7	-1	0	-1	5.0
8	1	0	-1	5.7
9	1	1	1	4.1
10	-1	1	-1	5.3
11	0	0	0	5.1
12	1	0	1	4.0

3.3.5.3. DoE – Results & Discussion:

ANOVA table for response Rs-1 is represented in Table 3.3.T8. According to the ANOVA for response Rs-1, the model F-value was 52.396, implying that the model is significant. Values of "Prob > F" for the terms C were 'less than 0.0500' indicating that these model terms are significant. The fraction of variation by means of coefficient of determination R2 was also evaluated. The "Pred R-Squared" of 0.7435 is in reasonable agreement with the "Adj R-Squared" of 0.8061. The curvature and lack fit values are insignificant (Table.3.3.T9).

Table: 3.3.T9. ANOVA Table for response Rs-1

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model *significant*	4.35	1	4.35	52.39	< 0.0001
C-pH	4.35	1	4.35	52.39	< 0.0001
Curvature Insignificant	0.18	1	0.18	2.21	0.1711
Residual	0.75	9	0.083		
Lack of Fit not *significant*	0.55	6	0.091	1.37	0.4297
Pure Error	0.20	3	0.067		
Cor Total	5.28	11			
Std. Dev.	0.31			R-Squared	0.8237
Mean	4.63			Adj R-Squared	0.8061
C.V. %	6.60			Pre R-Squared	0.7435
PRESS	1.36			Adeq Precision	11.84

The model is significant and significantly influenced by factor-C. The resolution between CLP and Imp-11 (Rs-1) was affected by factor-C (pH of mobile phase-A), when compared to other individual factors. Fig.3.3.F4 represents the responses that were plotted in the form of three-dimensional response surfaces in order to easily and more precisely define the chromatographic behavior of the investigated substances.

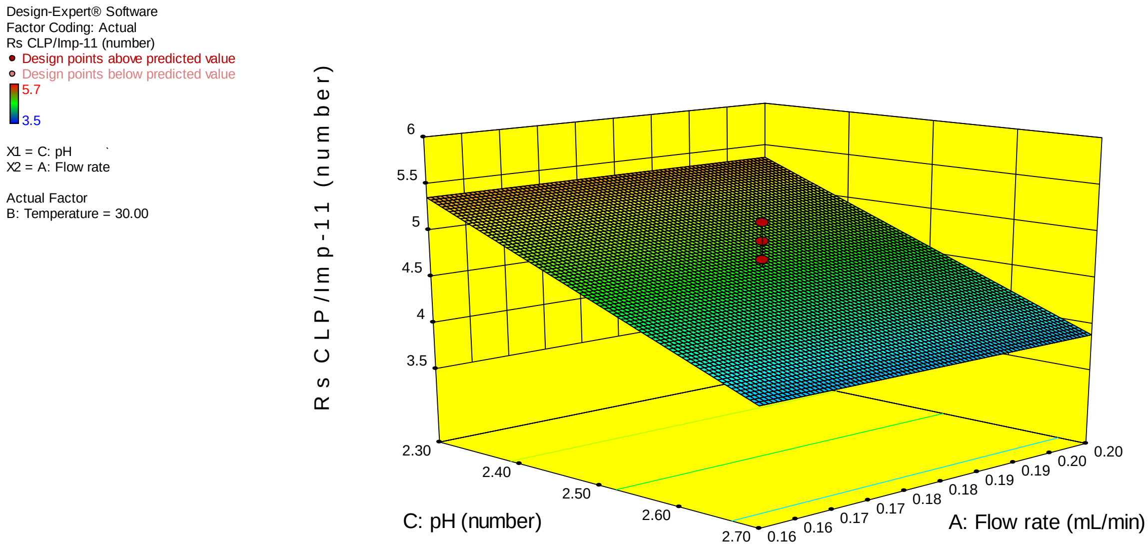


Fig: 3.3.F3. 3D plots of the response surface for the resolution

A) The variation of response (Rs-1;CLP/Imp-11) as a function of pH of mobile phase-A and Flow rate ; fixed factor is Column temperature =30°C

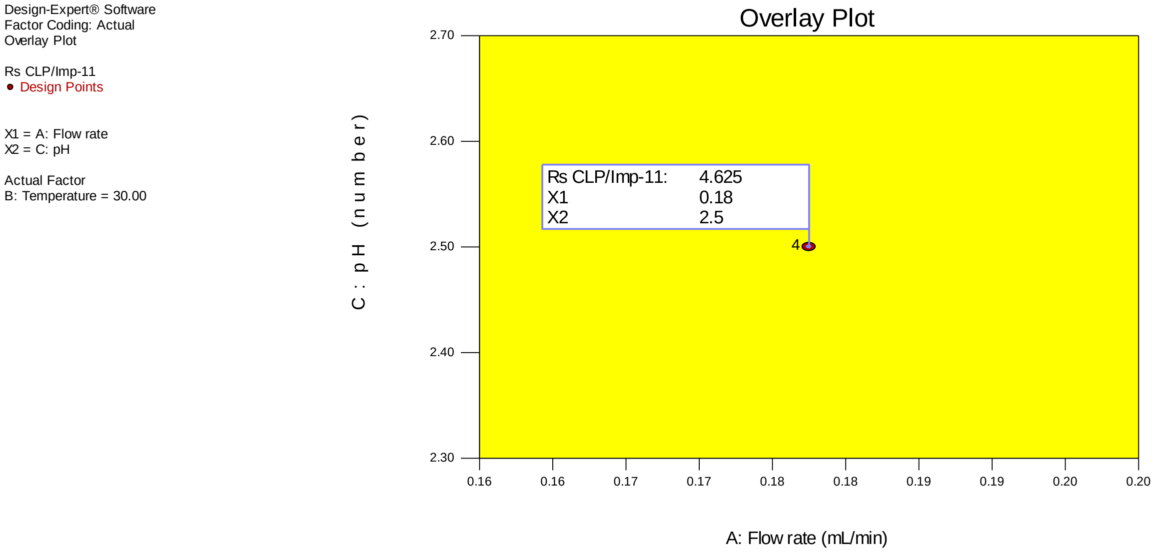


Fig: 3.3.F4. Overlay plot

A) Overlay plot for response (Rs-1;CLP/Imp-11) as a function of pH of mobile phase-A and Flow rate ; fixed factor is Column temperature =30°C (Fig.3.3.F4)

From the overplot it may be concluded that the method fulfils its intended purpose when operated within the range of the selected factors. Data analysis led to the conclusion that the final pH of the mobile phase-A should be maintained at 2.5. The temperature of the column should be maintained at 30°C and the flow rate should be maintained at 0.18mL/min.

3.3.6. Optimized chromatographic conditions

Optimized chromatographic conditions for related substances estimation of CLP and quantification of CLP in drug substance is given in Table 3.3.T10.

Table: 3.3.T10. Final chromatographic conditions of CLP method

Column	BEH Sheild C18 column, 100 mm, 2.1mm, 1.7 µm particle size
Buffer for Mobile phase-A	0.1% ortho phosphoric acid in 1000mL of milli-Q water, pH adjusted to 2.5 with dilute KOH solution
Buffer for Mobile phase-B	0.1% ortho phosphoric acid in 1000mL of milli-Q water
Mobile phase	Mobile phase-A : Buffer : Methanol : 70: 30 (v/v)
	Mobile phase-B : Buffer : Acetonitrile : Methanol 20: 70:10 (v/v/v)
Mode of elution	Gradient
Flow rate	0.18 mL min^{-1}
Column temperature	30°C
Wavelength of detection	220 nm
Injection volume	3 µL
Run time	18 min
Diluent	Acetonitrile and Buffer A in the ratio of 2:8
Gradient program	Time (min) / % Mobile phase- B: 0.01/5, 2.5/5, 6.0/40, 11.0/90, 11.50/100,17.0/100,17.01/5.0 and 18.0/5.
Concentration	150 µg mL^{-1}

The retention times (RTs) and relative retention times (RRTs) of all the known compounds were presented in Table 3.3.T11. Typical chromatogram representing CLP and its known and unknown impurities are presented in Figure 3.3.F6.

Table: 3.3.T11. RTs and RRTs of known and unknown components in finalized method

S.No.	Name of the Analyte	Retention time (min)	Relative retention time w.r.t. CLP peak
1	Imp-1	1.49	0.14
2	Imp-2	2.89	0.27
3	Imp-3	3.86	0.36
4	Imp-4	4.97	0.47
5	Imp-5	5.45	0.52
6	Imp-6	6.21	0.59
7	Imp-7	6.83	0.65
8	Imp-8	7.65	0.72
9	Imp-9	9.66	0.91
10	Imp-10	9.98	0.94
11	Imp-11	11.13	1.05
12	Imp-12	15.08	1.42
13	CLP	10.59	1.00

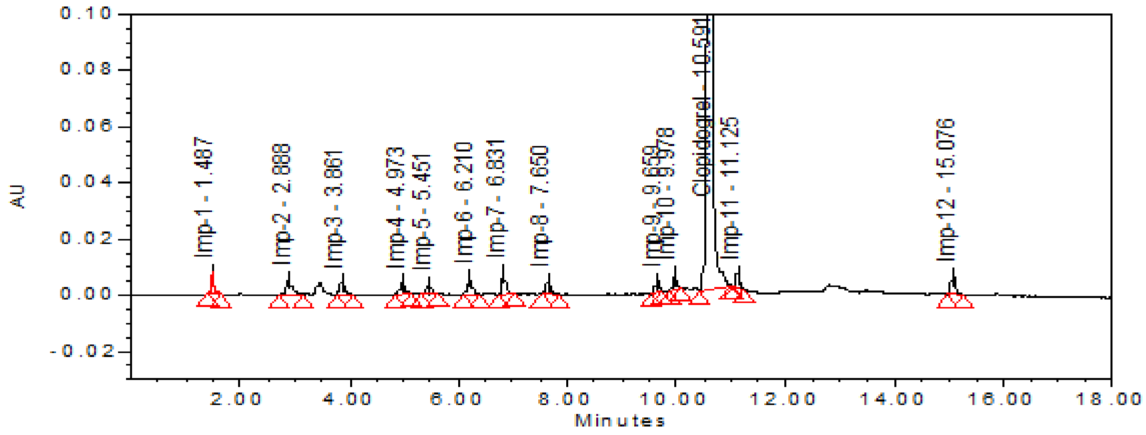


Figure: 3.3.F5. Typical chromatogram representing CLP and its known impurities

3.4. Discussion on Forced degradation studies

Forced degradation samples were analyzed in HPLC method by using PDA detector. The homogeneity of CLP peak was confirmed in all the degradation samples.

3.4.1. Degradation of CLP during oxidation

CLP drug substance was subjected to oxidation by using 3.0% Hydrogen peroxide at room temperature. The oxidized sample solution was analyzed and monitored for possible degradation products. No significant degradation was observed in oxidation conditions. Based on peak purity data, it was confirmed that the main component in oxidized sample is homogeneous. Chromatogram of CLP sample after oxidation and peak purity table are presented in Fig.3.4.F1 and Table 3.4.T1 respectively.

Fig: 3.4.F1. Chromatogram of oxidized CLP sample

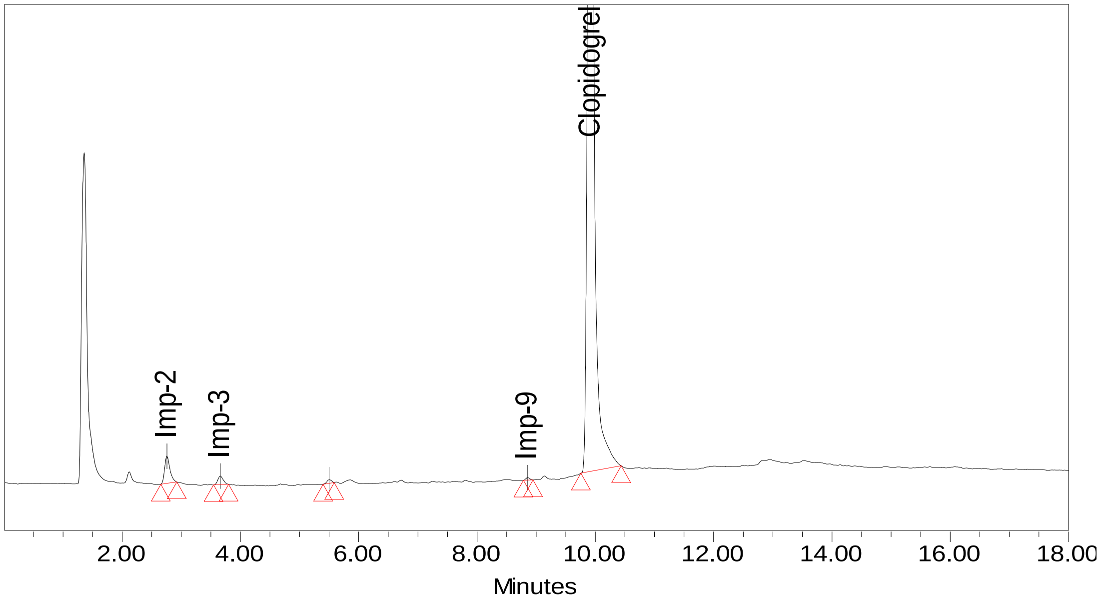


Table: 3.4.T1. Peak purity Table for CLP peak in oxidized sample

Name	**Retention Time**	**Purity1 Angle**	**Purity1 Threshold**	**Area**	**% Area**	**Height**
Imp-1	1.527					
Imp-2	2.757	1.731	3.895	70484	1.84	11403
Imp-3	3.659	6.144	8.559	22255	0.58	3584
Imp-4	4.719					
Imp-5	5.206					
	5.498	11.526	15.625	8158	0.21	1528
Imp-6	5.923					
Imp-7	6.582					
Imp-8	7.249					
Imp-9	8.857	11.777	19.282	4584	0.12	1005
Imp-10	9.285					
Clopidogrel	9.922	1.679	2.070	3722559	97.24	618258
Imp-11	10.519					
Imp-12	14.877					

3.4.2. Degradation during base hydrolysis

CLP drug substance was subjected to base hydrolysis by using 0.01N Sodium hydroxide at room temperature. The hydrolyzed sample solution was analyzed and monitored after 1h. The sample was degraded upto 10% under basic conditions, major degradent being imp-3, which was further confirmed by performing co-injection of Imp-3. Based on peak purity, the data confirmed that the main component and Imp-3 in base hydrolyzed sample is homogeneous. Chromatogram of base hydrolyzed CLP sample and purity table are presented in Fig.3.4.F2 and Table 3.4.T2 respectively.

Fig: 3.4.F2. Chromatogram of base hydrolysis CLP sample

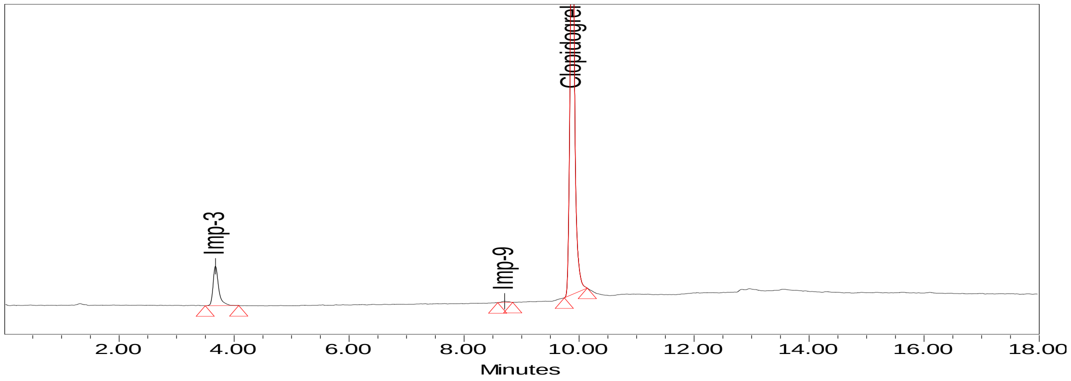


Table: 3.4.T2. Peak purity table for CLP peak in base hydrolysis

Name	Retention Time	Purity1 Angle	Purity1 Threshold	Area	% Area	Height
Imp-1	1.527					
Imp-2	2.828					
Imp-3	3.675	1.377	4.160	176678	10.07	26277
Imp-4	4.719					
Imp-5	5.206					
Imp-6	5.923					
Imp-7	6.582					
Imp-8	7.249					
Imp-9	8.705	8.336	13.150	6070	0.35	817
Imp-10	9.285					
Clopidogrel	9.881	0.653	3.101	1571774	89.58	285867
Imp-11	10.519					
Imp-12	14.877					

3.4.3. Degradation during Acid hydrolysis

CLP drug substance was subjected to base hydrolysis by using 0.1N HCl at 60 °C. The hydrolyzed sample solution was analyzed and monitored after 24h. The sample was degraded up to 0.8% under acidic conditions, major degradent being imp-3, which was further confirmed by performing co-injection of Imp-C. Based on peak purity data, it was confirmed that the main component and Imp-3 in acid hydrolyzed sample is homogeneous. Chromatogram of acid hydrolyzed CLP sample and purity table are presented in Fig.3.4.F3 and Table 3.4.T3 respectively.

Fig: 3.4.F3. Chromatogram of Acid hydrolysis CLP sample

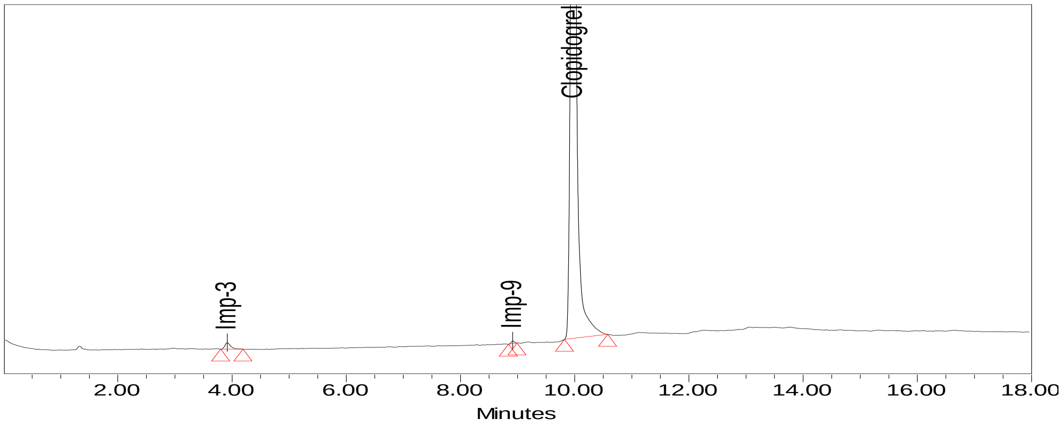


Table: 3.4.T3. Peak purity Table for CLP peak in acid hydrolysis

Name	Retention Time	Purity1 Angle	Purity1 Threshold	Area	% Area	Height
Imp-1	1.527					
Imp-2	2.828					
Imp-3	3.916	8.671	18.900	27228	0.77	3892
Imp-4	4.719					
Imp-5	5.206					
Imp-6	5.923					
Imp-7	6.582					
Imp-8	7.249					
Imp-9	8.915	15.897	46.030	6035	0.17	1621
Imp-10	9.285					
Clopidogrel	9.978	0.703	3.148	3516319	99.06	585480
Imp-11	10.519					
Imp-12	14.877					

3.4.4. Degradation during water hydrolysis

CLP drug substance was subjected to hydrolysis by using water at 60 °C. The aqueous hydrolysis sample solution was collected after 24 h and analyzed.

Fig: 3.4.F4. Chromatogram of water hydrolysis CLP sample

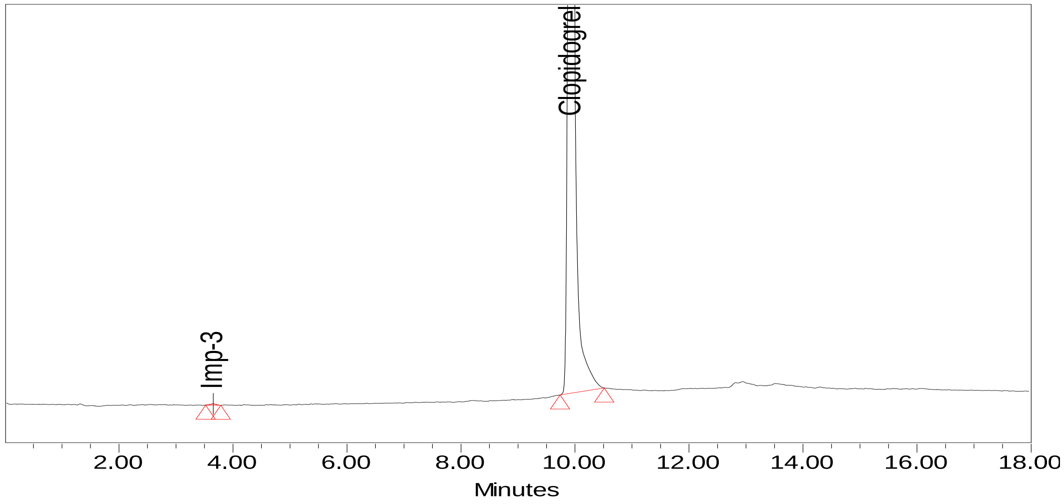


Table: 3.4.T4. Peak purity table for CLP peak in water hydrolysis

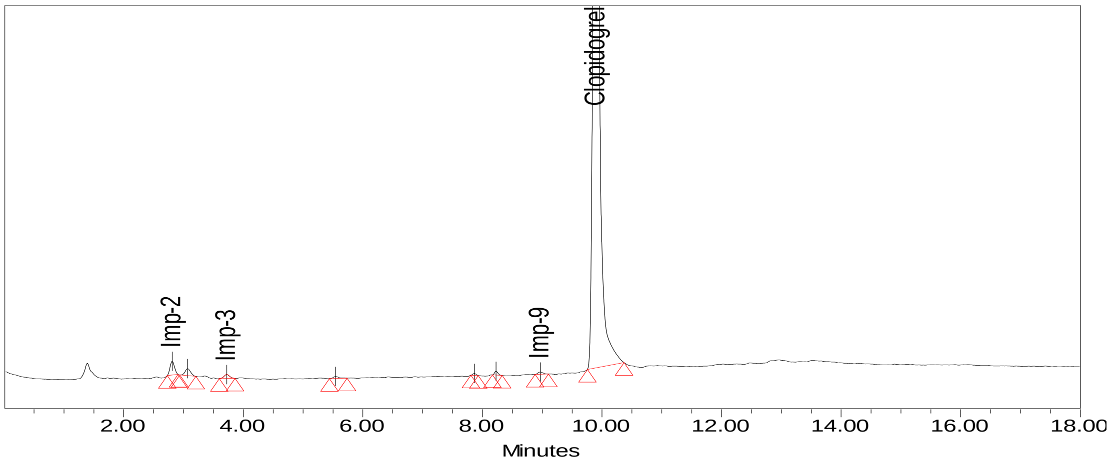
Name	Retention Time	Purity1 Angle	Purity1 Threshold	Area	% Area	Height
Imp-1	1.527					
Imp-2	2.828					
Imp-3	3.654	25.437	35.523	4423	0.09	668
Imp-4	4.719					
Imp-5	5.206					
Imp-6	5.923					
Imp-7	6.582					
Imp-8	7.249					
Imp-9	8.977					
Imp-10	9.285					
Clopidogrel	9.936	2.367	3.047	4743876	99.91	763358
Imp-11	10.519					
Imp-12	14.877					

No significant degradation was observed in water hydrolysis conditions. Peak purity data confirmed that the main component in sample is homogeneous. Chromatogram of stressed sample and purity table are presented in Fig.3.4.F4 and Table 3.4.T4 respectively.

3.4.5. Thermal degradation

CLP drug substance was exposed to a temperature of 90 °C for 10 days; Imp-2 and Imp-3 were the major degradation products. CLP peak in thermally degraded sample was found to be spectrally pure. Chromatogram of stressed sample and peak purity plot is shown in Fig.3.4.F5 and Table 3.4.T5.

Fig: 3.4.F5. Chromatogram of Thermal degradation CLP sample



3.4.6. Photolytic degradation

CLP drug substance was exposed to UV radiations at 254nm and 365nm for 10 days. No considerable degradation was observed. To confirm the stability of CLP drug substance towards photolytic condition, peak purity of CLP peak was verified in degraded sample and it was found that the product was homogeneous. Chromatogram of photolytic degradation sample and peak purity plot is shown in Fig. 3.4.F6 and Table 3.4.T6 respectively.

Table: 3.4.T5. Peak purity table for CLP peak in Thermal degradation Sample

Name	**Retention Time**	**Purity1 Angle**	**Purity1 Threshold**	**Area**	**% Area**	**Height**
Imp-1	1.527					
Imp-2	2.809	6.062	7.335	39113	1.00	7536
	3.069	3.108	4.130	24510	0.63	3835
Imp-3	3.722	9.327	18.388	13598	0.35	2159
Imp-4	4.719					
Imp-5	5.206					
	5.545	17.702	39.944	7273	0.19	1115
Imp-6	5.923					
Imp-7	6.582					
Imp-8	7.249					
	7.863	8.972	20.916	3876	0.10	1065
	8.226	6.776	12.178	8323	0.21	2131
Imp-9	8.967	20.459	39.211	7227	0.19	1207
Imp-10	9.285					
Clopidogrel	9.902	0.844	1.106	3790798	97.33	630476
Imp-11	10.519					
Imp-12	14.877					

Fig: 3.4.F6. Chromatogram of Photolytic degradation CLP sample

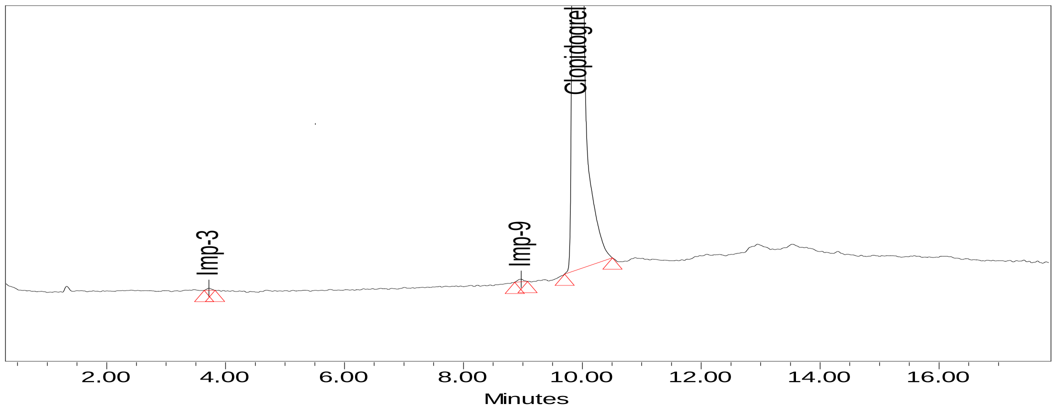


Table: 3.4.T6. Peak purity table for CLP peak in photolytic degradation sample

	Name	Retention Time	Purity1 Angle	Purity1 Threshold	Area	% Area	Height
1	Imp-1	1.527					
2	Imp-2	2.828					
3	Imp-3	3.719	29.835	90.000	2490	0.05	439
4	Imp-4	4.719					
5	Imp-5	5.206					
6	Imp-6	5.923					
7	Imp-7	6.582					
8	Imp-8	7.249					
9	Imp-9	8.971	35.244	90.000	2626	0.06	451
10	Imp-10	9.285					
11	Clopidogrel	9.914	0.919	3.136	4583183	99.89	738224
12	Imp-11	10.519					
13	Imp-12	14.877					

3.4.7. Results of Forced degradation studies

The peak purity results from forced degradation studies proved the stability indicating nature of the method and the method is suitable for determination of all the related substances and degradation products in CLP drug substance during stability testing. Percentage of all specified related compounds, in all the stressed samples are presented in Table 3.4.T7.

3.5. Analytical method validation

The developed and optimized LC method was fully validated as per ICH and USP guidelines.

3.5.1. Precision

Precision study evaluated by performing repeatability study. To ensure the repeatability of related substances method, prepared six individual preparations of CLP drug substance spiked with Imp-1 to Imp-12 at specification level (0.15%) with respect to test concentration. The %RSD was calculated for the area of each individual known impurity. Results are tabulated in Table 3.5.T1.

Table: 3.4.T7. Results of forced degradation studies

Degradation condition	**Time**	**RS by HPLC % degradation**	**Remarks/observation**
HCl- 0.1N at (60°C)Acid hydrolysis)	24hr	0.9%	Impurity-3 as a degradation product
NaOH-0.01N RT (Base hydrolysis)	1 hr	10.4%	Impurity-3 as a degradation product
Water hydrolysis at (60°C)	24hrs	No degradation	No degradation observed.
Oxidation by H_2O_2- 3.0% RT	10days	2.8%	Imp-2 and Imp-3 as degradation products.
Thermal (90°C) for 10 days	10days	2.7%	Imp-2 and Imp-3 as degradation products.
UV at 254nm & 365nm for 10 days	10days	No degradation	No degradation observed.

Table: 3.5.T1. Results of CLP related substances method precision

S.No	**Area of Related substances**											
	Imp-1	**Imp-2**	**Imp-3**	**Imp-4**	**Imp-5**	**Imp-6**	**Imp-7**	**Imp-8**	**Imp-9**	**Imp-10**	**Imp-11**	**Imp-12**
Pre-1	6635	9080	27616	5816	4189	6210	6294	5082	6365	9749	6554	7395
Pre-2	6694	9473	27080	5880	4092	6213	6397	5125	6643	9745	6494	7424
Pre-3	6741	9234	26846	5848	4183	6278	6247	5102	6388	9862	6603	7348
Pre-4	6660	9060	26832	5859	4259	6254	6323	5067	6478	9898	6549	7343
Pre-5	6660	9121	27202	5965	4248	6248	6323	5046	6405	9775	6411	7385
Pre-6	6696	9029	27161	5906	4251	6200	6250	5200	6361	9748	6593	7381
Mean	6681	8293	26143	5879	4204	6234	6306	5104	6440	9796	6534	7379
SD	33.4	179.9	174.7	46.6	70.9	31.7	62.2	59.8	113.7	70.1	79.2	32.7
%RSD	0.50	2.17	0.67	0.79	1.69	0.51	0.99	1.17	1.77	0.72	1.21	0.44

The above results prove that the method is repeatable within acceptable limits of % RSD for six preparations of related substances between 0.4-2.2.

3.5.2. Sensitivity

Sensitivity of the method was demonstrated in terms of the Limit of Quantitation (LOQ) and Limit of Detection (LOD) values of specified analytes. LOQ, LOD values were established for Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11, Imp-12 and CLP based on Linearity method.

3.5.2.1 Limit of quantification (LOQ)

A series of dilutions of CLP, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and Imp-12 was prepared in different concentrations and injected into the liquid chromatograph. The calibration line was established, the residual standard deviation and Slope of the calibration curve were determined. The LOQ was also determined by using the formula given below:

$$\text{Limit of quantificatio} = \frac{10^{*}\ \text{Residual standard deviation}}{\text{Slope}}$$

LOQ values of the impurities are presented in Table 3.5.T2.

3.5.2.2. Limit of detection (LOD)

A series of dilutions of CLP, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and Imp-12 was prepared in different concentrations and injected into the liquid chromatograph.

The calibration line was established and the residual standard deviation and Slope of the calibration curve were determined. The LOD was also calculated using the formula given below.

$$\text{Limit of detection} = \frac{3.3 * \text{Residual standard deviation}}{\text{Slope}}$$

The limit of detection values of all the impurities and CLP presented in Table 3.5.T3.

Table: 3.5.T2. LOQ values of CLP and its related compounds

S.No.	Name of the analyte	LOQ in µg/mL	% w.r.t. test concentration
1	CLP	0.030	0.045
2	Imp-1	0.025	0.037
3	Imp-2	0.024	0.036
4	Imp-3	0.027	0.041
5	Imp-4	0.026	0.039
6	Imp-5	0.018	0.026
7	Imp-6	0.024	0.036
8	Imp-7	0.026	0.039
9	Imp-8	0.029	0.044
10	Imp-9	0.009	0.013
11	Imp-10	0.008	0.012
12	Imp-11	0.054	0.081
13	Imp-12	0.017	0.025

Table: 3.5.T3. LOD values of the impurities and CLP peak

S.No.	Name of the analyte	LOD in µg/mL	% w.r.t. test concentration
1	CLP	0.015	0.010
2	Imp-1	0.012	0.008
3	Imp-2	0.012	0.008
4	Imp-3	0.013	0.009
5	Imp-4	0.013	0.009
6	Imp-5	0.009	0.006
7	Imp-6	0.012	0.008
8	Imp-7	0.013	0.009
9	Imp-8	0.014	0.010
10	Imp-9	0.004	0.003
11	Imp-10	0.004	0.003
12	Imp-11	0.027	0.018
13	Imp-12	0.008	0.005

3.5.2.3. Precision at Limit of quantification level

Six preparations of CLP containing all impurities at Limit of quantificcation level were prepared and injected individually and the % RSD for the areas of each analyte was calculated. No significant variation observed in the area of each analyte for six consecutive injections; RSD of all the components ranges from 0.8 to 7.7 %. Results are summarized in 3.5.T4.

Table: 3.5.T4. Results of CLP related substances LOQ precision

S.No	Area of impurities												
	Imp-1	**Imp-2**	**Imp-3**	**Imp-4**	**Imp-5**	**Imp-6**	**Imp-7**	**Imp-8**	**Imp-9**	**Imp-10**	**CLP**	**Imp-11**	**Imp-12**
Pre-1	972	2062	1321	3440	2603	3139	1511	1932	1886	1234	1498	1033	2409
Pre-2	924	2064	1371	3455	2589	2966	1624	2000	1898	1258	1213	1162	2458
Pre-3	1070	2030	1309	3510	2613	3010	1572	1888	1912	1361	1517	1074	2441
Pre-4	948	2063	1332	3044	2670	2930	1575	1899	1875	1263	1430	1077	2414
Pre-5	942	2067	1398	3228	2504	3130	1588	1650	1865	1228	1437	1088	2409
Pre-6	988	2032	1342	3331	2591	3185	1483	1988	1869	1290	1456	1031	2412
Mean	974	2053	1346	3335	2595	3060	1559	1893	1884	1272	1425	1078	2424
SD	52.2	17.1	33.3	174.5	53.6	104.	52.1	127.	18.2	48.8	109.4	47.8	20.7
%RSD	5.4	0.8	2.5	5.2	2.1	3.4	3.3	6.7	1.0	3.8	7.7	4.4	0.9

3.5.2.4. Accuracy at LOQ level

CLP sample was injected in test concentration i.e. 150 µg mL^{-1} to estimate the content of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and Imp-12. Three different sample solutions (150 µg mL^{-1}) of CLP containing Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and Imp-12 at LOQ level were prepared and each solution injected once. From the corrected area of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and Imp-12, % recovery of each impurity was calculated. Results are summarized in Table 3.5.T5.

Table: 3.5.T5. Results of Accuracy at LOQ level

Name	Workup	Amount Added (µg/mL)	Amount obtained (µg/mL)	% Recovery	% Mean Recovery
Imp-1	1	0.0372	0.0394	105.7	108.4
	2		0.0411	110.3	
	3		0.0407	109.2	
Imp-2	1	0.0359	0.0369	103.0	103.2
	2		0.0367	102.2	
	3		0.0374	104.2	
Imp-3	1	0.0409	0.1134	105.1	103.4
	2		0.1148	108.5	
	3		0.1100	96.7	
Imp-4	1	0.0392	0.0391	99.7	99.3
	2		0.0385	98.3	
	3		0.0392	99.8	

Name	Workup	Amount Added (µg/mL)	Amount obtained (µg/mL)	% Recovery	% Mean Recovery
Imp-5	1	0.0265	0.0274	103.4	101.8
	2		0.0266	100.5	
	3		0.0269	101.6	
Imp-6	1	0.0363	0.0390	107.4	106.5
	2		0.0386	106.1	
	3		0.0385	105.9	
Imp-7	1	0.0393	0.0387	98.5	99.5
	2		0.0393	99.9	
	3		0.0393	100.0	
Imp-8	1	0.0437	0.0478	109.6	109.0
	2		0.0475	108.8	
	3		0.0475	108.7	
Imp-9	1	0.0134	0.0136	101.5	102.7
	2		0.0142	105.4	
	3		0.0136	101.3	
Imp-10	1	0.0122	0.0119	97.4	98.6
	2		0.0122	99.8	
	3		0.0120	98.7	
Imp-11	1	0.0814	0.0790	97.0	96.0
	2		0.0757	92.9	
	3		0.0798	98.0	
Imp-12	1	0.0249	0.0248	99.3	99.0
	2		0.0248	99.3	
	3		0.0245	98.3	

3.5.3. Linearity

3.5.3.1. Linearity of the method

A series of linearity solutions were prepared containing CLP, Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and Imp-12 standard solution at different concentrations i.e.

0.01% 0.02 %, 0.03 %, 0.04 %, 0.05 %, 0.075 %, 0.1125%, 0.15%, 0.1875 and 0.225 % of working concentration (150 µg mL^{-1}) by performing appropriate dilutions to achieve the targeted concentration. Each solution was injected once and calibration plots were drawn for the concentration of each component versus peak area of corresponding known component. Linearity plot of each analyte with best fit linear equation is shown in Fig.3.5.F1.-3.5.F13. Linear regression analysis was performed for each analyte and data presented in Table 3.5.T6. - 3.5.T18.

Table: 3.5.T6. Linearity of Imp-1

S.No.	**Conc. (µg mL^{-1})**	**Imp-1 peak area**	**Calculated response through trend line equation**	**Residual**	**Residual square**	**Sensitivity**
1	0.0151	445	393	-52	2704	29470
2	0.0302	854	856	2	3	28278
3	0.0453	1366	1319	-47	2250	30155
4	0.0604	1761	1781	20	414	29156
5	0.0755	2236	2244	8	66	29616
6	0.1133	3401	3401	0	0	30031
7	0.1699	5061	5137	76	5710	29792
8	0.2265	6696	6872	176	30981	29563
9	0.2831	8790	8607	-183	33319	31046
10	0.3398	10590	10343	-247	61050	31170
Regression coefficient		0.9995	Residual sum of squares		108603	
Slope		31081				
Intercept		-104				
% y-Intercept		-1.6	Linearity equation		y= 31081x-104	

Fig: 3.5.F1. Linearity plot for Imp-1

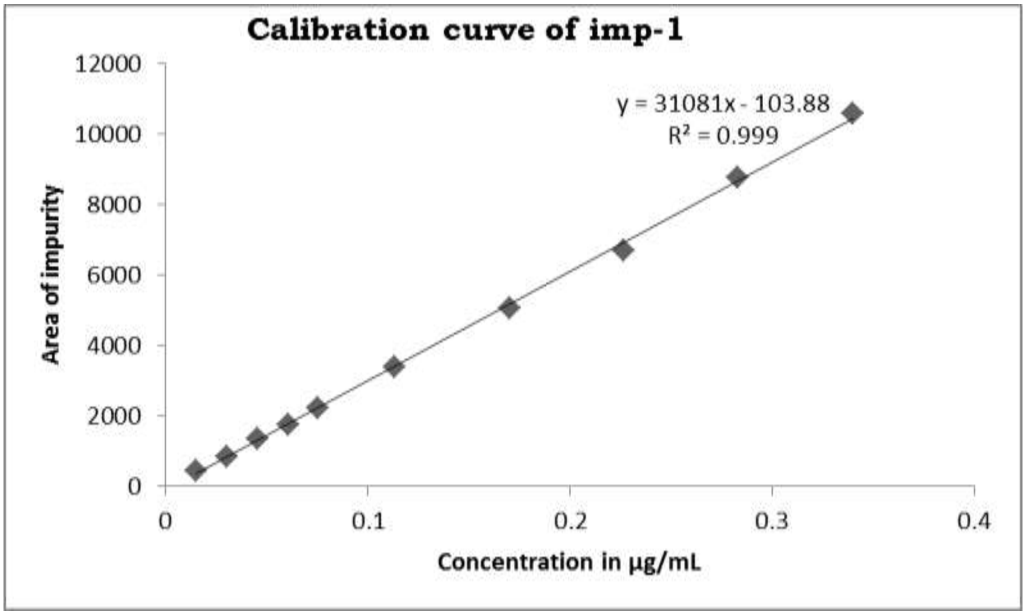


Table: 3.5.T7. Linearity of Imp-2

S.No.	Conc. ($\mu g\ mL^{-1}$)	Imp-2 peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.0150	382	406	24	563	25467
2	0.0300	770	803	33	1087	25667
3	0.0450	1277	1200	-77	5898	28378
4	0.0600	1547	1597	50	2544	25783
5	0.0750	1944	1995	51	2567	25920
6	0.1125	3137	2988	-149	22275	27884
7	0.1688	4507	4477	-30	878	26708
8	0.2250	5790	5967	177	31328	25733
9	0.2813	7527	7457	-70	4953	26763
10	0.3375	8955	8946	-9	77	26533
Regression coefficient		0.9996	Residual sum of squares		72170	
Slope		26482				
Intercept		9				
% y-Intercept		0.15	Linearity equation		y= 26482.19x+9	

Fig: 3.5.F2. Linearity plot for Imp-2

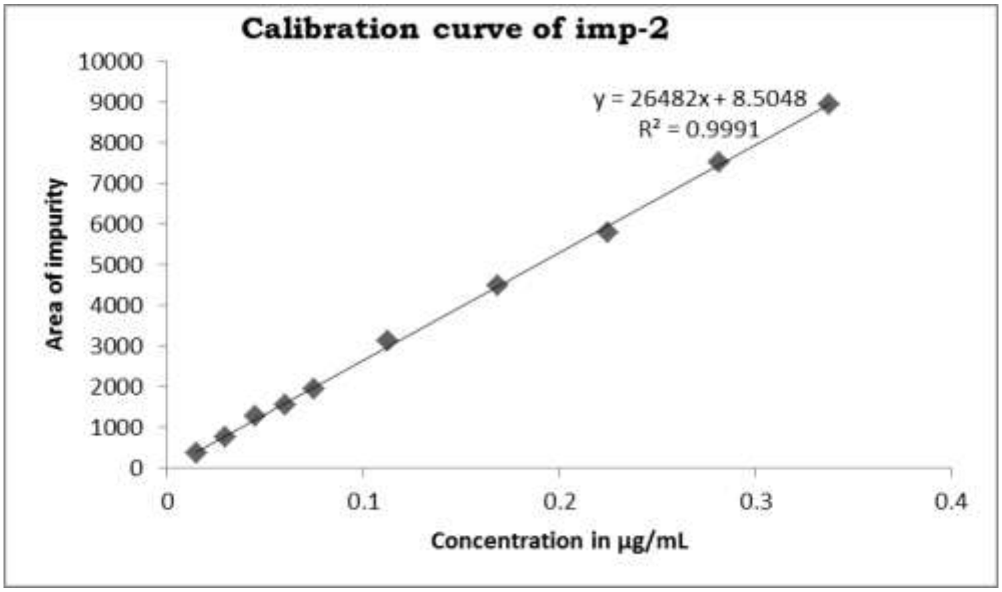


Table: 3.5.T8. Linearity of Imp-3

S.No.	Conc. (µg mL^{-1})	Imp-3 peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.0151	456	466	10	91	30299
2	0.0301	923	922	-1	1	30664
3	0.0452	1374	1379	5	25	30432
4	0.0602	1868	1836	-32	1037	31030
5	0.0753	2269	2293	24	555	30153
6	0.1129	3434	3434	0	0	30423
7	0.1693	5195	5147	-48	2274	30683
8	0.2258	6950	6860	-90	8071	30786
9	0.2822	8280	8573	293	85857	29342
10	0.3386	10447	10286	-161	25965	30851
Regression coefficient		0.9994	Residual sum of squares		123875	
Slope		30350				
Intercept		9				
% y-Intercept		0.13	Linearity equation		y= 30349.5x+9	

Fig: 3.5.F3. Linearity plot for Imp-3

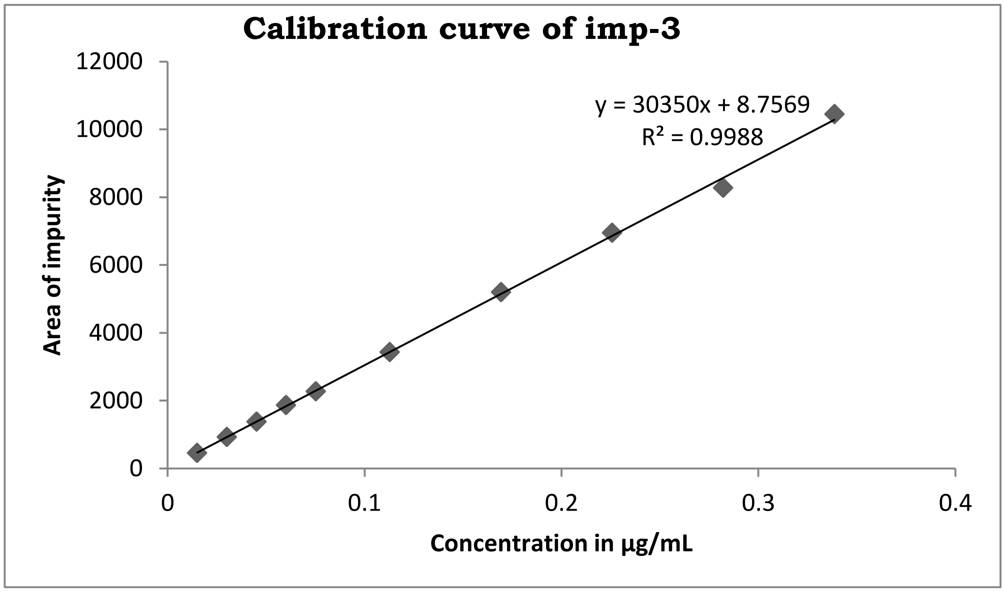


Table: 3.5.T9. Linearity of Imp-4

S.No.	Conc. (µg mL^{-1})	Imp-4 peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.0150	376	373	-3	8	25083
2	0.0300	792	772	-20	415	26418
3	0.0450	1154	1170	16	259	25662
4	0.0600	1508	1569	61	3665	25150
5	0.0750	2006	1967	-39	1522	26765
6	0.1124	2999	2963	-36	1287	26676
7	0.1686	4419	4457	38	1468	26204
8	0.2249	5857	5952	95	8932	26048
9	0.2811	7679	7446	-233	54426	27321
10	0.3373	8818	8940	122	14860	26145
Regression coefficient		0.9995	Residual sum of squares		86842	
Slope		26581				
Intercept		-25				
% y-Intercept		-0.43	Linearity equation		y= 26581.2x-25	

Fig. 3.5.F4. Linearity plot for Imp-4

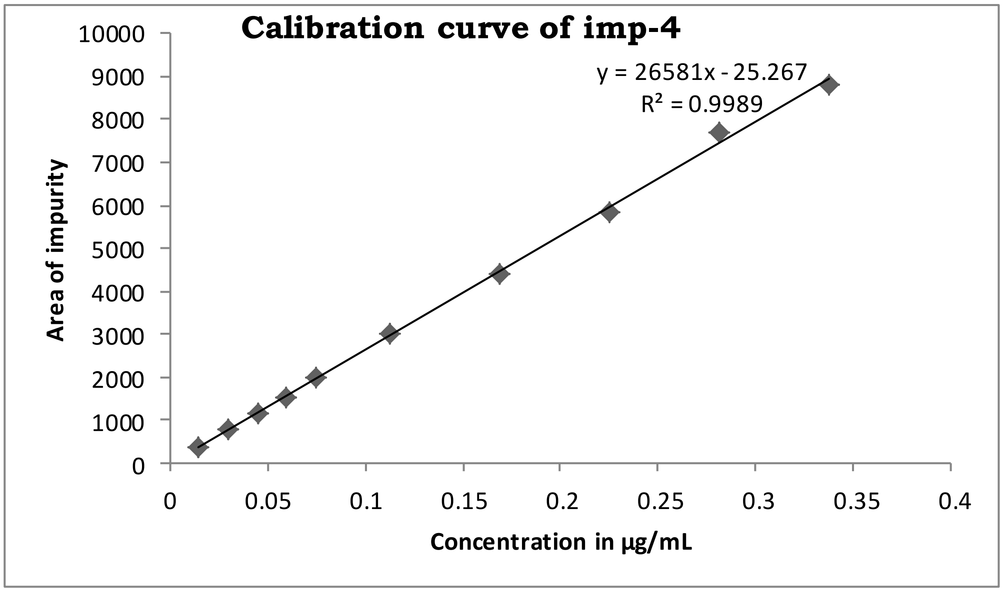


Table: 3.5.T10. Linearity of Imp-5

S.No.	Conc. (µg mL^{-1})	Imp-5 peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.0152	271	281	10	109	17888
2	0.0303	561	561	0	0	18515
3	0.0455	838	841	3	9	18438
4	0.0606	1162	1121	-41	1704	19175
5	0.0758	1386	1400	14	210	18297
6	0.1136	2106	2100	-6	38	18535
7	0.1704	3182	3149	-33	1092	18670
8	0.2273	4149	4198	49	2404	18257
9	0.2841	5162	5247	85	7244	18172
10	0.3409	6378	6296	-82	6692	18711
Regression coefficient		0.9998	Residual sum of squares		19502	
Slope		18466				
Intercept		2				
% y-Intercept		0.04	Linearity equation		y= 18465.7x+2	

Fig. 3.5.F5. Linearity plot for Imp-5

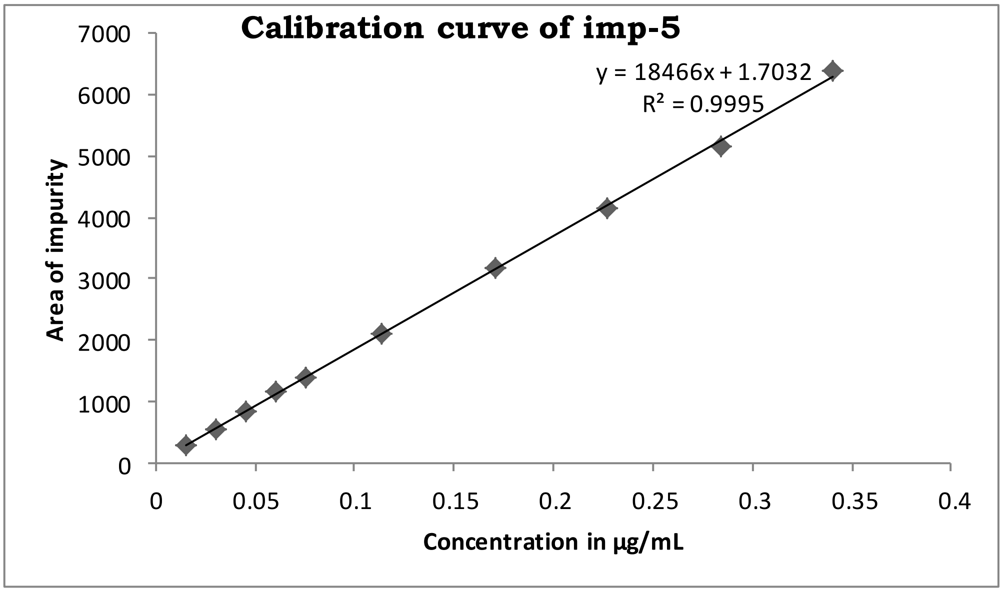


Table: 3.5.T11. Linearity of Imp-6

S.No.	Conc. (µg mL^{-1})	Imp-6 peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.0150	372	294	-78	6108	24800
2	0.0300	710	710	0	0	23667
3	0.0450	1112	1127	15	219	24711
4	0.0600	1509	1543	34	1176	25150
5	0.0750	2003	1960	-43	1869	26707
6	0.1125	3038	3001	-37	1371	27004
7	0.1688	4378	4563	185	34145	25944
8	0.2250	6041	6125	84	6987	26849
9	0.2813	7858	7686	-172	29448	27940
10	0.3375	9236	9248	12	149	27366
Regression coefficient		0.9995	Residual sum of squares		81472	
Slope		27765				
Intercept		-123				
% y-Intercept		-2.0	Linearity equation		y= 27765.5x-123	

Fig. 3.5.F6. Linearity plot for Imp-6

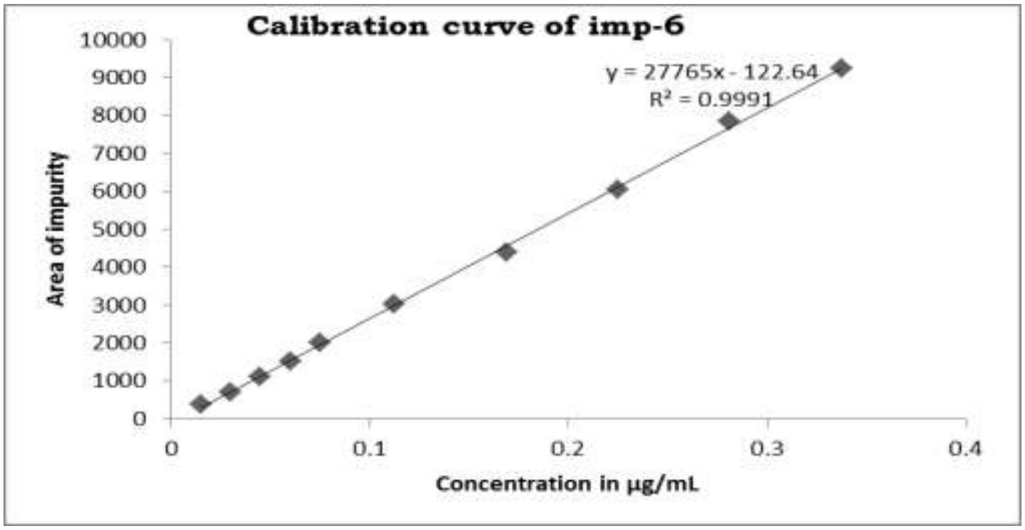


Table: 3.5.T12. Linearity of Imp-7

S.No.	Conc. (µg mL^{-1})	Imp-7 peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.0150	406	362	-44	1920	27013
2	0.0301	821	794	-27	727	27312
3	0.0451	1275	1226	-49	2412	28277
4	0.0601	1610	1658	48	2279	26780
5	0.0752	2164	2090	-74	5536	28796
6	0.1127	3192	3169	-23	519	28317
7	0.1691	4618	4789	171	29127	27311
8	0.2255	6239	6408	169	28598	27674
9	0.2818	8021	8028	7	43	28462
10	0.3382	9824	9647	-177	31330	29050
Regression coefficient		0.9995	Residual sum of squares		102491	
Slope		28733				
Intercept		-70				
% y-Intercept		-1.1	Linearity equation		y= 28732.6x-70	

Fig: 3.5.F7. Linearity plot of Imp-7

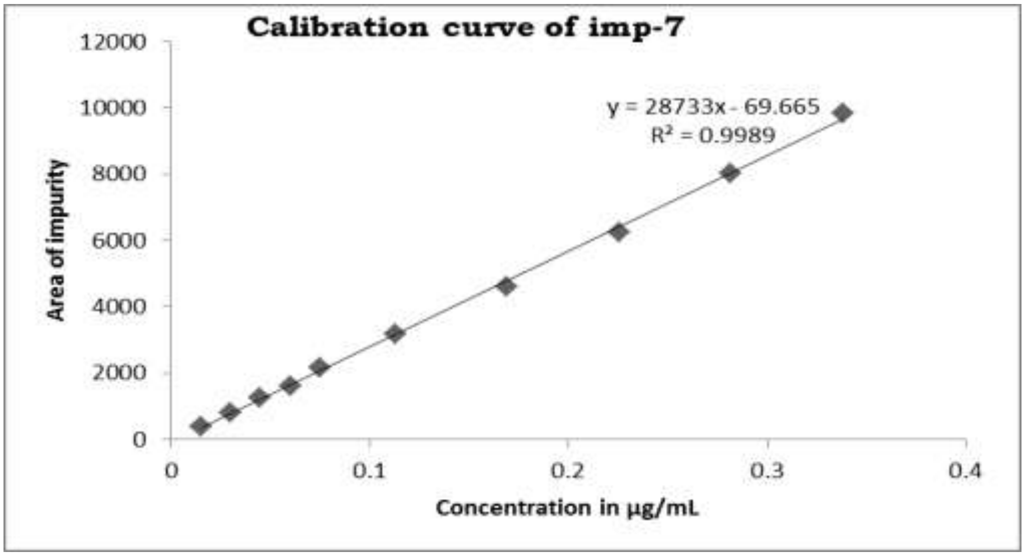


Table: 3.5.T13. Linearity of Imp-8

S.No.	Conc. (µg mL^{-1})	Imp-8 peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.0151	314	281	-33	1082	20864
2	0.0301	634	621	-13	158	21063
3	0.0452	960	962	2	3	21262
4	0.0602	1257	1302	45	2036	20880
5	0.0753	1644	1642	-2	2	21847
6	0.1129	2475	2493	18	335	21927
7	0.1693	3919	3770	-149	22333	23147
8	0.2258	4826	5046	220	48320	21378
9	0.2822	6354	6322	-32	1019	22517
10	0.3386	7655	7598	-57	3210	22606
Regression coefficient		0.9993	Residual sum of squares		78498	
Slope		22613.				
Intercept		-59				
% y-Intercept		-1.2	Linearity equation		y= 22613.7x-59	

Fig: 3.5.F8. Linearity plot of Imp-8

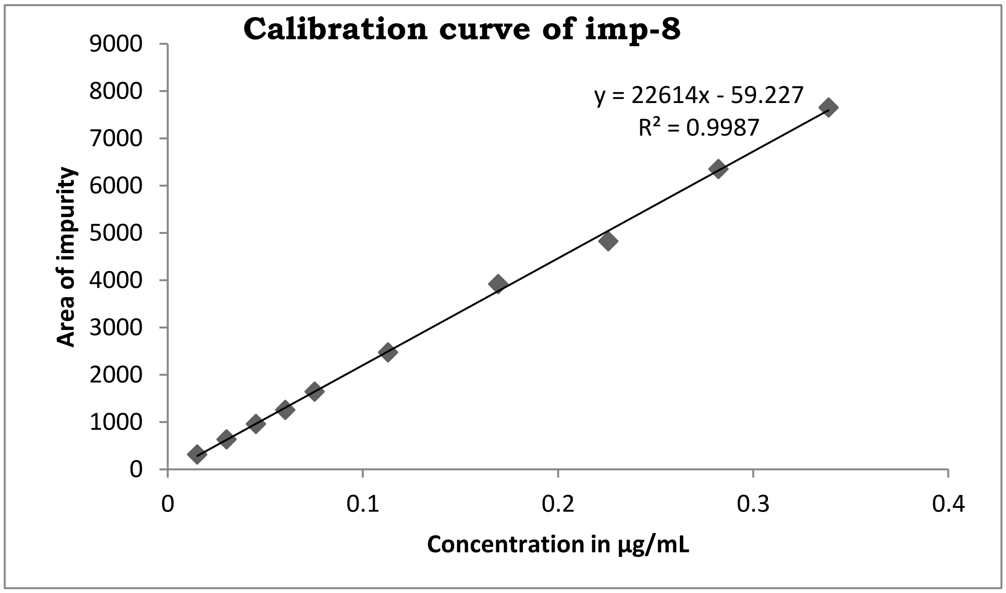


Table: 3.5.T14. Linearity of Imp-9

S.No.	Conc. (µg mL^{-1})	Imp-9 peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.0150	423	416	-7	50	28162
2	0.0300	850	845	-5	25	28296
3	0.0451	1273	1274	1	1	28251
4	0.0601	1709	1703	-6	34	28445
5	0.0751	2116	2132	16	263	28176
6	0.1127	3188	3205	17	286	28300
7	0.1690	4868	4814	-54	2926	28809
8	0.2253	6345	6423	78	6070	28162
9	0.2816	8078	8032	-46	2124	28684
10	0.3380	9635	9641	6	35	28510
Regression coefficient		0.9999	Residual sum of squares		11814	
Slope		28566				
Intercept		-13				
% y-Intercept		-0.21	Linearity equation		y= 28566x-13	

Fig: 3.5.F9. Linearity plot of Imp-9

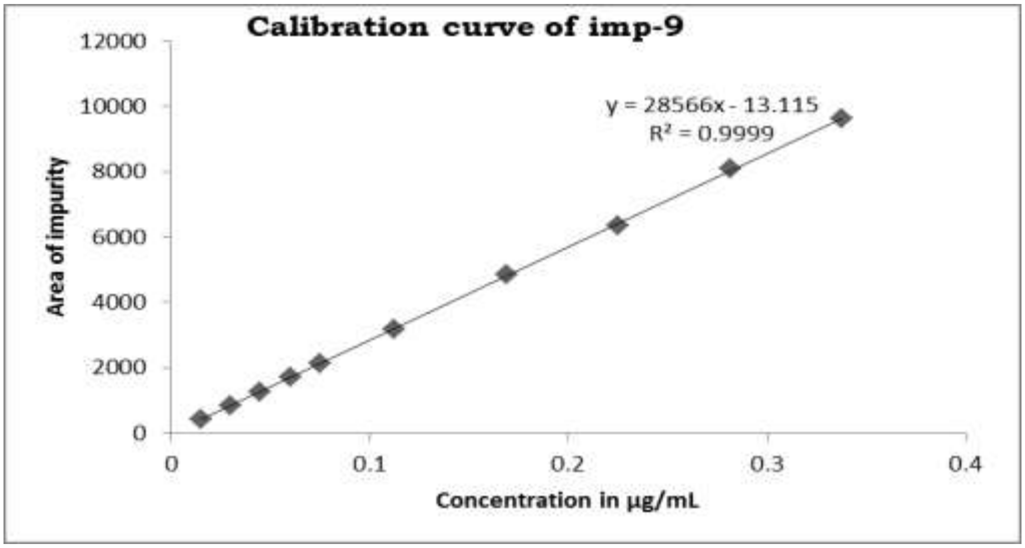


Table: 3.5.T15. Linearity of Imp-10

S.No.	Conc. (µg mL^{-1})	Imp-10 peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.0150	657	632	-25	610	43684
2	0.0301	1282	1293	11	112	42620
3	0.0451	1968	1953	-15	230	43617
4	0.0602	2633	2613	-20	396	43767
5	0.0752	3253	3273	20	414	43258
6	0.1128	4945	4924	-21	441	43839
7	0.1692	7362	7400	38	1443	43511
8	0.2256	9766	9876	110	12093	43289
9	0.2820	12436	12352	-84	7065	44099
10	0.3384	14842	14828	-14	198	43859
Regression coefficient		0.9999	Residual sum of squares		23001	
Slope		43900				
Intercept		-28				
% y-Intercept		-0.29	Linearity equation		y= 43900.36x-28	

Fig: 3.5.F10. Linearity plot of Imp-10

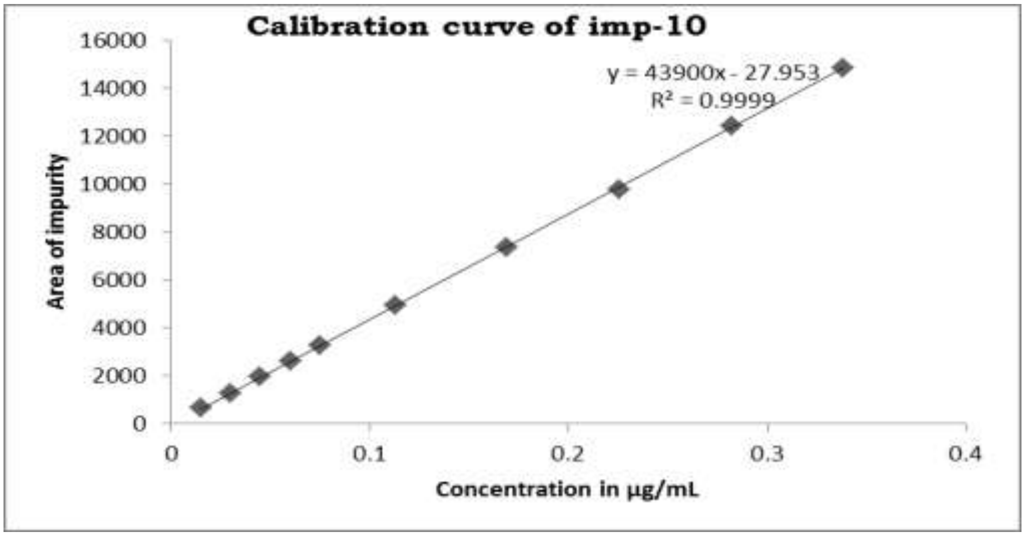


Table: 3.5.T16. Linearity of Imp-11

S.No.	**Conc. (µg mL-1)**	**Imp-11 peak area**	**Calculated response through trend line equation**	**Residual**	**Residual square**	**Sensitivity**
1	0.0150	490	714	224	50368	32645
2	0.0300	1066	1124	58	3416	35510
3	0.0450	1529	1534	5	30	33955
4	0.0600	2074	1944	-130	16772	34544
5	0.0751	2607	2355	-252	63749	34737
6	0.1126	3450	3380	-70	4961	30646
7	0.1689	4762	4917	155	24071	28200
8	0.2252	6440	6455	15	217	28603
9	0.2814	8023	7992	-31	942	28507
10	0.3377	9505	9530	25	620	28144
Regression coefficient		0.9990	Residual sum of squares		165146	
Slope		27316.6				
Intercept		304				
% y-Intercept		4.7	Linearity equation		y= 27231.8x+304	

Fig: 3.5.F11. Linearity plot of Imp-11

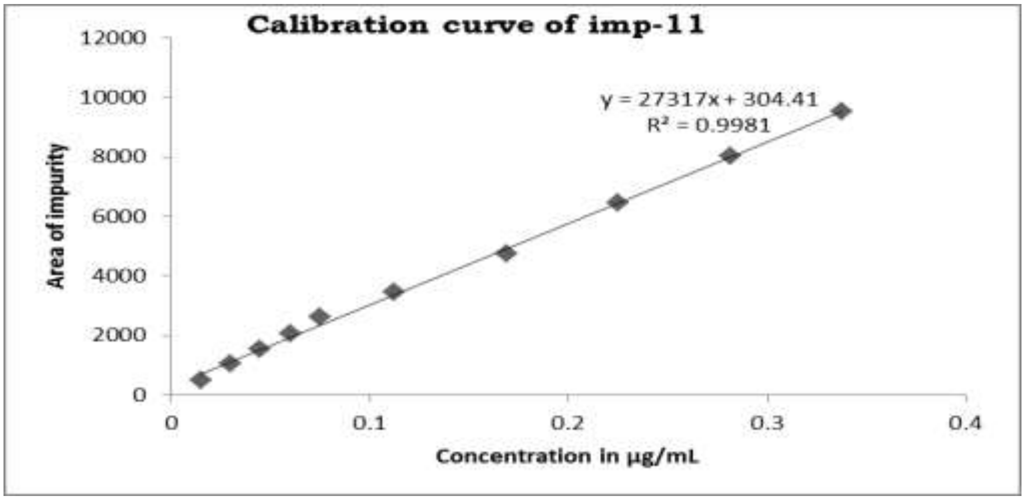


Table: 3.5.T17. Linearity of Imp-12

S.No.	Conc. (µg mL^{-1})	Imp-12 peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.0151	467	467	0	0	31009
2	0.0301	946	949	3	7	31408
3	0.0452	1452	1430	-22	468	32138
4	0.0602	1936	1912	-24	574	32138
5	0.0753	2401	2394	-7	53	31886
6	0.1130	3587	3598	11	119	31757
7	0.1694	5273	5404	131	17203	31123
8	0.2259	7354	7210	-144	20614	32554
9	0.2824	8917	9017	100	9938	31579
10	0.3389	10871	10823	-48	2309	32082
Regression coefficient		0.9998	Residual sum of squares		51283	
Slope		31983				
Intercept		-15				
% y-Intercept		-0.20	Linearity equation		y= 31983x-15	

Fig: 3.5.F12. Linearity plot of Imp-12

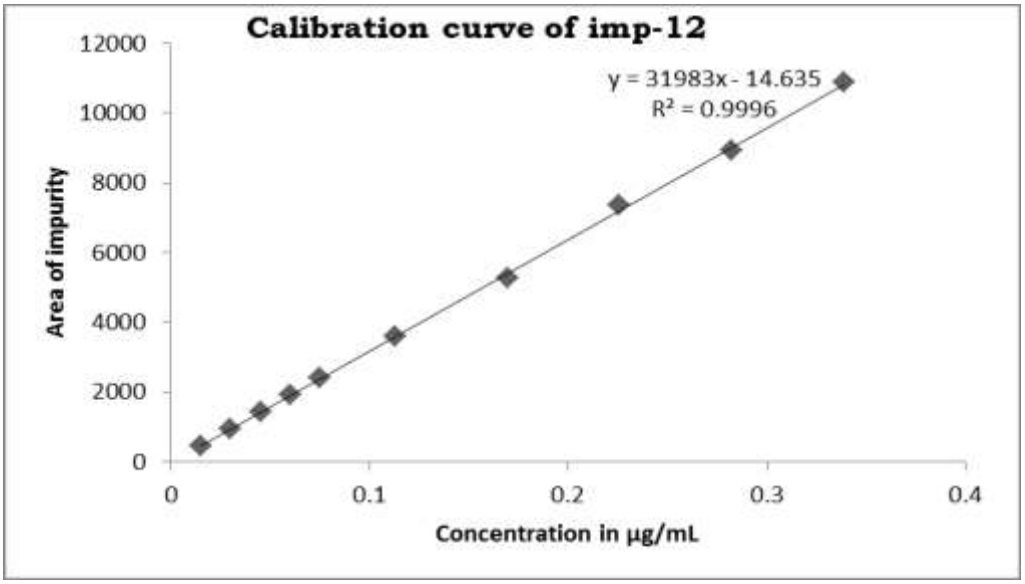
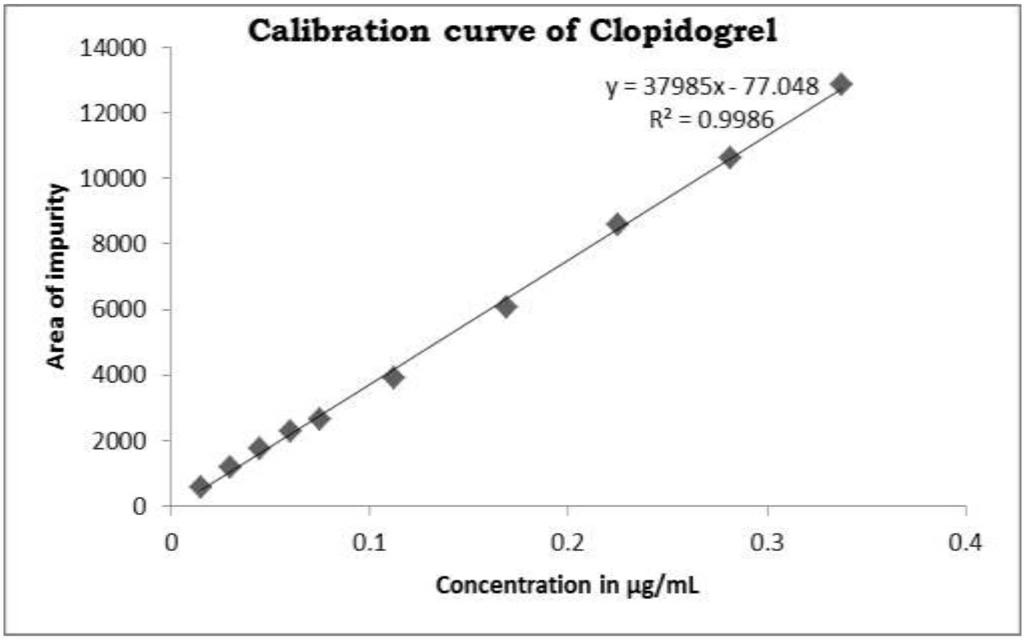


Table: 3.5.T18. Linearity of CLP

S.No.	Conc. (µg mL^{-}1)	CLP peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.0150	573	493	-80	6384	38175
2	0.0300	1182	1063	-119	14101	39374
3	0.0450	1745	1633	-112	12454	38752
4	0.0600	2296	2204	-92	8547	38241
5	0.0751	2668	2774	106	11173	35550
6	0.1126	3916	4199	283	80132	34786
7	0.1689	6076	6337	261	68193	35982
8	0.2252	8603	8475	-128	16333	38210
9	0.2814	1062	10613	-10	95	37746
10	0.3377	1286	12751	-110	12029	38081
Regression coefficient		0.9993	Residual sum of squares		229440	
Slope		3798				
Intercept		-77				
% y-Intercept		-0.90	Linearity equation		y= 37984.7x-77	

Fig: 3.5.F13. Linearity plot of CLP



Regression coefficients for concentration against peak area of all the related compounds of CLP and CLP peak from 0.01% to 0.225% level is more than 0.99. The % y-bias with respect to 100% specification of known compounds is within the limit of ± 5.0 which indicates that the method for the estimation of related substances of CLP is linear.

3.5.4. Accuracy

Solutions of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and Imp-12 at three different concentration levels i.e. 50 %, 100 % and 150 % w.r.t. the specification limit (0.15%) of working concentration was spiked with CLP sample solution (conc. 150 µg mL^{-1}), each level was prepared in triplicate and each solution was injected once.

Amount of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9, Imp-10, Imp-11 and Imp-12 obtained in each solution was calculated as percentage recovery. Accuracy results at three levels are summarized in Table 3.5.T19.

Table: 3.5.T19. Accuracy results

Name of the analyte	Concentration of analyte w.r.t. specification limit	Amount of impurity spiked to CLP (µg mL-1)	Amount of impurity recovered (µg mL-1)	%Mean recovery ± SD
Imp-1	50%	0.1133	0.1132	99.2±0.8
	100%	0.2265	0.2247	99.2±0.8
	150%	0.3398	0.3276	96.4±0.4
Imp-2	50%	0.1125	0.1147	101.9±0.3
	100%	0.2250	0.2333	103.7±3.3
	150%	0.3375	0.3682	109.9±0.9
Imp-3	50%	0.1129	0.1216	107.7±2.2
	100%	0.2258	0.2234	99.0±5.6
	150%	0.3386	0.3396	100.3±0.6
Imp-4	50%	0.1124	0.1088	96.8±1.5
	100%	0.2249	0.2215	98.5±0.5
	150%	0.3373	0.3575	106.0±0.6
Imp-5	50%	0.1136	0.1165	102.5±1.7
	100%	0.2273	0.2236	98.4±1.3
	150%	0.3409	0.3569	104.7±3.8
Imp-6	50%	0.1125	0.1127	100.2±0.8
	100%	0.2250	0.2284	101.5±0.6
	150%	0.3375	0.3597	106.6±0.4
Imp-7	50%	0.1127	0.1126	99.9±0.5
	100%	0.2255	0.2234	99.1±1.2
	150%	0.3382	0.3671	108.6±2.4
Imp-8	50%	0.1129	0.1174	104.0±1.9
	100%	0.2258	0.2354	104.3±0.4
	150%	0.3386	0.3537	104.4±0.1

Imp-9	50%	0.1127	0.1158	102.8±1.0
	100%	0.2253	0.2286	101.5±2.4
	150%	0.3380	0.3446	102.0± 0.0
Imp-10	50%	0.1128	0.1141	101.2±0.4
	100%	0.2256	0.2296	101.8±0.7
	150%	0.3384	0.3393	100.3±0.7
Imp-11	50%	0.1126	0.1136	100.9±0.8
	100%	0.2252	0.2296	102.0±0.8
	150%	0.3377	0.3419	101.2±0.2
Imp-12	50%	0.1130	0.1149	101.7±1.1
	100%	0.2259	0.2280	100.9±0.5
	150%	0.3389	0.3336	98.4±4.9

% Recoveries obtained for three different levels range from 96.8 to 109.9. Standard deviation for the average of % recoveries of each individual analyte is less than 5.6. The above accuracy reveals that the method is highly accurate.

3.5.5. Range

As evident from Linearity, accuracy and precision study of related substances method, range has been established for all the analytes i.e. CLP, Imp-1to Imp-12 from LOQ to 150 % of specification limit.

3.5.6. Conclusion from analytical method validations

The RP-LC method developed for quantitative determination of related substances of CLP in drug substance is precise, accurate, selective and linear as per the ICH recommended guidelines.

3.6 Conclusion

The proposed RP-HPLC method is sensitive, linear, precise and accurate for quantitation of related substances of CLP and its degradation product. As the method was fully validated as per ICH and proved the stability indicating power, it can be used for estimation of impurities of CLP for routine analysis, stability testing in pharmaceutical quality control labs.

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Chapter – 4

Stability indicating UPLC method for the determination of Ramelteon and their degradation products in Active pharmaceutical ingredients

4.1. Introduction to Ramelteon and details of available analytical literature

Ramelteon (RAM) is an orally active hypnotic with the chemical formula (S)-N-(2-(1,6,7,8-tetrahydro-2*H*-indeno[5,4-b]furan-8-yl)ethyl) propionamide (Fig.4.1.F1.). Ramelteon is highly selective melatonin MT_1 and MT_2 receptor agonist used for sleep disorders [1-10]. Empirical formula of RAM is $C_{16}H_{21}NO_2$ and molecular weight of RAM is 259.35. This drug is marketed with a brand name of ROZEREM. First time this drug was marketed by USA based pharmaceutical industry, Takeda pharmaceutical America, Inc. Complete drug information of RAM given in Table 4.1.T1.

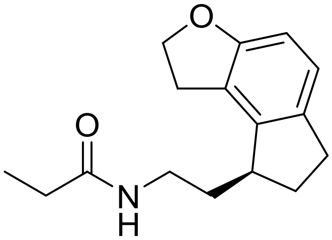


Fig: 4.1.F1. Chemical structure of RAM

Table: 4.1.T1. RAM Drug information

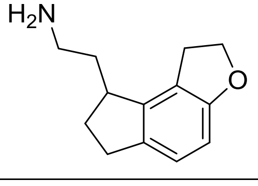
Molecular weight	:	259.35
Molecular formulae	:	$C_{16}H_{21}NO_2$
Brand name	:	ROZEREM
Dosage strengths	:	8mg Tablet
Dosage forms	:	Tablet
Maximum daily dosage	:	8 mg

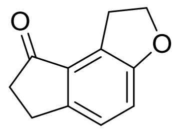
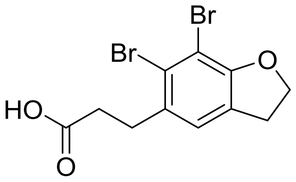
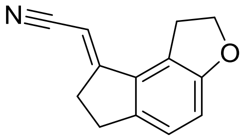
Analytical literature survey revealed that very few methods were reported for the chiral purity determination of Ramelteon (11-24) but not for related substances.

To the best of our knowledge, none of the currently available analytical methods can separate and quantify all the known related compounds and degradation impurities of Ramelteon API. Furthermore, there is no stability-indicating HPLC/UPLC method that was reported in the literature that can adequately separate and accurately quantify Ramelteon API. It is, therefore, felt necessary to develop a new stability indicating method for the related substance determination of Ramelteon. We intend to opt for a faster chromatographic technique UPLC, for the said study. An attempt has been made to determine whether UPLC can reduce analysis times without compromising the resolution and sensitivity.

Hence a reproducible stability-indicating RP UPLC method was developed for the quantitative determination of Ramelteon and its four impurities namely Imp - A, B, C and D. The structures of possible impurities and degradation products are provided in Table 4.1.T2.

Table: 4.1.T2. Details of related substances of RAM drug substance

Chemical structure of impurity	Chemical name
Imp-A 	2-(1,6,7,8-Tetrahydro-2H-indeno[5,4-b]furan-8-yl)ethylamine

Chemical structure of impurity	Chemical name
Imp-B 	1,2,6,7-tetrahydro-8H-indeno[5,4-b]furan-8-one
Imp-C 	3-(6,7-dibromo-2,3-dihydrobenzofuran-5-yl)propanoic acid
Imp-D 	2-(1 ,2,6,7-tetrahydro-8H-indeno[5,4-b]furan-8-ylidene)acetonitrile

4.2. Experimental

4.2.1. Materials

Materials or chemicals used for this study were listed in below Table 4.2.T1.

Table: 4.2.T1. Details materials and chemicals

S.No.	Name of the compound	Grade	Procured from../synthesized by..
1.	RAM drug substance samples	--	Dr.Reddy's Laboratories, INDIA
2.	Acetonitrile	Chromatography grade	Merck, India
3.	Trifluoro acetic acid	AR grade	ACROS ORGANICS , Belgium

S.No.	Name of the compound	Grade	Procured from../synthesized by..
4.	Water	High pure	Purified by Milli-Q plus system, USA
5.	Imp-A, Imp-B, Imp-C & Imp-D	--	Dr.Reddy's Laboratories, INDIA

4.2.2 Equipments

For method development and forced degradation, studies were carried out on Waters Acquity UPLC system equipped with 2996 photodiode array detectortor. The output signal was monitored and processed by Empower software. For analysis of forced degradation samples to identify m/z values of degradants, Agilent 1200 series liquid chromatograph coupled with Applied Biosystems 4000 Q Trap triple quadruple mass spectrophotometer with Analyst 1.4 software, MDS, SCIEX, USA was used.

4.2.3. Preparation of solutions

4.2.3.1. Preparation of impurity standard solution

Impurity stock solution was prepared by dissolving appropriate amount of all the known impurities i.e. Imp-A, Imp-B, Imp-C and Imp-D in diluent (Acetonitrile and Solvent A in the ratio of 10:90, v/v) to get the final concentration of each impurity in stock solution as 20 µg mL^{-1}.

As per ICH Q3A guidelines, specification limit of each known impurity in drug substance should not be more than 0.15 %, where the maximum daily dosage of drug is less than or equal to 2.0 g[25-26]. As described in Table 4.1.T1, maximum daily dosage of RAM is less than 2.0 g. Hence, considered specification limit for all the known impurities is 0.15 % and prepared impurity blend solution of 0.15 % by spiking appropriate volume of impurity stock solution in µL to 200 µg mL^{-1} RAM drug substance test solution.

4.2.3.2. Preparation of RAM test solution

RAM drug substance stock solution was prepared by weighing 20 mg of drug substance in 100 mL volumetric flask dissolved and diluted to volume with diluent. From this solution, 1 mL was transferred into 100 mL volumetric flask and diluted upto mark. 1mL of this solution was further diluted to 10 with diluent. Final concentration of the solution was 0.2 µg mL^{-1} of RAM drug substance, which was used for related substances estimation.

4.2.4 Preparation of forced degradation samples

As per ICH guidelines to generate degradation samples, one lot of RAM drug substance was selected and subjected to different stress conditions like acid hydrolysis, base hydrolysis, water hydrolysis, oxidation, photo degradation and Thermal degradation.

4.2.4.1. Preparation of Photo degradation sample

For photo degradation study, RAM drug substance was taken in Petri dish and kept in UV cabinet. Both lamps of Ultra Violet and Visible radiations were kept in “ON” mode to expose the sample to both 254 nm and 365 nm. After 10 days, sample was taken-off from UV cabinet and test solutions were prepared to get the final concentration of 200 μg mL^{-1}.

4.2.4.2. Preparation of Thermal degradation sample

RAM drug substance was placed in Petri dish and spread uniformly. The Petri dish was kept in an oven and temperature of the oven was maintained at 60 °C for 10 days. After 10 days, RAM samples were taken-off from oven and the test solutions were prepared to get the final concentration of 200 μg mL^{-1}.

4.2.4.3. Preparation of oxidative degradation sample

200 mg of RAM drug substance was transferred to 100 mL volumetric flask, and dissolved in 10 mL of Acetonitrile and made upto volume with 3 % aqueous peroxide solution. Placed magnetic stirrer in the solution and kept it at 60°C temperature under continuous stirring for 24 h. Diluted 1 mL of above stressed solution to 10 mL for the testing of related substances.

4.2.4.4. Preparation of water hydrolysis sample

200 mg of RAM drug substance was transferred to 100 mL volumetric flask, and dissolved in 10 mL of Acetonitrile and made upto the volume with water. Placed magnetic stirrer in the solution and kept solution at 60 °C temperature under continuous stirring for 24 h. Diluted 1 mL of above stressed solution to 10 mL for the testing of related substances.

4.2.4.5. Preparation of Acid hydrolysis sample

200 mg of RAM drug substance was transferred to 100 mL volumetric flask, and dissolved in 10 mL of Acetonitrile and made upto mark with 1N hydrochloric acid. Placed magnetic stirrer in the solution and kept solution at 60°C temperature under continuous stirring for 4 h. Inorder to prepare test solution for related substances estimation transferred 1 mL of above stressed sample solution into 10 mL volumetric flak, neutralized with 1 N sodium hydroxide and made upto mark with diluent.

4.2.4.6. Preparation of Base hydrolysis sample

200 mg of RAM drug substance was transferred to 100 mL volumetric flask, and dissolved in 10 mL of Acetonitrile and made upto mark with 0.5N Sodium hydroxide solution. Placed magnetic stirrer in the solution and kept solution at 60°C temperature under continuous stirring for 24 h. Diluted 1 mL of above stressed solution to 10 mL for the testing of related substances.

4.3. Sequential steps in method development and optimization

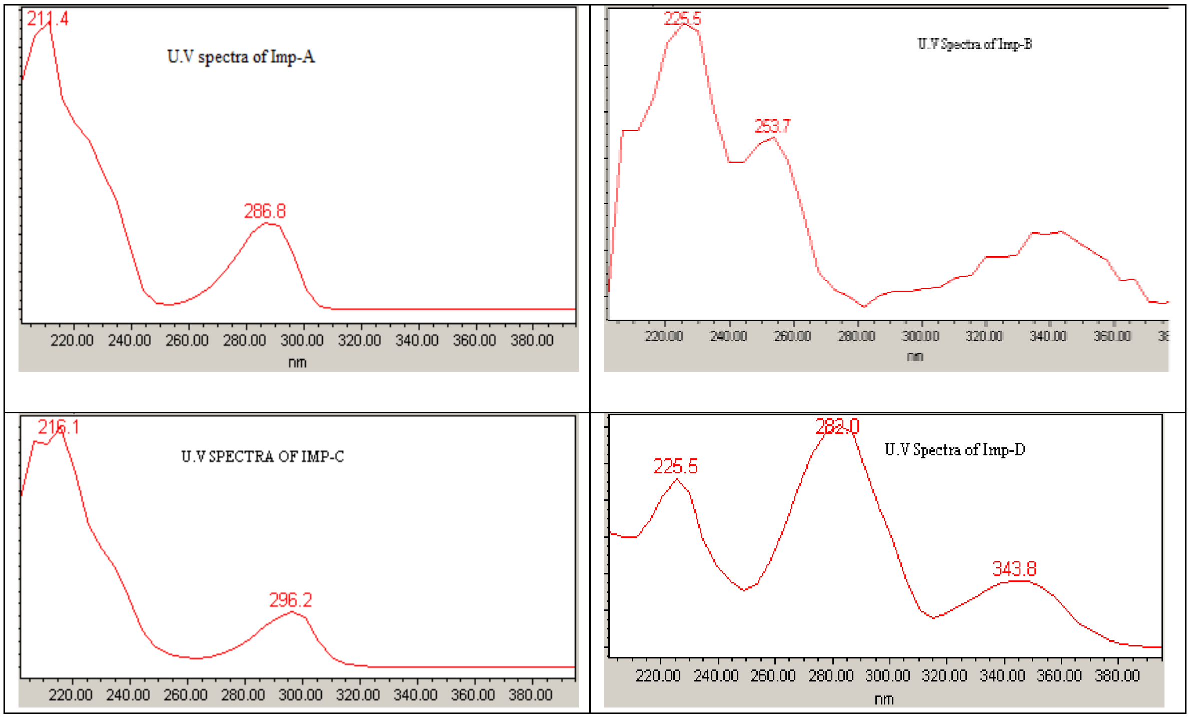
During initial development, impurity blend solution containing four potential impurities, Imp-A, Imp-B, Imp-C and Imp-D at 2 μg/mL spiked to 200 μg/mL of RAM drug substance was used. To confirm stability indicating power of the method, forced degradation samples were also considered for development.

4.3.1. Selection of Diluent

All the known impurities and drug substances were freely soluble in organic solvent, Acetonitrile at 1 mg mL^{-1} concentration level. Hence, initial development trials were made with Acetonitrile as a diluent. To get the gausian peak shape for Imp-A under optimization of chromatographic conditions, the sample is dissolved in 1.0mL of acetonitrile and made upto the volume with the solvent-A.

4.3.2. Selection of Wavelength

Injected Ramelteon, Imp-A, Imp-B, Imp-C and Imp-D in to the UPLC system and extracted the U.V spectra. U.V spectra of Ramelteon, Imp-A, Imp-B, Imp-C and Imp-D were presented as Fig. 4.3.F1. RAM and its compounds showing wavelength maximum around 230 nm, hence it has been selected as UV detector wavelength for LC method development.



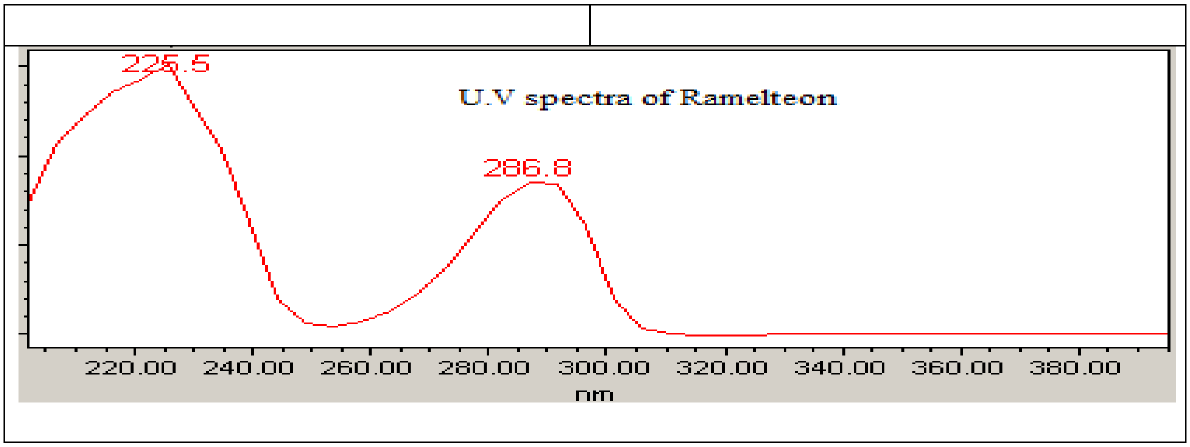


Fig: 4.3.F1. Typical UV spectra of RAM and its related impurities

4.3.3. Method development and optimization.

From the literature it was found that the pKa of the molecule is -0.84. Due to lower pKa of this molecule it was decided to adopt 0.1% Trifluoroacetic acid as solvent A. The blend containing 200 µg/ml of Ramelteon and 2 µg/ml of each impurity (four) was prepared in the mixture of Acetonitrile and solvent A (1:9, v/v). Ramelteon spiked solutions were subjected to separation by reverse-phase LC on a Waters Acquity BEH C18, 50 x 2.1 mm, 1.7µm column with 0.1% of Trifluoroacetic acid as solvent A and Acetonitrile, water (80:20, v/v) as solvent B. Flow rate was set at 0.3mL/min. The UPLC gradient program (T/%B) was set as 0.01/40, 8.0/90, 9.0/90, 9.01/40 and 10.0/40. Column temperature was maintained at 35°C (Trial-1). In this trial one of the unknown impurity closely eluting with Imp-A and other unknown impurity with Imp-C (Resolution<1.5) hence efforts were made to separate these closely eluting pair of compounds. In order to increase the resolution between these pairs of compounds buffer composition was increased from 60 to 90 in the initial gradient step. With this increased buffer composition the retention time of Ramelteon was increased but Imp-A and its adjacent peak was co-eluting. Efforts were made to separate the pairs of compounds on Waters Acquity BEH C18, 100 x 2.1 mm, 1.7µm column. The chromatographic conditions of Trial-1 were employed in this trial. With the increase in column length Imp-A and its adjacent peak were separated (Resolution >2) but the resolution between Imp-C and its adjacent peak was not improved. Various trials were made by changing the gradient compositions but none of the trial could serve the purpose. It was decided to change the column chemistry and Acquity UPLC BEH SHIELD RP18 100 mm, 2.1 mm, and 1.7µm column was used with the conditions mentioned in trial-1. It was found that all the peaks were separated with a resolution greater than 2.

System suitability parameters were evaluated for Ramelteon and its four impurities. Tailing factor for all four impurities and Ramelteon was found less than 1.2. USP Resolution of Ramelteon and four potential impurities was greater than 2.0 for all pairs of compounds.

4.3.4. Optimized chromatographic conditions

Optimized chromatographic conditions for related substances estimation in RAM and quantification of RAM in drug substance was given in Table 4.3.T1.

Table: 4.3.T1. Final chromatographic conditions of RAM method

Column	Waters Acquity UPLC BEH shield RP18, 100mm x 2.1mm, 1.7µ particle size.
Buffer	0.1 % of Trifluroroacetic acid in Milli-Q water
Mobile phase	Solvent-A : Degassed buffer
	Solvent-B : Water : Acetonitrile : 20: 80 (v/v)
Mode of elution	Gradient
Flow rate	0.3 mL min^{-1}
Column temperature	35 °C
Wavelength of detection	230 nm
Injection volume	1.0 µL
Run time	10 min
Diluent	Acetonitrile and Solvent-A in the ratio 1:9 (v/v)
Gradient program	Time (min)/ % Mobile phase- B: 0.01/40, 8.0/90, 9.0/90, 9.01/40 and 10.0/40.
Concentration	For related substances estimation : 200 µg mL^{-1}

The retention times (RTs) and relative retention times (RRTs) of all the known compounds are presented in Table 4.3.T2.

Table: 4.3.T2. RTs and RRTs of known components in finalized method

S.No.	Name of the Analyte	Retention time (min)	Relative retention time w.r.t. RAM peak
1	Imp-A	1.204	0.38
2	Imp-B	2.546	0.81
3	Imp-C	4.274	1.36
4	Imp-D	4.461	1.42
5	RAM	3.134	1.00

4.4. Discussion on Forced degradation studies

Analyzed RAM drug substances were spiked with all the known impurities, Imp-1, Imp-2, Imp-3 and Imp-4 at 0.15% level with respect to test concentration. The spectral homogeneity of main component was ensured in spiked solution. Spiked sample chromatogram with all known impurities and peak purity plot for RAM peak in spiked solution is presented in Fig.4.4.F1.–F2.

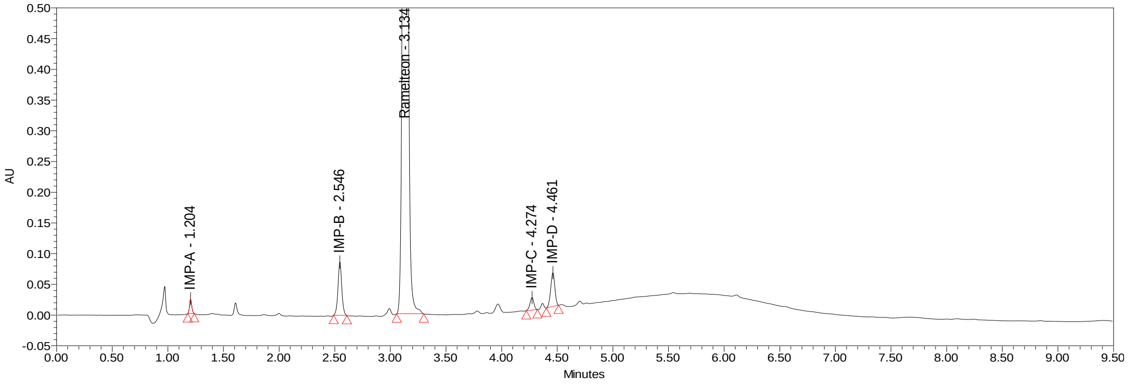


Fig: 4.4.F1. Chromatogram of RAM spiked with all known components

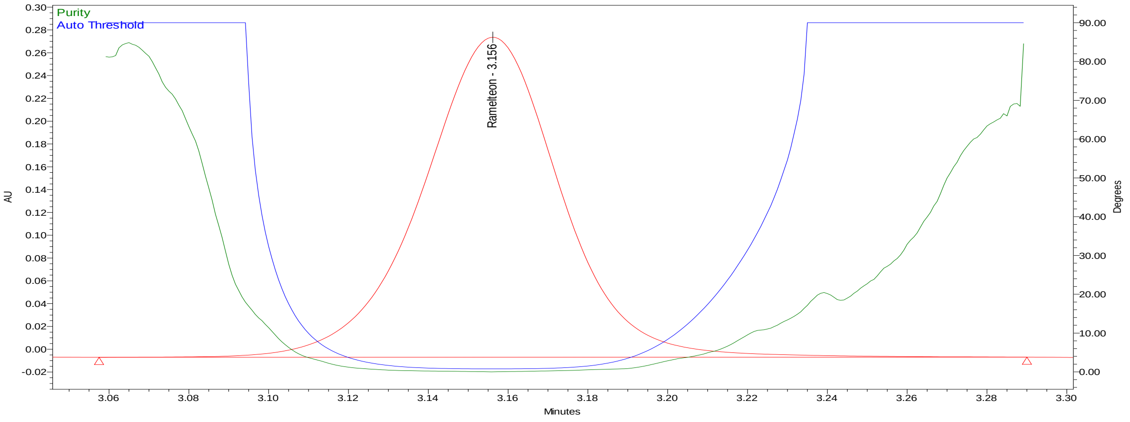


Fig: 4.4.F2. Peak purity plot for RAM peak in spiked solution

All the forced degradation samples were analyzed in HPLC method by using PDA detector. Homogenity of the RAM peak is confirmed in all the degradation samples.

4.4.1. Degradation of RAM during oxidation

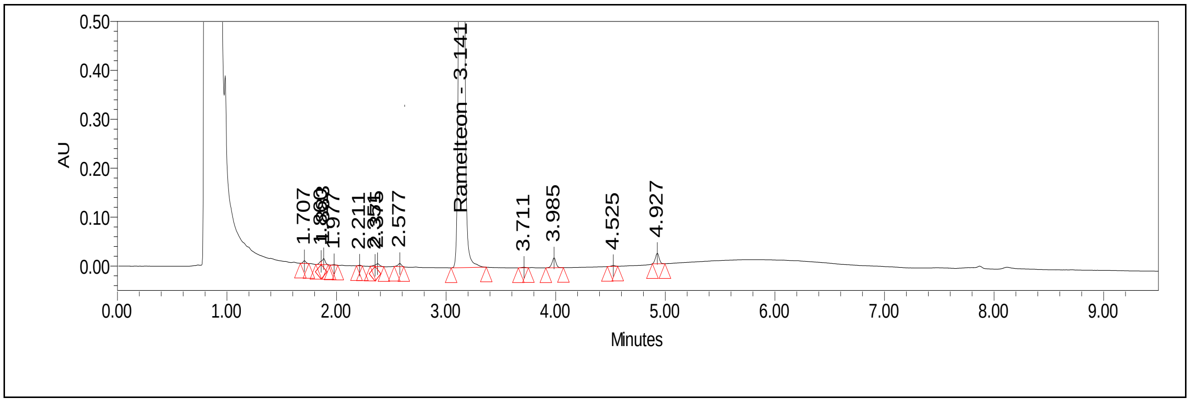


Fig: 4.4.F3. Chromatogram of oxidized RAM sample

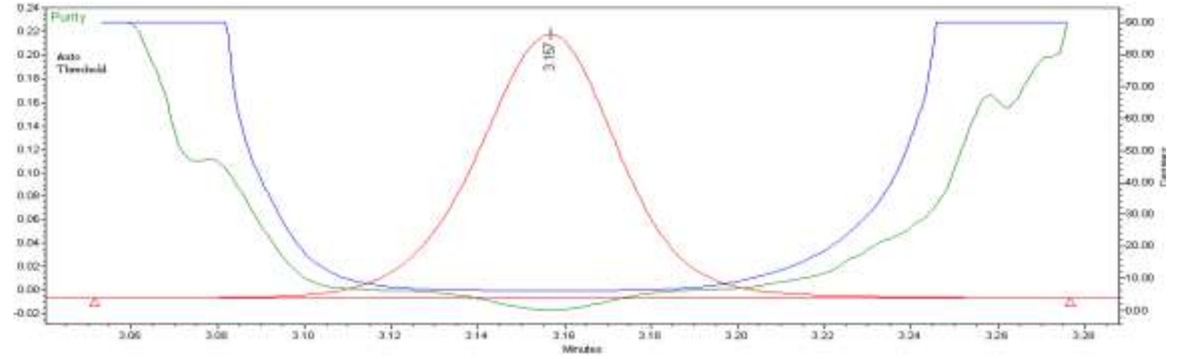


Fig: 4.4.F4. Peak purity plot for RAM peak in oxidized sample

RAM drug substance was sensitive towards the oxidation, it undergone degradation and formed impurity at RRT 1.26 and 1.56. Oxidative degradation conditions and time are discussed in section 4.2.4.3. Peak purity of RAM peak in oxidized drug substances was confirmed as homogeneous. Chromatogram of RAM sample after oxidation and peak purity plot of RAM peak are presented in Fig. 4.4.F3, 4.4.F4.

4.4.2. Degradation during base hydrolysis

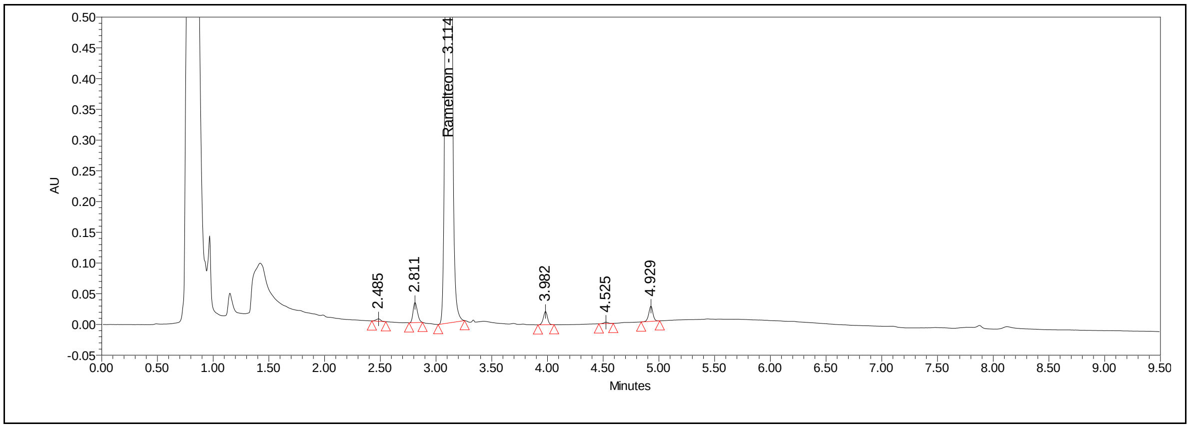


Fig: 4.4.F5. Chromatogram of base hydrolysis RAM sample

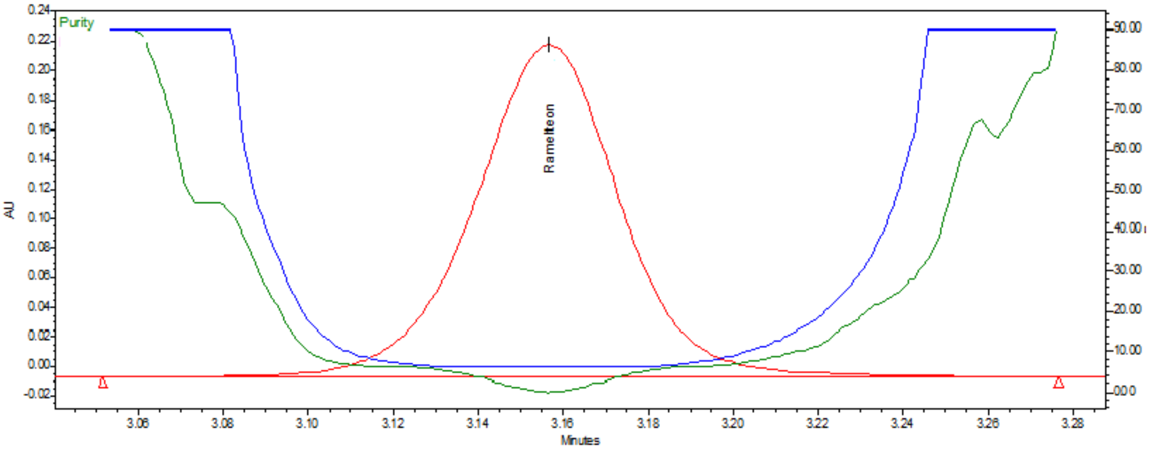


Fig: 4.4.F6. Peak purity plot for RAM peak in base hydrolysis

RAM drug substance was subjected to base hydrolysis by using 0.5N Sodium hydroxide at 60°C temperature. Hydrolyzed sample solution was analyzed and monitored upto 24 h. No significant degradation was observed in basic conditions. Based on peak purity data it was confirmed that the main component in base hydrolyzed sample is homogeneous.

Chromatogram of base hydrolyzed RAM sample and purity plots are presented in Fig.4.4.F5 and Fig.4.4.F6 respectively.

4.4.3. Degradation during Acid hydrolysis

When, RAM drug substance was subjected to acid hydrolysis by using 1 N Hydrochloric acid at 60°C, significant degradation was observed. After 4h of acid hydrolysis, around 12.6% of RAM was getting degraded leading to a major peak at 0.38RRT. Peak purity of RAM peak in acid hydrolyzed sample was found to be homogeneous. LC chromatogram of acid hydrolyzed sample and peak purity plots are shown in Fig. 4.4.F7. and Fig.4.4.F8. respectively.

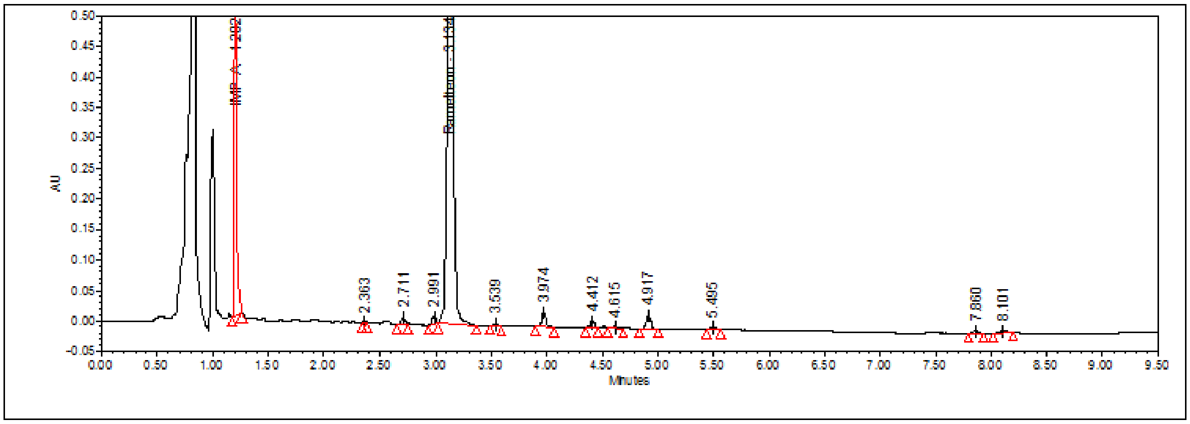


Fig: 4.4.F7. Chromatogram of Acid hydrolysis RAM sample

Impurity at RRT 0.38 was identified by LC-MS analysis. It exhibited molecular ion at m/z 204.4 amu $(M+H)^+$ and corresponds to Imp-A. LC-MS analytical conditions are described in below section 4.4.3.1. Mass spectral data of acid degradation is presented in Fig. 4.4.F9.

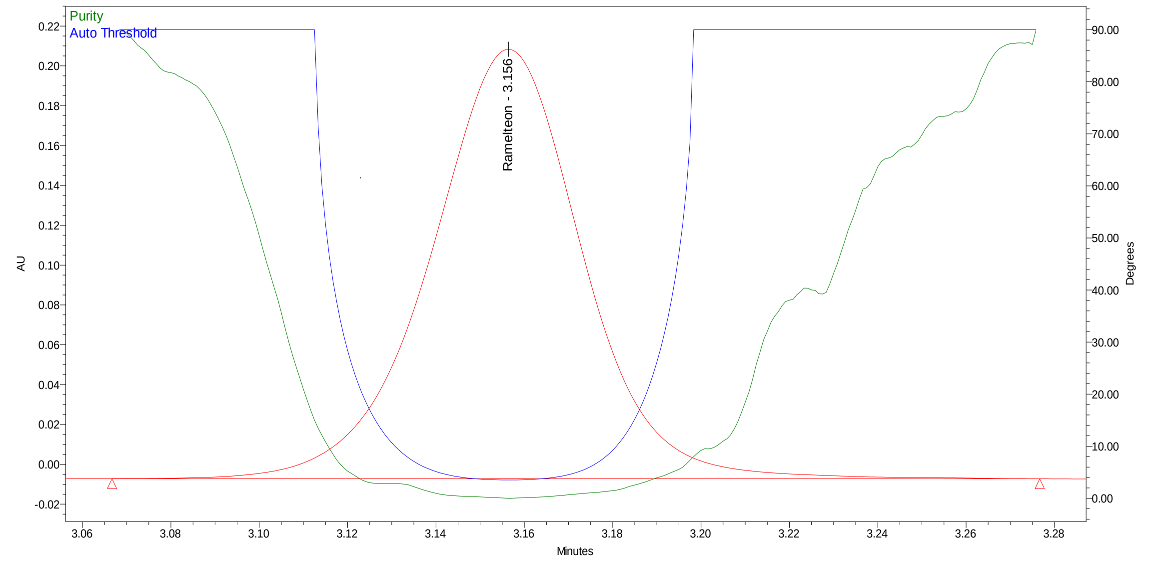


Fig: 4.4.F8. Peak purity plot for RAM peak in acid hydrolysis

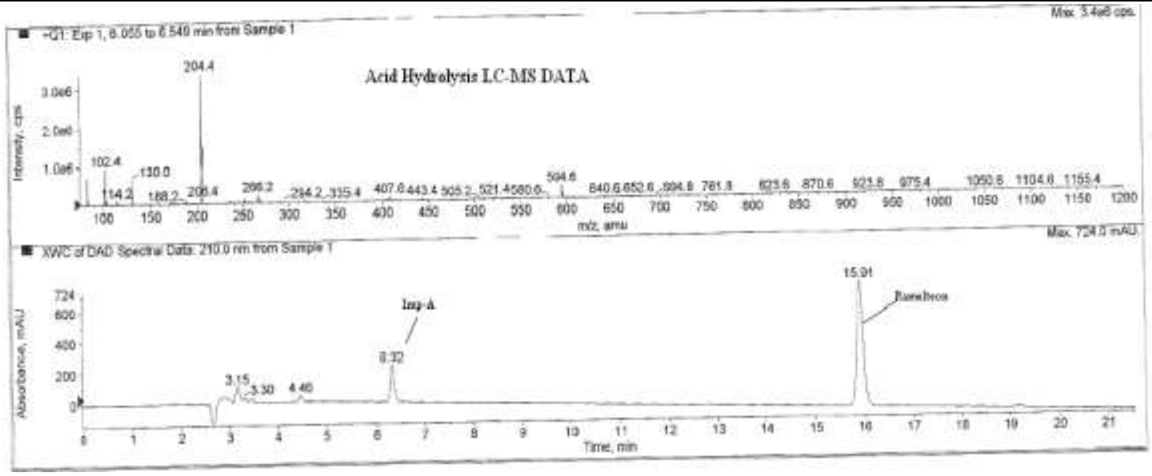


Fig: 4.4.F9. Mass spectra of degradant found during Acid hydrolysis.

4.4.3.1. LC-MS analytical conditions

LC–MS/MS system (Agilent 1200 series liquid chromatography coupled with Applied Biosystems 4000 Q Trap triple quadruple mass spectrometer with Analyst 1.4 software, MDS SCIEX,USA) was used for the generation of mass spectral data of unknown compounds formed during forced degradation studies. Develosil ODS MG-5, 250 x 4.6 mm, 5µm column (Nomura Chemical Co, Japan) was used as stationary phase. 0.1% Trifluoroacetic acid (ACROS ORGANICS, Geel, Belgium) was used as buffer. 100% Buffer was used as solvent A and buffer and acetonitrile in the ratio 15:85, v/v; was used as solvent B. The gradient program (T/%B) was set as 0.01/35, 20/70, 40/80, 45/95, 64/95, 65/35 and 70/35. Mixture of acetonitrile and Solvent A in the ration 1:9, v/v; was used as diluent. The flow rate was 1.0 ml/min. The analysis was performed in positive electro spray positive ionization mode. Ion Source voltage was 5000V. Source temperature was 450 °C. GS1 and GS2 are optimized to 30 and 35 psi respectively. Curtain gas flow was 20 psi.

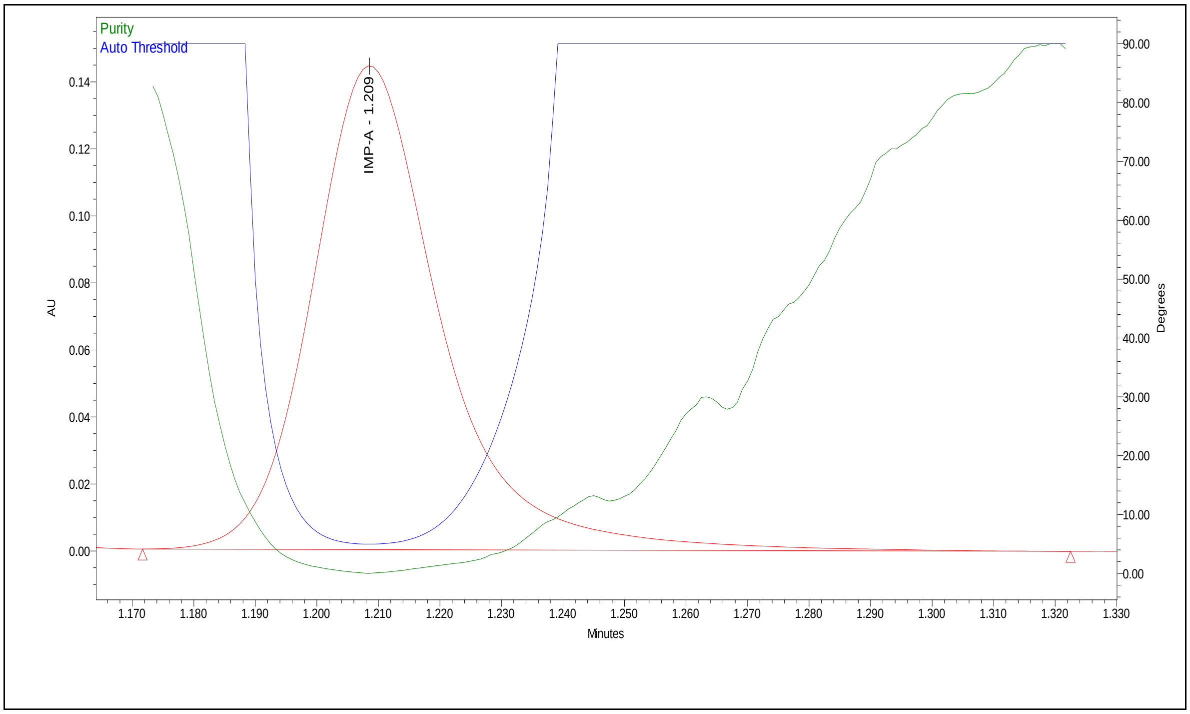


Fig: 4.4.F10. Peak purity plot for acid hydrolysis peak

To further confirm that the acid hydrolysis peak is Imp-A, spiked Imp-A to the acid hydrolysis sample and verified the peak purity. The peak was found to be spectrally pure, confirming that the acid hydrolysis of RAM leads to the formation of Imp-A. Peak purity plot of acid hydrolysis degradant is shown in Fig.4.4.F10.

4.4.4. Degradation during water hydrolysis

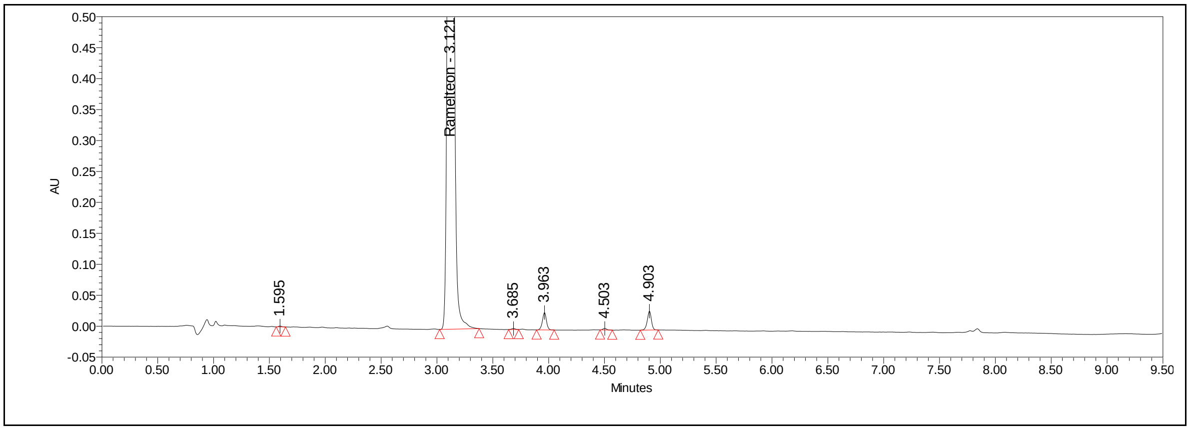


Fig: 4.4.F11. Chromatogram of water hydrolysis RAM sample

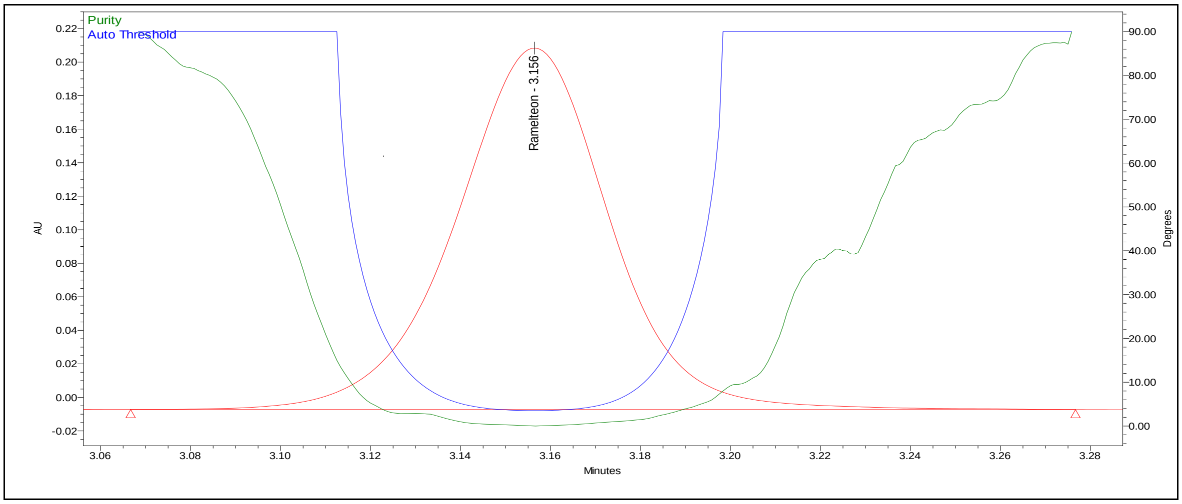


Fig: 4.4.F12. Peak purity plot for RAM peak in water hydrolysis

RAM drug substance is more stable towards water hydrolysis; RAM was not degraded even after 24h of water hydrolysis at 60°C. Peak purity data of RAM peak in final sample indicates that peak is homogeneous.

Chromatogram of stressed sample and peak purity plot of RAM are shown in Fig.4.4.F11. and Fig. 4.4.F12. respectively.

4.4.5. Thermal degradation

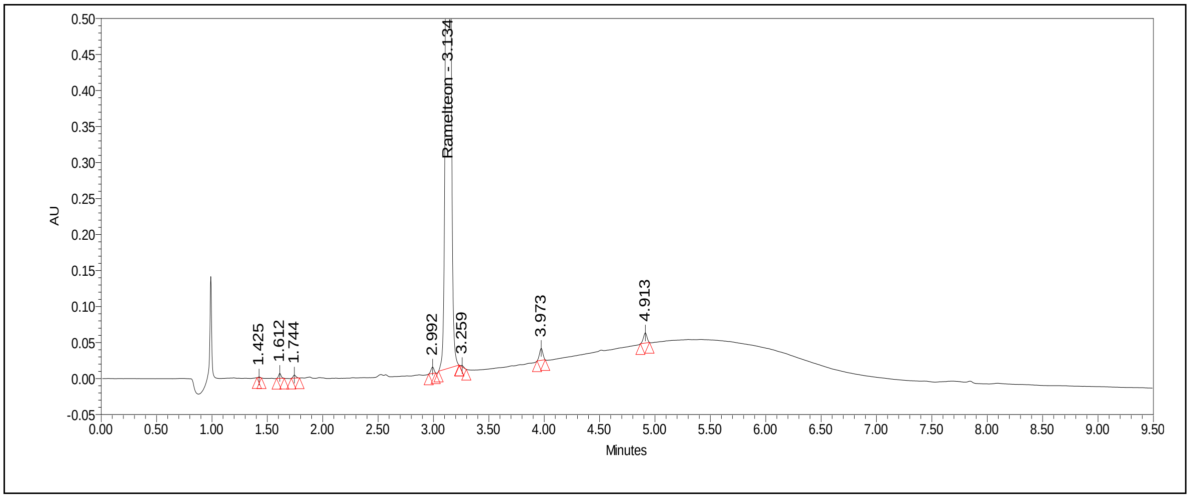


Fig: 4.4.F13. Chromatogram of Thermal degradation RAM sample

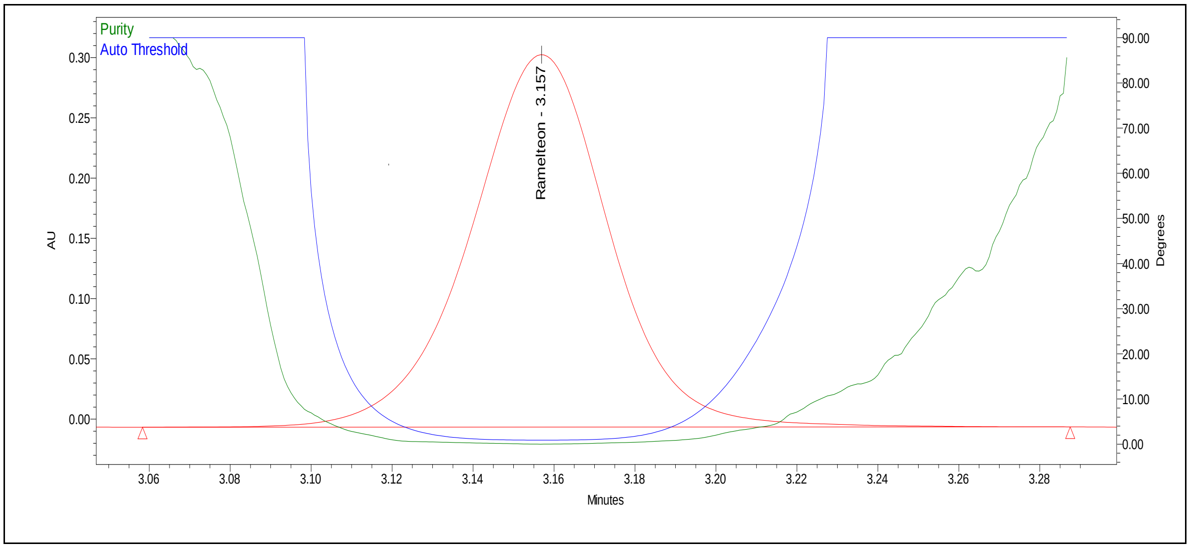


Fig: 4.4.F14. Peak purity plot for RAM peak in Thermal degradation Sample

RAM drug substance was thermally stable. The drug was exposed to 60 °C temperature for 10 days, practically it was not degraded. RAM peak in thermally degraded sample was found to be spectrally pure.

Chromatogram of stressed sample and peak purity plot is shown in Fig.4.4.F13. and Fig.4.4.F14.

4.4.6. Photolytic degradation

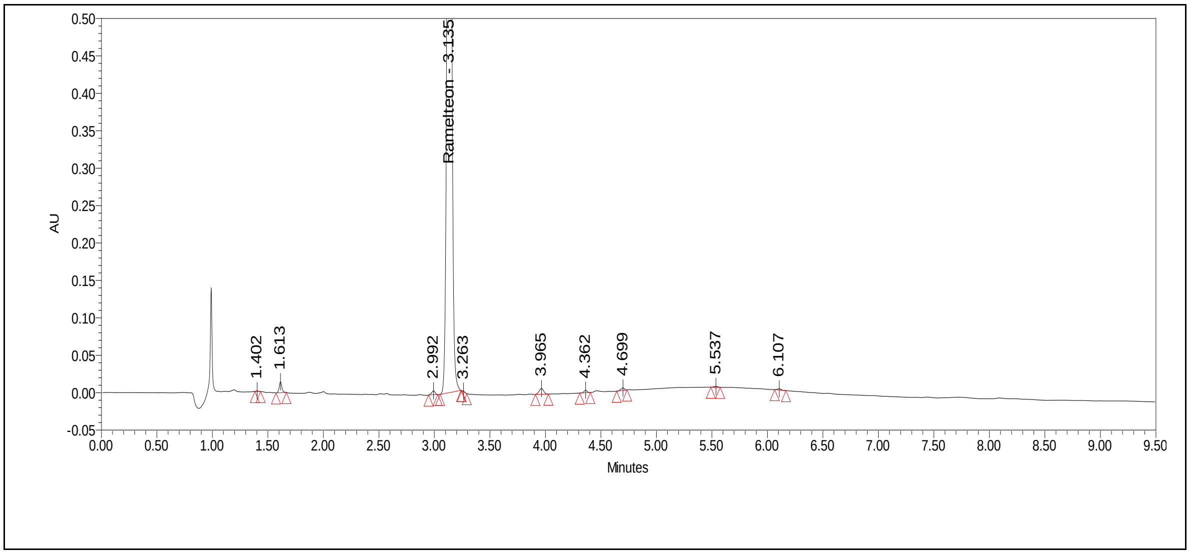


Fig: 4.4.F15. Chromatogram of Photolytic degradation RAM sample

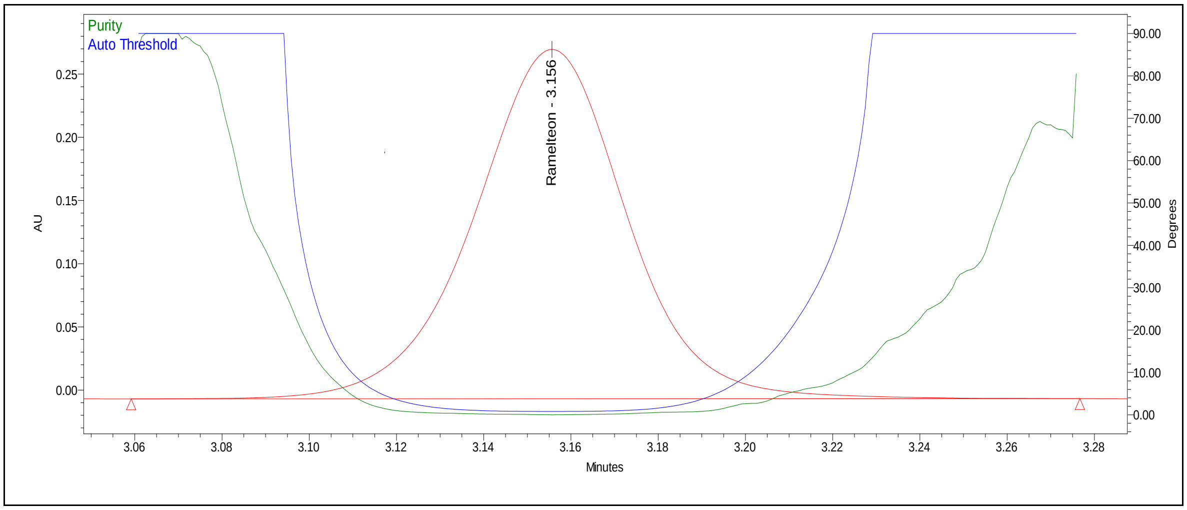


Fig: 4.4.F16. Peak purity plot for RAM peak in photolytic degradation sample

4.4.7. Results of Forced degradation studies

RAM drug substance was exposed to UV and visible radiations for 10 days. No considerable degradation was observed. To confirm the stability of RAM drug substance towards photolytic condition, peak purity of RAM peak was verified in degraded sample and found that it was homogeneous. Chromatogram of photolytic degradation sample and peak purity plot was put on display in Fig. 4.4.F15. and 4.4.F16. respectively. The summary of force degradation studies was reported in Table 4.4.T1.

Table: 4.4.T1. Results of forced degradation studies

Degradation condition	Time	RS by UPLC % of degradation	Remarks/observation
HCl- 1N 60 °C (Acid hydrolysis)	4hr	12.6%	Impurity-A formed
NaOH-0.5N 60 °C (Base hydrolysis)	24hrs	0.3%	No significant degradation observed
Water hydrolysis (60 °C)	24hrs	0.2%	No significant degradation observed
Oxidation by H_2O_2- 3.0% 60 °C	24hrs	1.5%	No significant degradation observed
Thermal (60 °C) solid	10days	0.2%	No degradation observed.
UV at 254nm & 365nm	10days	0.2%	No degradation observed.

4.5. Analytical method validation

The developed and optimized LC method was fully validated as per ICH and USP guidelines.

4.5.1. System suitability test (SST)

Prepared six different test solutions of RAM drug substance, they were spiked with all specified known impurities, Imp-A, Imp-B, Imp-C and Imp-D at 0.15 % level with respect to test concentration. These solutions were injected into LC system. System suitability results were tabulated in Table 4.5.T1. System suitability chromatogram is shown in Fig.4.5.F1.

Table: 4.5.T1. SST results for RAM related substances

Name of the Compound	Retention time (min)	RRT w.r.t. RAM peak
Imp-A	1.204	0.38
Imp-B	2.546	0.81
Imp-C	4.274	1.36
Imp-D	4.461	1.42
RAM	3.134	1.00

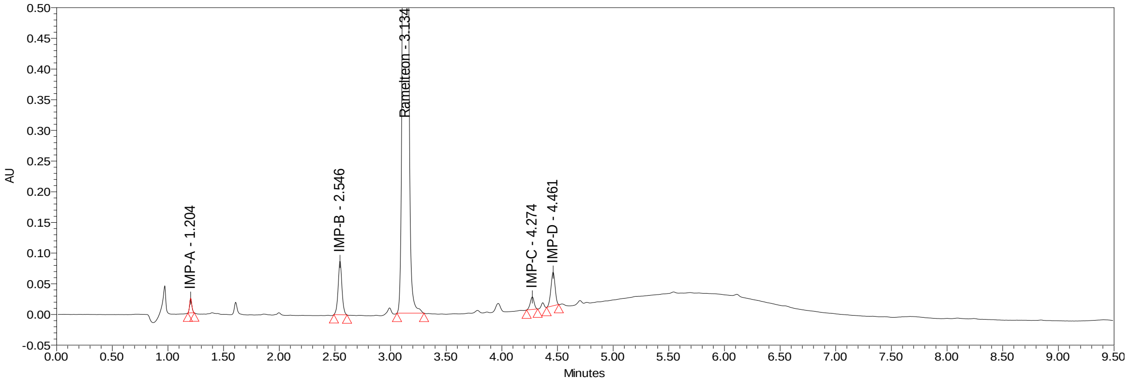


Fig: 4.5.F1. System suitability chromatogram

4.5.2. Precision

Precision study has been evaluated by performing both repeatability and intermediate precision.

To ensure the repeatability of related substances method, six individual preparations of RAM drug substance was prepared and spiked with Imp-A, Imp-B, Imp-C and Imp-D at specification level (0.15%) with respect to test concentration. Later calculated the %RSD for each individual known impurity content. Results are tabulated in Table 4.5.T2.

Table: 4.5.T2. Results of RAM related substances method precision

Sample solution	% of Related substances			
	Imp-A	Imp-B	Imp-C	Imp-D
Preparation-1	0.14	0.16	0.14	0.15
Preparation-2	0.13	0.16	0.14	0.15
Preparation-3	0.13	0.16	0.15	0.14
Preparation-4	0.13	0.16	0.14	0.15
Preparation-5	0.14	0.15	0.14	0.15
Preparation-6	0.14	0.16	0.14	0.15
Mean	0.14	0.16	0.14	0.15
%RSD	3.90	0.87	1.41	0.81

The above results are evidently show that the method is repeatable within acceptable limits of % RSD for six preparations of related substances; 0.81-3.90.

Intermediate precision or Ruggedness of RAM RS method was demonstrated by performing precision study as mentioned in repeatability testing on two different days, by a different analyst, using different lots of reagents, different column and by using different equipment. Then the % RSD for content of each impurity was calculated. Ruggedness of RAM RS method is tabulated in Table 4.5.T3.

Table: 4.5.T3. Results of Ruggedness for RAM RS method

Name of analyte	**%RSD for six different preparation**	
	Day-1	**Day-2**
Imp-A	3.90	1.57
Imp-B	0.87	0.77
Imp-C	1.41	1.10
Imp-D	0.81	1.06

Results have shown insignificant variation in measured response in two different days, which demonstrated that the method was highly precise for its intended use of estimation of related substances of RAM.

4.5.3. Sensitivity

Sensitivity of the method was demonstrated in terms of Limit of Quantitation (LOQ) and Limit of Detection (LOD) values of specified analytes. LOQ, LOD values were established for Imp-A, Imp-B, Imp-C, Imp-D and RAM based on signal to noise ratio of each peak.

Table: 4.5.T4. LOQ values RAM and its related compounds

S.No.	**Name of the analyte**	**LOQ in µg/mL**	**% w.r.t. test concentration**
1	Imp-A	0.07	0.035
2	Imp-B	0.04	0.020
3	Imp-C	0.08	0.040
4	Imp-D	0.06	0.030
5	RAM	0.05	0.025

4.5.3.1 Limit of quantification (LOQ)

Prepared a series of dilutions of RAM, Imp-A, Imp-B, Imp-C and Imp-D in different concentrations and injected them into the liquid chromatograph to get the signal to noise ratio 10 (i.e. 9.5 to 10.0). Limit of quantitation values of all the analytes are presented in Table 4.5.T4.

4.5.3.2. Limit of detection (LOD)

Prepared a series of dilutions of RAM, Imp-A, Imp-B, Imp-C and Imp-D in different concentrations and injected them into the liquid chromatograph to get the signal to noise ratio 2 to 3. Limit of detection values of all the analytes are presented in Table 4.5.T5.

Table: 4.5.T5. LOD values of the impurities and RAM peak

S.No.	Name of the analyte	LOD in µg/mL	% w.r.t. test concentration
1	Imp-A	0.022	0.011
2	Imp-B	0.014	0.007
3	Imp-C	0.025	0.013
4	Imp-D	0.021	0.011
5	RAM	0.018	0.009

4.5.3.3. Precision at Limit of quantification level

Six preparations of Imp-A, Imp-B, Imp-C and Imp-D at LOQ level were injected individually and the % RSD for the areas of each analyte was calculated. No significant variation was observed in the area of each analyte for six consecutive injections, RSD of all the components ranges from 0.8 to 8.3 %. Results are summarized in 4.5.T6.

Table: 4.5.T6. LOQ precision results of RAM related compounds

Preparation	Area of Imp-A	Area of Imp-B	Area of Imp-C	Area of Imp-D
Preparation-1	756	3355	1116	2848
Preparation-2	712	3347	1127	2801
Preparation-3	754	3225	1158	2853
Preparation-4	604	3339	1169	2824
Preparation-5	666	3431	1179	2868
Preparation-6	725	3372	1145	2853
Mean	702.8	3344.8	1149	2841
Stdev.	58.6	67.3	24.4	24.3
%RSD	8.3	2.0	2.1	0.8

Above results indicate that RAM RS method is precise at LOQ level.

4.5.3.4. Accuracy at LOQ level

Table: 4.5.T7. Results of Accuracy at LOQ level

Name	Workup	Amount Added (µg/mL)	Amount obtained (µg/mL)	% Recovery	% Mean Recovery
Imp-A	1	0.0672	0.0636	94.6	105.2
	2		0.0736	109.5	
	3		0.0749	111.5	
Imp-B	1	0.0413	0.0429	104.0	103.3
	2		0.0425	103.0	
	3		0.0425	103.0	
Imp-C	1	0.0758	0.0693	91.4	99.3
	2		0.0792	104.4	
	3		0.0774	102.2	
Imp-D	1	0.0630	0.0658	103.9	104.4
	2		0.0649	104.2	
	3		0.0666	105.1	

RAM sample was injected in test concentration i.e. 200 µg mL-1 to estimate the content of Imp-A, Imp-B, Imp-C and Imp-D. Three different sample solutions (200 µg mL-1) of RAM containing Imp-A, Imp-B, Imp-C and Imp-D at LOQ level were prepared and injected each solution once. From the corrected area of Imp-A, Imp-B, Imp-C and Imp-D, % recovery of each impurity was calculated. Results are summarized in Table 4.5.T7.

4.5.4. Linearity

A series of linearity solutions were prepared containing RAM, Imp-A, Imp-B, Imp-C and Imp-D solution at different concentrations i.e. 0.038 %, 0.075 %, 0.125 %, 0.15 %, 0.19 % and 0.225 % of working concentration (200 µg mL^{-1}) by performing appropriate dilutions to achieve the targeted concentrations.

Table: 4.5.T8. Linearity of Imp-A

S.No.	Conc. (µg mL-1)	Imp-A peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.075	2902	2953	51	2572	38693
2	0.150	5818	5981	163	26644	38787
3	0.225	9460	9010	-450	202731	42044
4	0.300	11794	12038	244	59662	39313
5	0.375	15111	15067	-44	1956	40296
6	0.450	18059	18095	36	1317	40131
Regression coefficient		0.9991	Residual sum of squares		294881	
Slope		40380	Intercept		-76	
% y-Intercept		-0.64	Linearity equation		$y = 40380x - 76$	

The above prepared solutions of RAM and its impurities are 25 %, 50 %, 100 %, 125 % and 150 % to known impurity specification limit i.e. 0.15 %. Each solution was injected once and calibration plots were drawn for concentration of each component versus peak area of corresponding known component. Linearity plot of each analyte with best fit linear equation is shown in Fig.4.5.F2.-4.5.F6. Linear regression analysis was performed for each analyte and data is presented in Table 4.5.T8. - 4.5.T12.

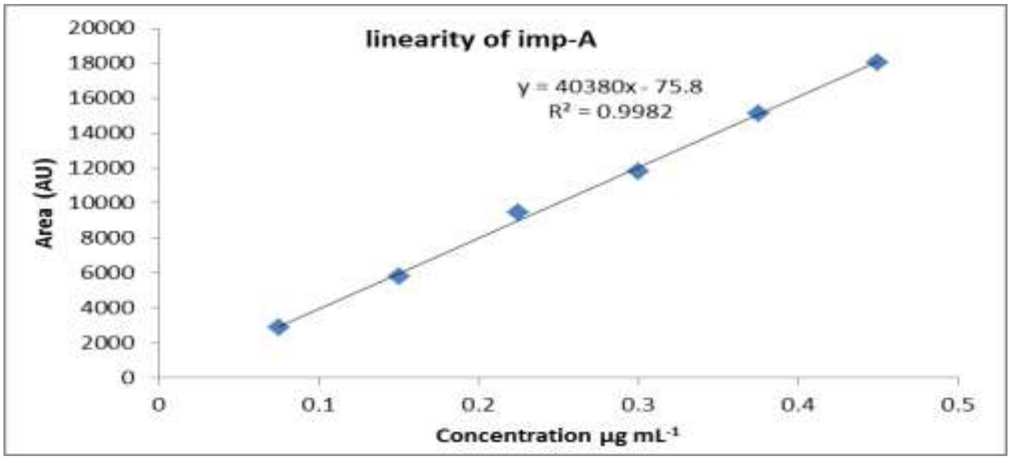


Fig: 4.5.F2. Linearity plot for Imp-A

Regression coefficients for concentration against peak area of all the related compounds of RAM and RAM peak from 25% to 150 % level to the specification limit were more than 0.99. And also % of y-bias with respect to 100 % specification of known compounds is within the limit of ± 5.0. This indicates that a developed related substances by LC method for RAM was linear.

Table: 4.5.T9. Linearity of Imp-B

S.No.	Conc. (µg mL^{-}1)	Imp-B peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.08625	18076	18494	418	175003	209577
2	0.1725	37826	37431	-395	156394	219281
3	0.25875	56962	56367	-595	354342	220143
4	0.345	74148	75303	1155	1333871	214922
5	0.43125	95273	94239	-1034	1068880	220923
6	0.5175	112724	113175	451	203702	217824
Regression coefficient		0.9997	Residual sum of squares		3292192	
Slope		219550				
Intercept		-442				
% y-Intercept		-0.60	Linearity equation		$y = 219550x - 442$	

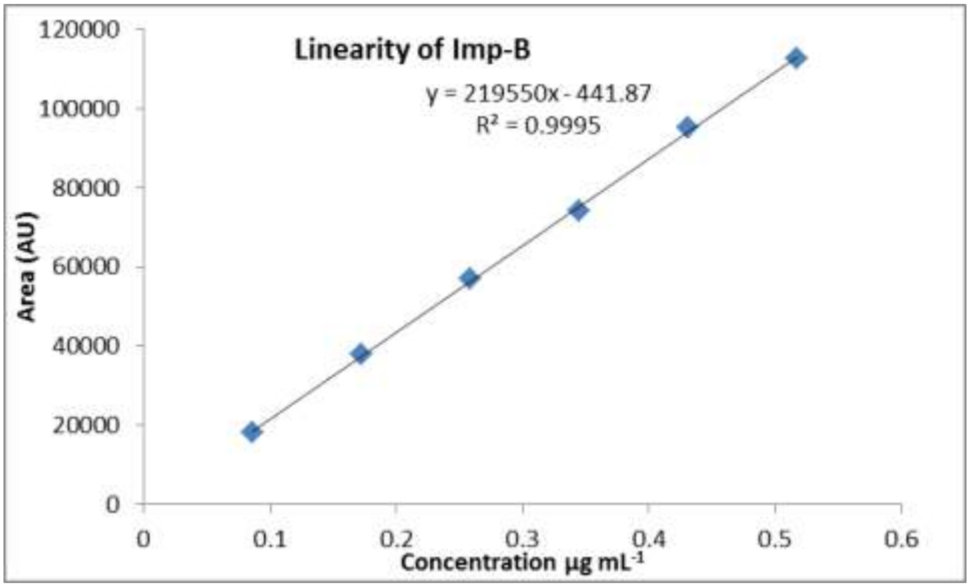


Fig: 4.5.F3. Linearity plot for Imp-B

Table: 4.5.T10. Linearity of Imp-C

S.No.		Conc. (µg mL-1)	Imp-C peak area	Calculated response through trend line equation	Residual	Residual square	Sensi tivity
1		0.084375	7024	6293	93	8693	73481
2		0.16875	12019	11968	-51	2641	71224
3		0.253125	18102	17642	-460	211618	71514
4		0.3375	23050	23316	266	70944	68296
5		0.421875	28404	28991	587	344245	67328
6		0.50625	35100	34665	-435	189142	69333
Regression coefficient			0.9993	Residual sum of squares		827282	
Slope			67252				
Intercept			619				
% y-Intercept			2.68	Linearity equation		y = 67252x +619	

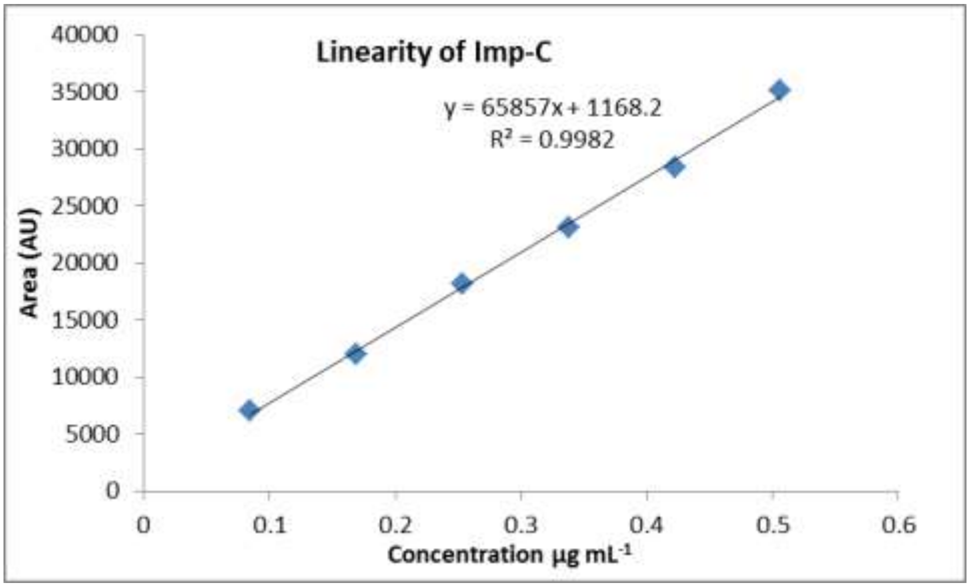


Fig: 4.5.F4. Linearity plot for Imp-C

Table: 4.5.T11. Linearity of Imp-D

S.No.	Conc. (µg mL-1)	Imp-D peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.084375	11332	11964	632	399484	134305
2	0.16875	22625	22559	-66	4335	134074
3	0.253125	33770	33154	-616	379116	133412
4	0.3375	43851	43749	-102	10324	129929
5	0.421875	55191	54345	-846	716554	130823
6	0.50625	63942	64940	998	995244	126305
Regression coefficient		0.9994	Residual sum of squares		2505057	
Slope		125572				
Intercept		1369				
% y-Intercept		3.12	Linearity equation		$y = 32578x - 195$	

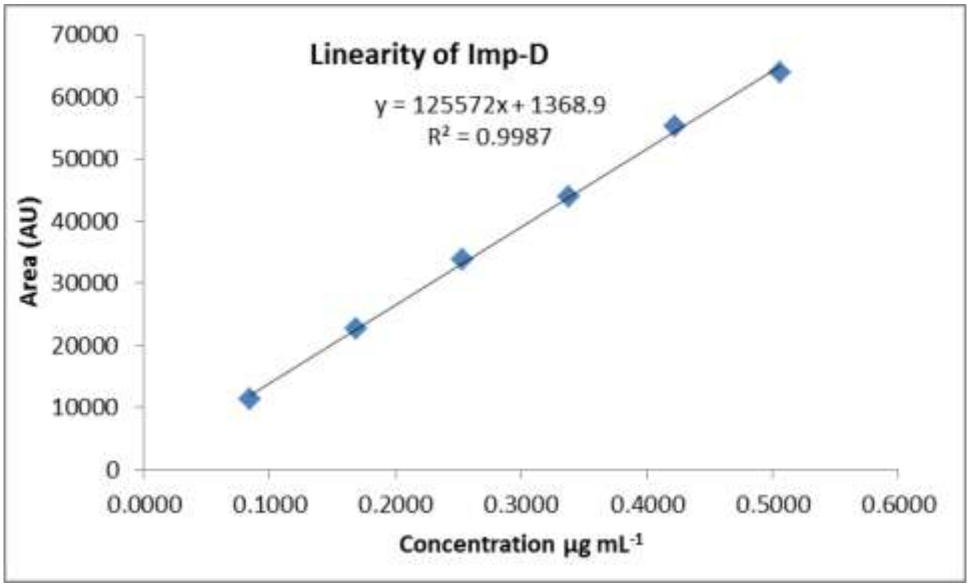


Fig: 4.5.F5. Linearity plot for Imp-D

Table: 4.5.T12. Linearity of RAM for RS method

S.No.	Conc. (µg mL^{-1})	RAM peak area	Calculated response through trend line equation	Residual	Residual square	Sensi tivity
1	0.0675	2416	2662	246	60352	35793
2	0.135	5430	5140	-290	83830	40222
3	0.2025	7685	7619	-66	4321	37951
4	0.27	10065	10098	33	1093	37278
5	0.3375	12516	12577	61	3705	37084
6	0.405	15040	15056	16	245	37136
Regression coefficient		0.9993	Residual sum of squares		153546	
Slope		36723				
Intercept		183				
% y-Intercept		1.82	Linearity equation		$y = 36723x + 183$	

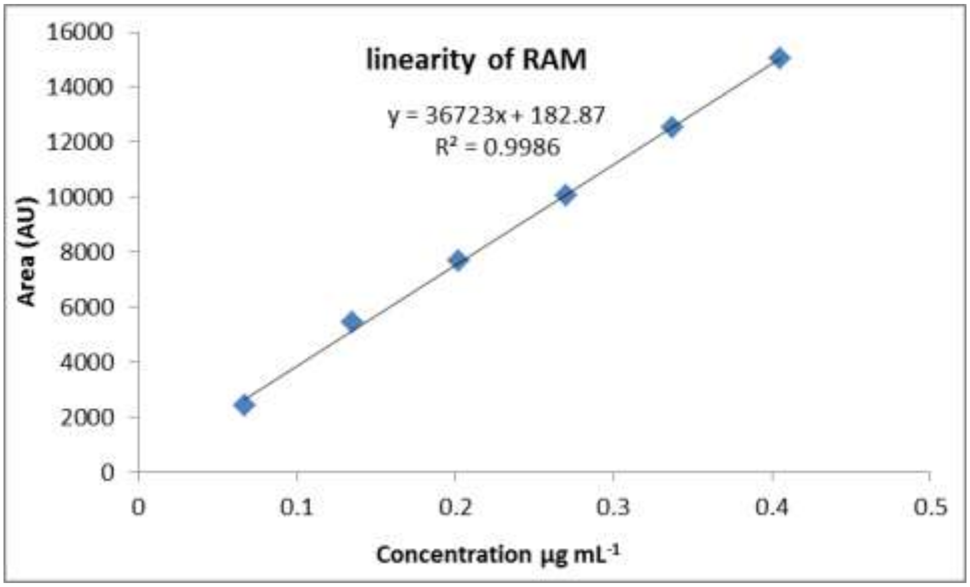


Fig: 4.5.F6. Linearity plot for RAM for RS method

4.5.5. Accuracy

Solutions of Imp-A, Imp-B, Imp-C and Imp-D at three different concentration levels i.e. 50 %, 100 % and 150 % w.r.t. the specification limit (0.15%) of working concentration was spiked with RAM sample solution (conc. 200 μg mL^{-1}), each level was prepared in triplicate and each of nine solutions were injected once. Amount of Imp-A, Imp-B, Imp-C and Imp-D obtained in each solution was calculated as % recovery. Accuracy results at three levels are summarized in Table 4.5.T13.

Table: 4.5.T13. Accuracy results

Name of the analyte	Concentration of analyte w.r.t. specification limit	Amount of impurity spiked to RAM*(μg mL-1)	Amount of impurity recovered (μg mL-1)	%Mean recovery ± SD
Imp-A	50%	0.1500	0.1470	98.0±4.6
	100%	0.3000	0.2867	95.6±3.7
	150%	0.4500	0.4691	104.2±0.2
Imp-B	50%	0.1725	0.1768	102.5±0.4
	100%	0.3450	0.3533	102.4±0.5
	150%	0.5175	0.5361	103.6±0.4
Imp-C	50%	0.1688	0.1840	109.0±3.7
	100%	0.3375	0.3553	105.3±1.5
	150%	0.5063	0.5278	104.2±0.5
Imp-D	50%	0.1688	0.1706	101.1±1.0
	100%	0.3375	0.3411	101.1±0.9
	150%	0.5063	0.5150	101.7±1.2

% recoveries obtained for three different levels ranged from 95.6 to 109. Stand deviation for the average of % recoveries of each individual analyte is less than 5.0. Above accuracy results reveal that this method is highly accurate.

4.5.6. Range

As evident from linearity, accuracy and precision study of related substances method, range has been established for all the analytes i.e. RAM, Imp-A, Imp-B, Imp-C and Imp-D from LOQ to 150 % of specification limit.

4.5.7 Robustness

To evaluate the influence of minute changes in finalized method parameters on separation of known components, robustness study was performed. Study was done by deliberately altering the method conditions from the original method parameters and verified RRTs of impurities and system suitability parameters of standard solution. Method parameters selected for the study were, flow rate (±0.03 mL/min) and column temperature (±5°C). Robustness study data of RAM related substances method is provided in Table 4.5.T14. Above results of RAM RS method robustness study reveal that no significant variation was found in the SST results and RRTs of RAM related substances. Hence, the developed method has been considered as robust.

4.5.8. Conclusion from analytical method validations

The developed RP-LC method developed for quantitative determination of related substances of RAM in drug substance is precise, accurate, selective and linear as per the ICH recommended guidelines. The

Robustness and ruggedness or intermediate precision study reveals that the method is highly rugged and robust for its intended use.

Table: 4.5.T14. Robustness study data

Parameter and Variation	RRTs of impurities				
		Imp-A	**Imp-B**	**Imp-C**	**Imp-D**
As such conditions		0.38	0.81	1.36	1.42
Flow rate (mL min^{-1})					
a. 0.27		0.39	0.82	1.35	1.41
b. 0.33		0.38	0.80	1.38	1.44
Column Temperature (°C)					
a. 30		0.38	0.82	1.38	1.44
b. 40		0.38	0.81	1.35	1.41

4.6. Conclusion

The proposed RP-HPLC method is sensitive, linear, precise and accurate for quantitation of related substances of RAM and its degradation product. As the method was fully validated as per ICH and proved the stability indicating power, it can be used for estimation of impurities in RAM for routine analysis, stability testing in Pharmaceutical quality control labs.

4.7. References

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CHAPTER - 5

Quality by Design approach for the separation of Naproxcinod and its related substances by fused core particle technology column

5.1 Introduction to Naproxcinod and details of available analytical literature

Naproxcinod (NAP), chemically known as (4-(nitrooxy) butyl-(2s)-2-(6-methoxy-2-naphthyl) propanoate (Fig.5.1.F1.), is a nitric oxide-donating cyclooxygenase inhibitor currently under development for Duchenne Muscular Dystrophy (DMD). DMD is the most serious forms of muscular dystrophy, a group of inherited diseases which cause muscle weakness and muscle loss. The molecular formula is $C_{18}H_{21}NO_6$ and molecular weight is 347.4 g/mol [1-12]. It is a derivative of naproxen with a nitroxybutyl ester to allow it to also act as a nitric oxide (NO) donor. This second mechanism of action makes naproxcinod the first in a new class of drugs, the cyclooxygenase inhibiting nitric oxide donators (CINODs), that are hoped to produce similar analgesic efficacy to traditional NSAIDs, but with less gastrointestinal and cardiovascular side effects.. This drug was developed by the French pharmaceutical company NicOx. Complete drug information of Naproxcinod (NAP) is given in Table 5.1.T1.

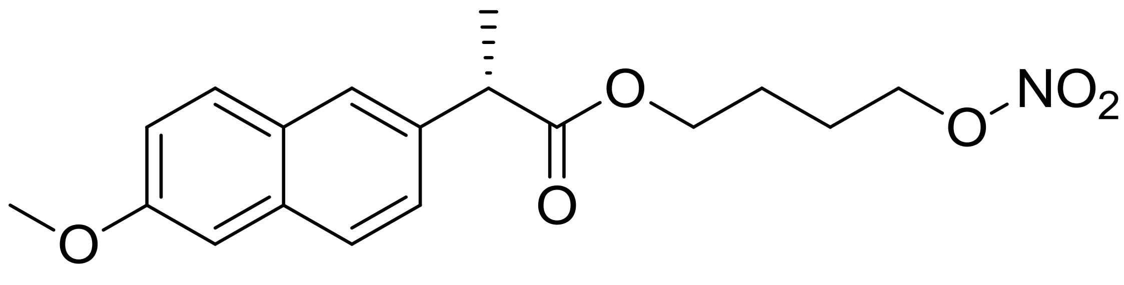


Fig: 5.1.F1. Chemical structure of NAP

Table: 5.1.T1. NAP Drug information

Chemical name	:	(S)-4-(nitrooxy)butyl 2-(6-methoxynaphthalen-2-yl)propanoate
Molecular weight	:	347.4
Molecular formulae	:	$C_{18}H_{21}NO_6$

Quality by design (QbD) is a key principle that has gained much discussion since its initiation as part of the U.S. Food and Drug Administration's vision for the 21st century cGMPs and the International Conference on Harmonisation (ICH) guidance on pharmaceutical development [13] and [14]. The fundamental principle of the initiative is to demonstrate both understanding and control of pharmaceutical processes to deliver high quality pharmaceutical products while affording opportunities for continuous improvement. While it is clear that the initiative is primarily intended for pharmaceutical product development, its use in the development of an integrated control strategy that involves analytical technology and methods should not be underestimated [15].

QbD is defined as a “systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management.” The scientific understanding gained during the method development process can be used to devise method control elements and to manage the risks identified [16]. Analytical literature survey revealed that a few Normal phase Liquid Chromatographic methods were available for determination of enantiomeric purity of Naproxcinod [17-41]. Furthermore, Shiva Raj et al. presented stability indicating Normal phase LC method for Naproxcinod by capturing only Impurities B, G and H [42]. But, RP-HPLC method for the separation and quantification of related substances (Imp-A to Imp-H) and degradation products of NAP is not available in the literature.

In the present study, along with the degradation products of NAP, related substances Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H were considered for analytical method development. The synthetic process (Fig: 5.1.F2.) for the preparation of Naproxcinod (NAP) involved the reaction of Naproxen (Imp-B) with 1-bromo-4-chloro butane to form an intermediate, (S)-4-chlorobutyl 2-(6-methoxynaphthalen-2-yl) propanoate (Imp-F) along with Imp-G as a byproduct. This intermediate (Imp-F) upon reaction with silver nitrate in a suitable solvent medium gave NAP.

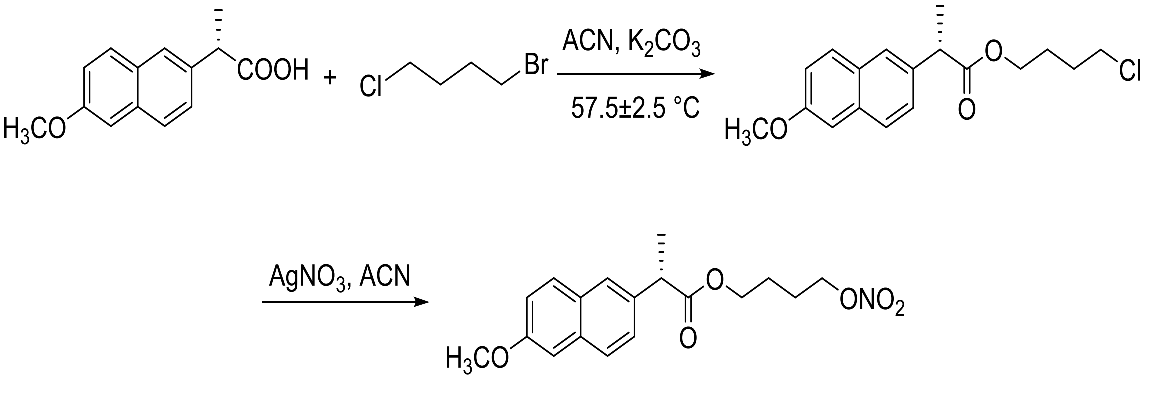
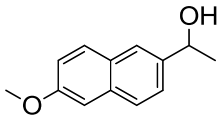
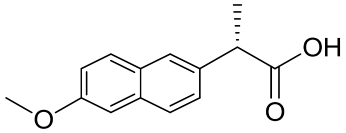
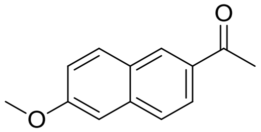
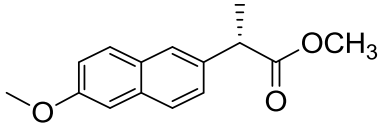
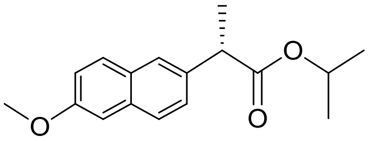
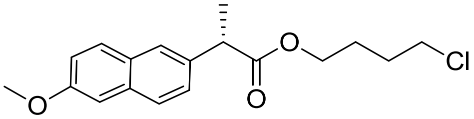
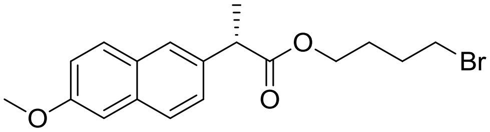
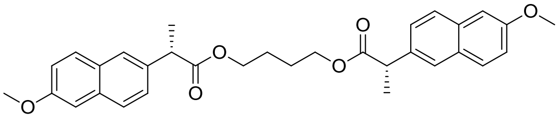


Fig: 5.1.F2. Synthetic scheme of NAP

Imp-C was a key starting material for the synthesis of Imp-B, and the reduction of the same gave Imp-A. As methanol and isopropyl alcohol were used during the synthesis of imp-B, the corresponding esters, i.e., Imp-D and Imp-E were captured. Imp-H was a dimer, formed due to the addition of Imp-B to the Imp-F. Impurities A, C and D were listed in European pharmacopoeia as part of Naproxen (Imp-B) monograph [43]. Chemical structures of possible impurities and degradation products are provided in Table 5.1.T2.

Table: 5.1.T2. Details of related substances of NAP drug substance

Chemical structure of impurity	Chemical name
Imp-A 	1-(6-methoxynaphthalen-2-yl)ethanol
Imp-B 	(S)-2-(6-methoxynaphthalen-2-yl)propanoic acid
Imp-C 	1-(6-methoxynaphthalen-2-yl)ethanone
Imp-D 	(S)-methyl 2-(6-methoxynaphthalen-2-yl)propanoate

Chemical structure of impurity	Chemical name
Imp-E 	(S)-isopropyl 2-(6-methoxynaphthalen-2-yl)propanoate
Imp-F 	(S)-4-chlorobutyl2-(6-methoxynaphthalen-2-yl)propanoate
Imp-G 	(S)-4-bromobutyl 2-(6-methoxynaphthalen-2-yl)propanoate
Imp-H 	(2S,2'S)-butane-1,4-diyl bis(2-(6-methoxynaphthalen-2-yl)propanoate)

Stability testing of drug substance as per ICH and USP specified guidelines [44] is an important task in Pharmaceutical industries. This testing of samples at certain defined intervals under specified climatic conditions like temperature and humidity gives the valuable information on shelf life of the drug substance. Hence, to establish the retest periods, storage conditions and shelf life of drug substance, stability studies have to be performed by using stability indicating analytical methods.

By considering all the cited impurities, a rapid stability indicating chromatographic method was developed and fully validated as per ICH guidelines [45].

5.2 Experimental

5.2.1. Materials

Materials or chemicals used for this study are listed in Table 5.2.T1.

Table: 5.2.T1. Details materials and chemicals

S.No.	Name of the compound	Grade	Procured from../synthesized by..
1.	NAP drug substance samples	--	Dr.Reddy's Laboratories, INDIA
2.	Acetonitrile	HPLC grade	Merck, India
3.	Potassium dihydrogen phosphate	AR grade	Merck, India
4.	Ortho phosphoric acid	AR grade	Merck, India
5.	Water	High pure	Purified by Milli-Q plus system, USA
6.	Imp-A to Imp-H	--	Dr.Reddy's Laboratories, INDIA

5.2.2 Equipments

LC analysis was carried out on a Waters Alliance 2695 HPLC with a 2998 photodiode array detector. The output signal was monitored and processed using Empowers Software. All experimental design work was carried out using 'Design Expert 8.0.6' software by Stat-Ease (Minneapolis, USA).

5.2.3. Preparation of solutions

5.2.3.1. Preparation of impurity standard solution

Impurity stock solution was prepared by dissolving appropriate amount of all the known impurities i.e. Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H in diluent (acetonitrile and water in the ratio of 3:1) to get the final concentration of each impurity in stock solution as 15 μg mL^{-1}. Specification limit for all the known impurities was considered as 0.15 % and prepared impurity blend solution of 0.15 % by spiking appropriate volume of impurity stock solution in μL to 150 μg mL^{-1} NAP drug substance test solution.

5.2.3.2. Preparation of NAP test solution

NAP drug substance stock solution was prepared by weighing 15 mg of drug substance in 100 mL volumetric flask dissolved and diluted to volume with diluent. Final concentration of the solution was 150 μg mL^{-1} of NAP drug substance, which was used for related substances estimation. A solution containing 0.15 μg mL^{-1} was prepared from this stock solution for quantification of related substances.

5.2.4 Preparation of forced degradation samples

As per ICH guidelines to generate degradation samples, one lot of NAP drug substance was selected and subjected to different stress conditions like acid hydrolysis, base hydrolysis, water hydrolysis, oxidation, photo degradation and Thermal degradation.

5.2.4.1. Preparation of Photo degradation sample

For photo degradation study, NAP drug substance was taken in Petri dish and kept in UV cabinet. The sample was exposed to 254 nm and 365nm. After 10 days, sample was taken-off from UV cabinet and test solution of 150 µg mL^{-1} was prepared for related substances analysis.

5.2.4.2. Preparation of Thermal degradation sample

NAP drug substance was placed in Petri dish and spread uniformly. Petri dish was kept in the oven and the temperature of oven was maintained at 90°C for 10 days. After 10 days, NAP samples were taken-off from oven and test solution was prepared to get the final concentration as 150 µg mL^{-1} for related substances by HPLC analysis.

5.2.4.3. Preparation of oxidative degradation sample

15mg of NAP drug substance was transferred to 100 mL volumetric flask, and dissolved in 25 mL of diluent and made up to mark with 3 % peroxide solution. Placed magnetic stirrer in the solution and kept solution at room temperature under continuous stirring for 24 h. The resultant solution was used for related substances testing.

5.2.4.4. Preparation of water hydrolysis sample

15 mg of NAP drug substance was transferred to 100 mL volumetric flask, and dissolved in 50 mL of diluent and made up to mark with water. Placed magnetic stirrer in the solution and kept solution at room temperature under continuous stirring for 24 h. Considered above stressed solution for related substances testing.

5.2.4.5. Preparation of Acid hydrolysis sample

15mg of NAP drug substance was transferred to 100 mL volumetric flask, and dissolved in 25 mL of diluent and made up to mark with 0.1N hydrochloric acid. Placed magnetic stirrer in the solution and kept solution at room temperature under continuous stirring for 24 h. The resultant solution was used for related substances testing.

5.2.4.6. Preparation of Base hydrolysis sample

15mg of NAP drug substance was transferred to 100 mL volumetric flask, and dissolved in 25 mL of diluent, and made up to mark 0.1N Sodium hydroxide solution. Placed magnetic stirrer in the solution and kept solution at room temperature under continuous stirring for 1 h. The final solutins of degradation were used for related substances testing.

5.3 Sequential steps in method development and optimization

During initial development, impurity blend solution containing potential impurities i.e., Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H at specification level in NAP drug substance was used. And to confirm stability indicating power of the method, forced degradation samples were also considered for development. Analytical method was developed by following Quality by Design approach.

5.3.1. Defining the method objectives:

An important consideration in developing impurity profiling methods is to appropriately define the requirements of the method. In a quality by design approach, this involves establishing what impurities need to be separated and eluted from the chromatographic column followed by detection. Examination of the route of synthesis for the compound of interest, and structurally similar compounds is often a good starting point to define the impurities that may be considered in method development. The objectives for development can be defined as 1) the need to retain Imp-B, a polar degradation product and to elute potential non-polar reaction by-product, Imp-H in a reasonable analysis time (2) resolve known and potential process related impurities from the API and (3) user-friendly methodology for quality control laboratories.

5.3.2. Selection of Diluent

Based on solubility of NAP and all the known impurities, Acetonitrile and water in the ratio of 3:1 was selected as diluent.

5.3.3. Selection of Wavelength

10 ppm solution of each known impurity and NAP Drug substance were prepared in Acetonitrile diluent. By using UV Visible spectrophotometer, the solution was scanned in the UV range of 200-400 nm against Acetonitrile as blank. Overlaid UV spectra of all the related components and NAP were given in Fig. 3.3.F1. NAP and its compounds have shown maximum wavelength of 230 nm, hence it has been selected as UV detector wavelength for LC method development.

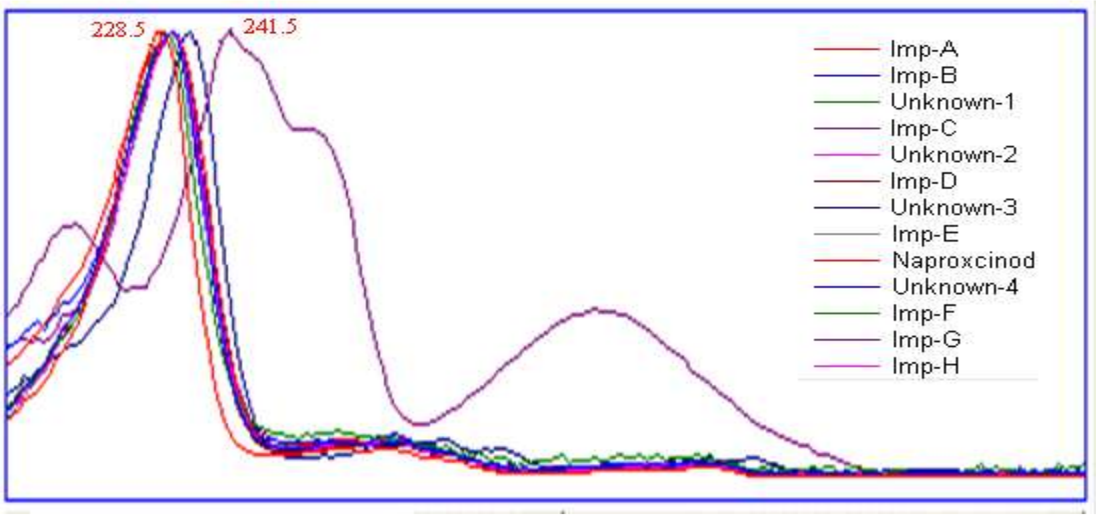


Fig: 5.3.F1. Typical UV spectra of NAP and its related impurities

5.3.4. Selection of Column

From the chemical structures of the impurities and NAP it was evident that they are hydrophobic in nature. Since the objective was to develop a rapid HPLC method, a C8 stationary phase was selected to reduce the hydrophobic interactions between the stationary phase and impurities.

Initial trials were made with 0.1% ortho phosphoric acid as mobile phase-A and methanol and water in the ratio 75:25(v/v) as mobile phase-B. The wavelength selected was 230nm and the column oven was maintained at 35°C. The gradient programme was (T/%B) 0.01/50, 15/85, 30/100, 54/100, 55/50, 60/50. Trials were made with different C8 columns. The resolution between two critical pairs, i.e., Imp-C/Unk-2 and Imp-E/NAP using different C8 columns is depicted in Table 3.3.T1. None of the trials met the objective of rapid resolution of impurities and

NAP as the NAP was retained up to 23 minutes and Imp-H was retained up to 40 minutes. However, based on this data, Ascentis express C8, 150 X 4.6, 2.7µm column was selected for further trials as the resolution achieved between the two critical pairs was more than 2.0.

Table: 5.3.T1. Trials on different C8 columns

S. No	**Column Name**	**Resolution**	
		Imp-C/Unk-2	**Imp-E/NAP**
1	Symmetry shield RP 8 150 X 4.6, 3.5µm	Less than 1.0	2.28
2	Zorbax Eclipse C8 150 X 4.6, 5µm	1.6	Less than 1.0
3	Inertsil C8-3 150 X 4.0, 5.0µm	1.1	Less than 1.0
4	Betasil C8 150 X 4.6, 5µm	1.2	Less than 1.0
5	YMC Pak Pro C8 150 X 4.6, 5µm	1.7	Less than 1.0
6	Xterra RP 8 150 X 4.6, 5µm	Less than 1.0	2.0
7	Ascentis Express C8 150 X 4.6, 2.7µm	3.5	2.5

5.3.5. Selection of buffer pH and gradient optimization:

Further trials were made with Ascentis express 150 X 4.6, 2.7 µm column employing a mixture of 0.02M KH2PO4 and 0.2% OPA as buffer (pH of the solution ~2.5). The mobile phase-A was buffer and acentonitrile in the ratio of 80:20(v/v). The mobile phase B was buffer and acentonotrile in the ratio 20:80(v/v). The initial gradient programme was (T/%B) 0.01/20, 25/100, 27/20, 30/20. The column oven was maintained at 35°C. The effect of pH of buffer on the elution of impurities was studied by injecting the impurity blend solution at buffer pH 2.5, 3.5 and 6.0. A graph representing the retention times of impurities at different buffer pH was shown in figure 3.3.F2. Based on results, pH 2.5 was selected. The final optimized gradient programme was (T/%B) 0.01/32.0, 17.0/90.0, 17.01/32.0, 20.0/32.0. Here all the peaks were separated with an Rs >1.5.

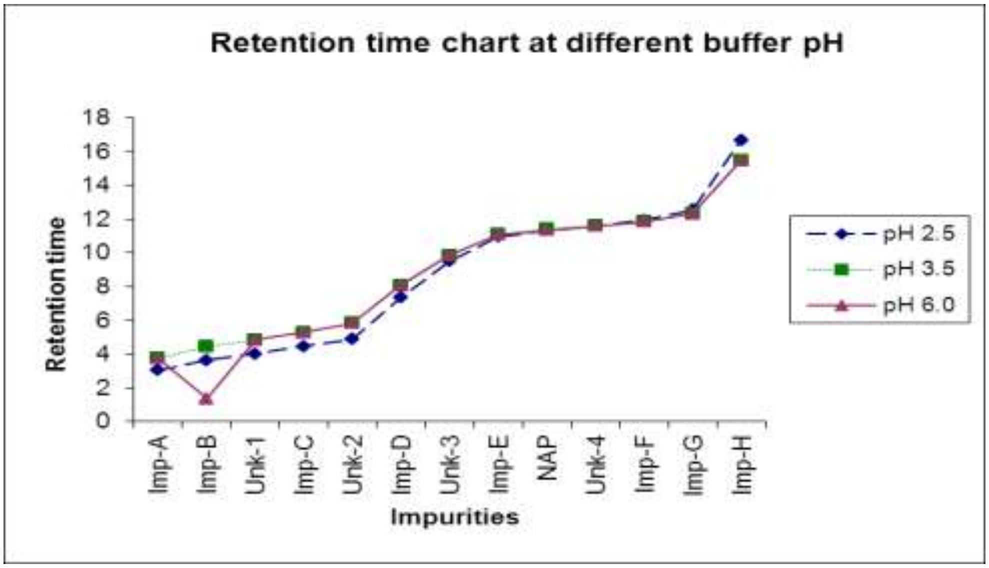


Fig: 5.3.F2. Effect of buffer pH on resolution

5.3.6. Design of Experiments

5.3.6.1. Identification of critical analytical method factors:

On the basis of the preliminary experiments, it was understood that, flow rate (factor A), Column temperature (factor B) and % of acetonitrile solvent-B (factor C) has huge influence on the separation of compounds.

5.3.6.2. Experiment design:

Factors and their “low” (−) and “high” (+) and “centre points” (0) levels are presented in the 5.3.T2.

Table: 5.3.T2. Factors and levels

Factors	**Levels**		
	-1	**0**	**+1**
Factor-A (Flow rate)	0.8mL/minute	1.0mL/minute	1.2mL/minute
Factor-B (Temperature)	30°C	35°C	40°C
Factor-C (% of acetonitrile in solvent-B)	72%	80%	88%

To screen the relative influence of these factors and their possible interactions in the experimental domain, Central composite design was chosen, which will study the effects of the selected three factors in twenty runs, including six centre points. As the parameters to define chromatographic behavior of investigated substance and its impurities, resolution between two critical pairs i.e., Unk-1, Impurity-C (Rs-1) and Impurity-E, NAP (Rs-2) was chosen. The design and the responses obtained against the experiments are presented in 5.3.T3. The same information gathered from CCD experiments provided the method robustness evaluation as well.

Table: 5.3.T3. Design of Experiments and responses

Run No	Factor-A (Flow rate)	Factor-B (Temperature)	Factor-C (% acetonitrile in solvent- B)	R_s-1	R_s-2
1	-1	0	0	2.0	1.6
2	0	0	0	1.7	1.9
3	-1	-1	1	2.8	1.6
4	1	1	-1	0.0	2.5
5	-1	-1	-1	2.5	1.8
6	-1	1	-1	1.1	1.7
7	0	-1	0	2.4	2.0
8	0	1	0	1.2	1.8
9	1	0	0	1.5	2.3
10	0	0	1	1.8	1.9
11	0	0	0	1.7	2.0
12	0	0	0	1.7	1.9
13	1	-1	-1	1.9	2.6
14	1	-1	1	2.2	2.3
15	-1	1	1	1.4	1.6
16	0	0	-1	1.4	2.2
17	1	1	1	1.1	2.1
18	0	0	0	1.6	1.9
19	0	0	0	1.7	1.9
20	0	0	0	1.7	1.9

5.3.5.3. DoE – Results & Discussion:

ANOVA table for response Rs-1 is represented in Table 5.3.T4. According to the ANOVA for the response Rs-1, the model F-value was 74.36, implied that the model is significant. Values of "Prob > F" for the terms A, B, C, AC, BC were 'less than 0.0500' indicated that these model terms are significant. The fraction of explained variation by means of coefficient of determination R2 was also evaluated. The "Pred R-Squared" of 0.8650 is in reasonable agreement with the "Adj R-Squared" of 0.9508.

Table: 5.3.T4. ANOVA Table for response Rs-1

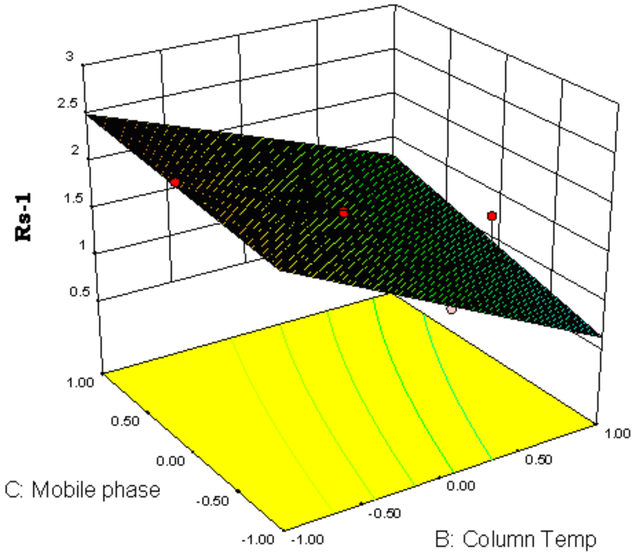
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model *significant*	6.53	5	1.31	74.36	< 0.0001
A-Flow rate	0.91	1	0.91	51.55	< 0.0001
B-Column Temp	4.89	1	4.89	278.00	< 0.0001
C-Mobile phase	0.57	1	0.57	32.23	< 0.0001
AC	0.084	1	0.084	4.78	0.0462
BC	0.092	1	0.092	5.26	0.0378
Residual	0.25	14	0.018		
Lack of Fit *significant*	0.24	9	0.027	17.59	0.0028
Pure Error	7.533E-003	5	1.507E-003		
Cor Total	6.78	19			
Std. Dev.	0.13			R-Squared	0.9637
Mean	1.67			Adj R-Squared	0.9508
C.V. %	7.92			Adj R-Squared	0.8650
PRESS	0.92			Adeq Precision	34.098

ANOVA table for response Rs-2 is represented in Table 5.3.T5. According to the ANOVA for the response Rs-2, the model F-value was 204.85, implied that the model is significant. Values of "Prob > F" for the terms A, B, C, AC, C2 were 'less than 0.0500' indicated that these model terms are significant. The fraction of explained variation by means of coefficient of determination R2 was also evaluated. The "Pred R-Squared" of 0.9611 is in reasonable agreement with the "Adj R-Squared" of 0.9817.

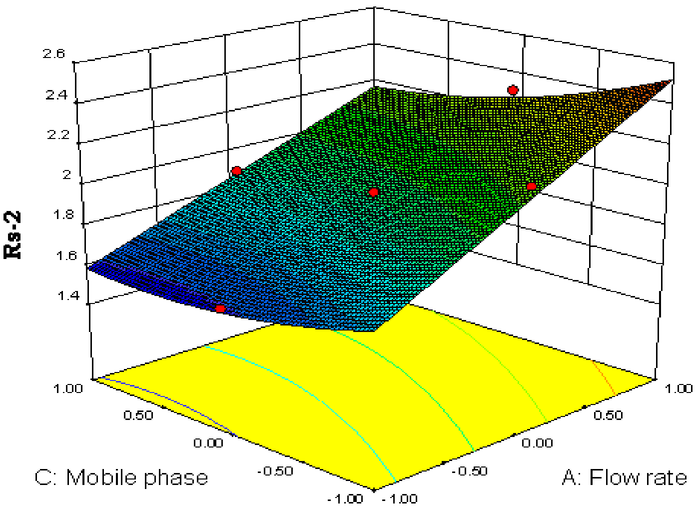
Table: 5.3.T5. ANOVA Table for response Rs-2

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model *significant*	1.43	5	0.29	204.85	< 0.0001
A-Flow rate	1.16	1	1.16	834.09	< 0.0001
B-Column Temp	0.059	1	0.059	42.53	< 0.0001
C-Mobile phase	0.16	1	0.16	115.69	< 0.0001
AC	0.017	1	0.017	12.27	0.0035
C^2	0.027	1	0.027	19.64	0.0006
Residual	0.020	14	1.394E-003		
Lack of Fit *not significant*	0.017	9	1.863E-003	3.39	0.0962
Pure Error	2.750E-003	5	5.500E-004		
Cor Total	1.45	19			
Std. Dev.	0.037			R-Squared	0.9865
Mean	1.97			Adj R-Squared	0.9817
C.V. %	1.90			Adj R-Squared	0.9611
PRESS	0.056			Adeq Precision	53.299

According to the values of coefficients of polynomial model of CCD, the resolution between Unk-1 and Impurity-C (Rs-1) was significantly influenced by factor-B (column temperature) and the resolution between Impurity-E and NAP (Rs-2) was affected by factor-A (flow rate), when compared to the other individual factors. The combined effect of the model term BC for the response Rs-1 and AC for the response Rs-2 was found to be more effective when compared to the other combinations of model terms. Figure 5.3.F3 represents the responses that were plotted in the form of three-dimensional response surfaces in order to easily and more precisely define the chromatographic behavior of the investigated substances.



A) The variation of response (Rs-1;Unk-1/Imp-C) as a function of acetonitrile content in solvent-b and column temperature; fixed factor is flow rate =1.0 mL/min



B) The variation of response (Rs-2; Imp-E/Nap)as a function of acetonitrile content in solvent-b and flow rate; fixed factor is column temperature T=35°C

Fig: 5.3.F3. 3D plots of the response surface for the resolution

The final equation for resolution Rs-1 and Rs-2 in terms of coded factors is represented as

Rs-1=1.67 - 0.30 X A - 0.70 X B + 0.24 X C + 0.10 X A X C+0.11 X B X C

Rs-2=1.93 + 0.34 X A - 0.077 X B - 0.13 * C - 0.046 * A * C + 0.074 * C2

In order to achieve the best separation performances and the reasonable retention of all substances in the gradient system, the data analysis led to the conclusion that the final composition of the mobile phase should contain 80% of Acetonitrile and 20% buffer. The temperature of the column should be maintained at 35°C and the flow rate should be maintained at 1.0mL/min.

5.3.7. Optimized chromatographic conditions

Optimized chromatographic conditions for related substances estimation in NAP and quantification of NAP in drug substance was given in Table 5.3.T6.

Table: 5.3.T6. Final chromatographic conditions of NAP method

Column	Ascentis express C-18 column, 150 mm, 4.6mm, 2.7 µm particle size
Buffer	Mixture of 0.02M KH2PO4 and 0.2% ortho phosphoric acid
Mobile phase	Mobile phase-A : Buffer : Acetonitrile : 80: 20 (v/v)
	Mobile phase-B : Buffer : Acetonitrile : 20: 80 (v/v)
Mode of elution	Gradient
Flow rate	1.0 mL min^{-1}
Column temperature	35 °C
Wavelength of detection	230 nm
Injection volume	10 µL
Run time	65 min
Diluent	Acetonitrile and water in the ratio of 3:1
Gradient program	Time (min) / % Mobile phase- B: 0.01/32, 17.0/90, 17.01/32 and 20.0/32.
Concentration	For related substances estimation : 150 µg mL^{-1}

The retention times (RTs) and relative retention times (RRTs) of all the known compounds are presented in Table 5.3.T7. Typical chromatogram representing NAP and its known and unknown impurities is presented in Figure 5.3.F4.

Table: 5.3.T7. RTs and RRTs of known and unknown components in finalized method

S.No.	Name of the Analyte	Retention time (min)	Relative retention time w.r.t. NAP peak
1	Imp-A	~2.8	~0.27
2	Imp-B	~3.3	~0.31
3	Unk-1	~3.8	~0.36
4	Imp-C	~4.0	~0.38
5	Unk-2	~4.4	~0.42
6	Imp-D	~6.7	~0.64
7	Unk-3	~8.7	~0.83
8	Imp-E	~10.1	~0.96
9	NAP	~10.5	-
10	Unk-4	~10.7	~1.02
11	Imp-F	~11.0	~1.05
12	Imp-G	~11.5	~1.10
13	Imp-H	~15.7	~1.50

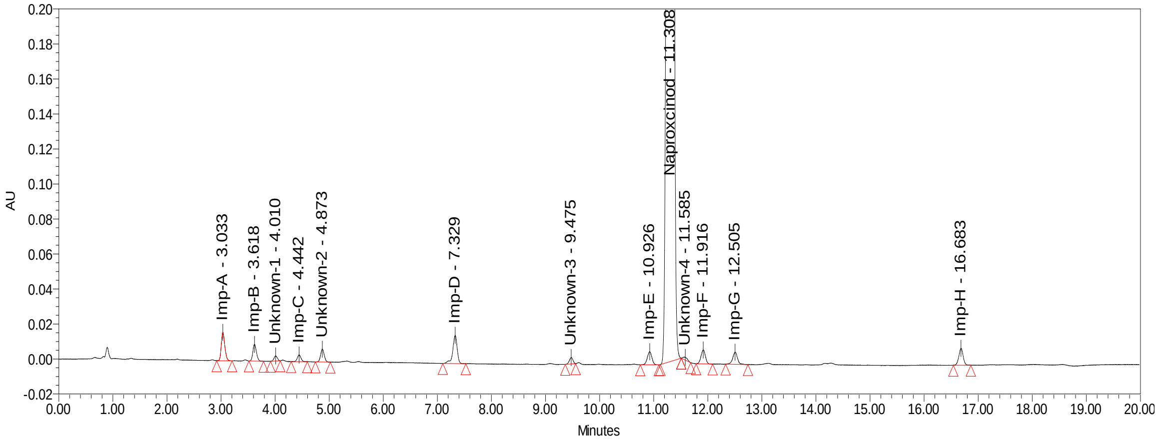


Figure: 5.3.F4. Typical chromatogram representing NAP and its known and unknown impurities

5.4 Discussion on Forced degradation studies

Forced degradation samples were analyzed by HPLC method having PDA detector. Homogeneity of NAP peak in all the degradation samples was confirmed.

5.4.1. Degradation of NAP during oxidation

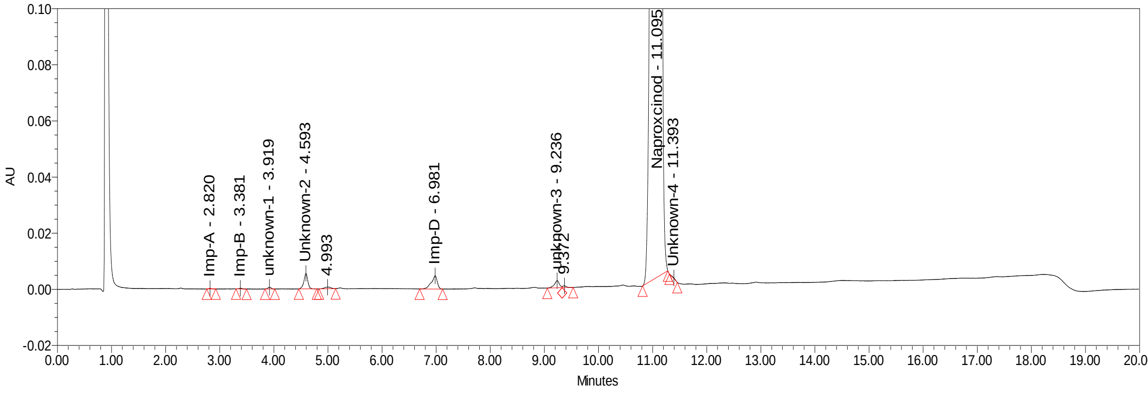


Fig: 5.4.F1. Chromatogram of oxidized NAP sample

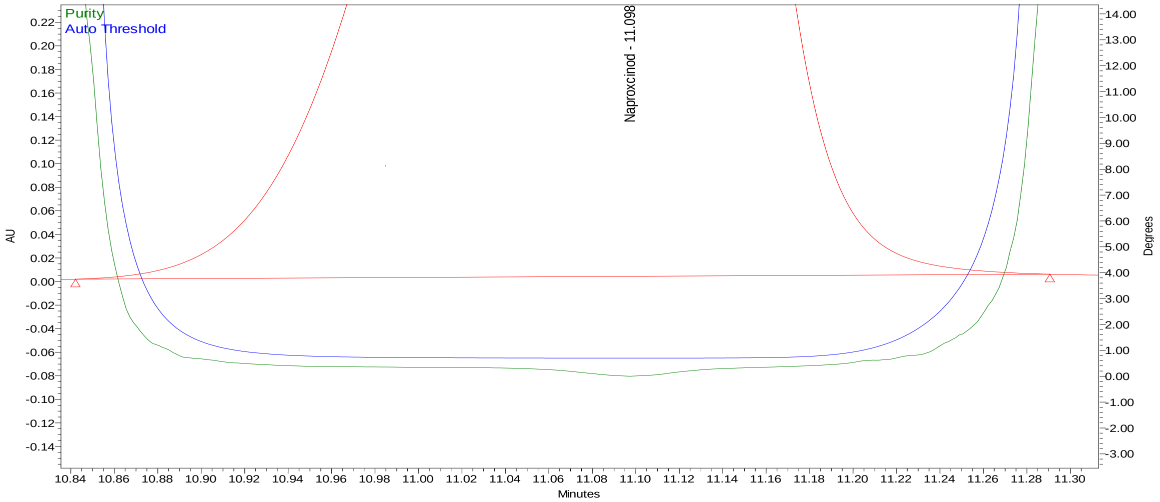


Fig: 5.4.F2. Peak purity plot for NAP peak in oxidized sample

NAP drug substance was subjected to oxidation by using 3.0% Hydrogen peroxide at room temperature. Later analyzed and monitored the oxidized sample solution up to 24 h. No significant degradation was observed in oxidation conditions. Based on peak purity data it was confirmed that the main component in oxidized sample is homogeneous. Chromatogram of oxidized NAP sample and purity plot is presented in Fig.5.4.F1 and Fig.5.4.F2 respectively.

5.4.2. Degradation during base hydrolysis

NAP drug substance was subjected to base hydrolysis by using 0.1N Sodium hydroxide at room temperature. Analyzed and monitored the hydrolyzed sample solution after 1h. Sample was degraded upto 3.1% under basic conditions, major degradent being imp-B, which was further confirmed by performing co-injection of Imp-B. Based on peak purity data it was confirmed that the main component and Imp-B in base hydrolyzed sample is homogeneous. Chromatogram of base hydrolyzed NAP sample and purity plots of NAP and Imp-B are presented in Fig.5.4.F3, Fig.5.4.F4 and Fig.5.4.F5 respectively.

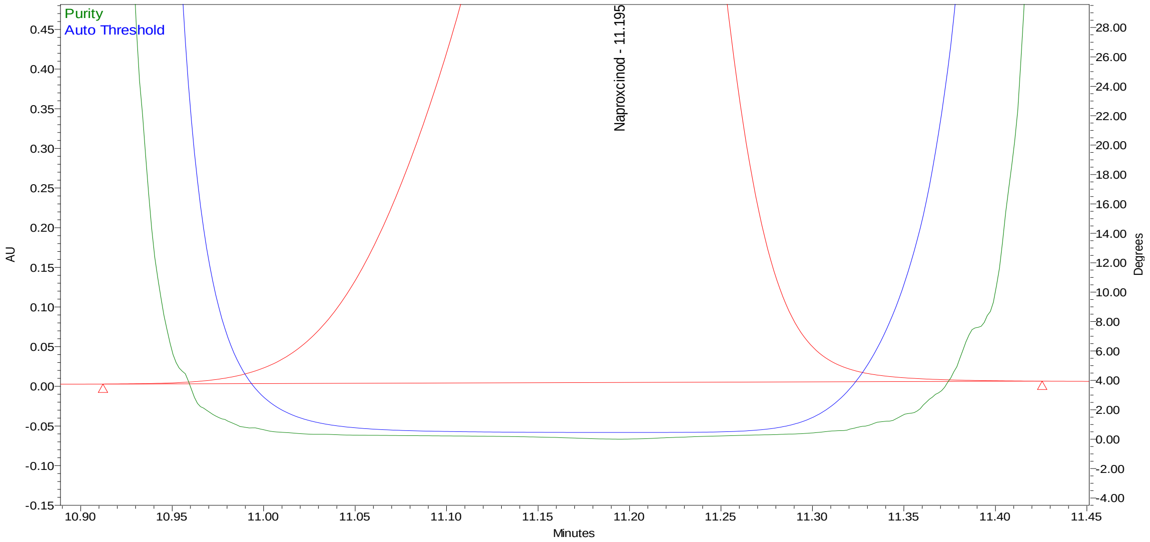


Fig: 5.4.F4. Peak purity plot for NAP peak in base hydrolysis

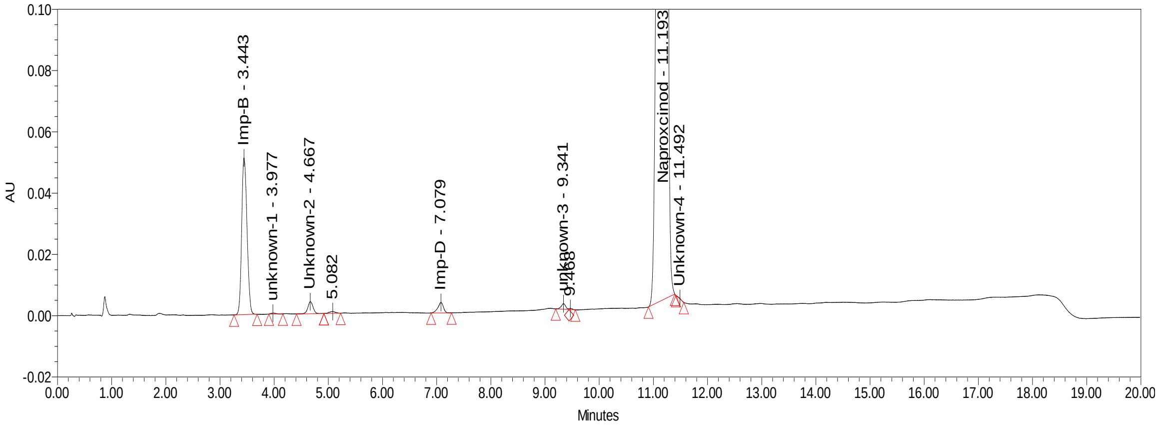


Fig: 5.4.F3. Chromatogram of base hydrolysis NAP sample

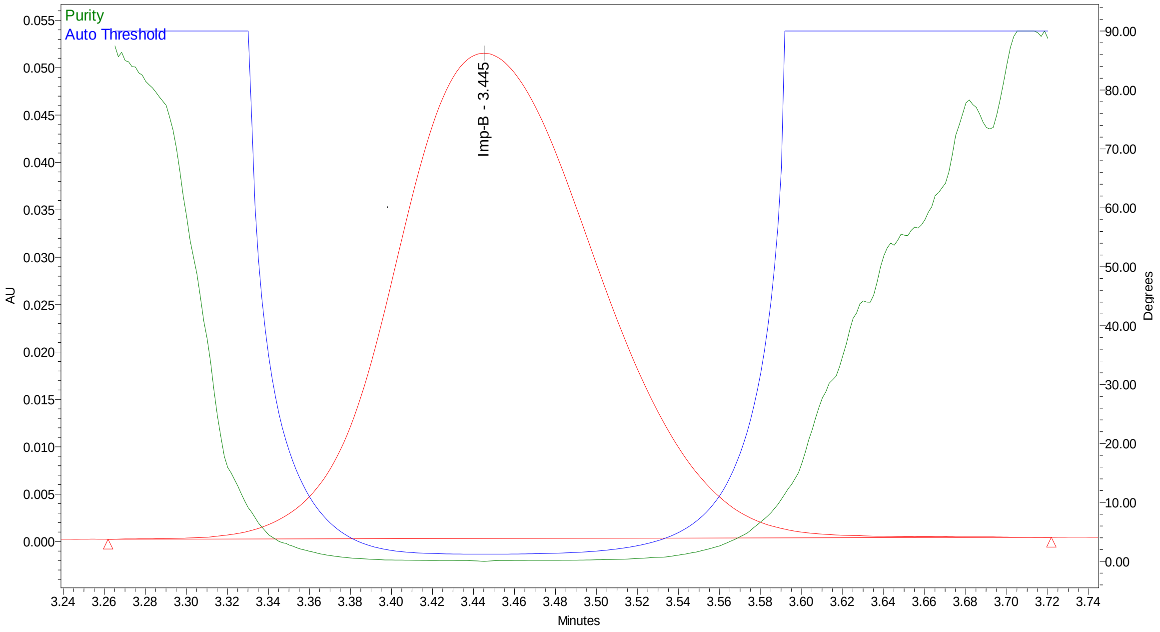


Fig: 5.4.F5. Peak purity plot for Imp-B peak in base hydrolysis

5.4.3. Degradation during Acid hydrolysis

NAP drug substance was subjected to acid hydrolysis by using 0.1N HCl at room temperature. Analyzed and monitored the hydrolyzed sample solution after 24h. Sample was degraded upto 1.9% under acidic conditions, major degradent being imp-B, which was further confirmed by performing co-injection of Imp-B. Based on peak purity data it was confirmed that the main component and Imp-B in acid hydrolyzed sample is homogeneous. Chromatogram of acid hydrolyzed NAP sample and purity plots of NAP and Imp-B are presented in Fig.5.4.F6, Fig.5.4.F7 and Fig.5.4.F8 respectively.

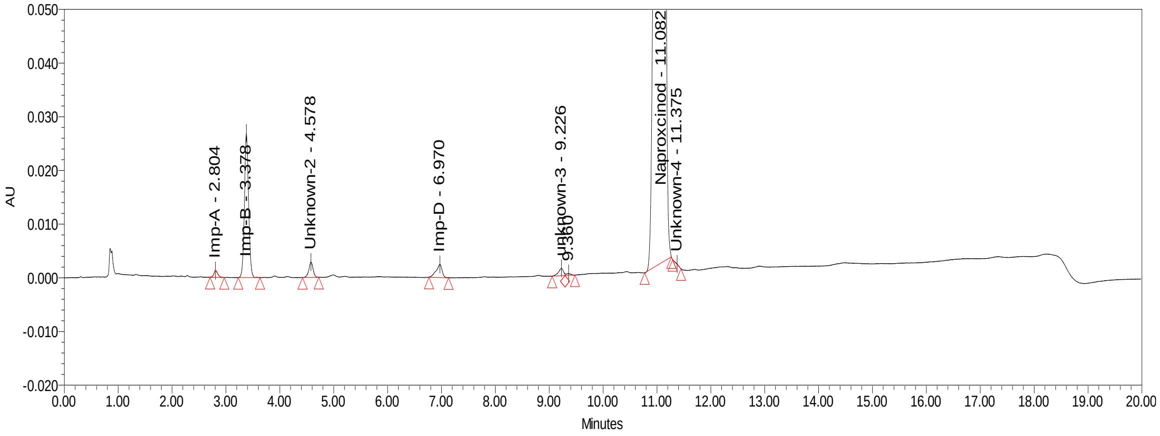


Fig: 5.4.F6. Chromatogram of Acid hydrolysis NAP sample

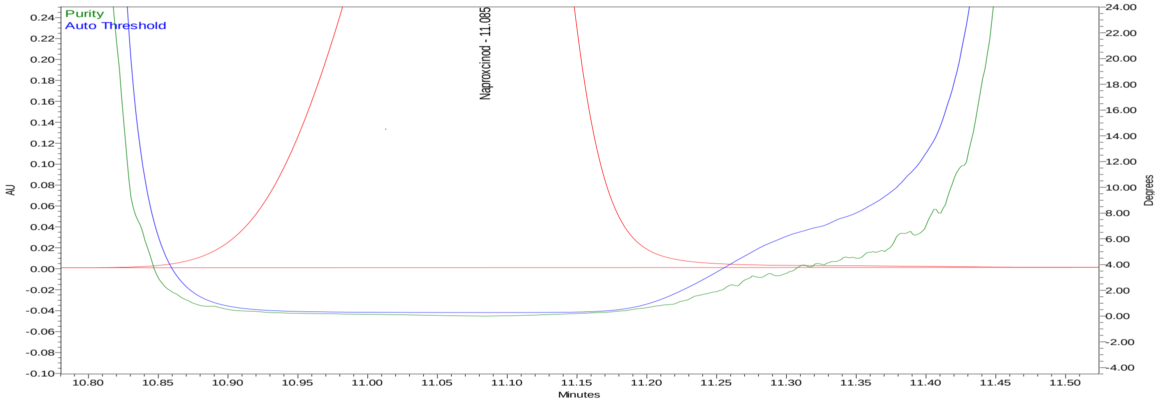


Fig: 5.4.F7. Peak purity plot for NAP peak in acid hydrolysis

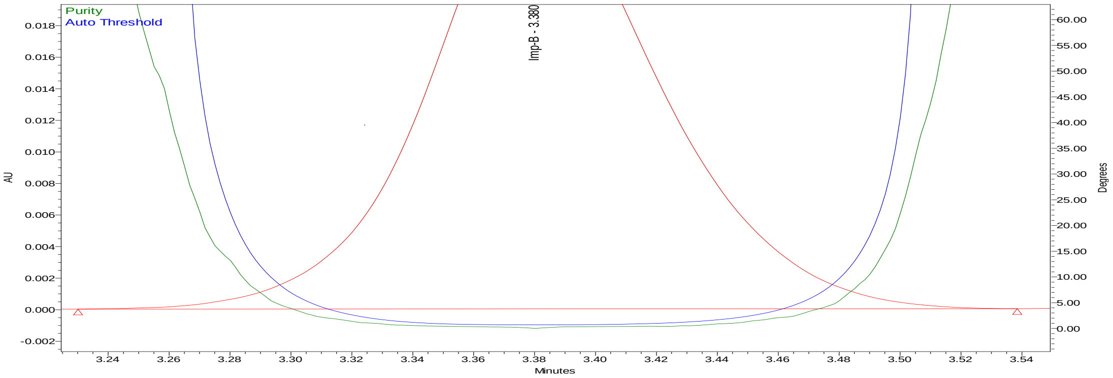


Fig: 5.4.F8. Peak purity plot for Imp-B peak in acid hydrolysis

5.4.4. Degradation during water hydrolysis

NAP drug substance was subjected to hydrolysis by using water at room temperature. Analyzed and monitored the sample solution upto 24h. No significant degradation observed in water hydrolysis conditions. Based on peak purity data it was confirmed that the main component in sample is homogeneous. Chromatogram of stressed sample and purity plots are presented in Fig.5.4.F9 and Fig.5.4.F10 respectively.

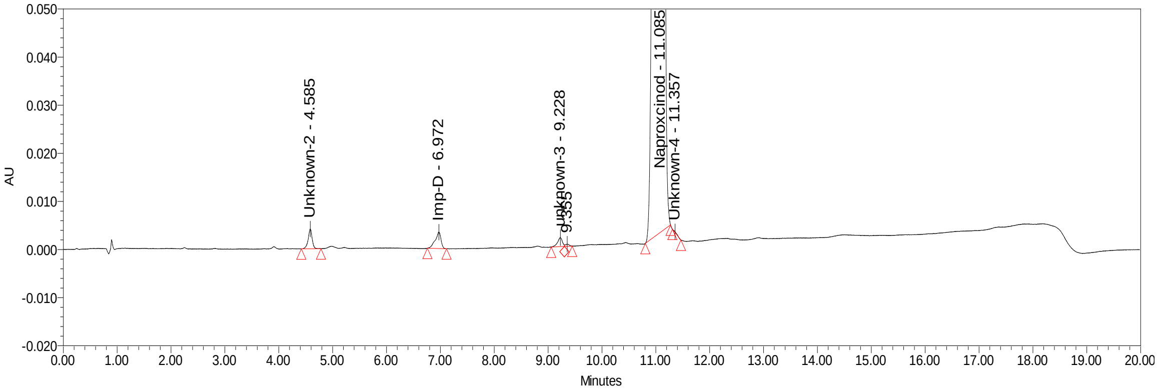


Fig: 5.4.F9. Chromatogram of water hydrolysis NAP sample

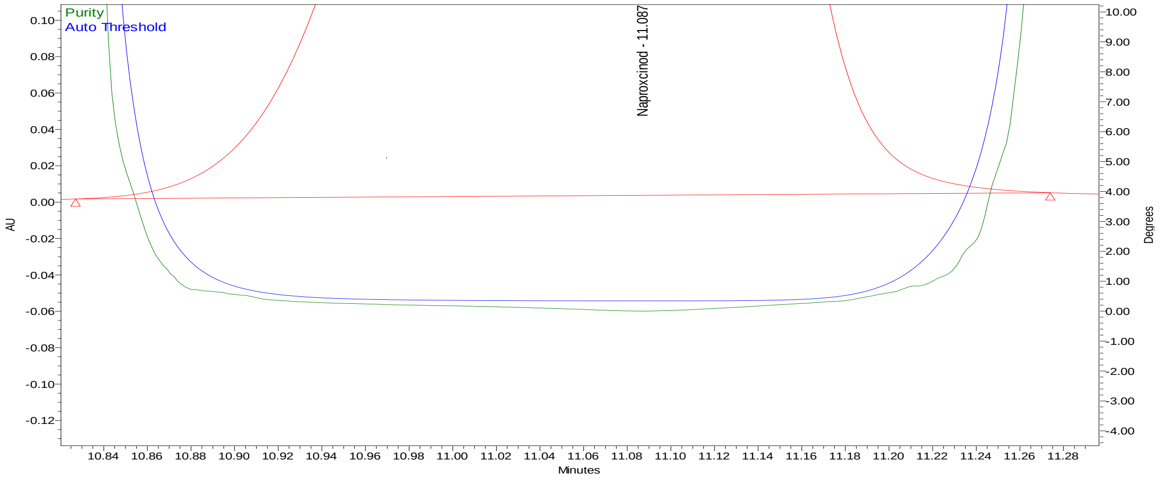


Fig: 5.4.F10. Peak purity plot for NAP peak in water hydrolysis

5.4.5. Thermal degradation

NAP drug substance was thermally stable. The drug was exposed to 90 °C temperature for 10 days, practically it was not degraded. NAP peak in thermally degraded sample was found to be spectrally pure. Chromatogram of stressed sample and peak purity plot is shown in Fig.5.4.F11 and Fig.5.4.F12.

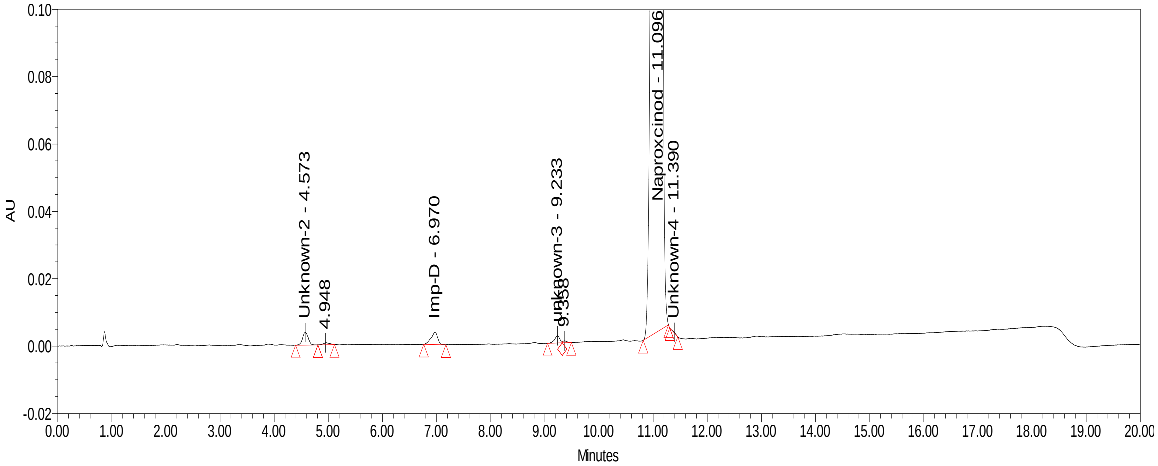


Fig: 5.4.F11. Chromatogram of Thermal degradation NAP sample

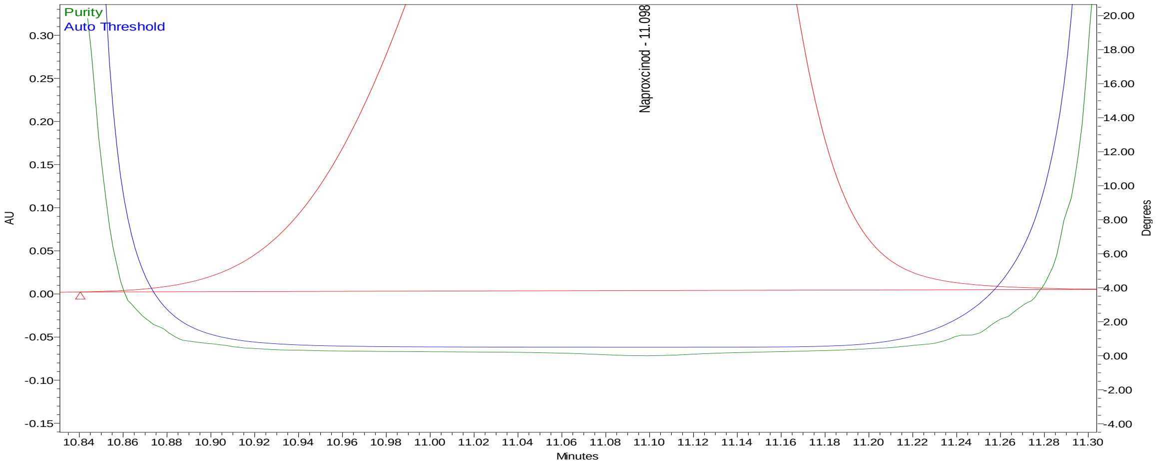


Fig: 5.4.F12. Peak purity plot for NAP peak in Thermal degradation Sample

5.4.6. Photolytic degradation

NAP drug substance was exposed to UV radiations at 254nm and 365nm for 10 days. No considerable degradation was observed. To confirm the stability of NAP drug substance towards photolytic condition, peak purity of NAP peak was verified in degraded sample and found that it was homogeneous. Chromatogram of photolytic degradation sample and peak purity plot was put on display as Fig. 5.4.F13 and 5.4.F14 respectively.

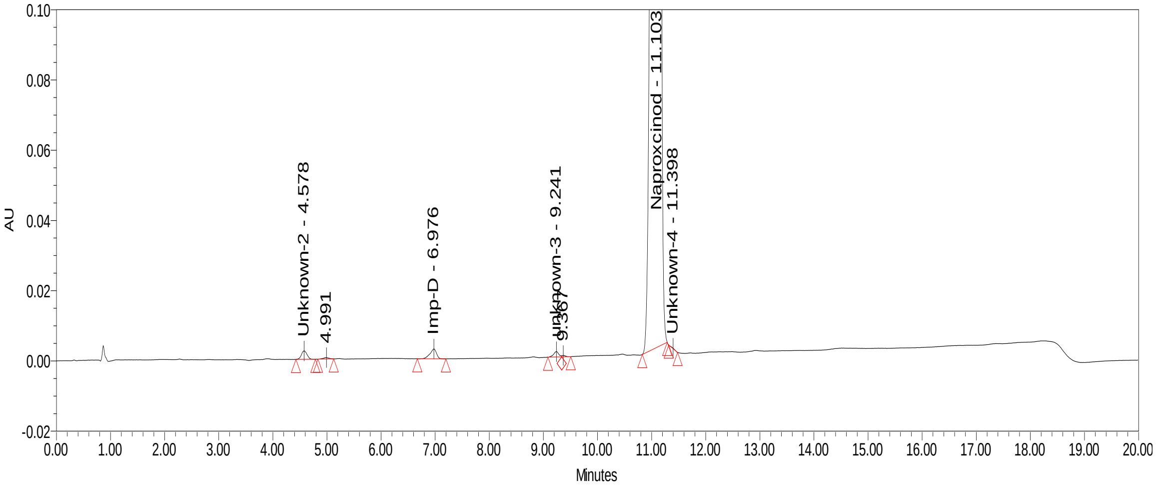


Fig: 5.4.F13. Chromatogram of Photolytic degradation NAP sample

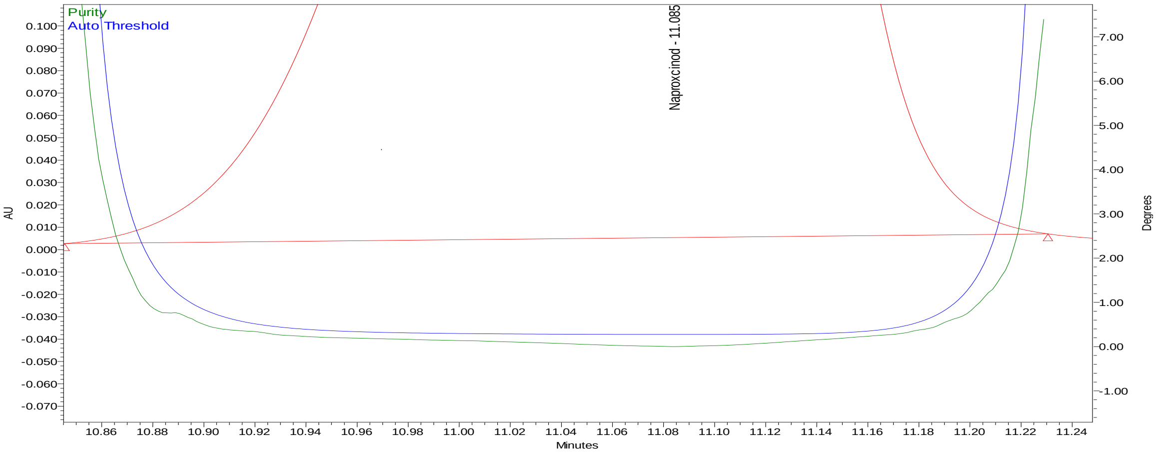


Fig: 5.4.F14. Peak purity plot for NAP peak in photolytic degradation sample

5.4.7. Results of Forced degradation studies

Table: 5.4.T1. Results of forced degradation studies

Degradation condition	**Time**	**RS by HPLC % degradation**	**Remarks/observation**
HCl- 0.1N RT (Acid hydrolysis)	24hr	1.9%	Impurity-B as a degradation product
NaOH-0.1N RT (Base hydrolysis)	1 hr	3.1%	Impurity-B as a degradation product
Water hydrolysis at RT	24hrs	No degradation	No degradation observed.
Oxidation by H_2O_2- 3.0% RT	24hrs	No degradation	No degradation observed.
Thermal (90°C)	10days	No degradation	No degradation observed.
UV at 254nm and 365nm	10days	No degradation	No degradation observed.

The peak purity results from forced degradation studies proved the stability indicating nature of the method and the method is suitable for determination of all the related substances and degradation products in NAP drug substance during stability testing. % of all specified related compounds, in all the stressed samples are presented in Table 5.4.T1.

5.5 Analytical method validation

The developed and optimized LC method was fully validated as per ICH and USP guidelines.

5.5.1. Precision

Precision study was evaluated by performing both repeatability and intermediate precision.

Table: 5.5.T1. Results of NAP related substances method precision

S.No	% of Related substances							
	Imp-A	**Imp-B**	**Imp-C**	**Imp-D**	**Imp-E**	**Imp-F**	**Imp-G**	**Imp-H**
Pre-1	46402	39272	11647	74070	21520	25652	27825	34576
Pre-2	46154	39129	11553	73849	20745	25248	27581	34484
Pre-3	46347	39271	11525	74043	21500	25468	27720	34645
Pre-4	46307	39200	11618	73994	21607	25566	27676	34590
Pre-5	46239	39247	11596	73929	21526	25493	27702	34582
Pre-6	46228	39202	11527	73967	21506	25432	27704	34547
Mean	46280	39220	11578	73975	21401	25477	27701	34571
%RSD	0.2	0.1	0.4	0.1	1.5	0.5	0.3	0.2

To ensure the repeatability of related substances method, prepared six individual preparation of NAP drug substance spiked with Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H at specification level (0.15%) with respect to test concentration. Calculated the %RSD for area of each individual known impurity. Results are tabulated in below Table 5.5.T1.

The above results show that the method is repeatable within acceptable limits of % RSD for six preparations of related substances 0.1-1.5. Intermediate precision or Ruggedness of NAP RS method was demonstrated by performing precision study as mentioned in repeatability testing on two different days, by a different analyst, different column and by using different equipment. Calculated the % RSD for area of each impurity. Results of Ruggedness of the NAP RS methods are tabulated in Table 5.5.T2.

Table: 5.5.T2. Results of Ruggedness for NAP RS method

Name of analyte	**%RSD for six different preparation**	
	Day-1	**Day-2**
Imp-A	0.2	0.3
Imp-B	0.1	0.4
Imp-C	0.4	0.7
Imp-D	0.1	0.2
Imp-E	1.5	0.6
Imp-F	0.5	0.3
Imp-G	0.3	1.5
Imp-H	0.2	0.2

Results have shown insignificant variation in measured response in two different days, which demonstrated that the method was highly precise for its indented use of estimation of related substances of NAP.

5.5.2. Sensitivity

Sensitivity of the method was demonstrated in terms of Limit of Quantitation (LOQ) and Limit of Detection (LOD) values of specified analytes. LOQ, LOD values were established for Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G, Imp-H and NAP based on signal to noise ratio of each peak.

5.5.2.1 Limit of quantification (LOQ)

Prepared a series of dilutions of NAP, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H in different concentrations and injected them into the liquid chromatograph to get the signal to noise ratio 10 (i.e. 9.5 to 10.0). Limit of quantitation values of all the analytes is presented in Table 5.5.T3.

Table: 5.5.T3. LOQ values NAP and its related compounds

S.No.	Name of the analyte	LOQ in µg/mL
1	NAP	0.045
2	Imp-A	0.045
3	Imp-B	0.045
4	Imp-C	0.068
5	Imp-D	0.045
6	Imp-E	0.045
7	Imp-F	0.045
8	Imp-G	0.045
9	Imp-H	0.045

Table: 5.5.T4. LOD values of the impurities and NAP peak

S.No.	Name of the analyte	LOD in µg/mL
1	NAP	0.015
2	Imp-A	0.015
3	Imp-B	0.015
4	Imp-C	0.025
5	Imp-D	0.015
6	Imp-E	0.015
7	Imp-F	0.015
8	Imp-G	0.015
9	Imp-H	0.015

5.5.2.2. Limit of detection (LOD)

Prepared a series of dilutions of NAP, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H in different concentrations and injected them into the liquid chromatograph to get the signal to noise ratio 2 to 3. Limit of detection values of all the analytes is presented in Table 5.5.T4.

5.5.2.3. Precision at Limit of quantification level

Six preparations of NAP, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H at LOQ level were injected individually and the % RSD for the areas of each analyte was calculated.

Table: 5.5.T5. LOQ precision results of NAP and its related compounds

S.No	Area of impurities							
	Imp-A	**Imp-B**	**Imp-C**	**Imp-D**	**Imp-E**	**Imp-F**	**Imp-G**	**Imp-H**
Pre-1	8578	7199	3290	5915	4919	5147	4780	6197
Pre-2	8546	7076	3405	5863	4897	4645	4675	6310
Pre-3	8629	7290	3329	5906	4902	4620	4761	6438
Pre-4	8512	7188	3416	5730	4866	4622	4599	6367
Pre-5	8593	7134	3436	5926	4911	4616	4719	6345
Pre-6	8506	7194	3373	5918	4925	4593	4709	6474
Mean	8561	7180	3375	5876	4903	4702	4707	6355
%RSD	0.6	1.0	1.7	1.3	0.4	4.6	1.4	1.5

Above results indicates that NAP RS method is precise at LOQ level. No significant variation observed in the area of each analyte for six consecutive injections, RSD of all the components ranges from 0.4 to 4.6 %. Results are summarized in 5.5.T5.

5.5.2.4. Accuracy at LOQ level

NAP sample was injected in test concentration i.e. 150 µg mL^{-1} to estimate the content of Imp-A to Imp-H. Three different sample solutions (150 µg mL^{-1}) of NAP containing Imp-A to Imp-H at LOQ level were prepared and injected each solution once. From the corrected area of Imp-A to Imp-H, % recovery of each impurity was calculated. Results are summarized in Table 5.5.T6.

Table: 5.5.T6. Results of Accuracy at LOQ level

Name	Workup	Amount Added (µg/mL)	Amount obtained (µg/mL)	% Recovery	% Mean Recovery
Imp-1	1	0.1125	0.1087	96.6	97.6
	2		0.1120	99.5	
	3		0.1089	96.8	
Imp-2	1	0.1547	0.1475	95.3	93.9
	2		0.1381	89.3	
	3		0.1501	97.0	
Imp-3	1	0.1301	0.1277	98.2	93.1
	2		0.1208	92.9	
	3		0.1147	88.2	
Imp-4	1	0.1372	0.1245	90.7	92.4
	2		0.1278	93.1	
	3		0.1268	92.4	

5.5.3. Linearity

A series of linearity solutions were prepared containing NAP, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H were prepared at different concentrations i.e. LOQ, 0.038 %, 0.075 %, 0.15 %, 0.15 %, 0.19 % and 0.225 % of working concentration (150 µg mL^{-1}) by performing appropriate dilutions to achieve the targeted concentrations. The above prepared solutions of NAP and its impurities are LOQ, 25 %, 50 %, 100 %, 125 % and 150 % to known impurity specification limit i.e. 0.15 %.

Each solution was injected once and calibration plots were drawn for concentration of each component versus average peak area of corresponding known component. Linearity plot of each analyte with best fit linear equation as Fig.5.5.F1.-5.5.F8. Linear regression analysis was performed for each analyte and data is presented in Table 5.5.T7. - 5.5.T14.

Table: 5.5.T7. Linearity of Imp-A

S.No.	Conc. (μg mL^{-1})	Imp-A peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.03	8555	8582	27	716	285167
2	0.05	14277	14653	376	141361	285540
3	0.075	22100	22242	142	20163	294667
4	0.1125	33867	33626	-241	58311	301040
5	0.15	45558	45009	-549	301345	303720
6	0.1875	57032	56393	-639	408861	304171
7	0.225	66891	67776	885	783409	297293
Regression coefficient		0.9997	Residual sum of squares		1714167	
Slope		303561	SE of slope			
Intercept		-525	SE of Intercept			
% y-Intercept		-1.2	Linearity equation		$y = 303561x - 525$	

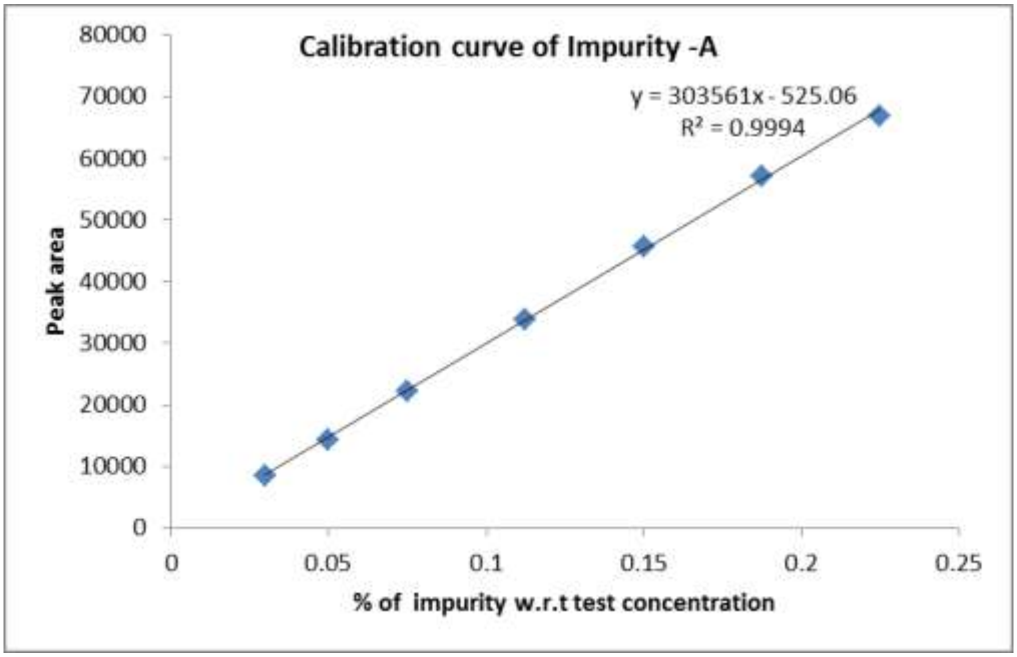


Fig: 5.5.F1. Linearity plot for Imp-A

Table: 5.5.T8. Linearity of Imp-B

S.No.	**Conc. (µg mL^{-1})**	**Imp-B peak area**	**Calculated response through trend line equation**	**Residual**	**Residual square**	**Sensitivity**
1	0.03	7355	7333	-22	472	245167
2	0.05	12178	12516	338	114239	243560
3	0.075	18852	18994	142	20272	251360
4	0.1125	28949	28712	-237	56187	257324
5	0.15	38887	38430	-457	209265	259247
6	0.1875	48555	48147	-408	166361	258960
7	0.225	57221	57865	644	414361	254316
Regression coefficient		0.9997	Residual sum of squares		981156	
Slope		259135	SE of slope			
Intercept		-441	SE of Intercept			
% y-Intercept		-1.13	Linearity equation		$y = 259135x - 441$	

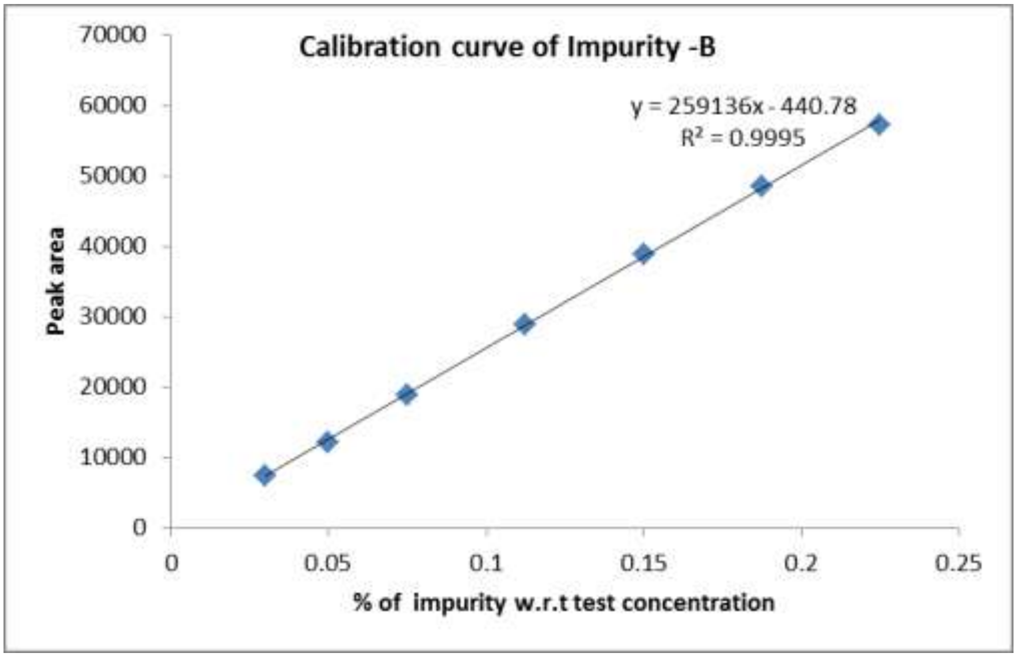


Fig: 5.5.F2. Linearity plot for Imp-B

Table: 5.5.T9. Linearity of Imp-C

S.No.	Conc. (µg mL^{-1})	Imp-C peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.03	2169	2066.0	-103.0	10600.1	72300.0
2	0.05	3370	3621.7	251.7	63358.4	67400.0
3	0.075	5581	5566.3	-14.7	216.2	74413.3
4	0.1125	8465	8483.2	18.2	330.2	75244.4
5	0.15	11708	11400.0	-308.0	94833.7	78053.3
6	0.1875	14336	14316.9	-19.1	363.8	76458.7
7	0.225	17059	17233.8	174.8	30556.0	75817.8
Regression coefficient		0.9995	Residual sum of squares		200259	
Slope		77783	SE of slope			
Intercept		-268	SE of Intercept			
% y-Intercept		-2.2	Linearity equation		$y = 77783x - 268$	

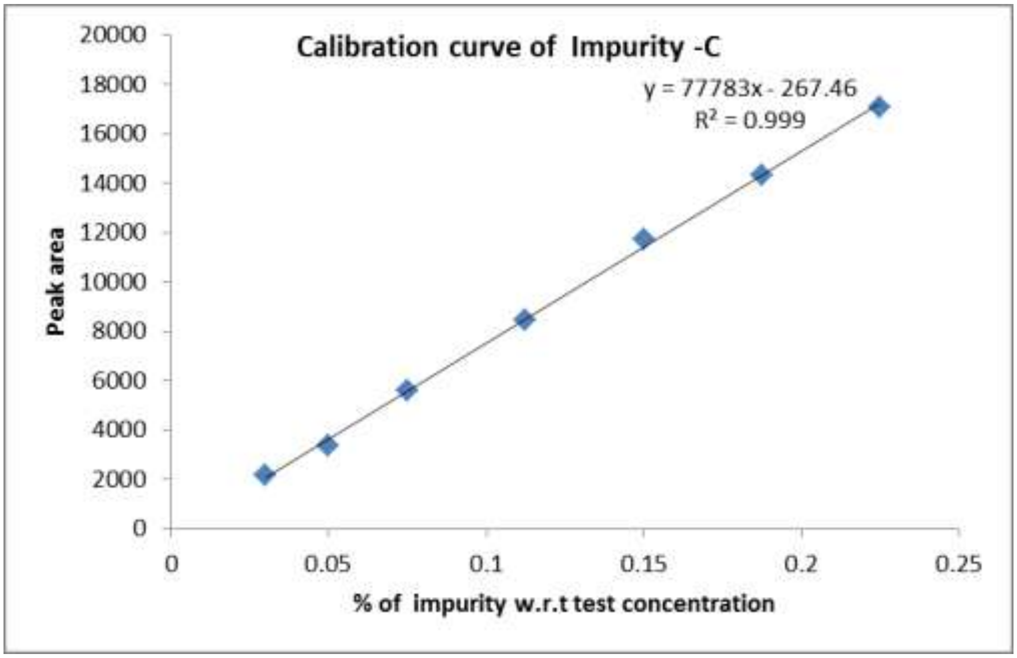


Fig: 5.5.F3. Linearity plot for Imp-C

Table: 5.5.T10. Linearity of Imp-D

S.No.	**Conc. (µg mL^{-1})**	**Imp-D peak area**	**Calculated response through trend line equation**	**Residual**	**Residual square**	**Sensitivity**
1	0.03	6098	6093	-5	21	203267
2	0.05	10143	10329	186	34690	202860
3	0.075	15425	15624	199	39644	205667
4	0.1125	23772	23566	-206	42275	211307
5	0.15	31882	31509	-373	139371	212547
6	0.1875	39729	39451	-278	77306	211888
7	0.225	46917	47393	476	226809	208520
Regression coefficient		0.9998	Residual sum of squares		560115	
Slope		211794	SE of slope			
Intercept		-260	SE of Intercept			
% y-Intercept		-0.82	Linearity equation		*y* = 211794*x* -260	

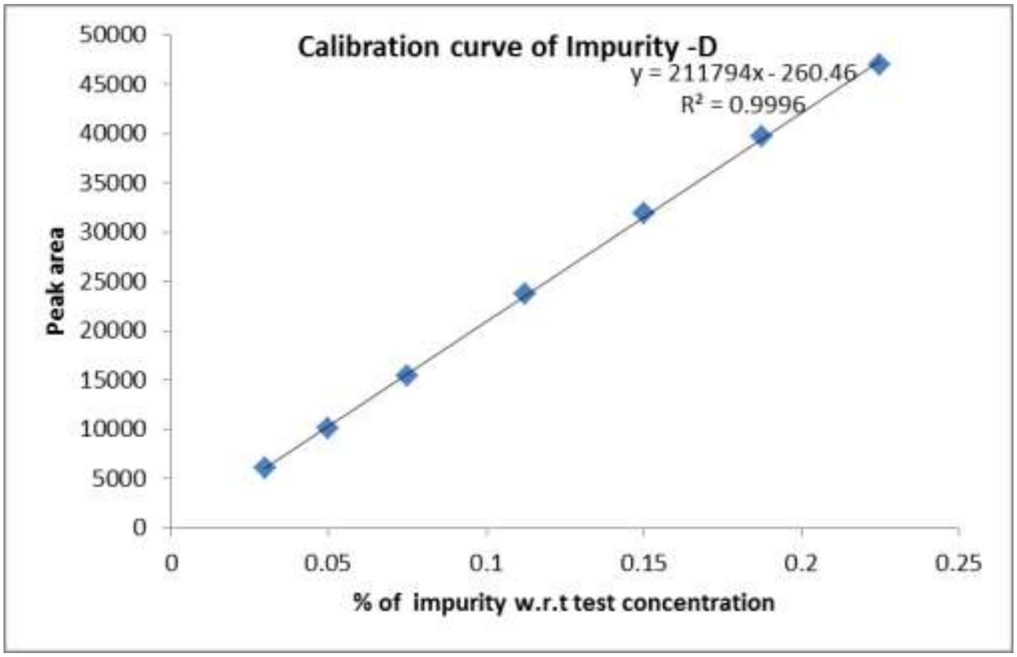


Fig: 5.5.F4. Linearity plot for Imp-D

Table: 5.5.T11. Linearity of Imp-E

S.No.	Conc. (μg mL^{-1})	Imp-E peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.03	4955	4960	5	21	165167
2	0.05	8305	8507	202	40935	166100
3	0.075	12808	12942	134	17941	170773
4	0.1125	19781	19594	-187	35015	175831
5	0.15	26565	26246	-319	101882	177100
6	0.1875	33202	32898	-304	92572	177077
7	0.225	39080	39550	470	220596	173689
Regression coefficient		0.9997	Residual sum of squares		508962	
Slope		177385	SE of slope			
Intercept		-362	SE of Intercept			
% y-Intercept		-1.36	Linearity equation		y = 177385x -362	

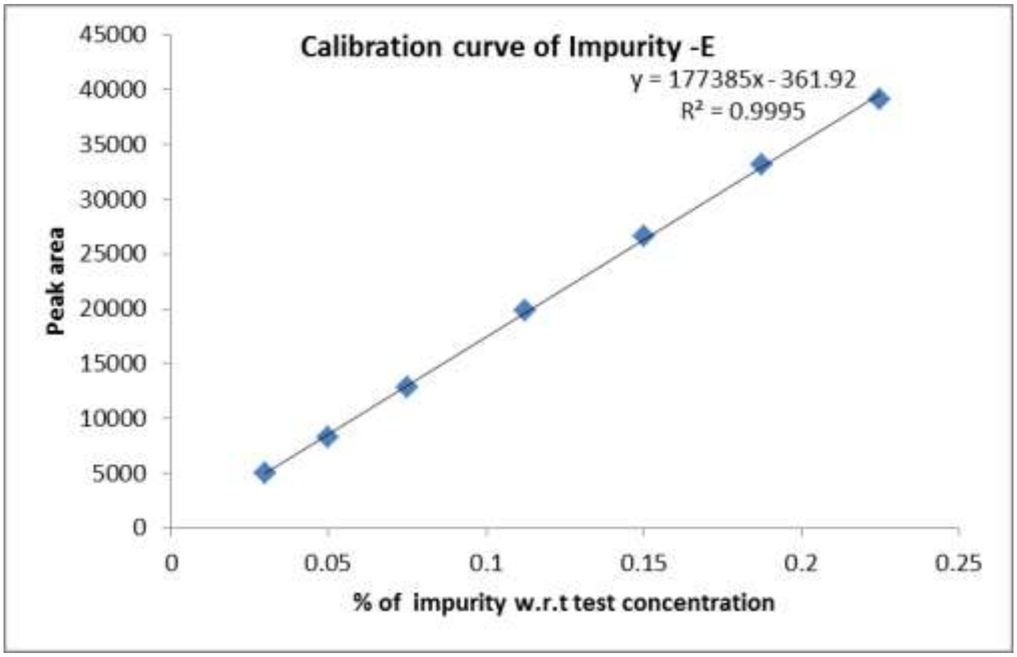


Fig: 5.5.F5. Linearity plot for Imp-E

Table: 5.5.T12. Linearity of Imp-F

S.No.	**Conc. (µg mL^{-1})**	**Imp-F peak area**	**Calculated response through trend line equation**	**Residual**	**Residual square**	**Sensitivity**
1	0.03	4682	4619	-63	3943	156067
2	0.05	8161	8362	201	40570	163220
3	0.075	12868	13041	173	30079	171573
4	0.1125	20139	20060	-79	6248	179013
5	0.15	27440	27078	-362	130701	182933
6	0.1875	34444	34097	-347	120414	183701
7	0.225	40640	41116	476	226113	180622
Regression coefficient		0.9998	Residual sum of squares		508962	
Slope		187161	SE of slope			
Intercept		-996	SE of Intercept			
% y-Intercept		-3.63	Linearity equation		*y* = 177385*x* -362	

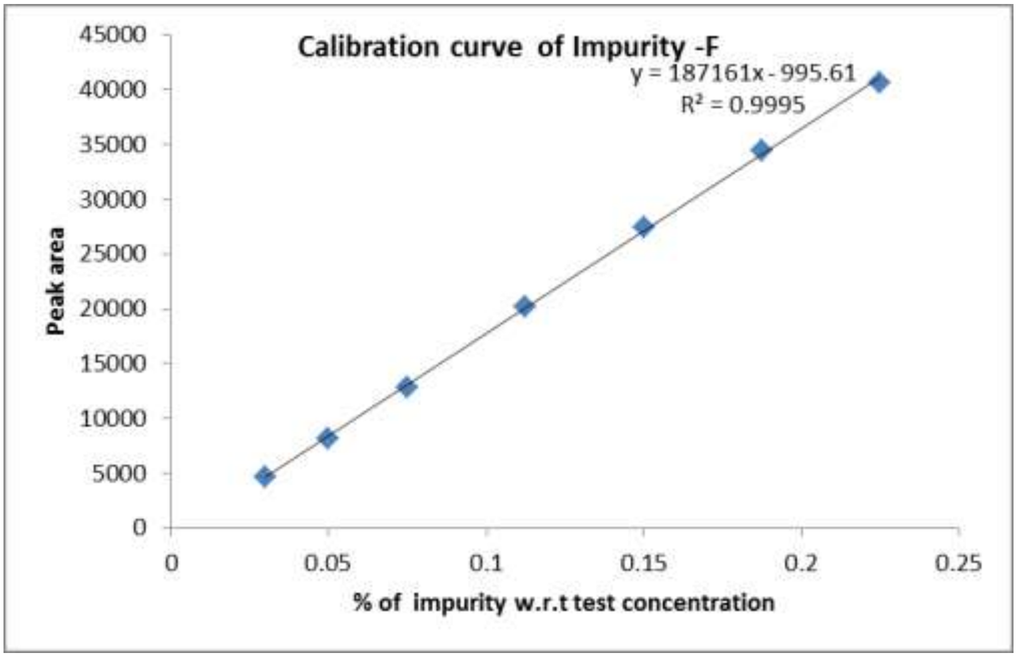


Fig: 5.5.F6. Linearity plot for Imp-F

Table: 5.5.T13. Linearity of Imp-G

S.No.	**Conc. (µg mL^{-1})**	**Imp-G peak area**	**Calculated response through trend line equation**	**Residual**	**Residual square**	**Sensitivity**
1	0.03	4885	5202	317	100446	162833
2	0.05	8216	8580	364	132364	164320
3	0.075	12462	12802	340	115720	166160
4	0.1125	20643	19136	-1507	2271914	183493
5	0.15	25422	25469	47	2233	169480
6	0.1875	32082	31803	-279	77960	171104
7	0.225	37418	38136	718	515988	166302
Regression coefficient		0.998	Residual sum of squares		3216625	
Slope		168894	SE of slope			
Intercept		135	SE of Intercept			
% y-Intercept		0.53	Linearity equation		$y = 168894x+135$	

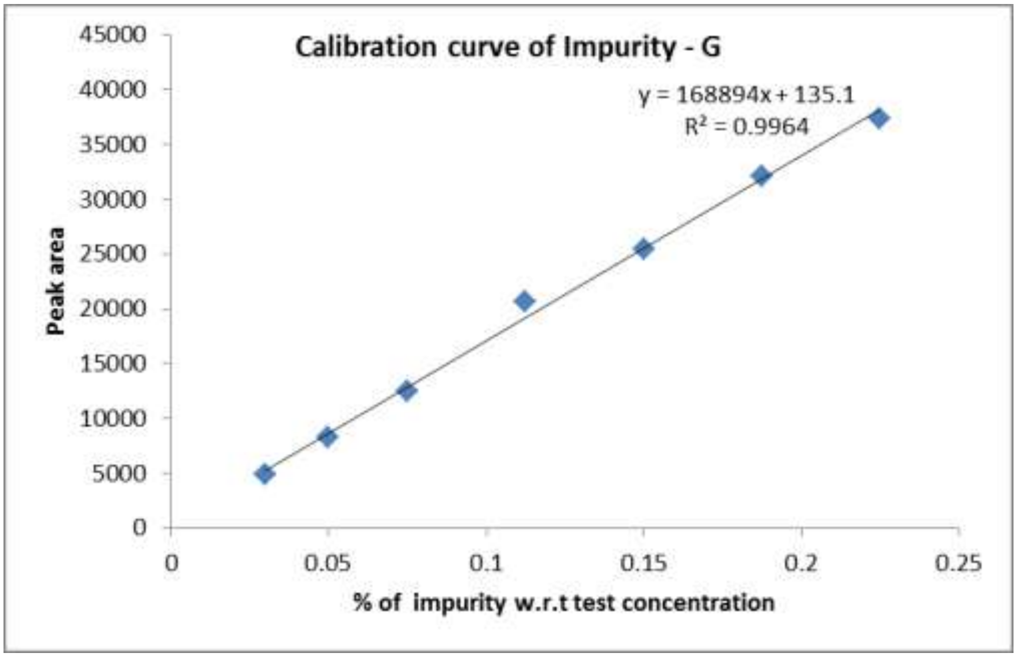


Fig: 5.5.F7. Linearity plot for Imp-G

Table: 5.5.T14. Linearity of Imp-H

S.No.	**Conc. (µg mL^{-1})**	**Imp-H peak area**	**Calculated response through trend line**	**Residual**	**Residual square**	**Sensitivity**
1	0.03	6477	6529	52	2703	215900
2	0.05	10725	11103	378	143243	214500
3	0.075	16602	16822	220	48217	221360
4	0.1125	26008	25399	-609	371192	231182
5	0.15	34339	33976	-363	131836	228927
6	0.1875	42914	42553	-361	130270	228875
7	0.225	50447	51130	683	466807	224209
Regression coefficient		0.9996	Residual sum of squares		1294267	
Slope		228724	SE of slope			
Intercept		-332	SE of Intercept			
% y-Intercept		-0.97	Linearity equation		$y = 228724x - 332$	

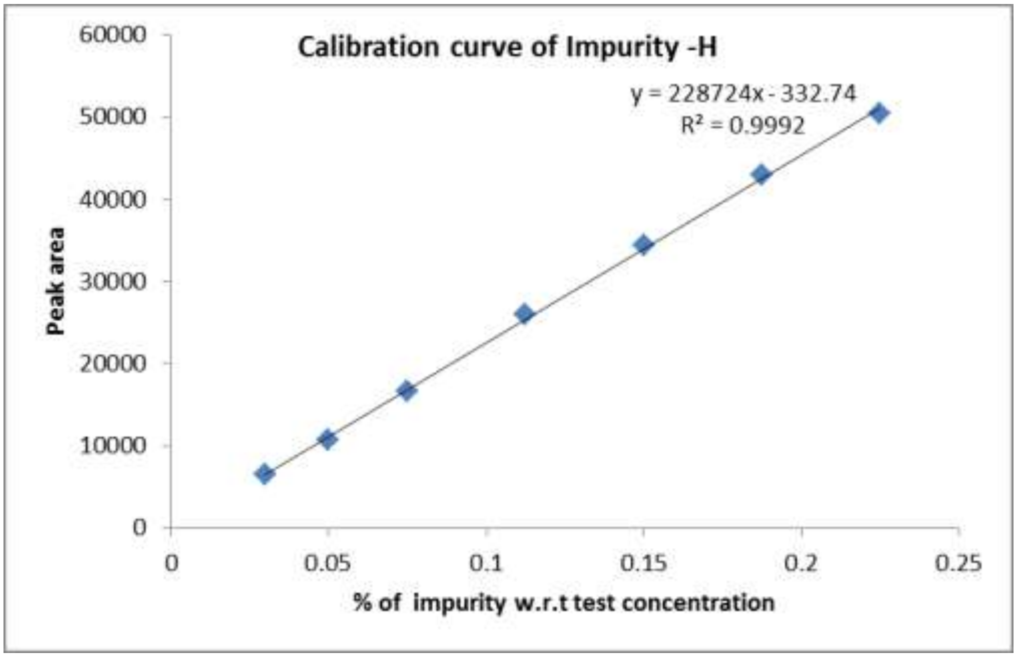


Fig: 5.5.F8. Linearity plot for Imp-H

Regression coefficients for concentration against peak area of all the related compounds of NAP and NAP peak from LOQ to 150 % level to the specification limit were more than 0.99. And also % y-bias with respect to 100 % specification of known compounds is within the limit of ± 2.0 which indicates that developed related substances by LC method for NAP were linear.

5.5.4. Accuracy

5.5.4.1. Accuracy of RS method

Solutions of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H at three different concentration levels i.e. 50 %, 100 % and 150 % w.r.t. the specification limit (0.15%) of working concentration was spiked with NAP sample solution (conc. 150 µg mL^{-1}), each level was prepared in triplicate and each of nine solutions were injected once.

Table: 5.5.T15. Accuracy results

Name of the analyte	Concentration of analyte w.r.t. specification limit	Amount of impurity spiked to NAP* (µg mL-1)	Amount of impurity recovered (µg mL-1)	%Mean recovery ± SD
Imp-A	50%	0.1133	0.1177	100.3±0.6
	100%	0.2265	0.2298	99.7±0.3
	150%	0.3398	0.3394	99.9±0.1
Imp-B	50%	0.1125	0.1153	102.5±1.6
	100%	0.2250	0.2269	100.9±0.2
	150%	0.3375	0.3397	100.7±0.1
Imp-C	50%	0.1140	0.1136	99.6±2.9
	100%	0.2280	0.2258	99.0±0.5
	150%	0.3420	0.3385	99.0±0.1
Imp-D	50%	0.1118	0.1069	95.7±4.3
	100%	0.2235	0.2183	97.7±0.4
	150%	0.3353	0.3252	97.0±0.6
Imp-E	50%	0.1148	0.1063	86.1±0.2
	100%	0.2295	0.2121	89.2±1.9
	150%	0.3443	0.3236	92.6±0.4
Imp-F	50%	0.1133	0.1027	90.7±0.4
	100%	0.2265	0.2105	93.0±0.7
	150%	0.3398	0.3225	99.9±0.9
Imp-G	50%	0.1118	0.1204	107.8±1.2
	100%	0.2235	0.2287	102.3±0.5
	150%	0.3353	0.3380	94.9±0.3
Imp-H	50%	0.1155	0.1175	101.7±0.4
	100%	0.2310	0.2323	100.5±0.2
	150%	0.3465	0.3482	100.8±0.2

Amount of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H obtained in each solution was calculated as % recovery. Accuracy results at three levels are summarized in Table 5.5.T15. % Recoveries obtained for three different levels were ranges from 86.1 to 107.8 Stand deviation for the average of % recoveries of each individual analyte is less than 5.0. Above accuracy results reveal that method is highly accurate.

5.5.6. Range

As evident from Linearity, accuracy and precision study of related substances method, range has been established for all the analytes i.e. NAP, Imp-A to Imp-H is from LOQ to 150 % of specification limit.

5.6. Conclusion from analytical method validations

The developed RP-LC method developed for quantitative determination of related substances of NAP in drug substance is precise, accurate, selective and linear as per the ICH recommended guidelines. The Robustness and ruggedness or intermediate precision study reveals that the method is highly rugged and robust for its intended use.

5.7 Conclusion

The proposed RP-HPLC method is sensitive, linear, precise and accurate for quantitation of related substances of NAP and its degradation product. The method was fully validated as per ICH and proved the stability indicating power, can be used for estimation of impurities of NAP.

5.8. References

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CHAPTER – 6

Development and Validation of Chiral Liquid Chromatographic Method for the Enantiomeric Separation of CPE, an Intermediate of Ticagrelor

6.1 Introduction to chiral intermediate and corresponding final drug substance

Chemical name of selected chiral intermediate is; 2-{[(3aR, 4S, 6R, 6aS)-6-amino-2,2-dimethyltetrahydro-3aH-cyclopenta[d][1,3]-dioxol-4-yl]oxy}-1-ethanol, L-tartrate (CPE) (Fig.6.1.F1.). Molecular formula is $C_{10}H_{19}NO_{4.}$ $C_4H_6O_6$ and molecular weight is 367.35. This is a key starting material for the synthesis of Ticagrelor, an anti coagulant drug. In October, 2005 this drug was approved by US FDA. Ticagrelor is chemically known as (1*S*, 2*S*, 3*R*, 5*S*)-3-(7-(((1*R*,2*S*)-2-(3,4-Difluorophenyl)cyclopropylamino)-5-(propylthio)-3*H*-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl)-5-(2-hydroxyethoxy)cyclopentane-1,2-diol. Ticagrelor is a direct –acting, reversibly-binding, oral $P2Y_{12}$ receptor antagonist used for the prevention of thrombotic events in patients with acute coronary syndromes [1-16]. Chemical structure of Ticagrelor is provided in Fig.6.1.F2.

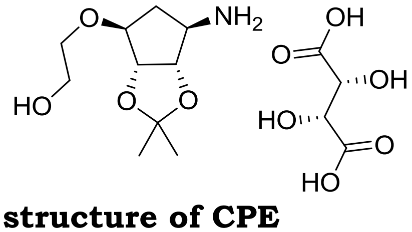


Fig: 6.1.F1. Chemical structure of CPE

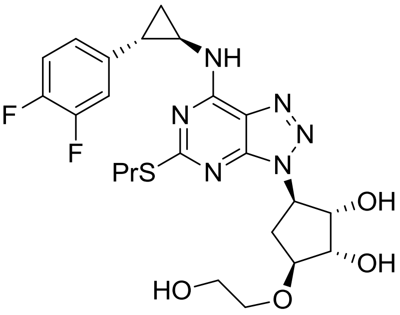


Fig: 6.1.F2. Chemical structure of Ticagrelor

Analytical literature survey revealed that no reported chiral methods are available for the estimation of CPE enantiomer in CPE. Present study deals with the chiral method development for separation of CPE-intermediate and its enantiomer impurity (Fig.6.1.F3.) by using chiral stationary phase. The developed method is fully validated as per ICH guidelines [17, 18].

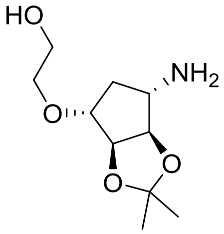


Fig: 6.1.F3. Chemical structure of CPE enantiomer

To get high pure drug substance of Ticagrelor, the CPE-intermediate should be as pure as possible. In fact, the source for the presence of undesired isomer Ticagrelor drug substance is from the presence of enantiomer in chiral intermediate stage i.e. CPE-intermediate. Hence, it is essential to ensure that the chiral purity of the intermediate and it is required to control/monitor the enantiomer impurity in CPE intermediate by using efficient validated chiral analytical method. Based on different experiments of process development team it has been decided that enantiomer impurity specification limit in CPE-intermediate is 0.10%. If, chiral impurity is more than 0.10% in intermediate stage, it will carry to API and would be responsible for formation of enantiomer in API.

6.2 Experimental

6.2.1 Materials

Material or chemicals used for this study is listed in Table 6.2.T1.

Table: 6.2.T1. Details of materials and chemicals

S.No.	Name of the compound	Grade	Procured from../synthesized by..
1.	CPE-intermediate and CPE enantiomer	Synthesized by in-house process.	Dr.Reddy's Laboratories, India
2.	$NaHCO_3$	HPLC Grade	SRL, India
3.	p-Toluene sulphonyl chloride	HPLC	AVRA, India
4.	Acetonitrile	HPLC	Merck, India
5.	Water	HPLC grade	In house

6.2.2. Equipments

During method development, Waters Alliance HPLC system equipped with 2690 model quaternary pump, auto injector and 2996 photo diode array detector was used. For analytical method validations HPLC equipped with e2695 model separation module and 2489 dual channel UV detector from Waters Corporation, USA was used.

6.2.3. Preparation of solutions

6.2.3.1. Preparation of blank solution

20 mg each of Sodium bicarbonate and para toluene sulphonyl chloride was weighed in a 10 mL volumetric flask, and dissolved in a few mL of diluent and made up to the mark with diluent. This solution was heated to 50°C in a water bath and maintained at this temperature for about 30 minutes with constant stirring.

For preparation of solutions mixture of water and acetonitrile in the ratio of 1:1 are used as diluent

6.2.3.2. Preparation of CPE enantiomer stock solution

5 mg of CPE enantiomer was dissolved in 100mL of diluent to get a final concentration of 50 μg mL^{-1}.

6.2.3.3. Preparation of 0.1% CPE enantiomer spiked test solution

20 mg each of CPE sample, Sodium bicarbonate and para toluene sulphonyl chloride was transferred into a 10mL volumetric flask. The contents were dissolved with the addition of 5mL of diluent and 0.4mL of above enantiomer stock solution was added and made upto the volume with the diluent. This solution was heated to 50°C in a water bath and maintained at this temperature for about 30 minutes with constant stirring.

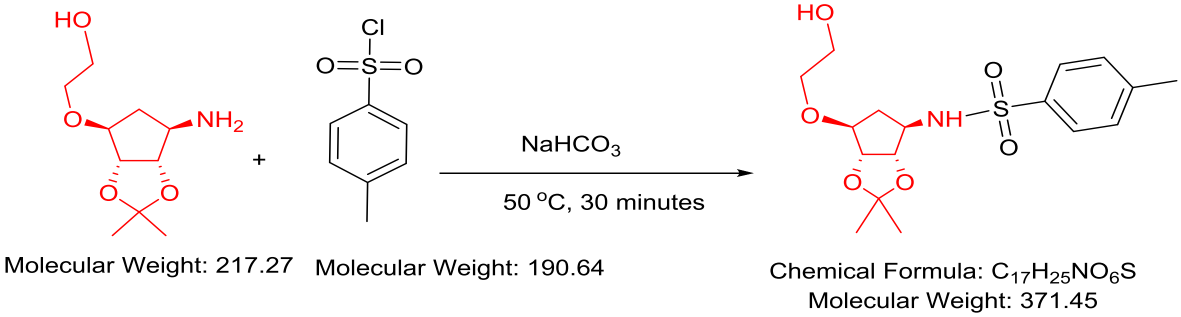
6.2.3.4. Preparation of CPE test solution

20 mg each of CPE sample, Sodium bicarbonate and para toluene sulphonyl chloride was transferred into a 10mL volumetric flask. The

contents were dissolved with the addition of 5 mL of diluent and made up to the volume with the diluent. This solution was heated to 50°C in a water bath and maintained at this temperature for about 30 minutes with constant stirring.

6.3 Discussion on chiral method development

Reported methods are available for the derivatization of primary amines with the p-Toluene sulphonyl chloride [19]. As the moiety is not having Chromophore in its structure, trials were made to derivatize it with p-toluene sulphonyl chloride to form a sulphonamide derivative which is having wavelength maximum at 230nm. Sodium bicarbonate was used as a base and temperature is maintained at 50°C for 30 minutes. For initiation of method development, chiral stationary phase (CSP) and reverse phase mode of chromatography were chosen due to the use of aqueous diluents.



The CSPs will interact with chiral analytes and form reversible transient diastereomers on stationary phase. Formation of diastereomers is based on hydrogen bonding or dipole-dipole or π- π interactions [20]. Based on the stability of the formed diastereomer, stereoisomers of analyte will get separate which indicates that more stable diastereomer will retain in CSP and less stable will elute form the column there by separation or resolution takes place. This is a widely used separation media for enantiomeric pharmaceutical molecules [21-26].

6.3.1 Selection of Diluent

To proceed for method development trials with reverse phase solvents as mobile phase, solubility of both the analytes, CPE intermediate and CPE-enantiomer were verified in polar solvents like water and acetonitrile and their combinations. Both compounds were dissolved in mixture of water and acetonitrile. Hence, a mixture of water and acetonitrile in the ratio of 1:1 was selected as diluent.

6.3.2 Selection of Wavelength

Racemic solution of CPE-intermediate was injected into liquid chromatograph equipped with PDA detector. Then extracted the UV spectra of both the isomers and found wavelength maxima at 230 nm (Fig.6.3.F1.). Hence, it has been selected as UV detector wavelength for LC method development.

6.3.3. Simultaneous optimization of Chiral stationary phase (CSP) and mobile phase

Trial-1: Amylose based chiral stationary phase i.e. Chiralpak AD-RH (amylase tris (3,5-dimethylphenyl carbamate)) column with 15 cm length and 4.6 mm internal diameter was used for first Trial. Later injected 0.1% spiked test solution by using water and acetonitrile in the ratio of

70:30, v/v as mobile phase. CPE-enantiomer was not separated from the CPE peak (Fig.6.3.F2.).

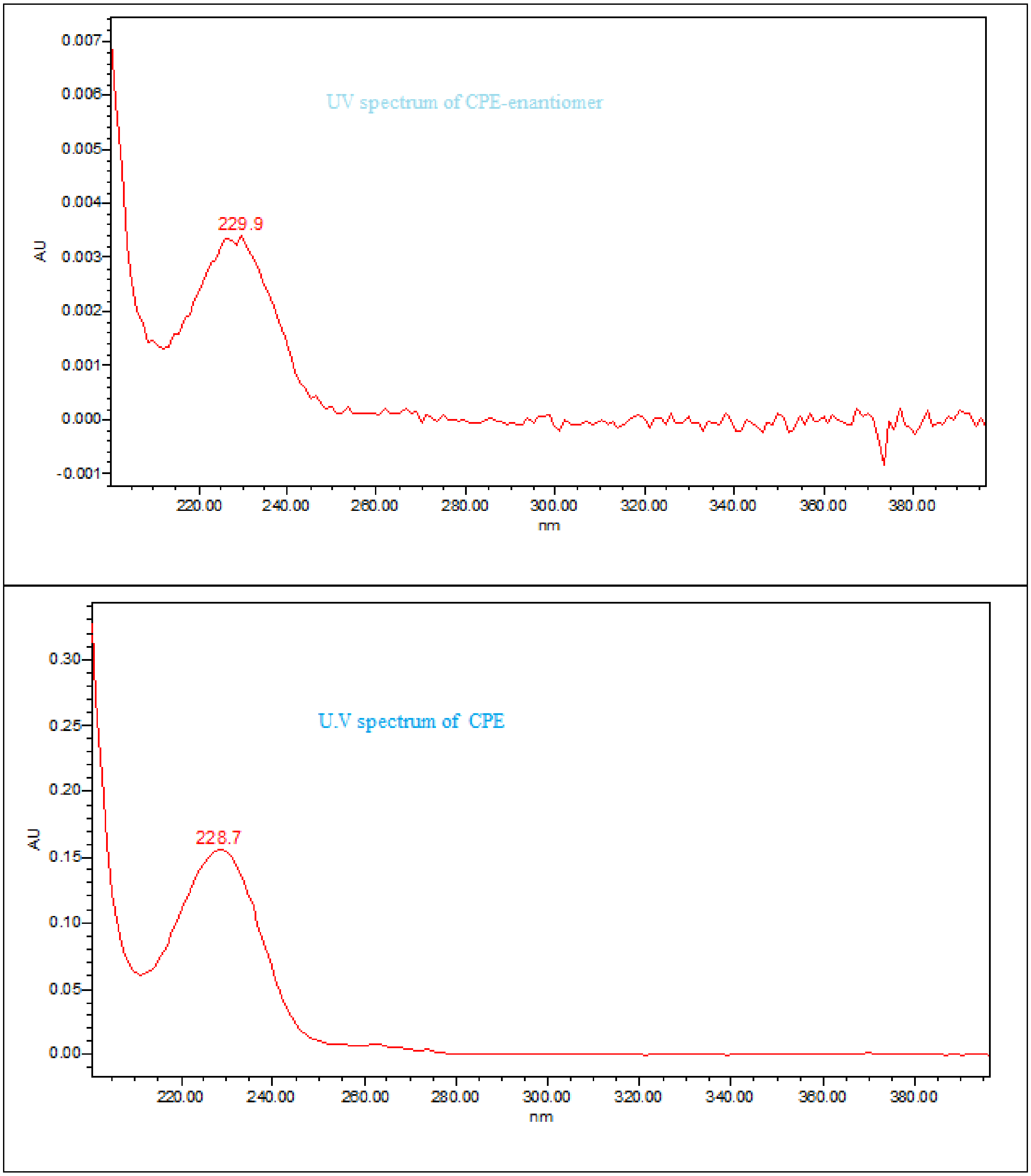


Fig: 6.3.F1. Typical UV spectra of CPE intermediate and CPE enantiomer

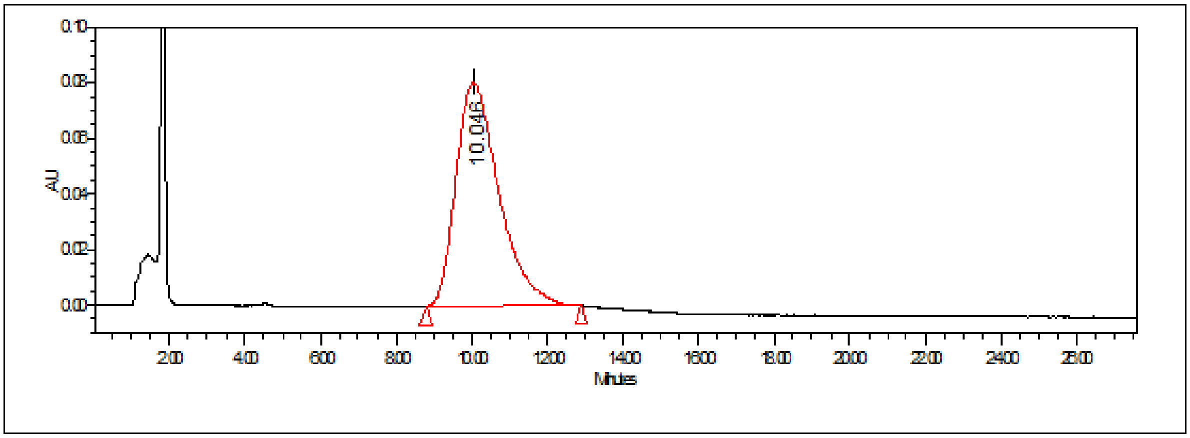


Fig: 6.3.F2. Chromatogram of spiked test solution in Trial-1

Trial-2: Next development trials were performed by using macrocyclic glycopeptide based stationary phase i.e. 250 mm length Chirobiotic TAG column with 4.6 mm internal diameter and 5 µm particle size. Then injected 0.1% enantiomer spiked test solution using the chromatographic conditions described in trial-1. No resolution was observed (Fig.6.3.F3.).

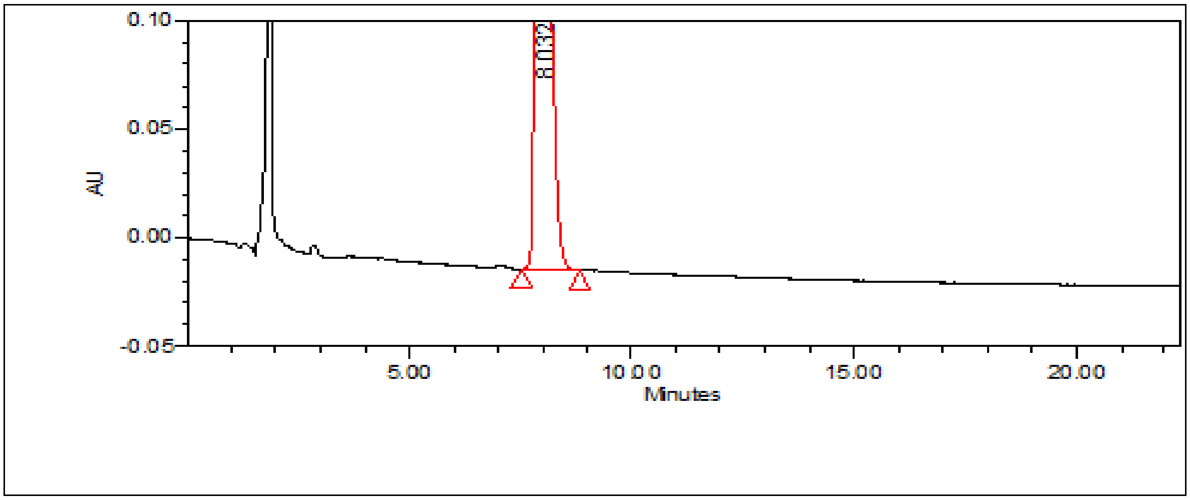


Fig: 6.3.F3. Chromatogram of spiked test solution in Trial-2

Trial-3: Since no selectivity was observed for the enantiomeric pair in the above mentioned columns, next trial was performed on Cellulose based coated column i.e Chiralcel OD-R (Cellulose tris(3,5-dimethylphenylcarbamate coated on 10µm silica-gel using the same mobile phase as mentioned in trial 1. CPE- enantiomer and CPE peak were separated with base to base separation. (Fig.6.3.F4.) Injected the individual components of the derivatization reaction and found that p-toluene sulphonyl chloride was not eluting with this composition of the mobile phase.

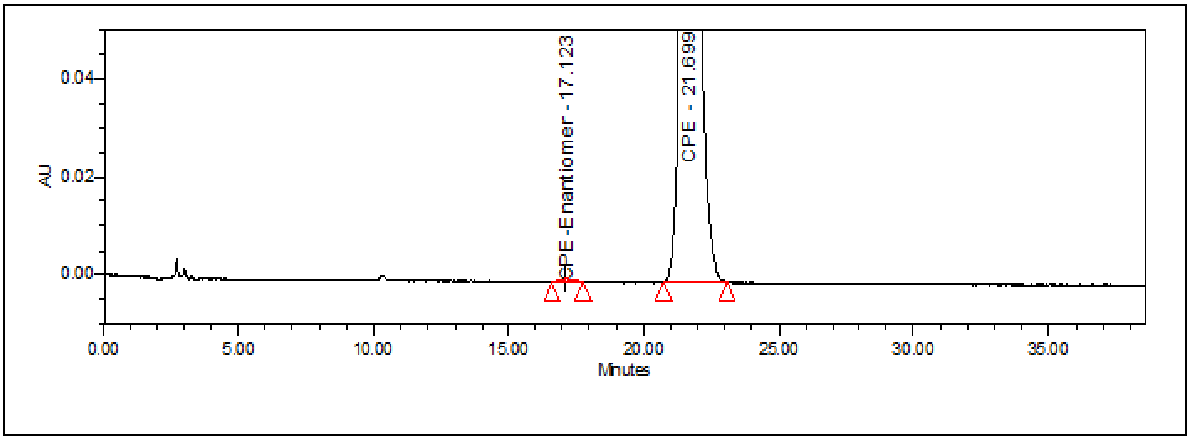


Fig: 6.3.F4. Chromatogram of spiked test solution in Trial-3

Trial-4: To elute the unreacted p-Toluene sulphonyl chloride, the composition of the mobile phase has been changed during the course of elution. A mixture of water and acetonitrile in the ratio of 70:30(v/v) have been used as mobile phase-A; water and acetonitrile in the ratio of 10:90(v/v) used as mobile phase-B. The gradient programme is shown in table 6.3.T1. Flow rate was 1.0 mL/min and the column oven temperature was maintained at ambient temperature.

Table: 6.3.T1. Gradient program for Trial-4

Time(m in)	0.01	15	20	30	31	45
%MP-A	95	95	5	5	95	95
%MP-B	5	5	95	95	5	5

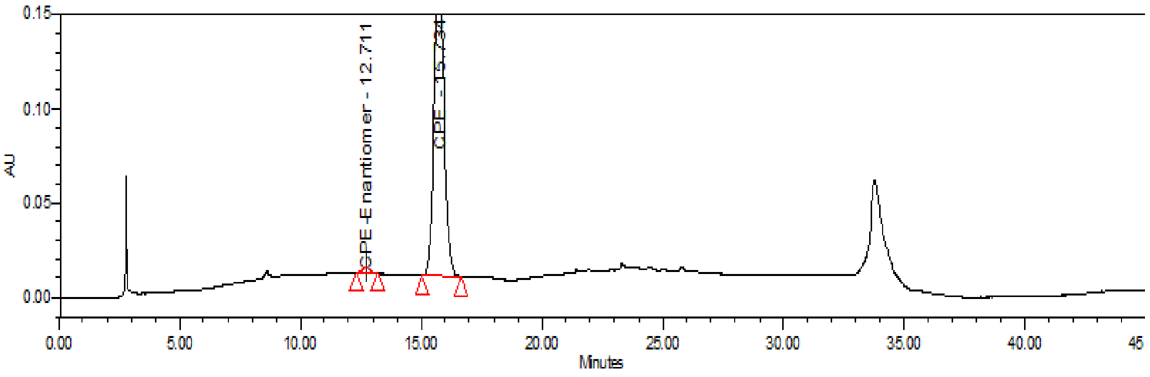


Fig: 6.3.F5. Chromatogram of spiked test solution in Trial-4

In the above trial CPE- enantiomer and CPE were separated with a base to base resolution greater than 4.0. Typical chromatograms of blank, system suitability and sample were presented in Fig.6.3.F6, Fig.6.3.F7, and Fig.6.3.F8. respectively.

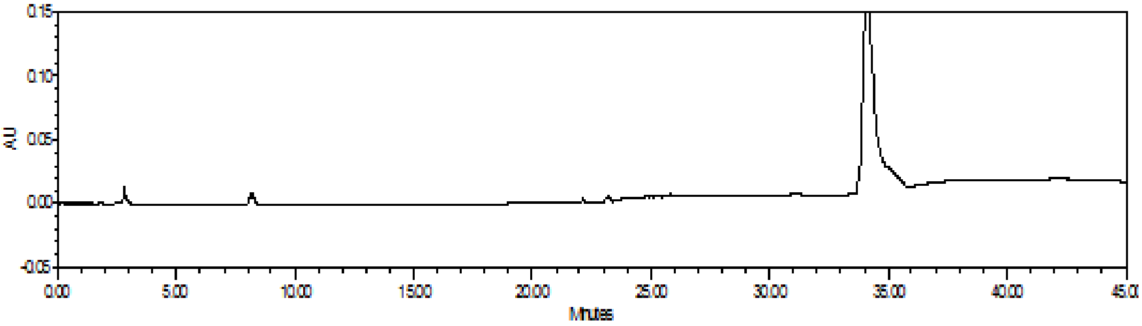


Fig: 6.3.F6. Chromatogram of Blank

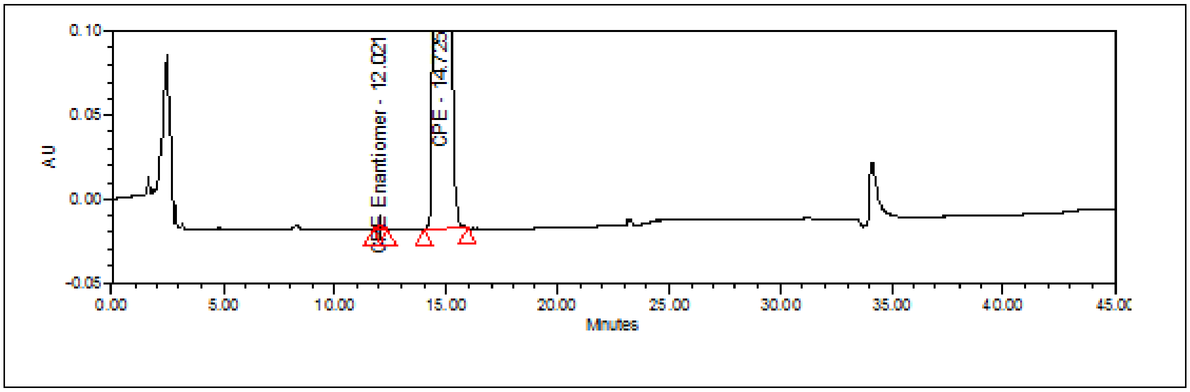


Fig: 6.3.F7. Chromatogram of system suitability

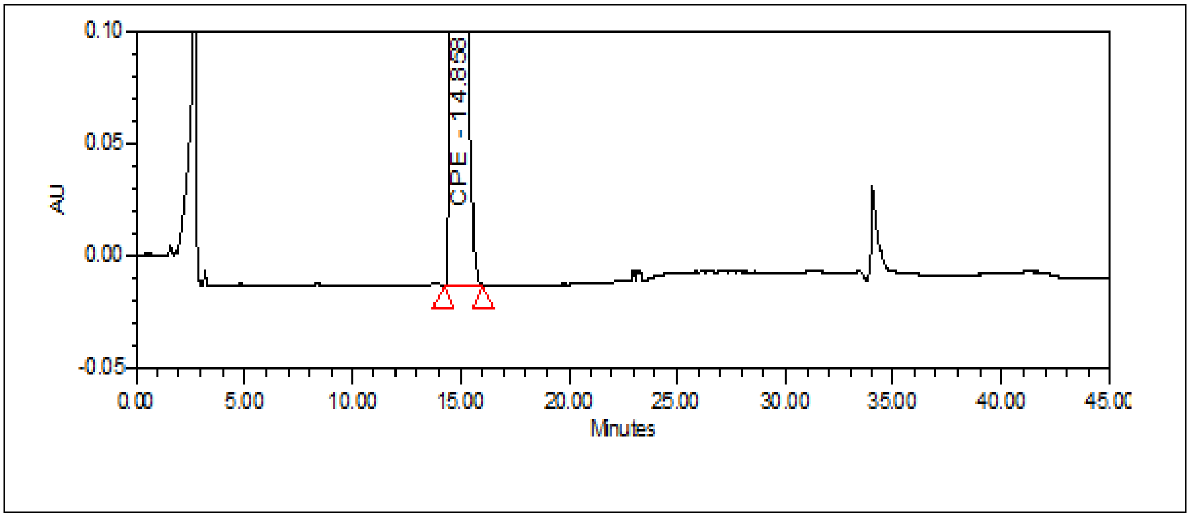


Fig: 6.3.F8. Chromatogram of test sample

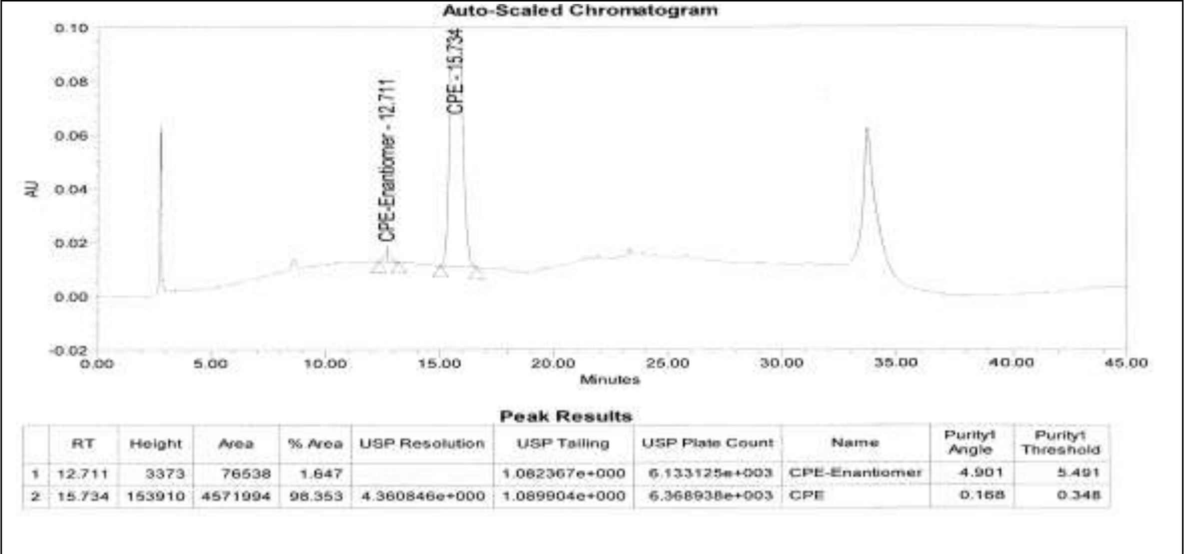
6.3.4. Specificity

Specificity of the method was verified by injecting spiked solution of CPE intermediate with 0.10 % enantiomer in the above method using PDA detector. Homogeneity of both CPE intermediate and CPE enantiomer was assured based on peak purity results (Table.6.3.T2.). Chromatogram of spiked solution and peak purity plot for CPE enantiomer in spiked solution is presented in Fig.6.3.F9. and Fig.6.3.F10. respectively.

Table: 6.3.T2. Peak purity results

S.No.	Name of the compound	Purity Angle	Purity Threshold	Results *
1.	CPE intermediate	0.168	0.348	Pass
2.	CPE enantiomer	4.901	5.491	Pass

*If, Purity angle < Purity threshold indicates peak is homogeneous



Peak Results

	RT	Height	Area	% Area	USP Resolution	USP Tailing	USP Plate Count	Name	Purity1 Angle	Purity1 Threshold
1	12.711	3373	76538	1.647		1.082367e+000	6.133125e+003	CPE-Enantiomer	4.901	5.491
2	15.734	153910	4571994	98.353	4.360846e+000	1.089904e+000	6.368938e+003	CPE	0.168	0.348

Fig: 6.3.F9. Chromatogram of spiked solution

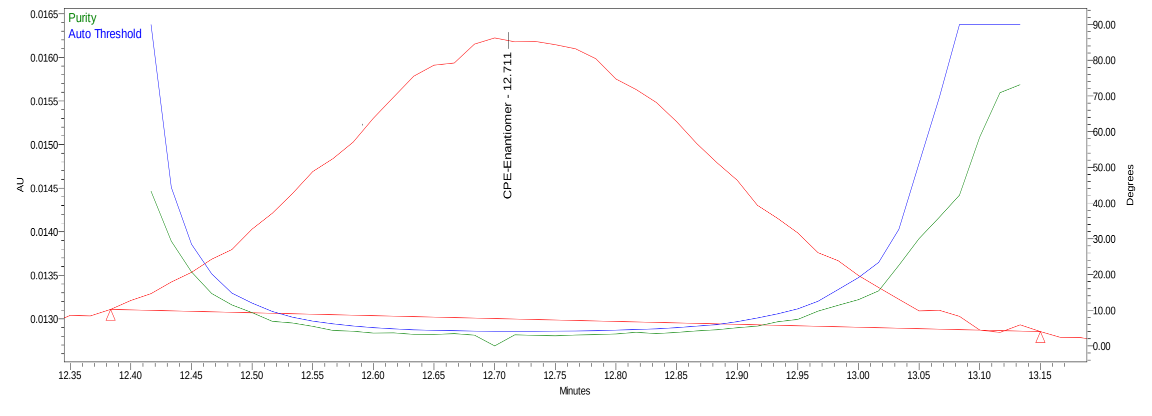


Fig: 6.3.F10. Purity plot for CPE -enantiomer

6.3.5. Optimized chromatographic conditions

The above Trial-5 was finalized for monitoring CPE enantiomer content in CPE intermediate. The finalized chromatographic conditions are listed in table 6.3.T3.

Table: 6.3.T3. Optimized chromatographic conditions

S.No.	Parameter	Chromatographic condition
1	Instrument	HPLC system with UV detector
2	Column	Chiral Cel OD-R, 250 x 4.6, 10 µm.
3	Column Temperature	Ambient (27 °C)
4	Mobile phase -A	Water and acetonitrile in the ratio of 700:300
	Mobile phase -B	Water and acetonitrile in the ratio of 100:900
5	Detector	UV detector
6	Wavelength	230 nm
7	Flow rate	1.0 mL/min
8	Injection volume	10.0 µL
9	Concentration	2 mg mL^{-1}
10	Diluent	Acetonitrile and water in the ratio of 50:50(v/v)
11	Elution mode	Gradient
12	Run time	45 min
13	Gradient programme (Time/% of B)	0.01/5,15.00/5, 20.00/95, 30.00/95, 31.00/5, 45.00/5

6.4. Analytical method validation

The developed and optimized chiral LC method was fully validated as per ICH and USP guidelines.

6.4.1. System suitability test

Prepared six different solutions of CPE intermediate in test concentration spiked with CPE Enantiomer at 0.10% level. Later these solutions were injected into LC system and ensured that the USP resolution (Rs) between CPE Enantiomer and CPE peaks should not be less than 3.0 and USP tailing factor for CPE intermediate peak should not be more than 2.0 in all the solutions. System suitability results were tabulated in Table 6.4.T1.

Table: 6.4.T1. System suitability results

Preparation	USP resolution between CPE Enantiomer and CPE peak	USP tailing factor for CPE intermediate peak
Preparation-1	3.1	1.38
Preparation-2	3.1	1.38
Preparation-3	3.1	1.37
Preparation-4	3.1	1.38
Preparation-5	3.1	1.38
Preparation-6	3.1	1.37

6.4.2. Precision

Precision study was evaluated by performing both repeatability and intermediate precision.

Repeatability of the method was verified by injecting six different preparations of CPE intermediate sample solution spiked with 0.10 % of CPE Enantiomer. %RSD for CPE enantiomer content was calculated. Repeatability test results are tabulated in Table 6.4.T2.

Table: 6.4.T2. Results of method precision

Sample preparation	% of CPE Enantiomer
Preparation-1	0.101
Preparation-2	0.099
Preparation-3	0.100
Preparation-4	0.099
Preparation-5	0.100
Preparation-6	0.101
Mean	0.100
Stdev.	0.001
%RSD	0.99

Intermediate precision or Ruggedness of developed chiral method was demonstrated by performing precision study as mentioned in repeatability testing on different day, by a different analyst, by using different lots of solvents, different column and using different equipment. Later % RSD for CPE enantiomer content from six different preparations was calculated. Results of Ruggedness of chiral method are tabulated in Table 6.4.T3.

Table: 6.4.T3. Results of Ruggedness study

Sample preparation	% of CPE Enantiomer in Day-1	% of CPE Enantiomer in Day-2
Preparation-1	0.101	0.099
Preparation-2	0.099	0.099
Preparation-3	0.100	0.099
Preparation-4	0.099	0.098
Preparation-5	0.100	0.100
Preparation-6	0.101	0.099
Mean	0.100	0.099
Stdev.	0.001	0.001
%RSD	0.99	0.71

% RSD of CPE Enantiomer content in ruggedness study which is less than1.0 shows insignificant variation in measured response in two different days, which demonstrated that the method was highly precise for its indented use of estimation of enantiomeric purity of CPE intermediate.

6.4.3. Sensitivity

Sensitivity of the method was established in terms of Limit of Quantitation (LOQ) and Limit of Detection (LOD) values of CPE Enantiomer impurity. LOQ, LOD values were established based on signal to noise ratio of CPE Enantiomer peak.

6.4.3.1. Limit of quantification (LOQ)

Prepared a series of dilutions of CPE Enantiomer peak in different concentrations and injected them into the liquid chromatograph to get the signal to noise ratio 10 (i.e. 9.5 to 10.0). Limit of quantitation results are given in Table 6.4.T4.

Table: 6.4.T4. Limit of Quantitation of CPE Enantiomer

S.No.	Impurity name	S/N Ratio	Conc. (µg mL^{-1})	Conc. (% w.r.t. test conc.)
1	CPE enantiomer	10.07	0.2856	0.014

6.4.3.2. Limit of detection (LOD)

Prepared a series of dilutions of CPE enantiomer in different concentrations and injected them into the liquid chromatograph until to get the signal to noise ratio between 2 to 3. Limit of detection results are given in Table 6.4.T5.

Table: 6.4.T5. Limit of detection of CPE enantiomer

S.No.	**Impurity name**	**S/N Ratio**	**Conc. (µg mL^{-1})**	**Conc. (% w.r.t. test conc.)**
1	CPE enantiomer	2.16	0.0803	0.004

6.4.3.3. Precision at Limit of quantification level

Six preparations of CPE enantiomer at LOQ level were injected individually and the % RSD for the area of impurity was calculated. Results are summarized in Table 6.4.T6.

Table: 6.4.T6. LOQ precision results of CPE enantiomer

S.No.	**Area of CPE enantiomer**
Preparation-1	4903
Preparation-2	5510
Preparation-3	5635
Preparation-4	5203
Preparation-5	5627
Preparation-6	4978
Mean	5309
Stdev.	326.63
%RSD	6.2

Above results indicate that method is extremely precise at limit of quantitation level.

6.4.3.4. Accuracy at LOQ level

CPE intermediate sample was injected in test concentration i.e. 2 mg mL^{-1} to estimate the content of CPE enantiomer. Three different sample solutions (2.0 mg mL^{-1}) of CPE intermediate containing CPE enantiomer at LOQ level were prepared and injected each solution once. From the corrected area of CPE enantiomer, % recovery of impurity was calculated. Results are summarized in Table 6.4.T7.

Table: 6.4.T7. Results for Accuracy CPE enantiomer at LOQ level

Workup	**Amount Added (µg/mL)**	**Amount obtained (µg/mL)**	**% Recovery**	**% Mean Recovery ± SD**
Prep-1	0.2856	0.2850	99.8	101.3± 2.8
Prep-2		0.2827	99.0	
Prep-3		0.3005	105.2	

% Recovery of 101.3 at Limit of quantitation level indicates that method is highly accurate at Limit of quantitation level.

6.4.4. Linearity

Linearity of the method was determined by analyzing CPE enantiomer in different concentrations ranging from LOQ to 150 % of specification limit, which is equivalent to 0.29 µg mL^{-1} to 2.68 µg mL^{-1}. Six linearity solutions were prepared by performing appropriate dilutions of the stock solution to achieve the targeted concentrations of 0.5, 0.75, 1.0, 1.25, 1.50 and 2.0 µg mL^{-1}. Each solution was injected once.

Table: 6.4.T8. Results of Linearity for CPE-Enantiomer

S.No.	Conc. (µg mL-1)	CPE Enantiomer peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.2856	4978	4679	-299	89212	1743
2	0.4463	6857	7483	626	392422	1536
3	0.8926	15456	15271	-185	34185	1731
4	1.3389	23486	23059	-427	182518	1754
5	1.7852	30854	30846	-8	57	1728
6	2.2315	38129	38634	505	255147	1708
7	2.6778	46635	46422	-213	45458	1741
Regression coefficient		0.99966	Residual sum of squares		999000	
Slope		17449.4				
Intercept		-304.2				
% y-Intercept		-0.99	Linearity equation		y = 17449.4x -304	

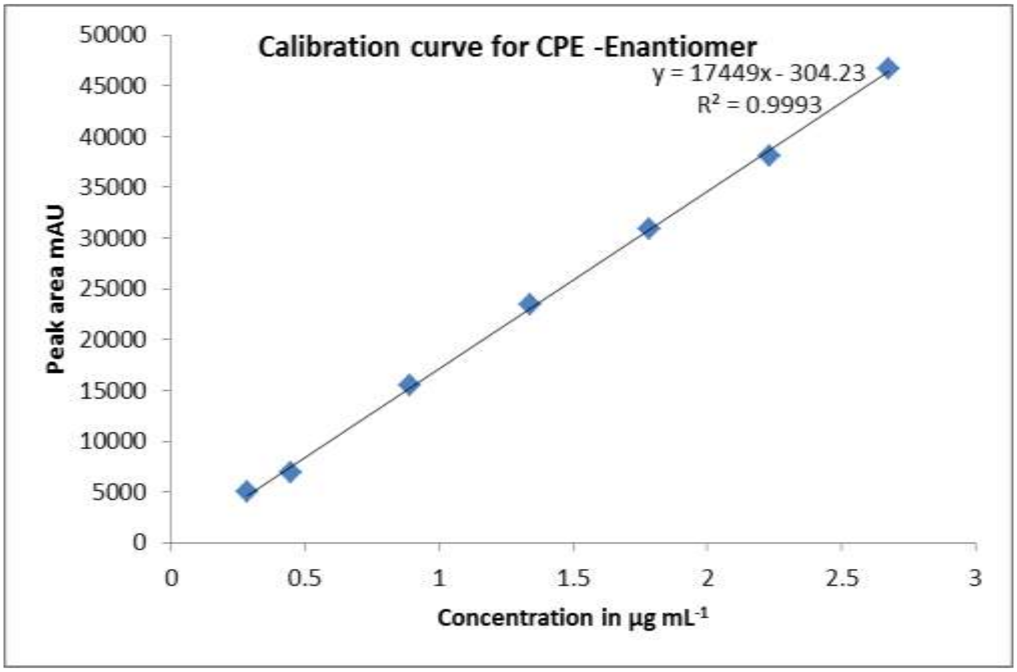


Fig: 6.4.F1. Linearity plot for CPE Enantiomer

Linearity graph was drawn with concentration of CPE-enantiomer on X-axis and mean area counts on Y-axis. The slope, y-intercept, Y-bias and correlation coefficient of the calibration curve were calculated. The results of linearity are summarized in Table 6.4.T8. The best fit linear equation obtained was Y = 17449.44x-304. The linearity graph is shown in Fig.6.4.F1.

Regression coefficient for concentration against peak area of CPE Enantiomer from LOQ to 150 % of specification limit i.e 0.10% was more than 0.99. And also % y-bias with respect to 100 % specification of CPE Enantiomer is within the limit of ± 2.0 indicates that developed chiral LC method for estimation of CPE Enantiomer in CPE intermediate is linear.

6.4.5. Accuracy

Solutions of CPE Enantiomer at three different concentration levels i.e. 50 %, 100 % and 150 % w.r.t. the specification limit (0.10%) of working concentration was spiked with CPE intermediate sample solution (conc. 2.0 mg mL^{-1}) each level was prepared in triplicate and each of nine solutions were injected once. Amount of CPE Enantiomer obtained in each solution was calculated as % recovery. Accuracy results at three levels are summarized in Table 6.4.T9. % recoveries obtained for three different levels ranged from 101.3 to 103.3 with standard deviation for the average of % recoveries of each individual analyte is less than 3.0. Above accuracy results reveal that the method is highly accurate.

Table: 6.4.T9. Accuracy result for CPE Enantiomer

Concentration of CPE Enantiomer	Amount of impurity spiked to CPE in percentage w.r.t test concentration	Amount of impurity recovered (µg mL^{-1})	% of Recovery	%Mean recovery ± SD
50% prep-1	0.05	0.052	104.0	103.3±3.1
50% prep-2		0.050	100.0	
50% prep-3		0.053	106.0	
100% prep-1	0.10	0.101	101.0	100.0±1.0
100% prep-2		0.099	99.0	
100% prep-3		0.100	100.0	
150% prep-1		0.152	101.3	101.3±0.0
150% prep-2		0.152	101.3	
150% prep-3		0.152	101.3	

6.4.6. Range

As evident from linearity, accuracy and precision study of the method, a range has been established for CPE enantiomer which is from LOQ to 150 % of specification limit. The validation results indicate that developed chiral method is linear, accurate and precise for the estimation of CPE enantiomer in CPE intermediate of Ticagrelor from LOQ to 0.15 % w.r.t. to test concentration.

6.4.7. Stability of analyte in solution form

To verify the solution stability of chiral LC method, analyzed freshly prepared solution of CPE intermediate sample was spiked with 0.10 % of CPE enantiomer. Later the content of CPE enantiomer was estimated at different time intervals upto 24 h. Solution stability results are listed in Table 6.4.T10.

Table: 6.4.T10. Solution stability data of CPE enantiomer

S.No.	Time Interval (hours)	% CPE enantiomer impurity
1	0 h	0.101
2	24 h	0.099

From the above data it can be concluded that solutions of sample solution were stable upto 24 h of study period. 0.002 % variation was observed in content of enantiomer impurity from initial to 24 h.

6.4.8. Robustness

To evaluate the influence of minute changes in finalized method parameters on separation of known components, robustness study was performed. Study was done by deliberately altering the method conditions from the original method parameters and verified USP resolution between known components.

Method parameters selected for the study were, flow rate (±0.2 mL/min), and column temperature (±5°C). Resolution between CPE intermediate and CPE enantiomer peaks under different method conditions are shown in Table 6.4.T11.

6.4.9. Conclusion from analytical method validations

The developed CPE intermediate chiral LC method by using cellulose based CSP is precise, accurate, sensitive and linear as per the ICH recommended guidelines.

The Robustness and ruggedness of intermediate precision study reveals that the method is highly rugged and robust for its intended use of enantiomer estimation in CPE intermediate.

Table: 6.4.T11. Resolution in Robustness study

Parameter and Variation	**USP resolution (Rs) between CPE intermediate and CPE Enantiomer**
As such conditions	3.08
Flow rate (mL/min)	
a. 0.8	3.30
b. 1.2	2.88
Temperature. (°C)	
a. 22	3.06
b. 32	3.02

6.5. Conclusion

The proposed chiral LC method is rapid, simple and sensitive for accurate and precise quantitation of enantiomer in CPE intermediate. This method can be effectively used for monitoring enantiomer in chiral intermediate of Ticagrelor. As the method was fully validated as per ICH guidelines, this method can be used for estimation of enantiomer for routine analysis, in quality control laboratories.

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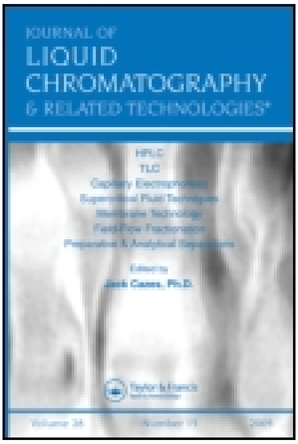
LIST OF PUBLICATIONS

List of Publications

1. **I. Ugandar Reddy,** K. Hima bindu, A. Madhuri, A. Rammohan Rao, Jaydeep lilakar, P. Nageswara Rao, V. Ranga Reddy. Development and Validation of a HPLC method for Separation and Simultaneous Determination of Process- Related substances of Irbesartan in Bulk Drugs. *Analytical chemistry An Indian journal.* 9(3) 2010 320-325.
2. **I. Ugandar Reddy,** P. Nageswara Rao, V. Ranga Reddy, K. V. V. Satyanarayana. Stability- indicating UPLC method for determination of Ramelteon and their degradation products in active pharmaceutical ingredients. *Journal of Liquid Chromatography & Related Technologie.* 35(5) 2012 688-699.
3. **I. Ugandar Reddy,** P. Nageswara Rao, V. Ranga Reddy. Quality by Design approach for the separation of Naproxcinod and its related substances by fused core particle technology column. *Journal of Chromatographic Science.* 51(5) 453- 459 2013.
4. K. Hima Bindu, **I. Ugandar Reddy,** A. Rammohan Rao, A. Madhuri, Y. Anjaneyulu, M.V. Suryanarayana. A Validated GC method for estimation of process related impurities in 3-(N-methyl-N-pentylamine) propionic acid hydrochloride (ISB), a precursor of ibandronate sodium. *Analytical chemistry An Indian journal.* 8(4) 2009 136-142.
5. Shiva Raj, K. Siva Kumari, A. Narasimha Rao, **I. Ugandhar Reddy** and M. Naga Raju. Development of a stability –indicating UPLC method for determination of olmesartan medoxomol and its degradation products in active pharmaceutical ingredient and dosage forms. *Journal of Liquid Chromatography & Related Technologies.* 35(8) 2012 1011-1026.
6. K. Hima Bindu, **I. Ugandar Reddy,** A Stability- indicating Ultra-Performance Liquid Chromatographic Method for Estimation of Related Substances and Degradants in Paliperidone Active Pharmaceutical Ingredient and its Pharmaceutical Dosage Forms. *Journal of Chromatographic Science,* 50(1) 2012 368-372.

REPRINTS



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STABILITY-INDICATING UPLC METHOD FOR DETERMINATION OF RAMELTEON AND THEIR DEGRADATION PRODUCTS IN ACTIVE PHARMACEUTICAL INGREDIENTS

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STABILITY-INDICATING UPLC METHOD FOR DETERMINATION OF RAMELTEON AND THEIR DEGRADATION PRODUCTS IN ACTIVE PHARMACEUTICAL INGREDIENTS

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☐ *A novel stability-indicating mass compatible gradient reverse phase ultra-performance liquid chromatographic (RP-UPLC) method was developed for the quantitative determination of purity of Ramelteon drug substance samples in the presence of its impurities and degradation products. The method was developed using Waters Acquity UPLC BEH SHIELD RP*$_{18}$ *(100 mm × 2.1 mm, 1.7 μm) column with mobile phase containing a gradient mixture of solvents A and B. The eluted compounds were monitored at 230 nm, the run time was 10 min within which Ramelteon and its four impurities were well separated. Ramelteon was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal, and photolytic degradation. Ramelteon was found to degrade significantly in acidic and slightly in oxidative stress conditions and stable in base, hydrolytic, and photolytic degradation conditions. The degradation products were well resolved from main peak and its impurities, proving the stability-indicating power of the method. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision, and robustness.*

Keywords forced degradation, LC-MS, Ramelteon, stability-indicating, UPLC, validation

INTRODUCTION

Though high-performance liquid chromatography (HPLC) is a well-established reliable technique used in controlling the quality and consistency of active pharmaceutical ingredients (API's) and dosage forms, it is often a slow technique because of the complexity of some of the samples, it could still be improved.

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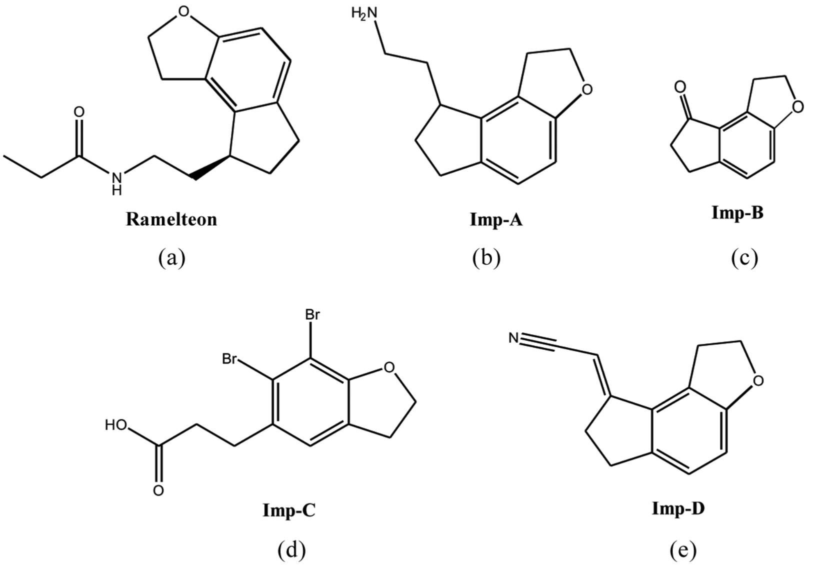


FIGURE 1 a: (S)-N-[2-(1,6,7,8-tetrahydro-2H-indeno-[5,4-b]furan-8-yl)ethyl] propionamide; b: 2-(1,6,7,8-Tetrahydro-2*H*-indeno[5,4-b]furan-8-yl)ethylamine; c: 1,2,6,7-tetrahydro-8H-indeno[5,4-b]furan-8-one; d: 3-(6,7-dibromo-2,3-dihydrobenzofuran-5-yl)propanoic acid; e: 2-(1,2,6,7-tetrahydro-8H-indeno[5,4-b] furan-8-ylidene)acetonitrile.

Ultra-performance liquid chromatography (UPLC) is a new category of separation technique based upon well-established principles of liquid chromatography, which utilizes sub-2 µm particles for stationary phase. These particles operate at elevated mobile phase linear velocities to affect dramatic increase in resolution, sensitivity, and speed of analysis. Owing to its speed and sensitivity, this technique is gaining considerable attention in recent years for pharmaceutical and biomedical analysis. In the present work, this technology has been applied to the method development and validation study of related substance determination of Ramelteon bulk drug.

The Ramelteon is an orally active hypnotic chemically designated as (S)-N-(2-(1,6,7,8-tetrahydro-2*H*-indeno[5,4-b]furan-8-yl)ethyl)propionamide and contains one chiral center (Figure 1a). Ramelteon is highly selective melatonin MT(1)/MT(2) receptor agonist used for sleep disorders.[1] Very few methods appeared in the literature for the determination of enantiomeric purity of Ramelteon on high-performance liquid chromatography (HPLC)[2] but not on the related substances.

To the best of our knowledge, none of the currently available analytical methods can separate and quantify all the known related compounds and degradation impurities of Ramelteon API. Furthermore, there is no stability-indicating HPLC/UPLC method reported in the literature that can adequately separate and accurately quantify Ramelteon API. It is, therefore, felt necessary to develop a new stability indicating method for the related substance determination of Ramelteon. We intend to opt for a faster chromatographic technique UPLC for the current study. An attempt was made to determine whether UPLC can reduce analysis times without compromising the resolution and sensitivity.

Hence, a reproducible stability-indicating RP UPLC method was developed for the quantitative determination of Ramelteon and its four impurities namely Imp-A, B, C, and D (Figure 1b–e).This method was successfully validated according to the International Conference on Harmonization (ICH) guidelines (Validation of Analytical Procedures: Test and Methodology Q2).[3]

EXPERIMENTAL

Materials and Reagents

Active pharmaceutical ingredient standards and samples were supplied by Dr. Reddy's Laboratories Limited, IPDO, Hyderabad, India. The HPLC grade acetonitrile was purchased from Merck, Darmstadt, Germany and analytical grade Trifluoroacetic acid 99%, extra pure was purchased from ACROS ORGANICS, Geel, Belgium. Water was prepared by using Millipore Milli-Q Integral 5 water purification system.

Chromatographic Conditions and Equipment

LC was carried out on a Waters Acquity UPLC with photodiode array detector. The output signal was monitored and processed using Empower software. The chromatographic column used was Waters Acquity UPLC BEH SHIELD RP_{18} 100 mm, 2.1 mm, and 1.7 µm particle size. The separation was achieved with a gradient method. The solvent A contains 0.1% Trifluoroacetic acid; and the solvent B contains a mixture of water and acetonitrile in the ratio 20:80 (v/v), respectively.

The flow rate of mobile phase was 0.3 mL/min. The UPLC gradient program (T/%B) was set as 0.01/40, 8.0/90, 9.0/90, 9.01/40, and 10.0/40. The column temperature was maintained at 35°C and the detection was monitored at wavelength 230 nm. The injection volume was 1.0 µL.

LC-MS/MS Conditions

LC–MS/MS system (Agilent 1200 series liquid chromatograph coupled with Applied Biosystems 4000 Q Trap triple quadruple mass spectrometer with Analyst 1.4 software, MDS SCIEX,USA) was used for the unknown compounds formed during forced degradation studies. Develosil ODS MG-5, 250 × 4.6 mm, 5 µm column (Nomura Chemical Co, Japan) was used as stationary phase. The 0.1% Trifluoroacetic acid (ACROS ORGANICS, Geel, Belgium) was used as buffer. The 100% buffer was used as solvent A and buffer and acetonitrile in the ratio 15:85, v/v was used as solvent B. The gradient program (T/%B) was set as 0.01/35, 20/70, 40/80, 45/95, 64/95, 65/35, and 70/35. Mixture of acetonitrile and Solvent A in the ratio 1:9, v/v was used as diluent. The flow rate was 1.0 mL/min. The analysis was performed in positive electro spray positive ionization mode. Ion Source voltage was 5000 V. Source temperature was 450°C. GS1 and GS2 are optimized to 30 and 35 psi, respectively. Curtain gas flow was 20 psi.

Preparation of Standard Solutions

A stock solution of Ramelteon (200 µg/mL) was prepared by dissolving an appropriate amount of drug in Acetonitrile:Solvent A 10:90 (v/v), respectively. Working solutions containing 0.2 µg/mL were prepared from this stock solution for determination of related substances. A mixed stock solution (20 µg/mL) of the impurities (denoted Imp-A to Imp-D) was also prepared in diluent.

Stress Studies

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities.[4] The specificity of the developed LC method for Ramelteon was carried out in the presence of its four impurities. Stress studies were performed at an initial concentration 200 µg/mL of Ramelteon drug substance to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress condition of UV light (254 nm), heat (60°C), acid (1 N HCl at 60°C), base (0.5 N NaOH at 60°C), hydrolytic (60°C), and oxidation (3.0% H_2O_2 at 60°C) to evaluate the ability of the proposed method to separate Ramelteon from its degradation products. For heat and light studies, the study period was 10 d; whereas for hydrolytic and base studies, the period was 24 hr; for acid studies, the period was 4 hr; and for oxidation studies, the period was 24 hr.

The purity of peaks obtained from stressed samples was checked by use of the PDA detector. The purity angle was within the purity threshold limit obtained in all stressed samples and demonstrates the analyte peak homogeneity.

METHOD VALIDATION

The described method has been extensively validated for related substances by UPLC determination.[3]

Precision

The repeatability of the related-substance method was checked by a six-fold analysis of 200 μg/mL Ramelteon spiked with 0.15% of each of the four impurities (Figure 2a). The RSD (%) of peak area was calculated for each impurity.

Inter- and intra-day variation and analyst variation was studied to determine intermediate precision of the proposed method. Intra-day precision was determined by six-fold analysis of 200 μg/mL Ramelteon spiked with 0.15% of each of the four impurities. The same protocol was followed for two different days to study inter-day variation ($n = 6$). Different analysts prepared different solutions on different days. The RSD (%) of peak area was calculated for each impurity.

Limit of Detection (LOD) and Quantification (LOQ)

The LOD and LOQ for Ramelteon and its impurities were determined at a signal-to noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at the LOQ level by injecting six ($n = 6$) individual preparations and calculating the RSD (%) of the area for each impurity.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the value found. For impurities, recovery was determined in triplicate for 0.075, 0.15, and 0.225% of the analyte concentration (200 μg/mL) on drug substance and recovery of the impurities was calculated.

Linearity of Response

Detector response linearity for all four impurities and Ramelteon was assessed by injecting six separately prepared solutions covering the range 25% to 150% (0.0375, 0.075, 0.1125, 0.15, 0.1875, 0.225) of the normal sample concentration (200 μg/mL). The correlation coefficients, slopes, and Y-intercepts of the calibration curve were determined.

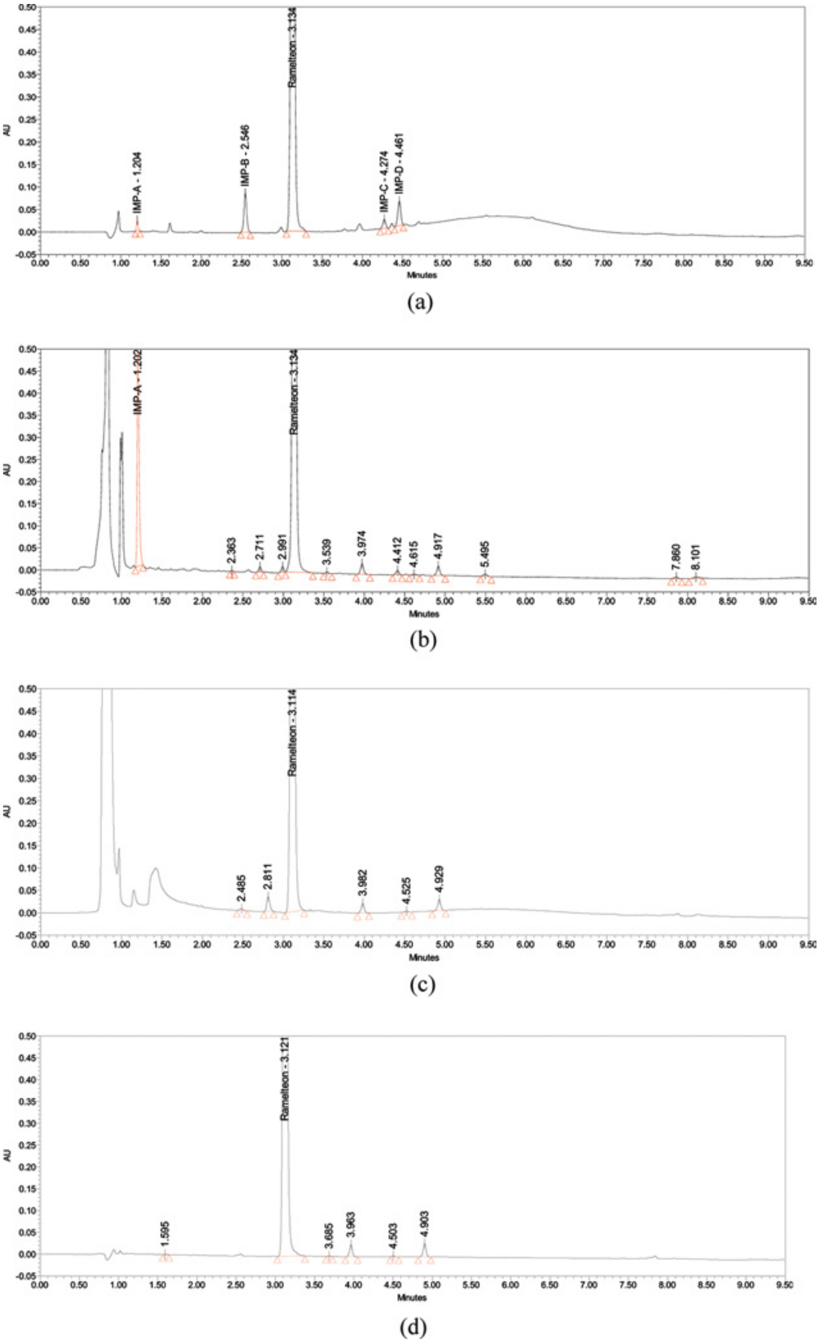
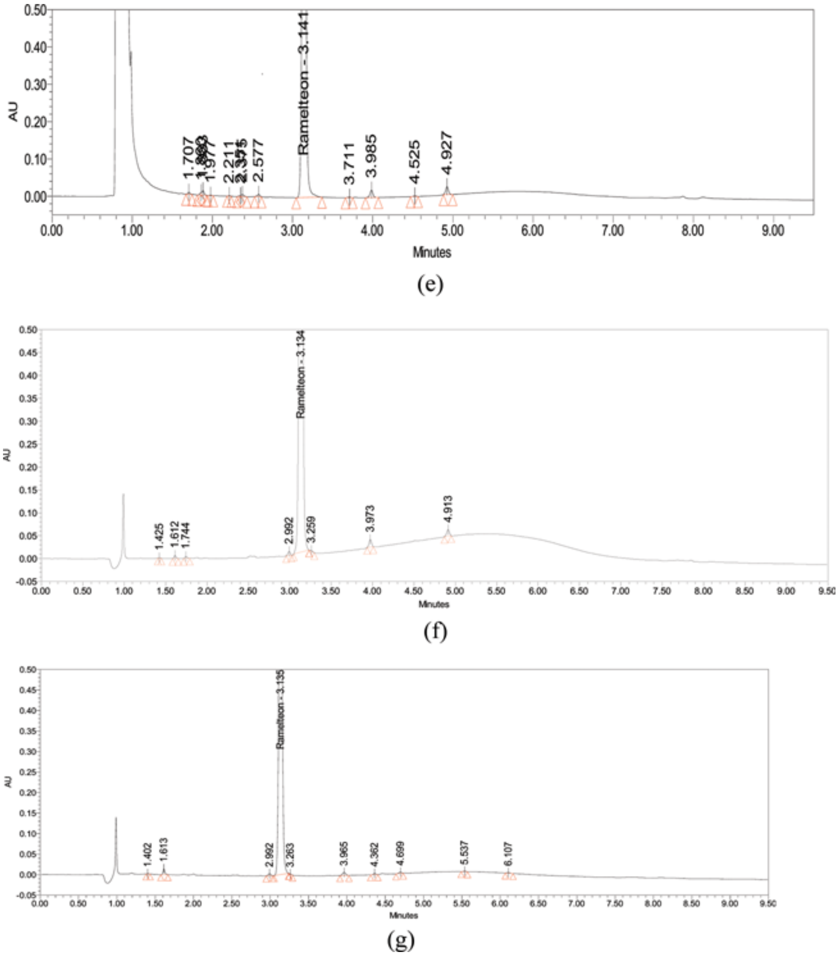


FIGURE 2 a: Impurities spiked chromatogram; b: Acid degradation chromatogram; c: Base degradation chromatogram; d: Water degradation chromatogram; e: Peroxide degradation chromatogram; f: Thermal degradation chromatogram; g: Photo degradation chromatogram. (Color figure available online.)



(e)

(f)

(g)

FIGURE 2 Continued.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

To determine the robustness of the method the experimental conditions were deliberately changed. The resolution of Ramelteon and the four impurities were evaluated. The mobile phase flow rate was 0.30 mL/min; to study the effect of flow rate on resolution it was changed to 0.27 and 0.33 mL/min. The effect of column temperature was studied at 30°C and 40°C.

Solution Stability and Mobile Phase Stability

Ramelteon solutions (spiked) prepared in diluent were injected at 0 hr, 24 hr, and 48 hr of time intervals, calculated the impurity content (Imp-A to Imp-D) and checked the consistency in the % area of the principal peak at each interval. Mobile phase prepared was kept constant during the study period.

The mobile phase stability was demonstrated by injecting the freshly prepared solution of Ramelteon and its impurities at different time intervals (0 hr, 24 hr, and 48 hr).

RESULTS AND DISCUSSION

Method Development and Optimization

From the literature it was found that the pKa of the molecule is −0.84. Due to the lower pKa of this molecule it was decided to adopt 0.1% Trifluoroacetic acid as solvent A. The blend containing 200 µg/mL of Ramelteon and 2 µg/mL of each impurity (four) was prepared in the mixture of Acetonitrile and solvent A (1:9, v/v). Ramelteon spiked solutions were subjected to separation by reverse-phase LC on a Waters Acquity BEH C18, 50 × 2.1 mm, 1.7 µm column with 0.1% of Trifluoroacetic acid as solvent A and Acetonitrile:water (80:20, v/v) as solvent B. Flow rate was set at 0.3 mL/min. The UPLC gradient program (T/%B) was set as 0.01/40, 8.0/90, 9.0/90, 9.01/40, and 10.0/40. Column temperature was maintained at 35°C (Trial-1). In this trial one of the unknown impurity closely eluting with Imp-A and other unknown impurity with Imp-C (Resolution <1.5). Efforts were made to separate these closely eluting pair of compounds. In order to increase the resolution between these pairs of compounds, buffer concentration was increased from 60 to 90 in the initial gradient step. With this increased buffer composition the retention time of Ramelteon was increased but Imp-A and its adjacent peak was co-eluting. Efforts were made to separate the pairs of compounds on Waters Acquity BEH C18, 100 × 2.1 mm, 1.7 µm column. The chromatographic conditions of Trial-1 were employed in this trial. With the increase in column length Imp-A and its adjacent peak were separated (Resolution >2) but the resolution between Imp-C and its adjacent peak was not improved. Various trials were made by changing the gradient compositions but none of the trial could serve the purpose. It was decided to change the column chemistry and Acquity UPLC BEH SHIELD RP_{18} 100 mm, 2.1 mm, and 1.7 µm column was used with the conditions mentioned in trial 1. It was found that all the peaks were separated with a resolution greater than 2.

System suitability parameters were evaluated for Ramelteon and its four impurities. Tailing factor for all four impurities and Ramelteon was found less than 1.2. USP Resolution of Ramelteon and four potential impurities was greater than 2.0 for all pairs of compounds.

Validation of the Method

Precision

The RSD (%) of peak area for the four impurities namely Imp-A, Imp-B, Imp-C, and Imp-D in the study of the repeatability is shown in Table 1. RSD (%) results of Ramelteon and its impurities for intermediate precision (intra- and inter-day repeatability) are within 4.0%. These results confirmed that the method was highly precise.

Limits of Detection and Quantification

The determined limit of detection, limit of quantification, values for Ramelteon and its four impurities are reported in Table 1.

Accuracy

The percentage recovery of four impurities of Ramelteon in bulk drug samples ranged from 87.0% to 104.9%.

Linearity

For all four impurities and Ramelteon, linear calibration curve was obtained ranging from 0.0375% to 0.225% (25%, 50%, 75%, 100%, 125%, and 150%). The correlation coefficient obtained was greater than 0.999 (Table 1). The results indicate excellent linearity.

Robustness

In all the deliberate varied chromatographic conditions (flow rate and column temperature), all analyte peaks were adequately resolved and elution orders remained unchanged.

Stability in Solution and in the Mobile Phase

No significant changes in the amounts of the four impurities were observed during solution stability and mobile phase experiments when performed using the related substances method. The results from solution stability and mobile phase stability experiments confirmed that standard solutions and solutions in the mobile phase were stable for up to 48 hr during determination of related substances.

Results from Forced Degradation Studies

All forced degradation samples were analyzed at an initial concentration 200 μg/mL of Ramelteon with UPLC conditions mentioned in conditions using PDA detector to ensure the homogeneity and purity of Ramelteon peak. Degradation was not observed when Ramelteon was subjected to

TABLE 1 Results of Validation

Regression and Precision Data

Parameter	Ramelteon	Imp-A	Imp-B	Imp-C	Imp-D
LOD (μg/ml)	0.018	0.022	0.014	0.025	0.021
LOQ (μg/ml)	0.05	0.07	0.04	0.08	0.06
Regression equation (y)					
Slope (b)	36722962.90	40380190.48	219550144.90	65856677.25	125571724.90
Intercept (a)	182.87	−75.80	−441.87	1168.20	1368.90
Correlation coefficient	0.9993	0.9990	0.9997	0.9991	0.9994
Y-intercept at 100% level	1.82%	−0.64%	−0.60%	5.06%	3.12%
Precision (% RSD)[a]	–	3.90	0.87	1.41	0.81
Intermediate precision (% RSD)#	–	1.57	0.77	1.10	1.06

Linearity range is 25%–150% with respect to 0.2 mg/mL of Ramelteon for impurities.

[a] $n =$ six determinations using 0.15% solution for impurities.

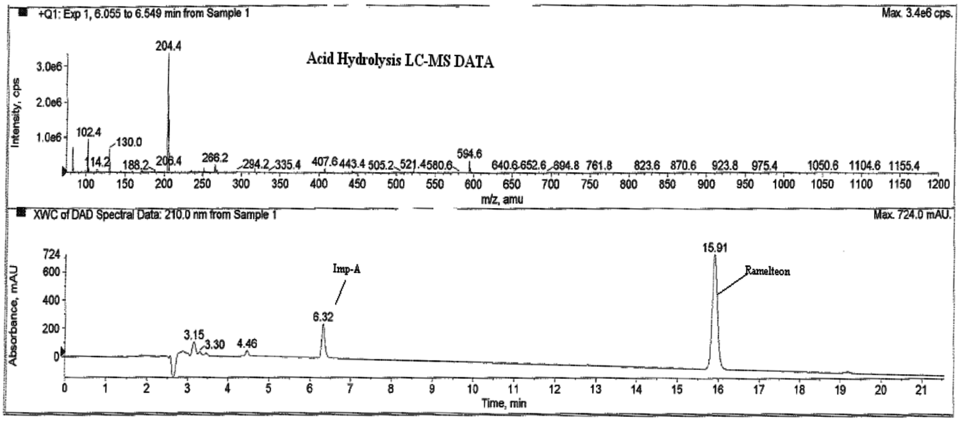


FIGURE 3 LC-MS spectra of acid hydrolysis of Ramelteon.

TABLE 2 Summary of Forced Degradation Results

Degradation Condition	Time	RS by UPLC % Degradation	Remarks/Observation
HCl-1N 60°C (Acid hydrolysis)	4 hr	12.6%	Impurity-A degradation product were formed
NaOH-0.5 N 60°C (Base hydrolysis)	24 hr	0.3%	No significant degradation observed
Water hydrolysis (60°C)	24 hr	0.2%	No significant degradation observed
Oxidation by H2O2-3.0% 60°C	24 hr	1.5%	Degradation observed
Thermal (60°C) solid	10 d	0.2%	No degradation observed
UV at 254 nm	10 d	0.2%	No degradation observed

hydrolytic (Figure 2d), base (Figure 2c), light (Figure 2g), and heat (Figure 2f) conditions. Slight degradation was observed when the drug was subjected to oxidative hydrolysis (Figure 2e) (3.0% H_2O_2 at 60°C for 24 hr) and significant degradation was observed in acid (1 N HCl at 60°C for 4 hr). Acid degradation was leading to the formation of Imp-A (Figure 2b). This was confirmed by co-injecting Imp-A standard to these degraded samples and by LC-MS/MS analysis. The m/z of the impurity was 204 which corresponded to Imp-A (Figure 3). Peak-Purity test results obtained by use of the PDA confirmed the Ramelteon peak obtained from all the degradation conditions was found to be homogenous and pure. Results from force degradation studies are presented in Table 2.

CONCLUSION

The rapid gradient RP-UPLC method developed for quantitative analysis of Ramelteon and related substances in bulk drugs is precise, accurate, linear, robust, and specific. Satisfactory results were obtained from validation of the method. The retention time (3.0 min) enables rapid determination of the drug. This method exhibited an excellent performance in terms of sensitivity and speed. The method is stability-indicating and can be used for routine analysis of production samples and to check the stability of samples of Ramelteon.

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Special thanks to my colleague Mr. B. Ramalinga Reddy from R&D and to the team members from AR&D.

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Article

Quality by Design Approach for the Separation of Naproxcinod and its Related Substances by Fused Core Particle Technology Column

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This paper describes the development of a rapid, novel, stability-indicating gradient reversed-phase high-performance liquid chromatographic method and associated system suitability parameters for the analysis of naproxcinod in the presence of its related substances and degradents using a quality-by-design approach. All of the factors that affect the separation of naproxcinod and its impurities and their mutual interactions were investigated and robustness of the method was ensured. The method was developed using an Ascentis Express C8 150 × 4.6 mm, 2.7 μm column with a mobile phase containing a gradient mixture of two solvents. The eluted compounds were monitored at 230 nm, the run time was 20 min within which naproxcinod and its eight impurities were satisfactorily separated. Naproxcinod was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. Naproxcinod was found to degrade significantly in acidic and basic conditions and to be stable in thermal, photolytic, oxidative and aqueous degradation conditions. The degradation products were satisfactorily resolved from the primary peak and its impurities, proving the stability-indicating power of the method. The developed method was validated as per International Conference on Harmonization guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness.

Introduction

Naproxcinod (NAP), chemically known as 4-(nitrooxy)butyl-(2s)-2-(6-methoxy-2-naphthyl) propanoate (Figure 1A), is a nitric oxide-donating cyclooxygenase inhibitor in development for osteoarthritis. Its molecular formula is $C_{18}H_{21}NO_6$ and its molecular weight is 347.4 g/mol (1–2).

Quality by design (QbD) is a key principle that has gained much discussion since its initiation as part of the U.S. Food and Drug Administration's vision for 21st century current Good Food Manufacturing Practices (cGMPs) and the guidance by the International Conference on Harmonization (ICH) on pharmaceutical development (3, 4). The fundamental principle of the initiative is to demonstrate both understanding and control of pharmaceutical processes to deliver high-quality pharmaceutical products while affording opportunities for continuous improvement. Although it is clear that the initiative is primarily intended for pharmaceutical product development, its use in the development of an integrated control strategy that involves analytical technology and methods should not be underestimated (5).

QbD is defined as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management." The scientific understanding gained during the method development process can be used to devise method control elements and to manage the risks identified (6).

Very few methods have appeared in the literature for the determination of the enantiomeric purity of NAP on high-performance liquid chromatography in normal phase mode (NP-HPLC) (7–8). A stability-indicating NP method was reported in the literature for NAP that only captured impurities B, G and H (9).

To the best of the authors' knowledge, there is no reported reversed-phase (RP)-HPLC method for the separation and quantification of related substances (Imp-A to Imp-H) and degradation products of NAP. Hence, an effort has been made to develop a rapid RP-HPLC method using the QbD concept.

A rapid, reproducible, stability-indicating RP-HPLC method was developed for the quantitative determination of NAP and its eight impurities; namely, Imp-A, B, C, D, E, F, G and H (Figures 1B-1I). The synthetic process for the preparation of NAP involved the reaction of naproxen (Imp-B) with 1-bromo-4-chlorobutane to form an intermediate, (*S*)-4-chlorobutyl 2-(6-methoxynaphthalen-2-yl) propanoate (Imp-F) along with Imp-G as a by-product. This intermediate (Imp-F), upon reaction with silver nitrate in a suitable solvent medium, gave NAP. Imp-C was a key starting material for the synthesis of Imp-B, and the reduction of the same gave Imp-A. Because methanol and isopropyl alcohol were used during the synthesis of Imp-B, the corresponding esters, i.e., Imp-D and Imp-E, were captured. Imp-H was a dimer, formed due to the addition of Imp-B to Imp-F. Impurities A, C and D were listed in the European Pharmacopoeia as part of a monograph on naproxen (Imp-B) (10).

This method was successfully validated according to ICH guidelines (12).

Experimental

Materials and reagents

Active pharmaceutical ingredient standards and samples were supplied by Dr. Reddy's Laboratories (Hyderabad, India). The HPLC-grade acetonitrile, potassium dihydrogen phosphate and ortho-phosphoric acid (approximately 88%) purchased from Merck (India). Water was prepared by using a Millipore Milli-Q Integral 5 water purification system.

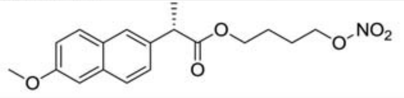
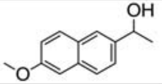
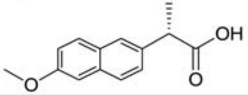
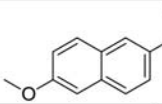
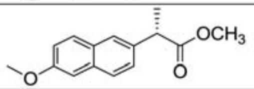
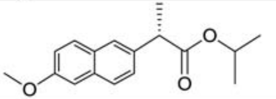
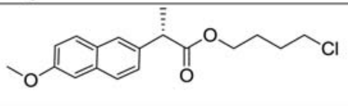
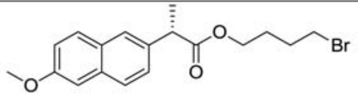
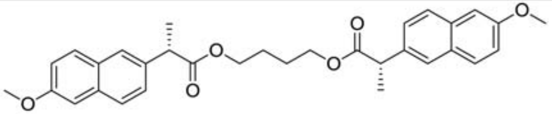
Figure	Name	Structure	Chemical name
A	Naproxcinod		(*S*)-4-(nitrooxy)butyl 2-(6-methoxynaphthalen-2-yl)propanoate
B	Impurity-A		1-(6-methoxynaphthalen-2-yl)ethanol
C	Impurity-B		(*S*)-2-(6-methoxynaphthalen-2-yl)propanoic acid
D	Impurity-C		1-(6-methoxynaphthalen-2-yl)ethanone
E	Impurity-D		(*S*)-methyl 2-(6-methoxynaphthalen-2-yl)propanoate
F	Impurity-E		(*S*)-isopropyl 2-(6-methoxynaphthalen-2-yl)propanoate
G	Impurity-F		(*S*)-4-chlorobutyl 2-(6-methoxynaphthalen-2-yl)propanoate
H	Impurity-G		(*S*)-4-bromobutyl 2-(6-methoxynaphthalen-2-yl)propanoate
I	Impurity-H		(2*S*,2'*S*)-butane-1,4-diyl bis(2-(6-methoxynaphthalen-2-yl)propanoate)

Figure 1. Structures of NAP and its eight impurities.

Chromatographic conditions and equipment

LC was conducted on a Waters Alliance 2695 HPLC with a 2998 photodiode array detector. The output signal was monitored and processed using Empower Software. All experimental design work was conducted using Design Expert 8.0.6 software by Stat-Ease (Minneapolis, MN). The chromatographic column used was an Ascentis express C8 column (150 × 4.6 mm, 2.7 μm particle size). The separation was achieved using a gradient method. KH_2PO_4 (0.02M) and 0.2% ortho phosphoric acid were used as buffer solution. Solvent A contained a mixture of buffer and acetonitrile in the ratio of 80:20 (v/v) and the solvent B contained a mixture of buffer and acetonitrile in the ratio 20:80 (v/v).

The flow rate of the mobile phase was 1.0 mL/min. The HPLC gradient program (time/percentage of solvent B) was set as: 0.01/32, 17.0/90, 17.01/32 and 20.0/32. The column temperature was maintained at 35°C and the detection was monitored at a wavelength of 230 nm, because all impurities and NAP exhibited λ_{max} at 230 nm. The injection volume was 10 μL. The diluent was a solution of acetonitrile and water (3:1)

Preparation of standard solutions

A stock solution of NAP (150 μg/mL) was prepared by dissolving an appropriate amount of the sample in diluent. Working solutions containing 0.15 μg/mL were prepared from this stock solution for the determination of related substances. A mixed stock solution (15 μg/mL) of the impurities (Imp-A to Imp-H) was also prepared in diluent.

Stress studies

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities (11). The specificity of the developed LC method for NAP was conducted in the presence of its impurities. Stress studies were performed at an initial concentration of 150 μg/mL of NAP to provide an indication of the stability-indicating properties and the specificity of the proposed method. Intentional degradation was attempted under stress conditions of ultraviolet (UV) light (254 nm), heat (90°C), acid (0.1N HCl at room temperature), base (0.1N NaOH at room temperature), aqueous (at room temperature) and oxidation (3.0% H_2O_2 at room temperature) to evaluate the ability of the proposed method to separate NAP from its degradation products. For heat and light studies, the study period was 10 days, whereas for base, it was 1 h; for hydrolytic, acid and oxidation, it was 24 h.

The purity of the peaks obtained from stressed samples was checked by use of a photodiode array (PDA) detector. The purity angle was within the purity threshold limit obtained in

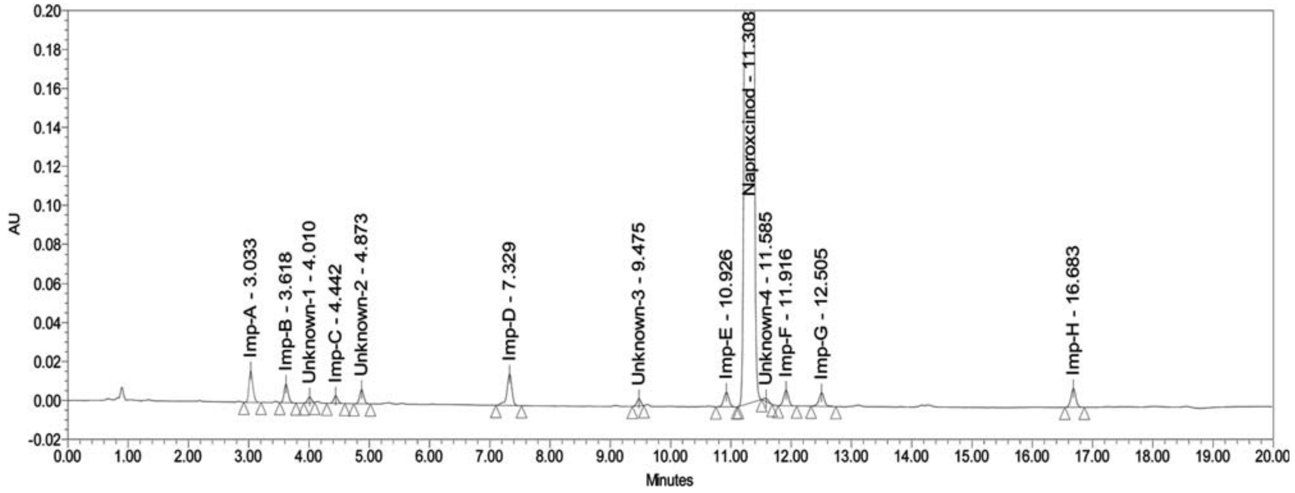


Figure 2. Naproxcinod spiked with 0.15% of all impurities.

all stressed samples and demonstrates the analyte peak homogeneity.

Method Validation

The described method was extensively validated for related substances by HPLC determination (12).

Precision

The precision of the method was verified by repeatability and intermediate precision. Repeatability was checked by injecting six individual preparations of NAP spiked with 0.15% of its eight impurities (Figure 2) with respect to the test concentration (150 µg/ mL).

The percent relative standard deviation (RSD) of the area was calculated for each impurity.

The intermediate precision of the method was checked by injecting six individual preparations of NAP spiked with 0.15% of its eight impurities with respect to the test concentration (150 µg/mL) by a different analyst using a different column on a different day.

Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) for NAP and its impurities were determined at signal-to-noise ratios of 3:1 and 10:1, respectively, by injecting a series of dilute solutions of known concentrations. A precision study was also conducted at the LOQ level by injecting six ($n = 6$) individual preparations and calculating the RSD (%) of the area for each impurity.

Accuracy

The accuracy of an analytical procedure expresses the agreement between the true value and the found value. For impurities, recovery was determined in triplicate at 0.075, 0.15, and 0.225% of the analyte concentration (150 µg/mL) on the drug substance and the recovery of the impurities were calculated.

Linearity of response

Linearity experiments were conducted by preparing and injecting solutions containing Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G, Imp-H and NAP covering the range from 25 to 150% (0.0375, 0.075, 0.1125, 0.15, 0.1875 and 0.225%) with respect to the specification limit (0.15%). The correlation coefficients, slopes and *Y*-intercepts of the calibration curve were determined.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage.

To determine the robustness of the method, the experimental conditions were deliberately changed. The resolution of NAP and its eight impurities were evaluated. The mobile phase flow rate was 1.0 mL/min; to study the effect of flow rate on resolution, it was changed to 0.80 and 1.2 mL/min. The effect of column temperature was studied at 30 and 40°C. The effect of acetonitrile content in solvent B was studied at levels of 90 and 110%.

Solution stability and mobile phase stability

NAP solutions (spiked) prepared in diluent were injected at 0, 12 and 48 h time intervals, the impurity content (Imp-A to Imp-H) was calculated and the consistency was checked in the percent area of the principal peak at each interval. The prepared mobile phase was kept constant during the study period.

The mobile phase stability was demonstrated by injecting the freshly prepared solution of NAP and its impurities at different time intervals (0, 12 and 48 h).

Results and Discussion

Method development and optimization

Defining the method objectives

An important consideration in developing impurity profiling methods is to appropriately define the requirements of the method. In a QbD approach, this involves establishing what impurities need to be separated and eluted from the chromatographic column, followed by detection. Examination of the route of synthesis for the compound of interest and structurally similar compounds is often a good starting point to define the impurities that may be considered in method development. The objectives for development can be defined as (i) to retain Imp-B, a polar degradation product, and to elute the potential non-polar reaction by-product, Imp-H, in a reasonable analysis time; (ii) to resolve known and potential process-related impurities from the active pharmaceutical ingredient (API); and (3) to determine a user-friendly methodology for quality control laboratories.

Column screening and method optimization

According to the chemical structures of the impurities and NAP, it is evident that they are hydrophobic in nature. Because the objective was to develop a rapid HPLC method, a C8 stationary phase was selected to reduce the hydrophobic interactions between the stationary phase and the impurities. The development started with 0.1% orthophosphoric acid (OPA) as mobile phase A, and methanol and water in the ratio of 75:25 (v/v) was used as mobile phase B. The selected wavelength was 230 nm and the column oven was maintained at 35°C. The gradient program was (time/percentage of mobile phase B) 0.01/50, 15/85, 30/100, 54/100, 55/50 and 60/50. NAP was retained up to 23 min and Imp-H was retained up to 40 min. Trials were made with different C8 columns and with different mobile phases (Table I). None of the trials met the objective of rapid resolution of impurities and NAP.

Trials were made with an Ascentis Express C8 150 × 4.6 mm, 2.7 µm column employing a mixture of 0.02M KH_2PO_4 and 0.2% OPA as buffer (pH of the solution: ~2.5). Mobile phase A was buffer and acetonitrile in the ratio of 80:20 (v/v). Mobile phase B was buffer and acetonitrile in the ratio 20:80 (v/v). The initial gradient program was (time/percentage of mobile phase B) 0.01/20, 25/100, 27/20 and 30/20. The column oven was maintained at 35°C. The effect of the pH of buffer on the elution of impurities was studied (Figure 3) and based on the results, a pH of 2.5 was selected. All of the peaks were separated with resolution (R_s) >1.5. Different gradient programs were attempted to optimize the retention of NAP and resolution between the impurities. The final optimized gradient program was (time/percentage of mobile phase B) 0.01/32.0, 17.0/90.0, 17.01/32.0 and 20.0/32.0.

Table I
Trials on Different C8 Columns

Sample	Column name	Resolution: Imp-C/Unknown-2	Resolution: Imp-E/NAP
1	Symmetry Shield RP8: 150 × 4.6 mm, 3.5 µm	Less than 1.0	2.28
2	Zorbax Eclipse C8: 150 × 4.6 mm, 5 µm	1.6	Less than 1.0
3	Inertsil C8-3: 150 × 4.0 mm, 5.0 µm	1.1	Less than 1.0
4	Betasil C8: 150 × 4.6 mm, 5 µm	1.2	Less than 1.0
5	YMC Pak Pro C8: 150 × 4.6 mm, 5 µm	1.7	Less than 1.0
6	Xterra RP8: 150 × 4.6 mm, 5 µm	Less than 1.0	2.0
7	Ascentis Express C8: 150 × 4.6 mm, 2.7 µm	3.5	2.5

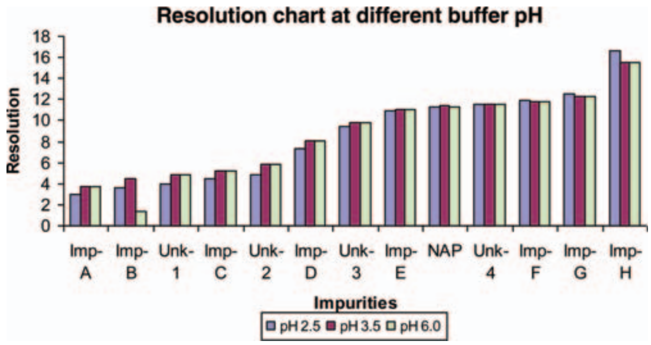


Figure 3. Effect of buffer pH on the resolution.

Table II
Factors and their Levels

Factors	Levels		
	−1	0	+ 1
Factor A (flow rate)	0.8 mL/min	1.0 mL/min	1.2 mL/min
Factor B (temperature)	30°C	35°C	40°C
Factor C (percentage of acetonitrile in solvent B)	72%	80%	88%

Design of experiments and identification of critical analytical method factors

On the basis of the preliminary experiments, the largest influences on the separation of compounds were flow rate (Factor A), column temperature (Factor B) and percentage of acetonitrile in solvent B (Factor C).

Factors and their low (−), high (+) and center point (0) levels are presented in Table II. To screen the relative influence of these factors and their possible interactions in the experimental domain, a central composite design (CCD) was chosen, which studies the effects of the selected three factors in 20 runs, including six center points. As the parameters to define chromatographic behavior of investigated substance and its impurities, resolution between two critical pairs; i.e., Unknown-1, Impurity-C (R_s-1) and Impurity-E, NAP (R_s-2) was chosen. The design and the responses obtained by the experiments are presented in Supplementary Table I. The information gathered from CCD experiments also provided an evaluation of the method's robustness.

According to the analysis of variance (ANOVA) for the response of R_s-1, the model's *F*-value was 74.36, which implies that the model is significant. Values of Prob > *F* for the terms A, B, C, AC and BC were less than 0.0500, which indicated that these terms of the model are significant. The fraction of explained variation by means of coefficient of determination, R^2, was also evaluated. The Pred *R*-Squared of 0.8650 is in reasonable agreement with the Adj *R*-Squared of 0.9508.

According to the ANOVA for the response R_s-2, the model's *F*-value was 204.85, which implies that the model is significant. Values of Prob > *F* for the terms A, B, C, AC and C2 were less

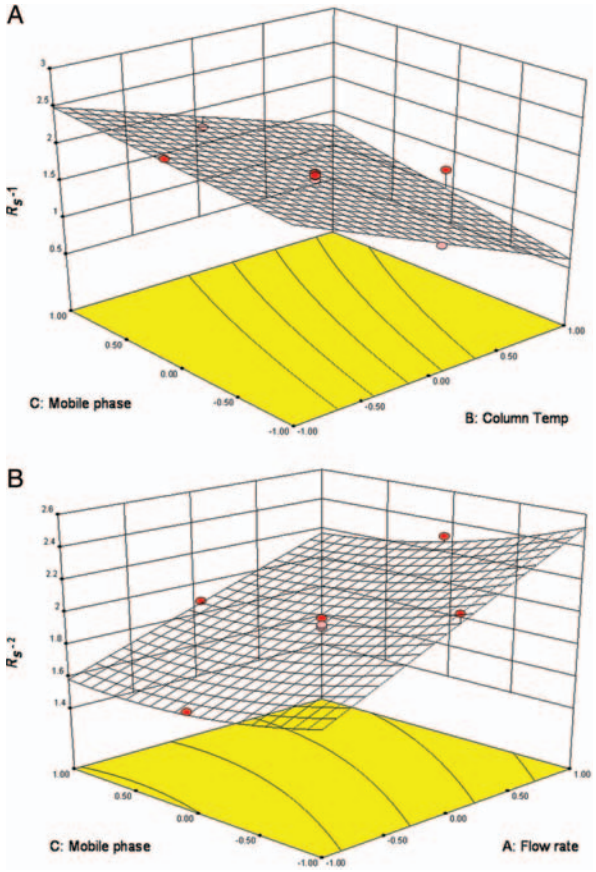


Figure 4. Three-dimensional plots of the response surface for the resolution: the variation of response (R_s-1; Unknown-1/Imp-C) as a function of acetonitrile content in solvent B and column temperature; the fixed factor is flow rate at 1.0 mL/min (A); the variation of response (R_s-2; Imp-E/NAP) as a function of acetonitrile content in solvent B and flow rate; the fixed factor is column temperature at 35°C (B).

than 0.0500, which indicates that these terms of the model are significant. The fraction of explained variation by means of R^2 was also evaluated. The Pred *R*-Squared of 0.9611 is in reasonable agreement with the Adj *R*-Squared of 0.9817. According to the values of coefficients of the polynomial model of CCD, the resolution between Unknown-1 and Imp-C (R_s-1) was significantly influenced by Factor B (column temperature) and the resolution between Imp-E and NAP (R_s-2) was affected by Factor A (flow rate), when compared to the other individual factors. The combined effect of the model term BC for the response R_s-1 and AC for the response R_s-2 was found to be more effective than the other combinations of model terms. The responses were plotted in the form of three-dimensional response surfaces to easily and more precisely define the chromatographic behavior of the investigated substances (Figure 4).

The final equation for resolution R_s-1 and R_s-2 in terms of coded factors was represented as

$R_s\text{-}1 = 1.67 - 0.30 \text{ X A} - 0.70 \text{ X B} + 0.24 \text{ X C} + 0.10 \text{ X A X C} + 0.11 \text{ X B X C}$

$R_s\text{-}2 = 1.93 + 0.34 \text{ X A} - 0.077 \text{ X B} - 0.13 \times \text{C} - 0.046 \times \text{A} \times \text{C} + 0.074 \times \text{C2}$

To achieve the best separation performances and reasonable retention of all substances in the gradient system, the data analysis led to the conclusion that the final composition of the mobile phase should contain 80% of acetonitrile and 20% of buffer. The temperature of the column should be maintained at 35°C and the flow rate should be maintained at 1.0 mL/min.

Validation of the method

Precision

The percent RSD of peak area for the eight impurities; namely, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H, in the study of the repeatability is shown in Table III. RSD (%)

Table III
Validation data

Parameter	NAP	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	Imp-F	Imp-G	Imp-H
Linearity									
r	0.9998	0.9997	0.9998	0.9995	0.9998	0.9997	0.9997	0.9982	0.9996
Slope	242.4	455.3	388.7	116.7	317.7	266.0	280.7	253.3	343.1
Y-Intercept	−3.1	−519.0	−435.7	−265.9	−256.3	−358.4	−991.9	138.4	−328.2
Accuracy (%Recovery)									
LOQ (= 3)	-	99.4	99.9	100.4	112.5	84.1	94.0	105.0	101.9
50% (= 3)	-	100.3	102.5	99.6	95.7	86.1	90.7	107.8	101.7
100% (= 3)	-	99.7	100.9	99.0	97.7	89.2	93.0	102.3	100.5
150% (= 3)	-	99.9	100.7	99.0	97.0	92.6	94.9	100.8	100.5
Precision (%RSD)									
LOQ (= 6)	0.8	0.6	1.0	1.7	1.3	0.4	4.6	1.4	1.5
100% (= 6)	-	0.2	0.1	0.4	0.1	1.5	0.5	0.3	0.2
150% (= 6)	-	0.2	0.2	0.3	0.4	0.8	0.4	0.2	0.2
Rugged ness: Different day and analyst (%RSD)									
100% (= 6)	-	0.3	0.4	0.7	0.2	0.6	0.3	1.5	0.2
Robustness (Resolution)									
Different flow 0.8mL/min	1.6	-	3.5	5.0	16.4	20.5	3.2	2.9	19.7
Different flow 1.2mL/min	2.3	-	3.1	4.4	14.7	18.5	3.1	3.1	21.7
Column temperature 30°C	2.1	-	3.6	4.9	16.2	19.3	3.3	3.1	20.8
Column temperature 40°C	1.8	-	3.0	4.7	14.9	17.7	3.0	2.9	20.8
90% organic ratio	2.2	-	3.6	4.8	15.8	18.6	3.2	3.0	21.1
110% organic ratio	1.9	-	3.2	4.7	15.8	18.9	3.2	2.9	20.4
Limit of Detection (concentration in μg/mL)									
-	0.015	0.015	0.015	0.025	0.015	0.015	0.015	0.015	0.015
Limit of Quantification(concentration in μg/mL)									
-	0.045	0.045	0.045	0.068	0.045	0.045	0.045	0.045	0.045

Table IV
Forced degradation data

Degradation condition	Time	RS by HPLC % degradation	Remarks/observation
HCl- 0.1N RT (Acid hydrolysis)	24hr	1.9%	Impurity-B as a degradation product
NaOH-0.1N RT (Base hydrolysis)	1 hr	3.1%	Impurity-B as a degradation product
Water hydrolysis at RT	24hrs	No degradation	No degradation observed.
Oxidation by H_2O_2- 3.0% RT	24hrs	No degradation	No degradation observed.
Thermal (90°C) for 10 days	10days	No degradation	No degradation observed.
UV at 254nm for 10 days	10days	No degradation	No degradation observed.

results of NAP and its impurities for intermediate precision (intra-day and inter-day repeatability) are within 1.5%. These results confirmed that the method was highly precise.

LOD and LOQ

The determined LOD, LOQ and precision at LOQ values for NAP and its eight impurities are reported in Table III.

Accuracy

The percentage recovery of eight impurities of NAP in bulk drug samples ranged from 86.1 to 112.5 % (Table III).

Linearity

For all eight impurities and NAP, a linear calibration curve was obtained, ranging from 0.0375 to 0.225% (25, 50, 75, 100, 125 and 150%) with respect to the specification limit (0.15%). The correlation coefficient obtained was greater than 0.99 (Table III). The results indicate excellent linearity.

Robustness

In all of the deliberate varied chromatographic conditions (flow rate, column temperature and variation of acetonitrile content in solvent B), all analyte peaks were adequately resolved and elution orders remained unchanged (Table III).

Solution stability and mobile phase stability

No significant changes in the amounts of the eight impurities were observed during solution stability and mobile phase experiments when performed using the related substances method. The results from solution stability and mobile phase stability experiments confirmed that standard solutions and solutions in the mobile phase were stable for up to 48 h during the determination of related substances.

Results from forced degradation studies

All forced degradation samples were analyzed at an initial concentration of 150 μg /mL of NAP with HPLC conditions mentioned previously, using a PDA detector to ensure the homogeneity and purity of the NAP peak. Degradation was not observed when NAP was subjected to aqueous, oxidative, light, and heat conditions. Significant degradation was observed when the drug was subjected to acidic hydrolysis (0.1N HCl for 24 h at room temperature) and in basic hydrolysis (0.1N NaOH at room temperature for 1 h). Acidic and basic degradation led to the formation of Imp-B. This was confirmed by co-injecting Imp-B standard with these degraded samples. The peak purity test results obtained from the PDA detector confirmed that the NAP peak obtained from all degradation conditions was homogenous and pure. Results from forced degradation studies are presented in Table IV.

This table shows that the ester functionality of NAP was susceptible to acidic and basic hydrolysis.

Conclusion

The rapid gradient RP-HPLC method developed for the quantitative analysis of naproxcinod and related substances in bulk drugs is precise, accurate, linear, robust and specific. Satisfactory results were obtained from the validation of the method .The retention time (approximately 10.0 min) enables rapid determination of the drug. This method exhibited an excellent performance in terms of sensitivity and speed. The method is stability-indicating and can be used for routine analysis of production samples and to check the stability of samples of naproxcinod.

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Development and validation of a HPLC method for separation and simultaneous determination of process-related substances of Irbesartan in bulk drugs

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ABSTRACT

A simple, inexpensive and rapid reversed-phase high-performance liquid chromatographic method has been developed for the separation and simultaneous determination of related substances of Irbesartan, an anti-hypertensive drug, in bulk drugs. Degradation studies were performed on the bulk drug by heating to 105°C, exposure to UV light at an energy of 200 Watt hours/m^2 and to Visible light at an illumination of not less than 1.2 million lux hours, acid (0.5 N Hydrochloric acid), base (0.05 N sodium hydroxide), aqueous hydrolysis and oxidation with 6.0% v/v hydrogen peroxide. Considerable degradation was observed under acid, base and oxidation conditions. Good resolution between the peaks corresponding to impurities produced during synthesis, degradation products and the analyte was achieved on a Symmetry shield RP 18 LC column using a mobile phase consisting of a mixture of aqueous potassium dihydrogen phosphate and acetonitrile. Validation of the method was carried out as per ICH requirements.

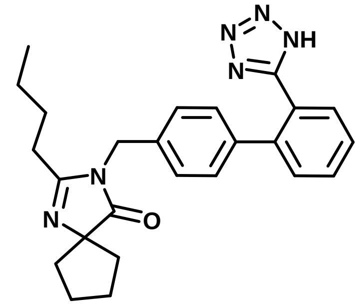
KEYWORDS

Column liquid chromatography;
Degradation studies;
Irbesartan;
Method validation.

INTRODUCTION

Irbesartan, 2-butyl-3-({4-[2-(2H-1,2,3,4-tetrazol-5-yl) phenyl] phenyl} methyl)-1,3-diazaspiro [4.4] non-1-en-4-one (Figure 1), is an angiotensin II receptor (AT_1 subtype) antagonist mainly for the treatment of hypertension. As shown in seven placebo controlled clinical trials, Irbesartan provides clinically significant dose related reductions in blood pressure in patients with mild-to-moderate hypertension. Once daily dosing provides full 24 h blood pressure control with blood pressure reductions equivalent to those of twice daily dosing, and long-term control with monotherapy in a high percentage of patients. The antihypertensive effect of Irbesartan is comparable to or exceeds that of leading antihypertensive agents[1]. The empirical formula for Irbesartan is $C_{25}H_{28}N_6O$, and the molecular weight is 428.53.

The International Conference on Harmonization (ICH) guidelines[2] require stress testing of drug substances, which can help identify the likely degradation products, can be useful in establishing degradation pathways and in validating the stability-indicating power of the analytical procedures used. Moreover, a validated



2-Butyl-3-[2′-(1H-tetrazol-5-yl)-biphenyl-4-ylmethyl]-1,3-diaza-spiro[4.4]non-1-en-4-one

Figure 1 : Structure and chemical name of Irbesartan

analytical method must be applied in stability studies[3].

Several methods have been described in the literature for the determination of Irbesartan in biological fluids. The techniques used include High-performance liquid chromatography (HPLC) with fluorescence detector[4,5]. UV[6,7], Diode-array[8]. LC-MS method was described for determining the levels of 4-methyl-2-cyano biphenyl and 4-bromomethyl-2-cyano biphenyl, which are key starting materials of Irbesartan[9]. HPLC method was mentioned in United states pharmacopeia[10] and European pharmacopoeia[11] for the estimation of one impurity. While studying the synthetic process we observed A, B, C, D, E, F, G and H as process-related impurities in the crude samples. Thus, there is a great need for development of analytical methods for separation and determination of related substances for the evaluation of quality of Irbesartan. It was simple method for the quantitative determination of process related impurities and degradents. The current work deals with the accelerated degradation of the drug substance under stress conditions like hydrolysis, oxidation, heating and UV light. The work also includes the validation of the stability-indicating method developed. This method can be used for quality control during manufacture and for assessment of the stability of bulk samples of Irbesartan.

EXPERIMENTAL

Chemicals and reagents

Samples of Irbesartan and its impurities namely Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H (Figure 2) were received from the Process Research Department of Integrated Product Development Operations of Dr. Reddy's Laboratories, Hyderabad, India. LC grade acetonitrile, Potassium dihydrogen orthophosphate, and Ortho-phosphoric acid were purchased from Merck, Schuchardt, Germany. High purity water was prepared by using a Millipore Milli Q plus purification system (Bedford, MA, USA).

Instrumentation

The LC system was a Waters model 2996 equipped with a PDA (Waters Corporation, Milford, USA) for specificity. The output signal was monitored and processed using Empower software (Waters Corporation, Milford, USA) on a Pentium computer (Digital Equipment Co). Validation Performed on a Agilent 1100 series HPLC with quaternary pump. The output signal was monitored and processed with Chemstation software.

Chromatographic conditions

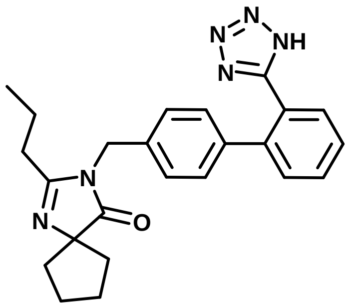
Chromatographic separation was achieved on a 5μm Symmetry shield RP18 column, (250 mm×4.6 mm), using a mobile phase with a buffer containing a mixture of 0.01 M aqueous Potassium dihydrogen orthophosphate . The mobile phase A was a mixture of buffer and acetonitrile (90:10, v/v) (Buffer pH adjusted to 2.6 using dilute ortho-phosphoric acid) and mobile phase B was a mixture of water and acetonitrile (20:80, v/v). The mobile phase was filtered and degassed through a 0.45μm nylon membrane filter. The mobile phase flow rate was 1.0mL min^{-1}. The LC gradient was time (min)/ %B: 0/30, 5/30, 43/80, 53/80, 55/30, and 60/30. The column was maintained at 30°C and the effluent was monitored at 220nm. The injection volume was 10μL. Acetonitrile and mobile phase-A (1:1) was used as diluent during the preparation of the standard and test samples.

Preparation of standard solutions

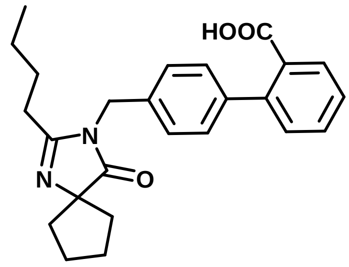
A solution of Irbesartan (1000μg mL^{-1}) was prepared by dissolving an appropriate amount in the diluent. A stock solution of impurities at 100μg mL^{-1} was also prepared in diluent.

Specificity

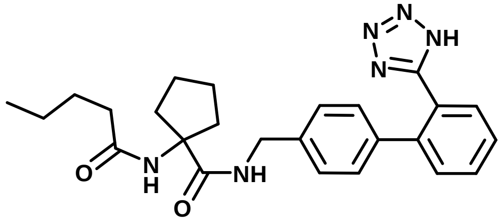
Specificity is the ability of the method to measure the analyte response in the presence of its potential im-



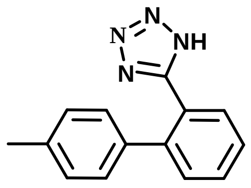
Imp-A : 2-propyl-3-[2'-(1H-tetrazol-5-yl)-biphenyl-4-ylmethyl]-1,3-diaza-spiro[4.4]non-1-en-4-one



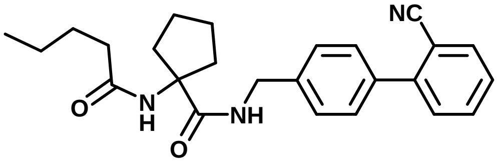
Imp-B : 4′-(2-butyl-4-oxo-1,3-diaza-spiro[4.4]non-1-en-3-ylmethyl)-biphenyl-2-carboxylic acid



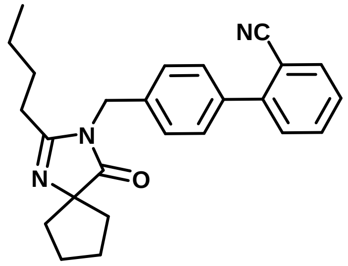
Imp-C : 1-pentanoylamino-cyclopentanecarboxylic acid [2′-(1H-tetrazol-5-yl)-biphenyl4-ylmethyl]-amide



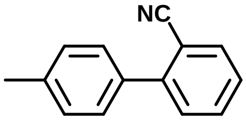
Imp-D : 5-(4′-methyl-biphenyl-2-yl)-1H-tetrazole



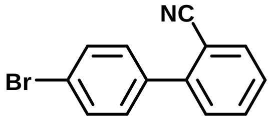
Imp-F : 1-pentanoylamino-cyclopentanecarboxylic acid (2′-cyano-biphenyl-4-ylmethyl)- amide



Imp-E : 4′-(2-butyl-4-oxo-1,3-diaza-spiro[4.4]non-1-en-3-ylmethyl)-biphenyl-2-carbonitrile



Imp-G : 4′-methyl-biphenyl-2-carbonitrile



Imp-H : 4′-bromo-biphenyl-2-carbonitrile

Figure 2 : Structures and chemical names of impurities

purities. Regulatory guidance in ICH Q2A, Q2B, Q3B and FDA 21CFR Sect. 211 requires the development and validation of a stability-indicating assay method. In order to determine whether the determination of impurities and the assay method were adequate, Irbesartan API sample was submitted to forced degradation studies. The specificity of the developed LC method for Irbesartan was carried out in the presence of its impurities.

The current regulatory guidelines do not indicate detailed degradation conditions in stress testing. However, the conditions used were found to effect a degradation of preferably not less than 5% but not complete degradation. Degradation conditions employed were UV light (200 Watt hours/m^2), Visible light(1.2 million lux hours), heating to 105°C, acid hydrolysis with 0.5 N HCl, base hydrolysis with 0.05 N NaOH, water hydrolysis and oxidative degradation using 6% H_2O_2. Peak purity testing was carried out on the stressed samples of Irbesartan by using the PDA detector.

Method validation

Precision

The precision of the determination of the impurities was checked by injecting six individual preparations of (1000μg mL^{-1}) Irbesartan spiked with 0.15% of Imp-A, Imp-B, Imp-C, Imp-D Imp-E, Imp-F, Imp-G and Imp-H and calculating the % RSD for the area of each impurity.

Limit of detection and limit of quantification

The LOD and LOQ for Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H were estimated at a S/N of 3:1 and 10:1 respectively, by inject-

TABLE 1 : LOD, LOQ, regression, precision and accuracy data

Parameter	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	Imp-F	Imp-G	Imp-H
LOQ (μgmL^{-1})	0.006	0.005	0.01	0.01	0.01	0.008	0.005	0.013
LOD (μgmL^{-1})	0.0020	0.002	0.003	0.0024	0.002	0.003	0.0016	0.0026
Regression equation (y)								
Slope (b)	0.7293	0.5288	0.5412	0.8367	0.6577	0.2922	1.2639	0.8978
Intercept (a)	-0.3089	-0.0406	-.3263	-0.9928	-0.9008	0.4824	0.2518	0.0074
Correlation coefficient	0.9999	0.9999	0.9999	0.9999	0.9969	0.9997	0.9998	0.9999
Y-intercept at 100% level	-0.42%	-0.08%	-0.61%	-1.21%	-1.39%	1.61%	0.20%	0.01%
R square value	0.9958	0.9988	0.999	0.9992	0.9997	0.9997	0.9984	0.9974
Precision(n = 6)								
(% RSD)#	5.44	4.99	5.22	2.93	7.90	4.58	4.43	3.96
Accuracy (n = 3)								
(% Recovery at LOQ)	105.3	105.1	107.8	98.3	105.3	91.6	102.2	99.1
(Recovery at 50%)	108.0	98.0	100.5	100.3	105.8	100.3	99.5	95.0
(Recovery at 100%)	99.2	99.7	98.9	100.2	100.6	100.5	100.5	98.7
(Recovery at 150%)	96.2	99.7	100.1	100.6	99.2	100.3	101.5	98.4

Linearity range is LOQ- 150 % with respect to 1.0mg/mL of Irbesartan for impurities, # six determinations using LOQ solution for impurities

ing a series of dilute solutions with known concentrations. Precision study was also carried at the LOQ level by injecting six individual preparations of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H, and calculated the %RSD for the areas.

Linearity

Linearity test solutions for the impurities were prepared by diluting the impurity stock solution to the required concentrations. The solutions were prepared at seven concentration levels from LOQ to 150% with respect to the impurities specification level of 0.15% (i.e. LOQ, 0.025, 0.050, 0.075, 0.10, 0.125 and 0.150%). The calibration curves were drawn by plotting the peak areas of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H against the corresponding concentration.

Accuracy

Accuracy of the determination of the impurities was carried out in triplicate at 0.05, 0.075, 0.10 and 0.15% of the Irbesartan concentration (1000µg mL^{-1}).The percentages recoveries for the impurities were calculated.

Robustness

To determine robustness, experimental conditions were purposely altered and the resolution between the impurities and Irbesartan peak was evaluated.

To study the effect of flow rate on the resolution between impurities and Irbesartan peak, it was changed by 0.2 units from 0.8 to 1.2mL min^{-1}. The effect of column temperature on the resolution between the impurities and Irbesartan peak was studied at 25 and 35°C. In all the above conditions, the components of the mobile phase were held constant.

Solution stability and mobile phase stability

The solution stability of Irbesartan and its impurities in the related substances method was carried out by leaving spiked sample solution in a tightly capped volumetric flask at room temperature for 48 h. Content of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H were determined at 24 h intervals. Mobile phase stability was also carried out for 48 h by injecting freshly prepared sample solutions at 24 h intervals. Content of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H were checked in test solutions. Mobile phase composition was kept constant during the study period.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Attempts were made by using different C_{18} and C_8

stationary phases[12]. The chromatographic conditions were optimized with respect to specificity, resolution and time of analysis. Effects of pH (2-7) and acetonitrile content were investigated using phosphate and acetate buffers. It was found that the retention time of Irbesartan shifts significantly at basic pH. The optimum conditions are given in 'Experimental'. Irbesartan, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H were well separated with a resolution of greater than 1.5.

Results of forced degradation studies

Degradation was not observed in Irbesartan bulk sample during stress conditions like photolytic and thermal degradation at 105°C. Slight degradation was observed in aqueous hydrolysis. Major degradation was observed in basic, acidic and oxidation conditions. Imp-C was the major degradent in basic degradation.

Peak purity test results confirmed that the Irbesartan peak was homogeneous and pure in all the analyzed stress samples.

Results of method validation studies

Precision

The RSD of area of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H were within 2.5%, confirming the good precision of the method.

Limit of detection and limit of quantification

The limits of detection (LOD) of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H were found to be in the range of 0.0016 to 0.003% (of analyte concentration 1000μg mL^{-1}) in each case for a 10μL sample size. The limits of quantification (LOQ) of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H were found to be in the range of 0.012 to 0.005%. The precision for Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H at LOQ level was below 8.0% RSD.

Linearity

Linear calibration plots for the impurities were obtained over the calibration ranges tested, i.e. LOQ to 015% for Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H. The correlation coefficients obtained were greater than 0.999. The results show that an excellent correlation between the peak area and concentration of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H.

Accuracy

The percentage recovery of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H in bulk drug samples ranged from 96.2 to 108.

Robustness

In all the deliberately varied chromatographic conditions (flow rate and column temperature), the resolution between the impurities and Irbesartan peak was more than 2.0. which reveals the adequate robustness of method.

Solution sand mobile phase stability

No significant change was observed in the content of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H during solution stability and mobile phase stability experiments. The solution stability and mobile phase stability experiments data confirm that sample solutions and mobile phase used during assays were stable up to 48 h.

CONCLUSIONS

The simple RP-LC method developed for the quantitative determination of Irbesartan and its possible degradation products and impurities is precise, accurate and specific for the analysis of bulk material. The method was fully validated, showing satisfactory results for all the parameters tested. The developed method is stability indicating and can be used for the routine analysis of production samples.

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