A New Quantification Methods of Drugs in Dissimilar Formulation

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First Impression: August 2023

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ISBN : 978-81-19477-19-7

Rs. 1000/- ($80)

Published by:
Nex Gen Publications

Preface

Analytical methodologies have taken on a significant role in pharmaceutical quality assurance. Changes in manufacturing techniques, as well as regulatory agencies' establishment of limits for individual and overall drug contaminants, are occurring on a regular basis. Pharmacological analytical approaches that include physical and chemical procedures are used to achieve this goal. We're working to create novel liquid chromatographic assays for specific medications as part of this study. The chemistry of chromomeric reagents, the reactions used in this study, is the focus of this study. New chromatographic procedures are often developed by optimising experimental circumstances (e.g. pH effects; reagent concentration and order; time and temperature retention between additions; solvent effects; colour development and stability; optical properties, etc.). Percent range of error; selectivity; precision; standard deviation; standard deviation; accuracy (comparison; assessment of significance by t-test and recovery experiments; selectivity; precision; standard deviation). Aside from studying the HPLC system components (solvent delivery systems and degassing systems, as well as gradient elution devices and sample introduction systems for liquid chromatography detectors), this research also focuses on HPLC performance calculations, such as the relative retention, theoretical plates per metre and the height of theoretical plates equal to the plate's height equivalent to theoretical plate (recovery, response function, sensitivity, precision and accuracy). The author attempted to create novel analytical techniques for several key substances in pure and pharmaceutical dose forms while keeping this in mind in the current work. Simple, fast, trustworthy, and dependable approaches are used in the thesis's methods section. Quality control and process development of bulk pharmaceuticals might benefit from the techniques.

Acknowledgement

I great fully acknowledge my sincere thanks to my Research Supervisor Prof. Rambabu, Head Department of Chemistry, RVR & JC College of Engineering, Chowdavaram Guntur, for encouraging me at every stage with exemplary patience and kindness.

We would also like to express our gratitude to the RVR & JC College of Engineering, Chowdavaram Guntur, for their support in every level in this book.

We would also like to express our gratitude who have shared their knowledge and experiences, both formally and informally.

We extend our thanks to Nex Gen Publications for publishing this edited book

Once again, thank you to all those who have played a part, big or small, in bringing this book to fruition. With heartfelt appreciation.

Dr. Raviteja. Gunturu

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 ISBN: 978-81-19477-19-7

Chapter - 1

Introduction to HPLC & UPLC and Development of Method

These last four years have been dedicated to the establishment and validation of HPLC and UPLC technologies for the chiral separation of specific enantiomeric pharmaceuticals at our lab. When conducting studies, it's crucial to understand how to apply chiral separation methods before delving into the details of the research and their findings. Chiral separation of racemic drugs, such as Rosiglitazone, Pioglitazone, Zaltoprofen, and Valganciclovir Hydrochloride, will be researched in this research group.

One of the most important characteristics of a chiral molecule is that it cannot be superimposed on itself. A chiral molecule's two mirror images are known as its enantiomers. The separation of chiral compounds has garnered considerable attention due to the prevalence of chiral molecules in bioorganic molecules. The chirality of an enantiomeric molecule is determined by the existence of chirality components (chirality axis, chirality plane, or chirality centre) in the molecule's structure. Chiral chemicals' biological activity is often reliant on their stereo selectivity since the live body is a highly chiral environment. Enantiomers, both commercial and experimental, make up the vast majority of pharmaceuticals on the market, and many of them demonstratesignificantenantioselective changes in their pharmacological characteristics. For quantitative study of chiral materials and for the assessment of enantiomeric purity, enantiomers exhibit almost identical physiochemical features. There is a lot of worry about chirality[**1-5**] in modern pharmaceutical businesses. Interest in the enantiomers of racemic drugs may be connected to this enhanced understanding of their potential effects on the body, as well as their ability to influence pharmacokinetics and pharmacodynamics.

Chiralityindrugtherapy[6,7]

There are a lot of chiral compounds in living organisms, such as DNA and enzymes, that are essential for life. In biology, chirality is critical, and enantiomers are crucial to the efficacy of medicines. Typically, a drug has only one enantiomer of interest since it must match a receptor in the cell. In cases when the enantiomer on the other hand is poisonous, it should be avoided. Table 1 (simplified). Pharmacologic properties of different isomers was shown in **[Table1.01]**

Table 1.01: Pharmacologicpropertiesofdifferentisomers

Drug	Renantiomer	Senantiomer
Propranolol	Betablocker	Inactive
Labetalol	RR-BetaBlocker	SR-alphablocker
Aminoacids	(-)tasteless/bitter	(+)sweet
Epinephrine	(-)10timesactive	(+)lessactive
Barbiturates	R(+)convulsant	S(-)depressant
Ibuprofen	Lessactive	1.4timesactive
Omeprazole	Lessactive	S(-)omeprazole(Superior)

The advantagesofusingsingleenantiomericdrugsare

- Thetotaldosecould bereduced
- Would make the dosage response connection easier to understand. simplifying the drug monitoring process
- In order to reduce the amount of inter-object variability,
- Toxicityfrominactivestereoisomerswouldbeminimized
- Pharmacokinetic complexity may be reduced by eliminating the metabolic, protein binding, transport or excretion differences between enantiomers.

Estimation of Rand Senantiomersintheirformulations

Enantiomers of racemic medications will interact with the body in a unique way and have a distinct pharmacological effect since the human body is very chiral selective. The required therapeutic action may be achieved by one isomer, whereas the other is either inactive or has undesirable side effects, depending on the specific situation.

It is necessary to digest the inactive enantiomer even if the negative effects are modest. In one chiral form of naproxen, the anti-inflammatory effectiveness is 28 times more than in the chiral relative. One isomer of dopamine is used to control tremors, whereas the other is toxic to nerve cells. Thalidomide was administered to pregnant women in the 1960s in racemic combinations to reduce morning sickness. The (R)-enantiomer, on the other hand, is the only one that can be used for treatment. There should be only one therapeutically active isomer of a chiral pharmaceutical on the market, and each of the enantiomers must be evaluated for its own pharmacological and metabolic pathways after the Thalidomide catastrophe. The (S)-enantiomer of thalidomide was discovered to be teratogenic only after hundreds of birth defects had occurred as a result of thalidomide administration. Additionally, a racemate of chiral drugs must be backed by a lengthy explanation. When it came to pharmaceutical research and development and the regulatory procedure, chirality issues had become essential.

Methods of separation of enantiomers

Singleenantiomericdrugisobtainedbyfollowingmethods

a. Chiralsynthesis

b. Indirect resolution of an achiral synthesis.

c. Chromatographicchiralseparation

a. Chiral Synthesis

A high purity chiral reagent or stereospecific reagent is required for chiral synthesis. Racemization may be avoided by meticulously monitoring the chiral purity throughout synthesis. With no waste of the undesired enantiomer and scalability to match production capacity, the long-term advantages are more obvious.

b. Achiral Synthesisfollowed byindirectresolution

Chiral syntheses are time-consuming and demanding, whereas achiral syntheses are more time-efficient and cost-effective. There are a wide range of options available.

i. Crystallization

ii. Enzymaticreaction

iii. Diastereoisomerformation followedbycrystallization.

An enantiomeric crystal (or an isomorphous substance) precipitates the racemic mixture in a supersaturated solution. This method has a significant downside since it requires a very enantioselective chiral selector. With a solution of racemic ammonium tartrate, Penicilliumglaucam (a mould), attacks the (+) form while developing, but stops if racemic modification is added.

The disadvantagesofthismethodare

- Dilutesolutionsmustbeusedandsotheamountsobtainedwillbesmall
- Oneformofisomerisdestroyed
- Itisnecessarytofindamicro-organismwhichwillattackonlyoneoftheenantiomers

It is possible to isolate the diastereoisomer formed from the racemic mixture by fractional crystallisation of the racemic mixture using optically active chemicals.

c. Chromatographicchiralseparations [8]

Using chromatography, an intriguing separation technology, enantiomeric separations may be made more efficient **[Fig. 1.01]**. For enantiomers to be separated, a particular set of factors must be taken into consideration. Enantiomers have the same physical and chemical properties as long as they only interact with achromatic substances. Sterochemicalorganisation is the only difference between them. To discriminate between the two enantiomers, chromatographic systems must be able to do this task with precision. In order to create diastereoisomer that differs for both enantiomers, a chiral component must exist in both the movable and stationary phases.

In HPLC, chiral Static phase additions or chiral mobile phase derivatization may be used to differentiate enantiomers. Routine estimations are most often made using the stationary phase method and chiral methods.

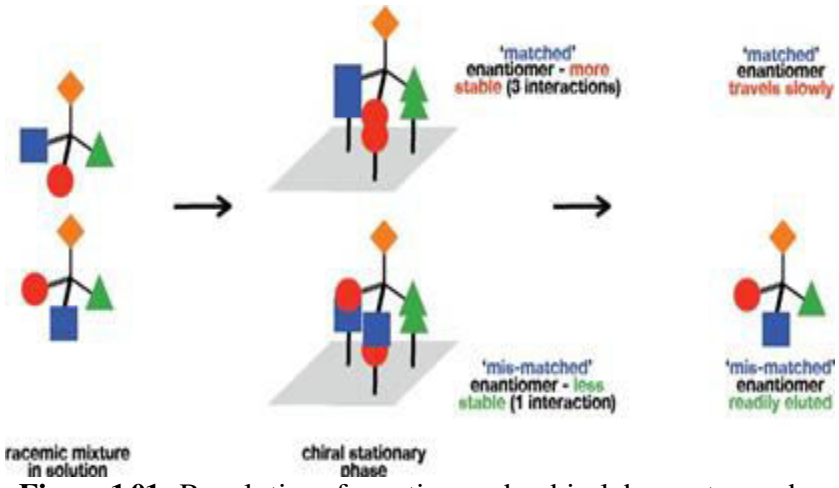

Figure1.01: Resolutionofenantiomersbychiralchromatography

Most pharmaceutical and pharmacological studies on stereo selectivity of chiral drugs employed pre-column derivatization of the enatiomers in normal phase mode of chromatography.

ThemethodsofseparatingchiralcompoundsbyHPLCare;

- Chiralstationaryphasemethod,
- Chiralmobilephaseadditivemethodand
- Derivatisationmethod.

The stationary phase of a chiral HPLC column is immobilised with a single enantiomer. The column packing surface must produce transient diastereoisomers in order to achieve resolution. Opposing enantiomers' diastereoisomer is less stable, hence it will elute sooner. For chiral recognition to work, there has to be at least three points of contact between the enantiomers.

Your mobile phase and temperature must be carefully optimised to provide maximum selectivity. Several interactions take happen at numerous stages in order to achieve separation in chromatography. Just 0.03 KJ/mol difference in free energy of contact is needed to separate enantiomers from the stationary phase.

A chiral recognition process includes intermolecular polar/ionic contacts, hydrophobic effects, pi-pi interactions, hydrophobic interactions and hydrogen bonds. Complex stages, such as inclusion complexes and binding to specific regions, such as peptide or receptor sites, may augment this even more. Intermolecular forces may be altered by changing the mobile phase pH.

Temperature has a significant impact on chiral HPLC. Lowering the temperature may actually make the chromatography worse since it alters the kinetics of mass transfer.

When it comes to enantiomer categorization, the kind of column used might be quite specific. Chiral columns are also pricey, making the choice to use one tough in the beginning. By analysing the structure of chiral phases and imagining how they can interact with the analyte, it is possible to drastically reduce the number of possible solutions. To discriminate between enantiomers and separate them in columns, a special connection between the solutes and solvents is required.

- Short-lived diastereoisomer produced by strong contacts such as co-ordinate bonds, covalent bonds, hydrogen bonds, etc..
- Close proximityofthebondstothe respectiveasymmetriccarbons.
- Two asymmetric carbons are brought into close contact, and more than one bond is needed to keep the solvent-solute molecules from interacting freely.minimise the non-contributing associative forms that don't bring the corresponding asymmetric centres close together.

Chiralstationaryphases[9]

Pirkle has pioneered the development of a new chiral stationary phase that employs derivatized and immobilised chiral agents as in-situ chiral discriminators throughout the chromatographic process. In order to reap the advantages of chromatographic separations, such as speed of analysis and capacity to analyse or purify enantiomers in complex mixtures, chiral stationary phases have been favoured. Analytical chromatographic systems may also be utilised to create enantiomers for preparative separations. In addition to its many practical uses, chiral stationary phases may be utilised to investigate the underlying mechanisms of molecule recognition. Analyzing interactions between enantiomers and stationary phases is possible because enantiomers are stored differently.

TypesofChiral StationaryPhases(CSP)

Classifying stationary phases based on how they interact with the solute is feasible. Following Wainer's publication of a system of classification.

To discriminate between the two enantiomers in type I stationary phases, carbamate-based chemicals are utilised. These include hydrogen bonding and interactions as well as dipole-to-dipole stacking.

A separation in chiral stationary phases of type II is induced through the application of attractive contacts and inclusion complexes. The bulk of type II phases are made up of cellulose derivatives.

The solute enters the chiral cavities and forms inclusion complexes in type III chiral stationary phases. Polytriphenyl methyl methacrylate and crown ether columns, for example, are common in this sector because they include inclusion complexes.

Type IV chiral stationary phases diasteroisomeric metal complexes are employed to separate them. "Chiral Ligand Exchange Chromatography" is another name for this technique.

Type V chiral stationary phases in proteins are formed through hydrophobic and polar interactions.

Chiralmobilephaseadditives

The following are benefits of chiral mobile phases over chiral stationary phases.

- Canusestandardcolumnsnamelysilica,C8,C18columns.
- Highbondingcapacityispossible
- It is possible to alter the solute's nature by ion pairing.
- WiderangeofadditivesavailableThedisadvantagesare
- After chromatography, the chiral selector must be removed.
- Divorce is a tough process to initiate.
- On a broad scale, additive recycling may be prohibitively costly.

Chiralderivitization

The reaction of an enantiomerically pure chiral derivatizing agent is required to form two diasteromers. The diastereomers may be separated in normal phase or in classic reversed phases. An optically pure chiral derivatizing agent is needed for the derivatization process to prevent the formation of racemic products (99 percent). Most chiral derivatizing agent reactions produce diasteriomeric amides, carbamates, and ureas. As seen in the **[Table1.02]**.

Table1.02: Functionalgroupsandtheirderivativesforchiralanalysis

Functionalgroups	**Derivative**
Aminogroups	Amides,Carbonates,urea,thiourea
Hydroxylgroups	Esters,carbamates,carbonates
CarboxylGroups	Esters,amides
Epoxides	Isothiocyanates,olefins
Thiols	Thioesters

Both reversed-phase and normal-phase systems have been employed for chiral derivatisation in liquid chromatography. To do chiral analysis, an optically active reagent must be used with a fluorescently-tagged target molecule. A chiral stationary phase may be more sensitive to chiral analyte if the achiral reagent is used in addition to the chiral molecules. Chiral reagents aid in the separation of molecules without distinct energy binding sites in a CSP.

Generalconsiderationsinchoosing aderivatising reagent are:

- Thederivatisingagentsmustbestable
- There should be no way to identify the derivatizing agent or any by-products that are generated during derivatization from the analyte..
- The derivatizing agent must react with the analyte in a convenient manner.
- Ifpossible,reagentsshouldbenon-toxic

Pirkle columns may be automated by using an achiral reagent for enantiomeric separation. When derivatization is carried out using an achiral reagent and a CSP column, no optically pure derivatizing reagent is required. A functional group near to the stereogenic centre of the analyte is more likely to help resolve the derivatized enantiomers **[Fig. 1.02]**. The most easily synthesised functional groups are alcohol, amines, and acids. Using chiral derivation for amines,

amino alcohols, amino acids, and thiols may increase enantioselectivity. CSP and analytes may be linked together by the use of suitable derivatives.

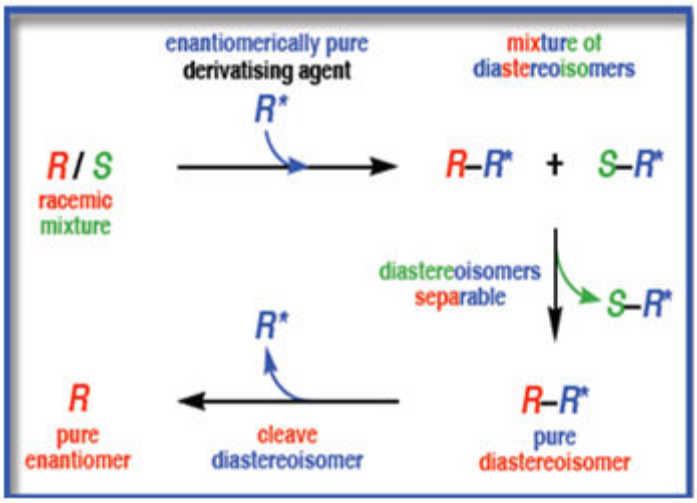

Figure1.02: Chiralderivatisingagents

Chromatographicmethods[10, 11]

This procedure is used to isolate and analyse certain sections. Depending on factors such as adsorption efficiency, partition coefficient, or molecule size, one of the system's two phases will flow in one direction or another. To separate two or more components, this technique makes use of a dynamic differential migration approach. Most often, HPLC and Liquid Chromatography/Mass Speedscreening are the chromatographic procedures used to separate and test the chiral medicine's enantiomers.

a. Highperformanceliquidchromatography

Finely divided stationary phase is used for separation in this method. For large flow rates, the mobile phase must be pressurised to at least hundreds of pounds per square inch. It is possible to utilise HPLC to separate a mixture of components for further investigation by utilizing a number of chemical and physical interactions between the columns and the material being studied.

Recently, new instruments have been developed that mix the sounds of many instruments into a single instrument. Combining a mass spectrometer (MS) with an instrument such as a gas chromatograph (GC) or high-performance liquid chromatography (LC-MS).

b. Liquidchromatography–massspectrometry [12]

This is a common method of hyphenating a phrase or word. A lab-ready analytical apparatus is born as a consequence. As long as the mixture can be dispersed, an HPLC column and mass spectrometer may be used to identify each peak component. The thermospray is one of the most often utilised HPLC-MS liquid injection interfaces. Methods that combine HPLC–MS with a thermospray interface to identify metabolites and medicines in body fluids have become more sophisticated.

TANDEM has two mass spectrometers this technique is used to separate the molecular ions of different components in a mixture and the second mass spectrometer is used to break down the first one. This combination of the three instruments is being employed in the most current and most sophisticated processes, with the goal of gaining greater power via the employment of these three instruments. Most of the GC effluent is split into two, with around 2% travelling directly to the MS and 98% going to FTIR. Each of the spectra is searched for separately in the library.

A Study of Chiral Stability by use of Forced Deterioration

A wide range of stress variables, including acid/base hydrolysis and oxidation are commonly included in these types of tests to see how they affect typical samples. Researchers may learn more about the chemistry of a medicinal molecule via these tests, and this knowledge can be used to improved formulations, storage conditions, and packaging. [**13-15**]. Degradation of the drug's ingredient and product may provide the following data.

- To discover how the medication substance and medication product undergo structural modifications.
- Low-concentration degradation products may be detected using this technique.
- In the presence of the intended product and product-related degradation agents.
- To distinguish between product-related degradants and those resulting from excipients and intact placebos. .
- To provide light on the likely course of degeneration.
- Accidental exposures to conditions outside of the typical range may be detected with this test.
- Explain the oxidative and photolytic breakdown mechanisms of the drug substance as well as the drug product.

Forced degradation studies are used to assess the stability of therapeutic substances and products, as well as discover putative degradation routes and the stability-indicating capacity of analytical techniques, according to ICH and FDA guideline manuals."

ExperimentalDesigntoForcedDegradationStudies-StudyProtoco[**16,17**]Figure 3 depicts two types of forced degradation test methods: those used for testing drug compounds and those used for testing drug products (hydrolysis, oxidation, etc).Forced degradation conditions for drug substances and drug products are shown in **[Fig. 1.03].**

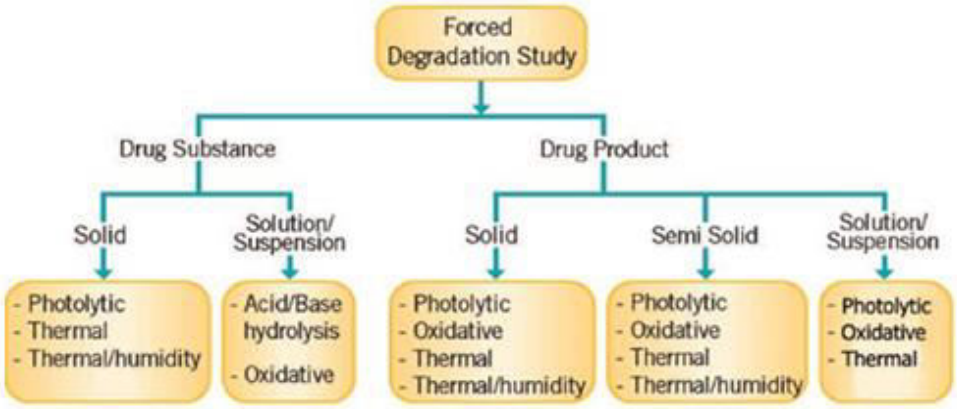

Figure 1.03: An illustrative flow diagram showing the different forceddegradationconditionsusedfordrugsubstanceanddrugproducts

Conditions under which stress testing may take place

In this initial attempt, a 10% degradation should be seen. Figure 1.03 shows a table summarising the most frequently utilised stress conditions and exposure durations for forced degradation. Degradation goals may be affected by sample solution drug concentrations that are too high or too low. Concentrated samples are less prone to degradation than diluted ones. A lower dosage might therefore help to speed up the

breakdown of the medicine.**[Table 1.03]** shows Conditions generally applied for forced degradation studies time line for conducting studies.

Table 1.03: Conditionsgenerallyappliedforforceddegradationstudiestimeline for conductingstudies

Degradation Type	Experimental Condition	Storage Condition	Sampling Time
Hydrolysis	Control API (no acid or base)	40 °C, 60 °C	1, 3, 5 days
	0.1N HCl	40 °C, 60 °C	1, 3, 5 days
	0.1N NaOH	40 °C, 60 °C	1, 3, 5 days
	Acid Control (no API)	40 °C, 60 °C	1, 3, 5 days
	Base Control (no API)	40 °C, 60 °C	1, 3, 5 days
	pH: 2, 4, 6, 8	40 °C, 60 °C	1, 3, 5 days
Oxidative	3% H_2O_2	25 °C, 40 °C	1, 3, 5 days
	Peroxide Control	25 °C, 40 °C	1, 3, 5 days
	Azobisisobutyronitrile (AIBN)	40 °C, 60 °C	1, 3, 5 days
	AIBN Control	40 °C, 60 °C	1, 3, 5 days
Photolytic	Light, 1 X ICH	NA	1, 3, 5 days
	Light, 3 X ICH	NA	1, 3, 5 days
	Light control	NA	1, 3, 5 days
Thermal	Heat Chamber	60 °C	1, 3, 5 days
	Heat Chamber	60 °C / 75% RH	1, 3, 5 days
	Heat Chamber	80 °C	1, 3, 5 days
	Heat Chamber	80 °C / 75% RH	1, 3, 5 days
	Heat Control	Room Temp.	1, 3, 5 days

The ICH guideline does not include forced deterioration tests in the early stages of establishment. For a number of good reasons, forced degradation studies of pharmaceutical substances should begin in phase I. As one of the most important reasons, the creation of a preliminary strategy that can identify the majority of likely degradation products is critical to this project's success. At this stage, a strategy with this level of reliability and strength would only need a minimal amount of testing. It is important to undertake forced degradation studies of both drug substance and product in order to identify the primary degradants before conducting stability studies prior to registration[**18,19**]

Laboratory experiments are used to verify or establish the accuracy, precision, and resilience of a technique as part of the validation process. Analytical techniques may be validated by verifying their applicability and repeatability when they are carried out by persons, laboratories, substances and equipment of the same or different kinds.

Variables in the validation process include:

- Accurateness,
- Precision(repeatabilityandreproducibility),
- Linearityandrange,
- Limitofdetection(LOD)/limitofquantification(LOQ),
- Selectivity/specificity,
- Ruggedness/robustness,
- Stabilityand
- System suitability

Accuracy

Analytical procedures are evaluated based on their capacity to provide results that are as close to the true value as feasible. Analytical techniques should be evaluated over their whole range to guarantee that they are reliable. Analyte recovery percentage, or the difference between the mean and the recognised true value, is used to assess the accuracy of

the test. Use the percentage of analyte that can be recovered by your test to evaluate how accurate your results are.

Precision [20]

Repeated testing of a procedure may reveal its accuracy by comparing the consistency of the test results obtained from the same homogeneous sample. When evaluating the accuracy of an analytical approach, the standard deviation or relative standard deviation (coefficient of variation) of a set of data is often utilised. An precise statistical estimation of the SD or RSD of a homogeneous sample is required before the validity of an analytical method's results can be determined. The accuracy of an assay, which is typically 0.3 to 3 percent, may be used to evaluate the reliability of a single measurement.

Specificity

As defined by the ICH guidelines, specificity is the ability to determine the presence or absence of components assumed to be present, such as pollutants and degradation products.

The test must be free of any impurities or excipients at all times in order to establish specificity. It is possible to show that the test results are not impacted by the presence of extraneous material by adding impurities or recipients to a substance or product.

Comparing the results of tests on materials with impurities or degradation products may demonstrate specificity. This comparison should contain samples that have been subjected to relevant stress. For example, the oxidation of light and heat.

Selectivity

Even if the analyte of interest happens to be present in the same matrices as other expected components of a sample, sophisticated analytical procedures may nevertheless accurately quantify it. Peak purity and the lowest resolution factor (Rs) of neighbouring peaks are often used to characterise HPLC selectivity. A peak purity measurement may be obtained by removing two wavelengths of data from a single chromatogram.

Samples with and without contaminants, degradation products, or placebo components may be used to test an analytical technique's selectivity.

Linearityandrange

If an analytical procedure can provide test results that are proportional to sample concentrations, it will perform well. This standard should contain all of the processes in the analysis. linearity may be described as the variation in the slope of a regression line's slope resulting from the results of tests on samples with varying concentrationsof analyte.

Analyte concentrations can be measured with a fair degree of precision, accuracy, and linearity utilising the method in question. The standard unit of measurement is used in most tests for the range. Percentage or ppm is the unit

Limitofdetection

Although it doesn't have to be quantitative, LOD stands for the lowest level of an analyte discovered in a sample. Sample detection limits may be calculated by determining the analyte's concentration (e.g., percent/ppm) in a sample.

Limitofquantification

When an analyte can be identified with adequate precision and accuracy under the experimental circumstances, it is known as the sample's LOQ. Percentage or parts per billion are used to describe the analyte concentration of a given sample (ppb).

Ruggedness
Reproducibility of test findings obtained from the same samples under multiple settings, including but not limited to distinct labs, analysts and equipment [**21**], a wide range of reagents, as well as varying assay times, temps, and days, etc., may be measured. In other words, an analytical technique is unable to be affected by operational and environmental circumstances.

Robustness [22]
Small but deliberate adjustments to process parameters are a good indicator of an analytical method's reliability when used routinely. Investigate how altering a few key elements of the procedure might alter the outcome of the separation. These characteristics are determined by a variety of variables, including mobile phase composition and pH, additives in the movable phase, column temperature, flow rate, and so on.

Stability
Samples, standards, and reagents must be kept in a stable state to provide consistent and reliable results in analytical techniques. Each analysis requires solutions and reagents to be stable for at least 24 hours. HPLC columns eventually degrade and lose their original performance, which is why long-term column stability is essential for method robustness.

Systemsuitabilitytests
System suitability assessment ensures an acceptable degree of precision and accuracy. Prior to conducting an analysis, USP parameters may be used to determine whether a system is appropriate. A column's performance may be assessed using a variety of metrics, including peak asymmetry, resolution, capacity factor, and even separation and relative standard deviation of peak area.

AIM AND OBJECTIVE
Liquid chromatography (HPLC and UPLC) technique development and validation for the measurement of pharmacological compounds in accordance with USP/ICH regulations.

1. RP-HPLC will be used to develop and validate a method for measuring Cidofovir and Famciclovir simultaneously.
2. For Mitomycin and Fluorouracil, to build and verify a stability-indicating method using UPLC technology.
3. Technique development and evaluation of Mobocertinib degradation for pharmaceutical dosage forms utilising a high pressure liquid chromatographic method.
4. Using Reverse phase liquid chromatography, this research aims to develop and validate Tepotinb techniques in bulk and pharmaceutical dosage forms.
5. A technique for the simultaneous estimation of Brigatinib and Alectinib may be designed and validated using Reverse Phase Ultra Performance Liquid Chromatography (RPUPLC).

Organization of Work:
The work presented in the thesis divided conveniently into six chapters as mentioned below.

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 ISBN: 978-81-19477-19-7

Chapter - 2

A Study of Development and Validation of a Method for Simultaneous Estimation of CIDOFOVIR and FAMCICLOVIR using RP-HPLC

2.1 Drug Profile

Drug Profile of Cidofovir

CMV and retinitis are the most common side effects of cidofovir, an injectable antiviral medication that is often used in persons with HIV to treat cytomegalovirus (CMV) [**1**]. Only one symptom, Cytomegalovirus retinitis, has been certified by authorities throughout the globe [**2**] This drug may also be used to treat HSV infections that are aciclovir-resistant [**3**]. Progress in the treatment of progressive multifocal leukoencephalopathy has been made with the use of cidofovir, and the results have been positive. In spite of this, randomised studies found that the medicine was ineffective [**4**] During a bioterror strike involving instances of smallpox, Cidofovir might have anti-smallpox characteristics and be employed on a limited basis. A much improved anti-smallpox agent, brincidofovir is a cidofovir analogue that may be used orally [**5,6**] Despite the lack of clinical investigations, it has been shown to reduce the replication of the varicella-zoster virus in vitro [**7,8**], which might be because safer options like aciclovir are readily available [**9**]. Cidofovir shows anti-BK virus action in a subset of transplant patients [**10,11**]. Adjunctive intralesional therapy for HPV-related papillomatosis might be Cidofovir [**12-14**].

2.1.1Structure of Cidofovir: [Fig. 2.01]

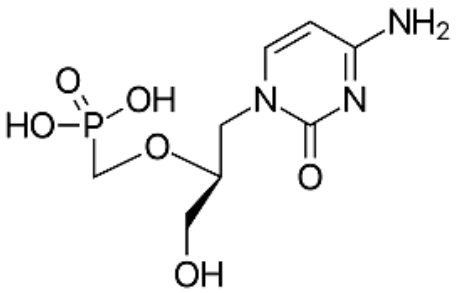

Figure 2.01: Cidofovir's chemical structure

2.1.2 Name of the IUPAC: [(2*S*)-1-(4-amino-2-oxopyrimidin-1-yl)-3-hydroxypropan-2-yl]oxymethylphosphonic acid

2.1.3Molecularformula: $C_8H_{14}N_3O_6P$

2.1.4Molecularweight: 279.19

2.1.5 Category

Antivirals are the class of medications Cidofovir belongs to. It does this by halting the virus's development. CMV retinitis cannot be cured with cidofovir, and it is possible that your condition may deteriorate both during and after the therapy.

2.1.6 Mechanism of Action

Cidofovir inhibits viral DNA polymerase in a targeted manner. An analysis of cidofovir diphosphate shows that it inhibits the CMV DNA polymerase in a specific manner. Cidofovir diphosphate inhibits herpesvirus polymerases by an 8- to 600-fold ratio when compared to the quantity necessary to block human cellular DNA polymerase alpha, beta, and gamma (1,2,3). Antiviral drug Ciofovir has been shown to slow down viral DNA production.

2.1.7 Side effects of Cidofovir:

- Vomiting
- Nausea
- Diarrhea

- Loss of appetite
- Headache
- Hair loss
- Sores on the lips, mouth, or throat

2.1.8 Contraindications:
There is a contraindication to Cidofovir use in individuals with a serum creatinine concentration of more than 1.5 mg/dl or a computed creatinine clearance of less than 55 ml/min, and in those with more than 2+ proteins in the urine (urine protein concentration more than or equal to 100 mg/l).

2.1.9 Absorption
Cytomegalovirus (CMV) replication is suppressed by cidofovir, which inhibits viral DNA synthesis. Viral DNA polymerase is selectively inhibited by Cidofovir. Cidofovir diphosphate, the active intracellular metabolite of cidofovir, has been shown in biochemical studies to block CMV DNA polymerase selectively.

2.1.10 Uses:
As an antiviral drug, cidofovir keeps the growth of specific viral cells in your body to a minimum. AIDS patients with cytomegalovirus retinitis (CMV) are treated with cidofovir (acquired immunodeficiency syndrome).

2.1.11 Adult dose:
Injectable solution75mg/ml

2.2 Famciclovir

Drug profile of Famciclovir:
Guanosine analogue antiviral medication Famciclovir is used to treat herpes zoster and other herpesvirus infections [**15,16**]. (shingles). Prodrug is a penciclovir with a better oral bioavailability. As a kind of antiviral medication, Famvir is known by its brand name, famciclovir (Novartis). It is used to treat immune-competent people with herpes zoster (shingles) and herpes simplex virus 2 (genital herpes) and to prevent recurrence in patients with these conditions [**17,18**], as well as patients with herpes labialis (cold sores). Persistent herpes simplex is also treated with it in HIV patients. Figure 1 depicts the chemical structures of Cidofovir and Famciclovir.

2.2.1 Structure of Famciclovir: [Fig. 2.02]

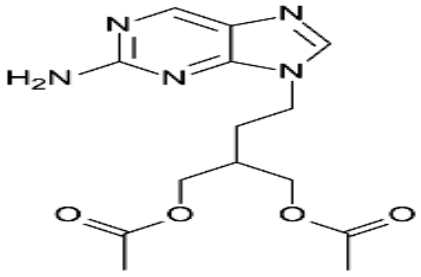

Figure 2.02: Famciclovir's chemical structure

2.2.2 Name of the IUPAC: [2-(acetyloxymethyl)-4-(2-aminopurin-9-yl)butyl] acetate

2.2.3Formulamolecular: $C_{14}H_{19}N_5O_4$

2.2.4Molecularweight: 321.33 $g \cdot mol^{-1}$

2.2.5 Category

In the family of drugs known as antivirals, famciclovir belongs. When the herpes virus is stopped from spreading, it has a positive effect on health.

2.2.6 Mechanism of Action:

Biotransformation of famciclovir[**19**] to penciclovir, an antiviral compound that suppresses herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and varicella zoster virus has been shown to take place (VZV). When cells are infected with HSV-1, HSV-2, or VZV, they create penciclovir monophosphate, which is phosphorylated by viral thymidine kinase and subsequently converted to penciclovir triphosphate by cell kinases. This drug is used to treat a variety of viruses. In vitro, penciclovir triphosphate competes with deoxyguanosine triphosphate to inhibit HSV-2 DNA polymerase. Herpes virus DNA synthesis and replication are particularly prevented as a result.

2.2.7 Side effects of Famciclovir:

- Headache
- Nausea
- Stomach pain
- Gas
- Diarrhea

2.2.8 Contraindications:

- All of the following antiviral drugs[**20**] have been linked to an increased risk of allergic reactions: hypersensitivity to acyclovir, valacyclovir, or valganciclovir.
- Dialysis, geriatric, renal failure, renal impairment.
- Children, infants, neonates.
- Herpes infection, varicella.

2.2.9 Absorption

Oral administration of famciclovir results in fast conversion to penciclovir (systemic availability: 77%) and good absorption (low intersubject variability). Aciclovir, on the other hand, has a sluggish and imperfect absorption, with a highly fluctuating systemic availability of just 10–20 percent.

2.2.10 Uses:

Herpes zoster may be treated with famciclovir (shingles; a rash that can occur in people who have had chickenpox in the past). Cold sores and fever blisters may also be treated with this medication if the patient has a healthy immune system[**21**].

2.2.11 Adult dose:

Tablet

- 125mg
- 250 mg
- 500 mg

2.3Literature Resurvey

Till today there is no literature on Cidofovir and Famciclovir

2.4 Experimental

2.4.1 Chemical and Reagents:

Merck (India) Ltd. Worli, Mumbai, India provided the acetonitrile, formic acid, and water. Glenmark Pharmaceuticals in Mumbai provided both Cidofovir and Famciclovir APIs used as reference standards.

2.4.2 Instrumentation:

HPLC makes: The chromatographic device used was the Waters alliance e-2695, which included a quaternary pump, a PDA detector-2996, and the chromatographic programme Empower-2.0.

2.4.3 Standard Solution Preparation

In a 100ml volumetric flask, weigh 75 mg of Cidofovir and 250 mg of Famciclovir working requirements, apply 70ml of diluents, sonicate for 15 minutes to remove the contents, and dilute volume with diluent. Using diluents, dilute 1 mL to 10 mL.

2.5 Method Development

Analytical Method Development:

A successful attempt has been made in the proposed project to establish a simple accuracy for Cidofovirand Famciclovir analysis using RP-HPLC.

2.5.1 Method Development Parameters:

Selection of following parameters in method development is very important.

- Mode of chromatography
- Wavelength
- Column
- Mobile phase composition
- Solvent delivery system
- Flow rate
- Injection volume

2.5.1.1 Selection of mode of chromatography:

Selected mode of chromatography	:	Reversed phase chromatography
Basis of selection	:	polarity of the molecule
Reason for selection	:	as Cidofovirand Famcicloviris polar

molecule it elutesat faster along with mobile phase

2.5.1.2 Detector wavelength selection:

The last stage in the analytical procedure is the choosing of the detector's wavelength. This higher-response API serves as the reference for figuring out the precise wavelength of the chromatographic system's PDA detector.

Selected wave length: 250 nm

Basis for selection: Maximum absorbance of analytes and impurities

Reason for selection: Cidofovirand Famciclovir having maximum absorbance

250nm.

2.5.1.3 Selection of column:

Column selected: Symmetry C-18 150x4.6mm, 3.5µ

Basis for selection: Based on the polarity, and chemical differences among analytics

Reason for selection:A wide range of bonding chemistries, high range of mechanical stability, excellent physiochemical surface properties and compatibility with a wide range of organic solvents

2.5.1.4 Selection of the mobile phase composition and of the buffer:
Peak symmetries and separation are heavily influenced by the buffer and its intensity. The ionic form may be altered during chromatography if the injection load on the column is not covered by the proper strength buffers.

Mobile phase preparation:
Solution A: Acetonitrile

Solution B: 0.1% Formic acid

2.5.1.5 Selection of the rate of flow:
Flow rate is expressed as an important factor, even in reverse phase separation for the resolution of small molecules. In large-scale inverted phase chromatography, the flow rate used during the loading of the sample solution is especially important but not crucial during analytical experiments. Depending on the flow rate used during sample loading, dynamic binding capacity can differ. In order to evaluate the optimum flow rate for loading, the dynamic binding capability should be calculated when scaling up the purification. In this system, the flow rate is set to 1 ml/min and is dependent on factors such as flow factor, retention duration, column composition, separation impurity, and peak symmetrical symmetry.

2.5.1.6 Selection of injection volume:
An injection volume of between 10 and 20 l is suggested for API estimations in most cases. However, since extraction proved to be problematic, the test concentration may be kept low and the injection volume increased to 50 l. Although it is crucial to make sure there is not too much pressure on the column volume that you set. This approach uses a 10 l injection volume for Cidofovir and Famciclovir.

2.5.1.7 Trials in optimization of chromatographic condition:

Trial-1 [Fig. 2.03]

Mobile phase	:	Acetonitrile and 0.1% OPA (80:20)
Column	:	X-bridge phenyl(250x 4.6mm, 5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	7min
Wavelength	:	200-400 nm
Observation	:	Peaks are not separated clearly

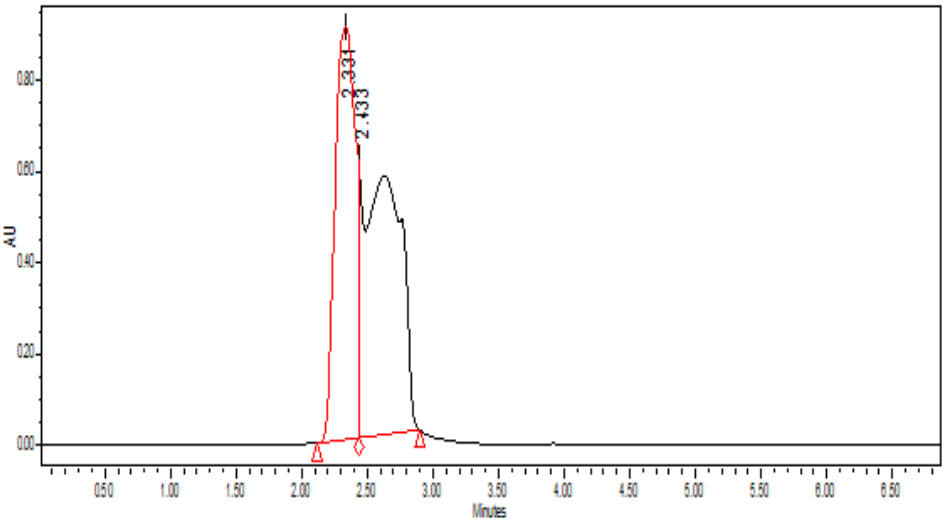

Figure 2.03: chromatogram of trial-1

	Name	RT	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		2.331	10728566	49.36		1.26	645
2		2.433	11006886	50.64	0.12	0.65	968

Trial-2 [Fig. 2.04]

Movable phase : Acetonitrile and 0.1% OPA (70:30)

Column : X-bridge phenyl (250x 4.6mm, 5µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 15 min

Wavelength : 250 nm

Observation : System suitability conditions are not within the limit

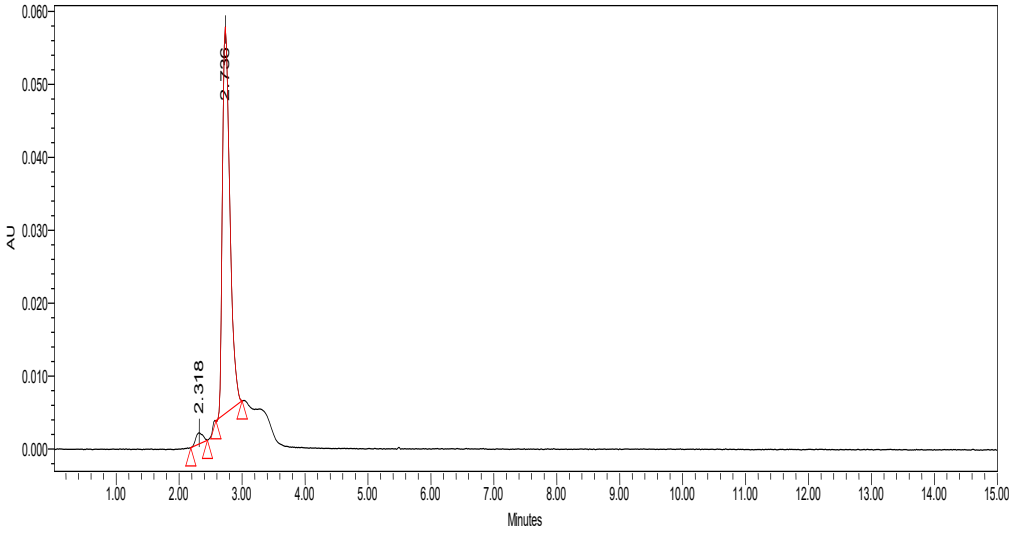

Figure 2.04: Chromatogram of trial-2

	Name	RT	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		2.318	12046	2.68		1.02	1763
2		2.736	437077	97.32	1.94	1.46	2442

Trial-3: [Fig. 2.05]

Movable phase : Acetonitrile and 0.1% OPA (65:35)

Column : X-bridge phenyl(250x 4.6mm, 5µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 5 min

Wavelength : 250 nm

Observation : Extra peak is observed

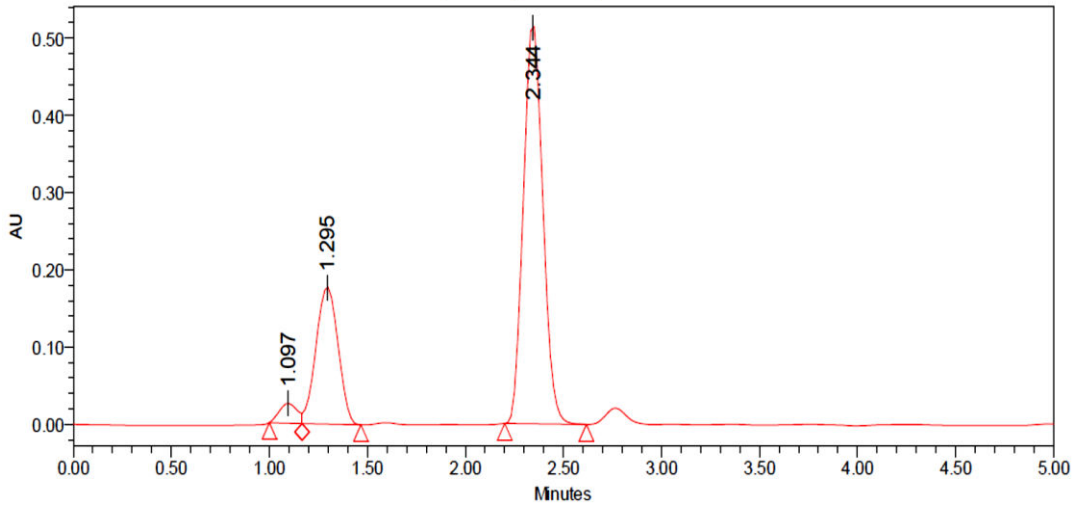

Figure 2.05: Chromatogram of trial-3

	Name	Retention Time	Area	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.097	8487	14.65		1.02	1220
2		1.295	148766	35.35	0.73	1.06	2537
3		2.344	98745633	51.38	3.87	1.01	5863

Trial-4 [Fig. 2.06]

Movable phase : Acetonitrile and 0.1% TFA (50:50)

Column : X-bridge phenyl(250x 4.6mm, 5µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 5 min

Wavelength : 250 nm

Observation : Retention time is not within the limit

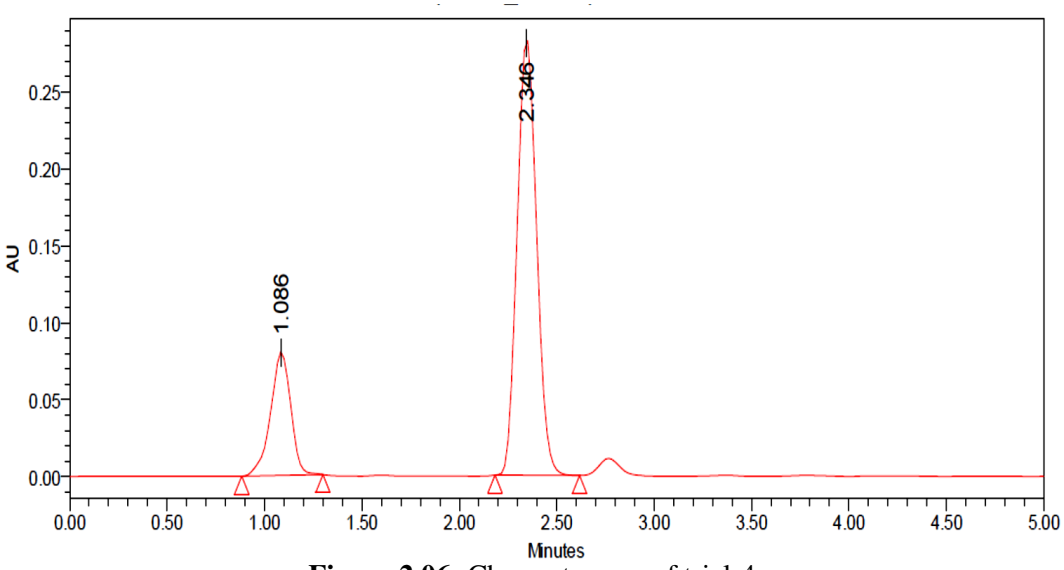

Figure 2.06: Chromatogram of trial-4

	Name	RT	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.086	95610	13.59		1.56	2388
2		2.346	2824564	86.41	4.59	1.48	5410

Trial-5 [Fig. 2.07]

Movable phase : Acetonitrile and 0.1% TFA (40:60)

Column : X-bridge phenyl(250x 4.6mm, 5µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 5 min

Wavelength : 250 nm

Observation : Peak height is not within the limit

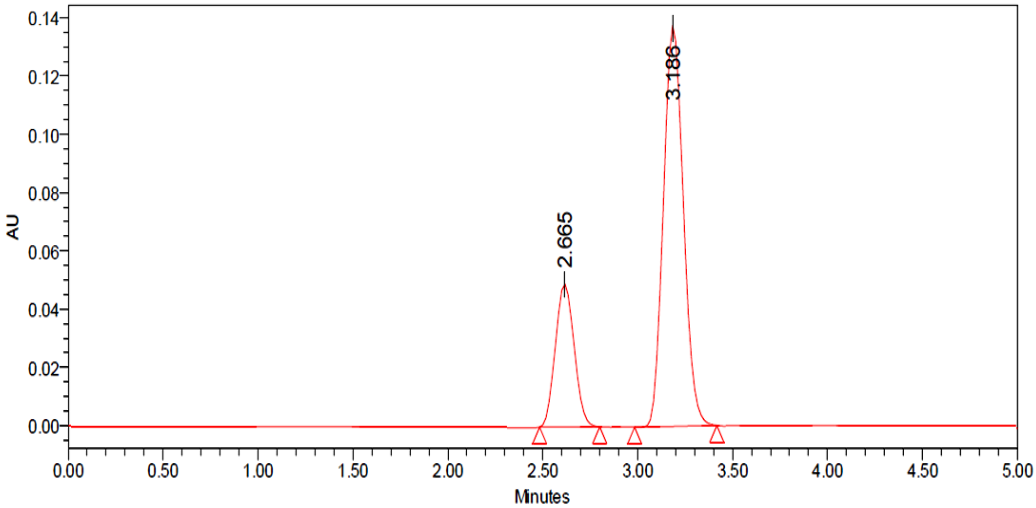

Figure 2.07: Chromatogram of trial-5

	Name	RT	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		2.665	98685	13.51		1.08	4645
2		3.188	2864631	86.49	2.05	1.11	8858

Trial-6 [Fig. 2.08]

Movable phase : Acetonitrile and 0.1% TFA (30:70)

Column : Symmetry C_{18} (150x4.6mm, 3.5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 8 min

Wavelength : 250 nm

Observation : Resolution is not with the limit

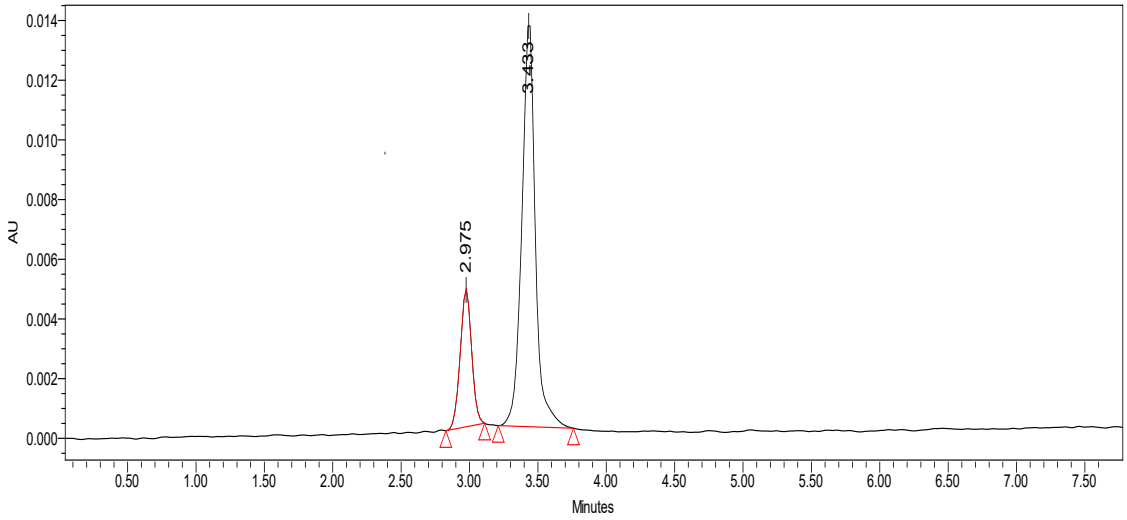

Figure 2.08: Chromatogram of trial-6

	Retention Time	Area	% Area	USP Resolution	USP Tailing	USP Plate Count
1	2.975	1627041	42.57		0.80	2221
2	3.433	2207626	57.43	1.86	1.31	5440

Trial-7 [Fig. 2.09]

Movable phase : Acetonitrile and 0.1% Formic acid (60:40)

Column : Symmetry C_{18} (150x4.6mm, 3.5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 9 min

Wavelength : 250 nm

Observation : Splitting of the peak is observed

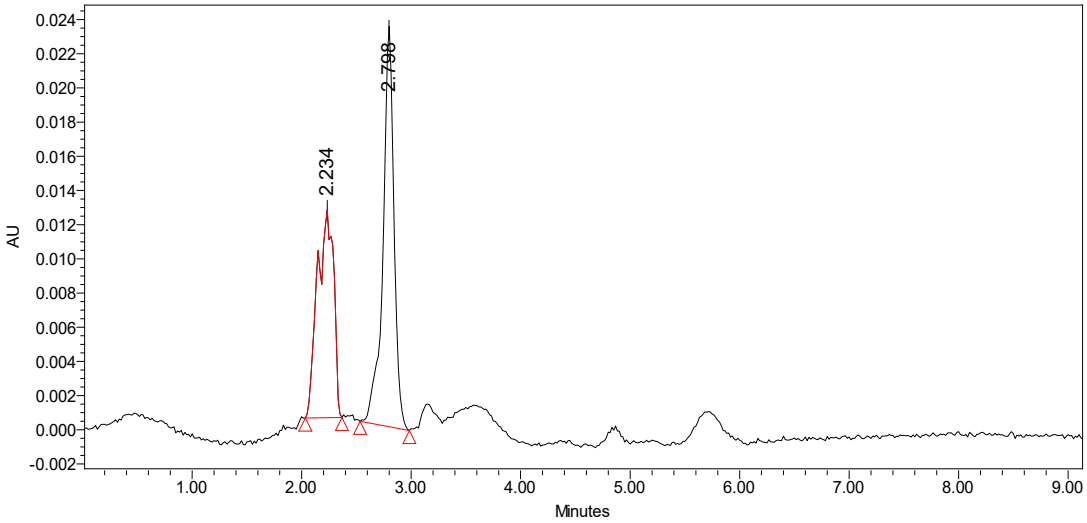

Figure 2.09: Chromatogram of trial-7

	Name	Retention Time	Area	% Area	USP Resolution	USP Tailing	USP Plate Count
1		2.234	126008	43.47		0.81	1163
2		2.798	163862	56.53	2.55	0.84	4306

Trial-8 [Fig. 2.10]

Movable phase : Acetonitrile and 0.1% Formic acid (50:50)

Column : Symmetry C_{18} (150x4.6mm, 3.5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 6min

Wavelength : 250 nm

Observation : Response of the peak is very high

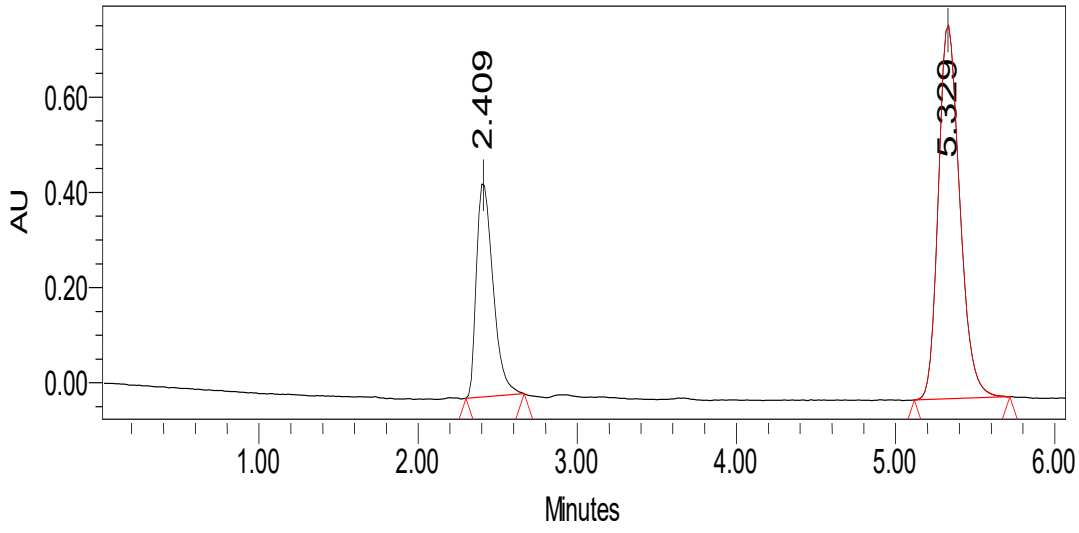

Figure 2.10: Chromatogram of trial-8

	Name	Retention Time	Area	% Area	USP Resolution	USP Tailing	USP Plate Count
1		2.409	3483674	31.82		1.64	2523
2		5.329	7463416	68.18	13.09	1.23	7490

Trial-9 [Fig. 2.11]

Movable phase : Acetonitrile and 0.1% Formic acid (45:55)

Column : Symmetry C_{18} (150x4.6mm, 3.5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 7 min

Wavelength : 250nm

Observation : Baseline is not sufficient

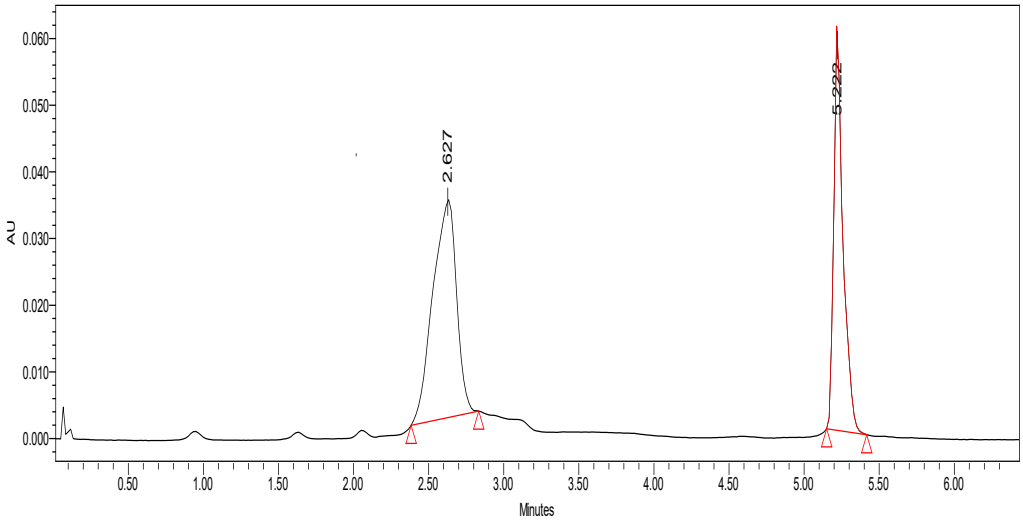

Figure 2.11: Chromatogram of trial-9

	Name	Retention Time	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		2.627	357744	58.14		0.82	8114
2		5.222	257606	41.86	12.07	1.52	10010

Trial-10 [Fig. 2.12]

Movable phase : Acetonitrile and 0.1% Formic acid (40:60)

Column : Symmetry C_{18} (150x4.6mm, 3.5 µ)

Rate of flow : 1 ml/min

Volume of injection : 10µl

Period of run : 8min

Wavelength : 250 nm

Observation : This method is suitable for validation

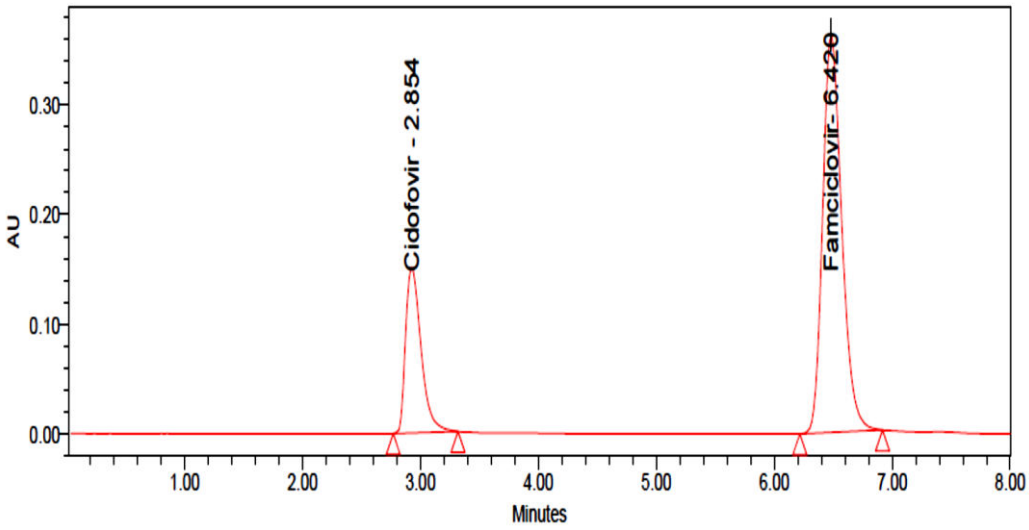

Figure 2.12: Chromatogram of trial-10

	Name	Retention time	Response	USP tailing	USP plate count	USP resolution
1	Cidofovir	2.854	1623947	1.01	2459	
2	Famciclovir	6.420	3655088	1.05	7365	13.23

2.5.1.8 Optimized Method: [Table 2.01]

Table 2.01: Optimized method chromatographic conditions

S.NO	Parameter	Chromatographic condition
1	Movable phase	Acetonitrile: 0.1% Formic acid (40:60)
2	Column	Symmetry C_{18} (150x4.6mm, 3.5 µ)
3	Rate of flow	1ml/min
4	Column temperature	Ambient temperature
5	Wavelength	250 nm
6	Volume of injection	10µl
7	Period of run	8min
8	Retention time	Cidofovir Retention time-2.854 Famciclovir retention time-6.420

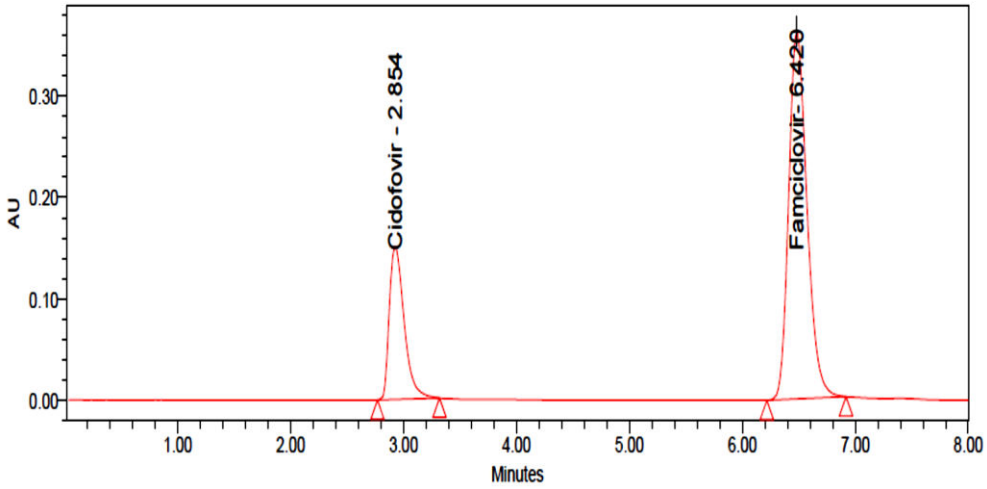

Figure 2.13: Chromatogram of standard

[Fig. 2.13] shows chromatogram of standard

2.6Validation of Method

Standard characteristics such as device compatibility, adequacy, specificity, precision and linearity were all verified using ICH Q2 (R1) standards as was the method's stability and forced deterioration.

2.6.1 Specificity: [Fig. 2.14]

One measure of analyte specificity is its ability to be detected even when the sample solution and standard reference solutions include unknown contaminants or excitations. Samples spiked with Cidofovir and Famciclovir were used to evaluate it.

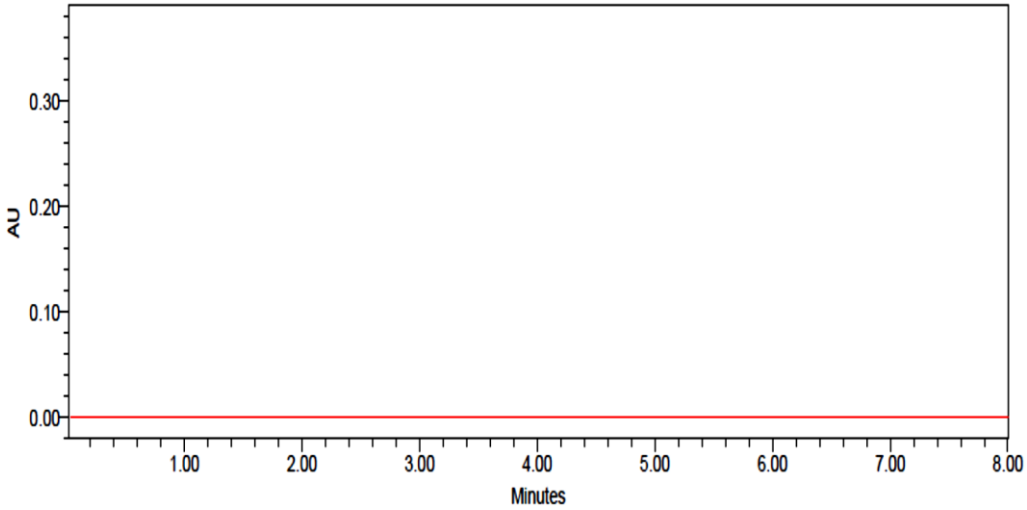

Figure 2.14: Chromatogram of blank

2.6.2 Linearity:

A study's ability to produce results within a specified framework is known as the linearity of empirical methodology. The peak area was inversely proportional to the concentration of analytes in the sample for the linearity spectrum assessment, and six sets of standard solutions were employed. Regression equations were developed by plotting the peak area of the calibration curve using the concentration of normal solution. The least squares method of least squares was used to get the slope and correlation coefficient.

Linearity Stock Solution Preparation:

Apply 70ml of diluents to 75mg of Cidofovir and 250mg of Famciclovir that have been weighed and transferred into a 100ml vacuum flask. For 15 minutes, sonicate the diluents to dissolve.

10 percent solution preparation: (7.5ppm of Cidofovirand 25ppm of Famciclovir)

Stock solution was diluted in a 10 ml vacuum flask with diluents up to the mark in a separate 10 ml flask containing 0.1 ml of the stock solution.

25 percent solution preparation: (18.75ppm of Cidofovir and 62.5ppm of Famciclovir)

0.25 ml of the stock solution was diluted in a 10 ml vacuum flask with the diluents up to the mark in a second flask.

50 percent solution preparation: (37.5ppm of Cidofovir and 125ppm of Famciclovir)

Diluting 0.5ml of the stock solution to the mark in another 10 ml vacuum flask, diluents were added.

100 percent solution preparation: (75ppm of Cidofovir and 250ppm of Famciclovir)

After diluting one millilitre of the stock solution to the desired concentration in another 10 millilitre vacuum flask, 1 millilitre of this stock solution was re-mixed with an equal amount of each of the following:

125 percent solution preparation: (93.75ppm of Cidofovirand 312.5ppm of Famciclovir)

Using the same stock solution, 1.25 ml of diluents were added to a 10 ml vacuum flask and the volume was brought up to the mark.

150 percent solution preparation: (112.5ppm of Cidofovir and 375ppm of Famciclovir)

1.5 ml of the above-mentioned stock solution was diluted with the diluents to the appropriate concentration in a separate 10 ml vacuum flask.

Procedure:
Use the chromatographic technique to inject and measure the peak response at each step. Coefficients of correlation may be calculated by drawing a line graph of peak area (Y-axis peak area) vs concentration (X-axis concentration). Linearity results are shown in [table 2.02], calibration plots are shown in [figs. 2.15, 2.16], and chromatograms showing linearity at 10%, 25%, 50%, 100%, 100%, 125 percent, and 150 percent are shown in [figs. 2.17-2.22].

Range:
Between the upper and lower analytical phases, there is a spectrum of analytical procedures that may be relied upon for accuracy, precision, and a linear relationship between results.

Inclusion Criteria:
Not less than 0.9999 is required for a correlation coefficient.

Table 2.02: Results of linearity

S. No.	Cidofovir		Famciclovir	
	Conc. (µg/ml)	Response	Conc. (µg/ml)	Response
Linearity-1	7.50	158204	25	348542
Linearity-2	18.75	425693	62.5	823021
Linearity-3	37.50	859647	125	1785496
Linearity-4	75.00	1652476	250	3618462
Linearity-5	93.75	2063021	312.5	4353621
Linearity-6	112.50	2458174	375	5278263
Slope	21873.41		14129.08	
Intercept	10127.10		5862.72	
CC	0.99988		0.99971	

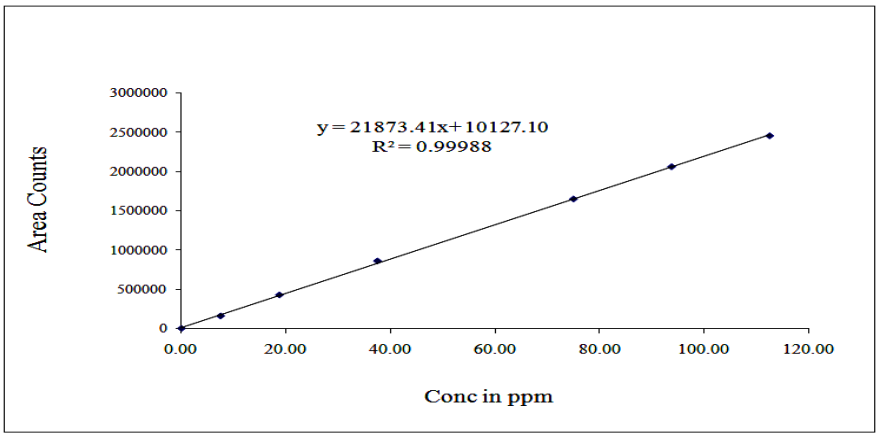

Figure 2.15: Calibration plot of Cidofovir

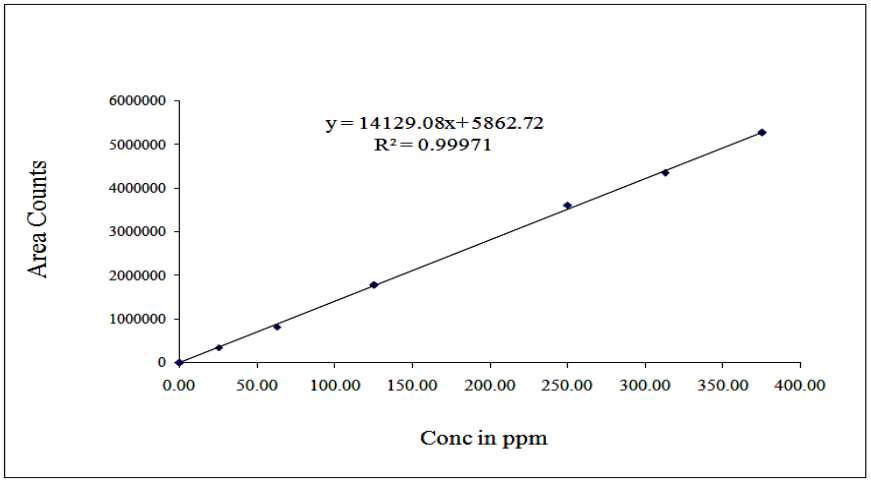

Figure 2.16: Calibration plot of Famciclovir

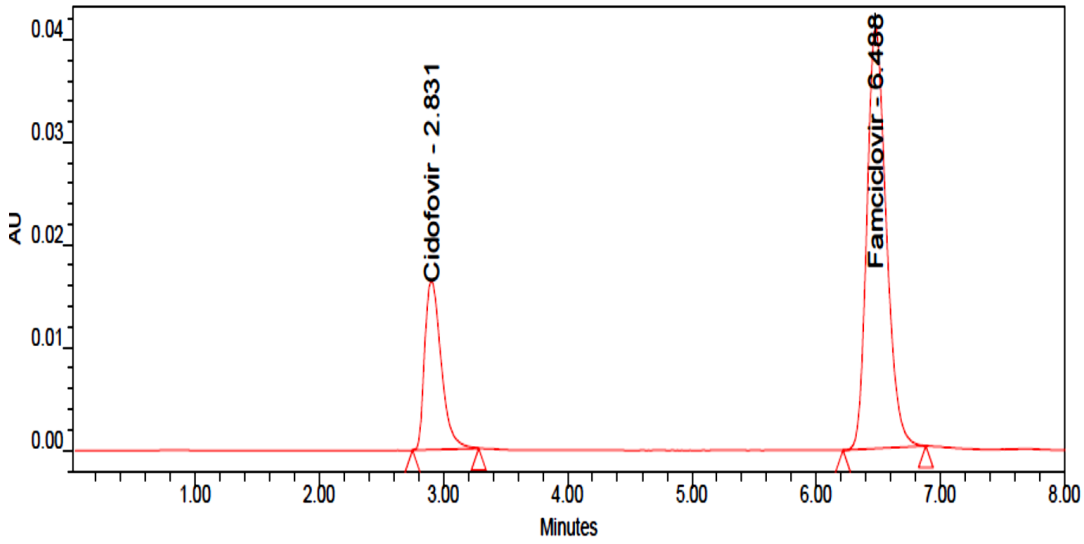

Figure 2.17: 10 percent linearity chromatogram

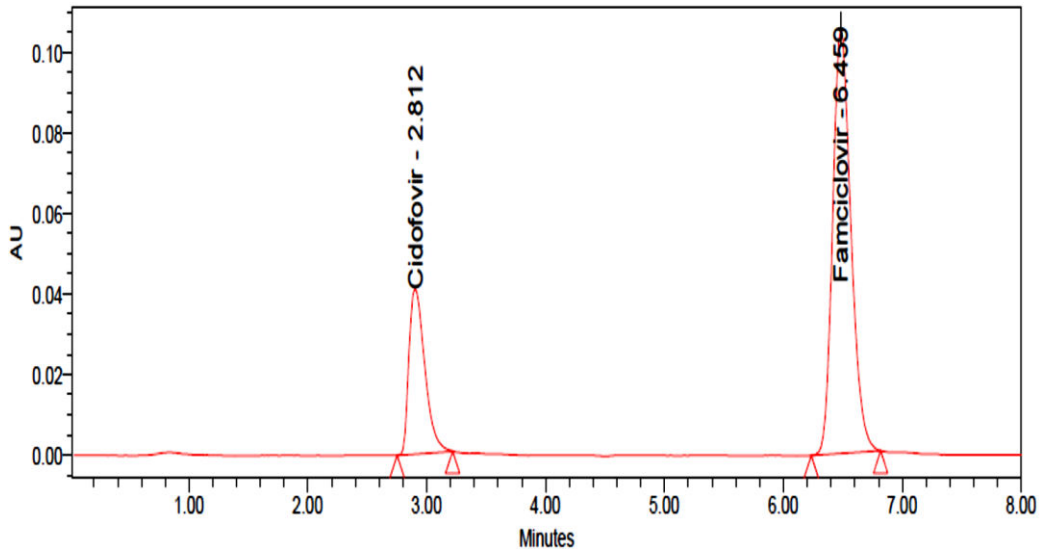

Figure 2.18: 25 percent linearity chromatogram

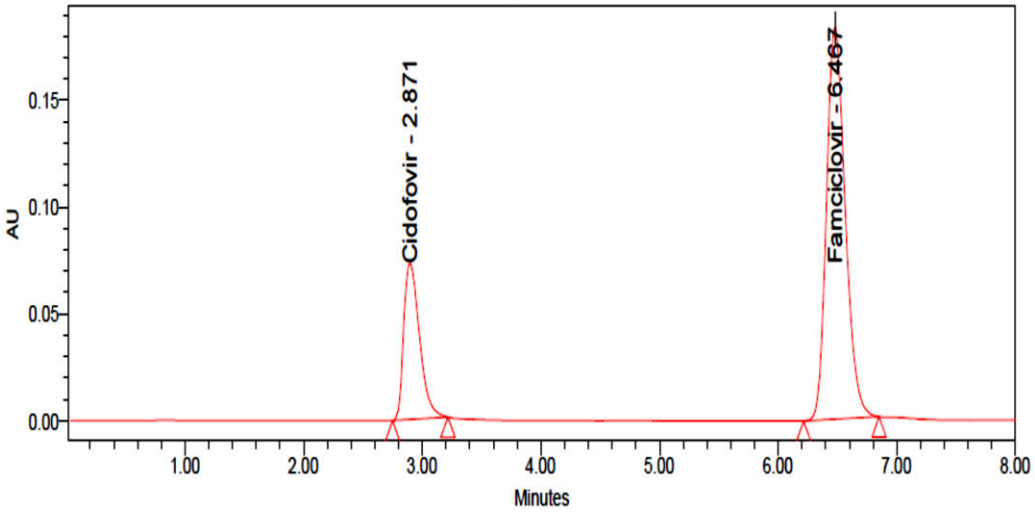

Figure 2.19: 50 percent linearity chromatogram

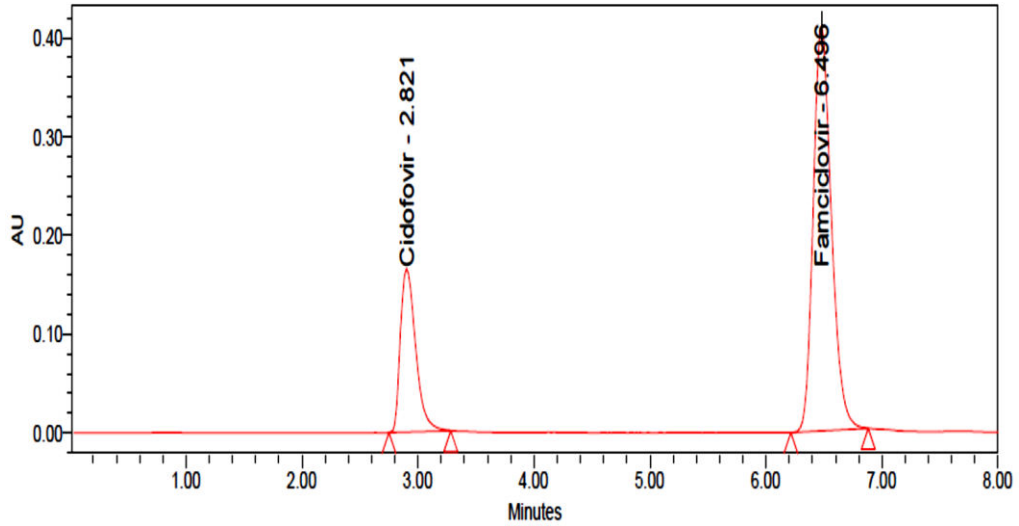

Figure 2.20: 100 percent linearity chromatogram

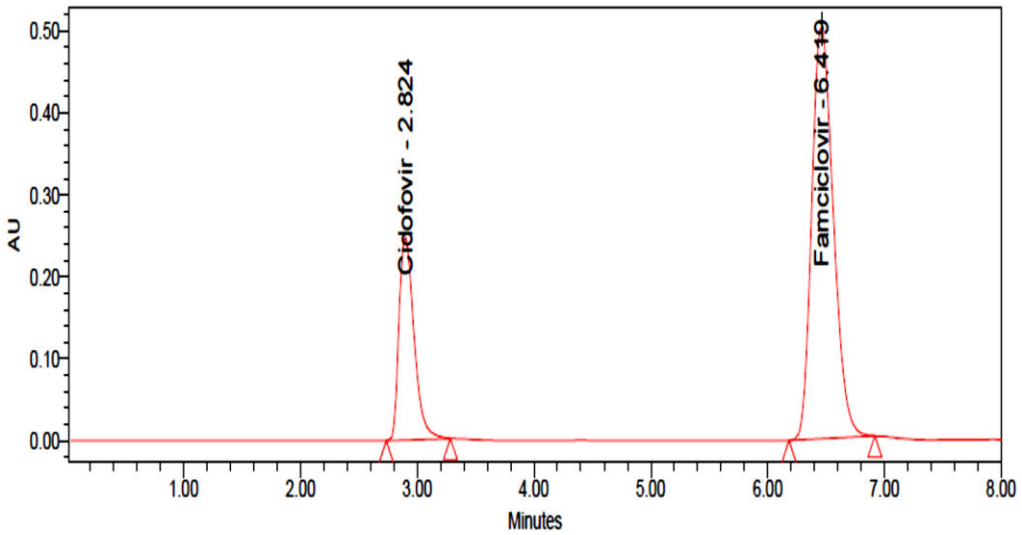

Figure 2.21: 125 percent linearity chromatogram

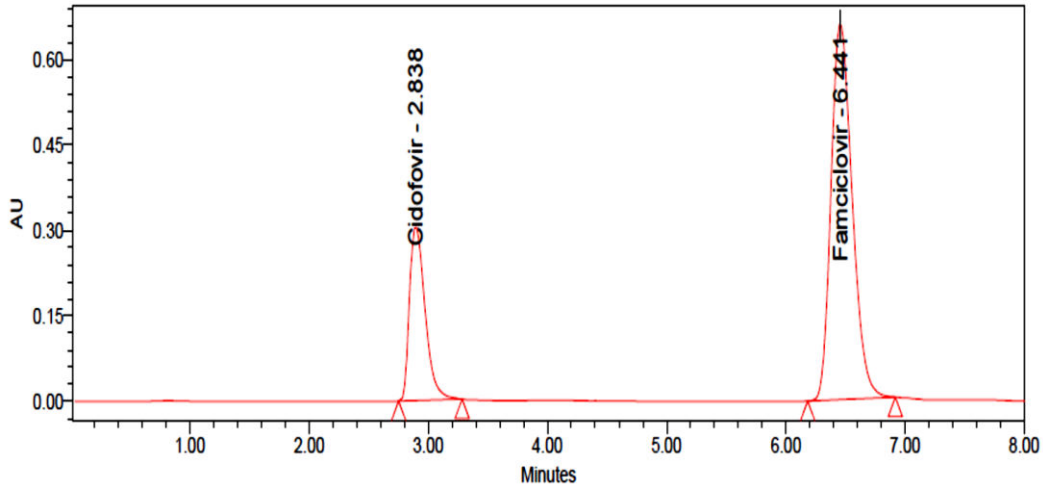

Figure 2.22: 150 percent linearity chromatogram

2.6.3 Accuracy:

50 percent solution preparation (with respect to the concentration of the target assay)
In a clean and dry volumetric flask, add 37.5 mg of Cidofovir and 125 mg ofFamciclovir and sonicate to dissolve well and bring the volume to the diluent level.Fill a 10 ml vacuum flask halfway with the given solution, then add diluents to get thedesired concentration. (37.5 parts per million of Cidofovir and 125 parts per million of Famciclovir)

100 percent solution preparation (with respect to target assay concentration)
As soon as the concentrations of the two drugs have been accurately measured and transferred to a volumetric flask, the diluents should be added and the solution sonicated to ensure that all of the active ingredients have been completely dissolved. You'll need to dilute the aforesaid solution using diluents until it reaches the desired volume in the volumetric flask. Cidofovir (75ppm) and Famciclovir (250ppm)

150 percent solution preparation (with respect to target assay concentration)
Add 112.5 mg of Cidofovir and 375 mg of Famciclovir to a clean, dry volumetric flask, add diluents, and then use a sonicator to dissolve and bring the diluents level to the desired point of dissolution for each.

Dose the volumetric flask with one millilitre of the aforesaid stock solution, then add diluents to dilute it to the desired concentration. Cidofovir (112.5ppm) with Famciclovir (375ppm)

Procedure:
Inject the standard solution, 50 percent accuracy, 100 percent accuracy, 150 percent accuracy solutions.**[Table 2.03 & 2.04]** shows the accuracy results of Cidofovir and Famciclovir and **[Fig. 2.23, 2.24 & 2.25]** shows the 50%, 100% and 150% accuracy chromatograms.

Acceptance Criteria:
The rate of recovery for each stage should be between 98-102 percent

Table 2.03: Accuracy results of Cidofovir

S. No.	% Level	% Recovery	Ave % Recovery
1	50	100.2	100.2
2		100.4	
3		100.1	
4	100	99.6	98.8
5		98.1	

6		98.6	
7	150	99.5	99.7
8		99.8	
9		99.8	

Table 2.04: Accuracy results of Famciclovir

S. No.	% Level	% Recovery	Ave %Recovery
1	50	98.6	99.2
2		99.7	
3		99.2	
4	100	100.2	99.4
5		99.7	
6		98.4	
7	150	100.4	100.4
8		100.7	
9		100.2	

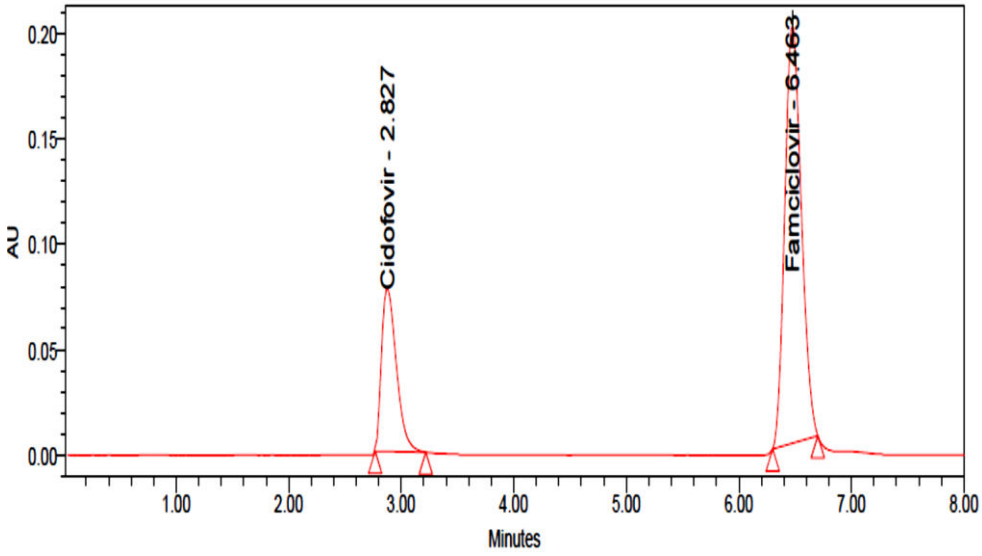

Figure 2.23: 50 percent accuracy chromatogram

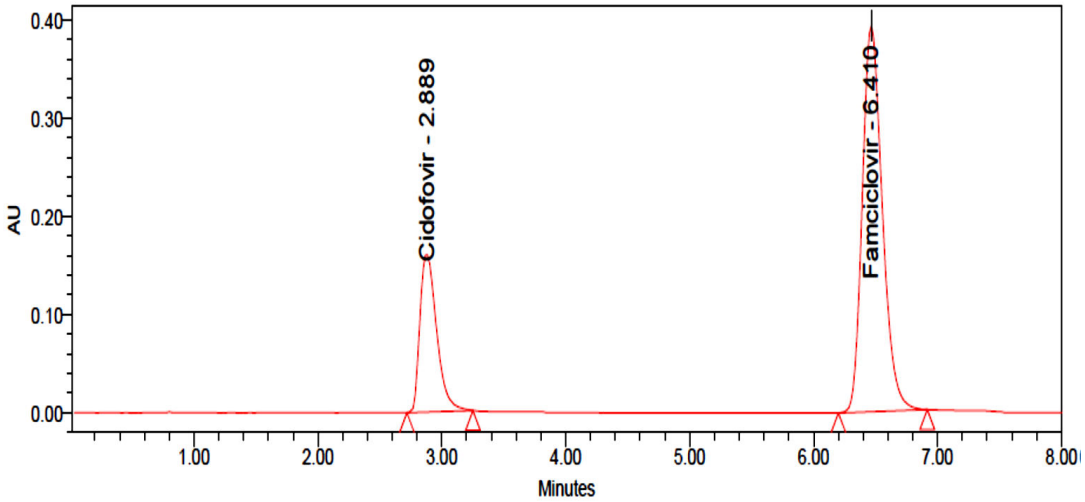

Figure 2.24: 100 percent accuracy chromatogram

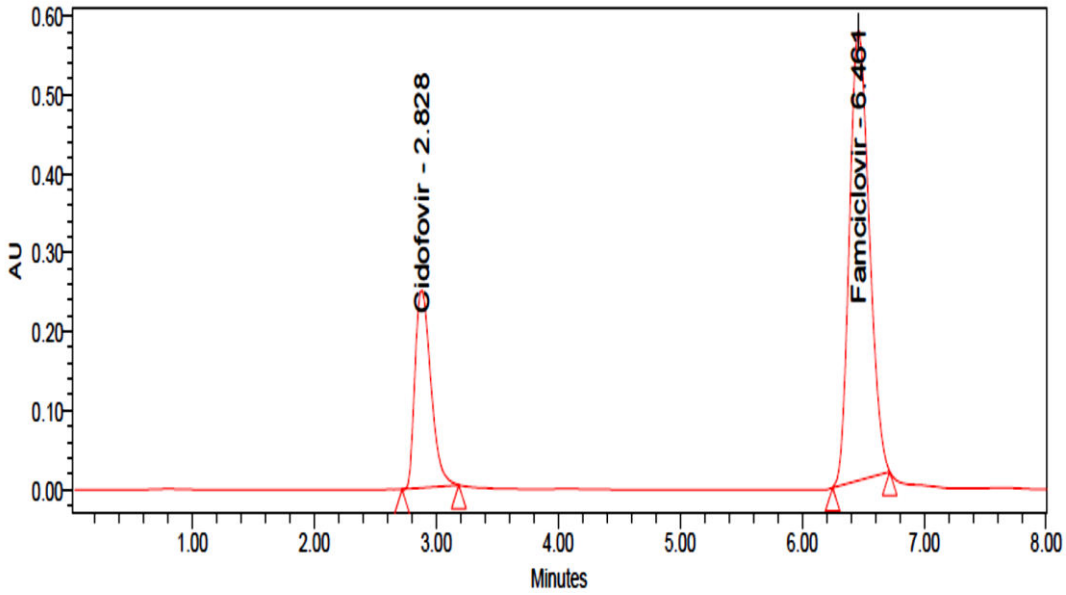

Figure 2.25: 150 percent accuracy chromatogram

2.6.4 Precision:

The accuracy of an analytical procedure is determined by the rate at which a set of measurements acquired from repeated homogenous samplings are in agreement (**22**). Cidofovir and Famciclovir were spiked into six different injections to ensure the precision of the injection technique. In [**Table 2.05**] and [**Fig. 2.26-2.31**], the system precision results and chromatograms are displayed.

Table 2.05: Results of system precision

S. No	System suitability parameter	Inclusion criteria	Medication Name	
			Cidofovir	Famciclovir
1	% RSD	NMT 2.0	0.55	0.14
2	USP Tailing	NMT 2.0	1.01	1.05
3	USP Plate count	NLT 3000	2459	7365

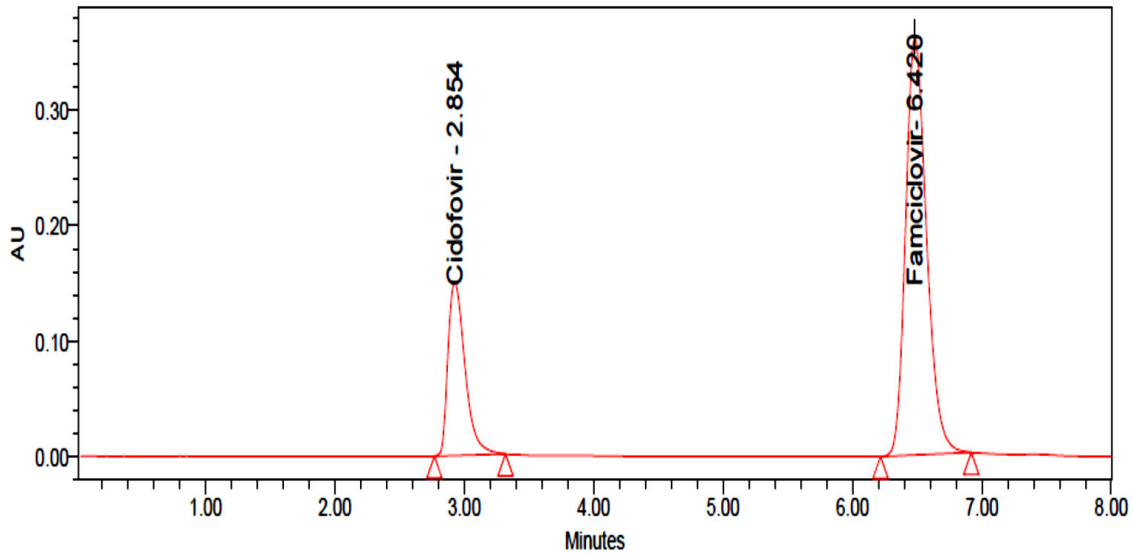

Figure 2.26: Chromatogram of system precision-1

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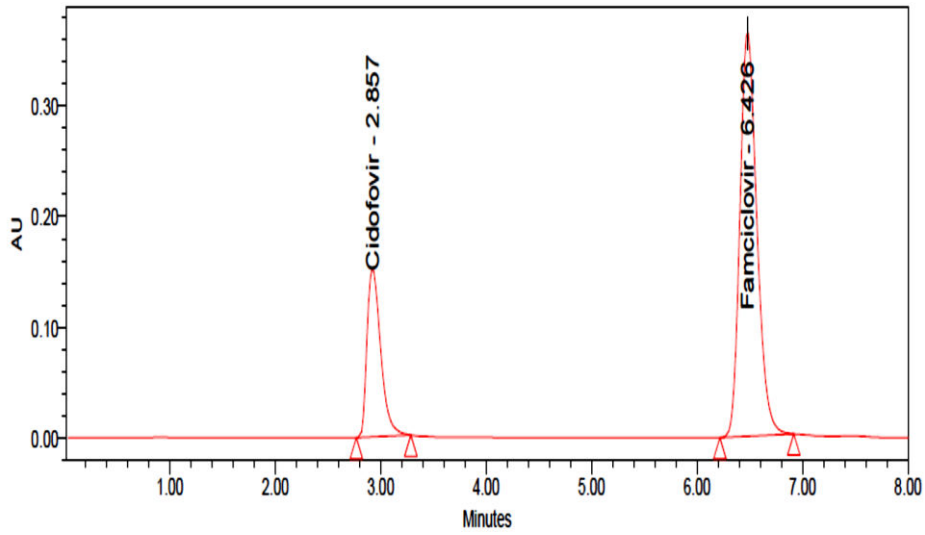

Figure 2.27: Chromatogram of system precision-2

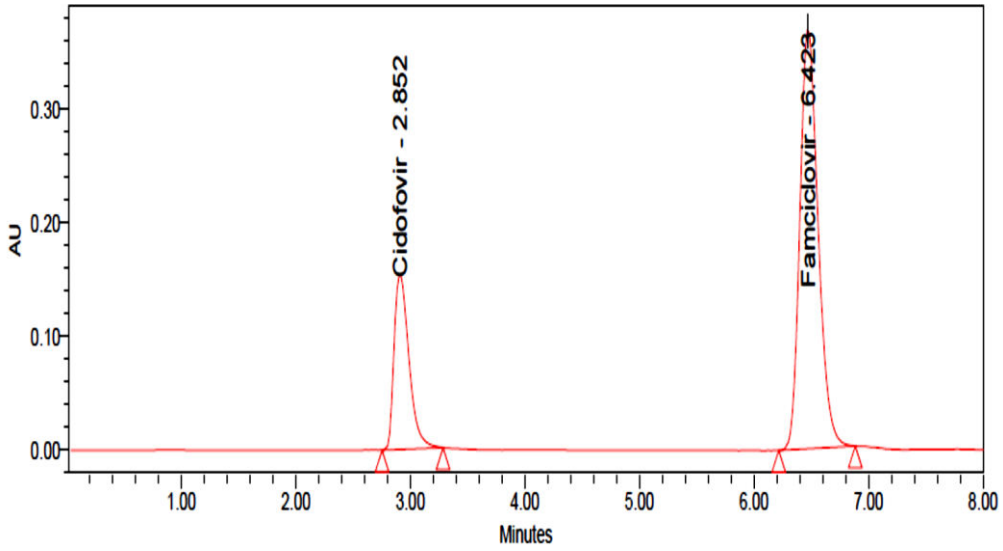

Figure 2.28: Chromatogram of system precision-3

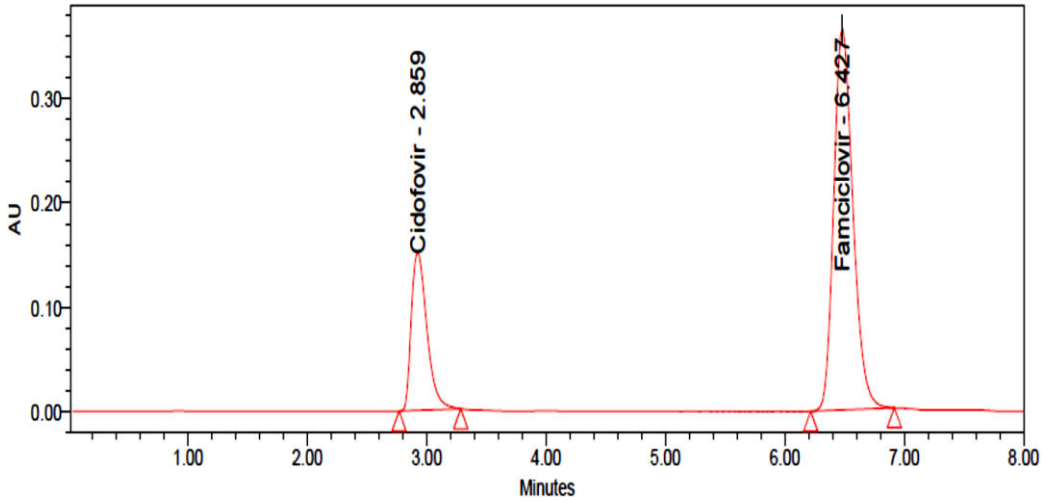

Figure 2.29: Chromatogram of system precision-4

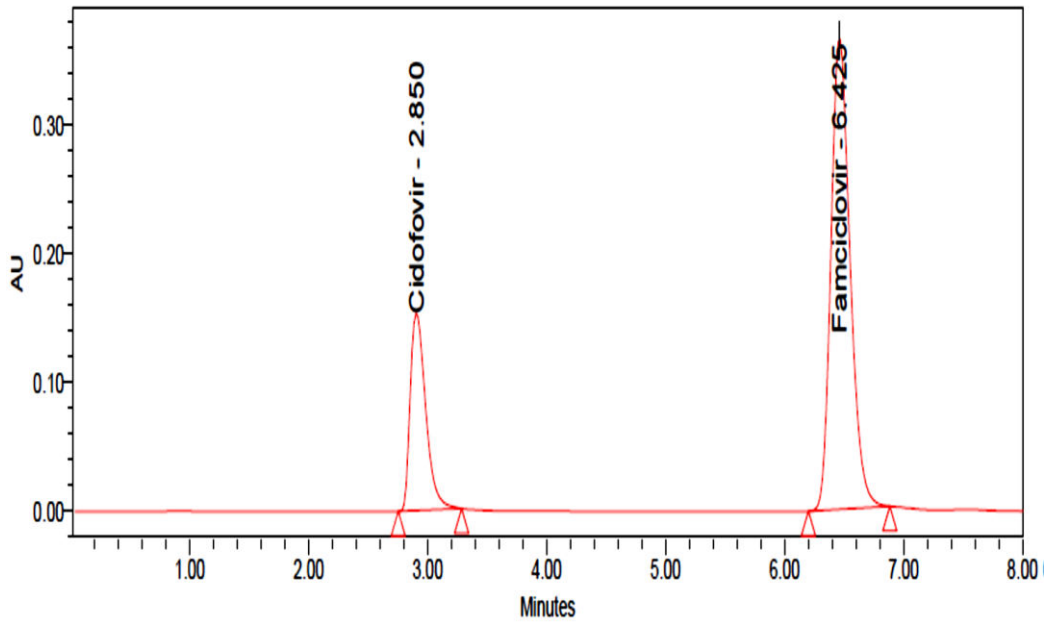

Figure 2.30: Chromatogram of system precision-5

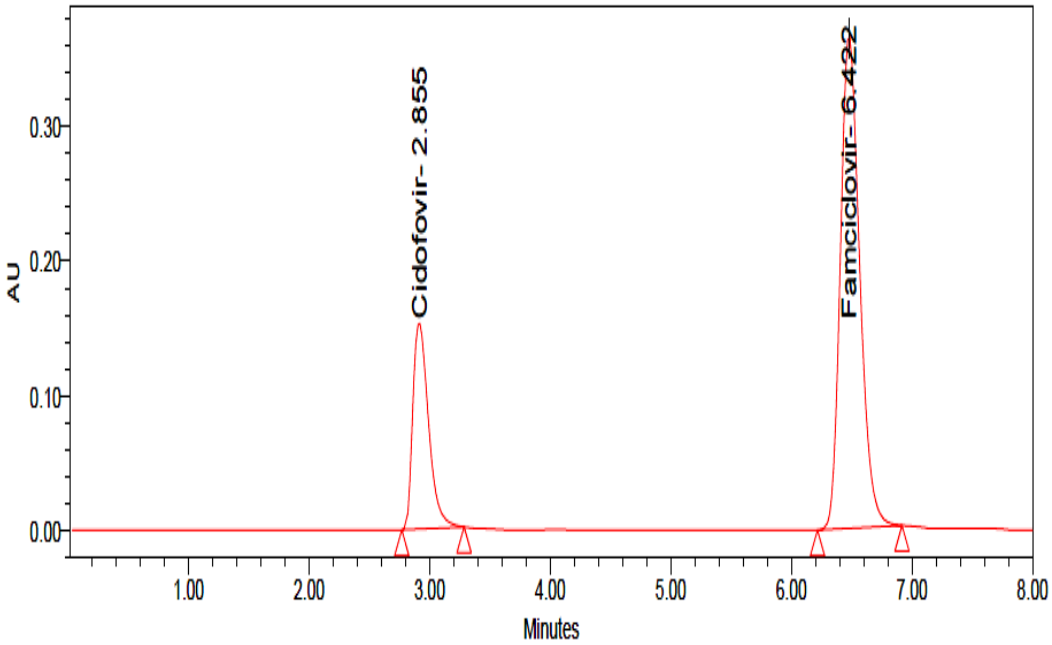

Figure 2.31: Chromatogram of system precision-6

Method precision: [Table 2.06 and Fig 2.32]

Table 2.06: Results of method precision

Analyte	Std Conc.	%RSD
Cidofovir	75	0.39
Famciclovir	250	0.47

Acceptance Criteria

For the area six standard injection findings, it is advised that the RSD percent be more than 2 percent.

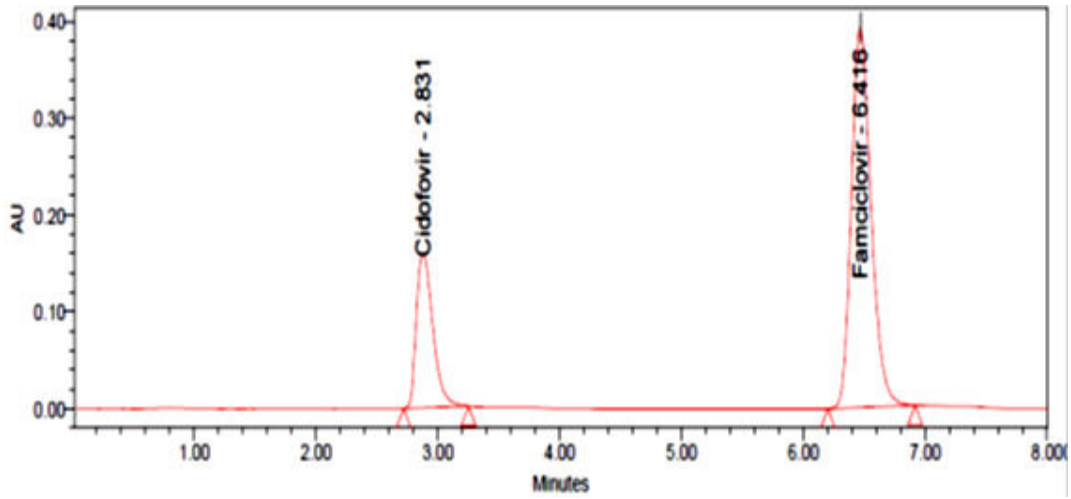

Figure 2.32: Chromatogram of method precision

Intermediate precision (Day-Day precision): [Table 2.07 and Fig 2.33]

Table 2.07: Results of intermediate precision

Analyte	Std. Conc.	%RSD
Cidofovir	75	0.58
Famciclovir	250	1.15

Acceptance Criteria

The RSD percentage for the six normal injection results should not be more than 2%.

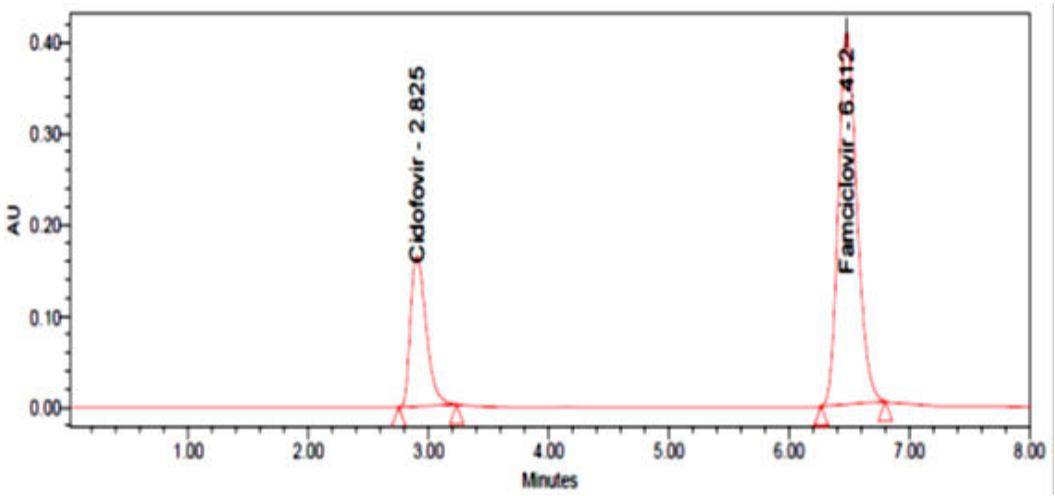

Figure 2.33: Chromatogram of intermediate precision

2.6.5 Limit of detection (LOD) and limit of quantification (LOQ):[Table 2.08 and Fig. 2.34 & 2.35]

Calibration curves were used to establish the LOD and LOQ (**23**), respectively. By injecting lower amounts of standard solutions, we were able to estimate the compound's LOD and LOQ using a well-established RP-HPLC procedure. S is the calibration curve slope and S the response standard deviation in this technique, which was used to calculate the levels of detection (LOD) and limits of quantification (LOQ). It was found that the LOD and LOQ concentrations of Cidofovir were 0.094% and 0.310%, respectively, while those of Famciclovir were 0.313% and 1.033%, respectively, in the same samples.

Table 2.08: Sensitivity parameter values

Cidofovir				Famciclovir			
LOD		LOQ		LOD		LOQ	
Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n
0.094	4	0.310	23	0.313	7	1.033	28

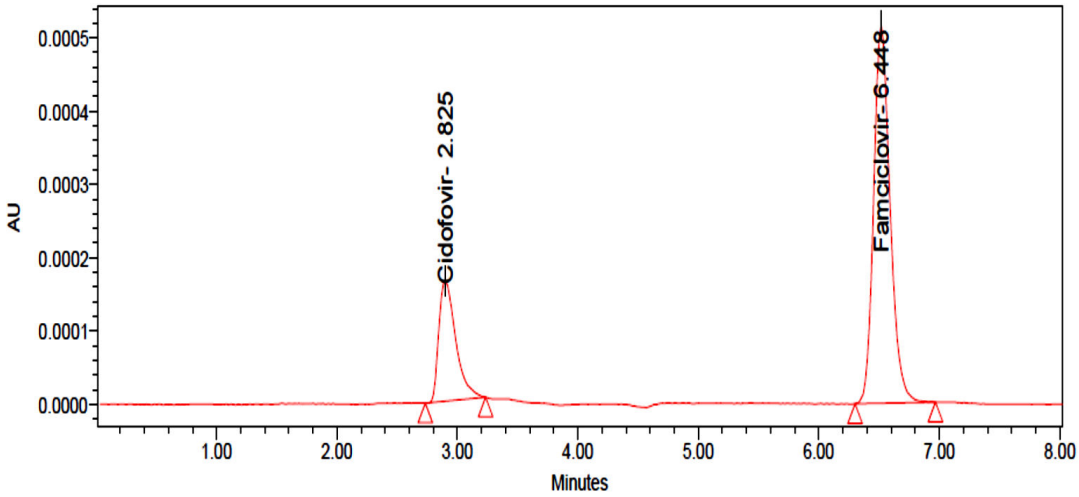

Figure 2.34: LOD chromatogram

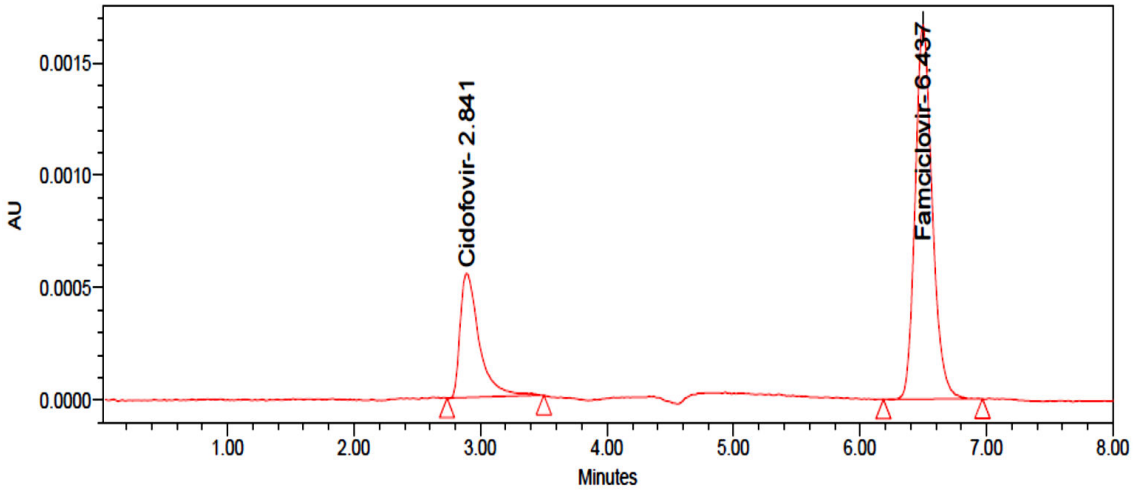

Figure 2.35: LOQ chromatogram

2.6.6 Robustness:

Tests were carried out on known systems, such as flow rate or movablestep in organic percentage, to see whether they could withstand these different circumstances. Active pharmaceutical components and contaminants were not considerably changed, and the retention time, theoretical plates and tailing factor were not significantly altered (**24**). As an output, this approach was dependable. Cidofovir and Famciclovir's robustness findings are shown in **[Table 2.09]**. Images of chromatograms from **[Fig. 2.36-2 .39]**

Table 2.09: Robustness results of Cidofovir and Famciclovir

S.No	Parameter name	% RSD for purity	
		Cidofovir	Famciclovir
1	Flow (0.8ml/min)	1.45	0.94

2	Flow (1.2ml/min)	1.01	0.23
3	Organic solvent (+10%) (44:56)	0.69	1.64
4	Organic solvent (-10%) (36:64)	0.88	0.25

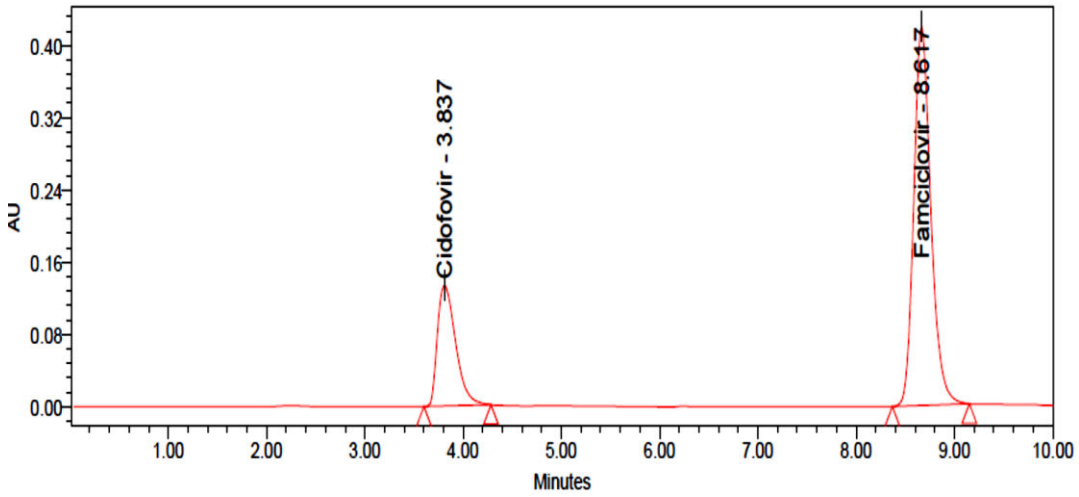

Figure 2.36: Less flow rate chromatogram (0.8ml/min)

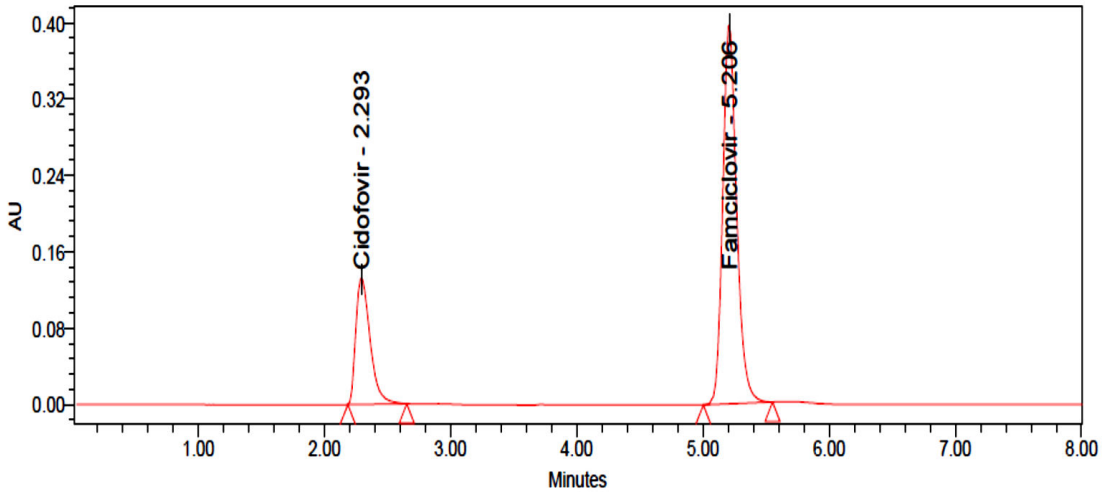

Figure 2.37: More flow rate chromatogram (1.2ml/min)

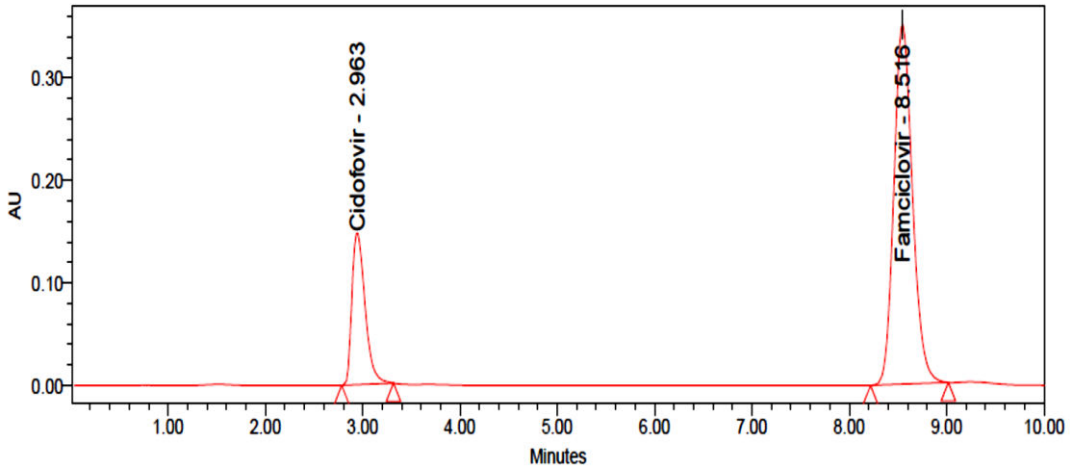

Figure 2.38: Less organic chromatogram (36:64)

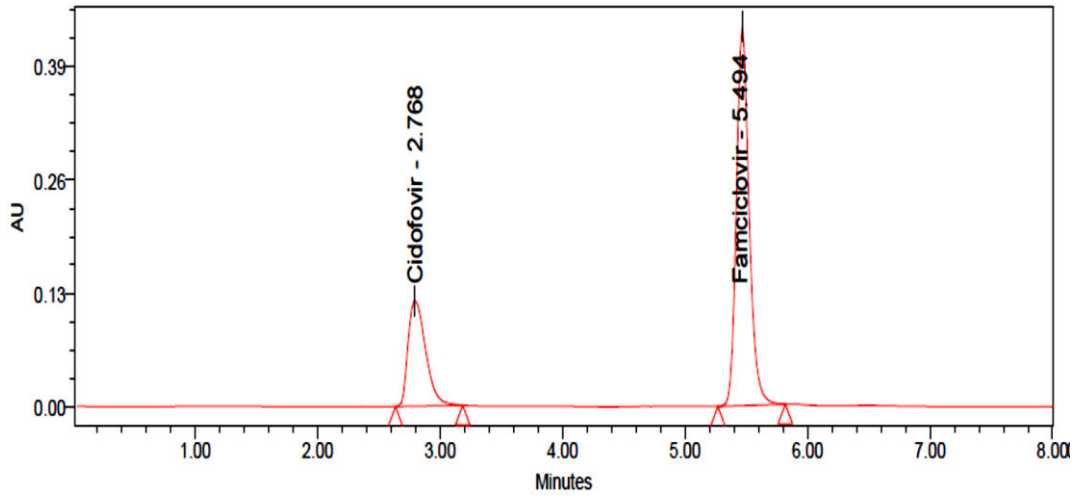

Figure 2.39: More organic chromatogram (44:56)

2.6.7 Forced Degradation Studies:

In order to partly breakdown the chemical, several conditions of forced degradation were applied to the Cidofovir and Famciclovir samples. Experiments with degrading materials have shown that the technique is suitable for use. Also we included the details of the medication in which condition it is unstable, so that actions may be made to mitigate probable instability during formulation.

Stock Solution Preparation:

In a vacuum flask with a capacity of 100 mL, weigh out 75 mg of Cidofovir and 250 mg of Famciclovir, add 70 mL of diluents, and sonicate to dissolve for 30 minutes to make up for the diluents mark.

Acid Degradation

The acid degradation procedure begins with the addition of 5 ml of standard solution to a 50 ml vacuum flask, followed by the addition of 1 ml of 1N HCl and 30 minutes of heating at 60°C before 1 millilitre of 1N NaOH is added and the flask is marked. A 0.45 nylon syringe filter is then utilized to filter the solution. In acid degradation condition, Cidofovir shows 14.6% and Famciclovir shows 13.4% of degradation.

Alkali Degradation

5ml of a standard solution is placed in a vacuum flask and heated to 60°C for 30 minutes before being treated with 1ml of 1N NaOH and marked with diluent in the alkali degradation procedure. A 0.45 nylon syringe filter is then used to filter the solution. In alkali degradation condition, Cidofovir shows 14.9% and Famciclovir shows 14.2% of degradation.

Peroxide Degradation

The following steps were taken in the breakdown process: Normal solution is brought in to the 50 mL vacuum flask, a half-teaspoon of 30 percent hydrogen peroxide in a half-teaspoon of H2O2 is added, and the mixture is heated for 30 minutes at 60°C. Filter the fluid using a 0.45 nylon syringe filter. In peroxide degradation condition, Cidofovir shows 15.2% and Famciclovir shows 14.7% of degradation.

Reduction Degradation

The following was the degrading procedure: A 50-mL vacuum flask holds 5 mL of a normal solution and 1 mL of 30 percent sodium bicarbonate solution. The flasks are heated to 60°C for 15 minutes and then cooled to room temperature before the diluent is added. Use a nylon syringe filter of 0.45 microns to remove the solution from the sample. In reduction degradation condition, Cidofovir shows 13.9% and Famciclovir shows 12.8% of degradation.

Thermal Degradation

Cidofovir and Famciclovir were both subjected to 105°C for three hours, and the resulting standard solution was tested. Five milligrams of normal solution were put into a 10 millilitre volumetric flask. Sonication was used to dissolve the five millilitres (mL) of diluent before diluting it to volume with diluent. For 60 minutes at 60°C, this solution is placed in an RB flask and refluxed. After then, let it to cool to room temperature. Dilute 1 ml to 10 ml using diluents. In Thermal degradation condition, Cidofovir shows 12.5% and Famciclovir shows 11.4% of degradation.

Photo Degradation

Sunlight exposure for 12 hours was followed by a 30 minute period of reflux at 60°C in the UV degradation process. Regular solution was used in the HPLC procedure. In photo degradation condition, Cidofovir shows 10.7% and Famciclovir shows 12.9% of degradation.

Hydrolysis Degradation

Using a 50-ml vacuum flask, add 5 ml of standard solution, 2 ml of HPLC water, and heat to 60°C for 15 minutes before cooling to diluent makeup. Filter the fluid using a 0.45 nylon syringe filter.

Table 2.10 gives results of forced degradation and the **figures** from **[2.40-2.46]** shows forced degradation chromatograms.

Table 2.10: Forced degradation results

Degradation Condition	% Degradation of Cidofovir	% Degradation of Famciclovir
Control Degradation	0.2	0.1
Acid Degradation	14.6	13.4
Alkali Degradation	14.9	14.2
Peroxide Degradation	15.2	14.7
Reduction Degradation	13.9	12.8
Thermal Degradation	12.5	11.4
Photolytic Degradation	10.7	12.9

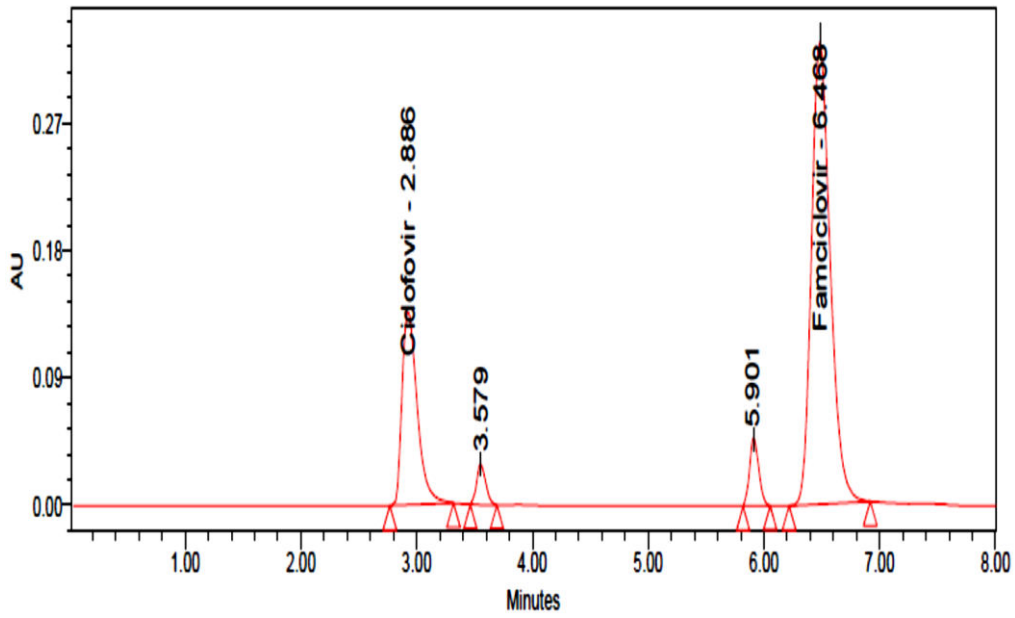

Figure 2.40: Chromatogram of acid degradation

 ISBN: 978-81-19477-19-7

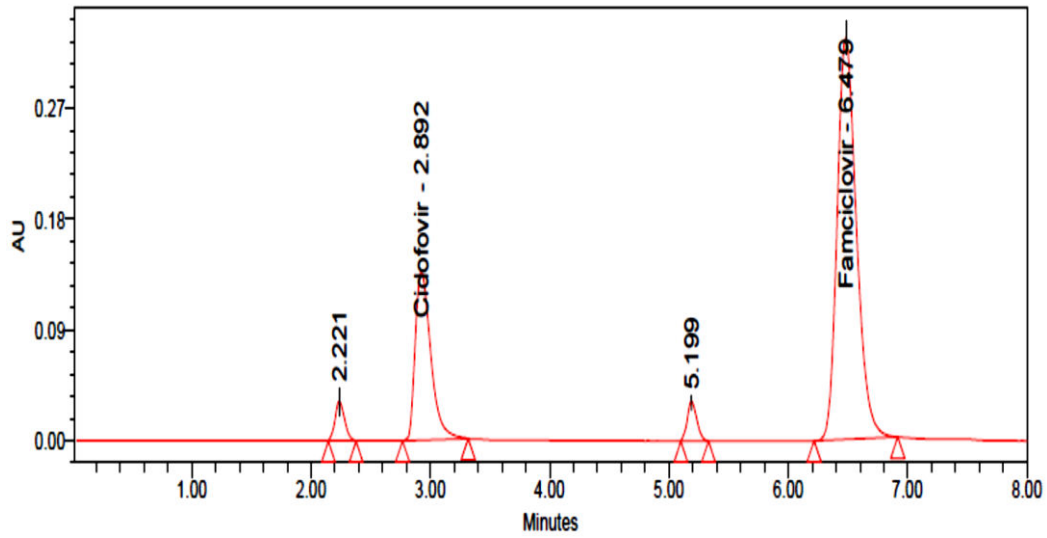

Figure 2.41: Chromatogram of alkali degradation

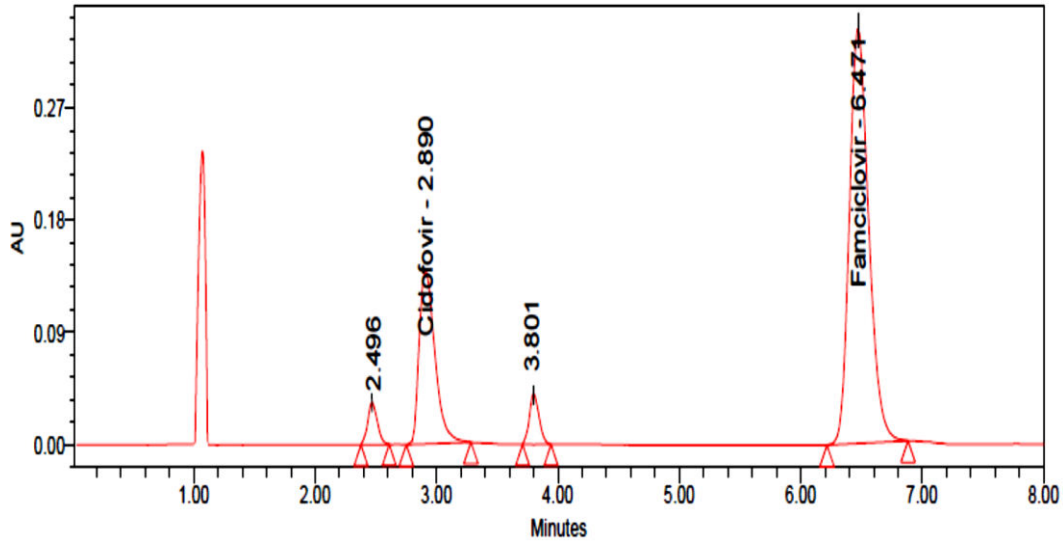

Figure 2.42: Chromatogram of peroxide degradation

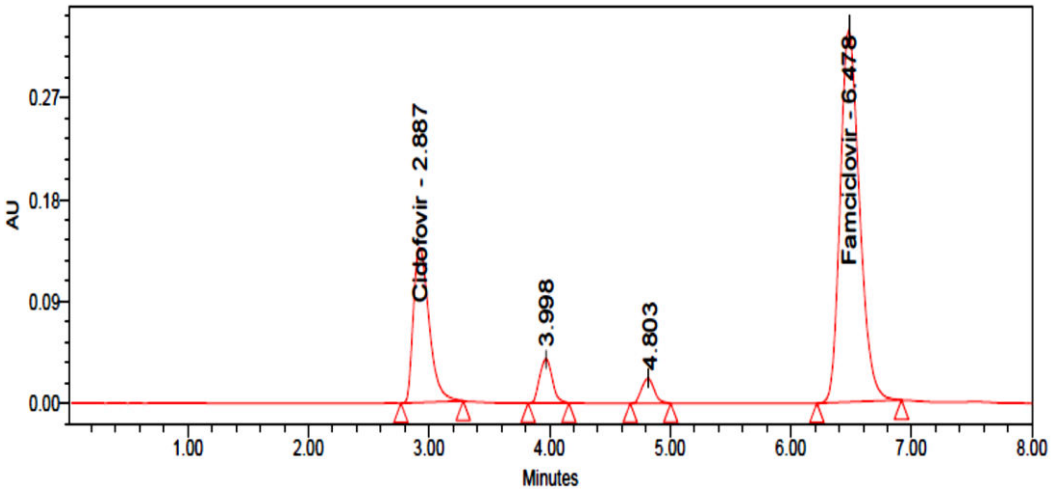

Figure 2.43: Chromatogram of reduction degradation

 ISBN: 978-81-19477-19-7

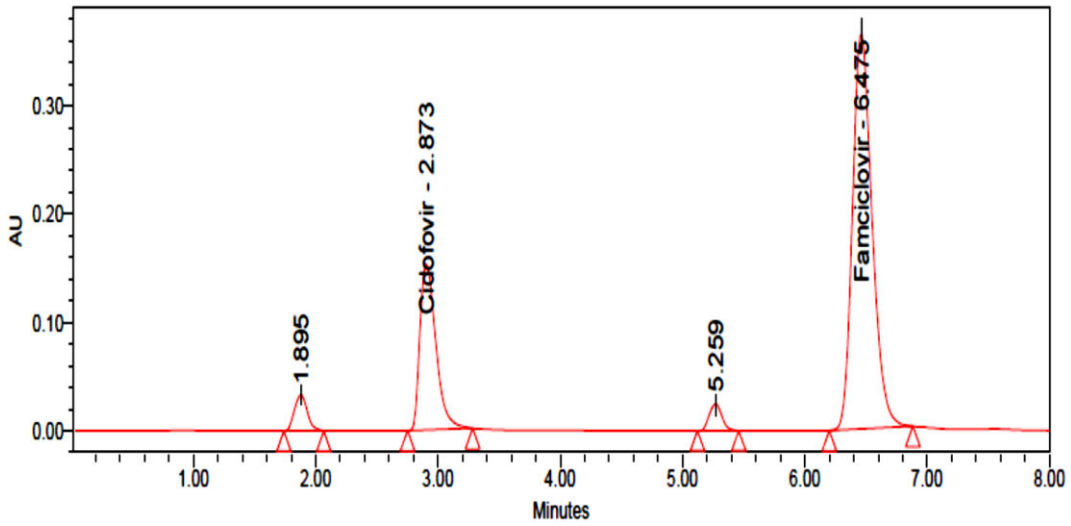

Figure 2.44: Chromatogram of thermal degradation

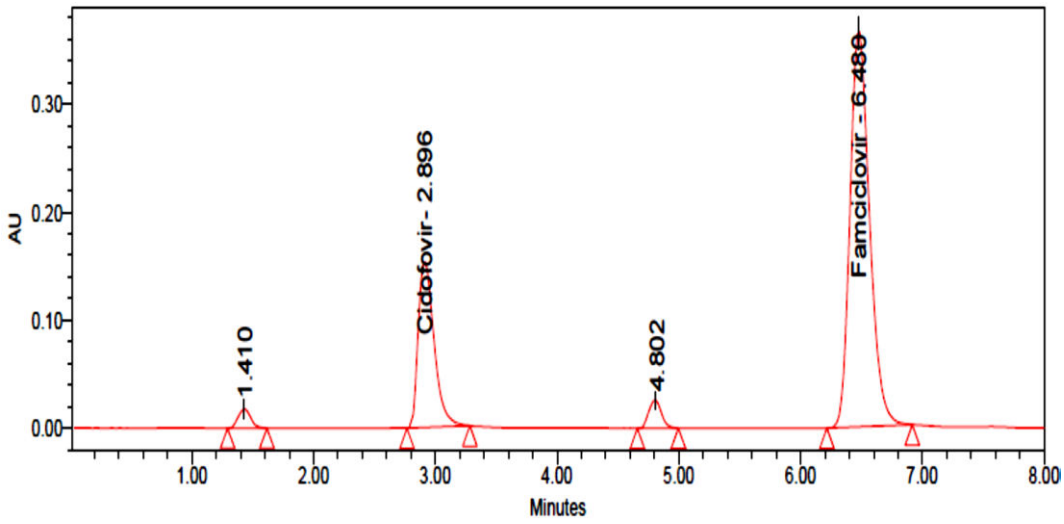

Figure 2.45: Chromatogram of Photo degradation

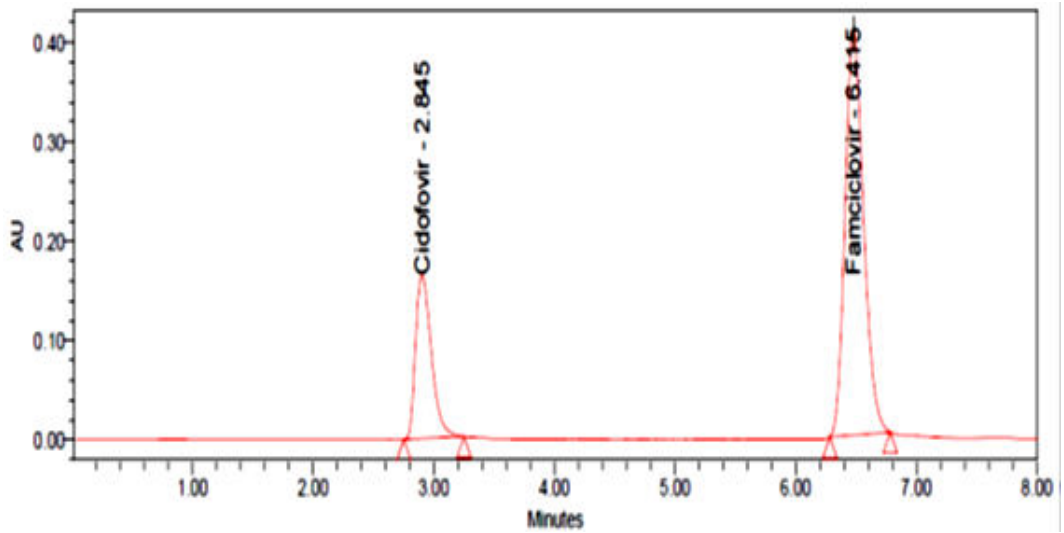

Figure 2.46: Chromatogram of control degradation

2.7 CONCLUSION

A proven RP-HPLC technique was used to construct a stable indication test for Cidofovir and Famciclovir. Under diverse circumstances, including acid, basic, and neutral, as well as oxidation, reduction, photolysis, and thermal stress, the drug's degradation behaviour was examined. The medicine was shown to be stable under thermally neutral settings but unstable in the remaining degradation conditions.

RP-HPLC isocratic methods for the detection of Cidofovir and Famciclovir have been developed. The regression line equation can reliably estimate Cidofovir's concentration in the range of 7.5-112.5 g/ml and Famciclovir's concentration in the range of 25-375 g/ml based on the peak area. The approach was tested and shown to be accurate, responsive, robust, and precise in detecting Cidofovir and Famciclovir.

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 ISBN: 978-81-19477-19-7

Chapter - 3

Stability Indicating Method Development and Validation of Mitomycin and Fluorouracil by using UPLC

3.1 Drug profile

Drug Profile of Mitomycin:
Streptomyces caespitosus and Streptomyces lavendulae produce the mitomycins, a class of aziridine-containing natural compounds**[1,2]**. Mitomycin A, B, and C are a few examples. It is most often referred to as mitomycin C when the word mitomycin is used alone. Mitomycin C is a drug **[3]** utilized to treat a variety of conditions linked to cell proliferation and spread. Mitomycin C causes transformation competence in the bacteria Legionella pneumophila**[4-6]**. Natural transformation is a method of bacterial sexual contact because it involves the transfer of DNA between cells **[8, 9]**. For example, in the fruit fly Drosophila melanogaster **[10, 11, 12]**, mitomycin C treatment enhances the rate of meiosis-related recombination **[12,13]**. A mutant mutant strain that lacks the genes required for recombination during meiosis and mitosis is hypersensitive to mitomycin C death in Arabidopsis thaliana **[15, 16]**. It has been shown that Borreliaburgdorferi stationary phase persisters may be treated with mitomycin C **[20, 21]**. It is being studied in clinical trials for its ability to cure gastrointestinal strictures **[22]**, wound healing following glaucoma surgery **[23]**, corneal excimer laser surgery **[24]**, and endoscopic dacryocystorhinostomy**[25]** symptoms, in addition to treating symptoms of pancreatic and stomach cancer.

3.1.1 Structure of Mitomycin: [Fig. 3.01]

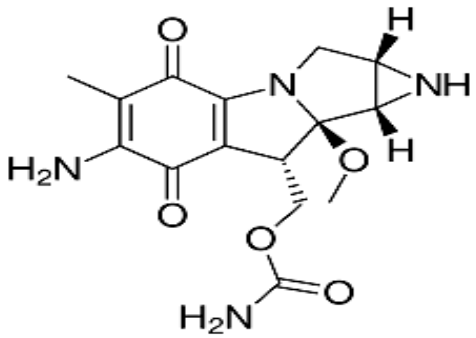

Figure 3.01: Mitomycin's chemical structure

3.1.2 Name of the IUPAC:
[6-Amino-8a-methoxy-5-methyl-4,7-dioxo-1,1a,2,4,7,8,8a,8b octahydroazireno[2',3':3,4]pyrrolo[1,2-a]indol-8-yl]methyl carbamate

3.1.3Molecularformula: $C_{15}H_{18}N_4O_5$

3.1.4Molecularweight:334.33

3.1.5Category
It is an anti-cancer chemotherapeutic medicine known as mitomycin ("antineoplastic" or "cytotoxic" in medical terminology). Antitumor antibiotics, such as this one, are what they're called. (For further information, read the part below under "How this medicine works").

3.1.6 Mechanism of Action
Inhibition of deoxyribonucleic acid production is achieved only by the use of mitomycin (DNA). Mitomycin-induced cross-linking is dependent on the amount of guanine and cytosine in the DNA. The medication also inhibits cellular RNA and protein production at high dosages.

3.1.7 Side effects of Mitomycin:
- Nausea and vomiting;
- Stomach/abdominal pain;

- Loss of appetite;
- Headache;
- Blurred vision, drowsiness;
- Temporary hair loss.

3.1.8 Contraindications:

- A bad infection.
- Hemolytic uremic syndrome, a condition that affects the kidney and the blood.
- Decreased function of bone marrow.
- Anemia.
- A higher than normal risk of bleeding as a result of blood coagulation issues.
- A greater chance of bleeding.
- Reduced platelet count.
- Low white blood cell counts.

3.1.9 Absorption

Intravesical infusion of mitomycin C results in absorption of the drug, and the extent of absorption depends on the extent of bladder injury. All of these individuals had no systemic effects on bone marrow or indications of deoxyribonucleic acid damage despite some absorption of the drug.

3.1.10 Uses:

Several forms of cancer may be treated with mitomycin, including stomach and pancreatic cancer. Cancer cell proliferation is slowed or halted as a result of this treatment.

3.1.11 Adult dose:

20mg/m^2 as a single *dose* every 6–8 weeks.

3.2 Fluorouracil

Drug Profile of Fluorouracil:

Fluorouracil (5-FU) is a cancer treatment drug available under many trade names, including Adrucil**[26]**. A vein injection is used to treat cancer of the colon **[27]**, esophageal cancer **[28]**, gastrointestinal cancers (including stomach cancers) **[29]**, pancreatic cancer **[30]**, breast cancer **[31]**, and cervical cancer **[32]**. Skin warts **[33]** and actinic keratosis **[34, 35]** are two conditions for which it is used as a cream. Most individuals get adverse effects if they are given the drug through injection. Some of the most frequent side effects are inflammation of the mouth, loss of appetite, low blood cell counts, hair loss, and inflammation of the skin. Skin irritation is frequent when this medication is used topically as a cream. Both forms of contraception should be avoided by pregnant women. It belongs to the antimetabolite **[36]** and pyrimidine analogue groups of drugs. It's not totally understood how it works, but it's widely assumed that it stops the creation of DNA by inhibiting the activity of thymidylate synthase **[37]**. In the WHO's list of essential medicines, which includes the safest and most effective drugs, it is included **[38]**. Fluorouracil has been given systemically for anal, breast, colorectal, oesophageal, stomach, pancreatic, and cutaneous malignancies (especially head and neck cancers). On the other hand, it has also been used as an eye drop for the treatment of the eye's surface squamous neoplasms (OSNS) and for the treatment of skin malignancies**[39]**. Additionally, ocular injections into a

previously produced bleb of trabeculectomy**[40]** may be used to lower intraocular pressure by inhibiting healing and scarring of tissue, thereby permitting appropriate aqueous fluid flow.

3.2.1 Structure of Fluorouracil: [Fig. 3.02]

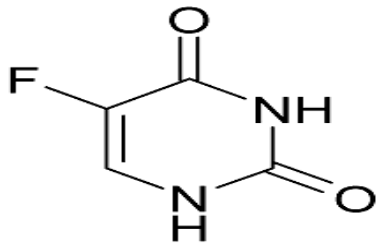

Figure 3.02: Fluorouracil's chemical structure

3.2.2 Name of the IUPAC: 5-Fluorodihydrouracil

3.2.3Formulamolecular: $C_4H_5FN_2O_2$

3.2.4Molecularweight: 132.09

3.2.5 Category
It is an anti-cancer (antineoplastic) or "cytotoxic" chemotherapy medication called fluorouracil. Fluorouracil is a "metabolite inhibitor." See the "How Fluorouracil Works" section below for further information.

3.2.6Mechanismofaction
The deoxy derivative of cytosine nucleotide is created when fluorouracil inhibits an enzyme that normally does so. Fluorouracil also inhibits DNA synthesis by preventing the thymidine nucleotide from being incorporated into the DNA strand.

3.2.7 Side Effects:
- Diarrhea
- Vomiting, which may or may not be accompanied by nausea
- Mouth ulcers
- Lack of desire to eat
- Watery eyes and light sensitivity (photophobia)
- A metallic taste in the mouth develops throughout the infusion process.

3.2.8 Contraindications
If a patient is in a low nutritional condition, has a decreased bone marrow function or is at risk of developing an infection, Fluorouracil Injection USP treatment is not recommended.

3.2.9 Absorption
Fluorouracil 5 percent cream is absorbed systemically at a rate of around 6%, with peak plasma concentrations being reached in about an hour after application. It's possible that medication absorption into cells is stronger in sick skin than in healthy skin.

3.2.10 Uses:
To treat certain breast cancers, fluorouracil is used in conjunction with other drugs, such as surgery or radiation treatment. Cancers of the pancreas and stomach are also treated with fluorouracil. Fluorouracil belongs to the antimetabolite family of medicines.

3.2.11 Adult Dose:
500 mg/m2 IV bolus on day 1

3.3 Literature Survey

Jaison Jose T, Subbareddy Y and Sankar K, A new assay method development and validation of two anti-cancer drugs by using effective liquid chromatographic method: The Symmetry C18 (4.6 x 150 mm, 3.5) column was used in a simple, sensitive, and rapid chromatographic technique to quantify Mitomycin and Fluorouracil in bulk and medicinal dosage form. Acetonitrile and OPA (Orthophosphoric acid) are mixed together in the mobile phase at 0.1 percent each. The absorbance at 260 nm was measured using photodiode array detectors and a flow rate of 1.0 ml/s. When the calibration curve was linear and the regression coefficient (R2) was 0.999, the mitomycin and fluorouracil concentrations ranged from 10–100 µg/ml and 5–75 µg/ml, respectively. LOD and LOQ for Fluorouracil and Mitomycin, respectively, were found to be 1 µg/ml and 0.5 µg/ml. For Mitomycin and Fluorouracil, there were at least 2000 theoretical plates and a maximum of two tailing factors. The method was found to be simple, cost-effective, suitable, and tested in line with ICH standards.

Angélique Saint, LudovicEvesque, et al. Mitomycin and 5-fluorouracil for second-line treatment of metastatic squamous cell carcinomas of the anal canal: Treatment options for anal canal metastasized Squamous Cell Carcinoma (SCC) after initial treatment has failed are not universal due to the rarity of the condition. 5-fluorouracil (5-FU) and mitomycin in combination with radiation is the standard therapy for locally advanced forms, although its usefulness at the metastatic stage has never been studied. After a platinum-based treatment failed, 5-FU and mitomycin were utilised to treat patients with metastatic SCC of the anal canal at our institution between 2000 and 2017. OS, response rate, and toxicity were the secondary endpoints. The main goal was to keep the patient alive for as long as possible (PFS). 15 females and four males were discovered in the study; the median age was 57. (range, 40-79 years). Patients received a total of one to seven doses of mitomycin 5-FU. There were six patients (31.6 percent) who needed dose reductions, one patient had to discontinue therapy owing to toxicity, and no deaths were reported as a consequence of the medication's adverse effects. Five patients (26.4 percent, 95 percent CI 6.6-46.2) had an objective response, and six patients (31.6 percent, 95 percent CI 10.7-52.5) had tumour stabilisation, with one patient reaching a full response. There was one patient who had an objective response of 100 percent! The median gap between PFS and OS was three months, with a 95 percent confidence interval (CI) spanning from one to five months. Patients previously treated with mitomycin and 5-FU at a local stage exhibited no notable difference in PFS or OS, with one patient's response lasting 23 months and the median response duration of 4 months (95 percent confidence interval [CI]: 1.8-6.1). The mitomycin and 5-FU combination is well tolerated by the tissues. Metastatic SCC of the anal canal is an option for patients if platinum-based chemotherapy fails. 5-FU was unintentionally left out of the Results and Conclusion section, but it has subsequently been reinserted after the initial online publication on 9 October 2019.

Ashish K Sethi. Concurrent Chemoradiation With 5-Fluorouracil and Mitomycin in Squamous Cell Carcinoma of the Rectum: This kind of cancer in the rectum is rare. The aetiology, prognosis, and treatment of rectal squamous cell carcinoma (SCC) are still poorly known. Non-surgical treatment options for rectal SCC include 5-FU and mitomycin 5-FU and mitomycin 5-FU. Treatment for SCC rectal cancer is based on its histological features, not its location. Remission in a 47-year-old Caucasian lady with rectal SCC after two years of mitomycin and 5-fluorouracil therapy (5-FU).

3.4 Experimental

3.4.1 Chemical and Reagents:

Merck India Ltd, Mumbai, India, supplied acetonitrile (HPLC quality), formic acid, and water (HPLC grade). Glen mark in Mumbai provided the APIs for Mitomycin and Fluorouracil standards.

3.4.2 Instrumentation

With the help of empower 2.0 software, the Waters Acquity model UPLC was used.

3.4.3 Standard Stock Solution Preparation

Take 20 mg Mitomycin and 50 mg Fluorouracil and place them in a volumetric flask with 70ml diluent. Sonicate the mixture for 10 minutes to dissolve the contents completely. Then add more diluent until desired concentration is reached.

Preparation of Standard Solution

5 ml of the normal stock solution is poured into a 50 ml volumetric flask and diluted to the required concentration.

3.5 Method Development

Analytical Method Development:

A successful attempt has been made in the proposed project to establish a simple accuracy for Mitomycin andFluorouracil analysis using UPLC.

3.5.1 Method Development Parameters:

Selection of following parameters in method development is very important.

- Mode of chromatography
- Wavelength
- Column
- Mobile phase composition
- Solvent delivery system
- Flow rate
- Injection volume

3.5.1.1 Selection of Mode of Chromatography:

Selected mode of chromatography	:	Reversed phase chromatography
Basis of selection	:	polarity of the molecule
Reason for selection	:	as MitomycinandFluorouracil is polar

molecule it elutes at faster along with mobile phase

3.5.1.2 Detector Wavelength Selection:

The last stage in the analytical procedure is the choosing of the detector's wavelength. PDA detector and wavelength are used to determine precise wavelength of the standard API, which is manufactured and injected into the chromatographic system using PDA detector and wavelength.

Selected Wave Length: 255 nm

Basis for Selection: Maximum absorbance of analytes.

Reason for Selection: Mitomycinand Fluorouracil having maximum absorbance 255 nm.

3.5.1.3 Selection of Column:

Column Selected: Inertsil ODS column of 250 x 4.6mm, 5 µ

Basis for Selection: Based on the polarity, and chemical differences among analytics

Reason for Selection

A wide range of bonding chemistries, high range of mechanical stability, excellent physiochemical surface properties and compatibility with a wide range of organic solvents

3.5.1.4 Selection of the Mobile Phase Composition and of the Buffer:
Peak symmetries and separation are heavily influenced by the buffer and its intensity. The ionic form may be altered during chromatography if the injection load on the column is not covered by the proper strength buffers.

Mobile Phase Preparation:
Solution A: Acetonitrile

Solution B: 0.1% formic acid

3.5.1.5 Selection of the Rate of Flow:
Flow rate is expressed as an important factor, even in reverse phase separation for the resolution of small molecules. In large-scale inverted phase chromatography, the flow rate used during the loading of the sample solution is especially important but not crucial during analytical experiments. Depending on the flow rate used during sample loading, dynamic binding capacity can differ. In order to evaluate the optimum flow rate for loading, the dynamic binding capability should be calculated when scaling up the purification. In this system, the flow rate is set to 1 ml/min and is dependent on factors such as flow factor, retention duration, column composition, separation impurity, and peak symmetrical symmetry.

3.5.1.6 Selection of Injection Volume:
An injection volume of between 10 and 20 l is suggested for API estimations in most cases. However, since extraction proved to be problematic, the test concentration may be kept low and the injection volume increased to 50 l. Although it is crucial to make sure there is not too much pressure on the column volume that you set. This approach uses a 10 l injection volume for Mitomycin and Fluorouracil.

3.5.1.7 Trials in Optimization of Chromatographic Condition:

Trial-1 [Fig. 3.03]

Mobile phase	:	Acetonitrile and 0.1% TFA (80:20)
Column	:	X-Bridge phenyl 150 x 4.6 mm, 3.5µm
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	10 min
Wavelength	:	200-400 nm
Observation	:	Peaks are not separated

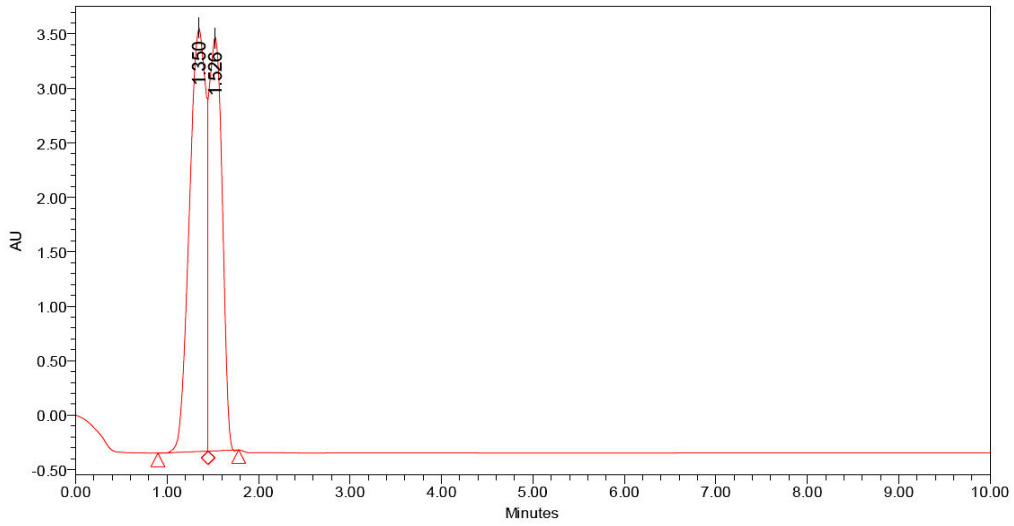

Figure 3.03: chromatogram of trial-1

	RT	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1	1.350	683589	54.25		1.05	2536
2	1.526	272341	45.75	1.09	1.23	3597

Trial-2 [Fig. 3.04]

Movable phase : Acetonitrile and 0.1% TFA(70:30)

Column : X-Bridge phenyl 150 x 4.6 mm, 3.5µm

Rate of flow : 1 ml/min

Volume of injection : 10µl

Period of run : 5 min

Wavelength : 255 nm

Observation : Resolution is not within the limit

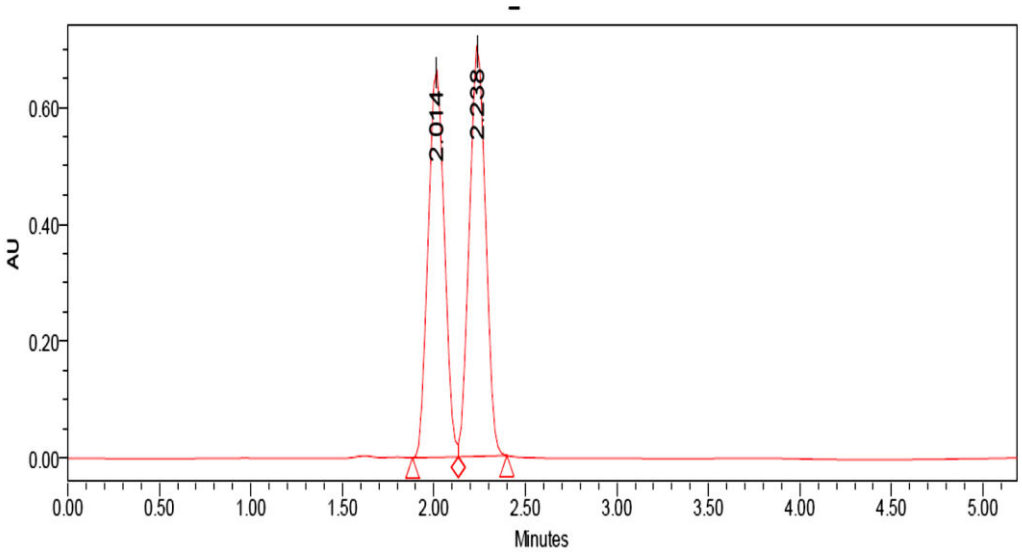

Figure 3.04: Chromatogram of trial-2

Peak Results

	Name	RT	Area	% Area	USP Tailing	USP Plate Count	USP Resolution
1		2.014	4101404	48.74	1.04	2191	
2		2.238	4313086	51.26	1.02	2618	1.29

Trial-3: [Fig. 3.05]

Movable phase : Acetonitrile and 0.1% TFA (60:40)

Column : X-Bridge phenyl 150 x 4.6 mm, 3.5µm

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 10min

Wavelength : 255 nm

Observation : Baseline is not sufficient

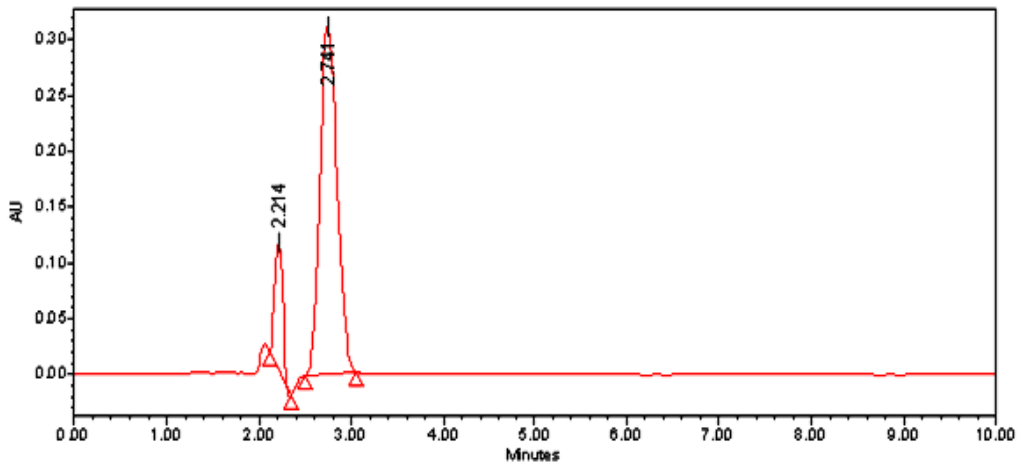

Figure 3.05: Chromatogram of trial-3

	Name	**RT**	**Response**	**% Area**	**USP Resolution**	**USP Tailing**	**USP Plate Count**
1		2.214	224981	16.62		2.05	2013
2		2.741	3085117	51.23	2.56	1.62	4563

Trial-4 [Fig. 3.06]

Movable phase : Acetonitrile and 0.1% OPA (30:70)

Column : X-Bridge phenyl 150 x 4.6 mm, 3.5µm

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 10 min

Wavelength : 255 nm

Observation : Extra peak is observed

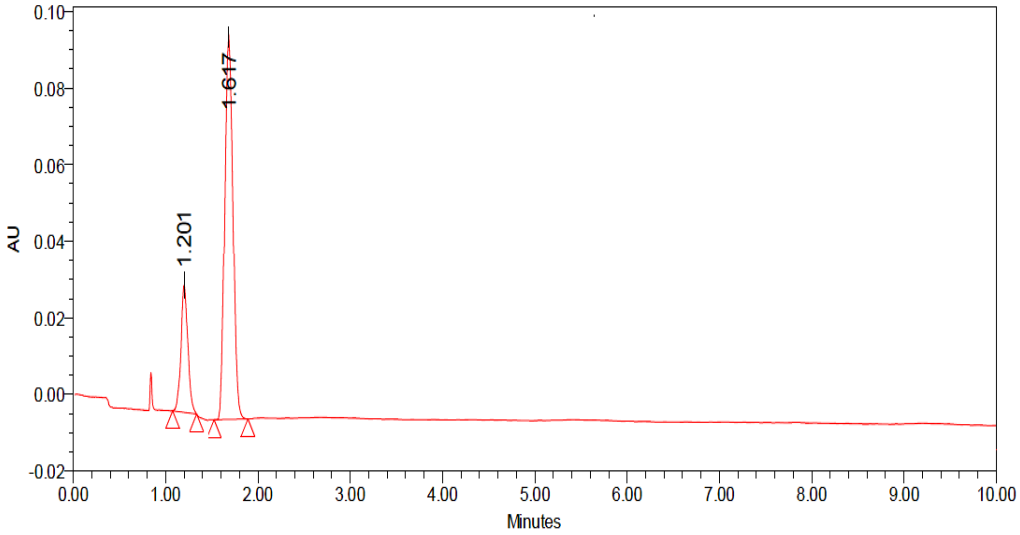

Figure 3.06: Chromatogram of trial-4

	Name	RT	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.201	25641	23.53		0.76	2339
2		1.617	8549611	76.47	2.13	1.90	2604

Trial-5 [Fig. 3.07]

Movable phase : Acetonitrile and 0.1% OPA (40:60)

Column : X-Bridge phenyl 150 x 4.6 mm, 3.5µm

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 11 min

Wavelength : 255 nm

Observation : Peak height is not within the limit

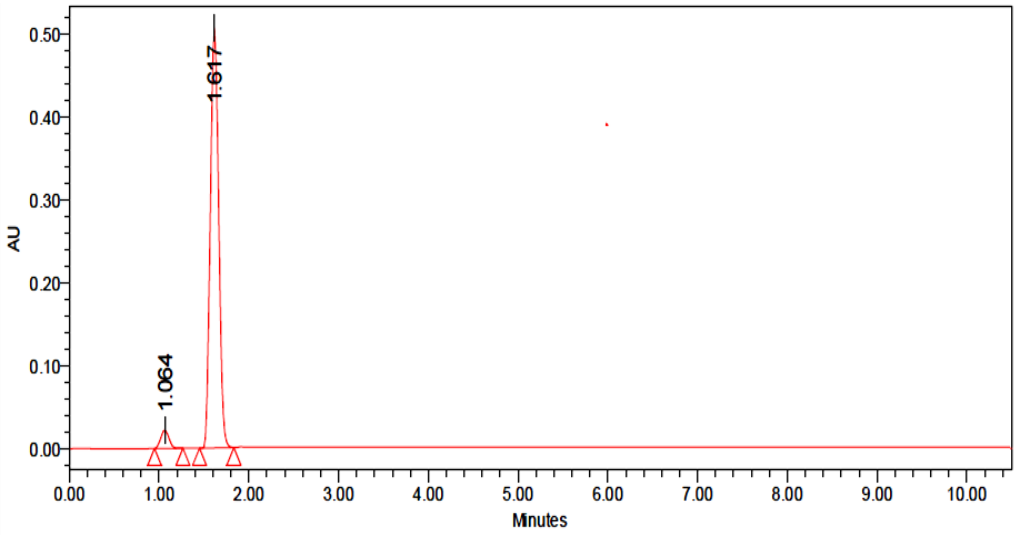

Figure 3.07: Chromatogram of trial-5

	Retention Time	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1	1.064	154122	12.00		0.54	3547
2	1.617	2846521	88.56	0.64	1.24	3825

Trial-6 [Fig. 3.08]

Movable phase : Acetonitrile and 0.1% OPA (50:50)

Column : Inertsil ODS (250x4.6mm, 5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 6min

Wavelength : 255 nm

Observation : Plate count is not within the limit

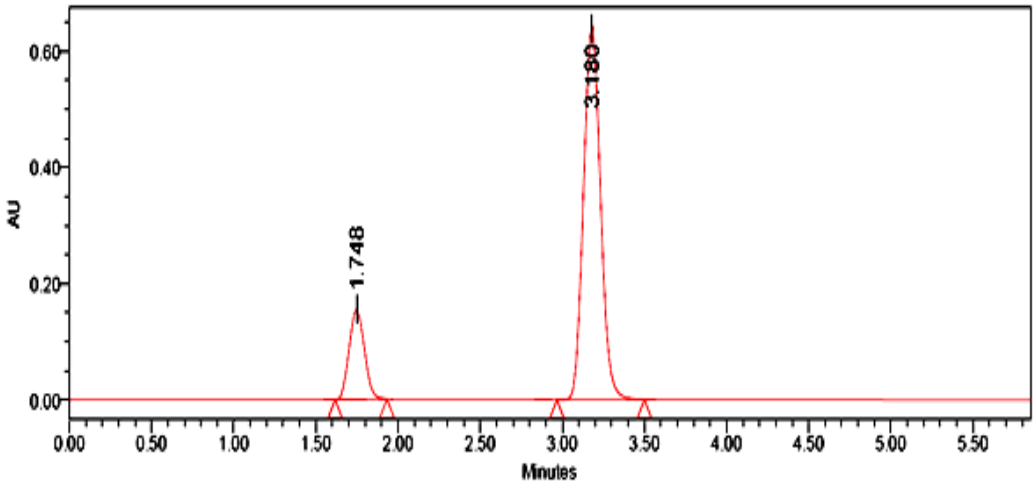

Figure 3.08: Chromatogram of trial-6

	Name	Retention Time	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.748	996980	30.58		1.09	1572
2		3.180	4622319	69.42	7.66	1.10	4369

Trial-7 [Fig. 3.09]

Movable phase : Acetonitrile and 0.1% formic acid (20:80)

Column : Inertsil ODS (250x4.6mm, 5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 6min

Wavelength : 255 nm

Observation : Tailing is not within the limit

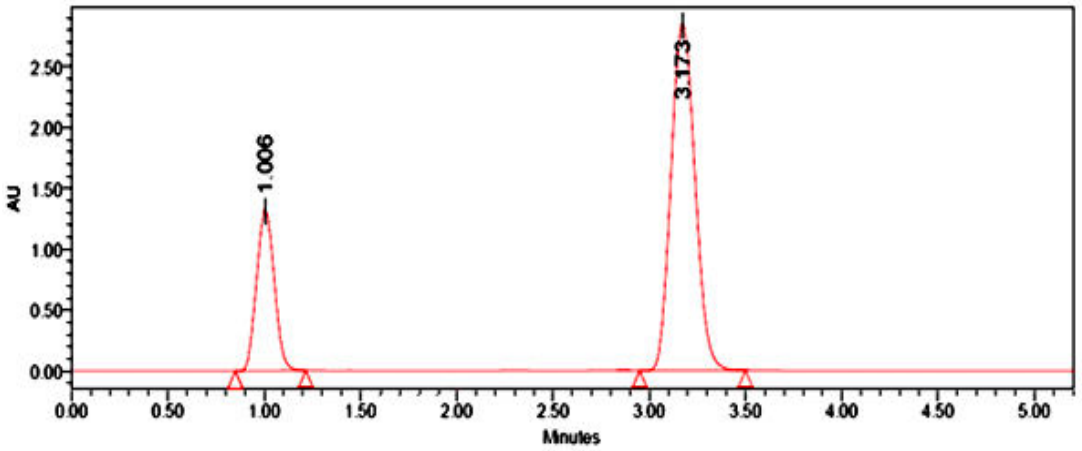

Figure 3.09: Chromatogram of trial-7

	Name	RT	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.006	8505649	40.79		1.08	2513
2		3.173	24739989	59.21	8.94	2.12	3003

Trial-8 [Fig. 3.10]

Movable phase : Acetonitrile and 0.1% formic acid (30:70)

Column : Inertsil ODS (250x4.6mm, 5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 3min

Wavelength : 255 nm

Observation : Broad peaks are observed

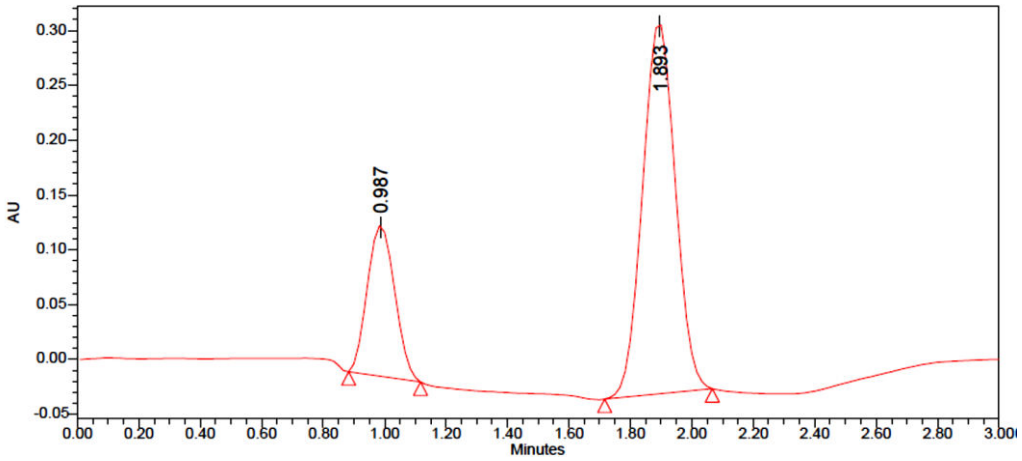

Figure 3.10: Chromatogram of trial-8

	Name	RT	Response	USP tailing	USP plate count	USP resolution
1		0.987	553184	1.06	2934	
2		1.893	2352679	1.02	5175	4.36

Trial-9 [Fig. 3.11]

Movable phase	:	Acetonitrile and 0.1% formic acid (35:65)
Column	:	Inertsil ODS (250x4.6mm, 5 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Period of run	:	6 min
Wavelength	:	255nm
Observation	:	Response of the peak is very high

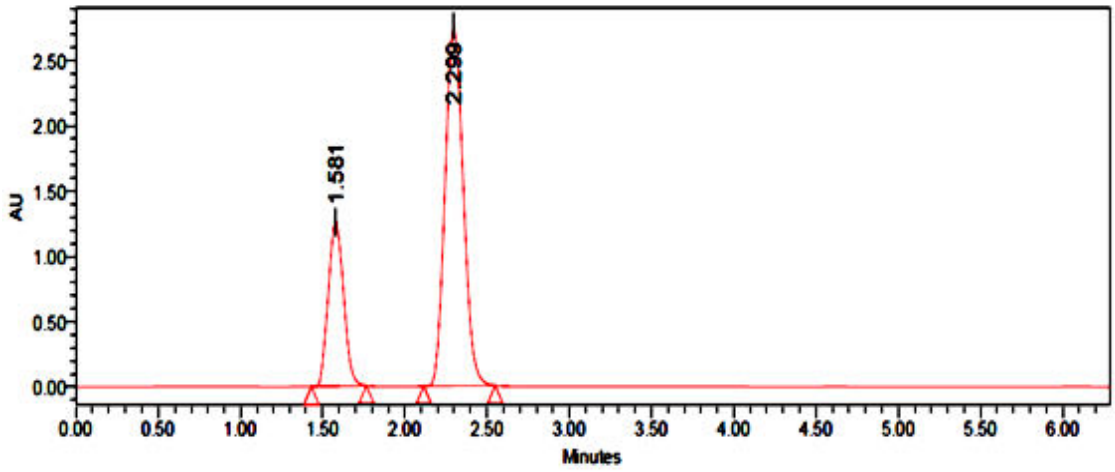

Figure 3.11: Chromatogram of trial-9

	Name	RT	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.581	8170668	21.49		1.10	2279
2		2.299	21222549	55.83	3.73	1.11	2012

Trial-10 [Fig. 3.12]

Movable phase	:	Acetonitrile and 0.1% formic acid (40:60)
Column	:	Inertsil ODS (250x4.6mm, 5 µ)
Rate of flow	:	1 ml/min
Volume of injection	:	10µl
Period of run	:	6 min
Wavelength	:	255 nm
Observation	:	This method is suitable for validation

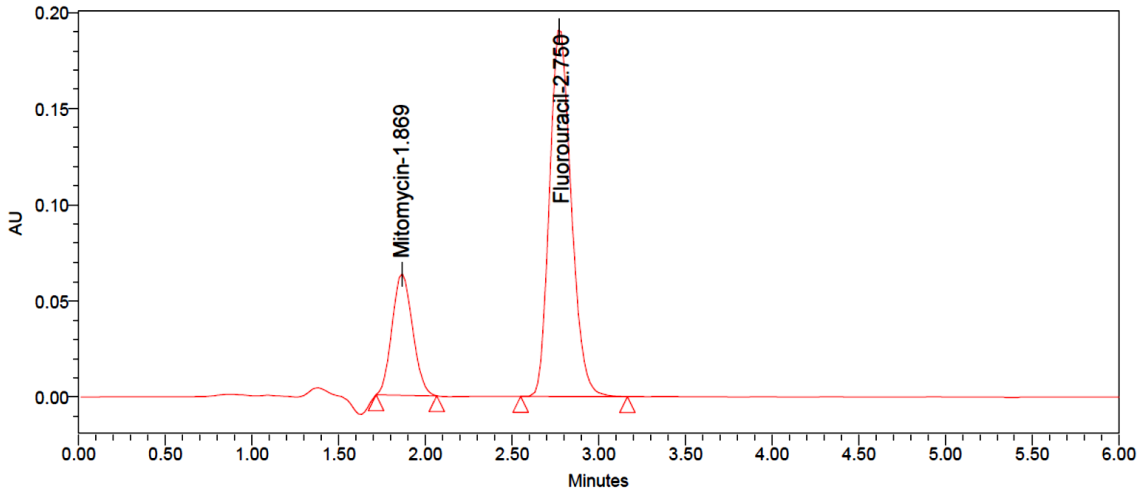

Figure 3.12: Chromatogram of trial-10

	Name	RT	Response	USP tailing	USP plate count	USP resolution
1	Mitomycin	1.869	192547	1.02	3628	
2	Fluorouracil	2.750	2454638	1.11	5487	8.64

3.5.2 Optimized Method: [Table 3.01]

Table 3.01: Optimized method chromatographic conditions

S.NO	Parameter	Chromatographic condition
1	Mobile phase	Acetonitrile: 0.1% formic acid (70:30)
2	Column	Inertsil ODS (250x4.6mm, 5 µ)
3	Rate of flow	1ml/min
4	Column temperature	Ambient temperature
5	Sample temperature	Ambient temperature
6	Wavelength	255 nm
7	Volume of injection	10µl
8	Period of run	6 min
9	Retention time	Mitomycin Retention time-1.869 Fluorouracil retention time-2.750

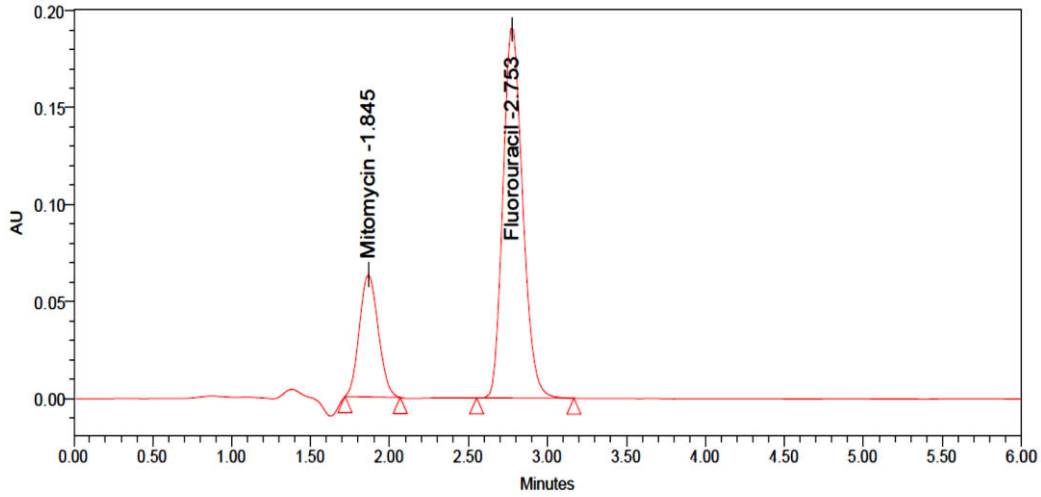

Figure 3.13: Chromatogram of standard

[Fig. 3.13] shows chromatogram of standard

3.6 Validation of Method

Standard characteristics such as device compatibility, adequacy, specificity, precision and linearity were all verified using ICH Q2 (R1) standards as was the method's stability and forced deterioration.

3.6.1 Specificity: [Fig. 3.14]

One measure of analyte specificity is its ability to be detected even when the sample solution and standard reference solutions include unknown contaminants or excitations. Blank samples and samples laced with Mitomycin and Fluorouracil were used to test it.

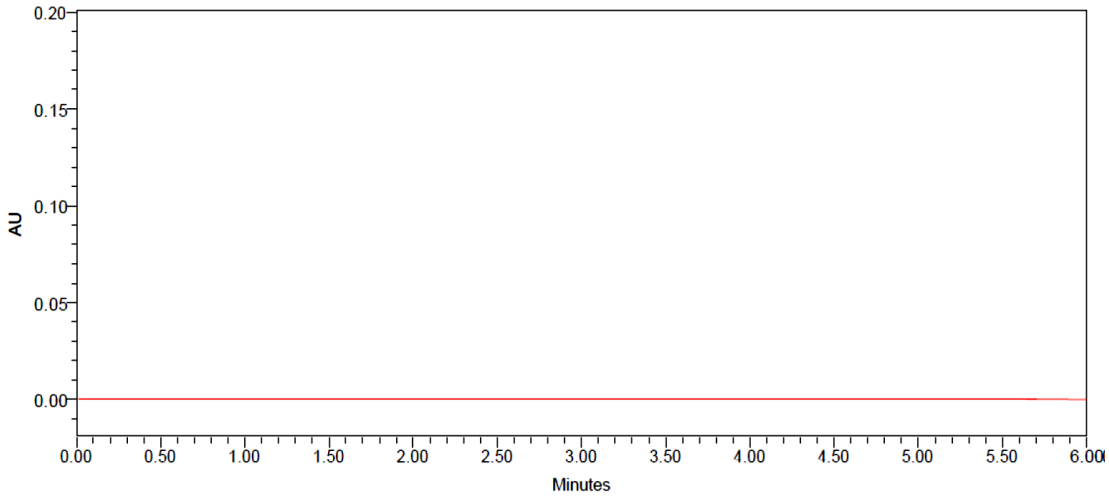

Figure 3.14: Chromatogram of blank

3.6.2 Linearity:

A study's ability to produce results within a specified framework is known as the linearity of empirical methodology. The peak area was inversely proportional to the concentration of analytes in the sample for the linearity spectrum assessment, and six sets of standard solutions were employed. Regression equations were developed by plotting the peak area of the calibration curve using the concentration of normal solution. The least squares method of least squares was used to get the slope and correlation coefficient.

The linearity peak areas of Mitomycin and Fluorouracil have been measured at 10, 25, 50, 100, 125, and 150 percent, respectively. In a linear regression analysis, the peak area was plotted against concentration. Calibration curve slope and y-intercept correlation coefficients were analysed as well as calculated. Covariance coefficients were all greater than 0.0001.

Procedure:

Use the chromatographic technique to inject and measure the peak area at each step. Coefficients of correlation may be calculated by drawing a line graph of peak area (Y-axis peak area) vs concentration (X-axis concentration). **[Figs. 3.17-3.22]** demonstrate linearity chromatograms with a 10 percent, 25 percent, 50 percent, 100 percent, 125 percent and 150 percent linearity range. The results of linearity are shown in **[table 3.02].**

Range:

Between the upper and lower analytical phases, there is a spectrum of analytical procedures that may be relied upon for accuracy, precision, and a linear relationship between results.

Acceptance Criteria:

When calculating correlation coefficients, they should not go below 0.999

Table 3.02: Results of linearity

S.no	Conc µg/ml	Mitomycin Response	Conc.µg/ml	Fluorouracil Response
1	2.00	17504	5.00	236501
2	5.00	45653	12.50	603257
3	10.00	95687	25.00	1205746
4	20.00	191546	50.00	2451068
5	25.00	228167	62.50	2825715
6	30.00	280568	75.00	3498601
CC		0.9996		0.9990
Slope		9328.11		46375.47
intercept		134.12		22075.56

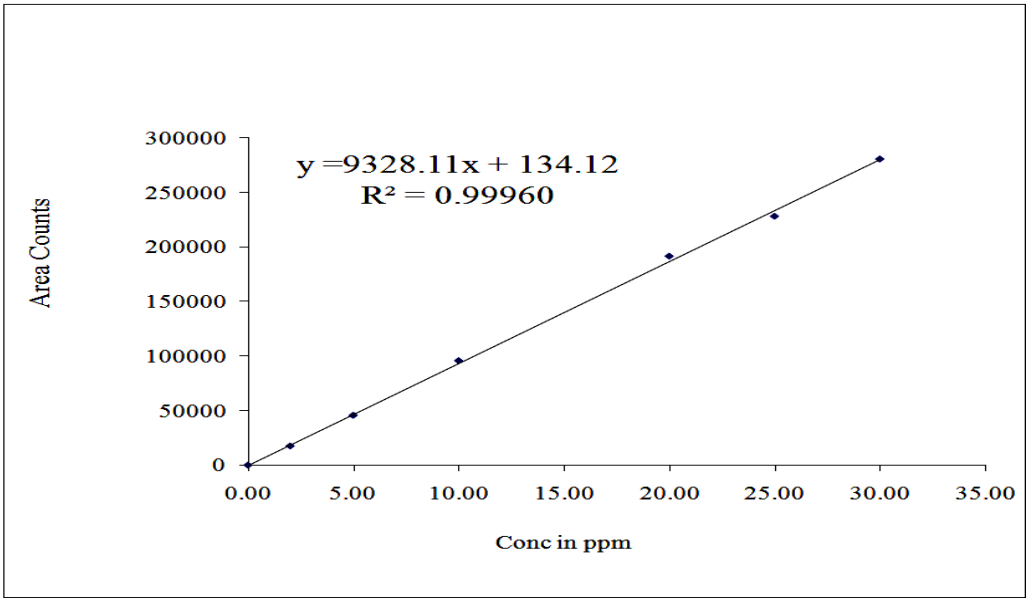

Figure 3.15: Mitomycincalibration plot

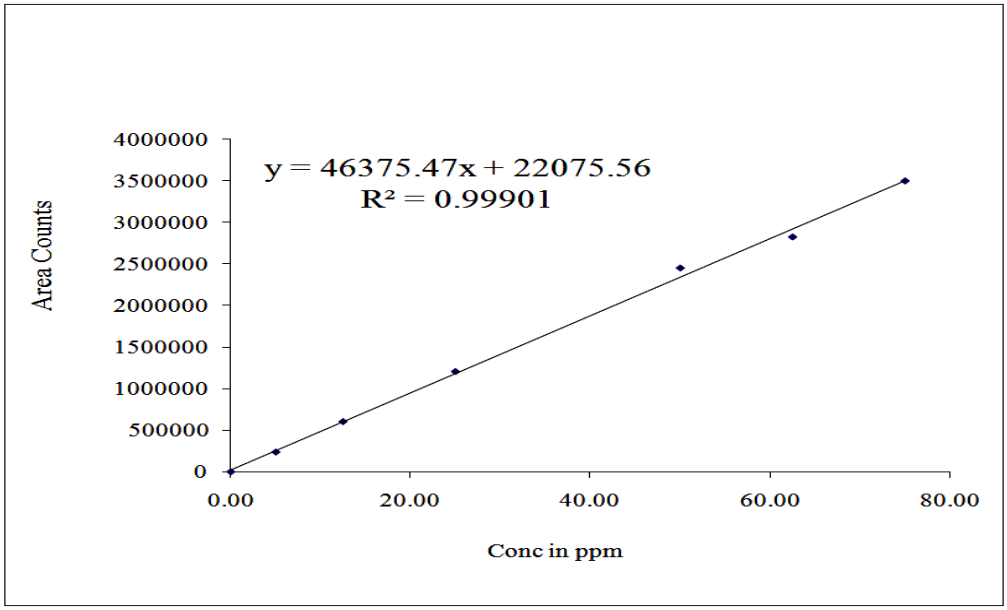

Figure 3.16: Fluorouracil calibration plot

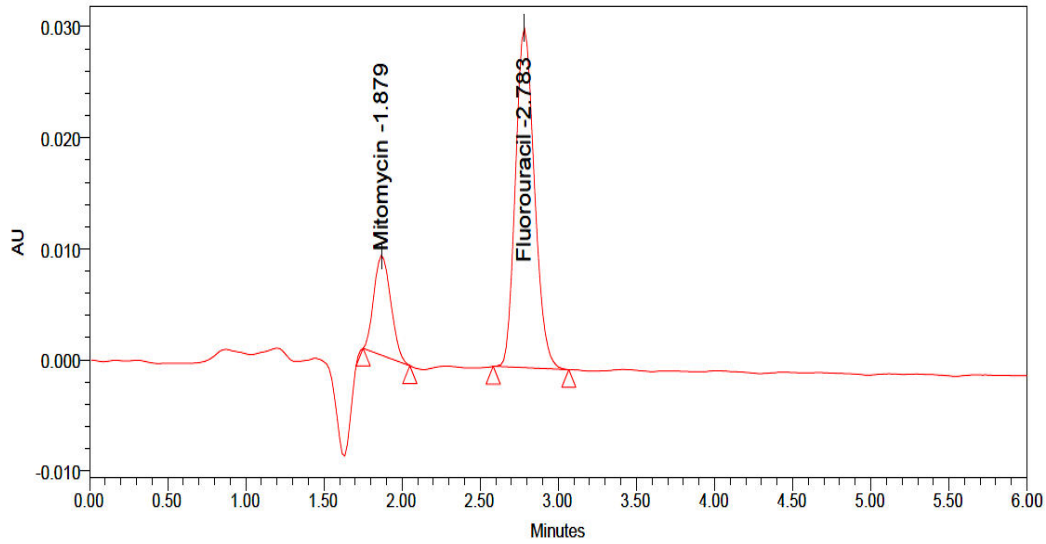

Figure 3.17: 10 percent linearity chromatogram

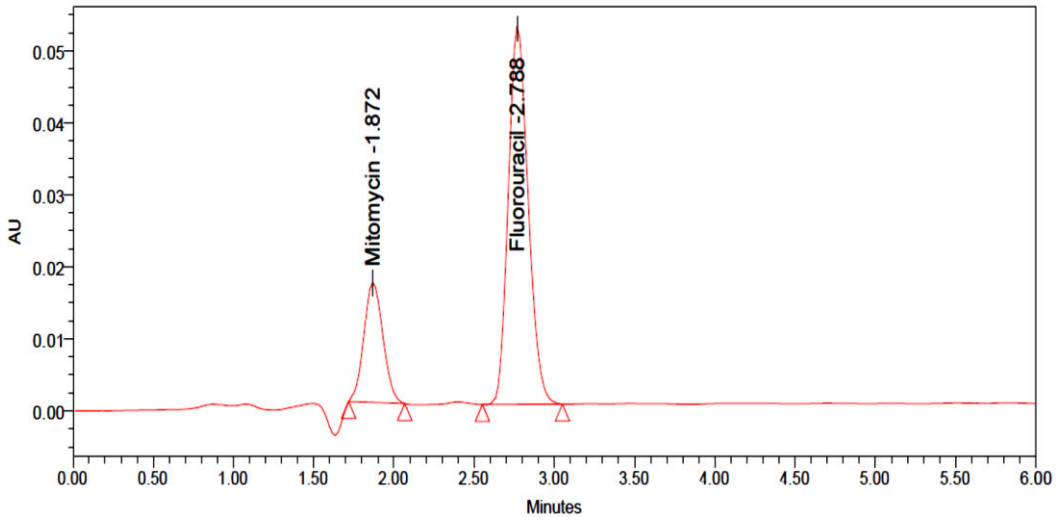

Figure 3.18: 25 percent linearity chromatogram

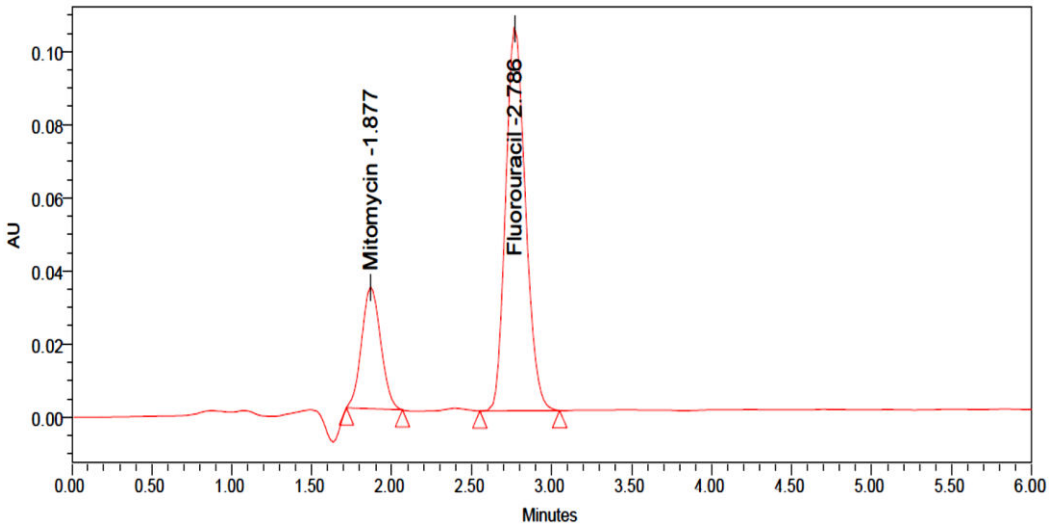

Figure 3.19: 50 percent linearity chromatogram

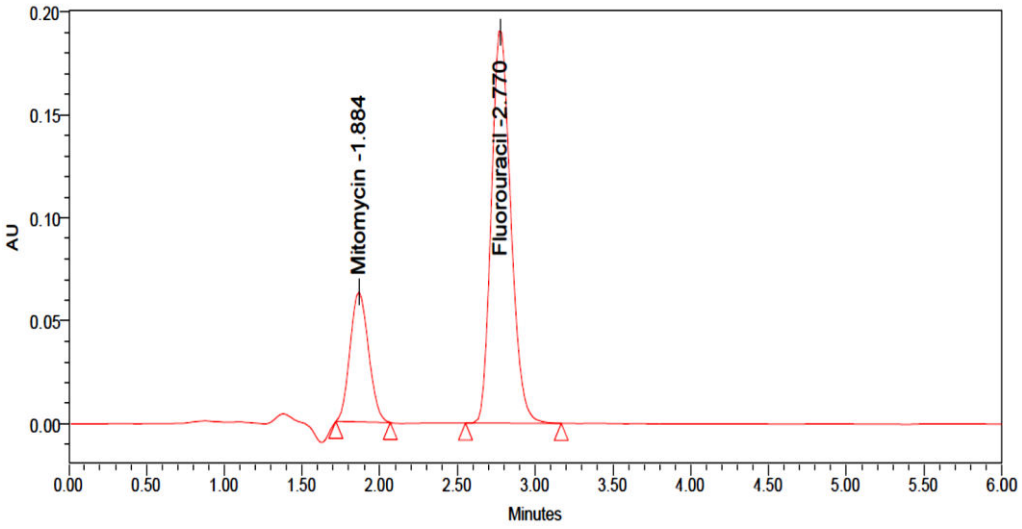

Figure 3.20: 100 percent linearity chromatogram

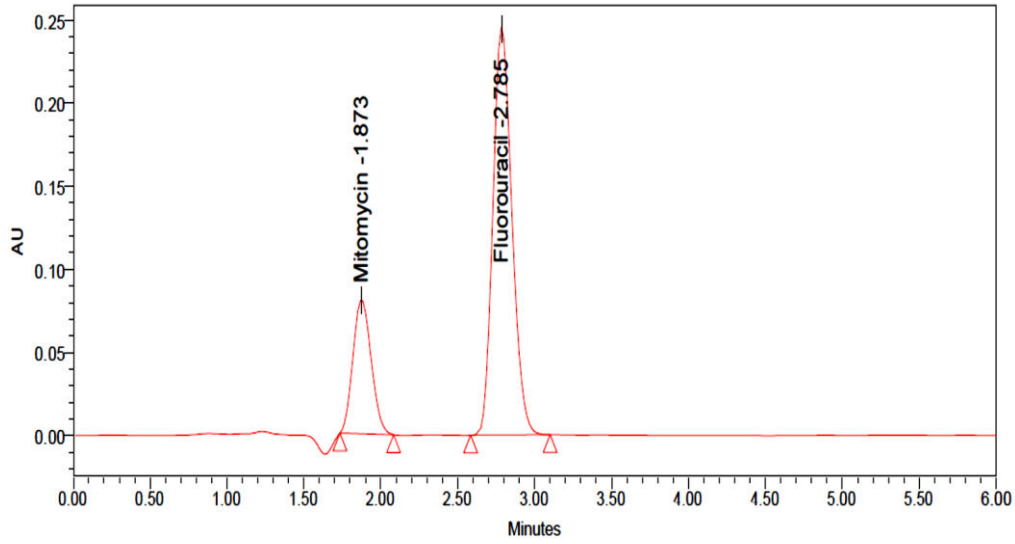

Figure 3.21: 125 percent linearity chromatogram

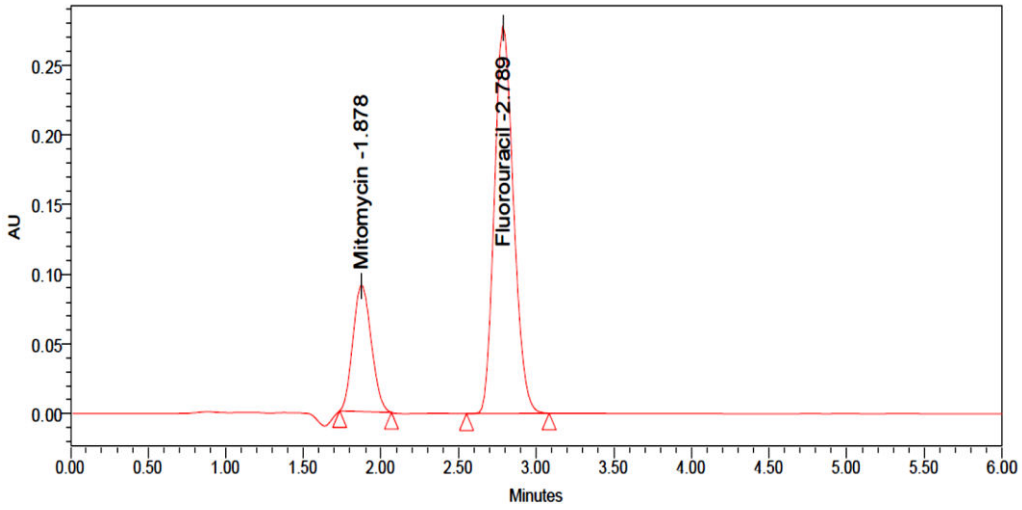

Figure 3.22: 150 percent linearity chromatogram

3.6.3 Accuracy:

50 percent solution preparation (with respect to the concentration of the target assay)
Apply diluents to a clean, dry volumetric flask containing 10 mg of Mitomycin and 25 mg of Fluorouracil and sonicate to dissolve well and bring up to the diluents level.

When the volumetric flask has been filled to the desired level, pipette 5 ml of the aforesaid solution into it. (Mitomycin, 10 ppm; Fluorouracil, 25 ppm)

100 percent solution preparation (with respect to target assay concentration)
Weigh and transfer exactly 20 mg of Mitomycin and Fluorouracil into a dry volumetric flask, then add the diluents and sonicate to dissolve them completely and bring the diluent level up to that of the diluents.

In a 50ml volumetric flask, pipette 5ml of the aforementioned solution and dilute it with diluents to the desired volume. (20 ppm Mitomycin and 50 ppm Fluorouracil)

150 percent solution preparation (with respect to target assay concentration)
Using an accurate balance, transfer 30 mg of Mitomycin and 75 mg of Fluorouracil to an airtight volumetric flask, diluents should be added, and the flask should be shaken before each addition to ensure complete dissolution.

Take 5ml of the aforesaid stock solution and dilute it to your desired concentration in a 50ml volumetric flask using diluents. Mitomycin (30 ppm) with Fluorouracil (75 ppm)

Procedure:
Inject the standard solution, 50 percent accuracy, 100 percent accuracy, and 150 percent accuracy solutions [**41**]. Mitomycin and Fluororacil accuracy values are shown in **[Table 3.03]** and the chromatograms for 50%, 100%, and 150% accuracy are shown in **[Fig. 3.23, 3.24 & 3.25]**.

Acceptance Criteria:
Each stage should have a recovery rate of 98-102 percent.

Table 3.03: Accuracy results of Mitomycin and Fluorouracil

S. No	% Level	Mitomycin % Recovery	Fluorouracil % Recovery
1	50	100.24	99.98
2	100	100.59	99.81
3	150	100.13	99.95
mean		100.32	99.91
SD		0.240	0.091

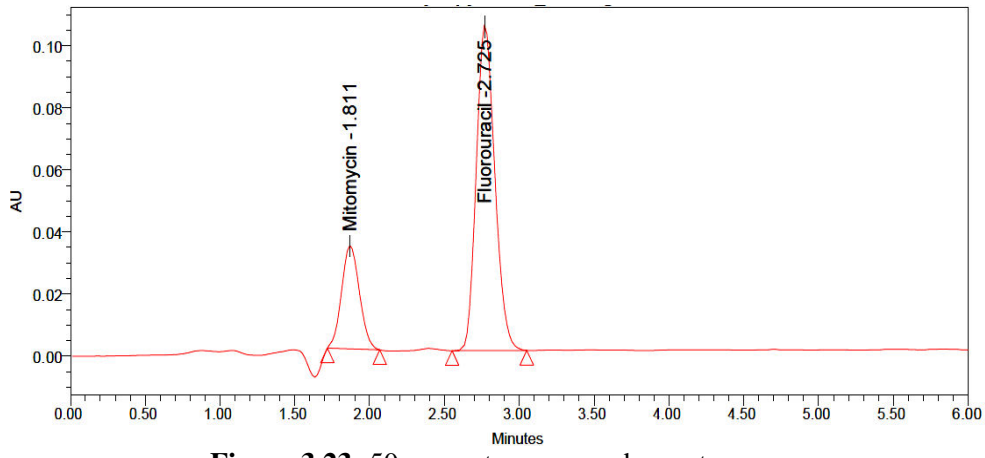

Figure 3.23: 50 percent accuracy chromatogram

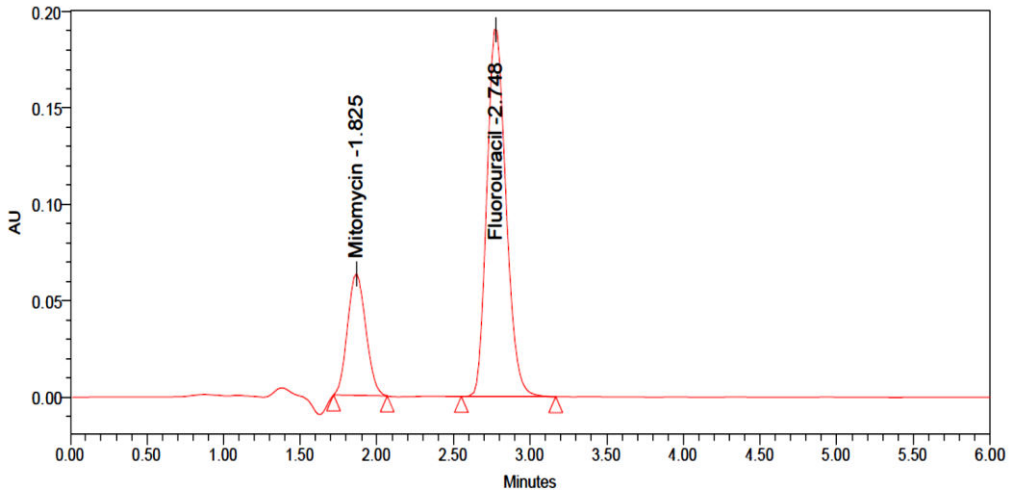

Figure 3.24: 100 percent accuracy chromatogram

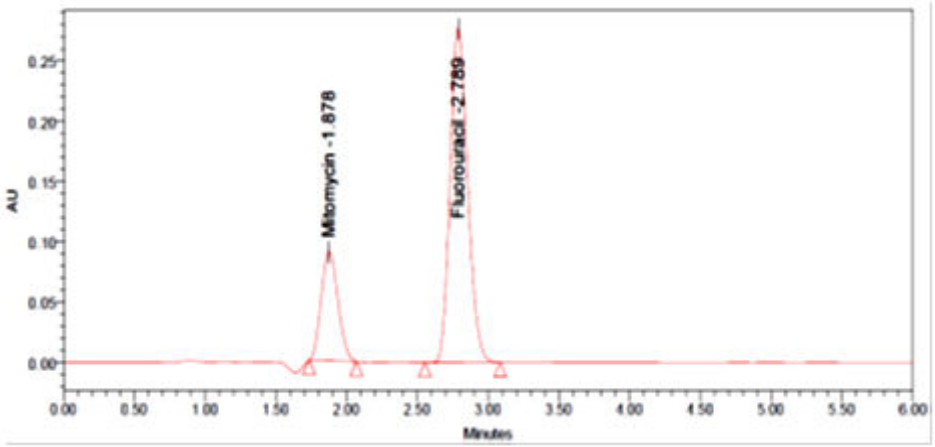

Figure 3.25: 150 percent accuracy chromatogram

3.6.4 Precision:

The accuracy of an analytical procedure is determined by the rate at which a set of measurements acquired from repeated homogenous samplings are in agreement. Fluorouracil (50ppm) and Mitomycin (20ppm) were each spiked six times to ensure the injection procedure used to test them was accurate [**42**]. In **[Table 3.04]** you can see the accuracy of the system, and in **[Fig. 3.26-31.31]** you can see the chromatograms.

Table 3.04: Results of system precision

System suitability parameter	Inclusion criteria	Medication name	
		Mitomycin	Fluorouracil
USP Plate Count	NLT 2000	3628	5487
USP Tailing	NMT 2.0	1.02	1.11
USP Resolution	NLT 2.0	-	8.64
% RSD	NMT 2.0	0.17	0.50

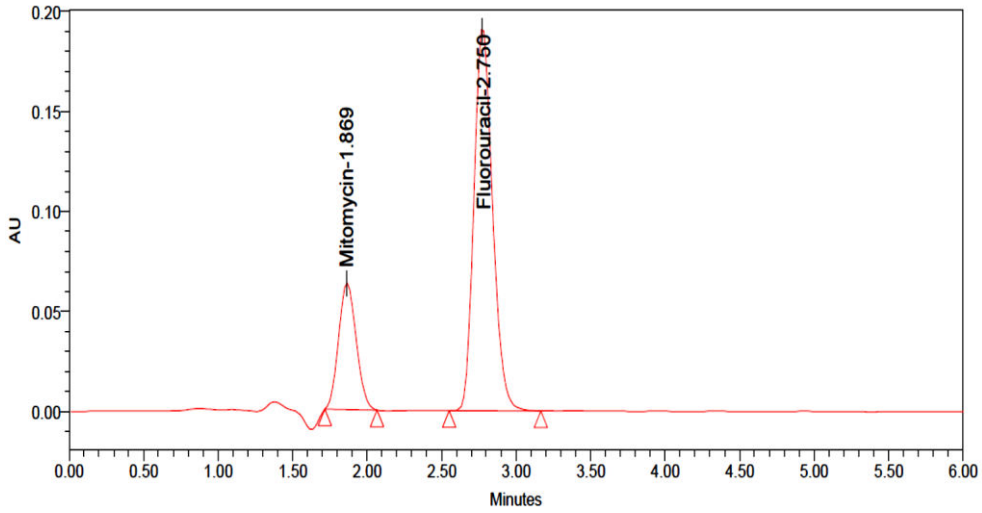

Figure 3.26: System Precision chromatogram-1

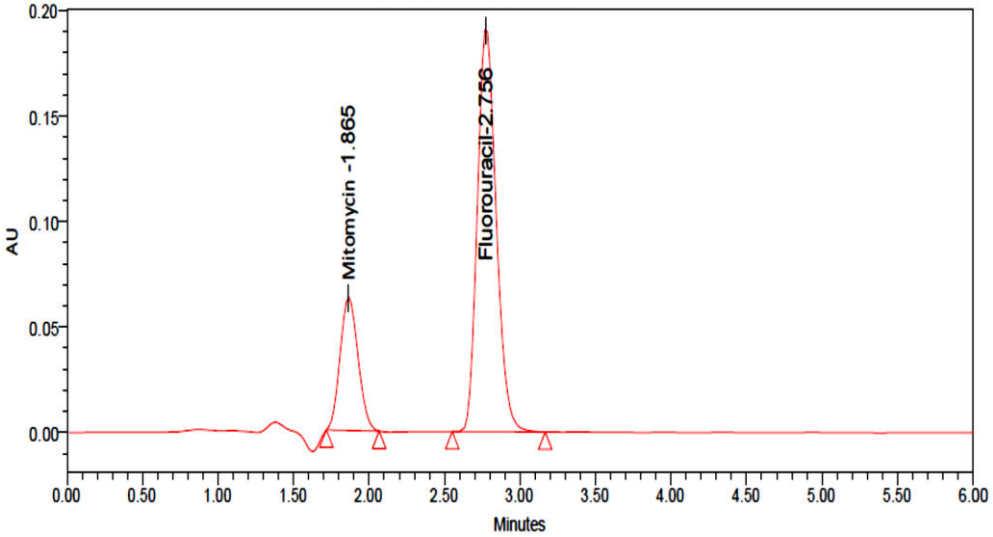

Figure 3.27: System Precision chromatogram-2

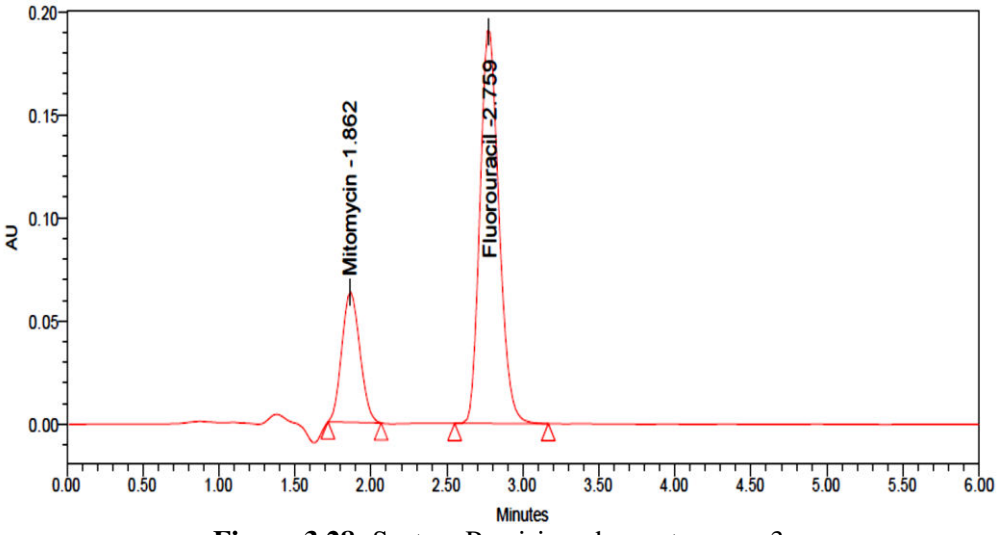

Figure 3.28: System Precision chromatogram -3

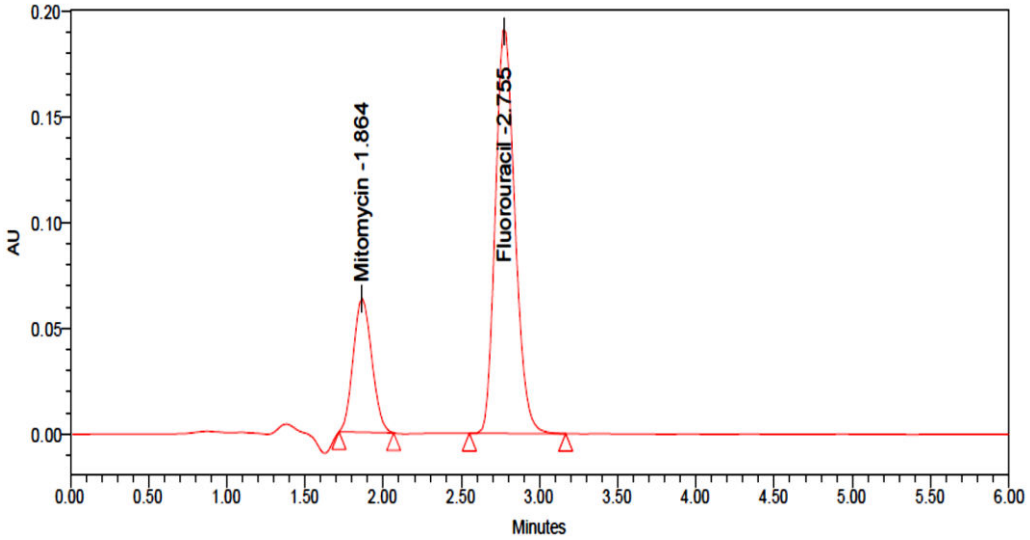

Figure 3.29: System Precision chromatogram -4

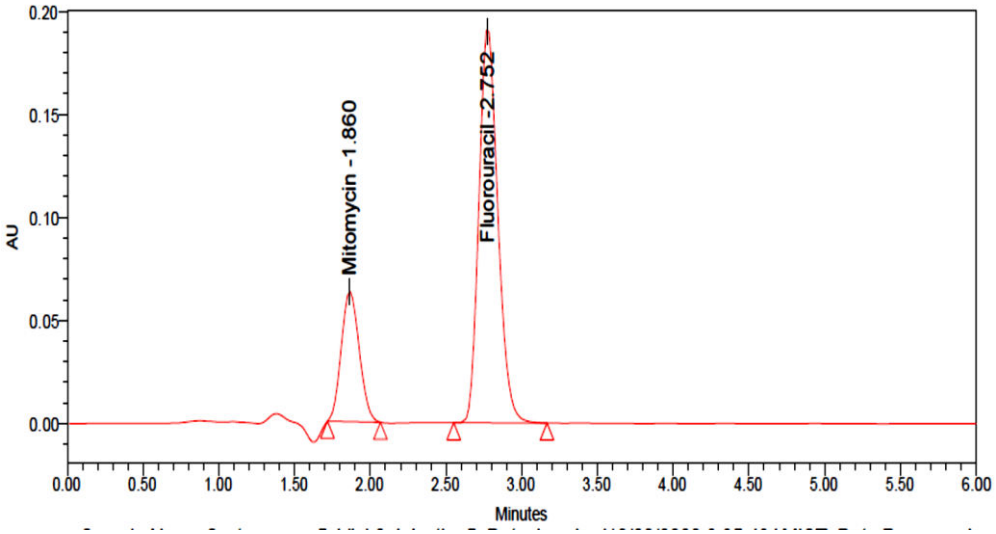

Figure 3.30: System Precision chromatogram-5

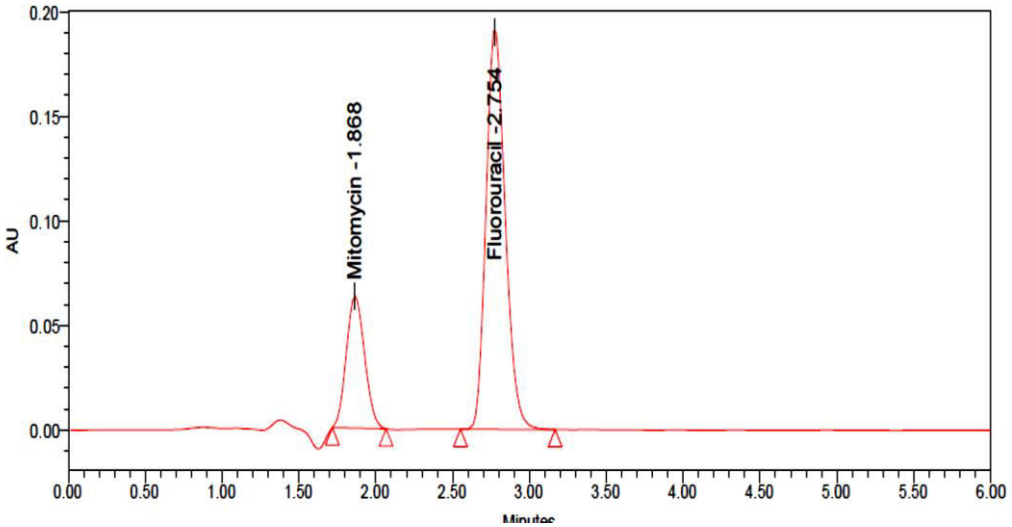

Figure 3.31: System Precision chromatogram-6

Method precision: [Table 3.05 and Fig 3.32]

Table 3.05: Results of method precision

Mitomycin				Fluorouracil		
S.No	Conc.(µg/ml)	Response	% assay as is	Conc. (µg/ml)	Response	% assay as is
1	20	191365	100	50	2451991	100.2
2		191143	99.9		2451387	100.1
3		191650	100.2		2435647	99.5
4		191554	100.1		2458475	100.4
5		190546	99.6		2455305	100.3
6		193341	101		2461250	100.5
% RSD	0.49		0.47	0.37		0.36
Mean	100.13			100.17		
SD	0.47188			0.35590		

Inclusion Criteria

The area six standard injection RSD percentage should not be less than 2%.

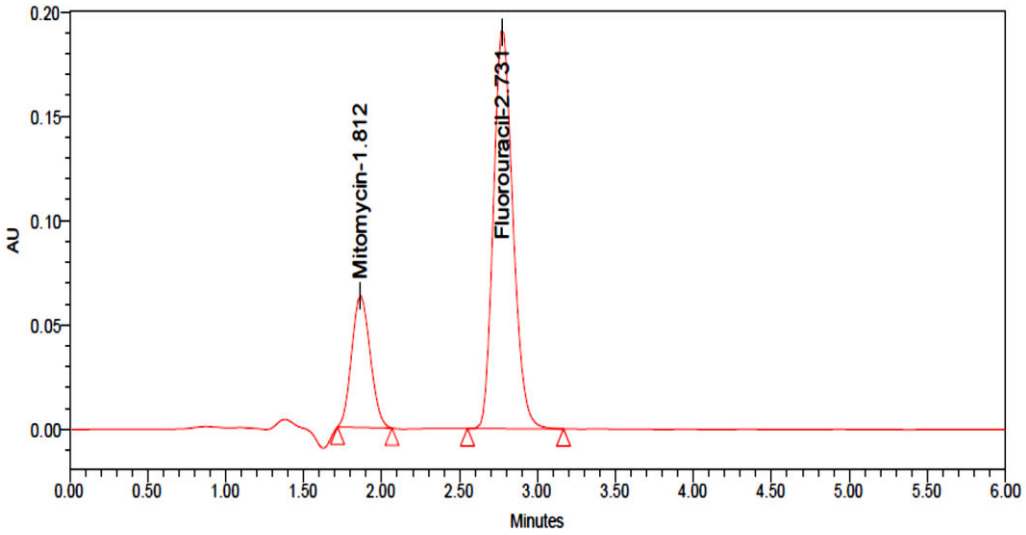

Figure 3.32: Chromatogram of method precision

Intermediate precision: [Table 3.06 and Fig 3.33]

Table 3.06: Results of intermediate precision

Mitomycin				Fluorouracil		
S.No.	Conc. (µg/ml)	Response	% assay as is	Conc. (µg/ml)	Response	% assay as is
1	20	191884	100.2	50	2451206	100.1
2		191327	100.0		2451954	100.2
3		191009	99.8		2434567	99.4
4		191567	100.1		2454877	100.3
5		191256	99.9		2448512	100
6		192368	100.5		2425457	99.1
%RSD	0.26		0.25	0.48		0.49
Mean	100.08			99.85		
SD	0.24833			0.48477		

Acceptance Criteria

The RSD percentage for the six normal injection results should not be more than 2%.

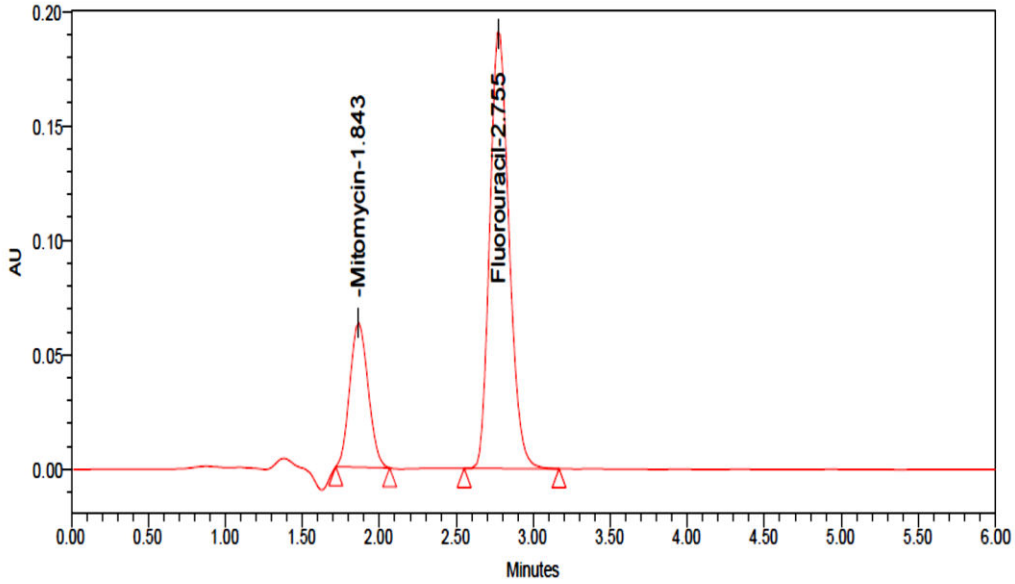

Figure 3.33: Chromatogram of intermediate precision

3.6.5 Limit of detection (LOD) and limit of quantification (LOQ): [Table 3.07 and Fig. 3.34 & 3.35]

Both the LOD and the LOQ were determined using the calibration curve approach. Compound LOD and LOQ were calculated using the RP-UPLC approach, which was developed. As you can see, the S/N ratios for LOD and LOQ are shown in this table. According to ICH standards, the process is validated.

Table 3.07: Sensitivity parameter values

Mitomycin				**Fluorouracil**			
LOD		**LOQ**		**LOD**		**LOQ**	
Conc.	s/n	Conc.	s/n	Conc.	s/n	Conc.	s/n
0.025µg/ml	4	0.083µg/ml	28	0.063µg/ml	7	0.208µg/ml	25

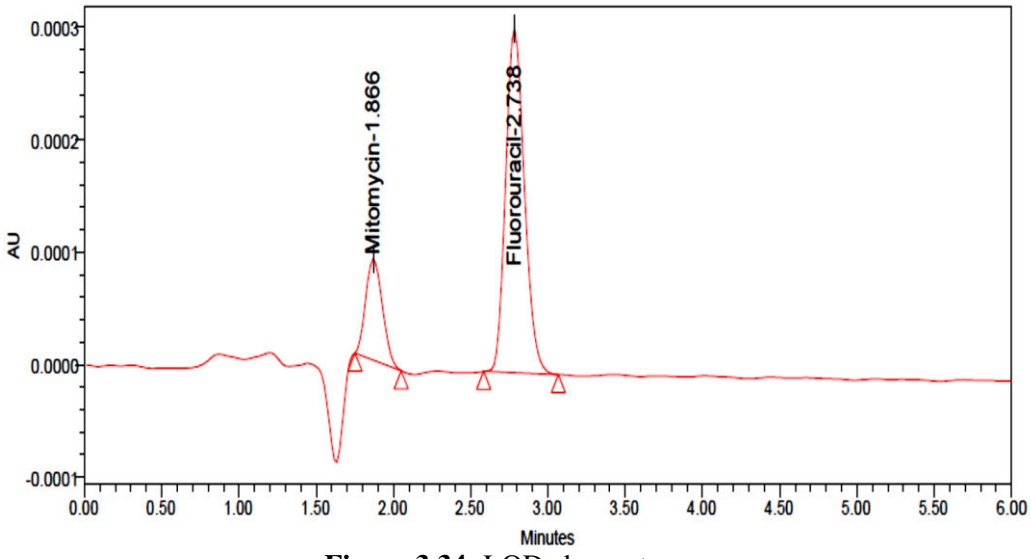

Figure 3.34: LOD chromatogram

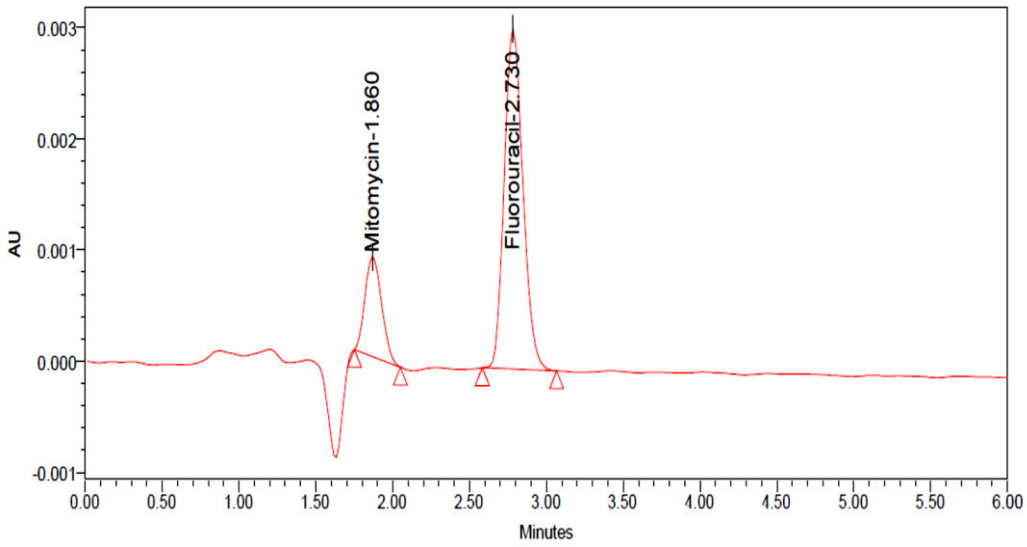

Figure 3.35: LOQ chromatogram

3.6.6 Robustness:

Tests were carried out on known systems, such as flow rate or movable phase in organic percentage, to see whether they could withstand these different circumstances. Active pharmaceutical components and contaminants were not considerably changed, and the period of retention, plate count, and tailing factor were not significantly altered[**43**]. As a result, this approach was dependable. **[Table 3.08]** compares the tensile strength of Fluorouracil with Mitomycin. **[Fig. 3.36-3.39]**Chromatograms.

Table 3.08: Robustness results

Parameter name	% RSD	
	Mitomycin	Fluorouracil
Flow minus (0.8 ml/min)	0.32	0.26
Flow plus (1.2 ml/min)	0.24	0.40
Organic minus (-10%)	0.21	0.68
Organic plus (+10%)	0.10	0.85

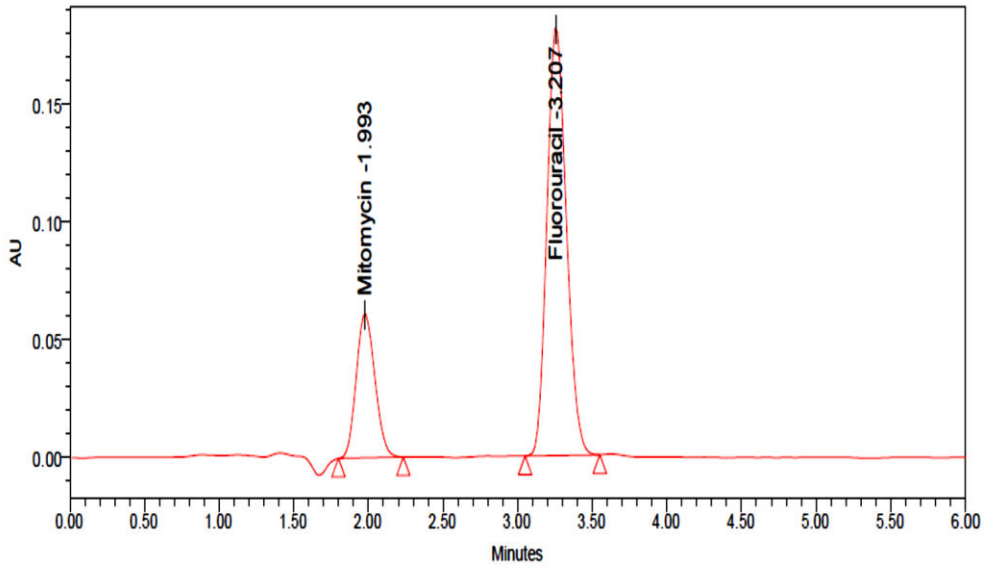

Figure 3.36: Less flow rate chromatogram (0.8ml/min)

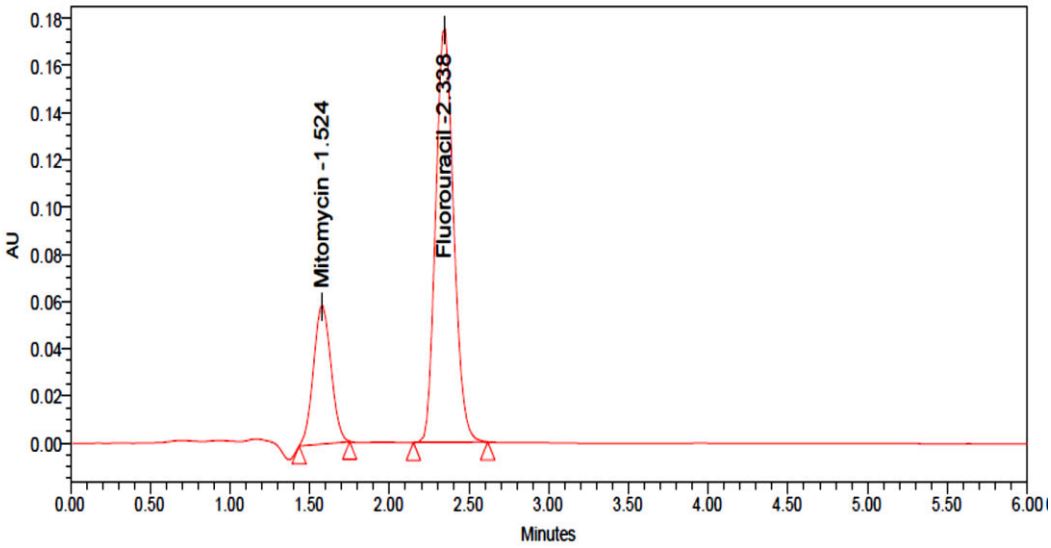

Figure 3.37: More flow rate chromatogram (1.2ml/min)

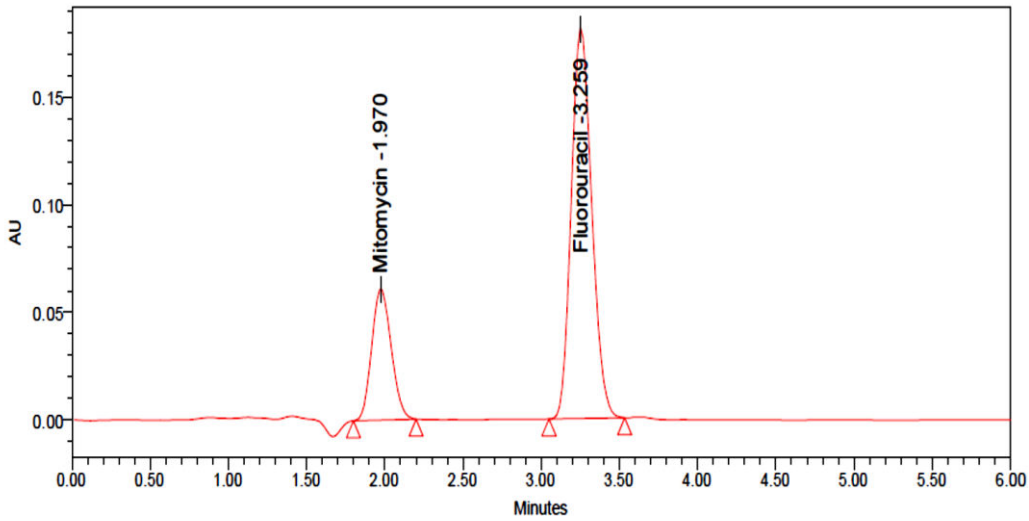

Figure 3.38: Less organic chromatogram

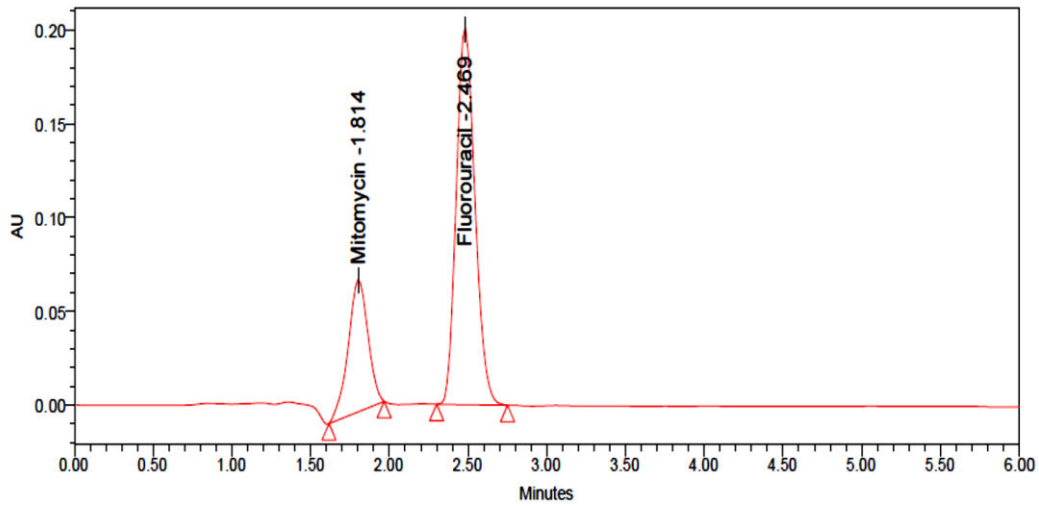

Figure 3.39: More organic chromatogram

3.6.7 Degradation Studies

An attempt was made to partially degrade the Fluorouracil and Mitomycin sample by putting it through a series of forced degradation settings. In order to determine whether forced degradation is appropriate for products of degradation, researchers have conducted a number of studies. Researchers may learn more about what makes a medicine unstable from this research and take precautions to prevent this from happening in the future.

Acid Degradation

A volumetric flask holds 10 ml of standard stock solution, 1 ml of 1N HCl, and the mixture is allowed to sit for 15 minutes. Using 1N NaOH and diluents to get the desired concentration, add 1ml after 15 minutes.

Alkali Degradation

For 15 minutes, a 10 ml volumetric flask was filled with 1 ml of standard stock solution and 1 ml of 1N NaOH. Then, after 15 minutes, add 1 ml of 1N HCl and dilute to the desired concentration using diluents to complete the reaction.

Peroxide Degradation

One millilitre of standard stock solution was diluted with 0.3 millilitres of 30% hydrogen peroxide in a 10 ml volumetric flask.

Reduction Degradation

For each 10 ml volumetric flask, one millilitre of 30 percent sodium bi sulphate solution was diluted with one millilitre of the standard stock solution before use.

Thermal Degradation

For six hours, the standard solution was baked at 105^{o}C in an oven. To get the final product, we used an ultrapure water column (UPLC).

Table 3.09 gives results of forced degradation and the **figures** from **[3.40-3.45]** shows forced degradation chromatograms.

Table 3.09: Forced degradation results of Mitomycin and Fluorouracil

Degradation condition	Mitomycin		Fluorouracil	
	% assay	% Degradation	% assay	% Degradation
Acid degradation	84.7	15.2	83.2	16.5
Alkali degradation	86.9	13.1	83.3	16.7
Peroxide degradation	86.3	13.7	87.7	12.3
Reduction degradation	88.5	11.5	85.4	14.6
Thermal degradation	89.1	10.9	88.9	11.1

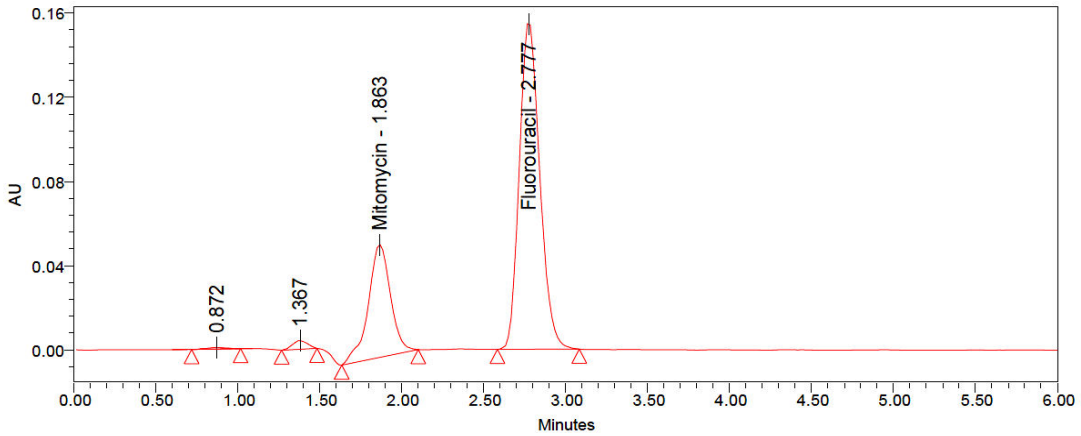

Figure 3.40: Chromatogram of acid degradation

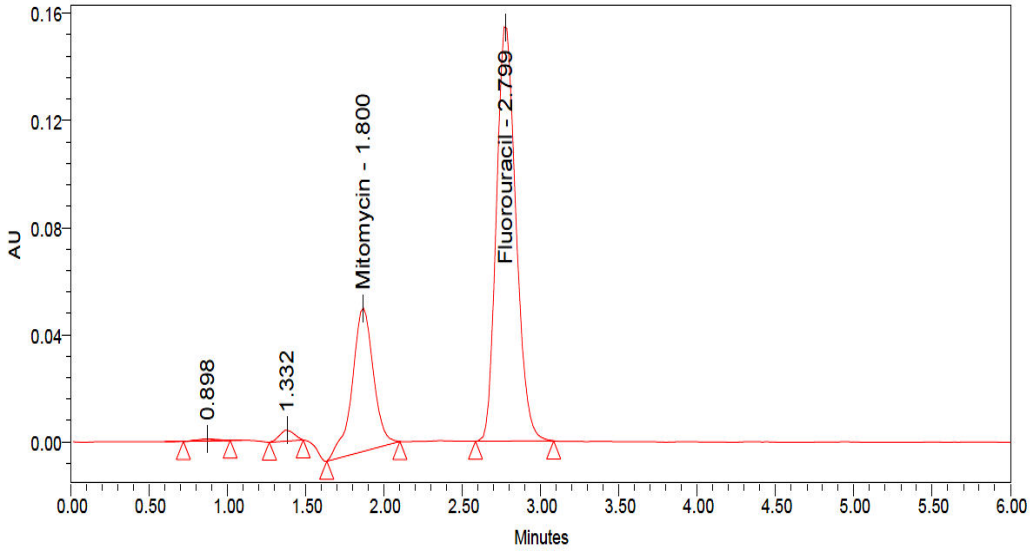

Figure 3.41: Chromatogram of alkali degradation

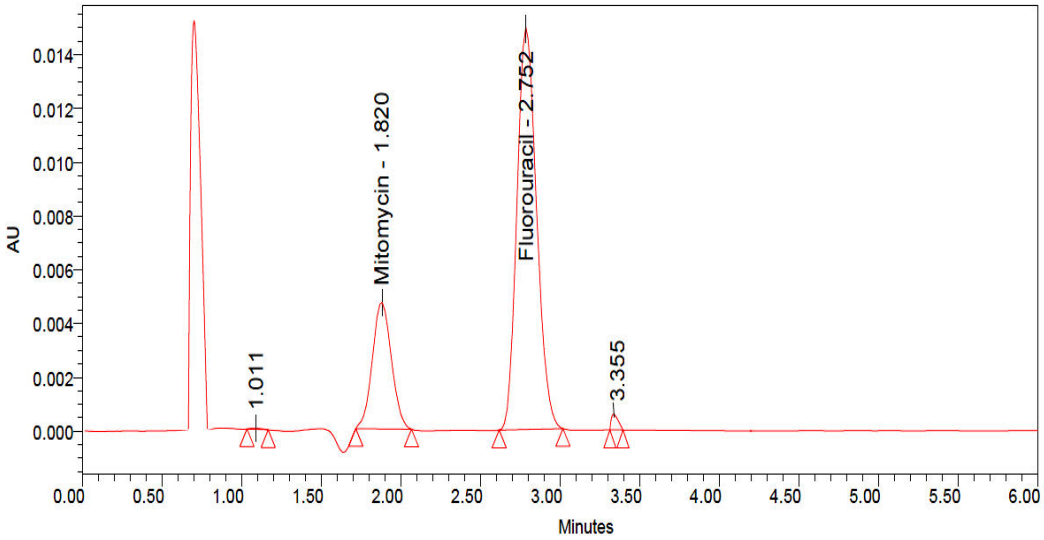

Figure 3.42: Chromatogram of peroxide degradation

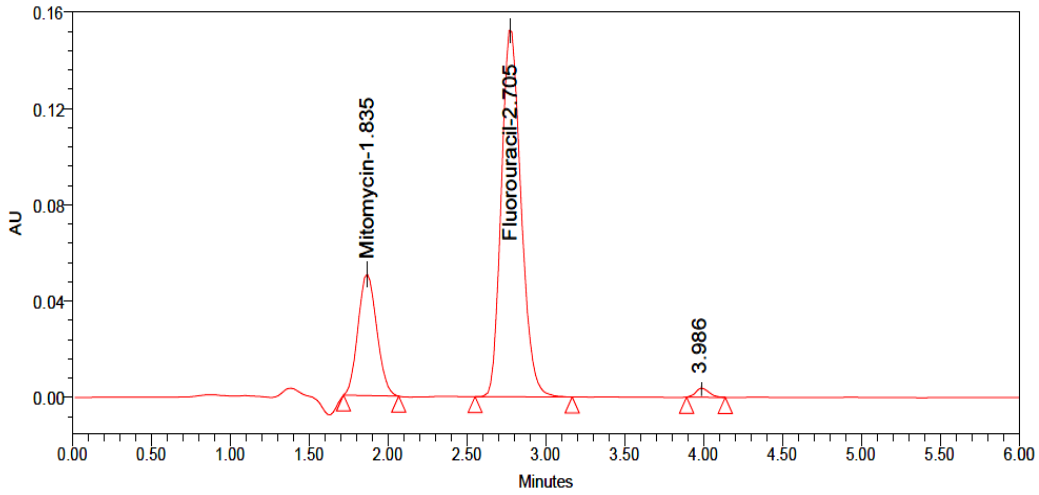

Figure 3.43: Chromatogram of reduction degradation

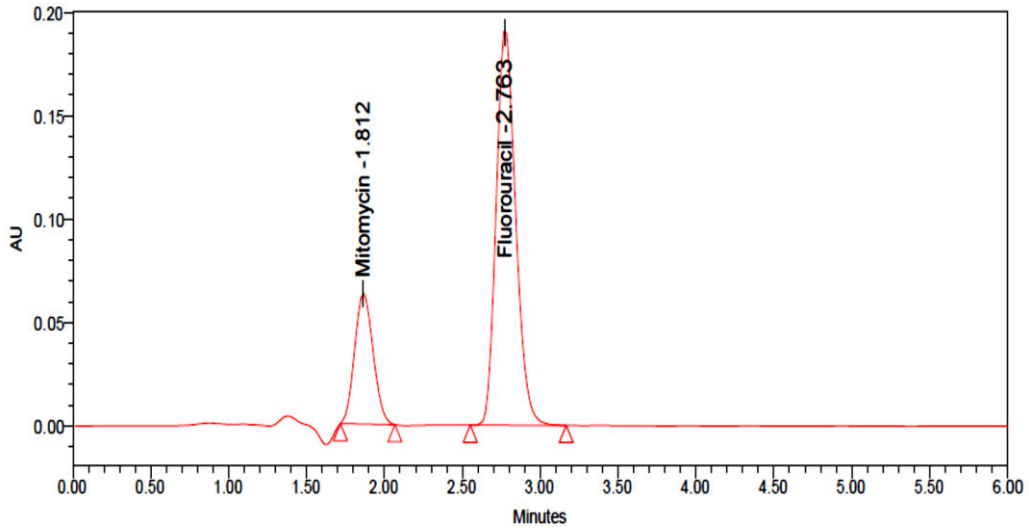

Figure 3.44: Chromatogram of thermal degradation

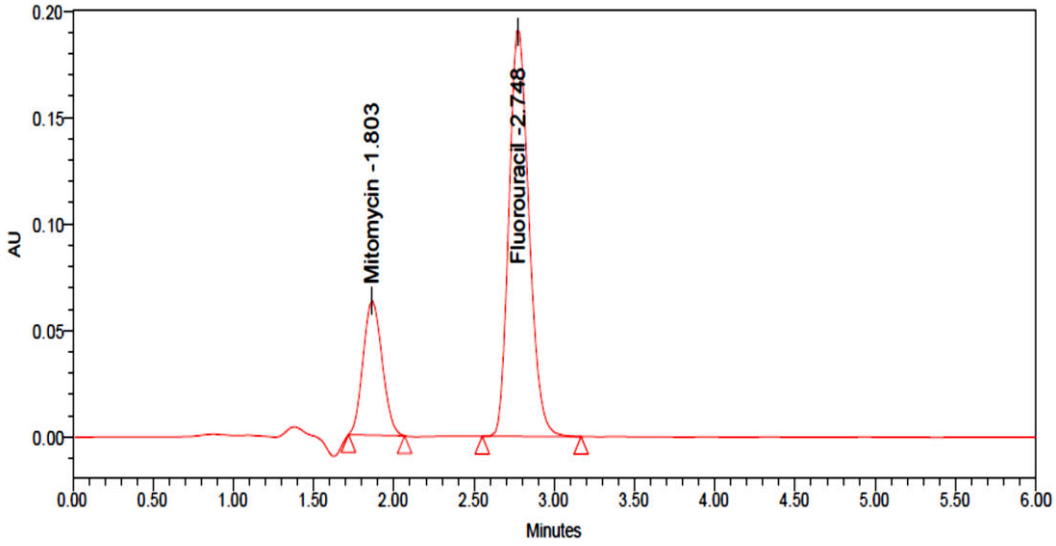

Figure 3.45: Chromatogram of control degradation

3.6.8 Stability:

At room temperature and between 2-8oC for up to 24 hours, the normal and sample solutions were stored. They were then poured into the apparatus, which calculated their departure from their original values over the course of 24 hours. During the study, there was no substantial variation and it was determined that the solutions were stable for 24 hours. Table 3.10 and 3.11 exhibit the results of Stability, while the chromatograms from [3.46-3.54] show the findings of Stability.

Table 3.10: Results of stability at RT

Stability	Mitomycin		Fluorouracil	
	Purity	% of deviation	Purity	% of deviation
Initial	100	0.00	100	0.00
6 Hrs	99.9	-0.10	99.9	-0.10
12 Hrs	99.8	-0.20	99.2	-0.80
18 Hrs	99.7	-0.30	98.8	-1.20
24 Hrs	99.6	-0.40	98.4	-1.60

Table 3.11: Stability results of Mitomycin and Fluorouracil at 2-8°C

Stability	Mitomycin		Fluorouracil	
	Purity	**% of deviation**	**Purity**	**% of deviation**
Initial	100.2	0.00	100	0.00
6 Hrs	100.1	-0.10	99.8	-0.20
12 Hrs	99.9	-0.30	99.3	-0.70
18 Hrs	99.8	-0.40	98.9	-1.10
24 Hrs	99.7	-0.50	98.4	-1.60

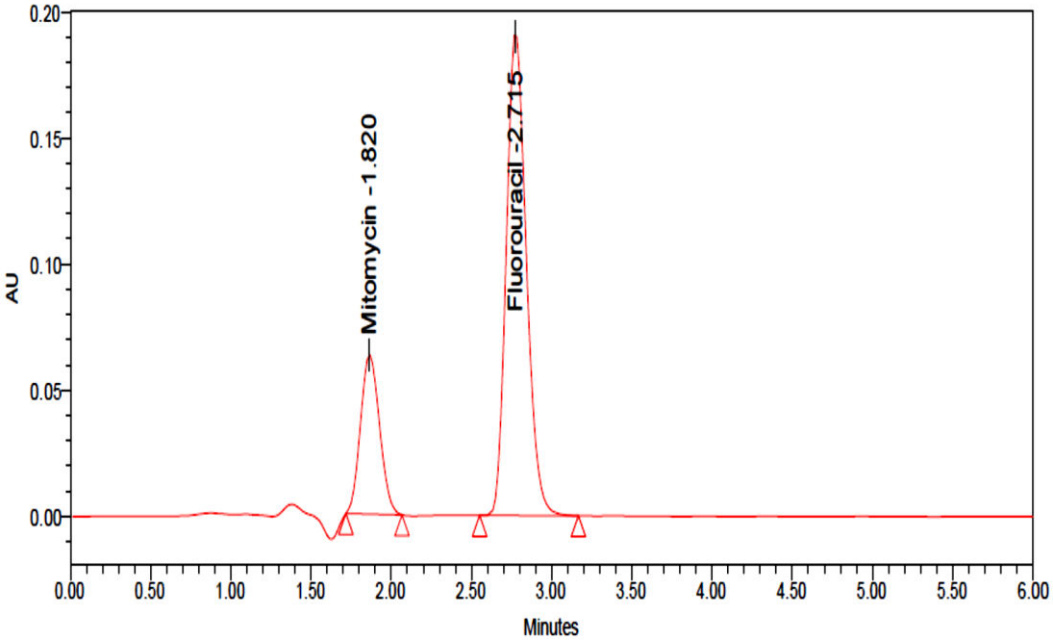

Figure 3.46: Chromatogram of stability initial RT

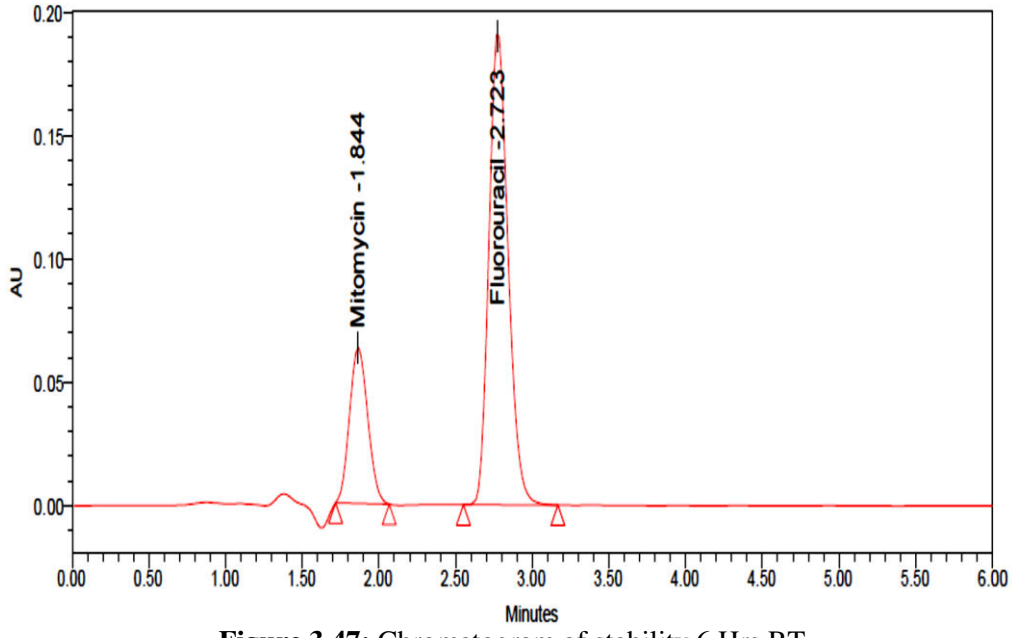

Figure 3.47: Chromatogram of stability 6 Hrs RT

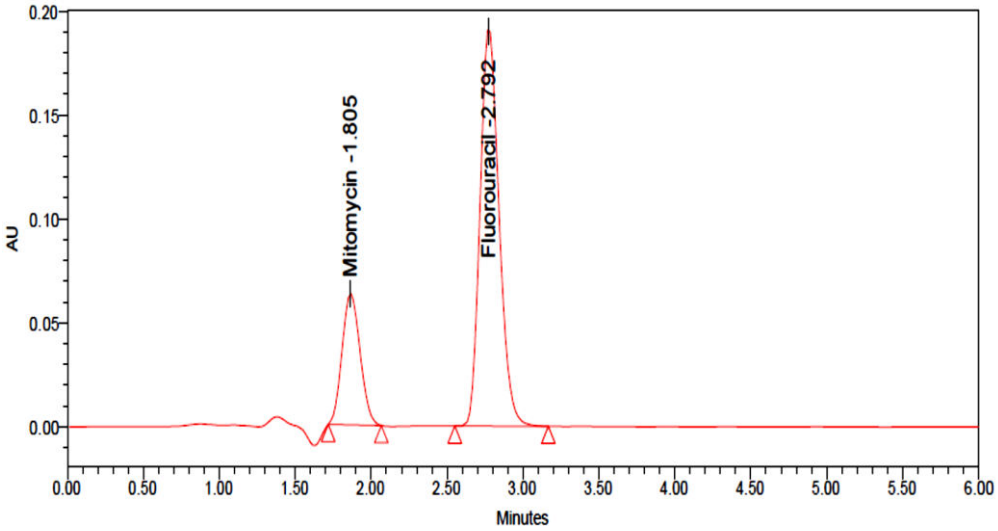

Figure 3.48: Chromatogram of stability 12 Hrs RT

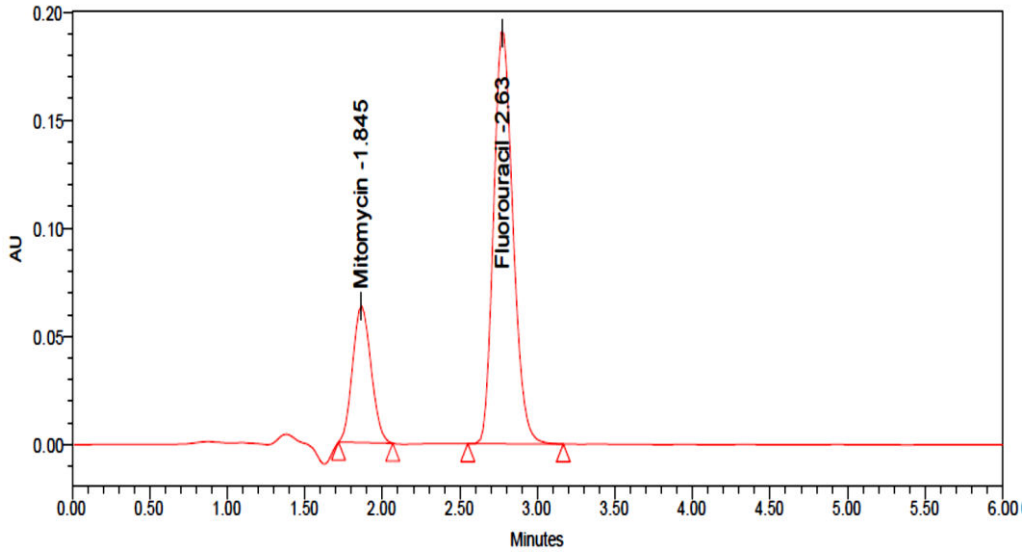

Figure 3.49: Chromatogram of stability 18 Hrs RT

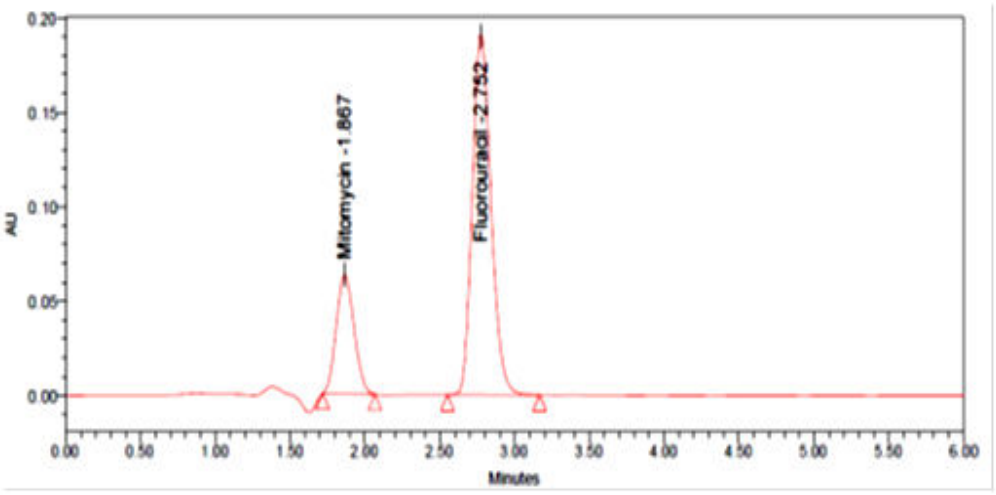

Figure 3.50: Chromatogram of stability 24 Hrs RT

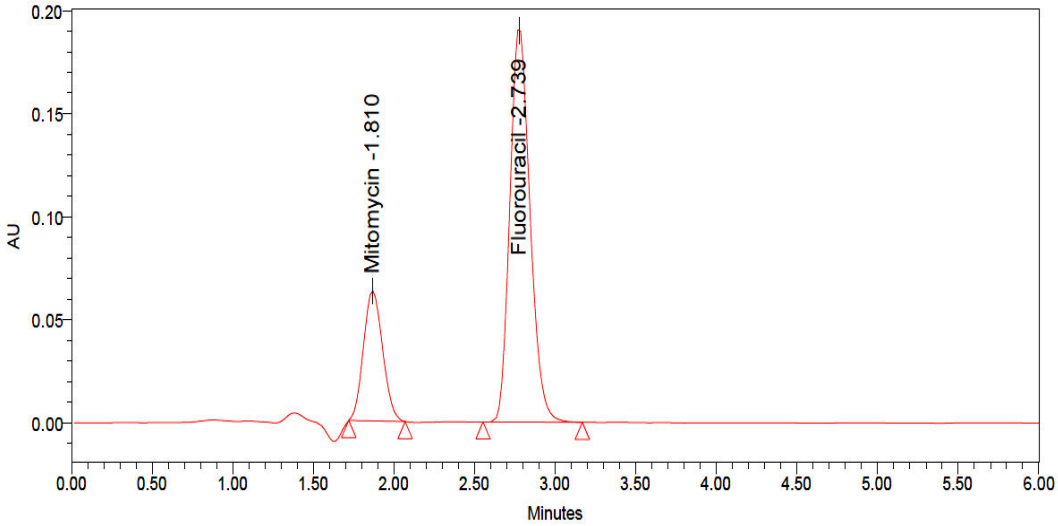

Figure 3.51: Chromatogram of stability 6 Hrs2-8

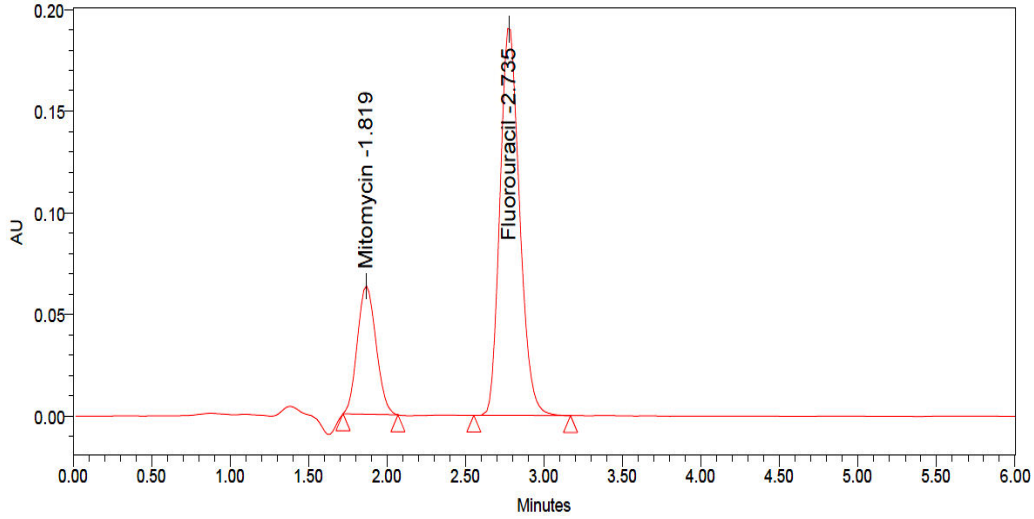

Figure 3.52: Chromatogram of stability 12 Hrs2-8

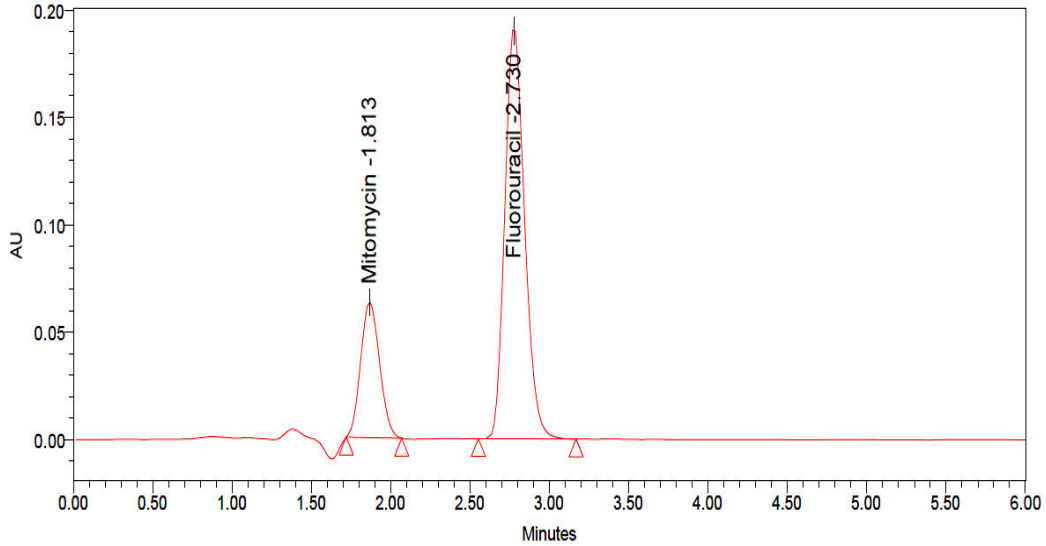

Figure 3.53: Chromatogram of stability 18 Hrs2-8

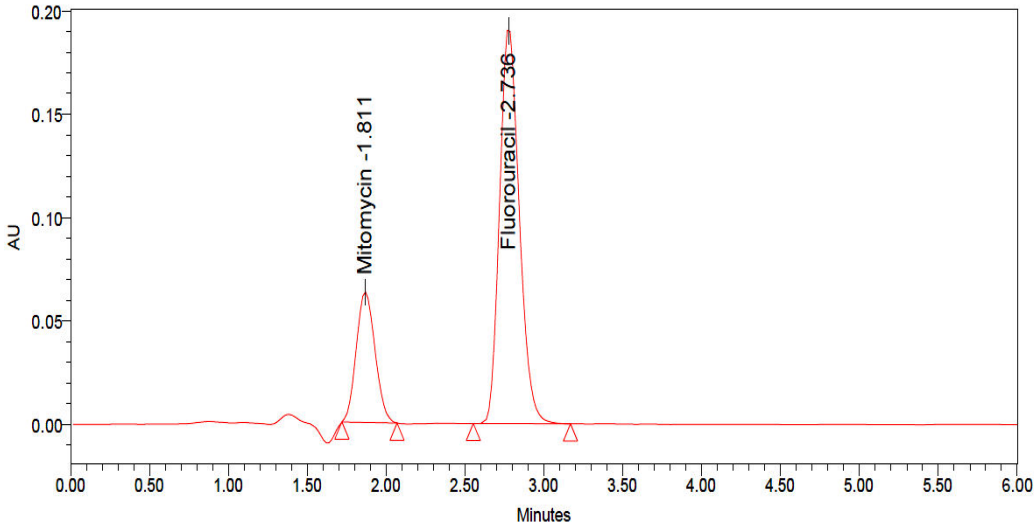

Figure 3.54: Chromatogram of stability 24 Hrs2-8

3.7 CONCLUSION

For quantitative detection of Mitomycin and Fluorouracil, the method is an easy, selective, verified, and well-defined approach using gradient RP-UPLC. There were no degradation products or active pharmaceutical components formed under stress, and peaks were well resolved and separated with an appropriate retention length, suggesting that the recommended technique is quick, simple, feasible, and inexpensive under assay conditions. Stability studies and routine testing of production samples may be performed using this method, which can be utilised for both.

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 ISBN: 978-81-19477-19-7

Chapter - 4

High Pressure Liquid Chromatographic Method for the Determination of Mobocertinib in Pharmaceutical Dosage form and Study of its Degradation

4.1.1 Drug Profile:

[1–2]Exkivity, a brand of mobocertinib, is used to treat non-small cell lung cancer. Aside from diarrhoea**[3,4]**, nausea**[5]**, stomatitis**[6]**, vomiting**[7],** reduced appetite**[8]**, and paronychia**[9]**, the most prevalent adverse effects include dry skin**[10]**, weariness**[11,12]**, and musculoskeletal discomfort **[13,14]**. It is a small molecule tyrosine kinase inhibitor **[16]**. The epidermal growth factor receptor (EGFR) **[17]**, which has alterations in the exon 20 region **[18]**, is its molecular target. EGFR Exon20 insertion mutations are the initial target for this first-in-class oral therapy. Non-small cell lung cancer (NSCLC) patients with exon 20 insertion mutations of the epidermal growth factor receptor (EGFR) are eligible for treatment with mobocertinib if their illness has progressed on or after platinum-based chemotherapy**[19]**. EGF receptors ErbB1**[20]** and HER 2**[21]** are selectively targeted by mobocertinib (human epidermal growth factor receptor 2, ErbB2).

4.1.2 Drug Profileof Mobocertinib [Fig 4.01] and [Table 4.01]:

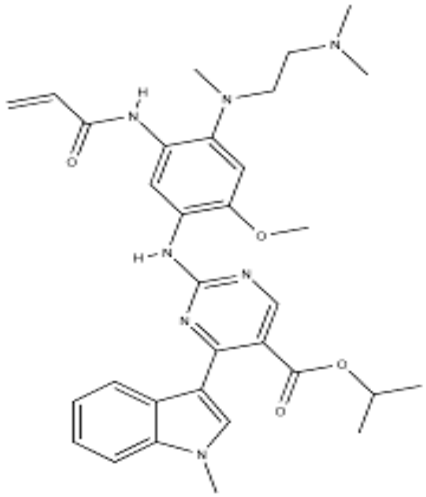

Figure. 4.01: Molecular structure of Mobocertinib

Table. 4.01: Drug profile of Mobocertinib

IUPAC name	propan-2-yl 2-[4-[2-(dimethylamino)ethyl-methylamino]-2-methoxy-5-(prop-2-enoylamino)anilino]-4-(1-methylindol-3-yl)pyrimidine-5-carboxylate
Molecular Formula	$C_{32}H_{39}N_7O_4$
Molecular Weight	585.709g.mol^{-1}
Half life	Based on the drug's accumulation in the body, pharmacokinetic data showed that mobocertinib had a geometric mean effective half-life of 11–17 hours and a median Tmax of 4 hours for daily doses of 20 to 160 mg.
Description	If a patient's cancer has advanced on or after platinum-based chemotherapy, the FDA-approved test indicates that he or she may be suitable for treatment with mobocertinib.

4.1.3 Uses of Mobocertinib

Lung cancer patients use this medicine. kinase inhibitors are the class of medications that include mobocertinib. Cancer cell proliferation is slowed or halted as a result of this treatment. This protein is found in certain cancers, and it binds to this protein.

4.1.4 Mechanism of Action

One of the most effective TKIs, mobocertinib has been developed to target and suppress the mutations that occur in exon 20 of the EGFR gene, which are more common in cancer patients with an EGFR exon 20 insertion in their gene than in those with the wild-type gene.

4.1.5 Side Effects:

Common side effects:

- Rash
- Nausea
- Sores and inflammation in the mouth
- Vomiting
- Decreased appetite
- Nail infection/inflammation

4.2 Literature Survey

Li Bo , Wang Jin, Xinyao Dou, Xinjie Zhang, Xianbei Xue , Qiao Xu , Wenwen Ran 6, Shan Xiong. Development and Validation of a LC-MS/MS Method for Quantification of Mobocertinib (TAK-788) in Plasma and its Application to Pharmacokinetic Study in Rats:

It was the goal of this study to establish an analytical method for testing the presence of mobocertinib in the plasma of rats using high performance liquid chromatography- tandem mass spectrometry (LC-MS). What you need and how to do it: When protein precipitation was utilised on plasma samples, a methanol solution of osimertinib was used as an internal standard (IS). An Inertsil ODS-3 column with an inner diameter of 5 m was utilised for chromatographic separation at a temperature of 40°C. The mobile phase consisted of a gradient of water and methanol pumped at a rate of 0.5 mL/min. To detect the mobocertinib and osimertinib mass transitions, mass spectrometry was performed in the SRM mode using a positive electrospray technique. Selectivity, linearity, accuracy and precision, extraction recovery and matrix effect, stability and carryover, as well as selectivity are some of the bioanalytical technique validation recommendations (FDA, 2018). Oral gavage was used to give mobocertinib at doses of 2 mg/kg, 6 mg/kg, and 18 mg/kg. Plasma samples from 18 rats were collected and examined for a total of 216 samples. The findings showed good linearity in the range of 1-1000 ng/mL (R2 = 0.9957). The precision ranged from 5.49 to 10.46 percent, while the intra-batch accuracy ranged from 94.65 to 102.09%. From 7.54 to 10.13 percent, the inter-batch accuracy varied between 97% and 102%. Extraction recovery and matrix factor were effective in mobocertinib'sbioanalysis. As a side benefit, the researchers noticed that mobocertinib stayed stable under the test conditions. pharmacokinetics of mobocertinib were linear in rats at oral doses ranging from 2.0-18.0 mg/kg. The newly created and validated approach was shown to be beneficial in pharmacokinetic studies in rats when mobocertinib doses of 2, 6, and 18 mg/kg were delivered.

Francois Gonzalvez, Sylvie Vincent, Theresa E. et al: EGFR Inhibitor for Non-Small Cell Lung Cancer Exon 20 Insertion Mutants TKIs approved to treat NSCLC do not work on some of the most frequent non-small cell lung cancer (NSCLC) drivers (TKI). Mobocertinib (TAK-788), a wholly new EGFR TKI, has been created to address the limitations of existing drugs targeting EGFR-mutated NSCLC, specifically tailored to block oncogenic variants of the EGFR gene with selectivity over wild-type EGFR. A broad spectrum of EGFRex20ins mutations, both in vitro and in vivo, were shown to be responsive to mobocertinib. When used in xenografts and orthotopic animal models, mobocertinib

efficiently reduced Ex20ins-driven cell lines better than any licenced EGFR TKI could. The clinical development of mobocertinib for EGFRex20ins-mutated NSCLC is supported by these data.

4.3 Experimental

4.3.1 Chemical and Reagents:

Acetonitrile, Ortho phosphoric acid (OPA), and water were acquired from Merck (India) Ltd., Mumbai, India (HPLC grade). A reference standard API for Mobocertinib was given by Glenmark Pharmaceutical Private Ltd., Andheri (E), Mumbai, India.

4.3.2 Instrumentation:

We used a quaternary pump and a PDA with an e-2695 chromatographic system and a PDA 2996 detector. The chromatographic data was analysed using Empower software version 2.0.

4.3.3 Preparation of Standard Solution

The diluent was used to dilute the solution to the appropriate volume after 60 mg of Mobocertinib working standard was added to 100 ml of the flask. Make a 50 ml solution by diluting 5 ml of the produced solution using dilution diluents.

4.3.4 Preparation of Sample Solution

Mobocertinib sample (138 mg) is mixed with 70 ml of diluents and sonicated to dissolve it, then centrifuged. Diluents are then added to the mark in the last 30 ml. Mix well with extra diluents to dilute the sample solution.

4.4 Method Development

4.4.1Analytical Method Development:

Mobocertinib analysis using RP-HPLC has been attempted in the proposed project and has been shown to be accurate.

4.4.2 Method Development Parameters:

Selection of following parameters in method development is very important.

- Mode of chromatography
- Wavelength
- Column
- Movable phase composition
- Solvent delivery system
- Rate of flow
- Volume of injection

4.4.2.1 Selection of Mode of Chromatography:

Selected mode of chromatography	:	Reversed phase chromatography
Basis of selection	:	polarity of the molecule
Reason for selection	:	As Mobocertinibis polar molecule it elutes at faster along with mobile phase

4.4.2.2 Detector Wavelength Selection:

In order to complete the analysis, it is necessary that the detector wavelength be selected correctly. The standard API is prepared and injected into the chromatographic system utilising PDA detector and wavelength to determine the exact wavelength.

Selected wave length: 224 nm

Basis for selection: Maximum absorbance of analytes and impurities

Reason for selection: Mobocertinibhaving maximum absorbance 224 nm.

4.4.2.3 Selection of Column:
Column selected: X-Bridge phenyl Column(150x4.6mm, 3.5 µ)

Basis for selection: Based on the polarity, and chemical differences among analytics

Reason for selection: It has good physiochemical surface qualities and is compatible with many different organic solvents, as well as an extensive variety of bonding chemistries.

4.4.2.4 Selection of the mobile phase composition and of the buffer:
To determine peak symmetries and separation, buffer and buffer intensity are critical factors. Peak tailings during chromatography can change the ionic form if the appropriate buffer strength is not used to cover the column injection load.

Mobile phase preparation:

Solution A: Acetonitrile

Solution B: 0.1percent Orthophosphoric acid

4.4.2.5 Selection of the rate of flow
Even in reverse phase separation for the resolution of tiny molecules, flow rate is cited as a crucial element. The flow rate used to load the sample solution is very critical in large-scale inversion phase chromatography, although it is not crucial for analytical research. Dynamic binding capacity might vary based on the flow rate utilised to load the sample. It is necessary to assess the dynamic binding capacity while increasing the purification scale before determining the optimal flow rate for loading. Based on flow factor, retention duration, column composition, separation impurity, and peak symmetry, a flow rate of 1 ml/min has been chosen in this system.

4.4.2.6 Selection of injection volume:
For API estimate, a volume of injection of 10 to 20 l is often advised. Extraction has been a problem thus the test concentration may be kept low and injection volume can be increased to 50 l. Be careful not to overflow the specified column volume, however. Mobocertinib is injected in a volume of 10 l using this technique.

4.4.2.7 Trials in optimization of chromatographic condition:

Trial-1 [Fig 4.02]

Movable phase	:	Acetonitrile and 0.1% formic acid (80:20)
Column	:	Symmetry C18 (250x 4.6mm, 5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Wavelength	:	200-400 nm
Observation	:	Extra peak is observed

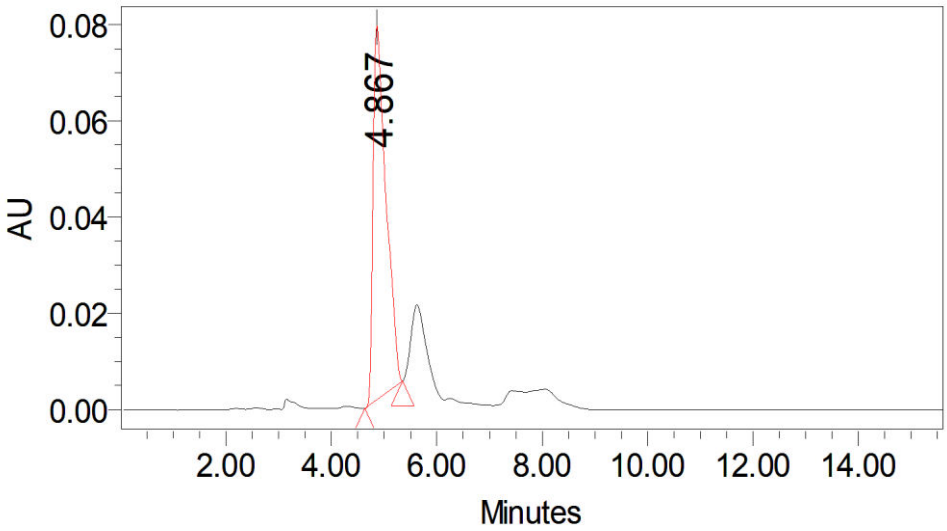

Figure 4.02: chromatogram of trial-1

	Name	RT	Response	% Area	USP Tailing	USP Plate Count
1		**4.867**	**546321**	**100.02**	**4.56**	**8563**

Trial-2 [Fig 4.03]

Movable phase : Acetonitrile and 0.1% formic acid (70:30)

Column : Symmetry C18 (250x 4.6mm, 5µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Wavelength : 224 nm

Observation : System suitability conditions are not within the limit

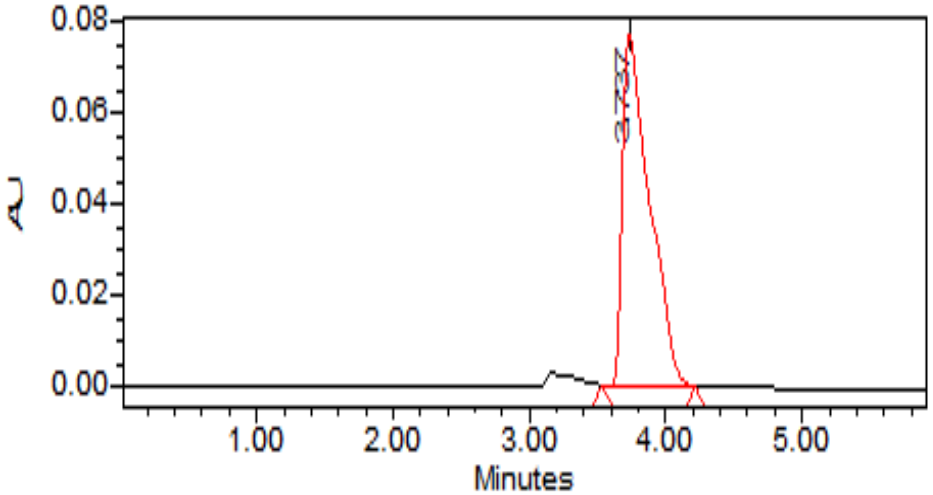

Figure 4.03: Chromatogram of trial-2

	Name	RT	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
		3.737	581263	100.23		2.12	1382

Trial-3: [Fig 4.04]

Movable phase : Acetonitrile and 0.1% formic acid (65:35)

Column : Symmetry C18 (250x 4.6mm, 5µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Wavelength : 224 nm

Observation : Peak height is not within the limit

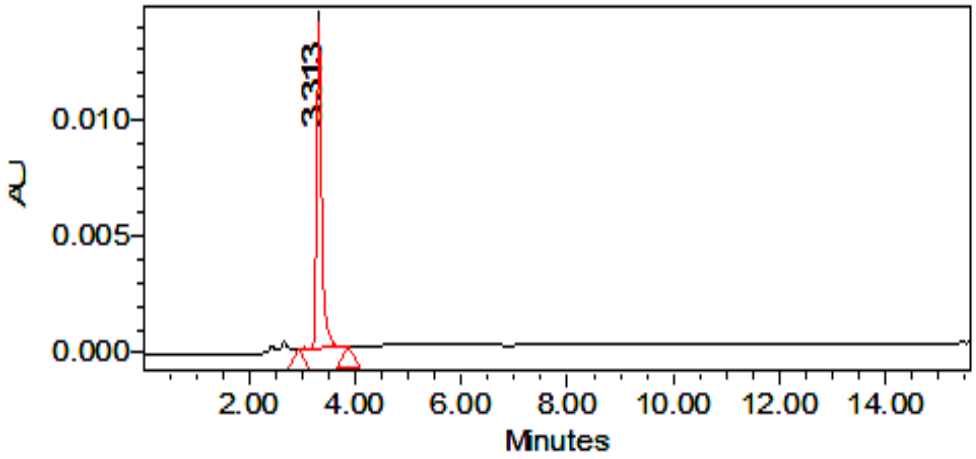

Figure 4.04: Chromatogram of trial-3

	Name	RT	Response	% Area	USP Tailing	USP Plate Count
1		3.313	102854	100.00	1.62	1888

Trial-4 [Fig 4.05]

Movable Phase : Acetonitrile and 0.1% formic acid (60:40)

Column : Symmetry C18 (250x 4.6mm, 5µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Wavelength : 224 nm

Observation : Peak shape is not good and response of the peak is very high

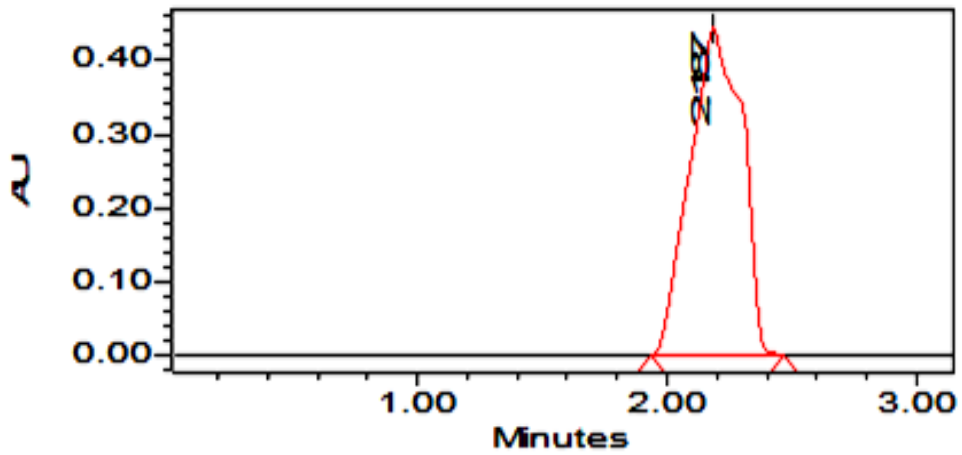

Figure 4.05: Chromatogram of trial-4

	Name	RT	Response	% Area	USP Tailing	USP Plate Count
1		2.187	6429928	100.00	1.58	2452

Trial-5 [Fig 4.06]

Movable phase	:	Acetonitrile and 0.1% formic acid (40:60)
Column	:	Symmetry C18 (250x 4.6mm, 5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Wavelength	:	224 nm
Observation	:	Broad peak is observed

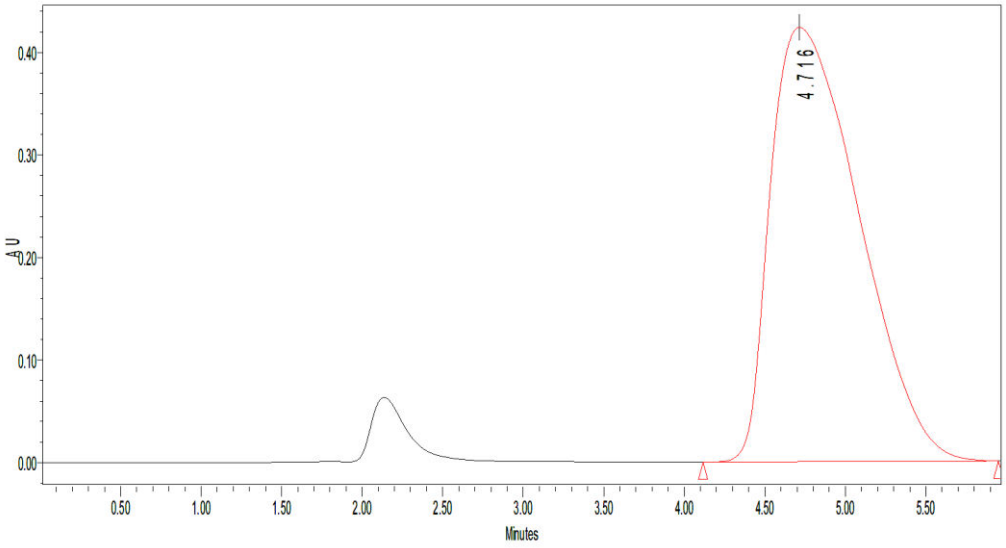

Figure 4.06: Chromatogram of trial-5

	Name	RT	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		4.718	16217023	100.00		1.72	2055

Trial-6 [Fig 4.07]

Movable phase : Acetonitrile and 0.1% formic acid (60:40)

Column : X-Bridge Phenyl Column(150x4.6mm, 3.5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Wavelength : 224 nm

Observation : Less retention time is observed.

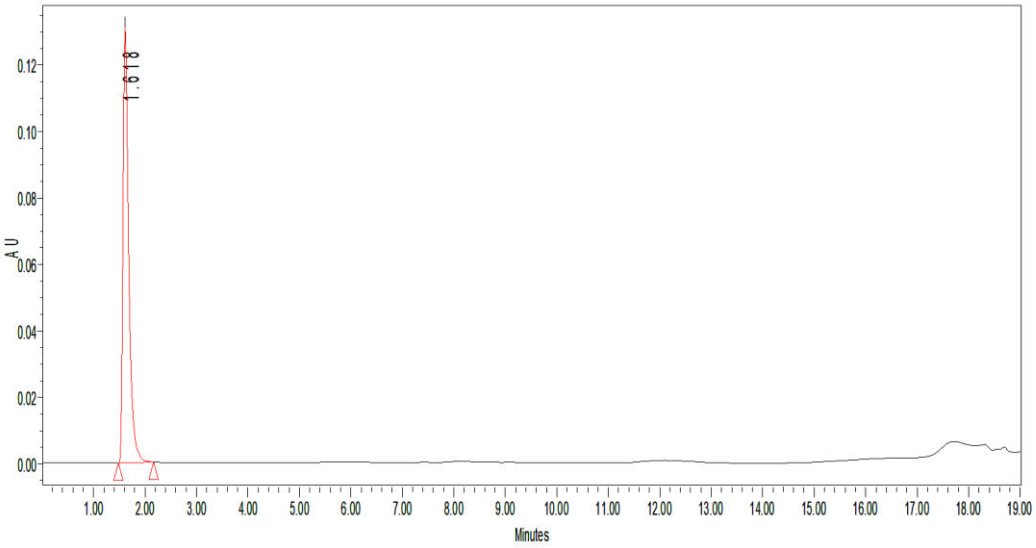

Figure 4.07: Chromatogram of trial-6

	Name	RT	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.618	1008168	100.00		1.58	2088

Trial-7 [Fig 4.08]

Movable phase : Acetonitrile: 0.1% formic acid (70:30)

Column : X-Bridge Phenyl Column(150x4.6mm, 3.5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Wavelength : 224 nm

Observation : Plate count is not within the limit

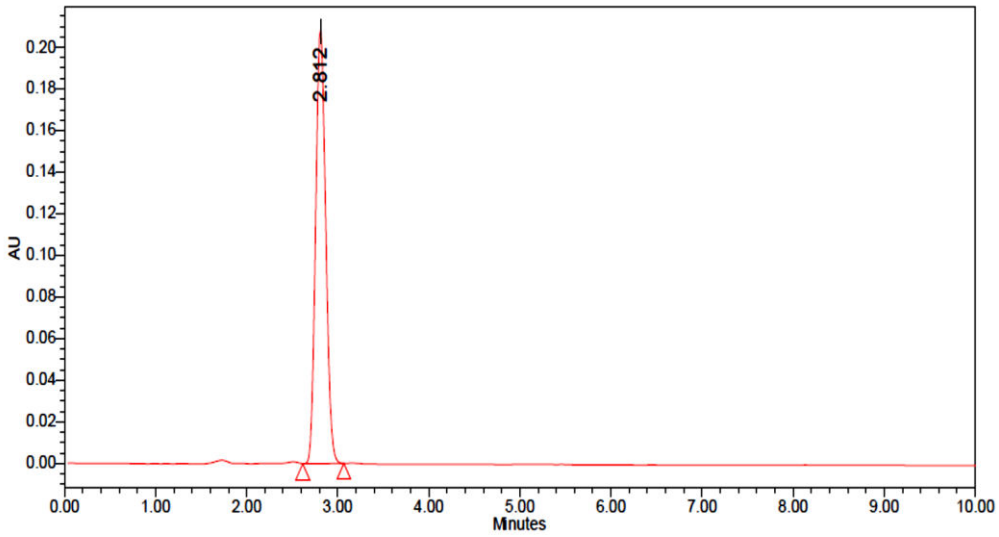

Figure 4.08: Chromatogram of trial-7

S.No	Name	RT	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		2.812	972544	100.00		1.99	1878

Trial-8 [Fig 4.09]

Movable phase : Acetonitrile: 0.1% OPA (50:50)

Column : X-Bridge Phenyl Column(150x4.6mm, 3.5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Wavelength : 224 nm

Observation : Peak shape is not good

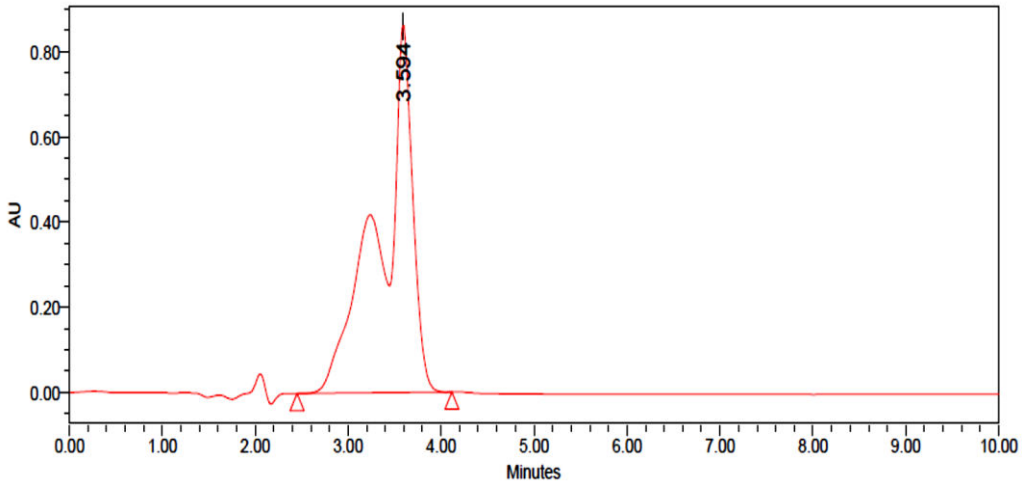

Figure 4.09: Chromatogram of trial-8

	Name	Retention Time	Response	USP Resolution	USP Tailing	USP Plate Count
1		3.594	21204282		0.66	2756

Trial-9 [Fig 4.10]

Movable phase : Acetonitrile: 0.1% OPA (55:45)

Column : X-Bridge Phenyl Column(150x4.6mm, 3.5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Wavelength : 224nm

Observation : Base line is not sufficient

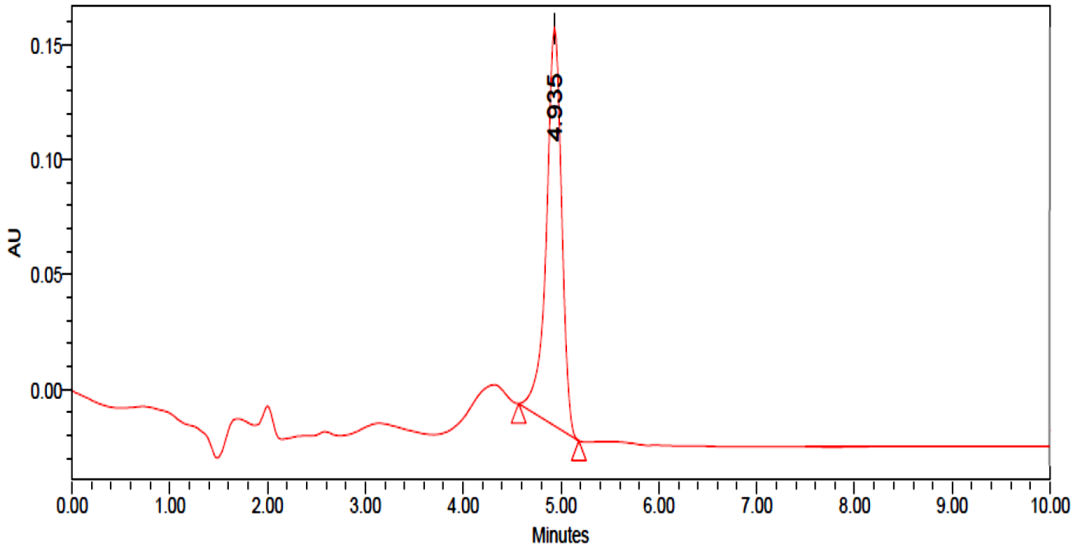

Figure 4.10: Chromatogram of trial-9

	Name	Retention Time	Response	USP Resolution	USP Tailing	USP Plate Count
1		4.935	1878175		0.90	5130

Trial-10 [Fig 4.11]

Movable phase : Acetonitrile: 0.1% OPA (60:40)

Column : X-Bridge Phenyl Column(150x4.6mm, 3.5 µ)

Rate of flow : 1 ml/min

Volume of injection : 10µl

Wavelength : 224 nm

Observation : This method is suitable for validation

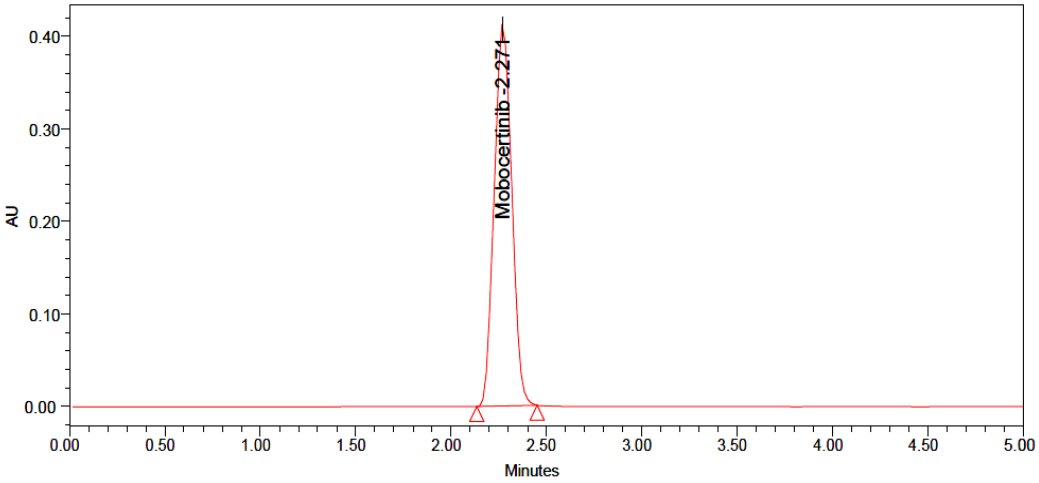

Figure 4.11: Chromatogram of trial-10

	Name	RT	Response	USP tailing	USP plate count	USP resolution
1	Mobocertinib	2.271	3854273	1.12	6851	

4.4.3 Optimized method: [Table 4.02]

Table 4.02: Optimized method chromatographic conditions

S.NO	Parameter	Chromatographic condition
1	Movable phase	Acetonitrile: 0.1% OPA (60:40)
2	Column	X-Bridge Phenyl Column(150x4.6mm, 3.5 µ)
3	Flow rate	1ml/min
4	Column temperature	Ambient temperature
5	Wavelength	224 nm
6	Volume of injection	10µl
7	Period of run	5 min
8	Retention time	2.271

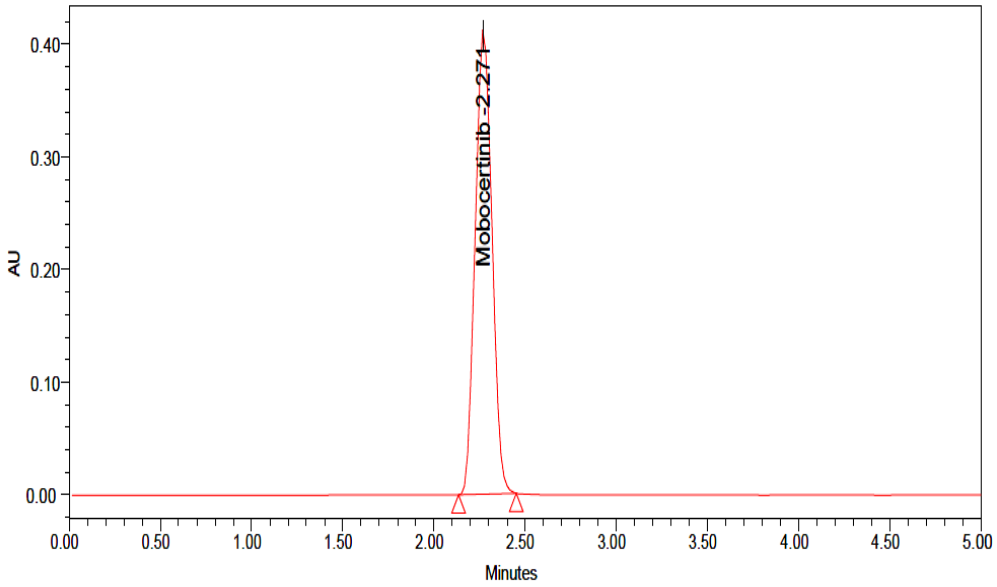

Figure 4.12: Chromatogram of standard

[Fig 4.12] Shows the chromatogram of Standard

4.5 Validation of Method

For example, ICH Q2 (R1) guidelines were used to evaluate the analytical procedure for characteristics such as device compatibility; accuracy; specificity; and precision; linearity and robustness; limits of detection and limits of quantification; and the ability to detect and quantify a particular substance (LOD and LOQ). **5.6.1**

4.5.1Specificity: [Fig 4.13]

Specificity [**22**] refers to the ability of the analyte to be examined in isolation from other components, such as contaminants or excitementsthat would be expected to be present in the sample solution and norm solution. The blank samples were spiked with mobocertinib.

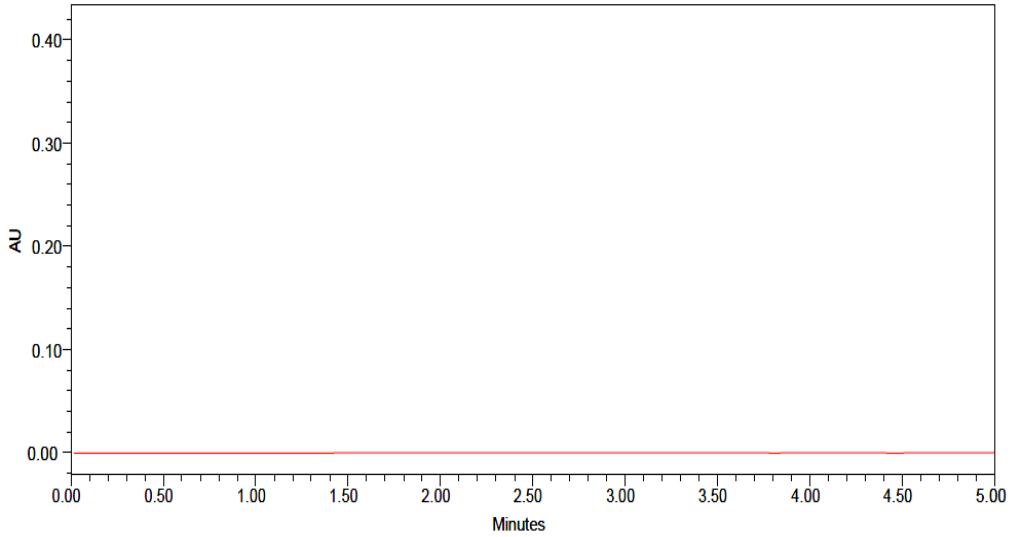

Figure 4.13: Chromatogram of blank

4.5.2 Linearity:

A reference solution of 60 micrograms per millilitre of Mobocertinib was produced to assess the tactic's linearity (100 percent of the targeted level of the assay concentration). To fix the problem, a series of dilutions of the prescribed solutions at concentrations ranging from 10%, 25%, 50%, 100%, 100%, 125%, and 1500% of the intended concentrations was necessary.. The fact that they were pumped makes them ideal for use in drawing calibration curves. The correlation between these two variables is 0.999. Results of the linearity tests and the calibration curve for Mobocertinib can be seen in Table 3 and Figure 4. The values of slope, intercept, and correlation coefficient were supplied by the linearity calculation sheet.

Procedure:

The chromatographic technique may be used to measure the peak area of each degree. Coefficients of correlation may be calculated by drawing a line graph of peak area (Y-axis peak area) and concentration (X-axis concentration). Results of linearity are in **[table 4.03]**, calibration plot is in **[fig 4.14]** and also **[Fig. 4.15-4.20]**shows 10%, 25%, 50%, 100%, 125% & 150% linearity chromatograms.

4.5.3 Range:

To put it another way: The range of analytic approaches encompasses the gap between the top and lower levels of analysis.

Inclusion Criteria:

Not less than 0.9999 is required for a correlation coefficient.

Table 4.03: Results of linearity

S. No	Mobocertinib	
	Concentration (µg/mL)	**Response**
1	6.00	396971
2	15.00	931459
3	30.00	1947935
4	60.00	3819390
5	75.00	4763458
6	90.00	5746340
CC	0.99996	
Slope	63682.47	
Intercept	4170.18	

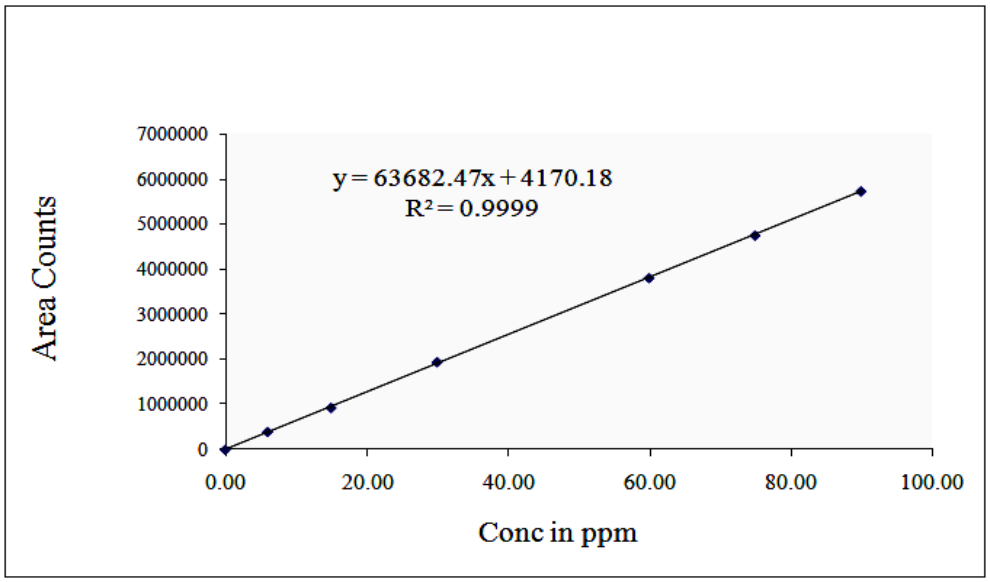

Figure 4.14: Calibration plot of Mobocertinib

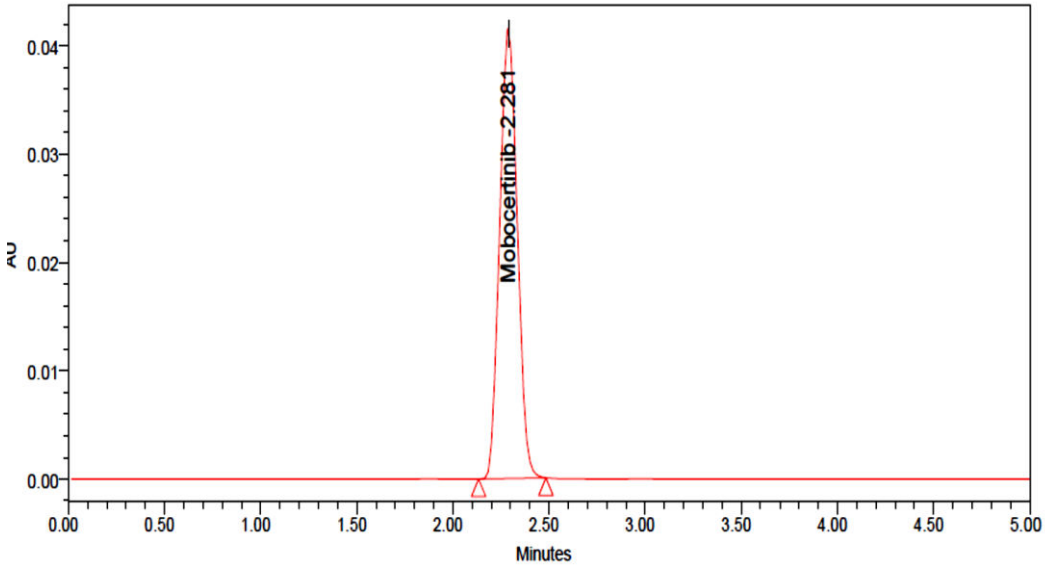

Figure 4.15: 10 percent linearity chromatogram

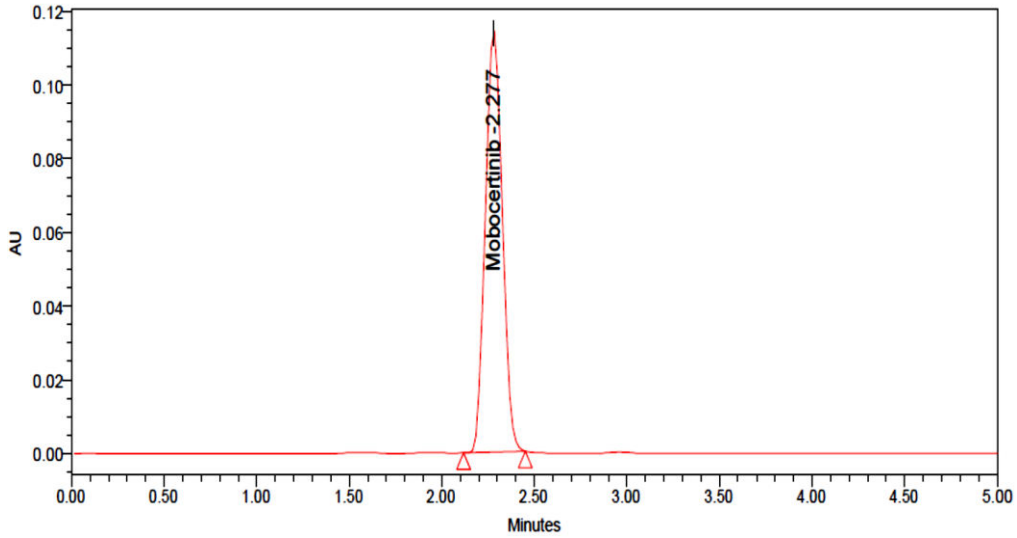

Figure 4.16: 25 percent linearity chromatogram

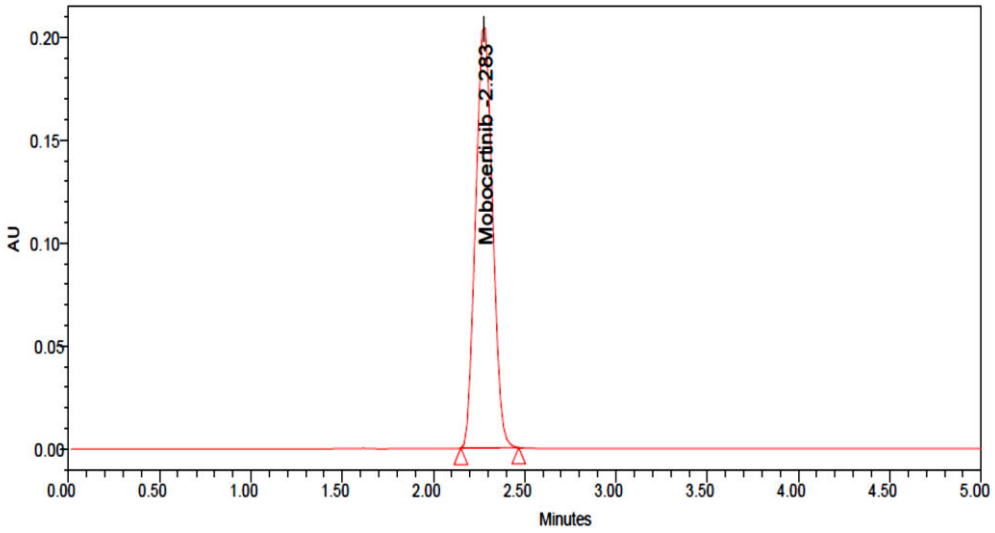

Figure 4.17: 50 percent linearity chromatogram

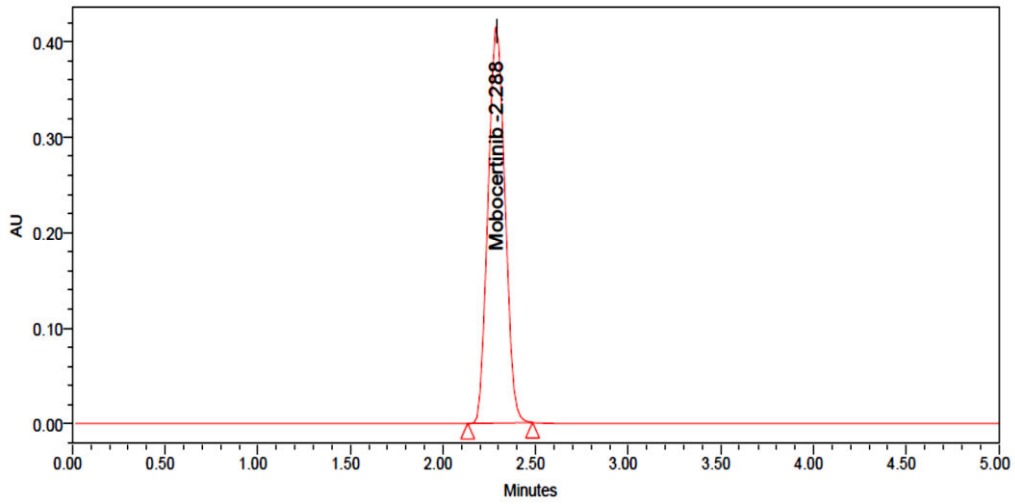

Figure 4.18: 100 percent linearity chromatogram

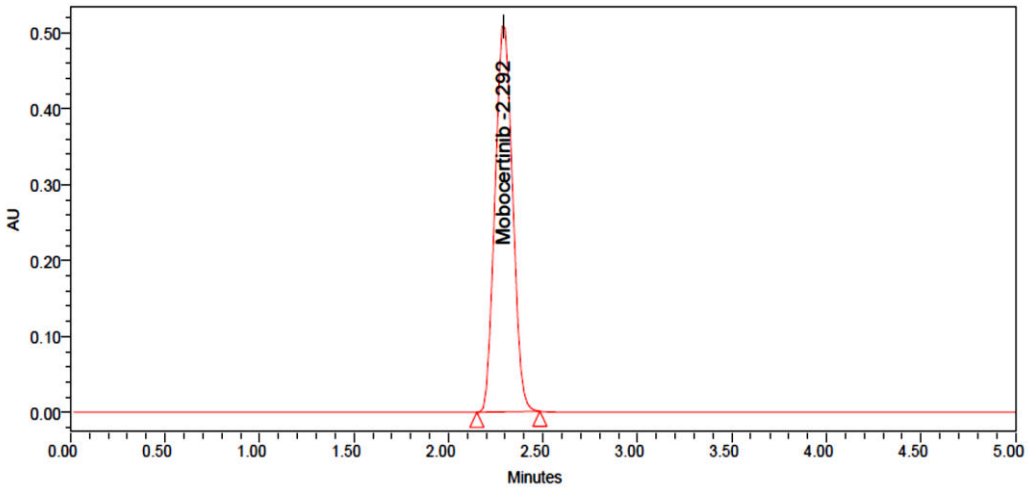

Figure 4.19: 125 percent linearity chromatogram

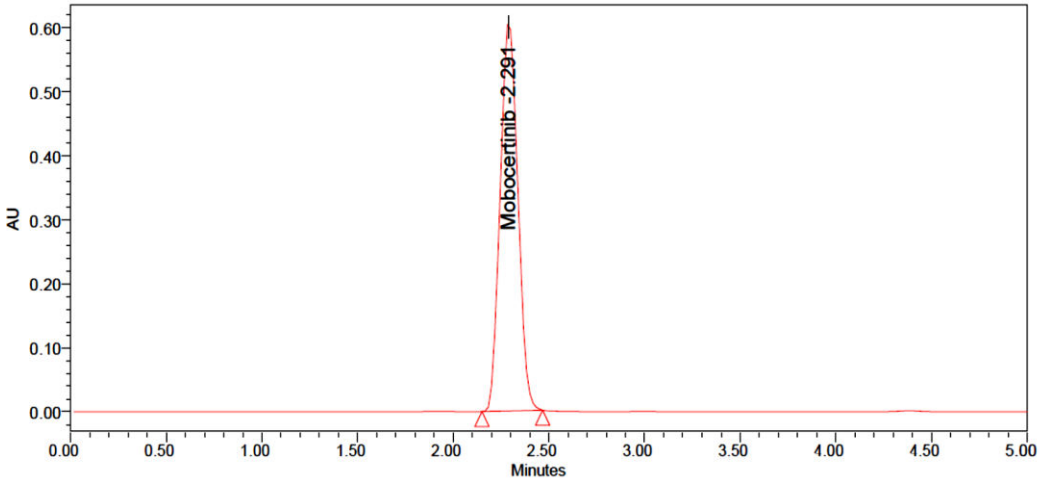

Figure 4.20: 150 percent linearity chromatogram

4.5.4 Accuracy:

50 percent solution preparation (with respect to the concentration of the target assay)
Sonicate30 mg of Mobocertinib in a clean and dry vaccum flask to dissolve it completely and bring the diluents level up to the diluents level, then transfer to a new vacuum flask.

Dose the volumetric flask with 5 ml of the aforementioned solution and dilute it to the desired concentration using the diluents provided. Mobocertinib at a concentration of thirty parts per million

100 percent solution preparation (with respect to target assay concentration)
Diluents should be utilized for dissolving 60 mg of Mobocertinib in a clean and dry vacuum flask. Sonicating the mixture will ensure that it is completely dissolved.

In a 50ml vacuum flask, pipette 5ml of the above mentioned solution and dilute it with diluents to the desired volume. This is the Mobocertinib60ppm dose.

150 percent solution preparation (with respect to target assay concentration)
In a clean and dry vacuum flask, accurately weigh and transfer 90mg of Mobocertinib. Then, add diluents and sonicate to dissolve thoroughly and bring the diluents level to the desired level.

Take 5ml of the aforesaid stock solution and dilute it to your desired concentration in a 50ml vacuum flask using diluents. Mobocertinib (90ppm of Mobocertinib)

Procedure:

The standard solution, 50 percent accuracy, 100 percent accuracy, and 150 percent accuracy solutions should be injected at the same time.**[Table 4.04]** shows the accuracy [**23**] results of Mobocertinib and **[Fig. 4.21, 4.22&4.23]** shows the 50%, 100% and 150% accuracy chromatograms.

Acceptance Criteria:

The rate of recovery for each stage should be between 98-102 percent

Table 4.04: Accuracy results of Mobocertinib

Accuracy	Amount of Mobocertinib	% Recovery
50%	30	99.7
100%	60	99.6
150%	90	100.3

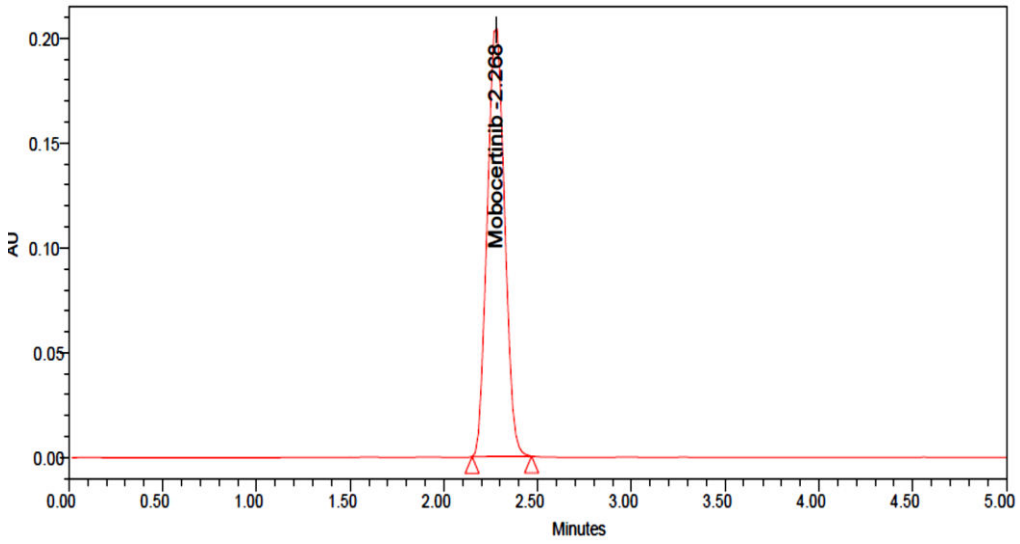

Figure 4.21: 50 percent accuracy chromatogram

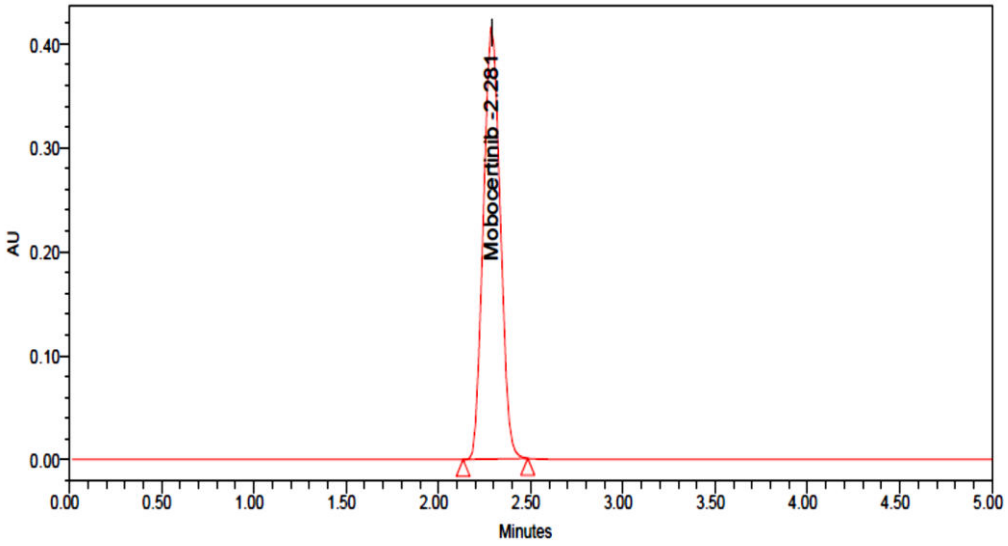

Figure 4.22: 100 percent accuracy chromatogram

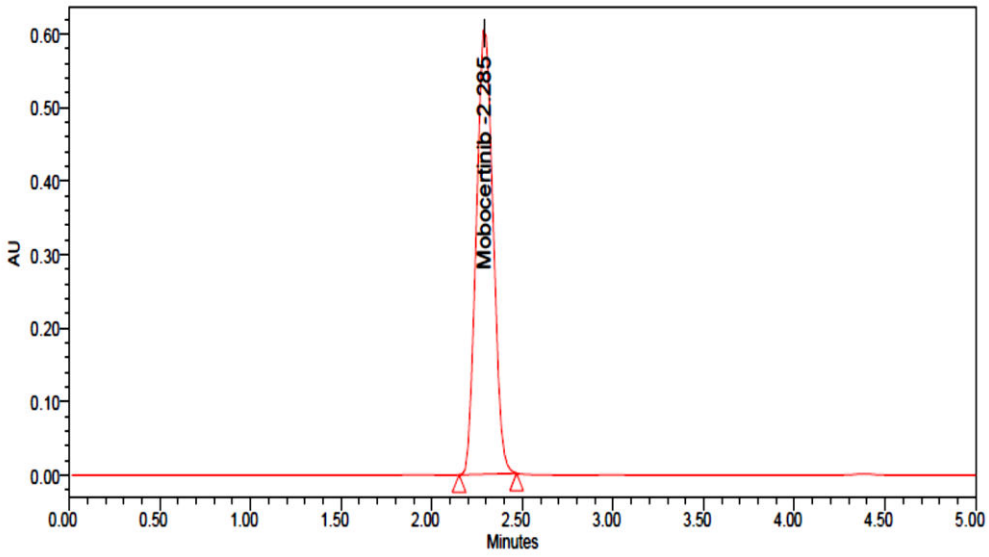

Figure 4.23: 150 percent accuracy Chromatogram

4.5.5 Precision:

In an analytical procedure, the rate at which repeated homogenous samplings provide similar results is a measure of accuracy. Mobocertinib (60ppm) was spiked into six different injections to ensure the accuracy of the injection technique. System precision results are shown in **[Table 4.05]**andChromatograms are shown in **[Fig. 4.24-4.29].**

Table 4.05: Results of system precision

Parameter	Mobocertinib
Theoretical plate count	6851
Tailing factor	1.12
Resolution	-
Retention time	2.271 min

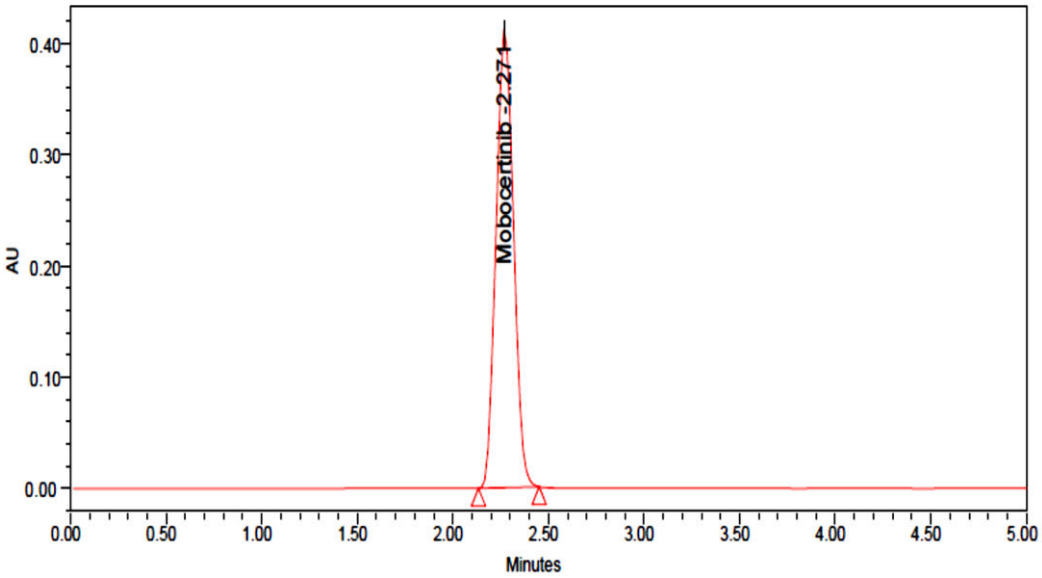

Figure 4.24: Chromatogram of system precision-1

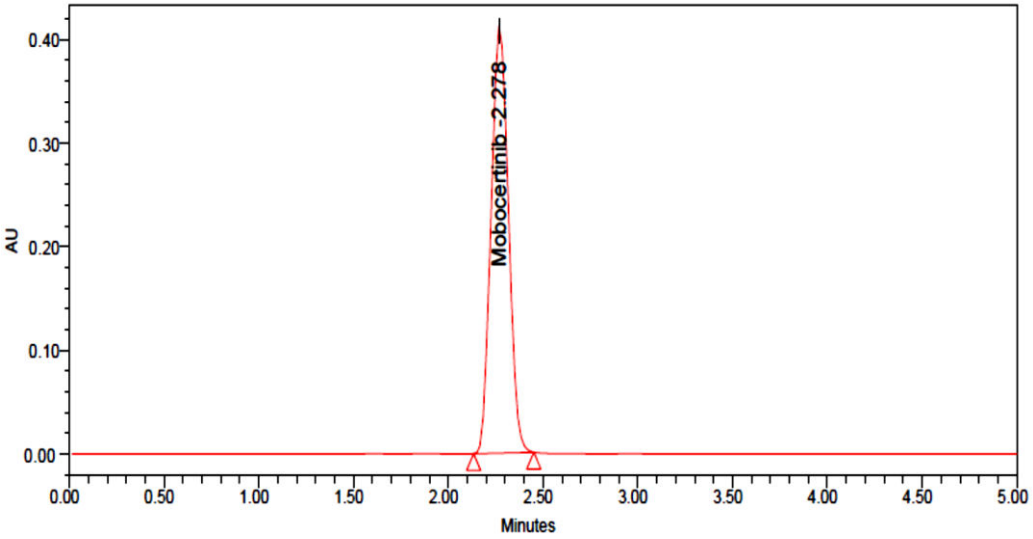

Figure 4.25: Chromatogram of system precision-2

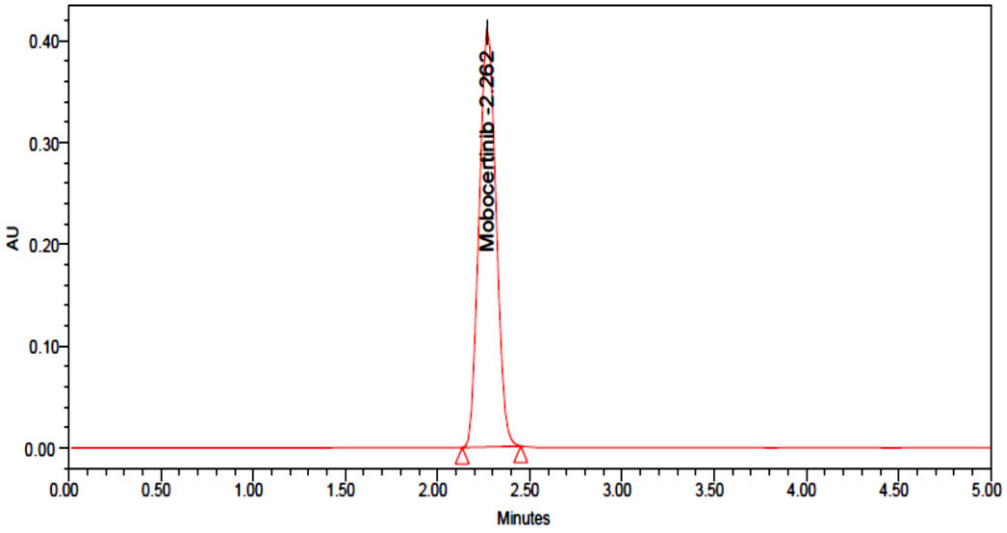

Figure 4.26: Chromatogram of system precision-3

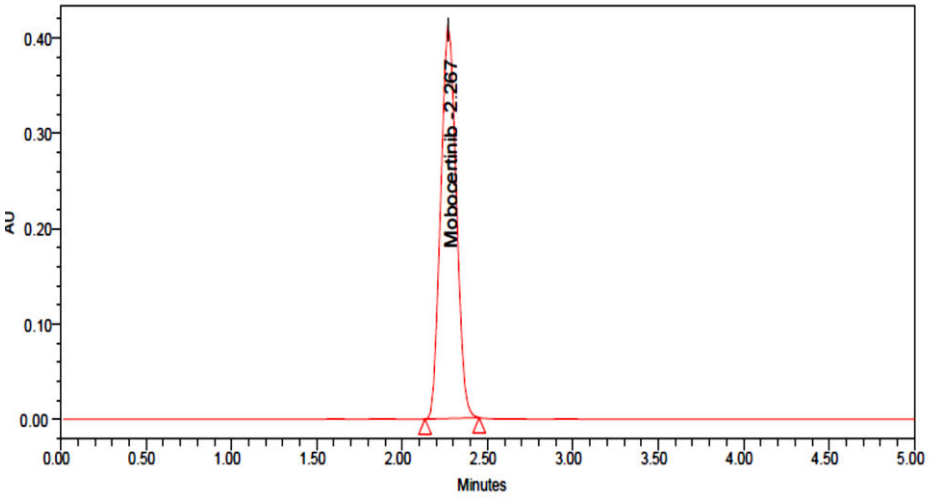

Figure 4.27: Chromatogram of system precision-4

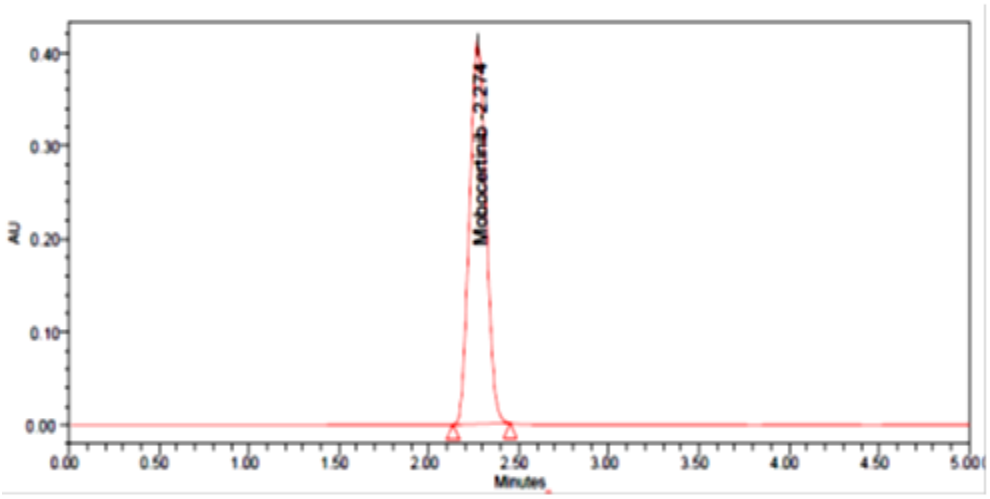

Figure 4.28: Chromatogram of system precision-5

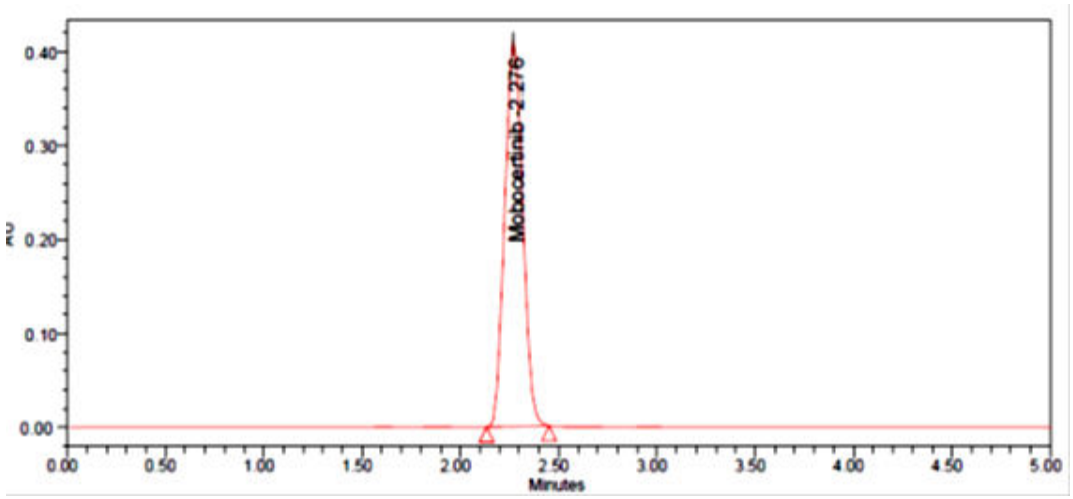

Figure 4.29: Chromatogram of system precision-6

Method precision: [Table 4.06 and Fig 4.30]

Table 4.06: Results of method precision

S. No.	Response of Mobocertinib
1	3832418
2	3801582
3	3814570
4	3828269
5	3817321
6	3851651
Mean	3824301.83
Std. dev	17258.1513
% RSD	0.45

Acceptance criteria

The area six standard injection RSD percentage should be more than 2%.

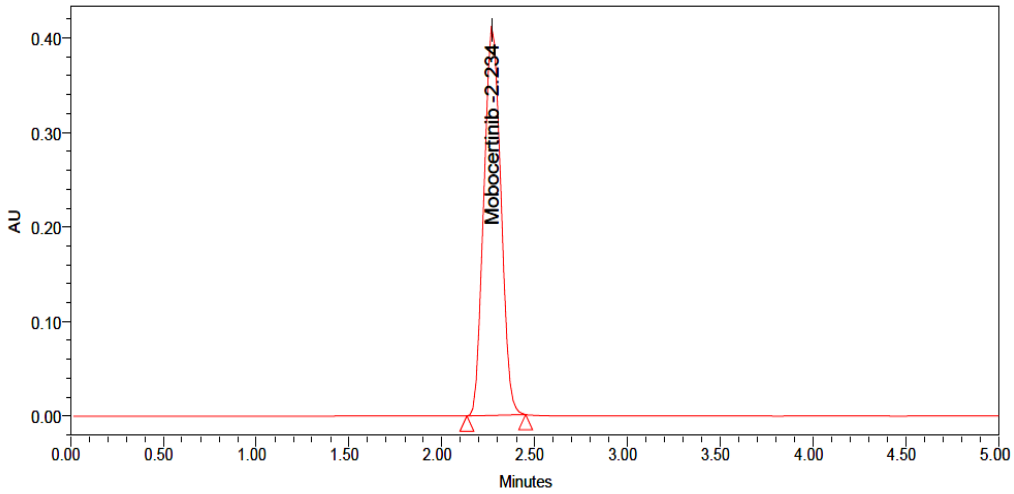

Figure 4.30: Chromatogram of method precision

Intermediate precision: [Table 4.07 and Fig 4.31]

Table 4.07: Results of intermediate precision

S.No.	Area of Mobocertinib	% RSD
1	3858214	0.79
2	3813206	
3	3815427	
4	3876539	
5	3804362	
6	3861488	

Acceptance Criteria

The RSD percentage for the six normal injection results should not be more than 2%.

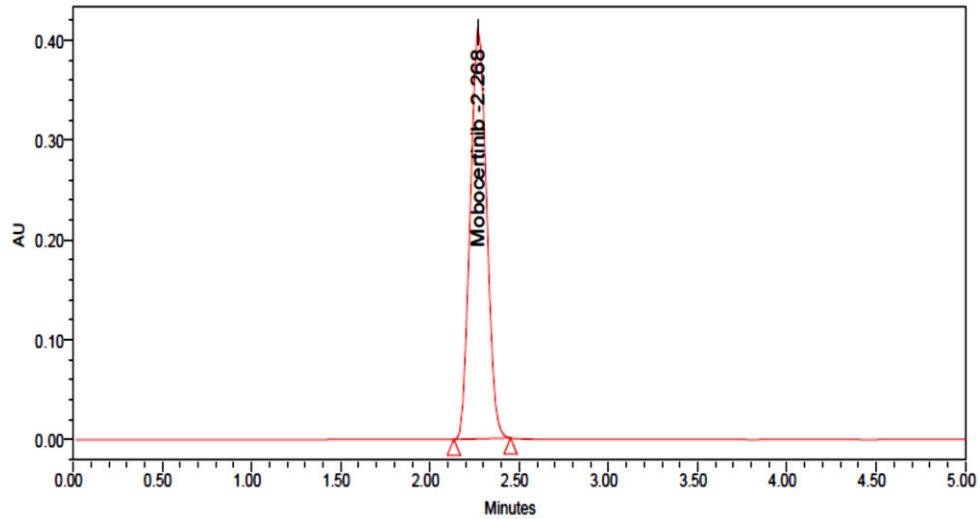

Figure 4.31: Chromatogram of intermediate precision

4.5.6 Limit of detection (LOD) and limit of quantification (LOQ): [Fig 4.32-4.33]

Only at the lowest concentrations of an analyte can its presence be reliably detected and quantified. The LOD concentration was 0.075 g/ml of mobocertinib, with a S/N ratio of 5. At 0.24 g/ml, Mobocertinib had an S/N value of 23 in the LOQ sample. It is possible to measure the S/N ratio.

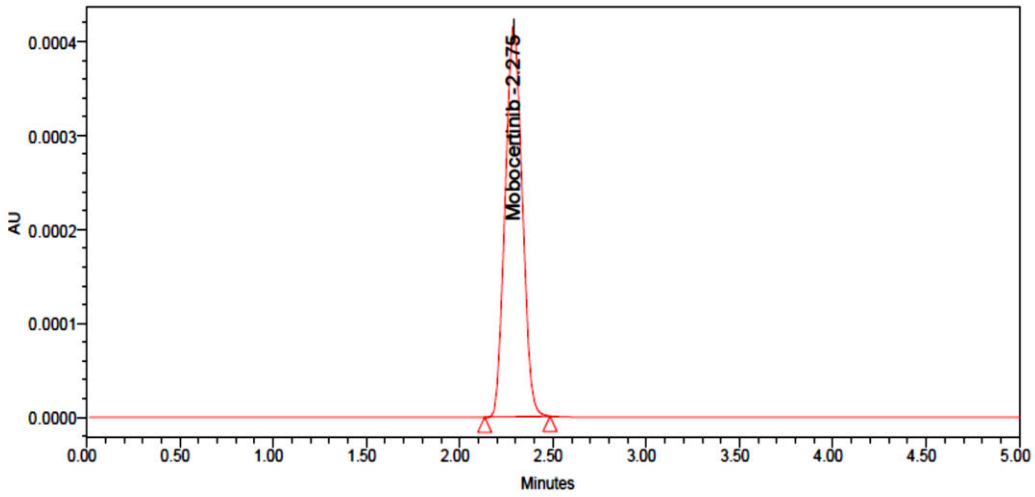

Figure 4.32: LOD chromatogram

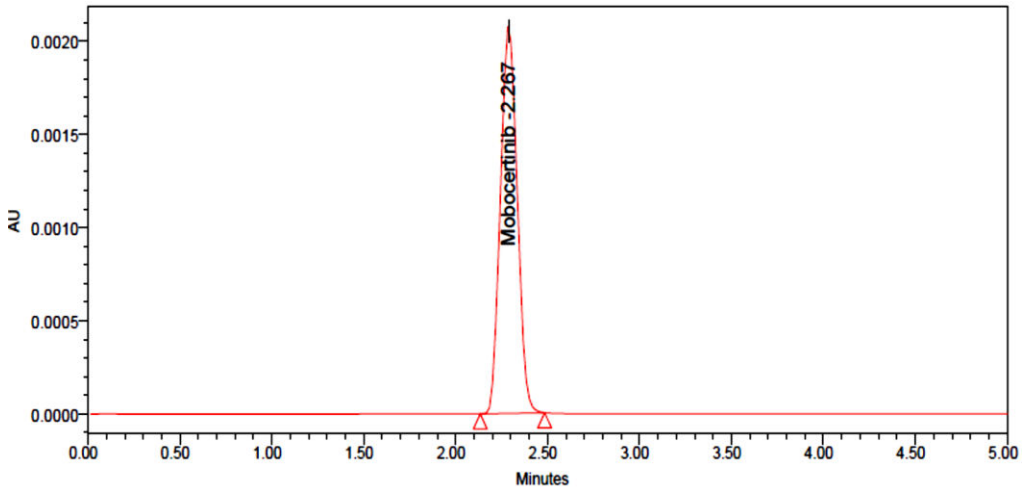

Figure 4.33: LOQ chromatogram

4.5.7 Robustness:

Flow rate, mobile phase organic percentage, and other experimental parameters were all included into the study. No major changes were made to the active pharmaceutical components and contaminants, and the retention time, plate count, and tailing factor were not significantly changed either. Thus, this method proved to be effective. Mobocertinib's robustness findings are shown in **Table 4.08. [Fig. 4.34-4.37]** Chromatograms.

Table 4.08: Robustness results of Mobocertinib

Parameter	% RSD of Mobocertinib
Flow (0.8 mL/min)	1.32
Flow (1.2 mL/min)	0.15
Organic phase (54:46)	0.11
Organic phase (66:34)	0.12

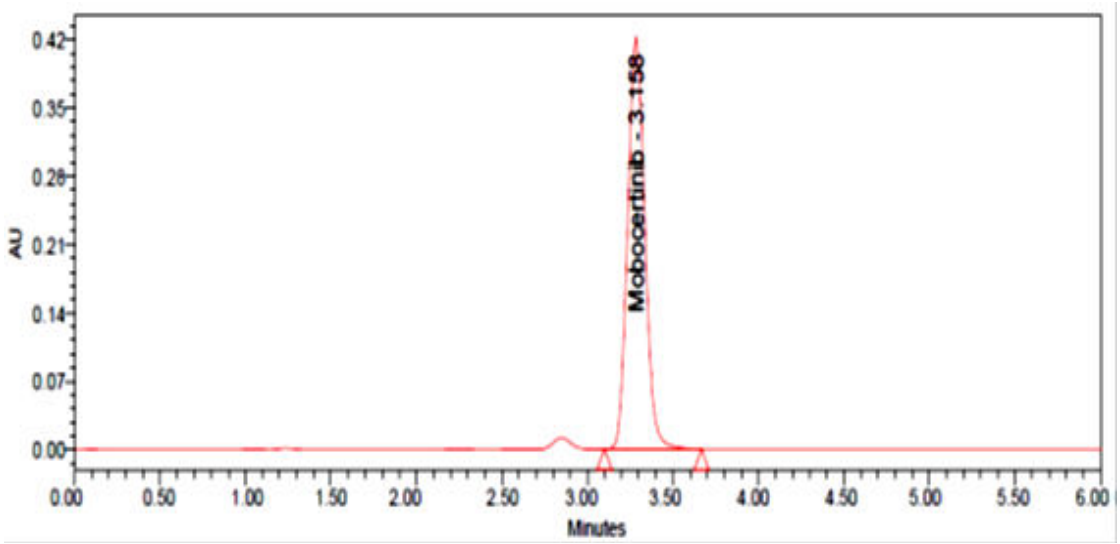

Figure 4.34: Less flow rate chromatogram (0.8ml/min)

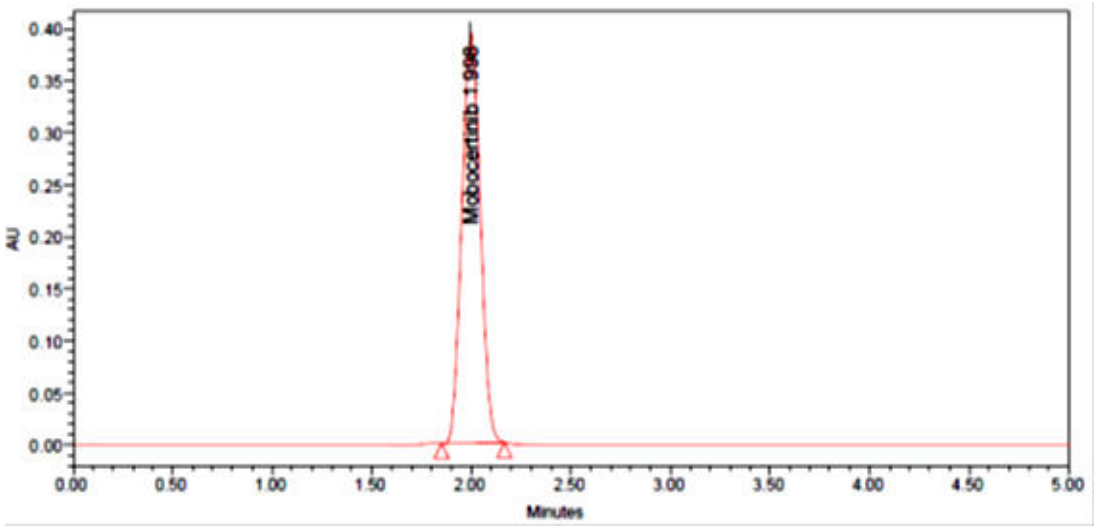

Figure 4.35: More flow rate chromatogram (1.2ml/min)

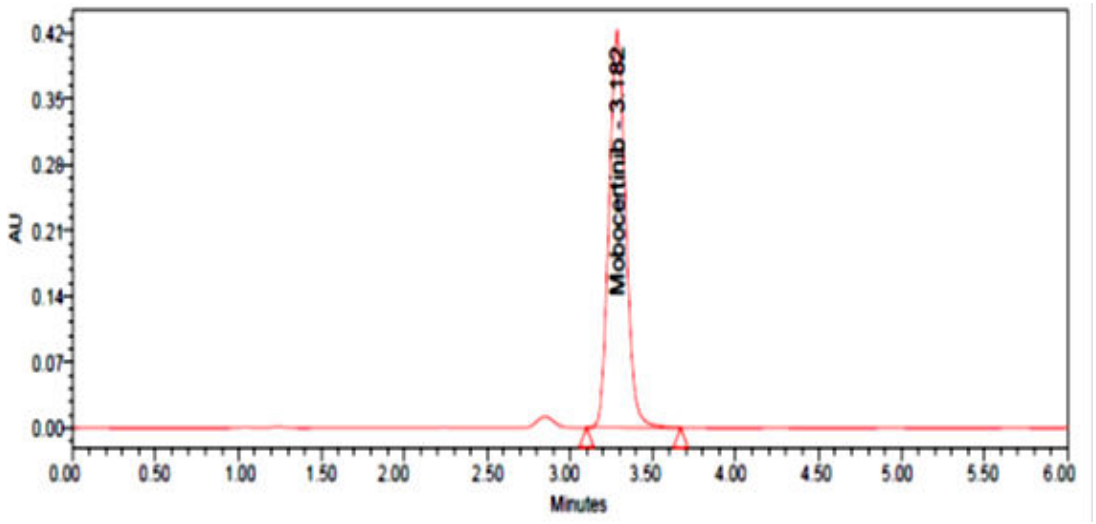

Figure 4.36: Less organic chromatogram

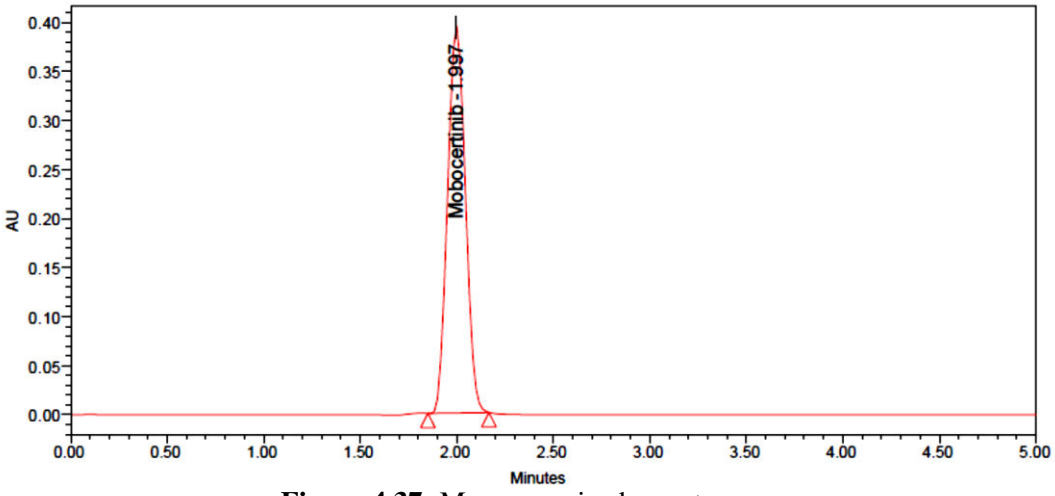

Figure 4.37: More organic chromatogram

4.5.8Forced Degradation Studies:

As a step forward from earlier methods, this methodology allows for both release and stability [**24**] experiments to be conducted simultaneously. Acid, base, oxidation, reduction, and heat degradation are all part of the process of forcing degradation. Finally, it seems that even though certain deteriorated peaks were found, since they are depending on the kind of chromatography utilised, the medications under examination were stable.**[Table 4.09] and [Fig. 4.38-4.44]** Chromatograms from forced degradation.

Acid Degradation

A volumetric flask containing 10 ml of the sample stock solution was used to hold 1 ml of 1N HCl, which was added and allowed to stand for 15 minutes. Make up to the diluent mark by adding 1ml of 1N NaOH after 15 minutes. Syringe filter the solution and inject it into the HPLC.

Alkali Degradation

Sample stock solution was transferred to the volumetric flask, and 1N NaOH was added to the mixture. The combination was let to stand for 15 minutes before being centrifuged at 10,000 rpm for 15 minutes. Adding 1 mL of 1N HCl to raise the solution to a desired concentration may be done after 15 minutes. Filter the solution using a syringe filter before injecting it into the HPLC apparatus.

Peroxide Degradation

Add 1 ml of a 30% hydrogen peroxide solution and dilute with diluents to produce up to 10 ml of sample stock solution. Syringe filter solution, inject into HPLC equipment, and see the results.

Reduction Degradation

Transfer 1 ml of the sample stock solution to a volumetric flask with a capacity of 10 ml, add 1 ml of a 30 percent sodium bisulphate solution, and then dilute with diluents to the necessary concentration. Filter the solution using a syringe filter before injecting it into the HPLC apparatus.

Thermal Degradation

The sample solution was held at 105°C for the duration of the 6-hour baking time. We next used a high-performance liquid chromatography equipment to separate out the resultant sample from the others.

Hydrolysis Degradation

An HPLC water solution of one millilitre was introduced to a volumetric flask that had a capacity of 10 millilitres, followed by the addition of one millilitre of sample stock solution. Filter the solution using a syringe filter before injecting it into the HPLC apparatus.

Table 4.09: Results of forced degradation

Stress Parameter	% Degradation of Mobocertinib
Acid degradation (1N HCl)	14.3
Alkali degradation (1N NaOH)	13.5
Peroxide degradation (30% Peroxide)	16.1
Reduction degradation (30% sodium bi sulphate)	13.4
Thermal (sample, 105°C, 6 Hrs)	4.1
Hydrolysis (1 ml HPLC water)	1.8

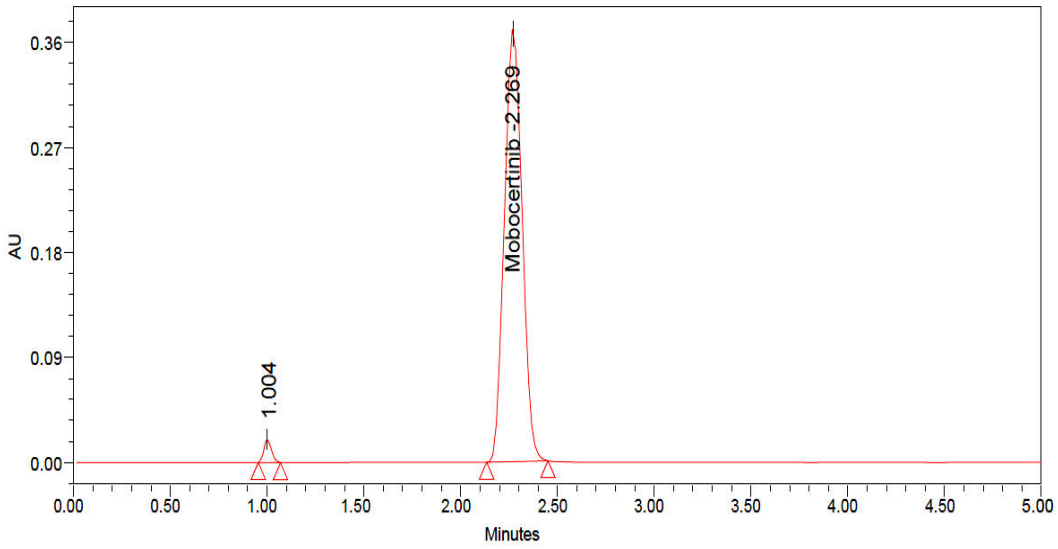

Figure 4.38: Acid degradation chromatogram

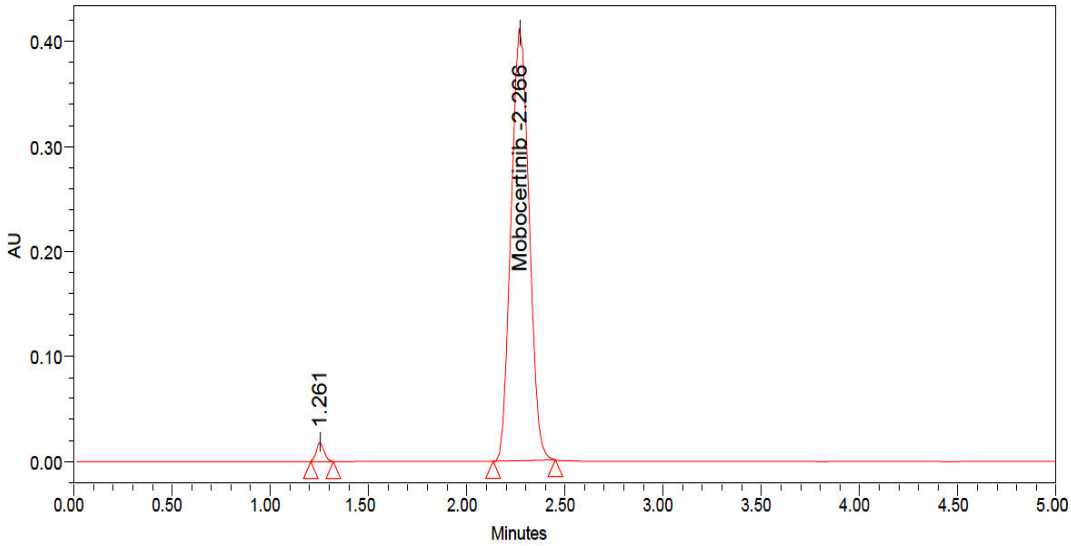

Figure 4.39: Alkali degradation chromatogram

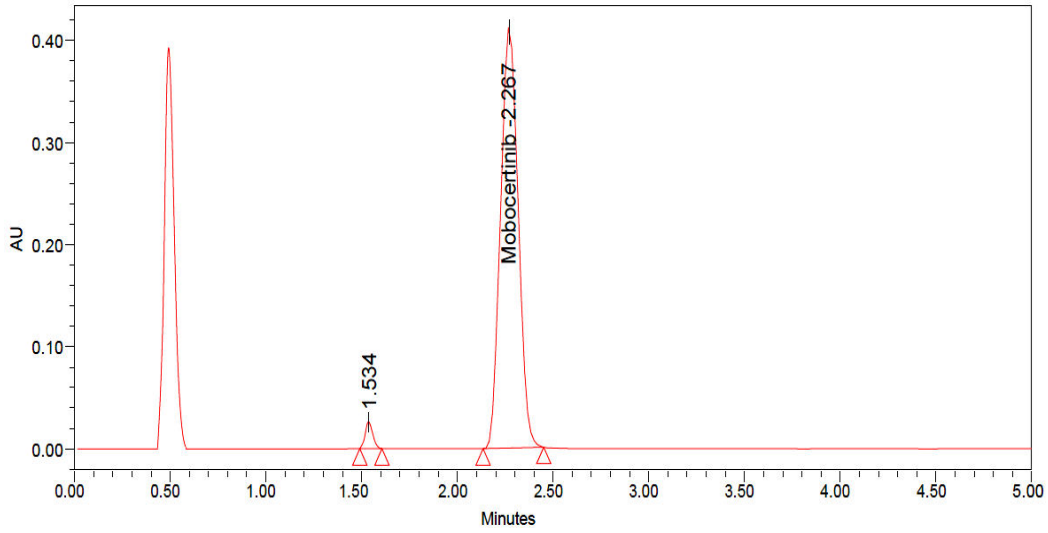

Figure 4.40: Peroxide degradation chromatogram

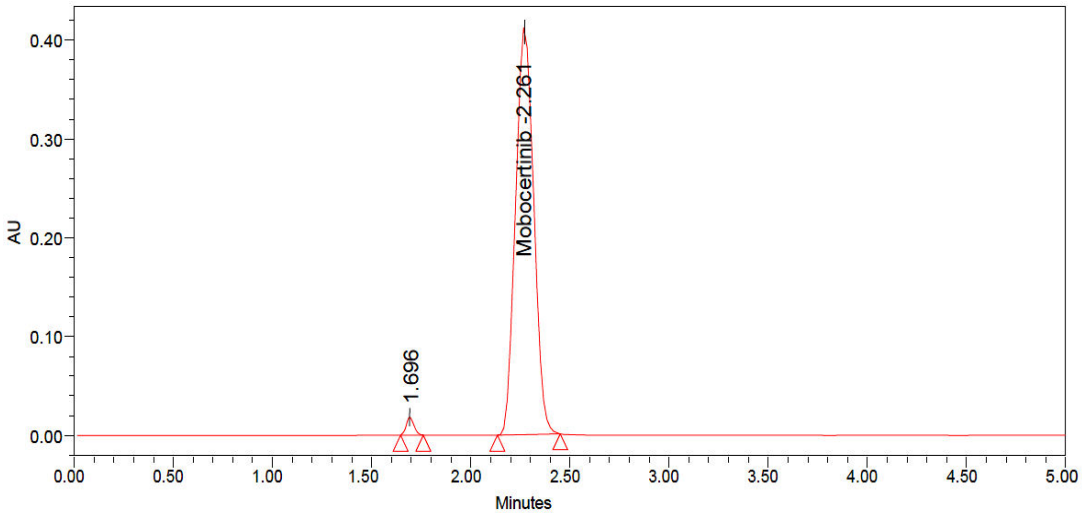

Figure 4.41: Chromatogram of reduction degradation

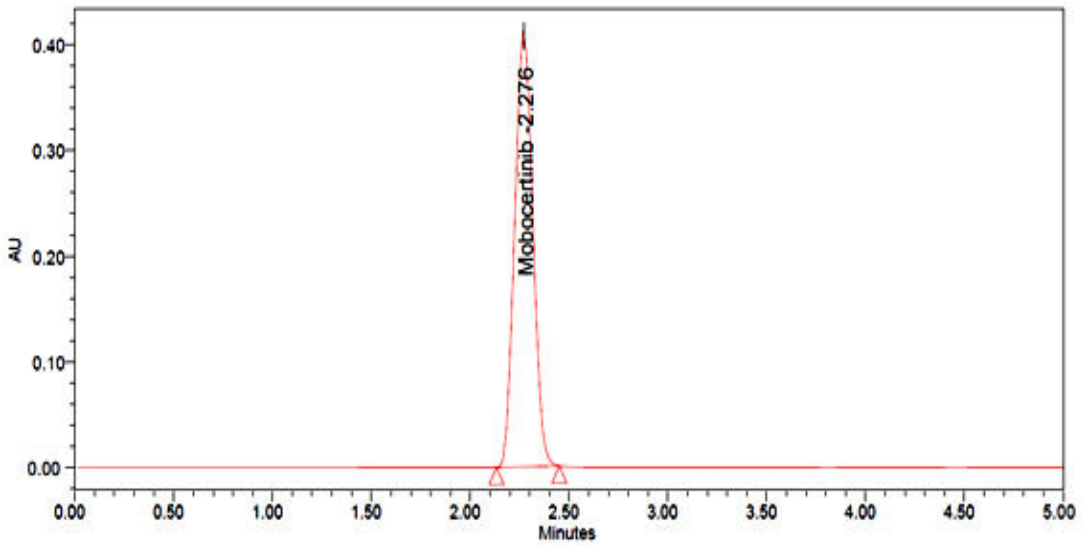

Figure 4.42: Chromatogram of Hydrolysis degradation

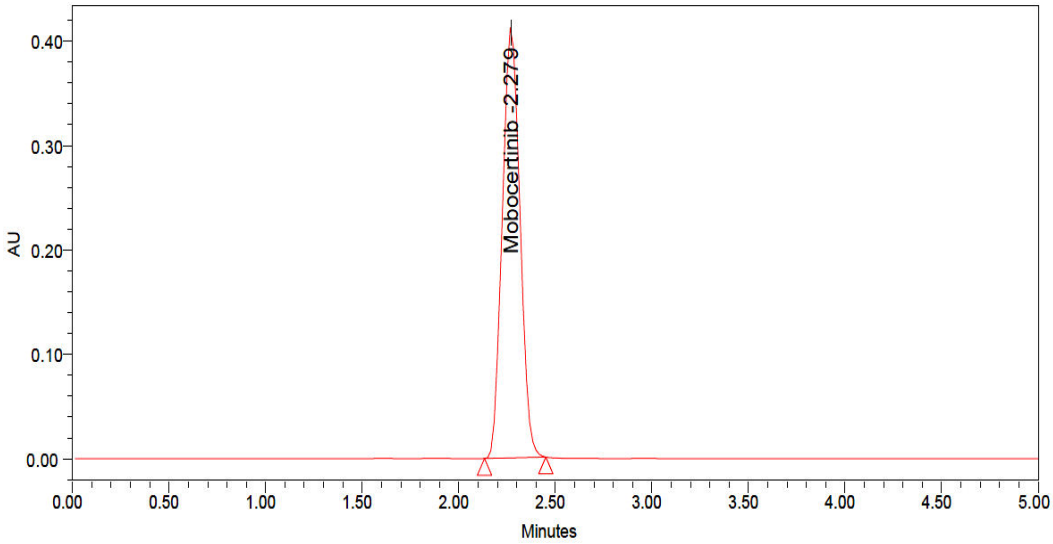

Figure 4.43: Chromatogram of Thermal degradation

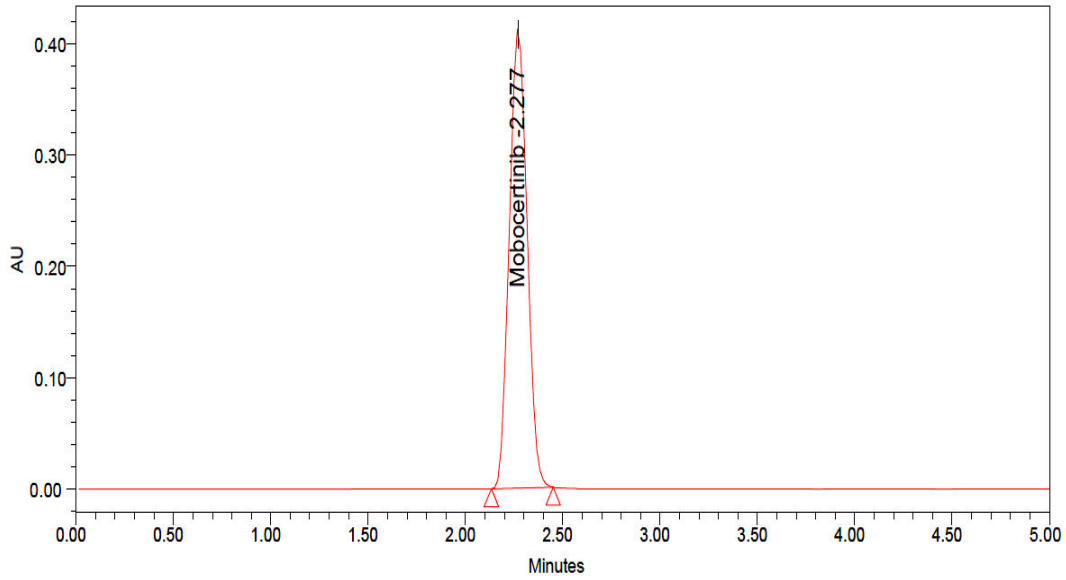

Figure 4.44: Chromatogram of Control degradation

4.5.9 Stability:

Samples and conventional solutions were kept at room temperature for up to 24 hours to study the stability of the solutions. There were many injections administered over a 24-hour period, with each one resulting in a 2% reduction in the test percentage. Mobocertinib has no impact under storage conditions. Stability data are shown in **[Table 4.10]** and chromatograms of Stability are shown in **[4.45-4.49].**

Table 4.10: Results of stability

Time intervals	Mobocertinib (% assay)	% Deviation
Initial	100.2	0.00
6 Hrs	99.3	-0.90
12 Hrs	99.1	-1.10
18 Hrs	98.5	-1.70
24 Hrs	97.3	-2.89

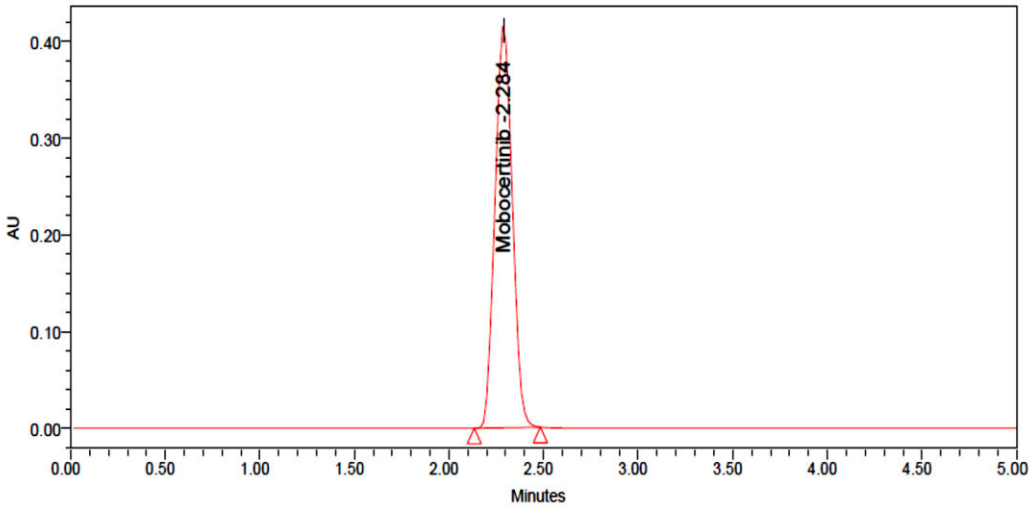

Figure 4.45: Chromatogram of stability initial

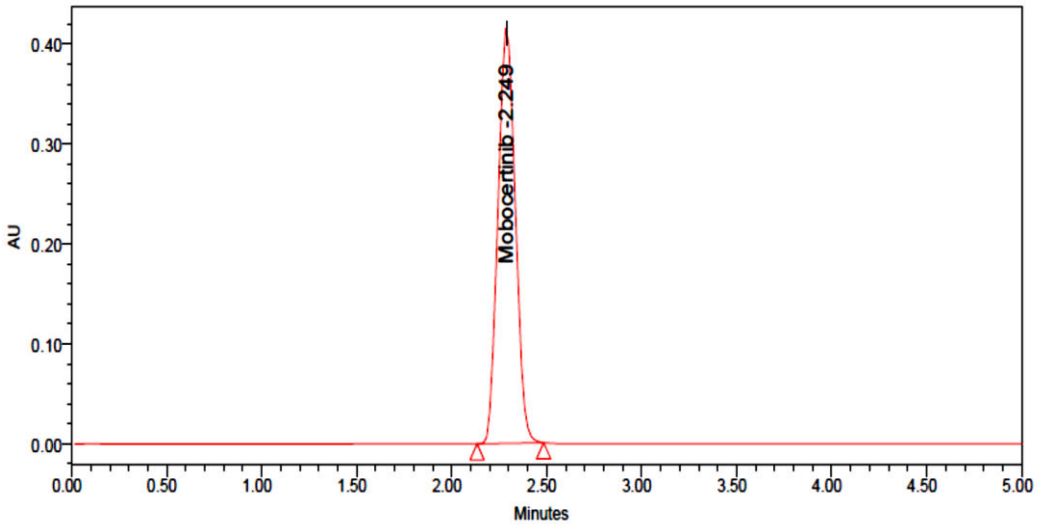

Figure 4.46: Chromatogram of stability 6 Hrs

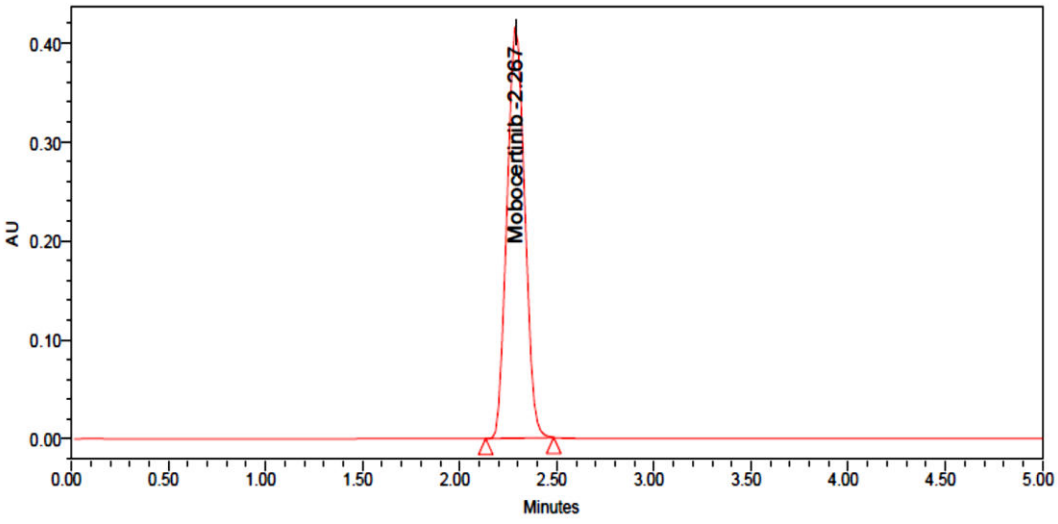

Figure 4.47: Chromatogram of stability 12 Hrs

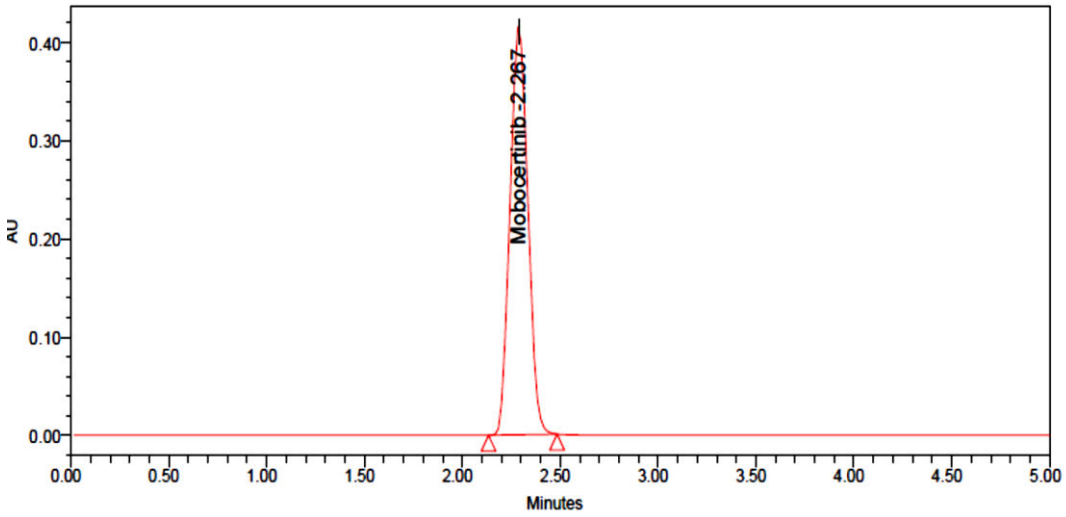

Figure 4.48: Chromatogram of stability 18 Hrs

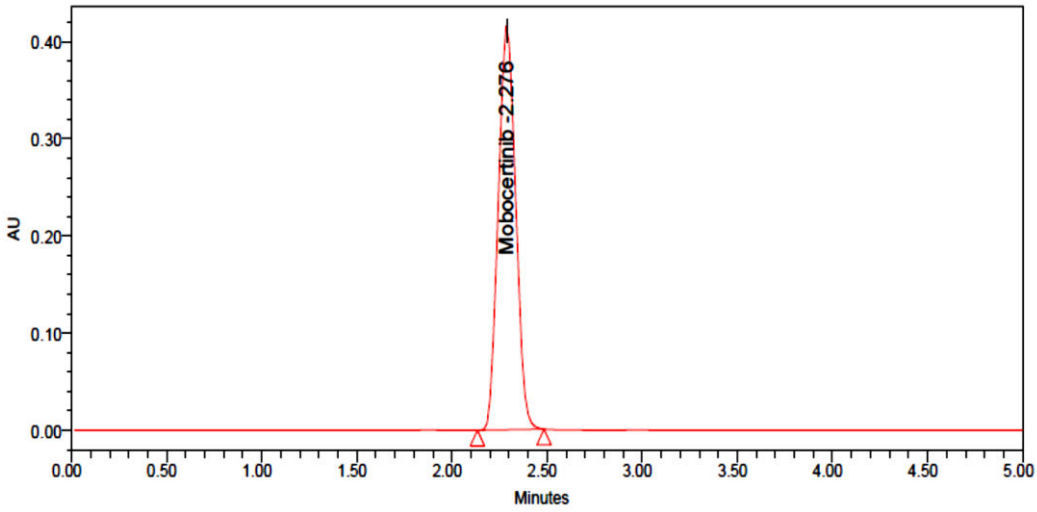

Figure 4.49: Chromatogram of stability 24 Hrs

4.6 CONCLUSION

In compliance with ICH guidelines, this approach describes how to quantify Mobocertinib in bulk and pharmaceutical dose form. An exact, precise, linear, and dependable demonstration of the correctness of the newly developed approach. Additional cost savings were achieved by using less costly reagents. The recommended HPLC conditions have been implemented in order to provide acceptable resolution. The data on accuracy and repeatability were found to be adequate based on the results of the tests. Chromatography-based screening for drugs has become commonplace.

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 ISBN: 978-81-19477-19-7

Chapter - 5

Method Development and Validation of Tepotinib by using Reverse Phase Liquid Chromatography in Bulk and Pharmaceutical Dosage Form

Drug Profile

Adults with metastatic non-small cell lung cancer (NSCLC) who have tumours with a mutation**[1,2]** leading to mesenchymal-epithelial transition (MET) exon 14 skipping may take Tepotinib**[3,4],** marketed under the trade name Tepmetko**[5,6]**. It is a sort of tyrosine kinase inhibitor known as a c-Met inhibitor **[7,8]**. It was discovered in clinical studies**[9,10]** that the most prevalent adverse effects were swelling **[11]**, exhaustion **[12,13]**, nausea **[14]**, diarrhoea **[15]**, muscular pains **[16]**, and shortness of breath **[17]**. In addition to causing interstitial lung disease **[18]** and liver damage **[19]**, tepotinib is also harmful to an unborn child **[20]**.

Structure of Tepotinib: [Fig. 5.01] & [Table 5.01]

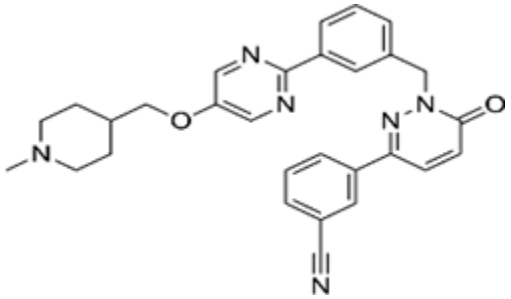

Figure. 5.01: Molecular structure of TepotinibTable.5.01: Drug profile of Tepotinib

IUPAC name	3-[1-[[3-[5-[(1-methylpiperidin-4-yl)methoxy]pyrimidin-2-yl]phenyl]methyl]-6-oxopyridazin-3-yl]benzonitrile
Molecular Formula	C29H31ClN6O3
Molecular Weight	492.6 g/mol
Description	The drug temobinib is used to treat metastatic cancer in NSCLC patients with tumours that contain an abnormal mesenchymal epithelial transition gene (cancer that has already spread). Your doctor will do a MET gene test on the medicine before you begin using it.
Half life	Approximately 32 hours

Uses of Tepotinib

It is possible to use Tepotinib to treat NSCLC that has spread to other parts of the body. A kinase inhibitor, Tepotinib falls within this category. It achieves its function by blocking the action of an abnormal protein that stimulates the proliferation of cancer cells.

Mechanism of Action

Tepotinib is one of many kinase inhibitors now on the market that specifically targets MET and its exon 14 skipping mutations. Tepotinib inhibits MET phosphorylation in both the HGF-dependent and -independent manner, as well as downstream signalling pathways that are MET-dependent.

Absorption:

The bioavailability of tepotinib after oral administration is around 72%. 7,3 At the recommended daily dosage of 450mg, the mean steady-state Cmax and AUC0-24h were 1,291 ng/mL and 27,438 ng•h/h, respectively.

Tepotinib's AUC and Cmax are increased by 1.6 and 2 times, respectively, when given with a high-fat, high-calorie meal.

Adult Dosage:

Tablet

- 225mg

Side Effects:

- Anxiety
- Chest pain or tightness
- Cough
- Dark urine
- Difficult or labored breathing

Literature Resurvey

No literatures are available on Method development and Validation of Tepotinib

Experimental

Chemical and Reagents:

It was decided to use HPLC-grade acetonitrile and tri-ethyl amine water from Merck (India) Ltd, Worli in Mumbai, India. Laurus Labs Pvt. Ltd., Hyderabad providedthe API for Tepotinib used as a reference standard.

Instrumentation:

PDA detector-2998 and quaternary pump were used in the HPLC system (Waters e2695 model). Empower 2.0 was used to do the data analysis.

Standard Solution Preparation

Tepotinib should be accurately weighed and transferred into a 100 ml volumetric flask with approx. 70 ml of diluent, sonicated for 30 minutes, and then brought up to the mark with diluent, according to the manufacturer's instructions. To continue, dilute the solution to a final volume of 50 ml using diluents from 5 ml.

Preparation of Sample Solution

Weight and transfer the Tepotinib sample to a 100-ml volumetric flask with precision, and then add 70 ml of diluent. Use a high-frequency sonicator to break down the substance and dilute it to the desired concentration. Filter the remaining solution through a 0.45-inch nylon syringe filter after diluting it to 50 millilitres with 5 ml of the aforementioned solution.

Method Development Analytical method development:

In the proposed study, RP-HPLC analysis of Tepotinib has been tried and shownto be accurate.

Method development parameters:

Selection of following parameters in method development is very important.

- Mode of chromatography
- Wavelength
- Column
- Movable phase composition
- Solvent delivery system
- Rate of flow
- Volume of injection

Selection of mode of chromatography:

Selected mode of chromatography	:	Reversed phase chromatography
Basis of selection	:	polarity of the molecule
Reason for selection	:	As Tepotinib is polar molecule it elutes at faster along with mobile phase

Detector wavelength selection:
In order to complete the analysis, it is necessary that the detector wavelength be selected correctly. A PDA detector and wavelength are used to identify the precise wavelength of the standard API after it has been produced and injected into a chromatographic machine.

Selected wave length: 254 nm

Basis for selection: Maximum absorbance of analytes and impurities

Reason for selection: Tepotinib having maximum absorbance 254 nm.

Selection of column:

Column selected: agilent eclipse C_{18} column (150x4.6mm, 3.5 µ)

Basis for selection: Based on the polarity, and chemical differences among analytics

Reason for Selection
It has good physiochemical surface qualities and is compatible with many different organic solvents, as well as an extensive variety of bonding chemistries.

Selection of the movable phase composition and of the buffer:
To determine peak symmetries and separation, buffer and buffer intensity are critical factors. Peak tailings during chromatography can change the ionic form if the appropriate buffer strength is not used to cover the column injection load.

Mobile phase preparation:

Solution A: Acetonitrile

Solution B: 0.1percent TEA

Selection of the Rate of Flow
Even in reverse phase separation for the resolution of tiny molecules, flow rate is cited as a crucial element. The flow rate used to load the sample solution is very critical in large-scale inversion phase chromatography, although it is not crucial for analytical research. Dynamic binding capacity might vary based on the flow rate utilised to load the sample. It is necessary to assess the dynamic binding capacity while increasing the purification scale before determining the optimal flow rate for loading. Based on flow factor, retention duration, column composition, separation impurity, and peak symmetry, a flow rate of 1 ml/min has been chosen in this system.

Selection of Injection Volume:
For API estimate, a volume of injection of 10 to 20 l is often advised. Extraction has been a problem thus the test concentration may be kept low and injection volume can be increased to 50 l. Be careful not to overflow the specified column volume, however. Tepotinib is injected in a volume of 10 l using this technique.

Trials in optimization of chromatographic condition:Trial-1 [Fig. 5.02]

Movable phase	:	Acetonitrile and 0.1% OPA (80:20)
Column	:	X-Bridge C8 (150x 4.6mm, 3.5µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Wavelength : 200-400 nm

Observation : System suitability conditions are not within the limit

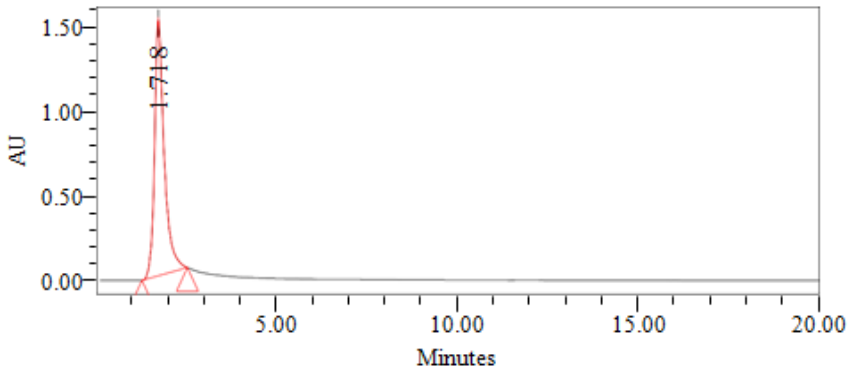

Figure 5.02: chromatogram of trial-1

	Name	Retention Time (min)	Response	USP Resolution	USP Tailing	USP Plate Count
1		1.718	34067232		1.80	215

Trial-2 [Fig. 5.03]

Movable phase : Acetonitrile and 0.1% OPA (70:30)

Column : X-Bridge C8 (150x 4.6mm, 3.5µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Wavelength : 254 nm

Observation : Plate count is not within the limit

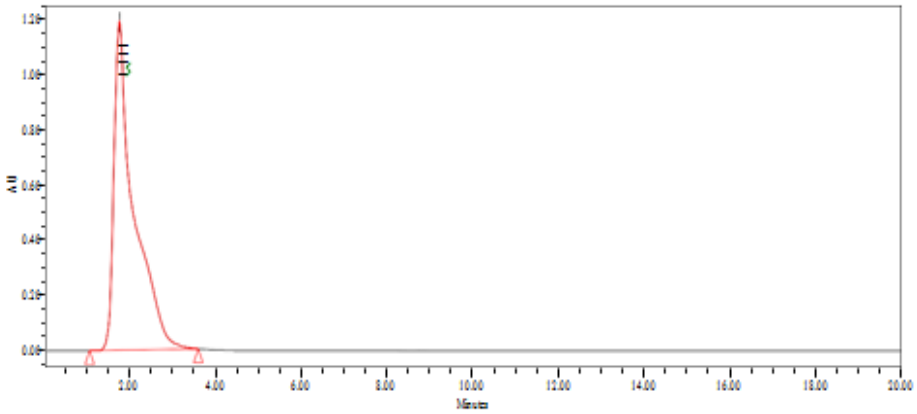

Figure 5.03: Chromatogram of trial-2

	Name	Retention Time (min)	Response	USP Resolution	USP Tailing	USP Plate Count
1		1.762	38344760		1.39	1028

Trial-3: [Fig. 5.04]

Movable phase	:	Acetonitrile and 0.1% OPA (65:35)
Column	:	X-Bridge C8 (150x 4.6mm, 3.5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Wavelength	:	254 nm
Observation	:	Broad peak is observed

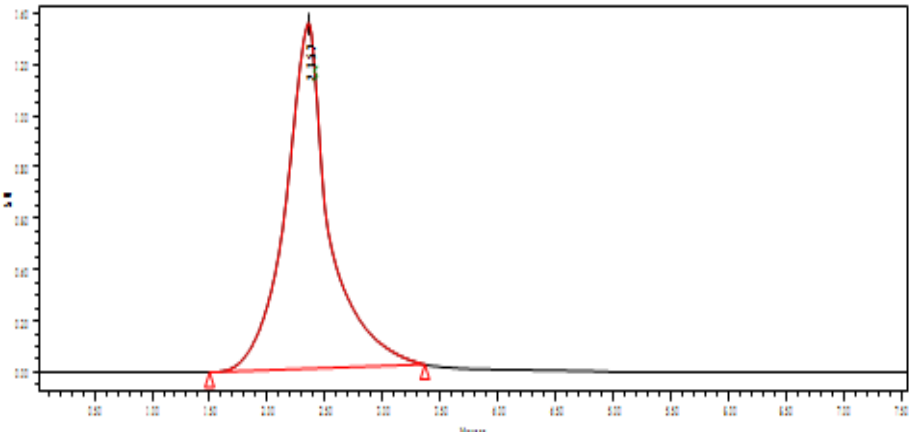

Figure 5.04: Chromatogram of trial-3

	Name	Retention Time (min)	Response	USP Resolution	USP Tailing	USP Plate Count
1		2.357	35609594		1.13	2643

Trial-4 [Fig. 5.05]

Movable Phase	:	Acetonitrile and 0.1% formic acid (60:40)
Column	:	Inertsil ODS (250x 4.6mm, 5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Wavelength	:	254 nm
Observation	:	Response of the peak is very high

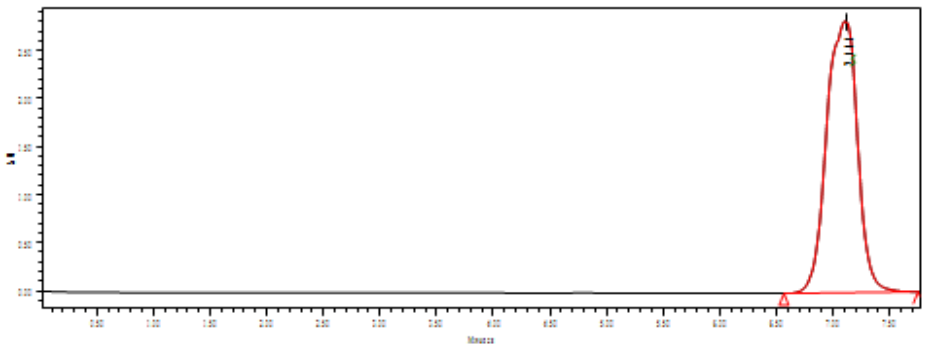

Figure 5.05: Chromatogram of trial-4

	Name	Retention Time (min)	Response	USP Resolution	USP Tailing	USP Plate Count
1		7.111	53541730		2.90	4023

Trial-5 [Fig. 5.06]

Movable phase	:	Acetonitrile and 0.1% Formic acid (65:35)
Column	:	Inertsil ODS (250x 4.6mm, 5µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Wavelength	:	254 nm
Observation	:	Tailing is not within the limit

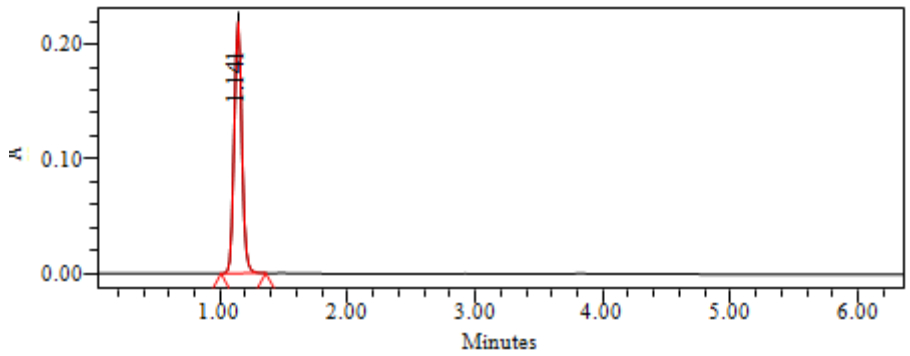

Figure 5.06: Chromatogram of trial-5

	Name	Retention Time (min)	Response	% Area	USP Tailing	USP Plate Count
1		1.141	7521263	100.00	2.56	2065

Trial-6 [Fig. 5.07]

Movable phase	:	Acetonitrile and 0.1% Formic acid (70:30)
Column	:	Agilent eclipse C_{18} (150x4.6 mm, 3.5 µ)
Rate of flow	:	1ml/min
Volume of injection	:	10µl
Wavelength	:	254 nm
Observation	:	Peak height is not within the limit

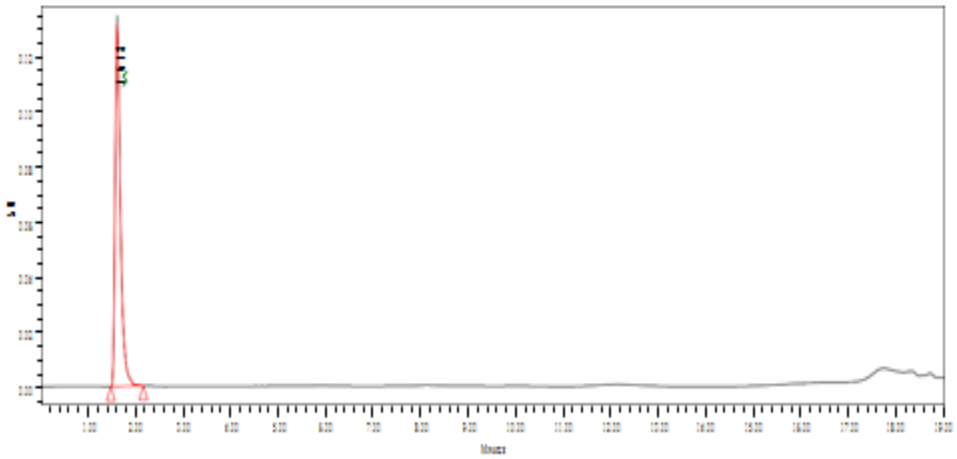

Figure 5.07: Chromatogram of trial-6

	Name	Retention Time (min)	Response	% Area	USP Tailing	USP Plate Count
1		1.816	524826	100.03	4.26	1263

Trial-7 [Fig. 5.08]

Movable phase : Acetonitrile: 0.1% TEA (50:50)

Column : Agilent eclipse C_{18} (150x4.6 mm, 3.5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Wavelength : 254 nm

Observation : Response of the peak is very high

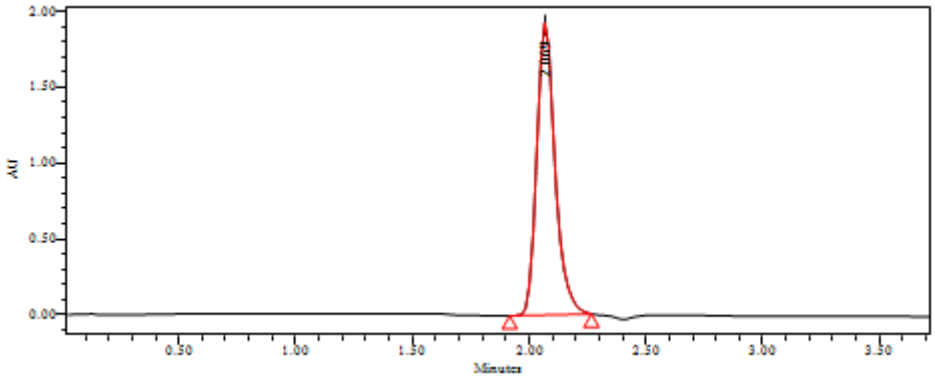

Figure 5.08: Chromatogram of trial-7

	Name	Retention Time (min)	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		2.068	21225632	100.23		5.24	4882

Trial-8 [Fig. 5.09]

Movable phase : Acetonitrile: 0.1% TEA (55:45)

Column : Agilent eclipse C_{18} (150x4.6 mm, 3.5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Wavelength : 254 nm

Observation : Extra peak is observed

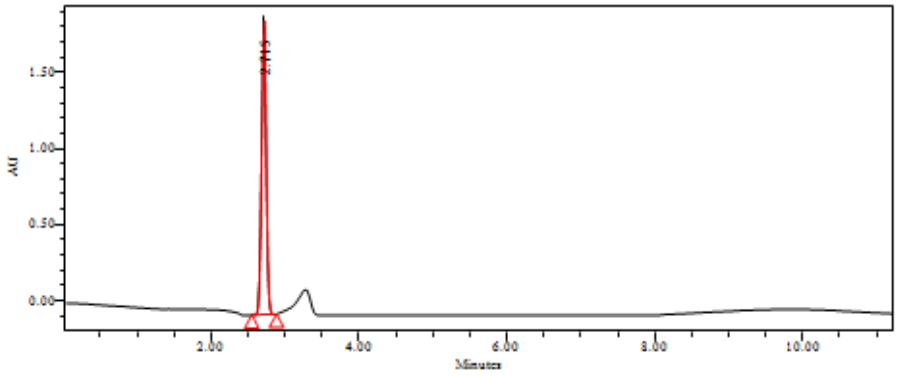

Figure 5.09: Chromatogram of trial-8

	Name	Retention Time (min)	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		2.715	2563148	100.12		1.44	2556

Trial-9 [Fig. 5.10]

Movable phase : Acetonitrile: 0.1% TEA (60:40)

Column : Agilent eclipse C_{18} (150x4.6 mm, 3.5 µ)

Rate of flow : 1ml/min

Volume of injection : 10µl

Wavelength : 254nm

Observation : Peak shape is not good

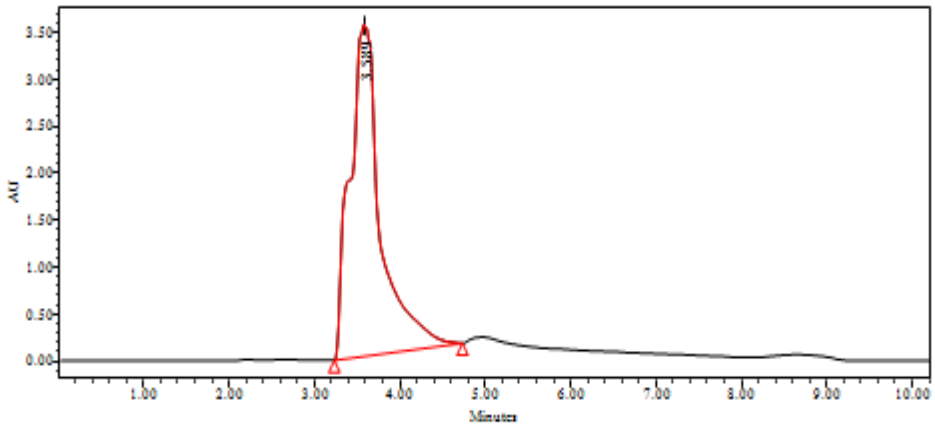

Figure 5.10: Chromatogram of trial-9

	Name	Retention Time (min)	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		3.589	32657856	100.28		4.26	6855

Trial-10 [Fig. 5.11]

Movable phase	:	Acetonitrile: 0.1% TEA (70:30)
Column	:	Agilent eclipse C_{18} (150x4.6 mm, 3.5 µ)
Rate of flow	:	1 ml/min
Volume of injection	:	10µl
Wavelength	:	254 nm
Observation	:	This method is suitable for validation

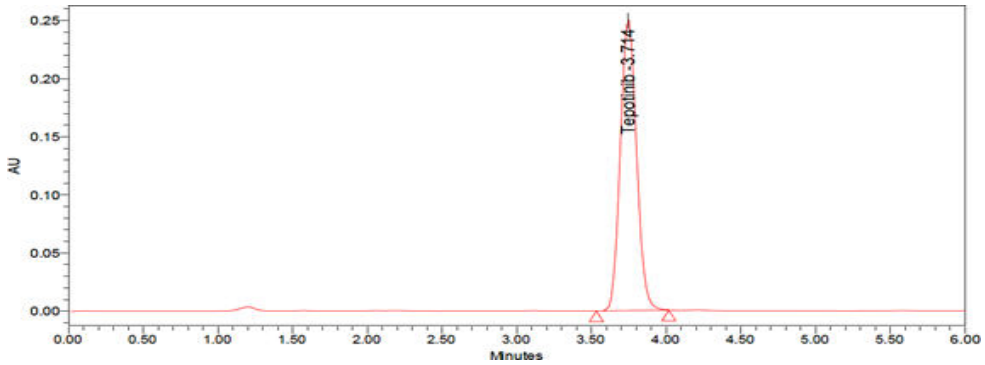

Figure 5.11: Chromatogram of trial-10

	Name	RT (min)	Response	USP tailing	USP plate count	USP resolution
1	Tepotinib	3.714	1894173	1.24	5324	

Optimized Method: [Table 5.02]

Table 5.02: Optimized method chromatographic conditions

S.NO	Parameter	Chromatographic condition
1	Movable phase	Acetonitrile: 0.1% TEA (70:30)
2	Column	Agilent eclipse C_{18}(150x4.6mm, 3.5 µ)
3	Rate of flow	1ml/min
4	Column temperature	Ambient temperature
5	Wavelength	254 nm
6	Volume of injection	10µl
7	Period of run	6 min
8	Retention time	3.714 min

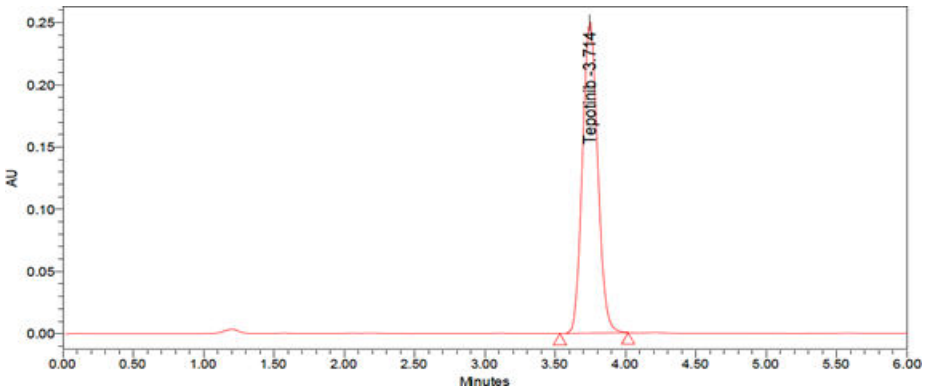

Figure 5.12: Chromatogram of standard[Fig. 5.12] shows chromatogram of standard

Validation of method

ICH Q2 (R1) guidelines were used to evaluate the analytical procedure forcharacteristics such as device compatibility, accuracy, specificity and precision as well as linearity and robustness as well as limits of detection and limits of quantification (LOD and LOQ).**5.6.1**

Specificity: [Fig. 5.13]

When the analyte can be tested in isolation from other components, such as contaminants or excitements that would be anticipated to be present in the sample solution and norm solution, this is called specificity. Tepotinib was used as a spike inthe blank samples.

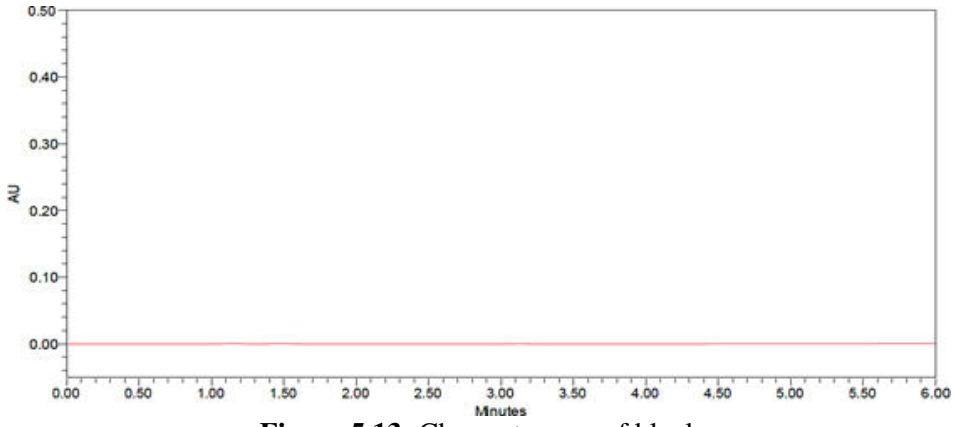

Figure 5.13: Chromatogram of blank

Linearity:

The linearity of the relationship between peak area and concentration was established using a calibration curve. Tepotinib concentrations of 22.5 to 337.5 g/ml were found to be linear in this calibration curve. An equation for the calibration curve of Tepotinib was Y=7831.06x+4819.23x (R2-0.9991)

Procedure

The chromatographic technique may be used to measure the peak area of each degree. Coefficients of correlation may be calculated by drawing a line graph of peak area (Y- axis peak area) and concentration (X-axis concentration). Results of linearity are in **table 5.03**, calibration plot is in **[fig 5.14]** and also **[Fig. 5.15-5.21]** shows 10%, 25%, 50%, 75%, 100%, 125% & 150% linearity chromatograms.

Range

To put it another way: The range of analytic approaches encompasses the gap betweenthe top and lower levels of analysis.

Inclusion Criteria

Not less than 0.9999 is required for a correlation coefficient.

Table 5.03: Results of linearity

S. No	Tepotinib	
	Concentration (µg/ml)	**Area**
1	22.50	184880
2	56.25	459113
3	112.50	896209
4	168.75	1256347
5	225.00	1834505
6	281.25	2178517
7	337.50	2655617

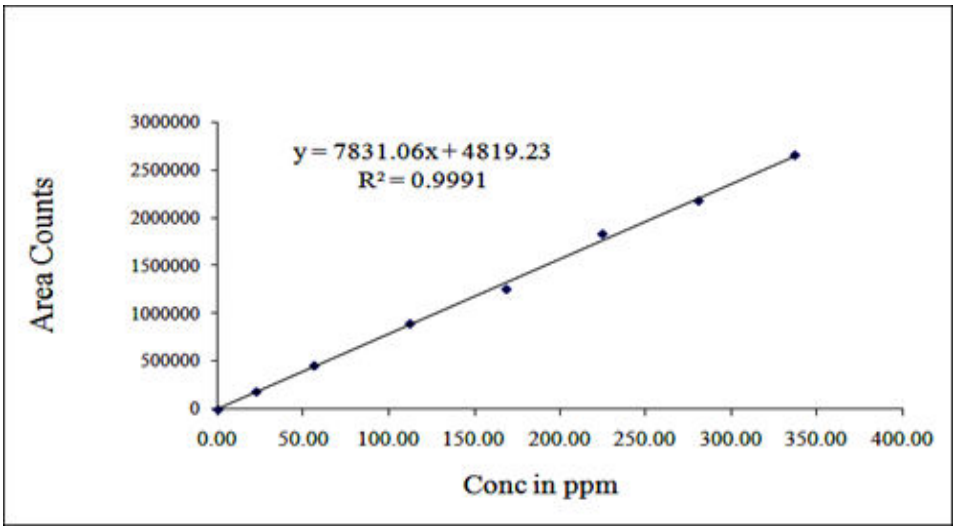

Figure 5.14: Calibration plot of Tepotinib

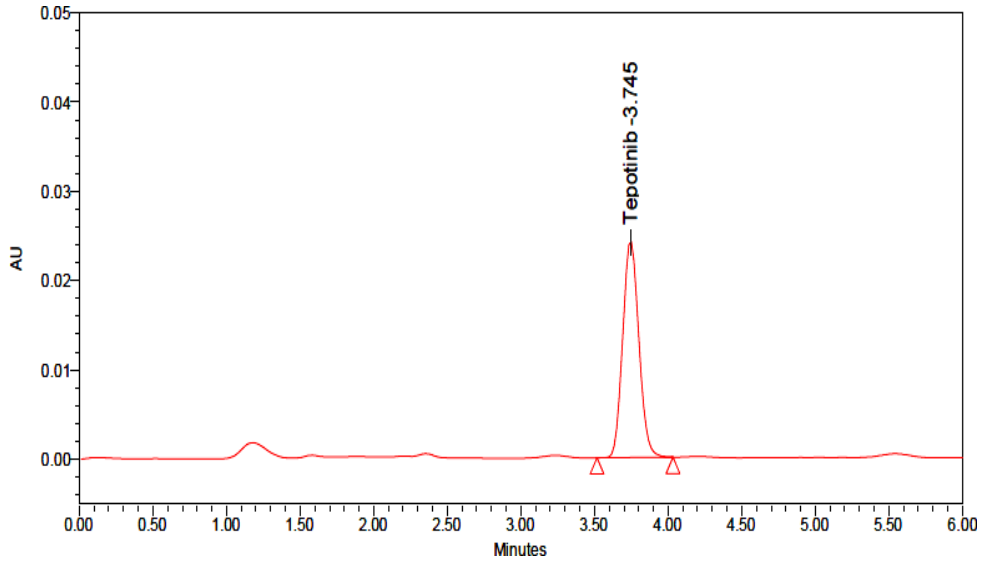

Figure 5.15: 10 percent linearity chromatogram

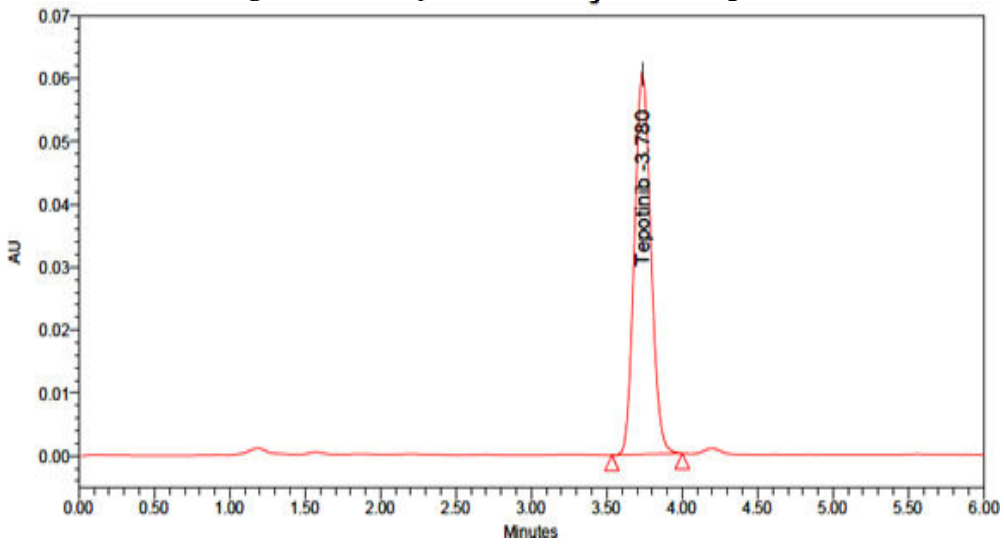

Figure 5.16: 25 percent linearity chromatogram

 ISBN: 978-81-19477-19-7

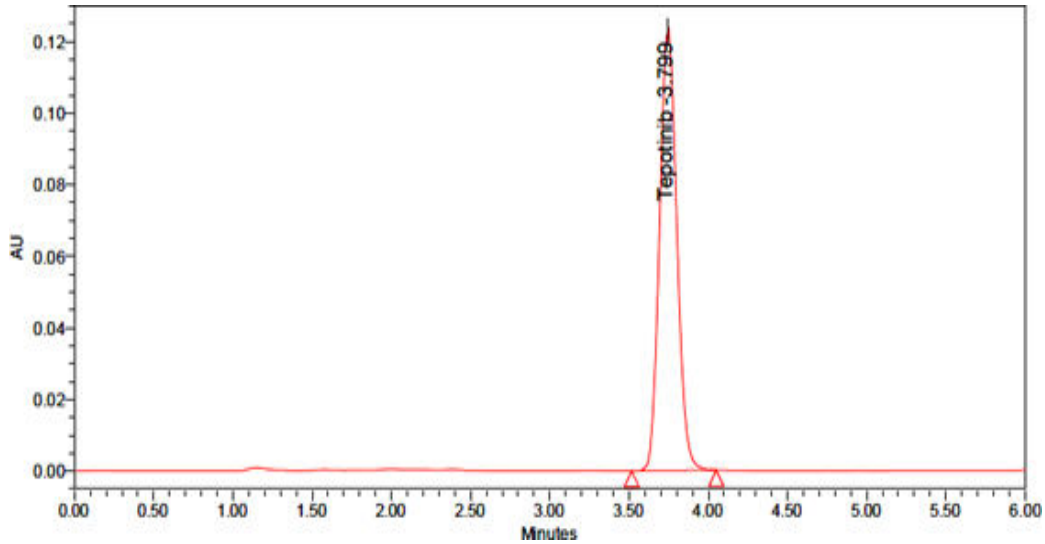

Figure 5.17: 50 percent linearity chromatogram

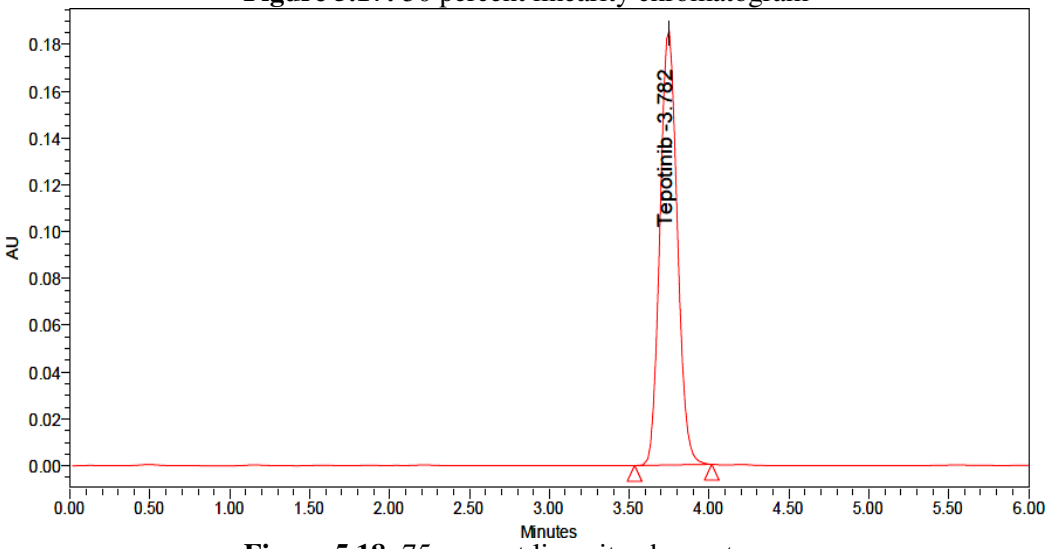

Figure 5.18: 75 percent linearity chromatogram

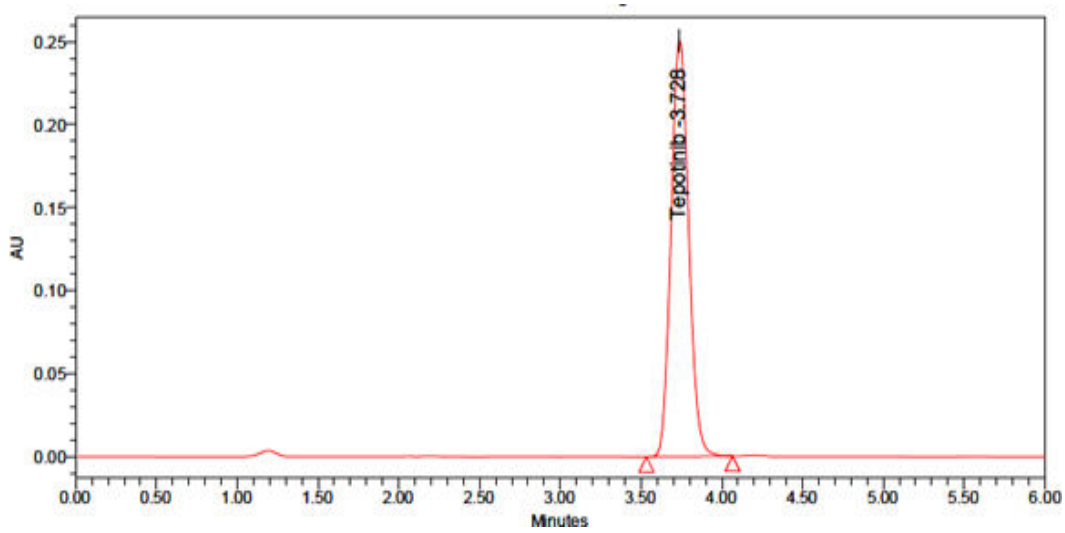

Figure 5.19: 100 percent linearity chromatogram

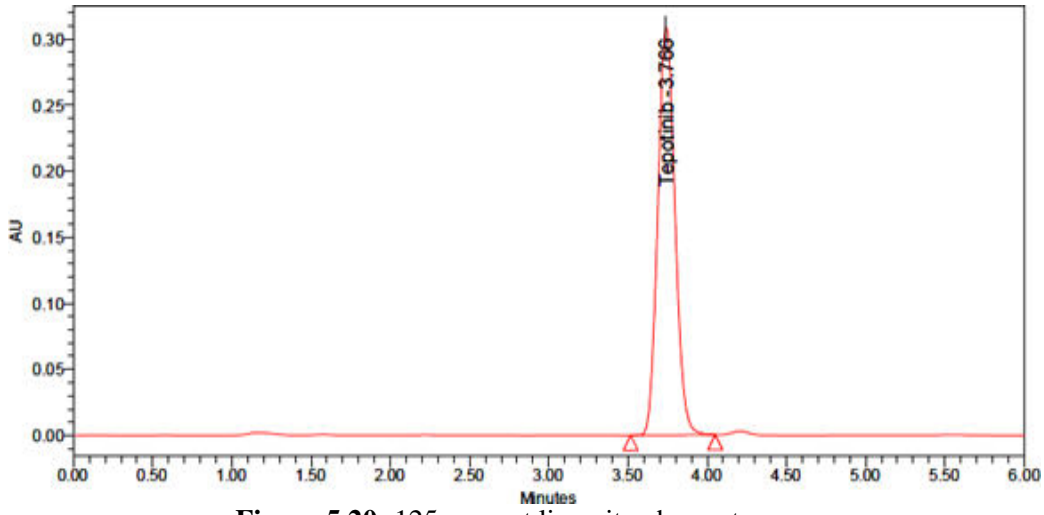

Figure 5.20: 125 percent linearity chromatogram

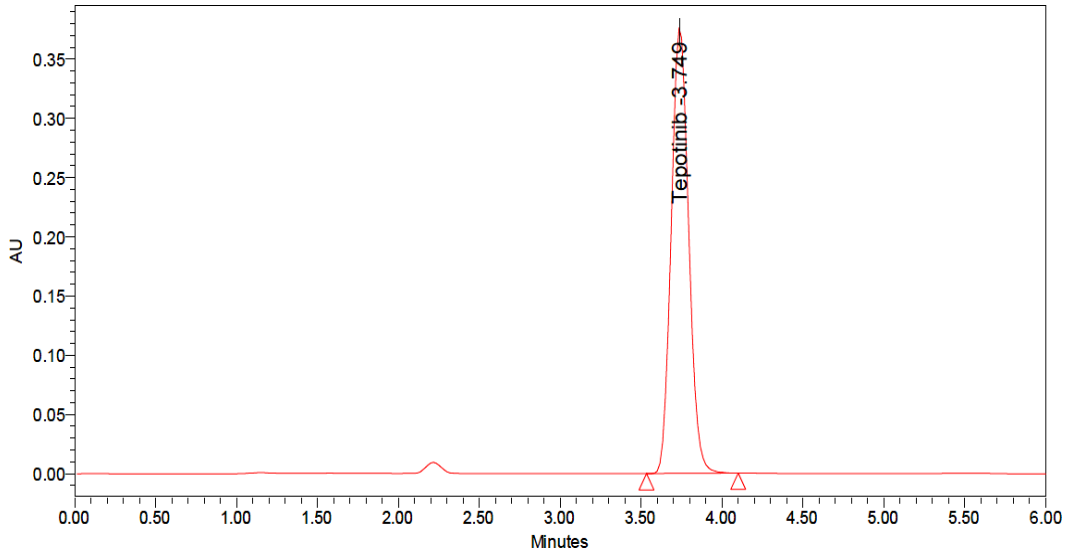

Figure 5.21: 150 percent linearity chromatogram

Accuracy:

50 percent solution preparation (with respect to the concentration of the targetassay)
Sonicate 112.5 mg of Tepotinib in a clean and dry vacuum flask to dissolve it completely and bring the diluents level up to the diluents level, then transfer to a new volumetric flask.

Dose the vacuum flask with 5 ml of the aforementioned solution and dilute it to the desired concentration using the diluents provided.

100 percent solution preparation (with respect to target assay concentration)
Diluents should be used for dissolving 225 mg of Tepotinib in a clean and dry vacuumflask. Sonicating the mixture will ensure that it is completely dissolved.

In a 50ml vacuum flask, pipette 5ml of the aforementioned solution and dilute it withdiluents to the desired volume.

150 percent solution preparation (with respect to target assay concentration)
In a clean and dry vacuum flask, accurately weigh and transfer 337.5mg of Tepotinib. Then, add diluents and sonicate to dissolve thoroughly and bring the diluents level tothe desired level.

Take 5ml of the aforesaid stock solution and dilute it to your desired concentration in a50ml vacuum flask using diluents.

Procedure:
The standard solution, 50 percent accuracy, 100 percent accuracy, and 150 percent accuracy solutions should be introduced at the same time. **[Table 5.04]** shows the accuracy outcomes of Tepotinib and **[Fig. 5.22, 5.23 & 5.24]** shows the 50%, 100% and 150% accuracy chromatograms.

Acceptance Criteria:
The rate of recovery for each stage should be between 98-102 percent

Table 5.04: Accuracy results of Tepotinib

Accuracy	Amount of Tepotinib	% Recovery
50	5	99.1
100	10	100.8
150	15	100.4

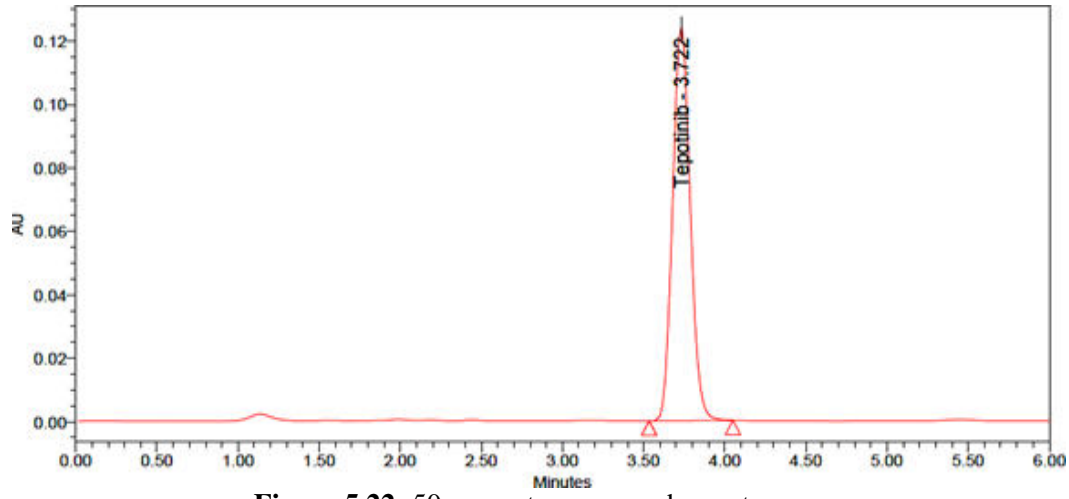

Figure 5.22: 50 percent accuracy chromatogram

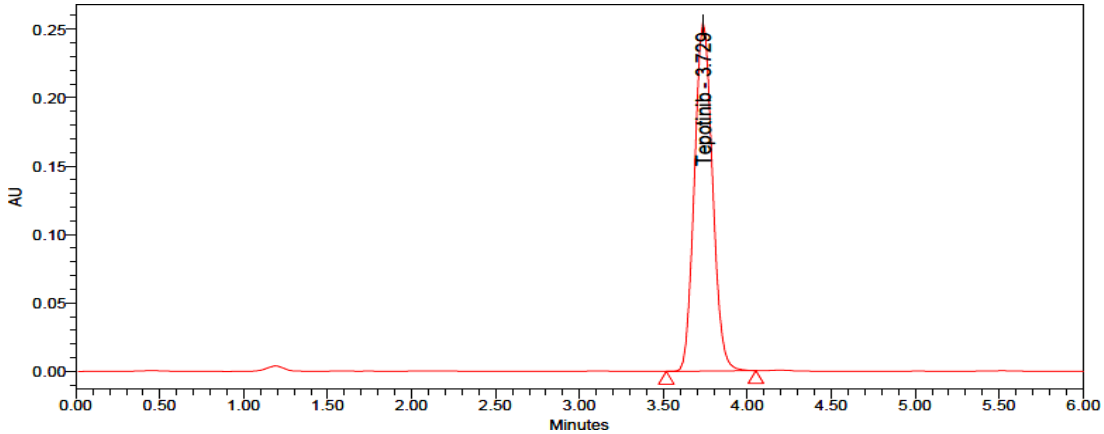

Figure 5.23: 100 percent accuracy chromatogram

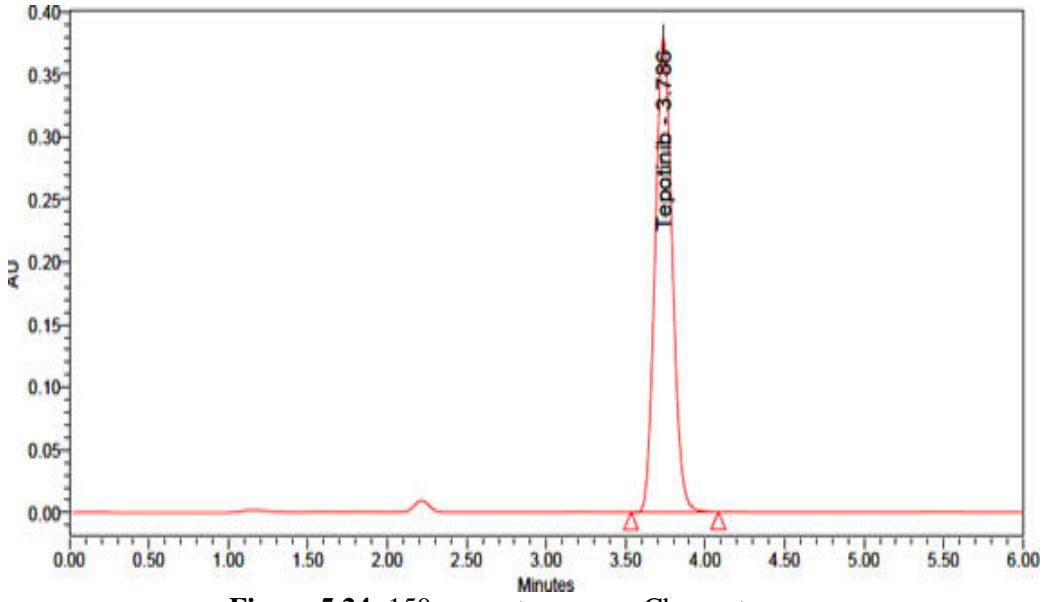

Figure 5.24: 150 percent accuracy Chromatogram

Precision:

In an analytical procedure, the rate at which repeated homogenous samplings provide similar results is a measure of accuracy. Tepotinib (60ppm) was spiked into six different injections to ensure the accuracy of the injection technique. System precision results are shown in **[Table 5.05]** and Chromatograms are shown in **[Fig. 5.25-5.30].**

Table 5.05: Results of system precision

Parameter	Tepotinib
Theoretical plate count	5324
Tailing factor	1.24
Resolution	-
Retention time (min)	3.714

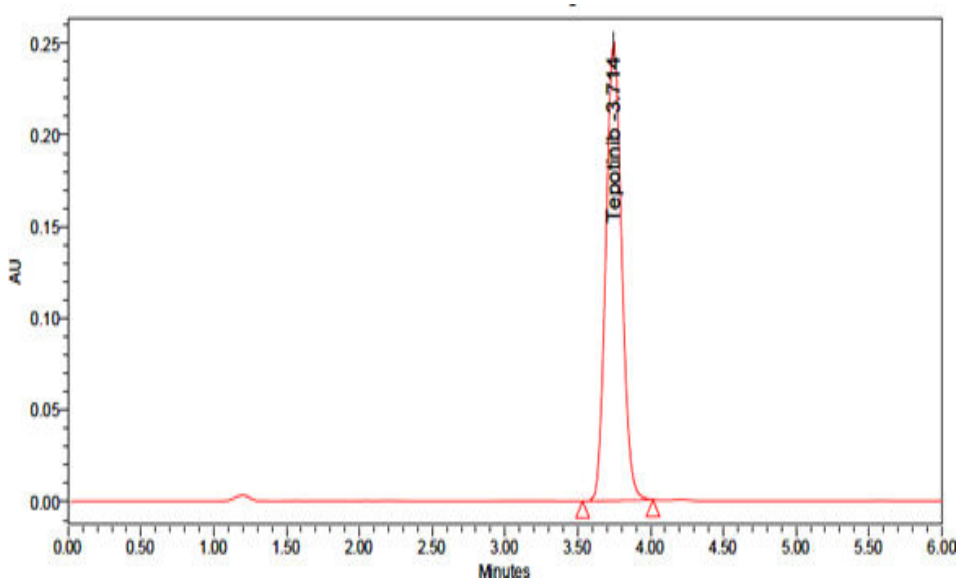

Figure 5.25: Chromatogram of system precision-1

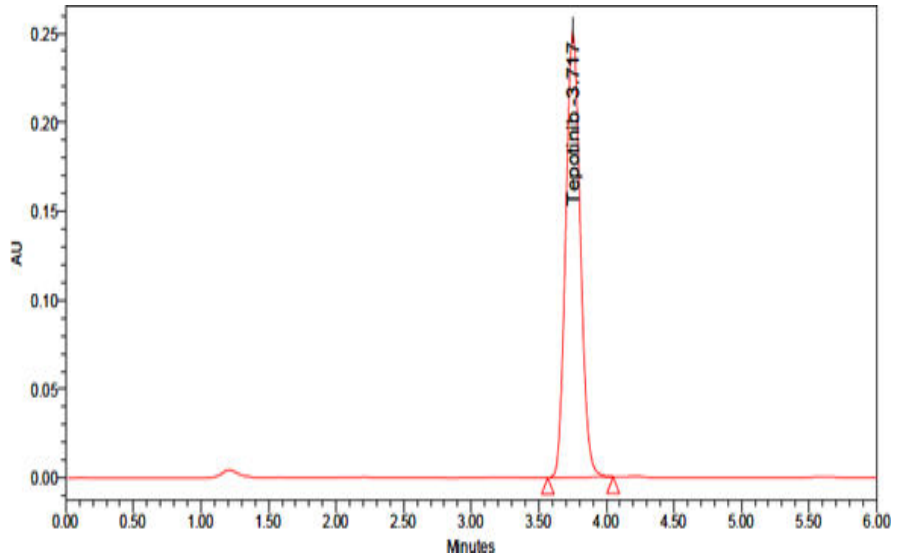

Figure 5.26: Chromatogram of system precision-2

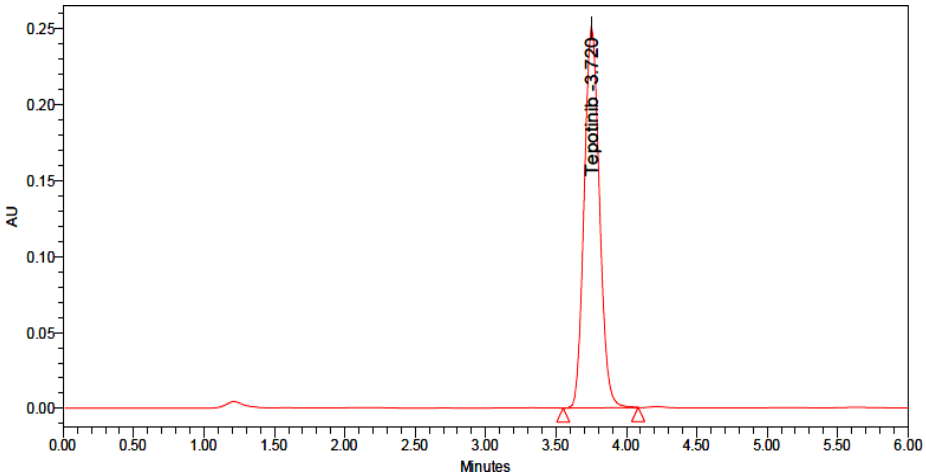

Figure 5.27: Chromatogram of system precision-3

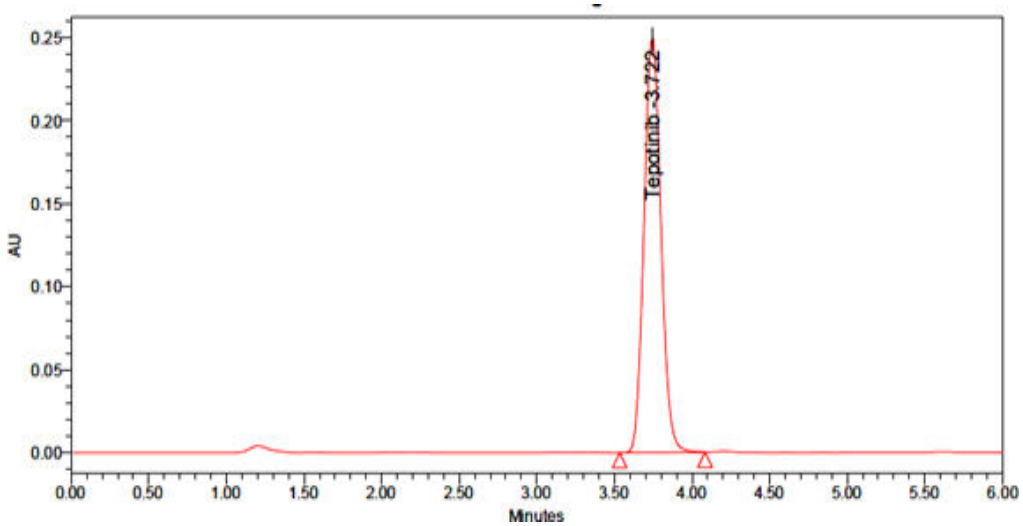

Figure 5.28: Chromatogram of system precision-4

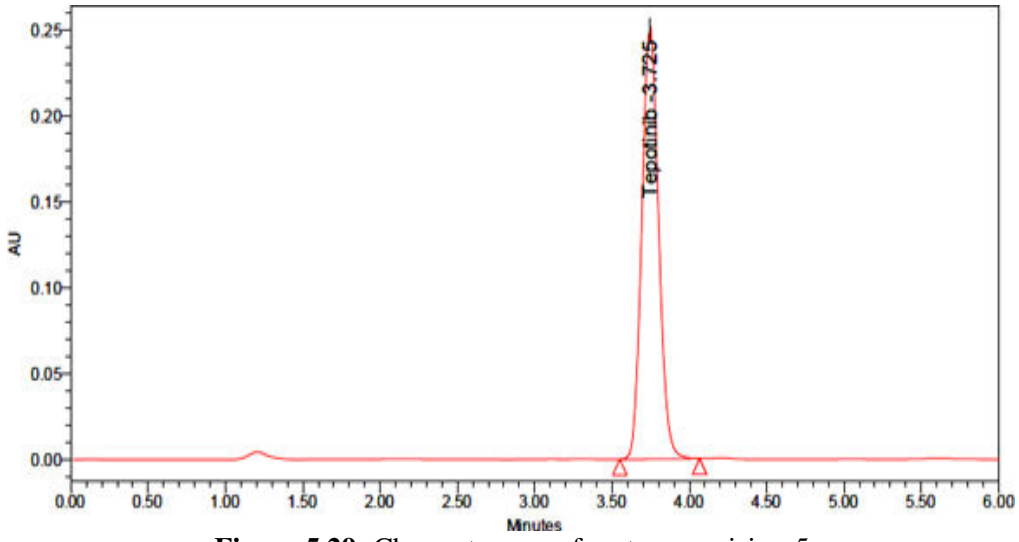

Figure 5.29: Chromatogram of system precision-5

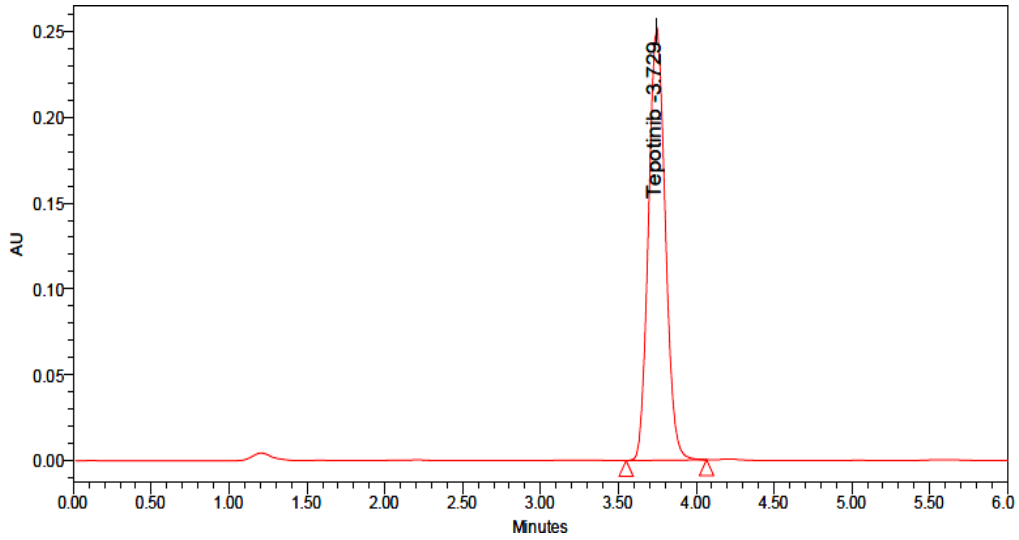

Figure 5.30: Chromatogram of system precision-6

Method Precision: [Table 5.06 and Fig 5.31]

Table 5.06: Results of method precision

S. No.	Area of Tepotinib
1	1900847
2	1897995
3	1903999
4	1907151
5	1903482
6	1902710
Mean	1902697
Std. dev	3088.07
% RSD	0.162

Acceptance Criteria: The area six standard injection RSD percentage should be more than 2%.

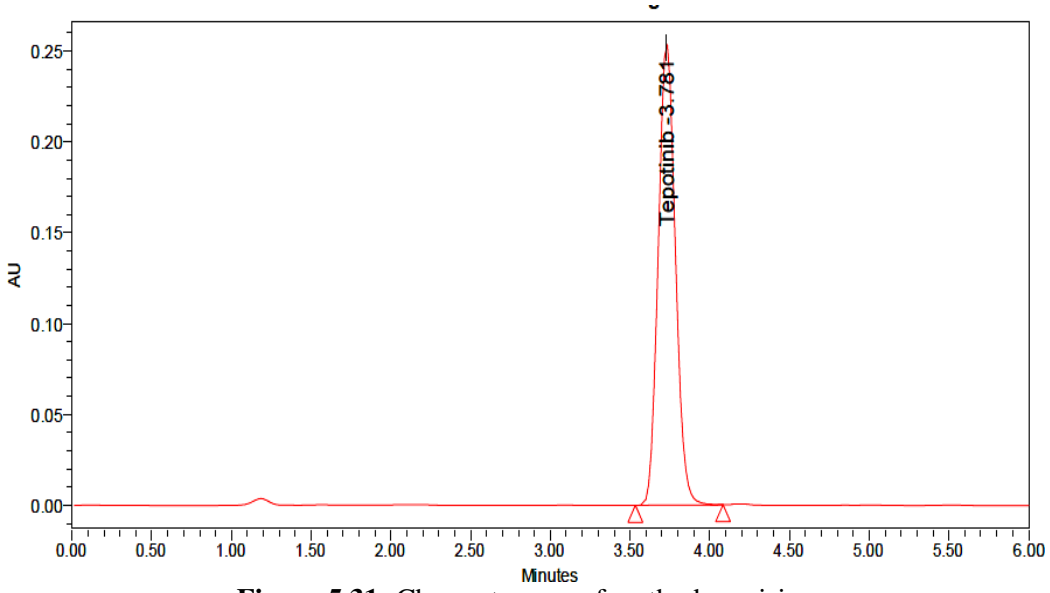

Figure 5.31: Chromatogram of method precision

Intermediate precision: [Table 5.07 and Fig 5.32]

Table 5.07: Results of intermediate precision

S.No.	Area of Tepotinib	% RSD
1	1900654	0.17
2	1897351	
3	1903262	
4	1905471	
5	1903031	
6	1902356	

Acceptance criteria: The RSD percentage for the six normal injection results should not be more than 2%.

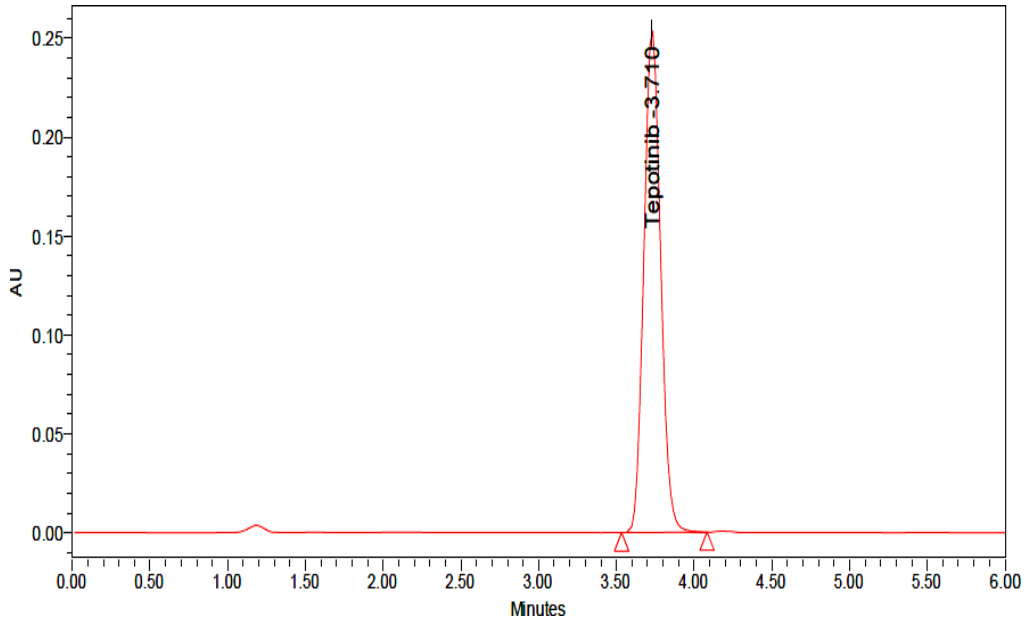

Figure 5.32: Chromatogram of intermediate precision

Limit of detection (LOD) and limit of quantification (LOQ): [Fig 5.33-5.34]

Only at the lowest concentrations of an analyte can its presence be reliably detected and quantified **[21]**. LOD and S/N values for Tepotinib were 6.75 g/ml and 6. The LOQ contained 22.5 g/ml of Tepotinib, with a S/N ratio of 25. An indicator of signal to noise ratio is the S/N.

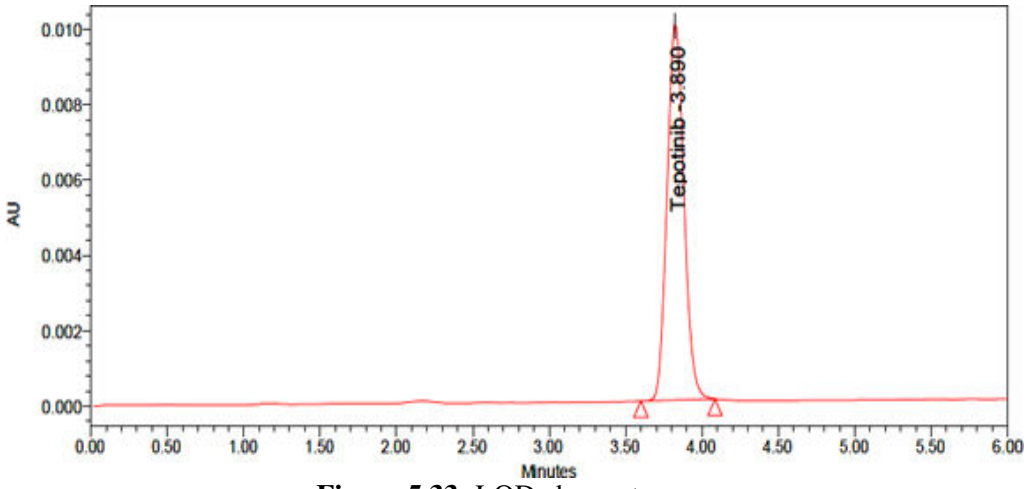

Figure 5.33: LOD chromatogram

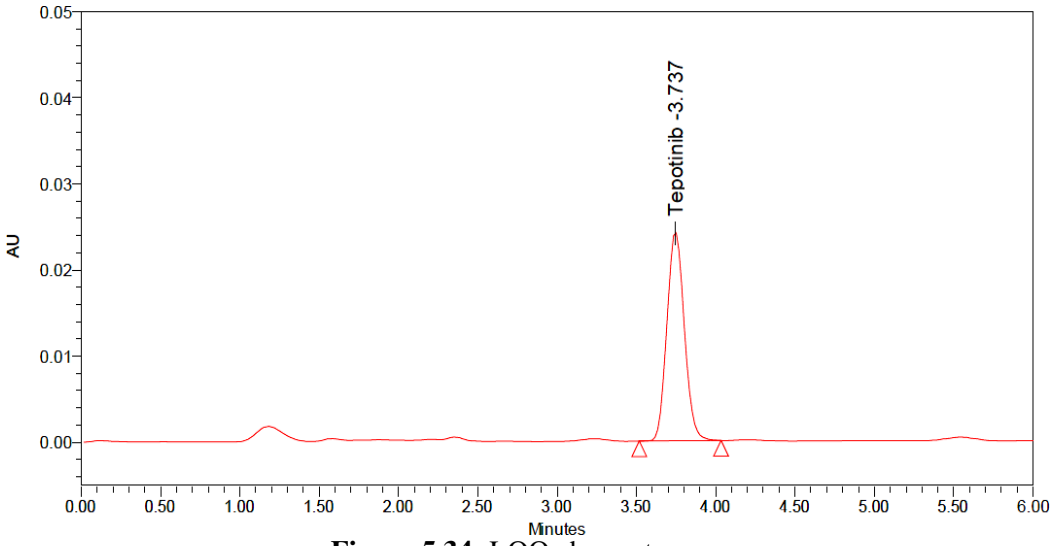

Figure 5.34: LOQ chromatogram

Robustness:

The purpose of the experiment was to assess the system's ability to withstand changes in flow rate, mobile phase organic percentage, and other parameters **[22]**. Active pharmaceutical components and contaminants were not considerably changed, and the period of retention, plate count, and tailing factor were not significantly altered. Thus, this strategy proved to be reliable. **[Table 5.08]** shows robustness results of Tepotinib. Chromatograms from **[Fig. 5.35-5.38]**

Table 5.08: Robustness results of Tepotinib

Parameter	% RSD of Tepotinib
Flow (0.8 ml/min)	0.22
Flow (1.2 ml/min)	0.31
Organic phase (45:55)	0.18
Organic phase (55:45)	0.06

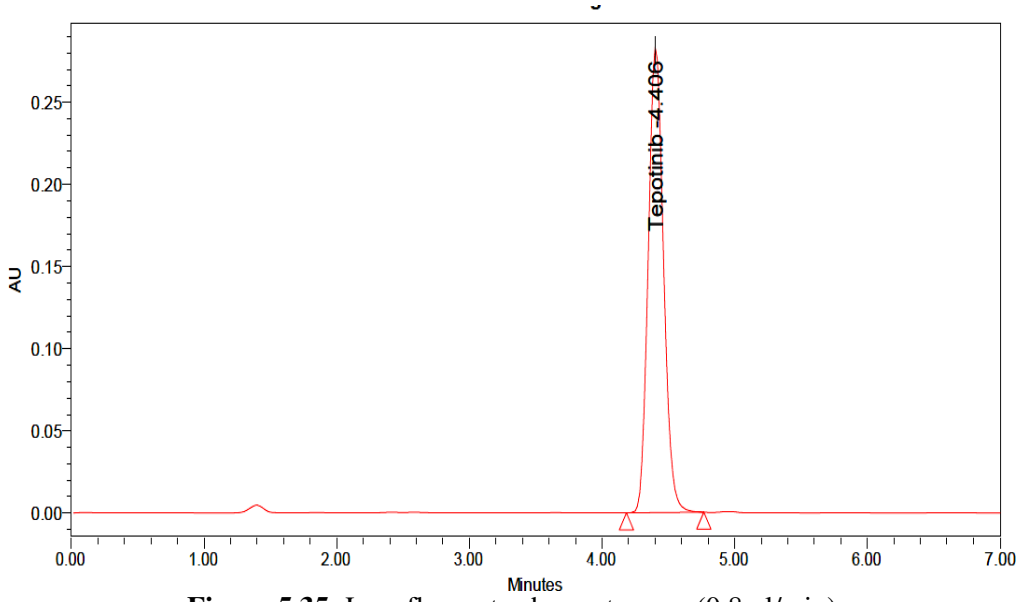

Figure 5.35: Less flow rate chromatogram (0.8ml/min)

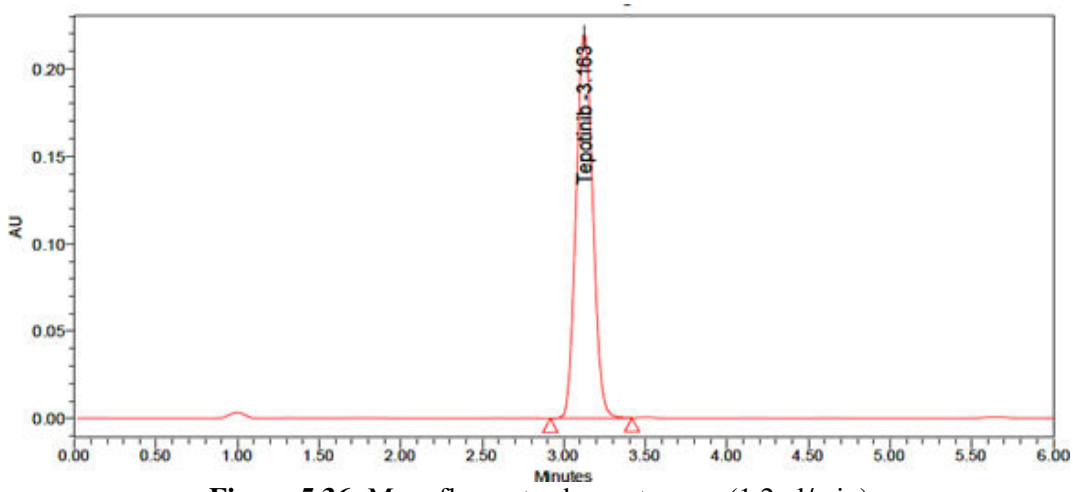

Figure 5.36: More flow rate chromatogram (1.2ml/min)

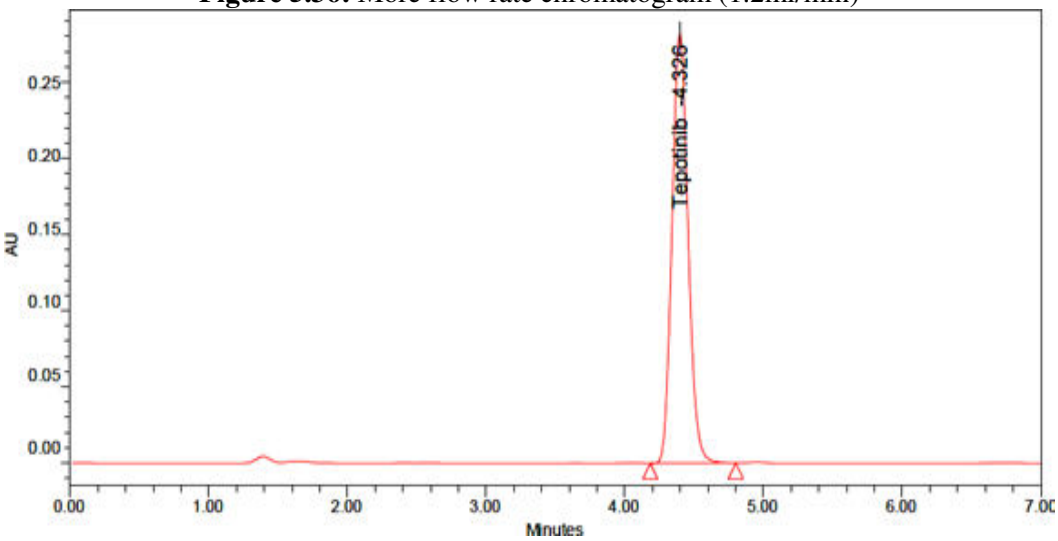

Figure 5.37: Less organic chromatogram (45:55)

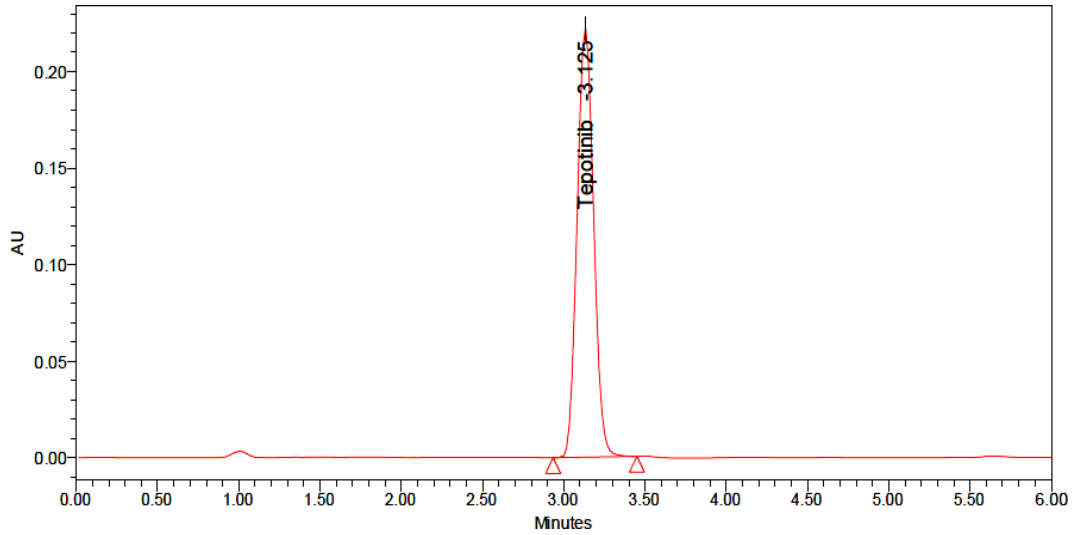

Figure 5.38: More organic chromatogram (55:45)

Forced Degradation Studies:

This technology is a leap ahead from previous methods, allowing simultaneous testing of both release and stability. Acid, base, oxidation, reduction, and heat degradation are all part of the process of forcing degradation. Despite the appearance of degradedpeaks, which are dependent on the kind of chromatography used, the drugs under investigation were found to be stable **[23]**. Effects of accelerated deterioration **[Table 5.09] and Chromatograms from [Fig. 5.39-5.43]**

Acid Degradation

A volumetric flask containing 10 ml of the sample stock solution was used to hold 1 ml of 1N HCl, which was added and allowed to stand for 15 minutes. Make up to the diluent mark by adding 1ml of 1N NaOH after 15 minutes. Syringe filter the solution and inject it into the HPLC.

Alkali Degradation

Sample stock solution was transferred to the volumetric flask, and 1N NaOH was added to the mixture. The combination was let to stand for 15 minutes before being centrifuged at 10,000 rpm for 15 minutes. Adding 1 mL of 1N HCl to raise the solution to a desired concentration may be done after 15 minutes. Filter the solution using a syringe filter before injecting it into the HPLC apparatus.

Peroxide Degradation

Add 1 ml of a 30% hydrogen peroxide solution and dilute with diluents to produce up to 10 ml of sample stock solution. Syringe filter solution, inject into HPLC equipment, andsee the results.

Reduction Degradation

After diluting with diluents to the desired concentration, transfer 1 ml of the sample stock solution to a volumetric flask with a capacity of 10 ml. Before injecting the solution into the HPLC system, filter it using a syringe filter.

Thermal Degradation

The sample solution was held at 105°C for the duration of the 6-hour baking time. We next used a high-performance liquid chromatography equipment to separate out the resultant sample from the others.

Hydrolysis Degradation

An HPLC water solution of one millilitre was introduced to a volumetric flask that had a capacity of 10 millilitres, followed by the addition of one millilitre of sample stock solution. Filter the solution using a syringe filter before injecting it into the HPLC apparatus.

Table 5.09: Forced degradation results

Stress Parameter	% Degradation of Tepotinib
Acid degradation (1N HCl+ reflux+ 24 hrs)	16.2
Alkali degradation (1N NaOH+ reflux+ 24 hrs)	16.6
Peroxide degradation (30% Peroxide+ reflux+ 24 hrs)	14.3
Thermal degradation (Sample + heat for 6 hrs)	17
Hydrolysis degradation (1 ml HPLC water + reflux + 24 hrs)	15

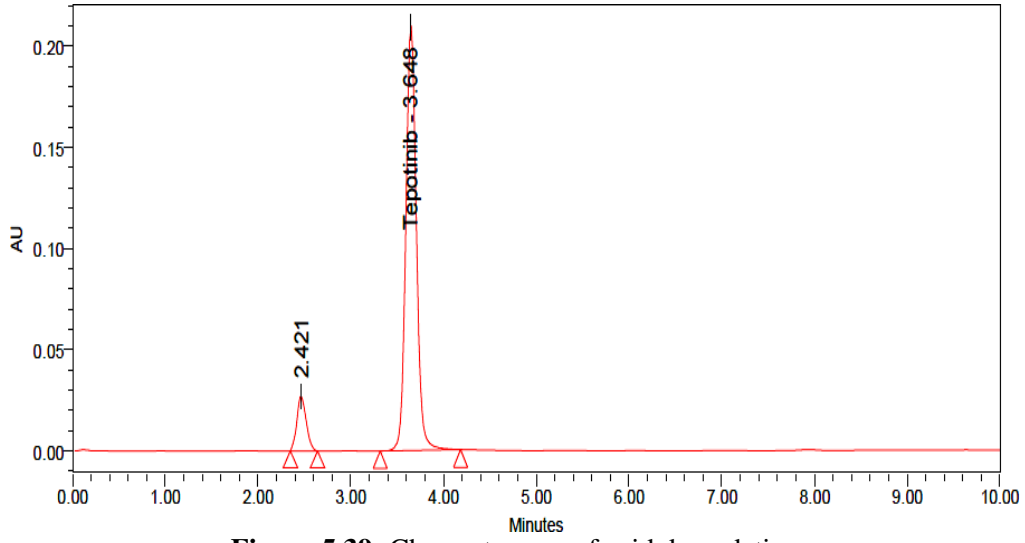

Figure 5.39: Chromatogram of acid degradation

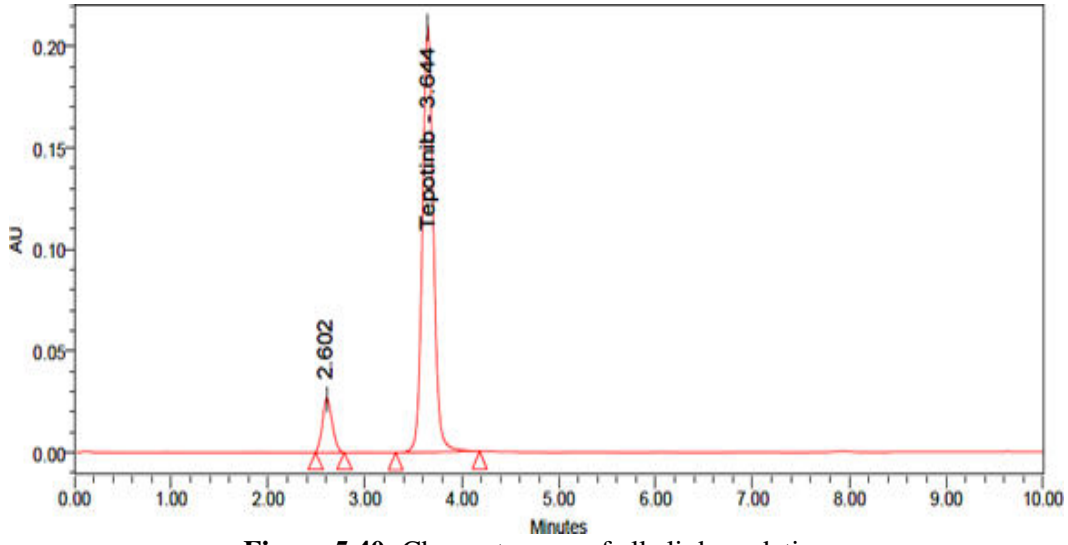

Figure 5.40: Chromatogram of alkali degradation

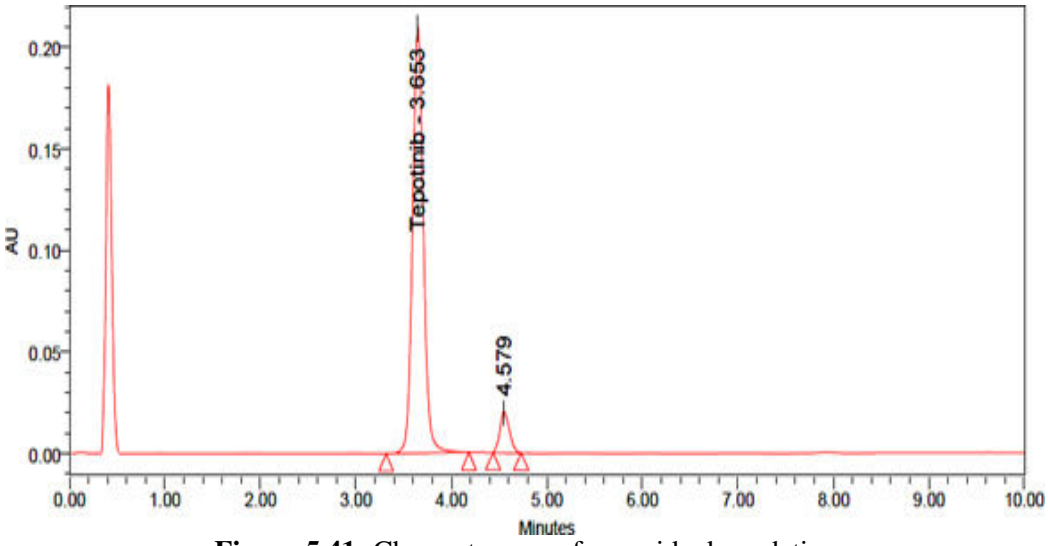

Figure 5.41: Chromatogram of peroxide degradation

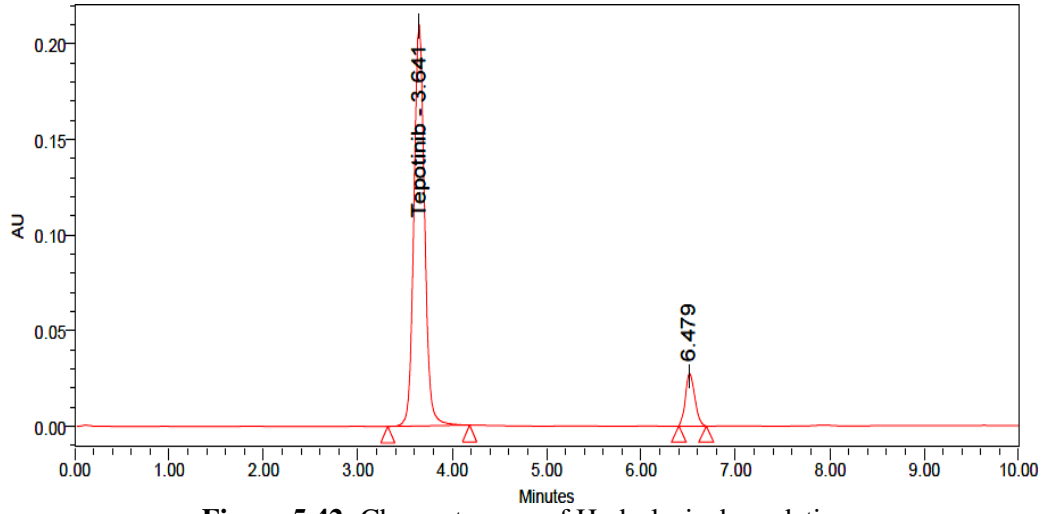

Figure 5.42: Chromatogram of Hydrolysis degradation

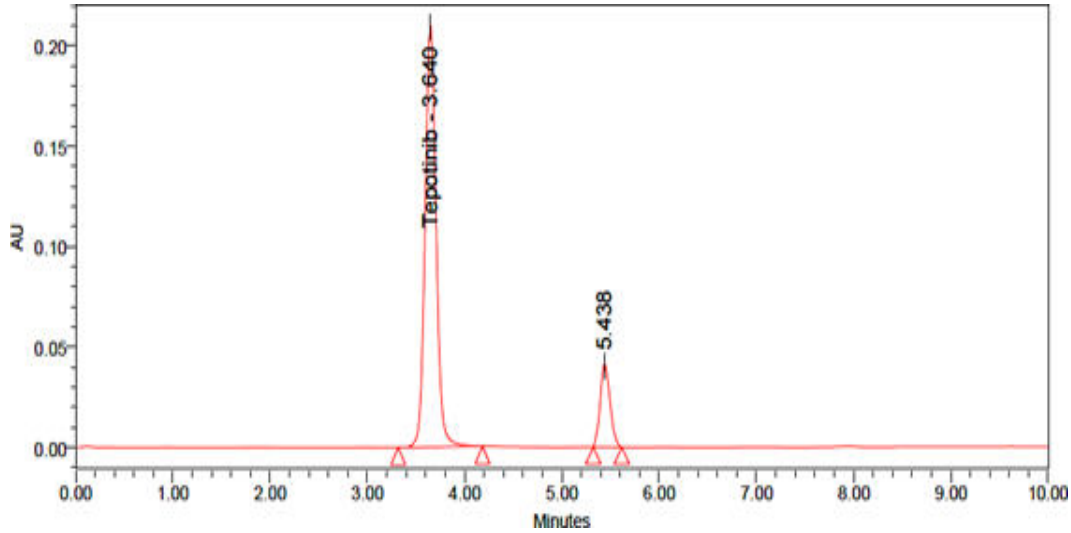

Figure 5.43: Chromatogram of Thermal degradation

CONCLUSION

Tepotinib was determined in bulk and pharmaceutical dosage form using a new, quick, inexpensive, sensitive, and conveniently accessible HPLC approach. In this approach, the run time is shorter, the price is cheap, the accessibility, sensitivity, dependability, and repeatability are all excellent features. Large numbers of samples need these characteristics. This method's robustness and robustness were both determined to be within acceptable limits throughout the validation of all of the method's many parameters. Because the RSD values for each parameter are less than 2%, this implies that the approach is reliable and the results achieved by this method are in good agreement. Consequently, the suggested approach may be used for regular analysis and pharmaceutical formulations of Tepotinib in quality control labs without the need for any prior separation.

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 ISBN: 978-81-19477-19-7

Chapter - 6

A Study of Development and Validation of a Method for Simultaneous Estimation of Brigatinib and Alectinib using Reverse Phase Ultra Performance Liquid Chromatography in Active Pharmaceutical Ingredient Form

6.1 Drug Profile

6.1.1 Drug profile of Brigatinib

ARIAD Pharmaceuticals, Inc. is developing a small-molecule targeted cancer medicine called Brigatinib, which is commercialised under the trade name Alunbrig and other names (Huang et al., 2016). Both ALK and the epidermal growth factor receptor (EGFR) are inhibited by the cancer drug brigatinib. (Mangieri et al., 2017; Mologni, 2012). (Wang et al., 2002; Stortelers et al., 2002). Cetuximab or panitumumab may be utilized in combination with osimertinib to address the EGFR C797S mutation-induced resistance to osimertinib (Sequist et al., 2011). The aggressive variant of large B-cell lymphoma (ALCL) was the first to show evidence of ALK as a chromosomal rearrangement (Quesada et al., 2019; Montes-Mojarro et al., 2018). Abnormal ALK expression has been linked to NSCLC (Sherwood et al., 2015; Nasser et al., 2020) and neuroblastomas (Maris et al., 2007; Friedman et al., 2007) in genetic studies, and these findings have been confirmed in studies of ALCL and ALK+ non-Hodgkin lymphoma. ALK is a possible cancer therapeutic target since it is seldom seen in normal adult tissues. A second, well-established target in NSCLC is the epidermal growth factor receptor (EGFR). It is estimated that roughly half of patients with the T790M "gatekeeper" mutation are resistant to first-generation EGFR treatments. Second-generation EGFR inhibitors are being developed, however toxicity issues associated with inhibiting the native (endogenous or unmutated) EGFR are keeping them from being used in clinical trials. T790M mutation of EGFR, which is a potential target for cancer treatment (Merlo and colleagues, 2006), is another intriguing molecular target for cancer therapy.

6.1.2Structure of Brigatinib: [Fig. 6.01]

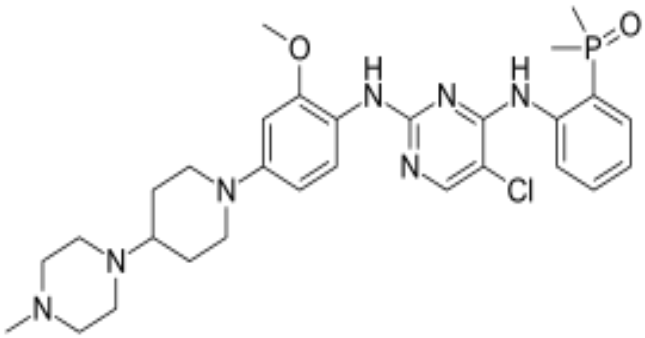

Figure 6.01: Brigatinib's chemical structure

6.1.3 Name of the IUPAC: 5-chloro-4-N-(2-dimethylphosphorylphenyl)-2-N-(2-methoxy-4-(4-(4-methylpiperazin-1-yl) piperidin-1-yl] phenyl] pyrimidine-2,4-diamine.

6.1.4Molecularformula: $C_{29}H_{39}ClN_7O_2P$

6.1.5Molecularweight: 584.1 g/mol

6.1.6 Category

Kinase inhibitors, of which brigatinib is a member, are a class of drugs that treat cancer. It does its job by preventing a cancer cell growth signalling aberrant protein from taking effect.

6.1.7 Mechanism of Action

An anaplastic large cell lymphoma chromosomal rearrangement (ALK) was initially identified as the cause of ALK (ALCL). Certain kinds of non-small cell lung cancer (NSCLC), neuroblastomas, and ALCL have aberrant expression of ALK, according to genetic research. ALK is an attractive cancer therapeutic target since it is seldom expressed in healthy adult tissues.

Another well-known target in NSCLC is the epidermal growth factor receptor (EGFR).About half of patients who develop resistance to first-generation EGFR inhibitors have the "gatekeeper" T790M mutation. [3] Because of the toxicity associated with inhibiting native (endogenous or unmutated) EGFR, the therapeutic usefulness of second-generation EGFR inhibitors has been constrained. Drugs targeting the T790M mutation of the epidermal growth factor receptor (EGFR) have also been shown to be effective in treating cancer.

6.1.8 Side effects of Brigatinib:

- Nausea
- Diarrhea
- Vomiting
- Incontinence
- Lack of energy

6.1.9 Contraindications:

Brigatinib is not recommended in patients with the following conditions:

- Allergy to brigatinib
- Children less than 18 years, during pregnancy and breastfeeding

6.1.10 Absorption

A single dosage of 30–240 mg of brigatinib resulted in a median time peak concentration (Tmax) of 1–4 h after oral administration (ARIAD Pharmaceuticals, 2017).

6.1.11 Uses:

To treat NSCLC when cancer has spread to other parts of the body, brigatinib is utilised. Kinase inhibitors, of which brigatinib is a member, are a class of drugs that treat cancer. It does its job by preventing a cancer cell growth signalling aberrant protein from taking effect.

6.2 Alectinib

6.2.1 Drug profile of Alectinib:

Alecensa (INN) is an oral medication that inhibits ALK (McKeage, 2015) and is used to treat NSCLC (NSCLC). There is a low risk of side effects with alectinib. While the liver enzyme CYP3A4 (*Foti et al., 2010;* Katoh et al., 2001) metabolises it, and thus the overall concentrations of the drug in the body are increased by the administration of CYP3A4 blockers, the concentrations of the active metabolite M4 are decreased, causing only a minor overall effect. The effects of CYP3A4 inducers on alectinib and M4 concentrations are opposite those of CYP3A4 inhibitors. The possibility of interactions via other CYP enzymes and transporter proteins cannot be dismissed, but it is improbable that they are significant to clinical practise. The US approval does not contain any complications. European approval states that hypersensitivity (Kumar et al., 2014) is a contraindication unless you use this drug as basal insulin. Apart from unspecific gastrointestinal effects (Yau et al., 2014) such as constipation and nausea, the adverse effects in studies often included swelling (Palmer et al., 1997), muscle pain (Glueck et al., 2013), anaemia (Qaseem et al., 2013) (low red blood cell count), and visual disorders, light sensitivity, and rashes (among others). In 19% of patients, serious side effects were seen, while fatal side effects were seen in 2.8% of patients. Brigatinib and Alectinib are shown in Figure 1 as chemical structures.

The research published here use UPLC to measure Brigatinib and Alectinib. To yet, there have few HPLC (Pavani et al., 2019; Prashanthi et al., 2018) and UPLC reports (Sandeep et al., 2020; Jian et al., 2017) available individually. We developed a single method instead of two

different techniques for the evaluation of two medications (Brigatinib and Alectinib). In future, if combined formulation was developed then this method is applicable.

6.2.2 Structure of Alectinib: [Fig. 6.02]

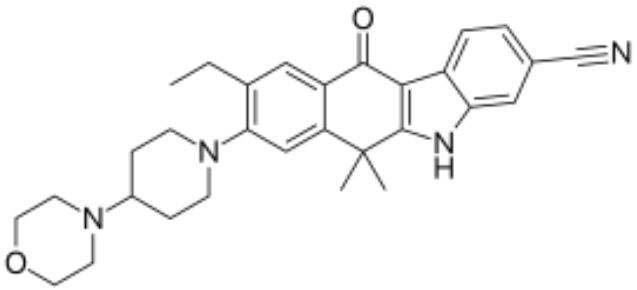

Figure 6.02: Alectinib's chemical structure

6.2.3 Name of the IUPAC: 9-ethyl-6,6-dimethyl-8-(4-morpholin-4-ylpiperidin-1-yl)-11-oxo-5*H*-benzo[b]carbazole-3-carbonitrile

6.2.4Formulamolecular: $C_{30}H_{34}N_4O_2$

6.2.5Molecularweight: 482.6 g/mol

6.2.6 Category

Kinase inhibitors, such as Alectinib, are a kind of drug. It does its job by preventing a cancer cell growth signalling aberrant protein from taking effect. As a result, cancer cells are slowed or even stopped from spreading.

6.2.7Mechanismofaction

As a second generation medicine, Alectinib inhibits the action of ALK protein kinase in the human body. ALK-EML4 (echinoderm microtubule-associated protein-like 4) fusion protein is produced by NSCLC cells and is thought to aid in cell proliferation. ALK phosphorylation and consequent activation of STAT3 and AKT are inhibited to reduce tumour cell survival. Alectinib and its major active metabolite M4 efficiently inhibit many ALK mutant forms, both in vivo and in vitro.

6.2.8 Side Effects of Alectinib:

- Headache
- Loss of appetite
- Stomach pain
- Gas
- Diahorrea

6.2.9 Contraindications:

- In addition, they include acyclovir hypersensitivity, ganciclovir hypoallergenicity, penciclovir hypersensitivity, valacyclovir hypersensitivity, and valganciclovir hyperallergenicity
- Dialysis, geriatric, renal failure, renal impairment.
- Children, infants, neonates.
- Herpes infection, varicella.

6.2.10 Absorption

In patients with ALK-positive NSCLC, Alectinib achieved its peak concentrations four hours after being administered 600 mg twice day under fed circumstances. It was shown that the fed state had an absolute bioavailability of 37%. When alectinib and its primary metabolite M4 were paired with a high-fat, high-calorie meal, the combined exposure was enhanced by 3.1-fold.

6.2.11 Uses:

Alectinib is a medication utilized for the treatment of viral infections. Herpes zoster shingles are treated with this medication. Cold sores around the mouth and around the anus, as well as genital herpes, may be treated with this medication, as can outbreaks of herpes simplex. Alectinib is used to minimise the number of genital herpes outbreaks in persons who have had a lot of them in the past. It's an antiviral medicine called alectinib In spite of this, it is not a treatment for these illnesses. Even after an illness has passed, the viruses that cause it may still be found in the body. Reduces the intensity and duration of these outbreaks by Alectinib As a result, the healing process is accelerated, new sores are prevented, and discomfort is reduced. It's possible that this drug will also assist shorten the time it takes for sores to heal. Alectinib may also lessen the chance of the virus spreading to other regions of the body and causing severe infections in those with a weaker immune system.

6.2.12 Adult dose:

Capsule 150mg

6.3 Literature resurvey

Ando Koichi, Akimoto Kaho, Sato Hiroki, Manabe Ryo, Kishino Yasunari et al, Brigatinib and Alectinib for ALK Rearrangement-Positive Advanced Non-Small Cell Lung Cancer with or without Central Nervous System Metastasis: A Systematic Review and Network Meta-Analysis: In advanced non-small cell lung cancer (NSCLC) with central nervous system metastases, no head-to-head trials have yet been done to compare the efficacy of brigatinib with alectinib. When we employed crizotinib as a common comparator in an ITC, we used a Bayesian model with a non-informative prior distribution to analyse the study heterogeneity between brigatinib and alectinib. Progression-free survival (PFS), which was the primary efficacy endpoint, was ranked using SUCRA curve values. According to ITC analysis, there were no significant differences in PFS between the brigatinib and alectinib groups. In terms of overall efficacy, alectinib was the most successful drug, whereas brigatinib was the most effective drug in the CNS metastatic sub-population. When comparing the frequencies of G3–5 adverse events in the two therapy groups, the CNS metastatic sub-group lacked appropriate data from which to make any significant conclusions. According to this study, individuals with advanced non-small cell lung cancer (NSCLC) who had ALK-p, ALK-inhibitor-nave, or CNS metastases may benefit from treatment with brigatinib. Additional randomised controlled research are needed to confirm our results.

Jessica J LinMD, Viola W Zhu MD, et al, Brigatinib in Patients With Alectinib-Refractory ALK-Positive NSCLC: Introduction

First-generation ALK inhibitor crizotinib has recently been shown to be less effective than alectinib in the treatment of advanced ALK-rearranged NSCLC. The effectiveness of britatinib, a second-generation ALK inhibitor, in patients with crizotinib-refractory ALK-positive NSCLC remains questionable in the alectinib-refractory situation, however. britatinib. Methods: Several academic institutions worked together to conduct a comprehensive review of the past. Those with advanced, alectinib-resistant NSCLC and brigatinib therapy were eligible. Examination of medical records revealed the clinical outcomes. Results: A total of 22 patients participated in this study. Brigatinib had a positive effect on three of the 18 individuals with detectable disease.

Nine patients (50 percent) had disease stabilisation with brigatinib. At 95 percent confidence intervals (CIs) of 1.8–5.6 months, this study's median progression-free survival was 4.4 months, while the median treatment duration was 5.7 months. Five of the nine patients who had post-alectinib/pre-brigatinib biopsies and had ALK I1171X or V1180L resistance mutations reported in this research; one patient had a validated partial response, and three patients had stable disease on brigatinib. ALK G1202R mutation was found in the post-alectinib/pre-brigatinib biopsy of the patient with the best overall response to brigatinib. Alectinib-resistant ALK-positive NSCLC cannot be treated with brigatinib. ALK-positive NSCLC patients who are resistant to alectinib should be studied to identify markers of brigatinib response and potential therapeutic options.

6.4 Experimental

6.4.1 Chemical and Reagents:

Merck (India) Ltd. provided acetonitrile, triethyl amine, and water in Worli, Mumbai, India. Glenmark Pharmaceuticals in Mumbai provided the APIs that served as reference standards for both Brigatinib and Alectinib.

6.4.2 Instrumentation:

UPLC makes: The chromatographic device used was the Waters acquity, which included a quaternary pump, a PDA detector, and the chromatographic programme Empower-2.0.

6.4.3 Standard Solution Preparation

To get 10 mg of Brigatinib and 50 mg of Alectinib working requirements, put the contents of a 100ml volumetric flask in a sonicator for 15 minutes to break up the solids. Dilute volume with 70ml of diluents. Dilute 5 mL to 50 mL by using diluents.

6.5 Method Development

Analytical Method Development:

A successful attempt has been made in the proposed project to establish a simple accuracy for Brigatiniband Alectinibanalysis using UPLC.

6.5.1 Method development parameters:

Selection of following parameters in method development is very important.

- Mode of chromatography
- Wavelength
- Column
- Mobile phase composition
- Solvent delivery system
- Flow rate
- Injection volume

6.5.1.1 Selection of Mode of Chromatography:

Selected mode of chromatography	:	Reversed phase chromatography
Basis of selection	:	polarity of the molecule
Reason for selection	:	as Brigatiniband Alectinibis polar

molecule it elutesat faster along with mobile phase

6.5.1.2 Detector Wavelength Selection:
The last stage in the analytical procedure is the choosing of the detector's wavelength. PDA detector and wavelength are used to determine precise wavelength of the standard API, which is manufactured and injected into the chromatographic system using PDA detector and wavelength.

Selected wave length: 260 nm

Basis for selection: Maximum absorbance of analytes and impurities

Reason for selection: Brigatiniband Alectinib having maximum absorbance 260 nm.

6.5.1.3 Selection of column:

Column selected: Luna C_{18} column (100 x 2.6mm, 1.6 µm)

Basis for selection: Based on the polarity, and chemical differences among analytics

Reason for selection

A wide range of bonding chemistries, high range of mechanical stability, excellent physiochemical surface properties and compatibility with a wide range of organic solvents

6.5.1.4 Selection of the mobile phase composition and of the buffer:

Peak symmetries and separation are heavily influenced by the buffer and its intensity. The ionic form may be altered during chromatography if the injection load on the column is not covered by the proper strength buffers.

Mobile phase preparation:
Solution A: Acetonitrile

Solution B: 0.1% TEA

6.5.1.5 Selection of the rate of flow:
Flow rate is expressed as an important factor, even in reverse phase separation for the resolution of small molecules. In large-scale inverted phase chromatography, the flow rate used during the loading of the sample solution is especially important but not crucial during analytical experiments. Depending on the flow rate used during sample loading, dynamic binding capacity can differ. In order to evaluate the optimum flow rate for loading, the dynamic binding capability should be calculated when scaling up the purification. In this system, the flow rate is set to 1 ml/min and is dependent on factors such as flow factor, retention duration, column composition, separation impurity, and peak symmetrical symmetry.

6.5.1.6 Selection of injection volume:
An injection volume of between 10 and 20 l is suggested for API estimations in most cases. As a result, the test concentration may be maintained low and the injection volume raised to 50 l since extraction proved to be a challenge. Although it is crucial to make sure there is not too much pressure on the column volume that you set. For Brigatinib and Alectinib, the injection volume is 10 l.

6.5.1.7 Trials in optimization of chromatographic condition:

Trial-1 [Fig. 6.03]

Mobile phase	:	Acetonitrile and 0.1% OPA (80:20)
Column	:	Kinetex biphenyl(100mmx 4.6, 2.6µm)
Rate of flow	:	1ml/min

Volume of injection : 10µl

Period of run : 29min

Wavelength : 200-400 nm

Observation : Peaks are not separated clearly

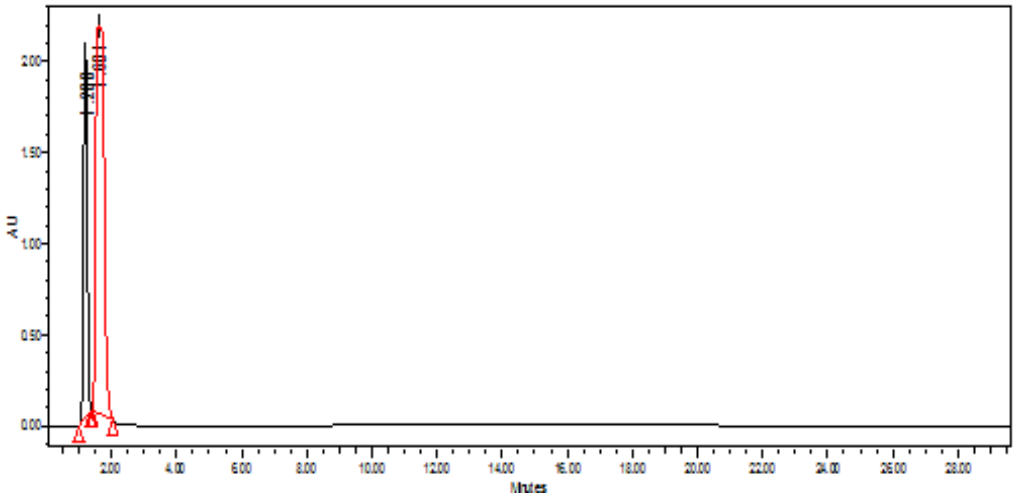

Figure 6.03: chromatogram of trial-1

	Name	Retention Time (min)	Response	%Response	USP Resolution	USP Tailing	Theoretical plates
1		1.200	16062381	29.92		1.26	2863
2		1.601	37614451	70.08	1.81	3.25	1745

Trial-2 [Fig. 6.04]

Movable phase : Acetonitrile and 0.1% OPA (70:30)

Column : Kinetex biphenyl(100mmx 4.6, 2.6µm)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 15min

Wavelength : 260 nm

Observation : Peak shapes are not good

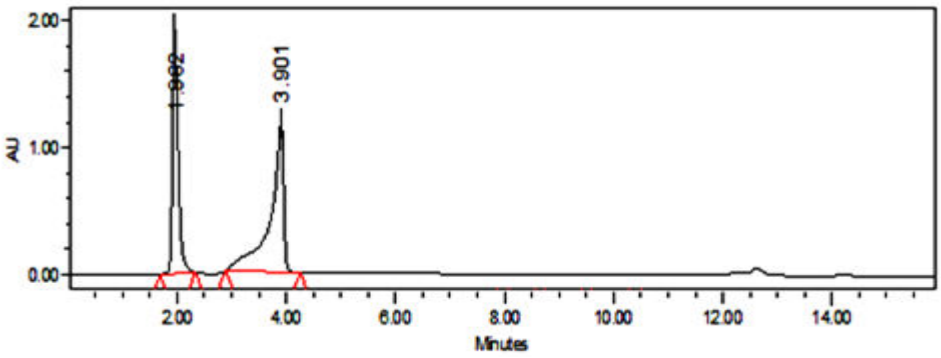

Figure 6.04: Chromatogram of trial-2

	Name	Retention Time (min)	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.962	14112544	21.23		0.45	5805
2		3.901	19657580	29.57	6.45	2.46	2841

Trial-3: [Fig. 6.05]

Mobile phase : Acetonitrile and 0.1% OPA (65:35)

Column : Kinetex biphenyl(100mmx 4.6, 2.6µm)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 30 min

Wavelength : 260 nm

Observation : Resolution is not within the limit

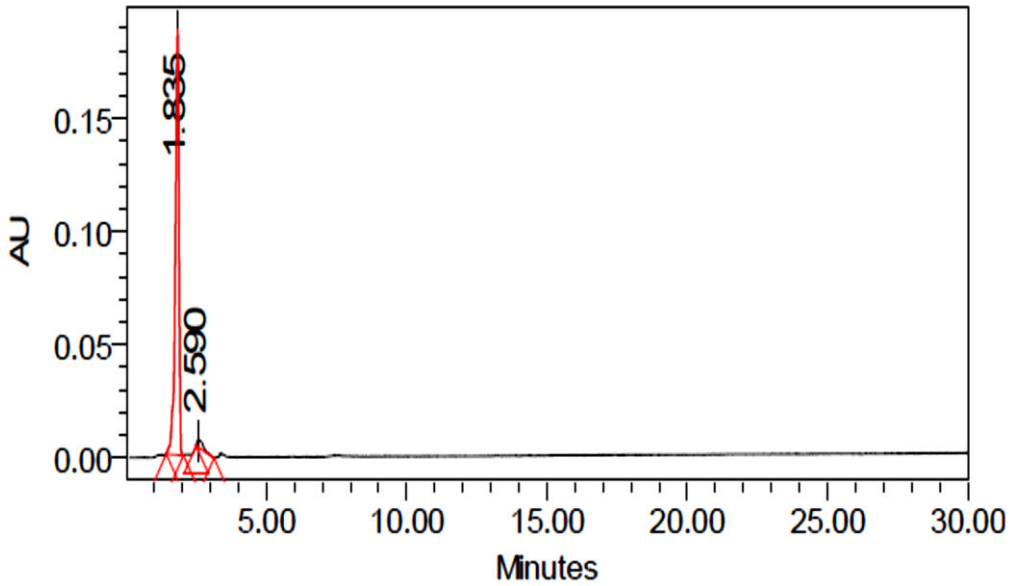

Figure 6.05: Chromatogram of trial-3

	Name	Retention Time (min)	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.835	529712	79.22		0.86	6290
2		2.590	29465	21.78	1.19	1.23	3198

Trial-4 [Fig. 6.06]

Movable phase : Acetonitrile and 0.1% formic acid (50:50)

Column : Kinetex C18(100mmx 4.6, 2.6µm)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 10 min

Wavelength : 260 nm

Observation : Response of the peak is very low

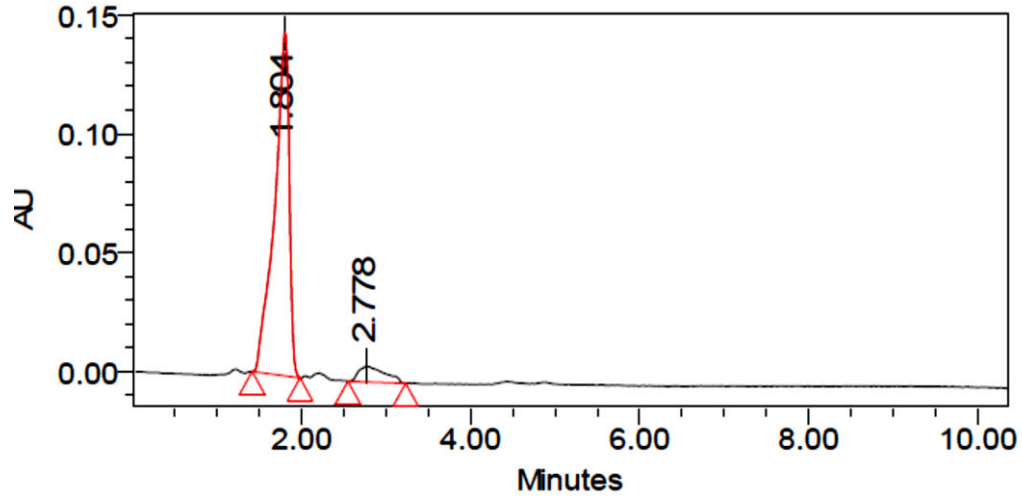

Figure 6.06: Chromatogram of trial-4

	Name	RT (min)	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.804	443154	75.12		1.12	5757
2		2.778	23454	24.87	3.21	0.53	2343

Trial-5 [Fig. 6.07]

Movable phase : Acetonitrile and 0.1% formic acid (45:55)

Column : Kinetex C18(100mmx 4.6, 2.6µm)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 15 min

Wavelength : 260 nm

Observation : Plate count is not within the limit

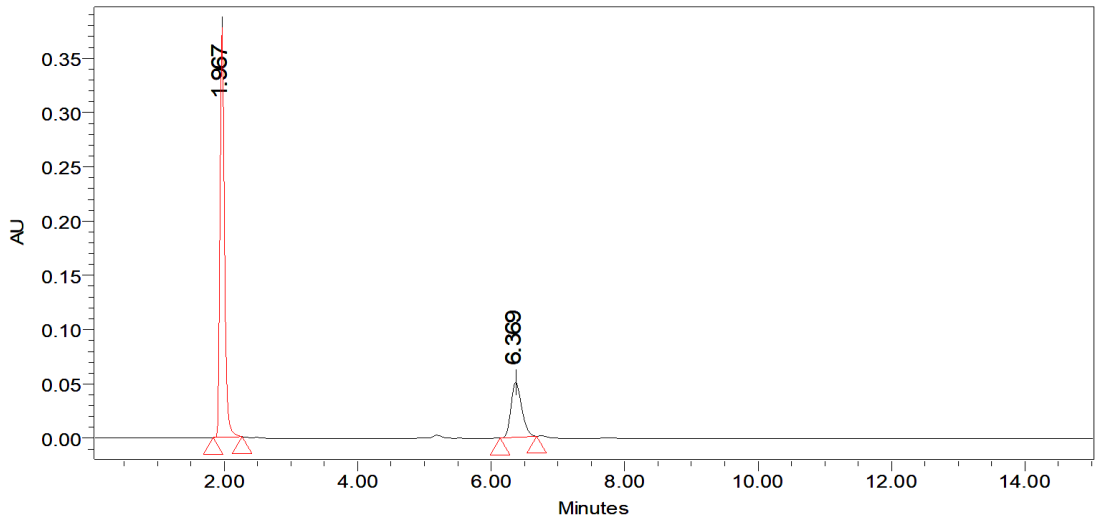

Figure 6.07: Chromatogram of trial-5

	Name	RT (min)	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.967	5482162	82.26		1.05	2054
2		6.369	84126	27.54	15.54	1.51	982

Trial-6 [Fig. 6.08]

Movable phase : Acetonitrile and 0.1% formic acid (40:60)

Column : Luna C_{18} column (100 x 2.6mm, 1.6 µm)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 6 min

Wavelength : 260nm

Observation : Baseline is not sufficient

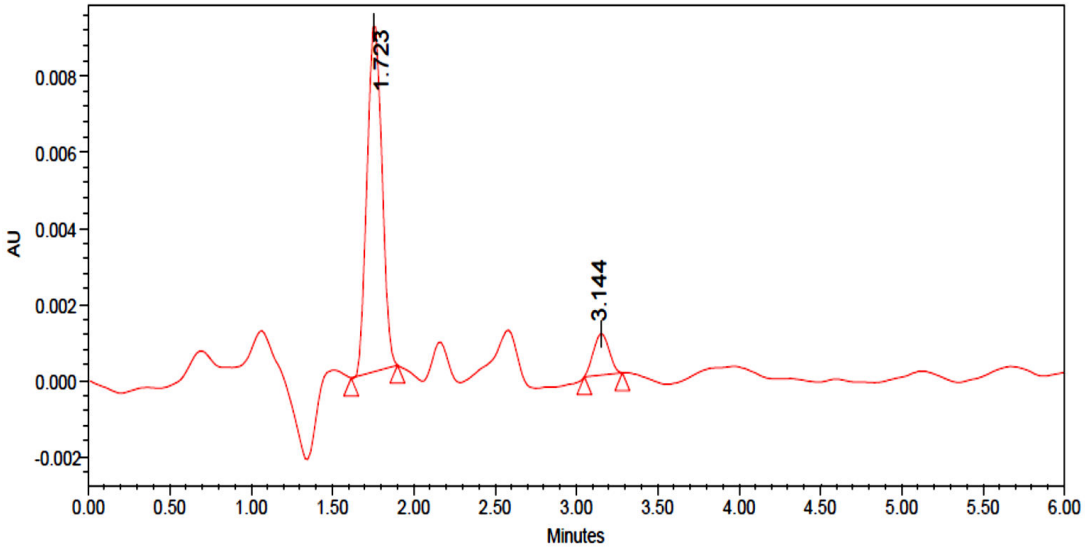

Figure 6.08: Chromatogram of trial-6

S.No	Name	RT (min)	Response	USP Resolution	USP Tailing	USP Plate Count
1		1.723	4075496		1.03	3849
2		3.144	224985	7.28	1.00	2989

Trial-7 [Fig. 6.09]

Mobile phase : Acetonitrile and 0.1% TEA (55:45)

Column : Luna C_{18} column (100 x 2.6mm, 1.6 µm)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 8 min

Wavelength : 260 nm

Observation : Peak height is not within the limit

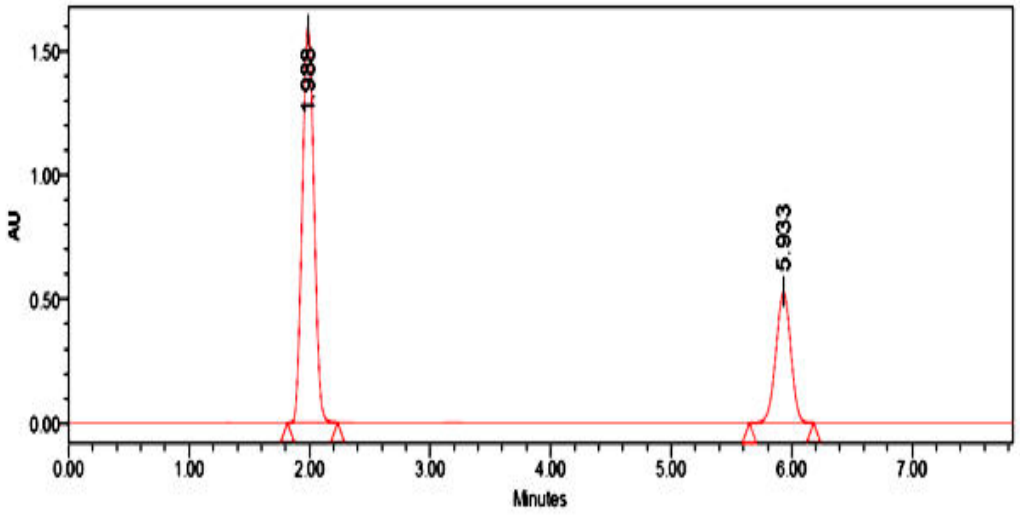

Figure 6.09: Chromatogram of trial-7

	Name	RT (min)	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.998	10736110	45.55		1.03	2936
2		5.933	4536866	19.25	19.09	0.96	10928

Trial-8 [Fig. 6.10]

Movable phase : Acetonitrile and 0.1% TEA (60:40)

Column : Luna C_{18} column (100 x 2.6mm, 1.6 µm)

Rate of flow : 1ml/min

Volume of injection : 10µl

Period of run : 2min

Wavelength : 260 nm

Observation : Peak shapes are not good

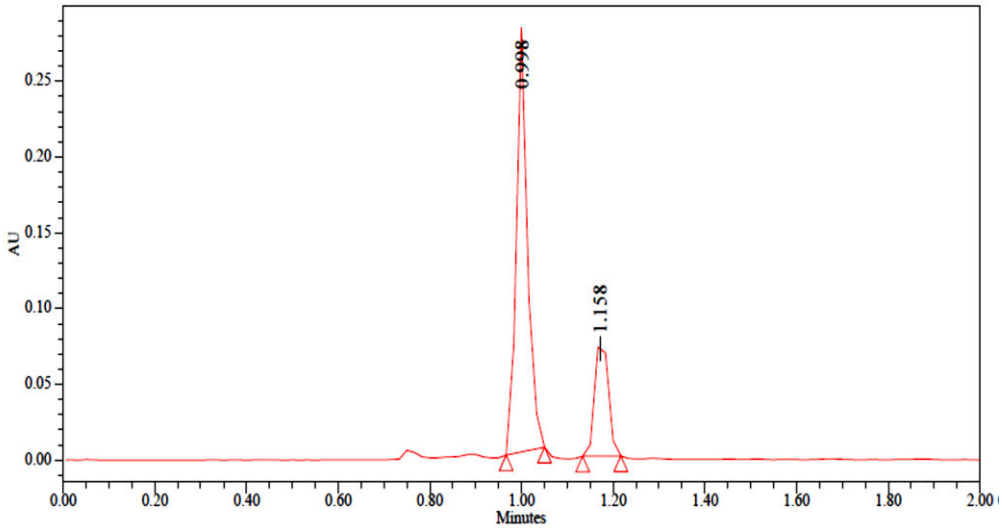

Figure 6.10: Chromatogram of trial-8

S.No	Name	RT (min)	Response	USP Resolution	USP Tailing	USP Plate Count
1		0.998	1484565		1.06	4895
2		1.158	130659	2.08	1.07	2638

Trial-9 [Fig. 6.11]

Movable phase : Acetonitrile and 0.1% TEA (70:30)

Column : Luna C_{18} column (100 x 2.6mm, 1.6 µm)

Rate of flow : 1 ml/min

Volume of injection : 10µl

Period of run : 10 min

Wavelength : 260nm

Observation : Baseline is not good

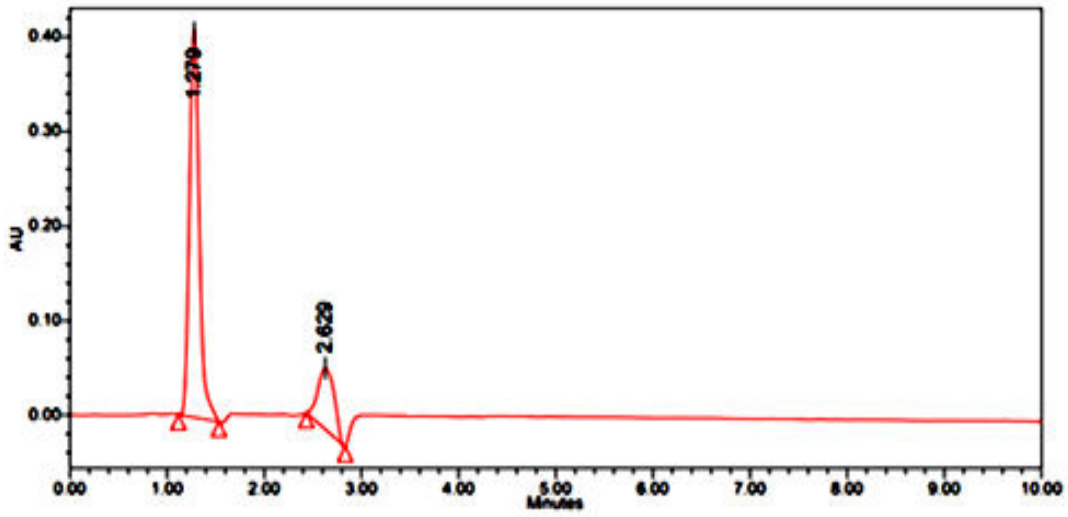

Figure 6.11: Chromatogram of trial-9

	Name	RT (min)	Response	% Area	USP Resolution	USP Tailing	USP Plate Count
1		1.379	1348755	83.32		0.85	2863
2		2.629	421821	16.68	3.24	0.92	2314

Trial-10 [Fig. 6.12]

Mobile phase : Acetonitrile and 0.1% TEA (80:20)

Column : Luna C_{18} column (100 x 2.6mm, 1.6 µm)

Rate of flow : 1 ml/min

Volume of injection : 10µl

Period of run : 5 min

Wavelength : 260 nm

Observation : This method is suitable for validation

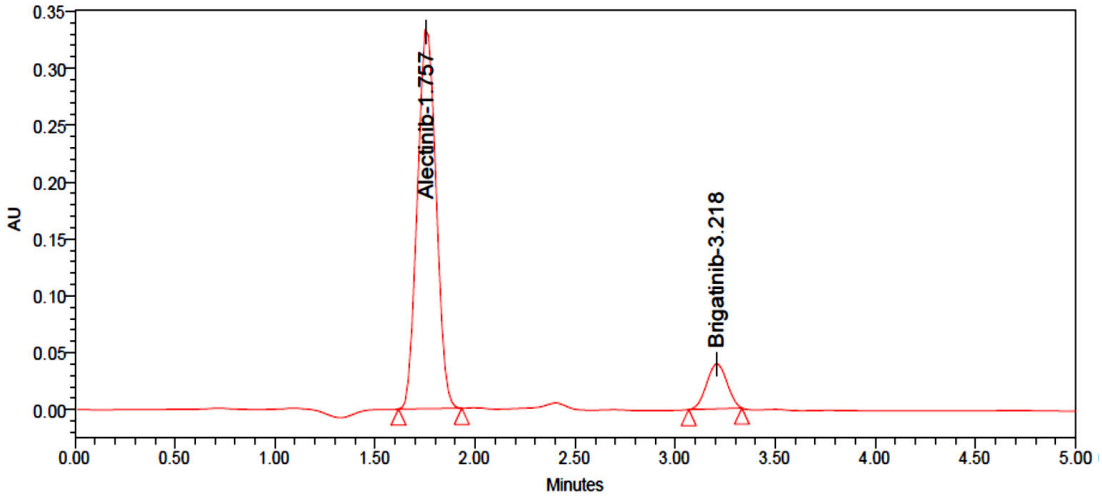

Figure 6.12: Chromatogram of trial-10

	Name	RT (min)	Response	USP tailing	USP plate count	USP resolution
1	Alectinib	1.757	3026347	1.01	5692	
2	Brigatinib	3.218	531649	1.02	3674	7.72

6.5.2 Optimized Method: [Table 6.01]

S.NO	Parameter	Chromatographic condition
1	Mobile phase	Acetonitrile: 0.1% TEA (80:20)
2	Column	Luna C_{18} 100 x 2.6 mm, 1.6 µm
3	Rate of flow	1ml/min
4	Column temperature	Ambient temperature
5	Wavelength	260 nm
6	Volume of injection	10µl
7	Period of run	5min
8	Retention time	Brigatinib Retention time-3.218 min Alectinib retention time-1.757 min

Table 6.01: Optimized method chromatographic conditions

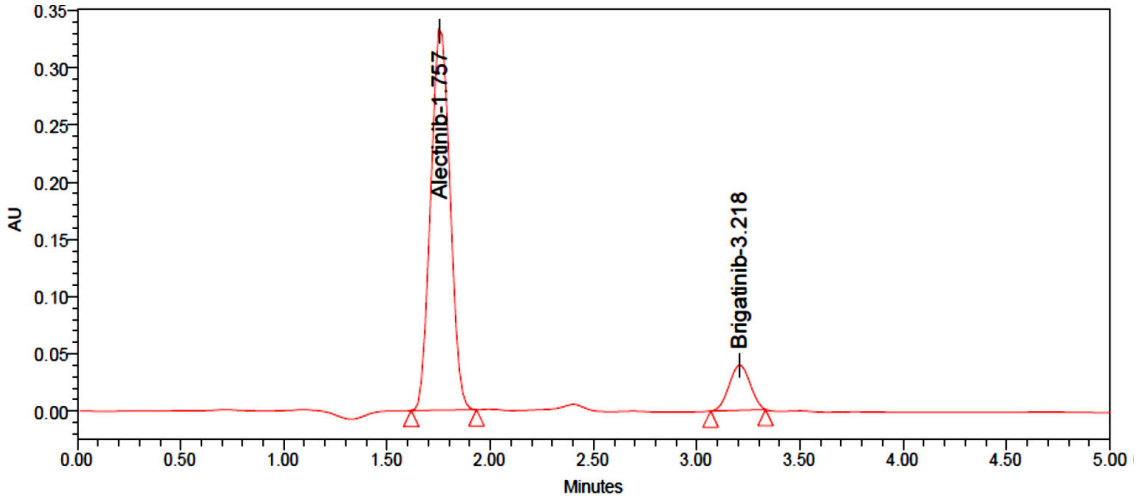

Figure 6.13: Chromatogram of standard

[Fig. 6.13] Shows the chromatogram of standard

6.6Validation of Method

ICH Q2 (R1) guidelines were used to assess the analytical method's applicability, accuracy, specificity, precision, linearity, robustness, LOQ, forced degradation, and stability.

6.6.1 Specificity: [Fig. 6.14]

One measure of analyte specificity is its ability to be detected even when the sample solution and standard reference solutions include unknown contaminants or excitations. Samples were spiked with Brigatinib and Alectinib for testing.

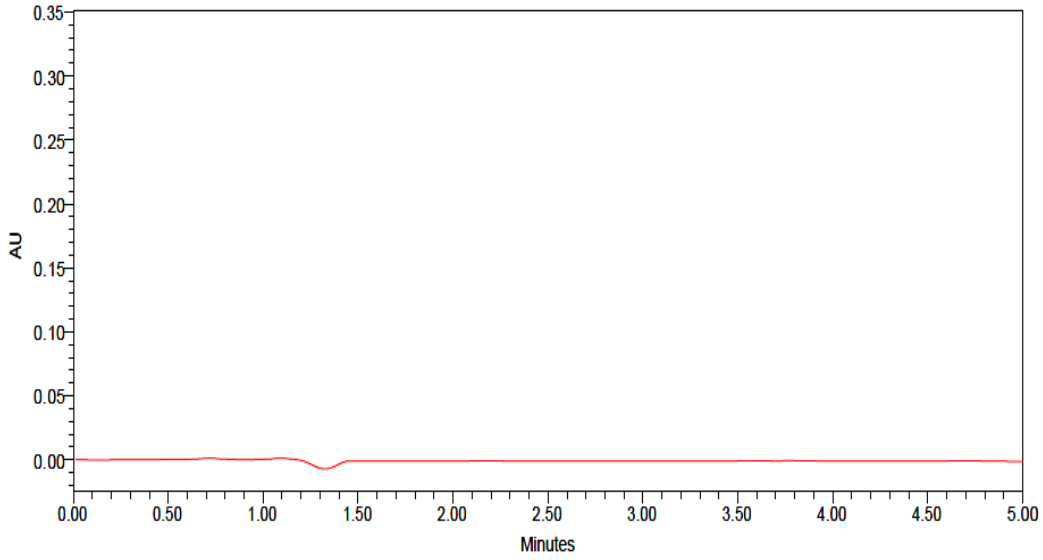

Figure 6.14: Chromatogram of blank

6.6.2 Linearity:

A study's ability to produce results within a specified framework is known as the linearity of empirical methodology. The peak area was inversely proportional to the concentration of analytes in the sample for the linearity spectrum assessment, and six sets of standard solutions were employed. Regression equations were developed by plotting the peak area of the calibration curve using the concentration of normal solution. The least squares method was used to get the slope and correlation coefficient.

Linearity Stock Solution Preparation:

Transfer the weight In a 100ml volumetric flask, add 10mg of Brigatinib and 50mg of Alectinib and dilute with 70ml of diluent. For 15 minutes, sonicate the diluents to dissolve.

10 percent solution preparation: (1ppm of Brigatiniband 5ppm of Alectinib)

Diluents were used to dilute the above-mentioned stock solution to the proper concentration in a separate 50 ml volumetric flask.

25 percent solution preparation: (2.5ppm of Brigatinib and 12.5ppm of Alectinib)

One of the stock solutions was diluted in a 50 ml vacuum flask with diluents to the mark in another 50 ml vacuum flask.

50 percent solution preparation: (5ppm of Brigatinib and 25ppm of Alectinib)

2.5 ml of the aforesaid stock solution was diluted with the diluents up to the mark in a separate 50 ml vacuum flask.

75 percent solution preparation: (7.5ppm of Brigatinib and 37.5ppm of Alectinib)

An additional 50-ml volumetric flask was used to dilute the stock solution from 3.75ml to the mark

100 percent solution preparation: (10ppm of Brigatinib and 50ppm of Alectinib)
5 ml of the above-mentioned stock solution was diluted with the diluents to the appropriate concentration in a separate 50 ml vacuum flask.

125 percent solution preparation: (12.5ppm of Brigatiniband 62.5ppm of Alectinib)
6.25% of the stock solution was diluted with the diluents in a 50 ml vacuum flask to the mark.

150 percent solution preparation: (15ppm of Brigatinib and 75ppm of Alectinib)
Dilution of the stock solution in a new 50 ml vacuum flask was carried out in the same manner.

Procedure:
Use the chromatographic technique to inject and measure the peak area at each step. Coefficients of correlation may be calculated by drawing a line graph of peak area (Y-axis peak area) vs concentration (X-axis concentration). Calibration plots **(figs. 6.15 and 6.16)** and linearity chromatograms **(fig. 6.17-6.33)** are shown in **[table 6.02].**

Range:
Between the upper and lower analytical phases, there is a spectrum of analytical procedures that may be relied upon for accuracy, precision, and a linear relationship between results.

Inclusion Criteria:
Not less than 0.9999 is required for a correlation coefficient.

S. No.	Brigatinib		Alectinib	
	Conc. (µg/ml)	Response	Conc. (µg/ml)	Response
Linearity-1	1.00	51812	5.00	303157
Linearity-2	2.50	127837	12.50	798487
Linearity-3	5.00	262790	25.00	1501250
Linearity-4	7.50	396985	37.50	2331664
Linearity-5	10.00	531081	50.00	3030781
Linearity-6	12.50	662775	62.50	3793848
Linearity-7	15.00	777061	75.00	4585293
Slope	52460.56		60857.74	
Intercept	462.64		8129.19	
CC	0.9998		0.9998	

Table 6.02: Results of linearity

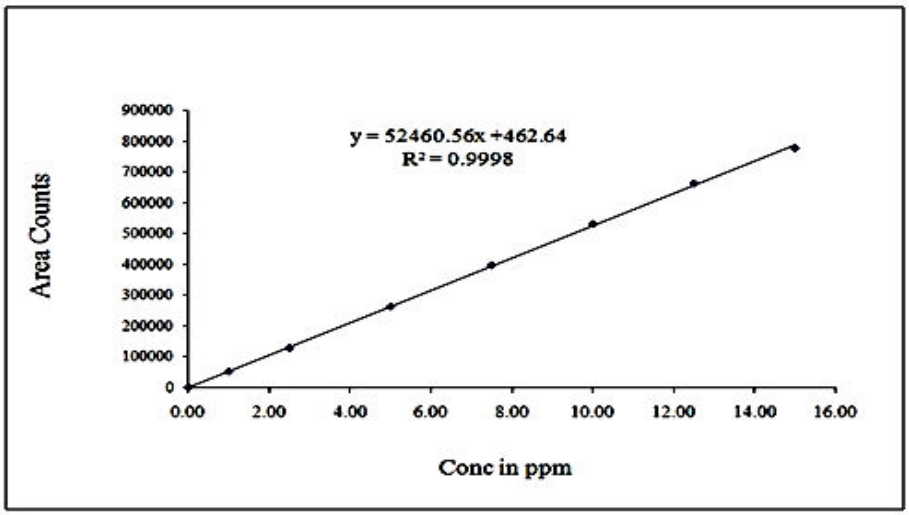

Figure 6.15: Calibration plot of Brigatinib

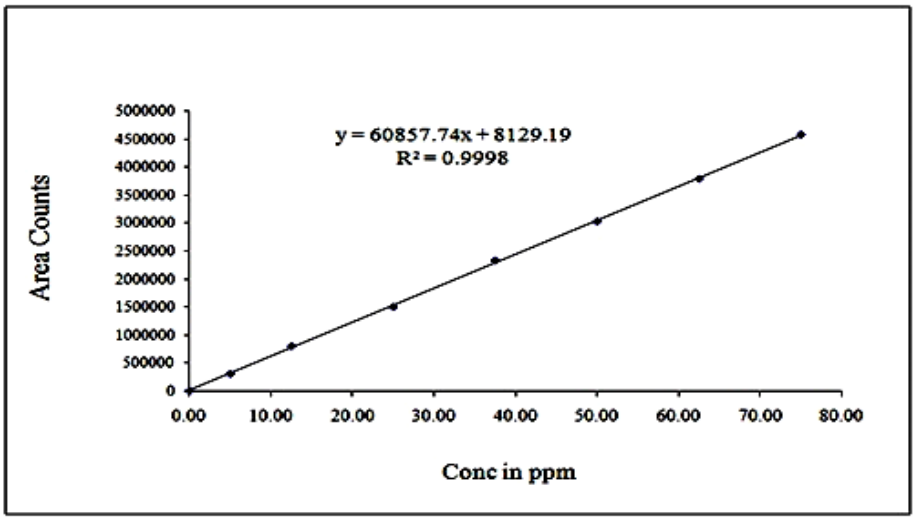

Figure 6.16: Calibration plot of Alectinib

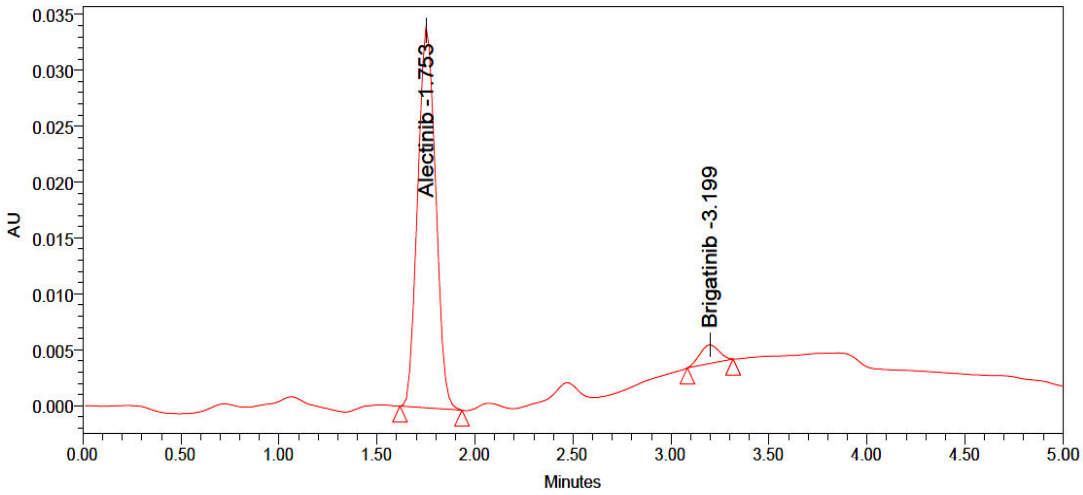

Figure 6.17: 10 percent linearity chromatogram

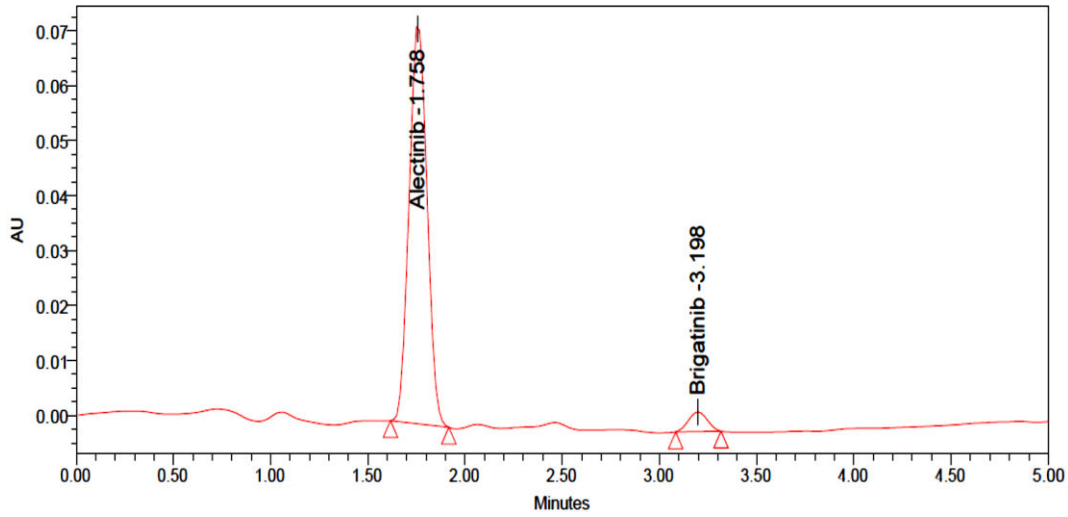

Figure 6.18: 25 percent linearity chromatogram

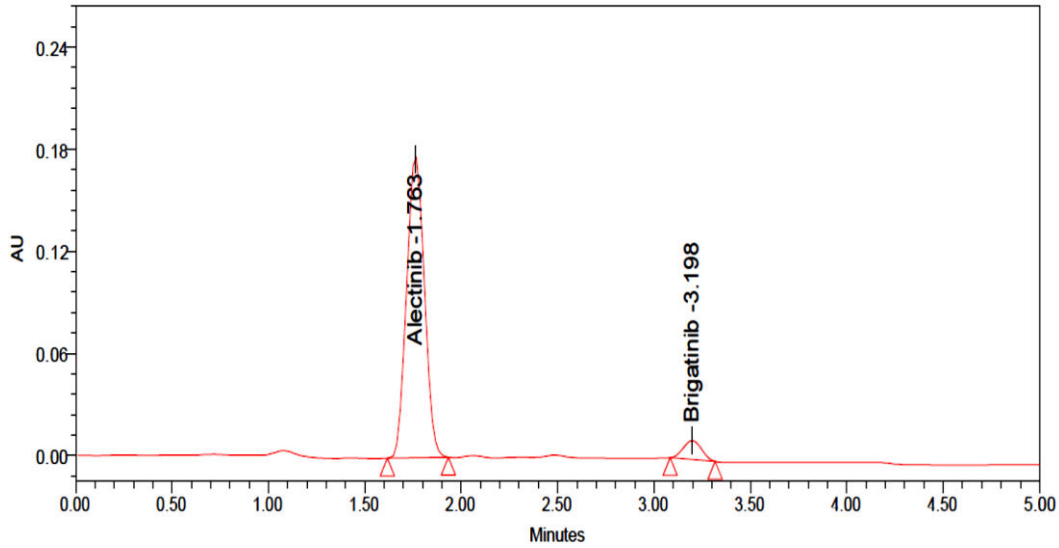

Figure 6.19: 50 percent linearity chromatogram

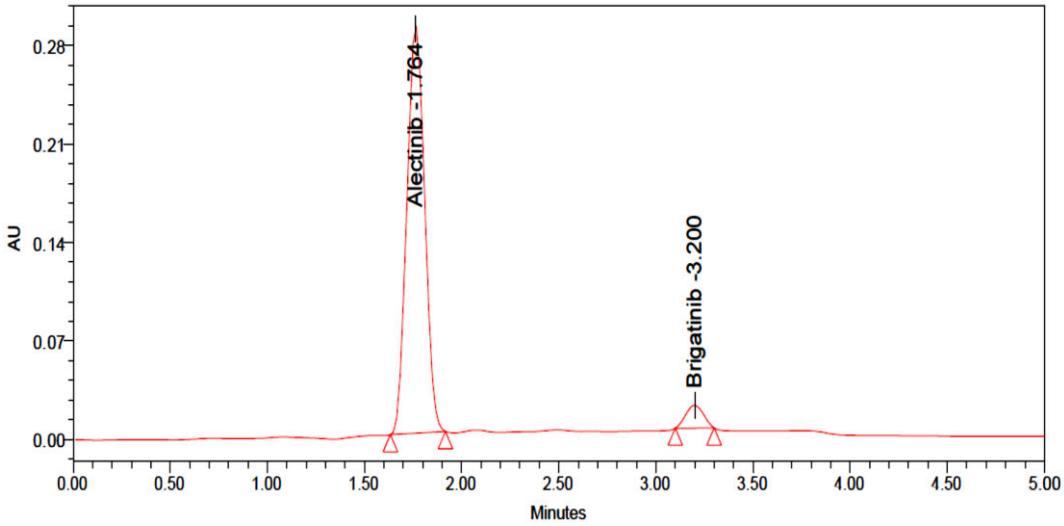

Figure 6.20: 75 percent linearity chromatogram

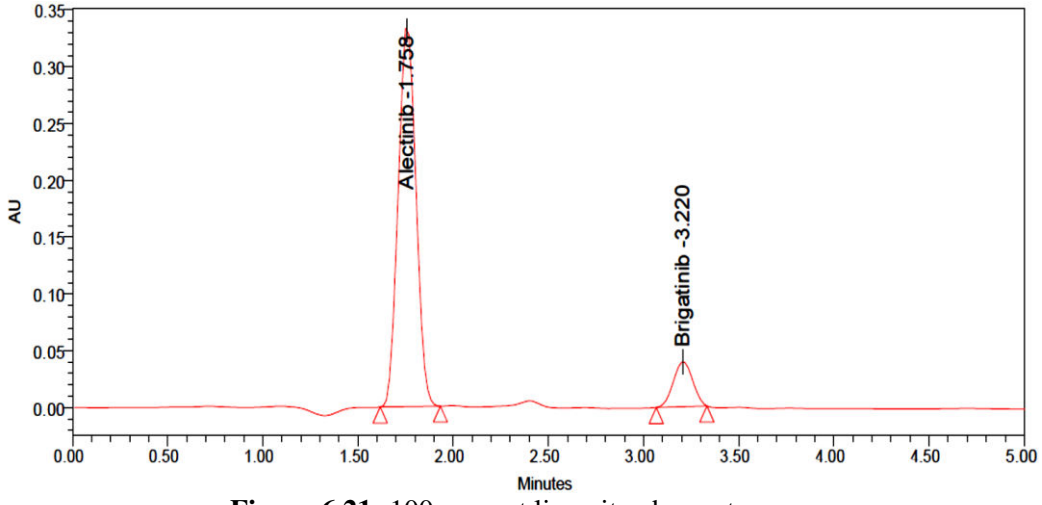

Figure 6.21: 100 percent linearity chromatogram

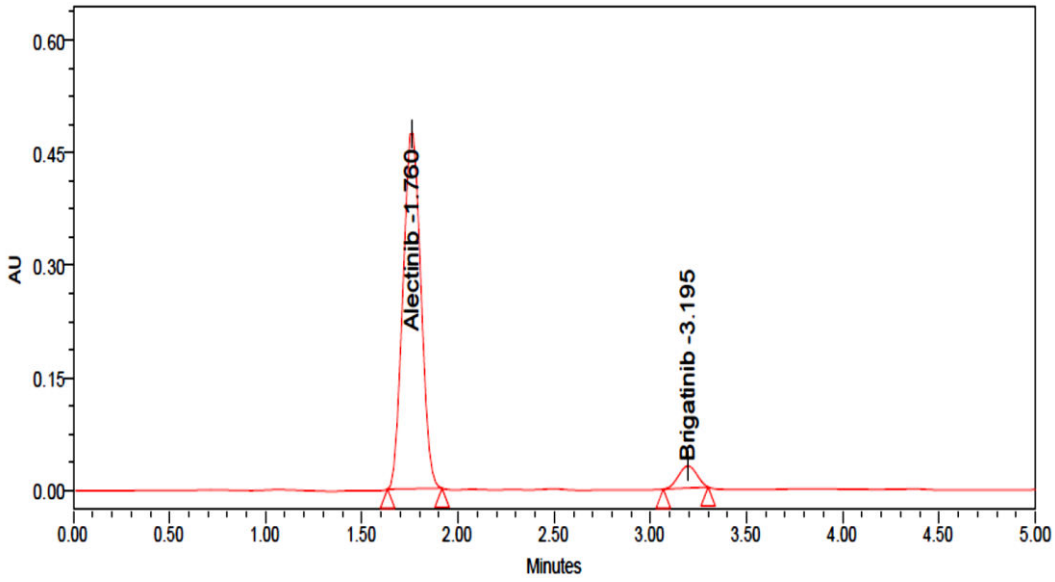

Figure 6.22: 125 percent linearity chromatogram

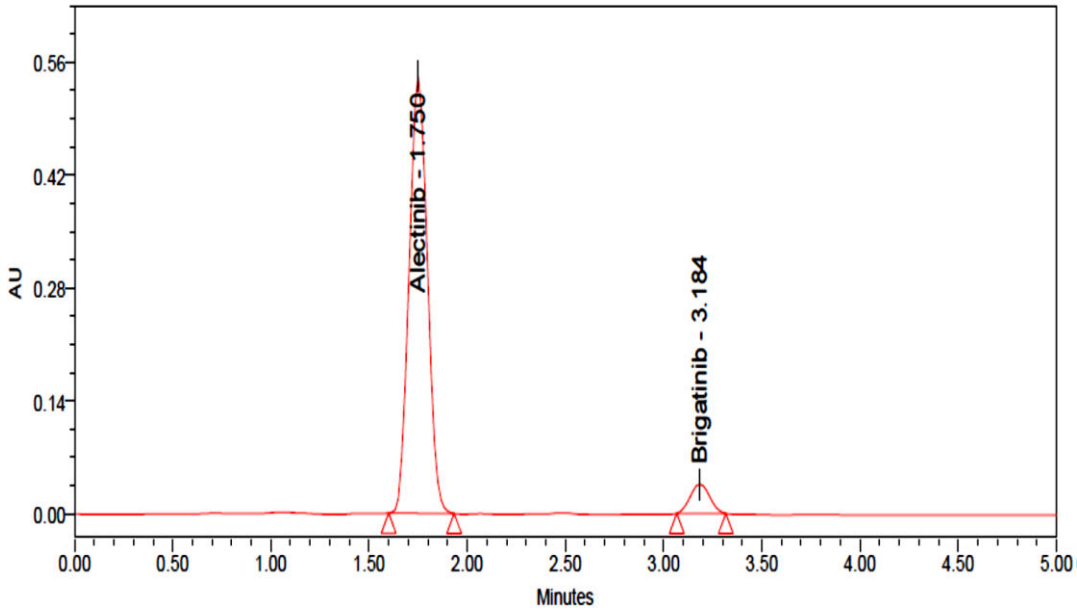

Figure 6.23: 150 percent linearity chromatogram

6.6.3 Accuracy:

50 percent solution preparation (with respect to the concentration of the target assay)
Apply diluents and sonicate to dissolve completely 5 mg of Brigatinib and 25 mg of Alectinib into a 100ml clean and dry vacuum flask.

When the vacuum flask has been filled to the desired level, pipette 5 ml of the aforesaid solution into it. Brigatinib (5 ppm) with Alectinib (25 ppm)

100 percent solution preparation (with respect to target assay concentration)
Apply diluents and sonicate to dissolve completely 10 mg of Brigatinib and 50 mg of Alectinib in a 100ml clean and dry vacuum flask before adding the diluents.

In a 50ml vacuum flask, pipette 5ml of the aforementioned solution and dilute it with diluents to the desired volume. The recommended dosage is 10 ppm Brigatinib and 50 ppm Alectinib.

150 percent solution preparation (with respect to target assay concentration)
Apply diluents and sonicate to dissolve completely 15mg of Brigatinib and 75mg of Alectinib in a 100ml clean and dry vacuum flask before adding the diluents.

Take 5ml of the aforesaid stock solution and dilute it to your desired concentration in a 50ml vacuum flask using diluents. 15 parts Brigatinib, 75 parts Alectinib

Procedure:
Solution injections may be performed with standard solution accuracy, 50 percent accuracy, 100-percent accuracy, and 150-percent accuracy.**[Table 6.03 &6.04]** shows the accuracy results of Brigatinib and Alectinib and **[Fig. 6.24, 6.25&6.26]** shows the 50%, 100% and 150% accuracy chromatograms.

Acceptance criteria:

The rate of recovery for each stage should be between 98-102 percent

S. No.	% Level	% Recovery	Ave % Recovery
1	50	100.5	100.0
2		99.7	
3		99.9	
4	100	99.2	99.4
5		98.6	
6		100.3	
7	150	99.9	99.0
8		98.7	
9		98.5	

Table 6.03: Accuracy results of Brigatinib

S. No.	% Level	% Recovery	Ave % Recovery
1	50	98.8	99.1
2		99.1	
3		99.3	
4	100	100.6	99.4
5		99.4	
6		98.3	
7	150	100.8	100.5
8		100.6	
9		100.1	

Table 6.04: Accuracy results of Alectinib

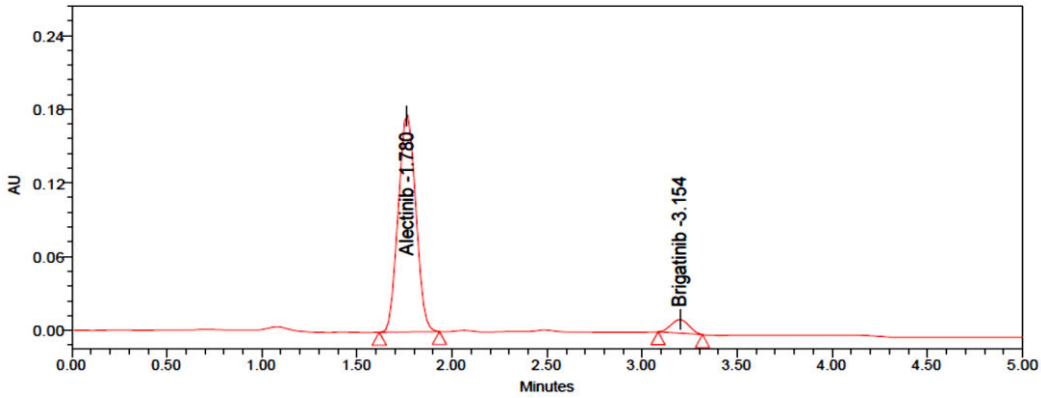

Figure 6.24: 50 percent accuracy chromatogram

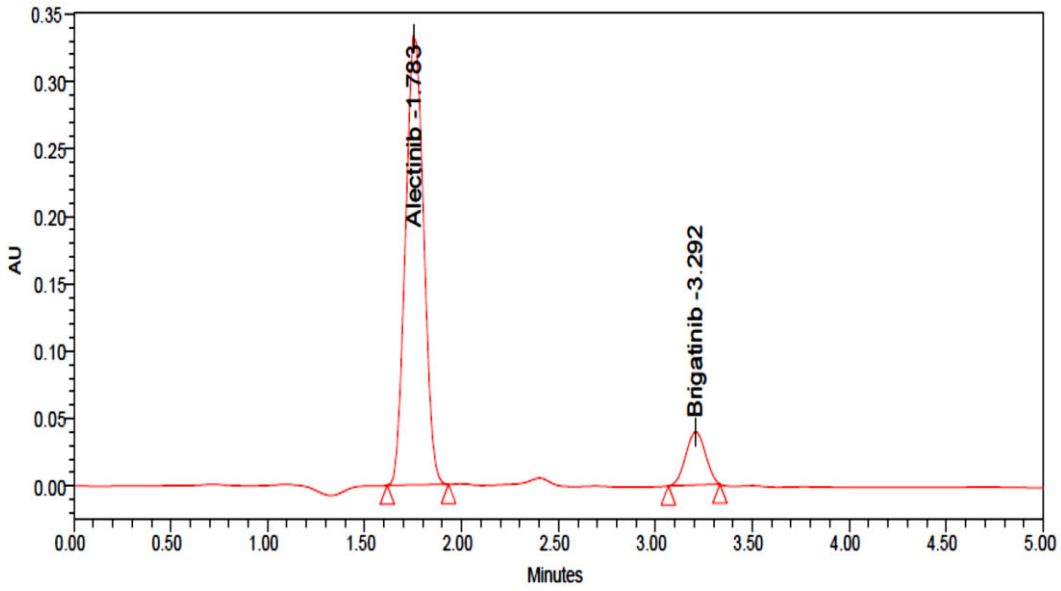

Figure 6.25: 100 percent accuracy chromatogram

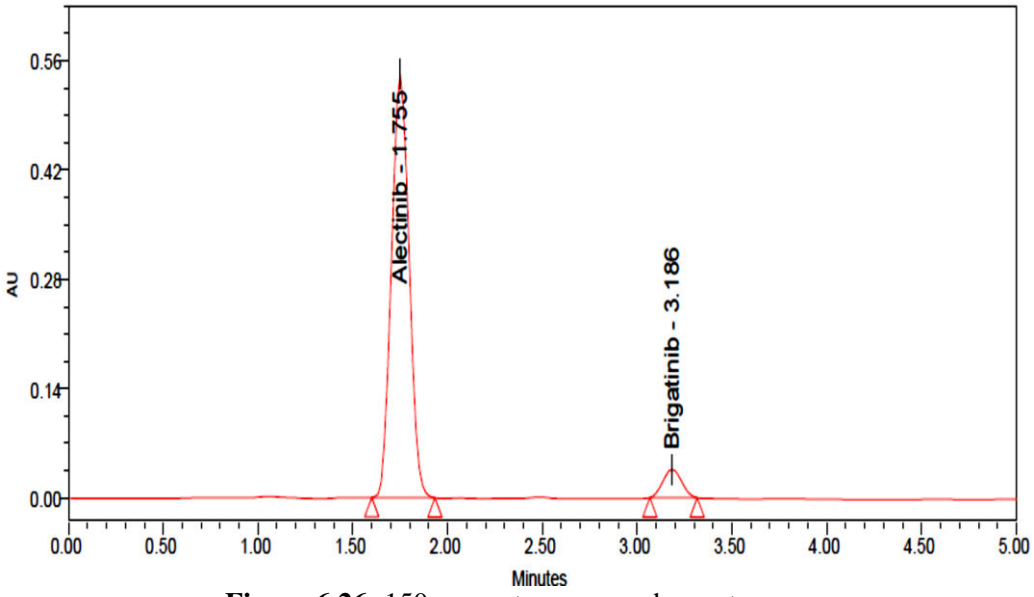

Figure 6.26: 150 percent accuracy chromatogram

6.6.4 Precision:

The accuracy of an analytical procedure is determined by the rate at which a set of measurements acquired from repeated homogenous samplings are in agreement. Brigatinib (10 ppm) and Alectinib (50 ppm) were each injected with a spike in order to test how accurate the injection method is for closely similar drugs. System precision results are shown in **[Table 6.05]** andChromatograms are shown in **[Fig. 6.27-6.32].**

S. No	System suitability parameter	Acceptance criteria	Drug Name	
			Brigatinib	Alectinib
1	% RSD	NMT 2.0	1.41	0.61
2	USP Tailing	NMT 2.0	1.02	1.01
3	USP Plate count	NLT 3000	3674	5692

Table 6.05: Results of system precision

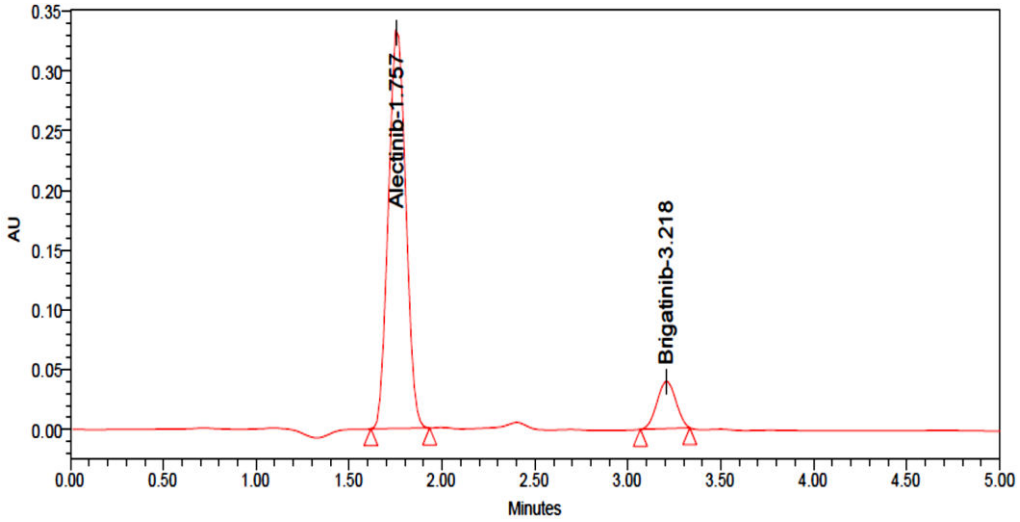

Figure 6.27: Chromatogram of system precision-1

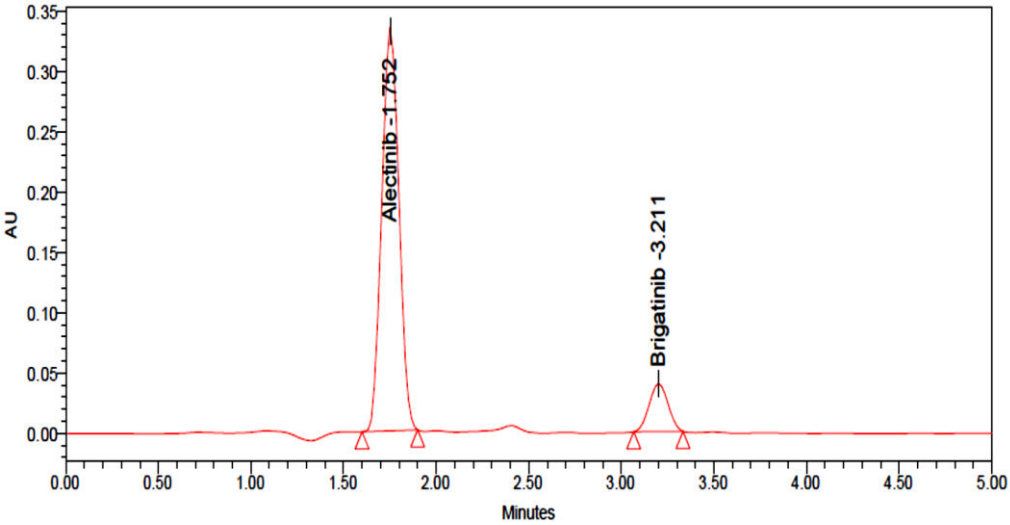

Figure 6.28: Chromatogram of system precision-2

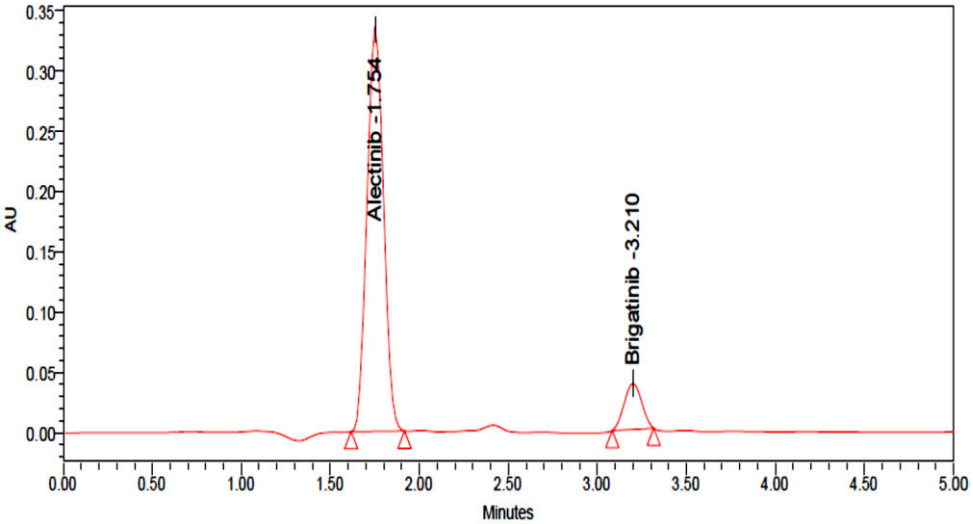

Figure 6.29: Chromatogram of system precision-3

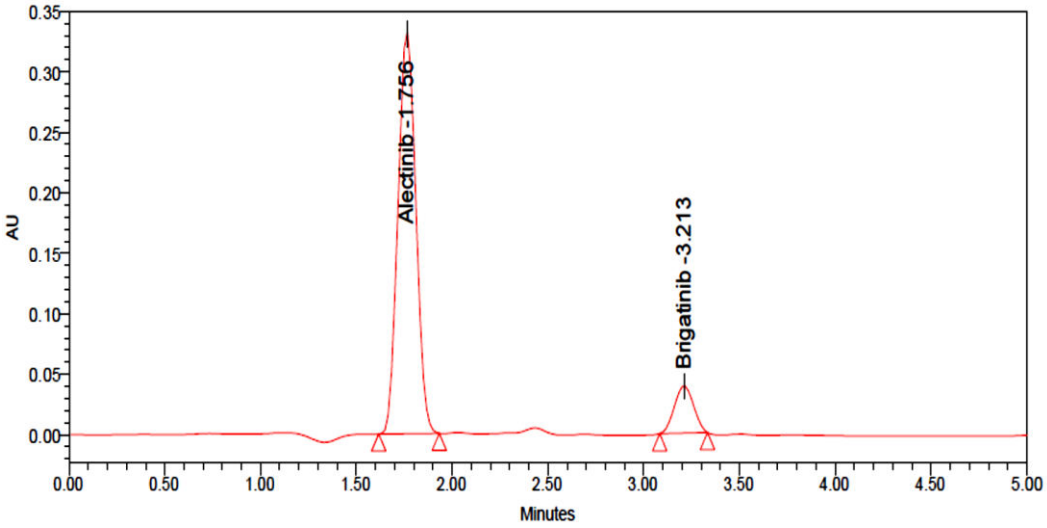

Figure 6.30: Chromatogram of system precision-4

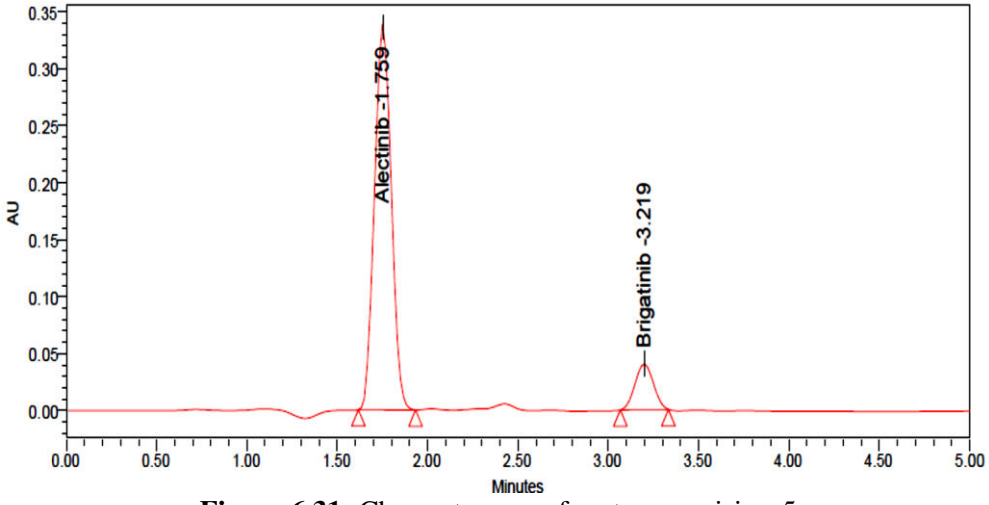

Figure 6.31: Chromatogram of system precision-5

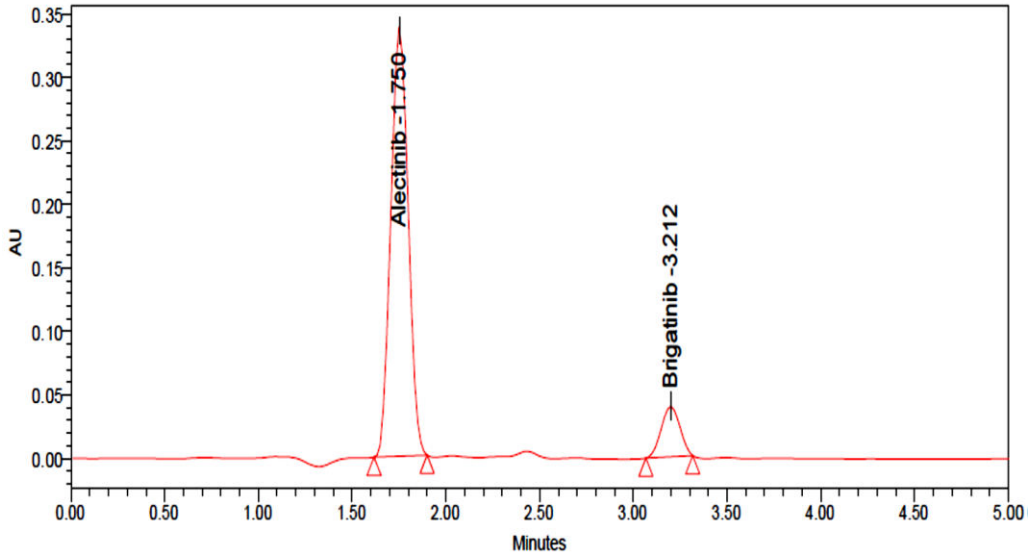

Figure 6.32: Chromatogram of system precision-6

Method precision: [Table 6.06 and Fig 6.33]

Analyte	Std Conc.	%RSD
Brigatinib	10	1.27
Alectinib	50	0.62

Table 6.06: Results of method precision

Acceptance Criteria

The area six standard injection RSD percentage should be more than 2%.

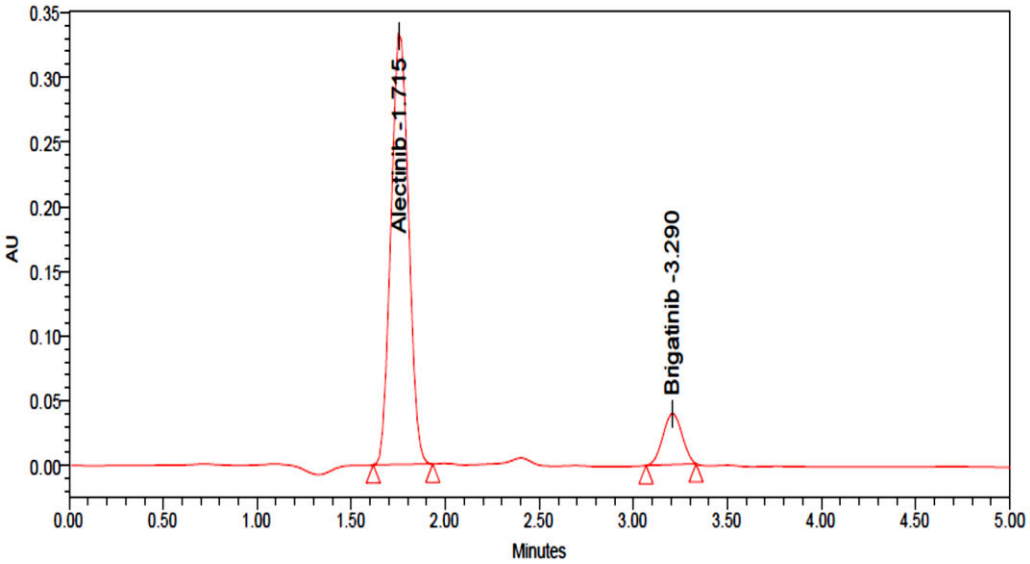

Figure 6.33: Chromatogram of method precision

Intermediate precision (Day-Day precision): [Table 6.07 and Fig 6.34]

Analyte	Std. Conc.	%RSD
Brigatinib	10	0.88
Alectinib	50	1.41

Table6.07: Results of intermediate precision

Acceptance Criteria

The RSD percentage for the six normal injection results should not be more than 2%.

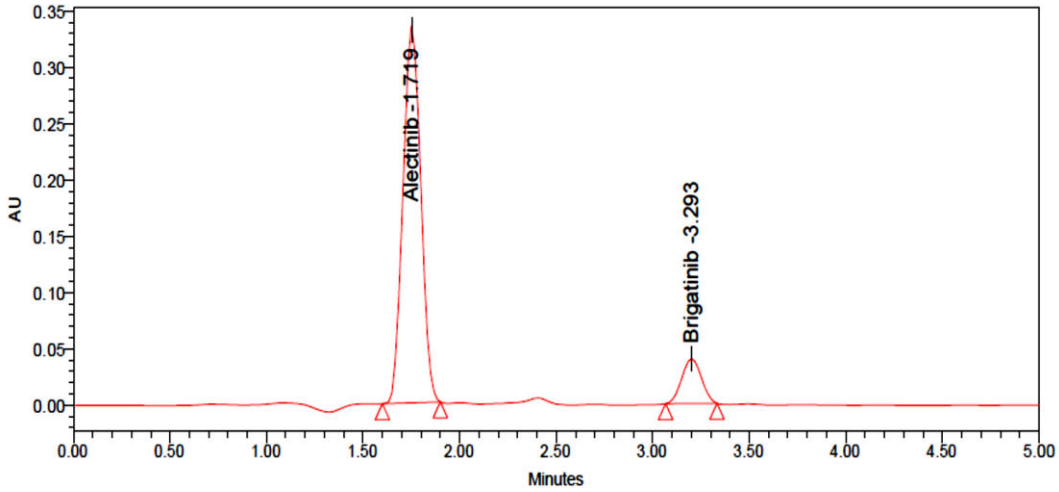

Figure 6.34: Chromatogram of intermediate precision

6.6.5 Limit of detection (LOD) and limit of quantification (LOQ): [Table 6.08 and Fig 6.35&6.36]

LOD and LOQ were calculated based on a calibration curve. The proven RP-UPLC method was used to inject decreasing quantities of standard solution in order to get the results. There is an s/n value of 3 for Brigatinib and a value of 9 for Alectinib at the LOD concentration of 0.063g/ml. S/N values range from 22 to 28 for Brigatinib, whereas Alectinib's concentration is 0.208 micrograms per millilitre (mg/ml).

Brigatinib				Alectinib			
LOD		LOQ		LOD		LOQ	
Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n	Conc. (µg/ml)	s/n
0.013	3	0.043	22	0.063	9	0.208	28

Table 6.08: Sensitivity parameter values

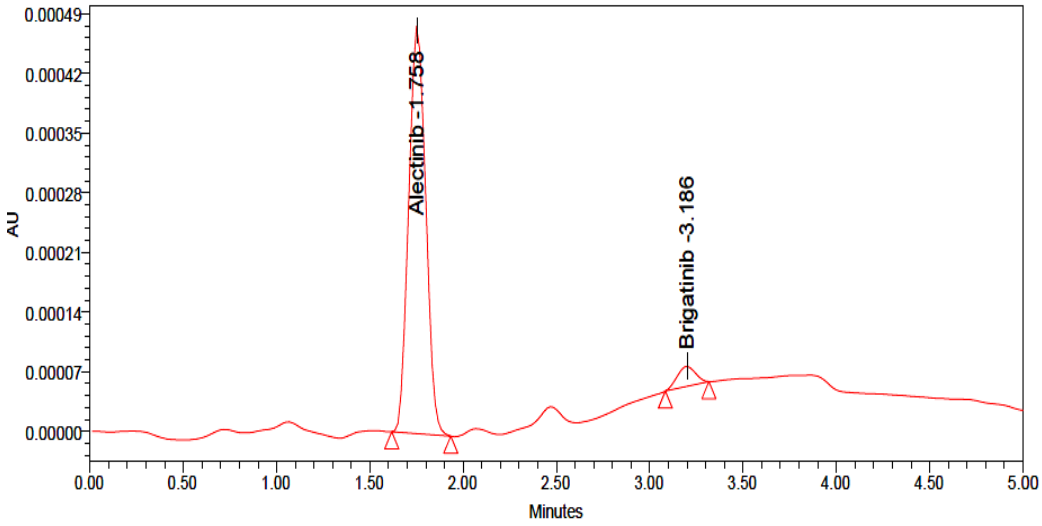

Figure 6.35: LOD chromatogram

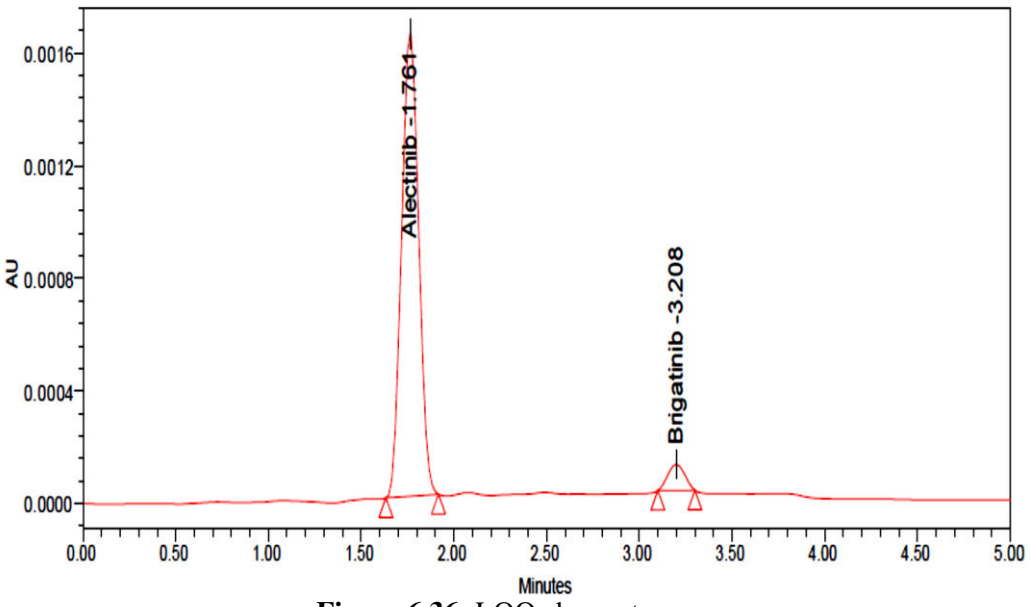

Figure 6.36: LOQ chromatogram

6.6.6 Robustness:

Tests were carried out on known systems, such as flow rate or mobile phase in organic percentage, to see whether they could withstand these different circumstances. Active pharmaceutical components and contaminants were not considerably changed, and the period of retention, plate count, and tailing factor were not significantly altered. As a result, this approach was dependable. **[Table 6.09]** shows robustness results of Brigatinib and Alectinib. Chromatograms from **[Fig. 6.37-6.40]**

S.No	Parameter name	% RSD for purity	
		Brigatinib	Alectinib
1	Flow (0.8ml/min)	1.32	0.99
2	Flow (1.2ml/min)	0.84	0.63
3	Organic solvent (+10%) (88:12)	0.57	0.82
4	Organic solvent (-10%) (72:28)	0.71	1.35

Table 6.09: Robustness results

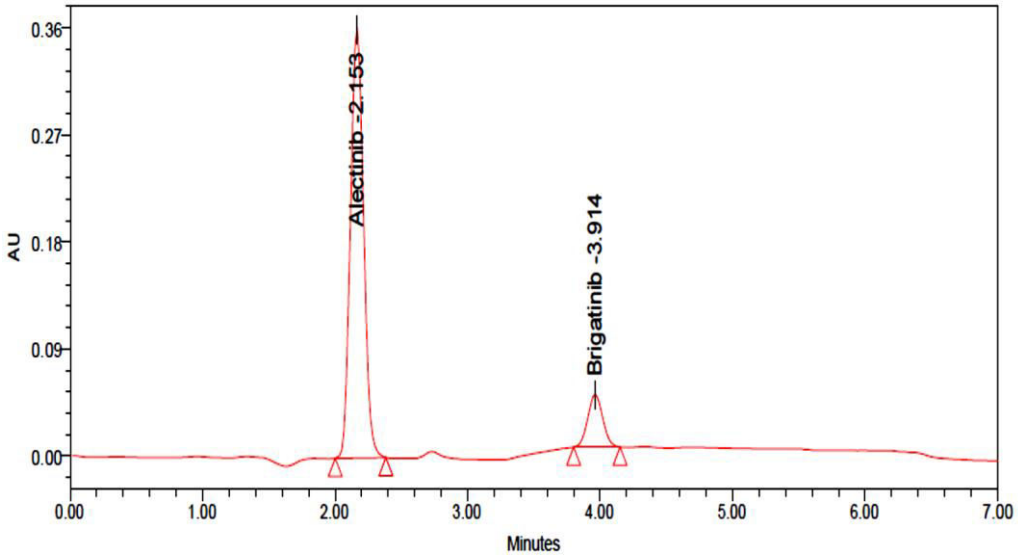

Figure 6.37: Less flow rate chromatogram (0.8ml/min)

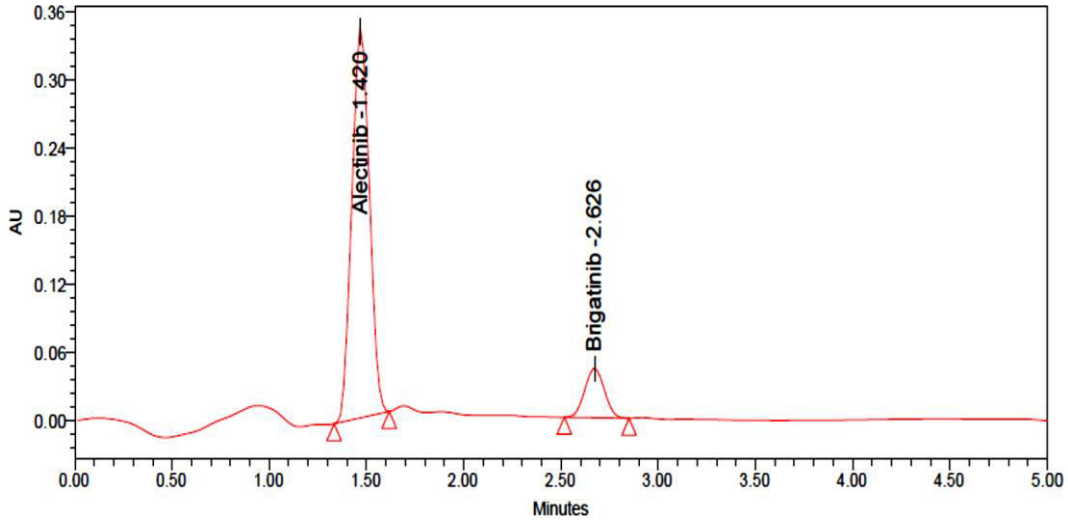

Figure 6.38: More flow rate chromatogram (1.2ml/min)

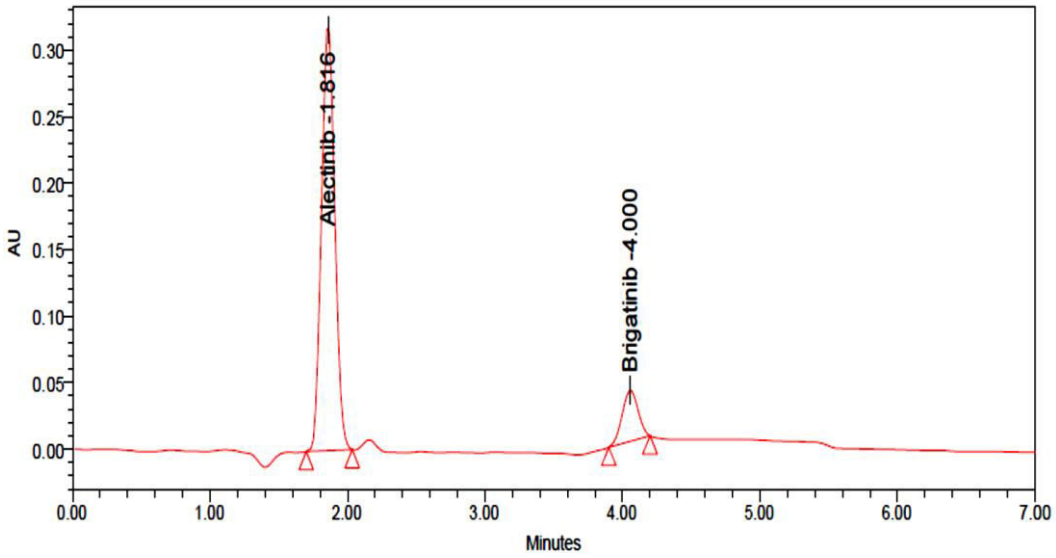

Figure 6.39: Less organic chromatogram (72:28)

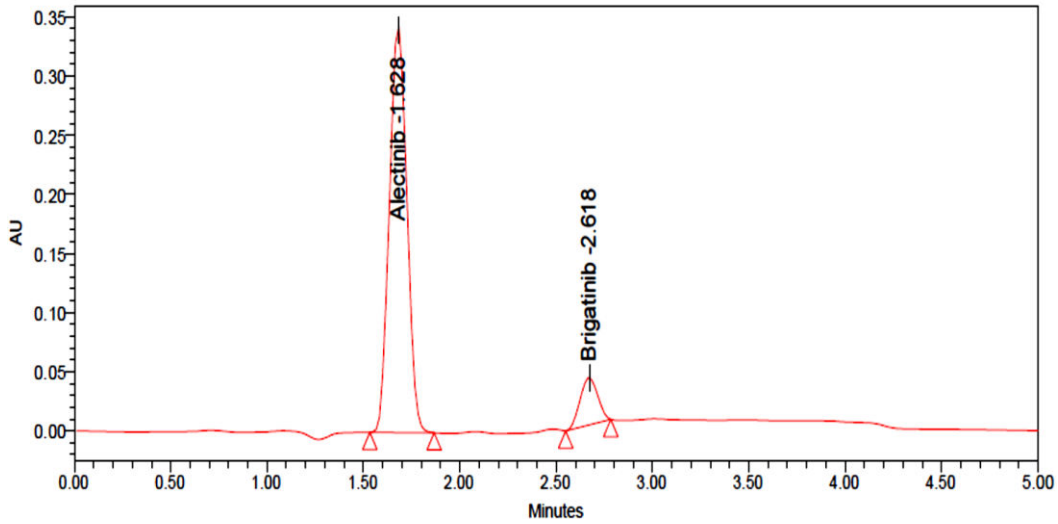

Figure 6.40: More organic chromatogram (88:12)

6.6.7 Forced Degradation Studies:

In order to partly breakdown the molecule, Brigatinib and Alectinib samples were each submitted to a separate set of forced degradation conditions. Experiments with degrading materials have shown that the technique is suitable for use. Also included are explanations of unstable scenarios in order to ensure that efforts are done to mitigate probable instability throughout the formulation process.

Stock Solution Preparation:

In a volumetric flask with a capacity of 100 ml, weigh out 10 mg of Brigatinib and 50 mg of Alectinib, add 70 ml of diluents, and sonicate for 30 minutes to dissolve the diluents.

Acid Degradation

The acid degradation method involves introducing 1ml of 1N HCl to a 50ml volumetric flask, heating the flask for 30 minutes at 60°C, then marking the flask with diluent before adding 1ml of 1N NaOH. The final product is obtained after filtering the solution using a 0.45 nylon syringe filter.

Alkali Degradation
First, a standard solution of 50 millilitres is measured, then 1 millilitre of 1N NaOH is added, and the mixture is heated at 60 degrees Celsius for 30 minutes. Afterwards, 1ml of 1N HCl is added, followed by diluting the liquid to the desired concentration. After filtering the fluid using a 0.45 nylon syringe filter, the final product is produced.

Peroxide Degradation
The following procedure was used to decompose the materials: The solutions, 5 mL of normal solution and 1 mL of 30% H2O2, are placed in volumetric flasks, then warmed for 30 minutes at 60°C and allowed to cool before combining with diluent. The solution can be filtered using a 0.45 nylon syringe filter.

Reduction Degradation
The degrading protocol was as follows: In a 50 mL volumetric flask, 5 mL of normal solution is put in, followed by 1 mL of 30% sodium bicarbonate solution. The entire contents are then heated to 60°C for 15 minutes, and then cooled down to 40°C. To filter the solution, use a 0.45-micron nylon syringe filter.

Thermal Degradation
The test product was put in an oven heated to 105°C for six hours and then refluxed for 30 min at 60°C. The solution was injected into the UPLC system as a result.

UV Degradation
A technique was performed where the standard solution was exposed to the sun for 12 hours, and then 60°C refluxed for 30 minutes. The UPLC technique requires normal water injection.

Hydrolysis Degradation
Standard solution of 5 ml is placed in to a 50 ml volumetric flask, and 2 ml of UPLC water is added. The flask is then heated to 60°C for 15 minutes before chilling with diluent. To filter the solution, use a 0.45-micron nylon syringe filter.

[Table 6.11] gives results of forced degradation and the **figures** from **[6.41-6.48]** shows forced degradation chromatograms.

Degradation Condition	% Degradation of Brigatinib	% Degradation of Alectinib
Unstressed Degradation	99.9	100.0
Acid Degradation	14.2	11.8
Alkali Degradation	13.5	12.5
Peroxide Degradation	13.2	15.4
Reduction Degradation	14.9	13.8
Thermal Degradation	1.5	1.9
Photolytic Degradation	0.7	1.1
Hydrolysis Degradation	0.9	1.5

Table 6.10: Forced degradation results

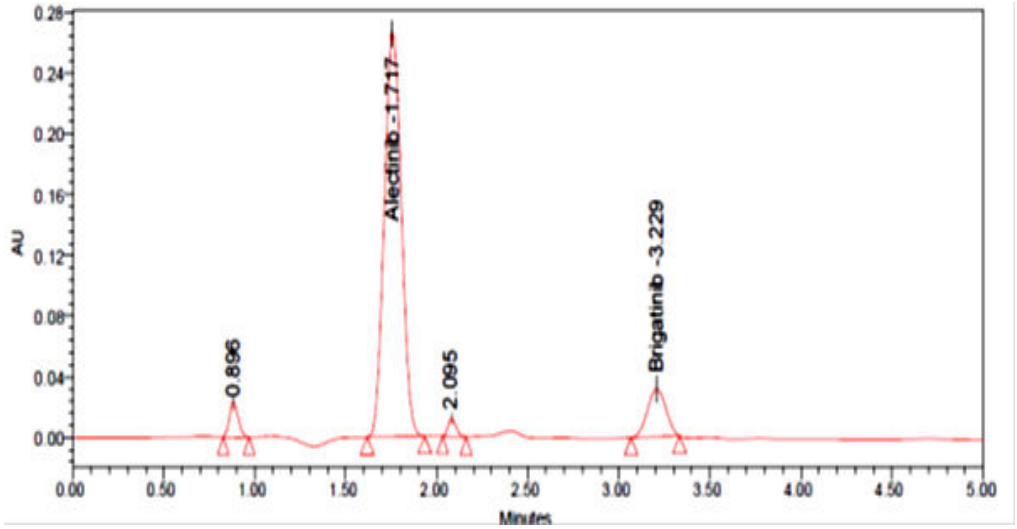

Figure 6.41: Chromatogram of acid degradation

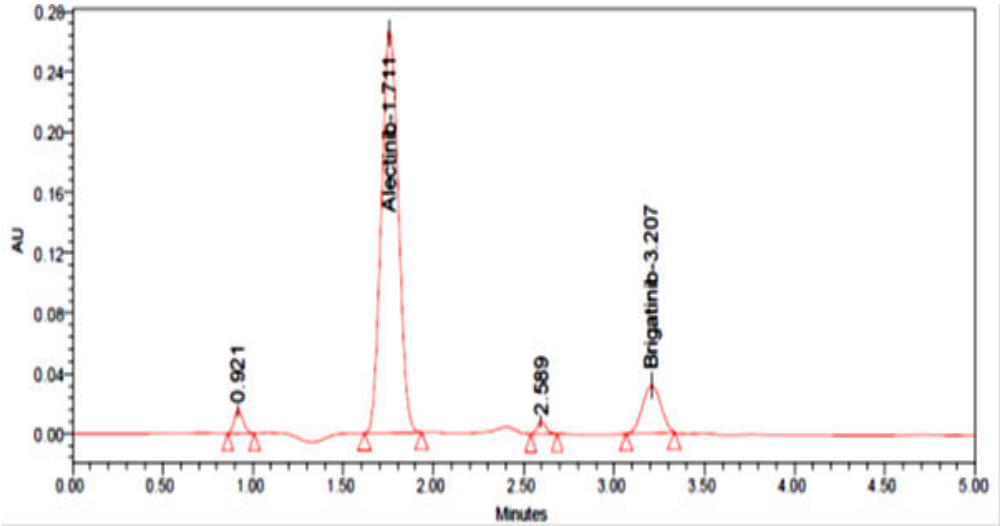

Figure 6.42: Chromatogram of alkali degradation

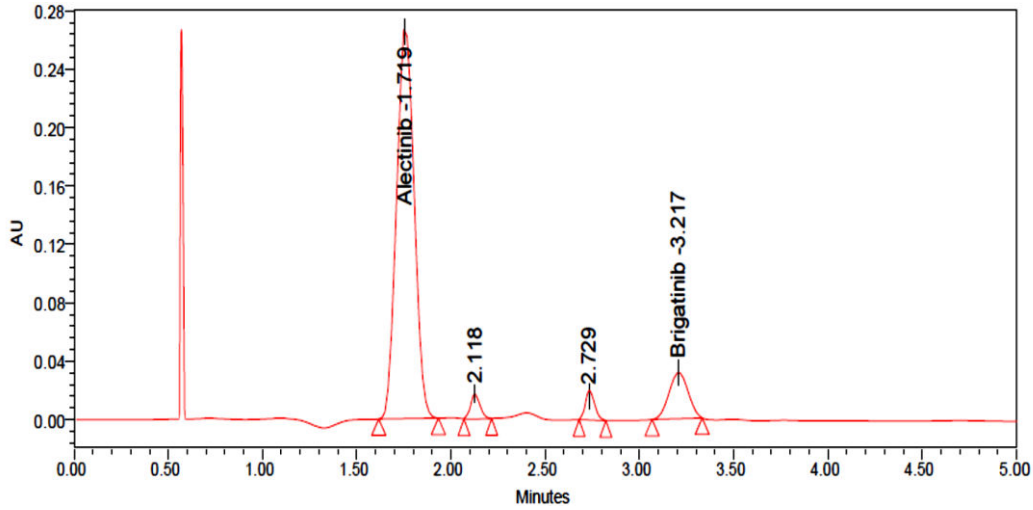

Figure 6.43: Chromatogram of peroxide degradation

 ISBN: 978-81-19477-19-7

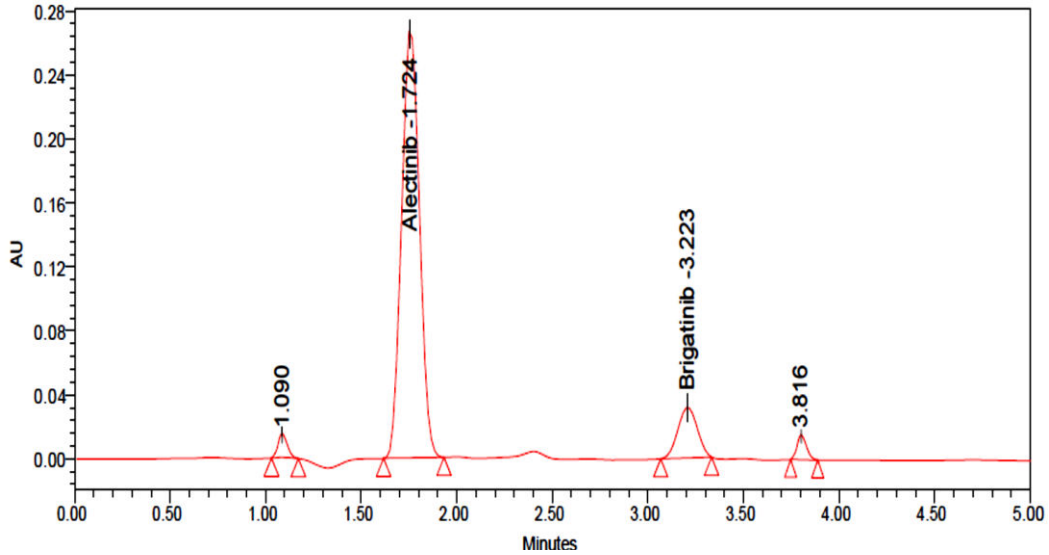

Figure 6.44: Chromatogram of reduction degradation

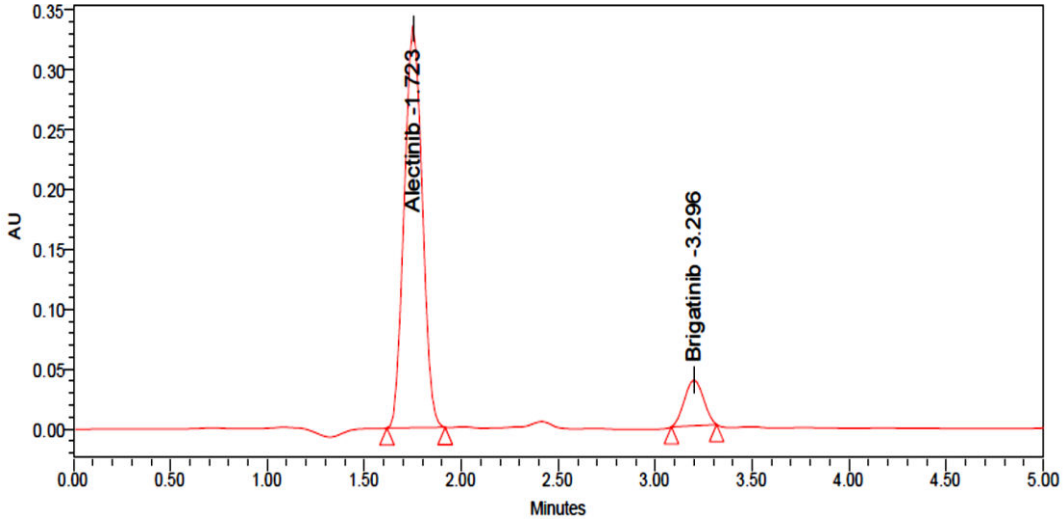

Figure 6.45: Chromatogram of thermal degradation

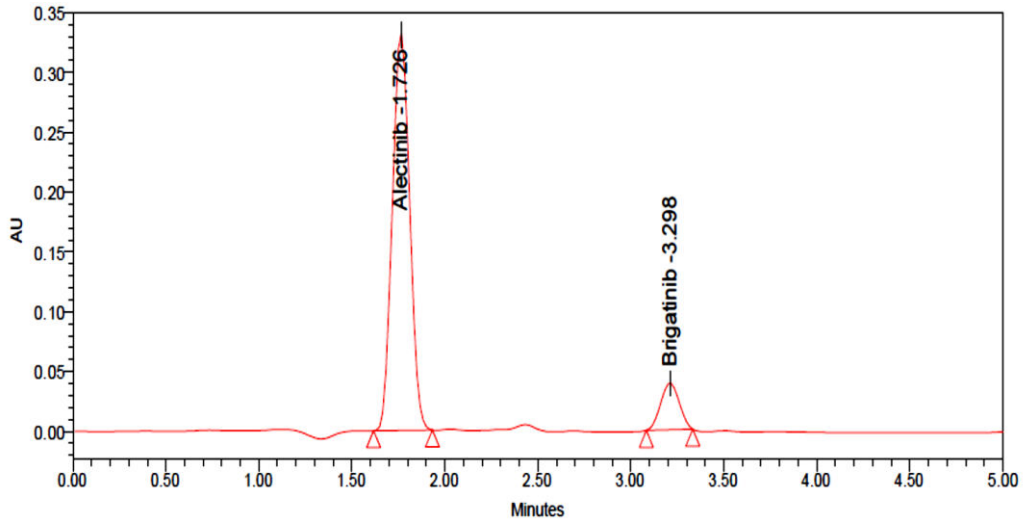

Figure 6.46: Chromatogram of Photo degradation

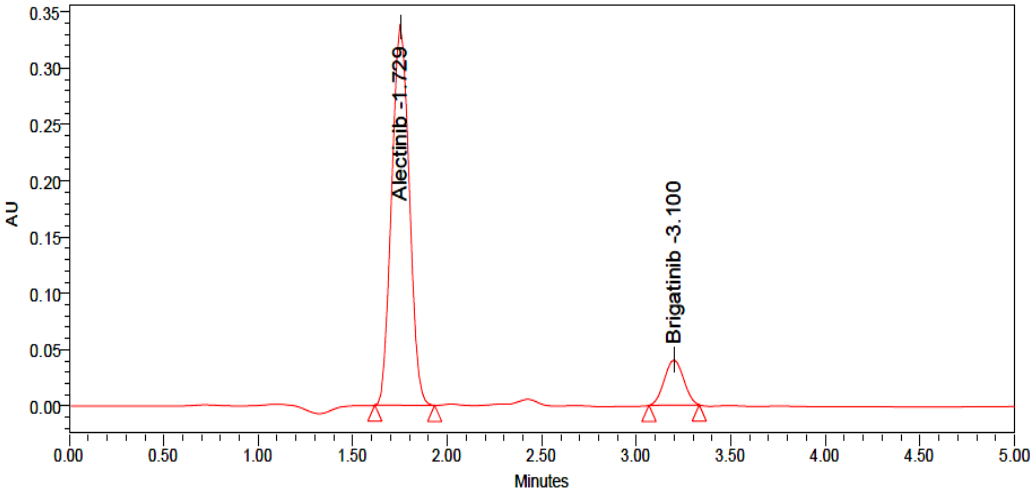

Figure 6.47: Chromatogram of Hydrolysis degradation

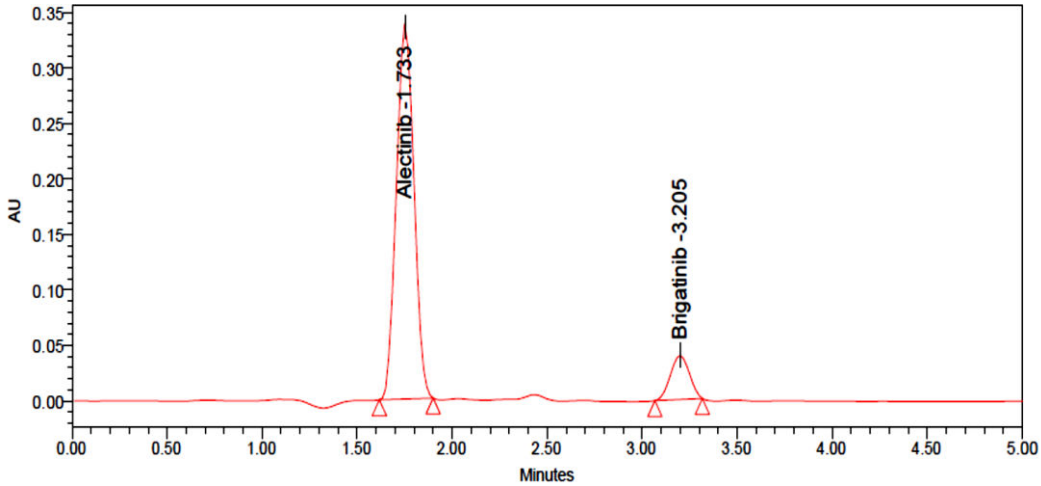

Figure 6.48: Chromatogram of control degradation

6.7 CONCLUSION

An RP-HPLC technique for the detection of active pharmaceutical ingredients (APIs) such as Brigatinib and Alectinib will be developed. Acidic, basic, and neutral environments, as well as oxidation, reduction, light, and heat stress, were studied for the drug's effects on its behaviour. Using neutral, thermal, and light conditions did not affect the medications, but the remaining degrading conditions did. Isocratic RP-UPLC has been designed to measure Brigatinib and Alectinib with high selectivity and precision. The concentrations of Brigatinib (1.0-15.0 g/ml) and Alectinib (5.0-75 g/ml) for which regression line equations were obtained in the peak region may be correctly predicted. Methods proven useful were able to reliably identify the two medications Brigatinib and Alectinib.

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 ISBN: 978-81-19477-19-7

Summary and Conclusion

Analytical methodologies have taken on a significant role in pharmaceutical quality assurance. Changes in manufacturing techniques, as well as regulatory agencies' establishment of limits for individual and overall drug contaminants, are occurring on a regular basis. Pharmacological analytical approaches that include physical and chemical procedures are used to achieve this goal.

We're working to create novel liquid chromatographic assays for specific medications as part of this study. The chemistry of chromomeric reagents, the reactions used in this study, is the focus of this study. New chromatographic procedures are often developed by optimising experimental circumstances (e.g. pH effects; reagent concentration and order; time and temperature retention between additions; solvent effects; colour development and stability; optical properties, etc.). Percent range of error; selectivity; precision; standard deviation; standard deviation; accuracy (comparison; assessment of significance by t-test and recovery experiments; selectivity; precision; standard deviation).

Aside from studying the HPLC system components (solvent delivery systems and degassing systems, as well as gradient elution devices and sample introduction systems for liquid chromatography detectors), this study also focuses on HPLC performance calculations, such as the relative retention, theoretical plates per metre and the height of theoretical plates equal to the plate's height equivalent to theoretical plate (recovery, response function, sensitivity, precision and accuracy).

The author attempted to create novel analytical techniques for several key substances in pure and pharmaceutical dose forms while keeping this in mind in the current work. Simple, fast, trustworthy, and dependable approaches are used in the thesis's methods section. Quality control and process development of bulk pharmaceuticals might benefit from the techniques.

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ABOUT THE BOOK

Analytical methodologies have taken on a significant role in pharmaceutical quality assurance. Changes in manufacturing techniques, as well as regulatory agencies' establishment of limits for individual and overall drug contaminants, are occurring on a regular basis. Pharmacological analytical approaches that include physical and chemical procedures are used to achieve this goal. We're working to create novel liquid chromatographic assays for specific medications as part of this study.

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