" ESTIMATION AND VALIDATION OF GEFITINIB BY USING RP- HIGH PERFORMANCE LIQUID CHROMATOGRAPHY "

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
CHENNAI

In partial fulfillment for the award of degree of

MASTER OF PHARMACY

IN

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

Submitted By

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Under the guidance of

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DEPARTMENT OF PHARMACEUTICAL ANALYSIS

CHERRAAN`S COLLEGE OF PHARMACY,

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(Affiliated to the Tamilnadu Dr.M.G.R medical university, Chennai)

Approved by The Govt. of Tamilnadu, Chennai
All India Council for Technical Education, New Delhi
Recognized by pharmacy council of India, New Delhi

CERTIFICATE

This is to certify that the Dissertation entitled "**ESTIMATION AND VALIDATION OF GEFITINIB BY USING RP- HIGH PERFORMANCE LIQUID CHROMATOGRAPHY"** Submitted to The Tamilnadu Dr. M.G.R Medical University, Chennai is a bonafide project work of **Reg No: 26117431** carried out in the Department of Pharmaceutical Analysis, Cherraan's college of pharmacy, Coimbatore for the partial fulfillment for the degree of Master of Pharmacy under my guidance during the academic year 2013-2014.

This work is original and has not been submitted earlier for the award of any other degree or diploma of this or any other university.

Place: Coimbatore

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ABSTRACT

In the present work, RP-HPLC method has been developed for the quantitative estimation of Gefitinib in Pharmaceutical formulations.

A rapid and sensitive RP-HPLC Method with UV detection (246 nm) for routine analysis of Gefitinib in Pharmaceutical formulation was developed using gradient pump (LC-10AT vp pump), The separations were achieved on a Agilents's 5µ C18 (2) 100A, 250X4.6mm, column with UV detection at 246 nm. Chromatography was performed with mobile phase containing a mixture of 0.1% Triflouroacetic acid and Methanol (35:65 v/v) with flow rate 1 ml/min and retention time at 3.8 min. In the range of 5 -30µg/ml, the linearity of Gefitinib shows a correlation co-efficient of 0.999. The proposed method was validated by determining sensitivity, accuracy, precision, linearity, selectivity and system suitability parameters.

Key words: *Gefitinib, RP- HPLC.*

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"***PARENTS ARE OUR LIVING GODS***" It gives us a sense of honor and pride to our heartfelt and soulful thanks to our dear most lovable parents who act as the guiding spirit behind all our activities. They sowed the seeds of hope on us and seeing their happiness in our eyes. Its they who carved and made us the person who we are today. Thanks a lot to my ***FATHER AND MOTHER***.

LIST OF ABBREVIATIONS

Abs	:	Absorbance
A.R.	:	Analytical Reagent
Conc	:	Concentration
Cm	:	Centimeter
FDA	:	Food and Drug Administration
gm	:	gram
HPLC	:	High Performance Thin Chromatography
ICH	:	International Conference on Harmonization
IP	:	Indian Pharmacopoeia
IUPAC	:	International Union of Pure and Applied Chemistry
LC	:	Liquid Chromatoraphy
LC-MS	:	Liquid Chromatography and Mass Spectroscopy
LOD	:	Limit of Detection
LOQ	:	Limit of Quantitation
Mm	:	Milli mole
mm	:	Millimeter
mV	:	Milli Volt
mL	:	Milli Litre
ng	:	Nano gram
nm	:	nano meter
NMT	:	Not More Than
NLT	:	Not Less Than

Abbreviations

OPA	:	Ortho Phosphric Acid
ppm	:	parts per million
pKa	:	Acid Dissociation Constant
QA	:	Quality Assurance
QC	:	Quality Control
r^2	:	Correlation Coefficient
R_f	:	Retardation Factor
R_t	:	Retention time
RP-HPLC :	:	Reversed Phase High Performance Liquid Chromatography
RSD	:	Relative Standard Deviation
SD	:	Standard Deviation
TLC	:	Thin Layer Chromatography
USP	:	United States Pharmacopoeia
v/v	:	Volume /Volume
w/v	:	Weight/ Volume
WHO	:	World Health Organisation
°C	:	Centigrade Temperature
µg	:	micro gram
µm	:	micro meter
EGFR	:	Epidermal Growth Factor Receptor
ATP	:	Adensine Triphosphate
INR	:	International Normalised Ratio
CAS	:	Chemical Abstracts Service

Abbreviations

CYP3A4	:	Cytochrome P450, family 3, subfamily A, polypeptide 4
CYP2D6	:	Cytochrome P450, family 2, subfamily D, polypeptide 6

EVALUATION CERTIFICATE

This is certify that the dissertation work entitled ***"ESTIMATION AND VALIDATION OF GEFITINIB BY USING RP- HIGH PERFORMANCE LIQUID CHROMATOGRAPHY"*** submitted by HITLER NINGTHOUJAM **Reg.no: 26117431** to The Tamilnadu Dr. M.G.R Medical University, Chennai, in the partial fulfillment for the degree of Master of Pharmacy in Pharmaceutical Analysis is a record of bonafide work carried out by the candidate at the Department of Pharmaceutical Analysis, Cherraan's College of Pharmacy, Coimbatore and was evaluated by us during the academic year 2013-2014.

Internal Examiner **External Examiner**

DECLARATION

The research work embodied in this work "***ESTIMATION AND VALIDATION OF GEFITINIB BY USING RP- HIGH PERFORMANCE LIQUID CHROMATOGRAPHY*****"** was carried out by me in the Department of Pharmaceutical Analysis, Cherraan's college of Pharmacy, Coimbatore under the direct supervision of **Dr**. **K.K SENTHIL KUMAR,** M.Pharm, PhD., HOD & Associate Professor of Pharmaceutical Analysis, Cherraan's College of Pharmacy, Coimbatore-39.

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Place: Coimbatore HITLER NINGTHOUJAM

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1. Analytical Chemistry Introduction:

Analytical chemistry is the science of obtaining, processing, and communicating information about the composition and structure of matter. In other words, it is the art and science of determining what matter is and how much of it exists.

Defining analytical chemistry as the application of chemical knowledge ignores the unique perspective that analytical chemists bring to the study of chemistry. The craft of analytical chemistry is not in performing a routine analysis on a routine sample which more appropriately is called chemical analysis, but in improving established analytical methods, in extending existing analytical methods to new types of samples and in developing new analytical methods for measuring chemical phenomena.

Analytical chemists perform qualitative and quantitative analysis use the science of sampling, defining, isolating, concentrating and preserving samples, set error limits, validate and verify results through calibration and standardization perform separations based on differential chemical properties create new ways to make measurements interpret data in proper content and communicate results.

They use their knowledge of chemistry, instrumentation, computers and statistics to solve problems in almost all areas of chemistry. For example, their measurements are used to assure compliance with environmental and other regulations to assure the safety and quality of food, pharmaceuticals and water to support the legal process to help physicians diagnose disease and to provide chemical measurements essential to trade and commerce.

Analytical chemists are employed in all aspects of chemical research in industry, academia and government. They do basic laboratory research develop processes and products design instruments used in analytical analysis, teach and work in marketing and law. Analytical chemistry is a challenging profession that makes significant contributions to many fields of science.

1.1 PRINCIPLE OF CHROMATOGRAPHY

Chromatography is a technique by which a mixture sample is separated into components. Although originally intended to separate and recover (isolate, purify and separation) the components of a sample today complete chromatography systems are often used to both separate and quantify sample components.

The term “chromatography" was coined by the Russian botanist, Tswett, who demonstrated that, when a plant extract was carried by petroleum ether through a column consisting of a glass tube packed with calcium carbonate powder, a number of dyes were separated. He named this analysis method "Chromatographie" after "chroma" and "graphos", which are Greek words meaning "color" and “to draw" respectively.

1.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. It also allows you to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture

The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive.

A HPLC system is basically composed of

- ✓ Mobile phase
- ✓ Pump
- ✓ Injector
- ✓ Column
- ✓ Column oven
- ✓ Detector.

1. MOBILE PHASE(SOLVENT RESERVOIR)

The most common type of solvent reservoir is a glass bottle. Most of the manufacturer's supply these bottles with special caps Teflon tubing and filters to connect to the pump inlet t and to the spurge gas (Helium) used to Remove Dissolved air. Filtration is needed to eliminate suspended Particles and organic impurities

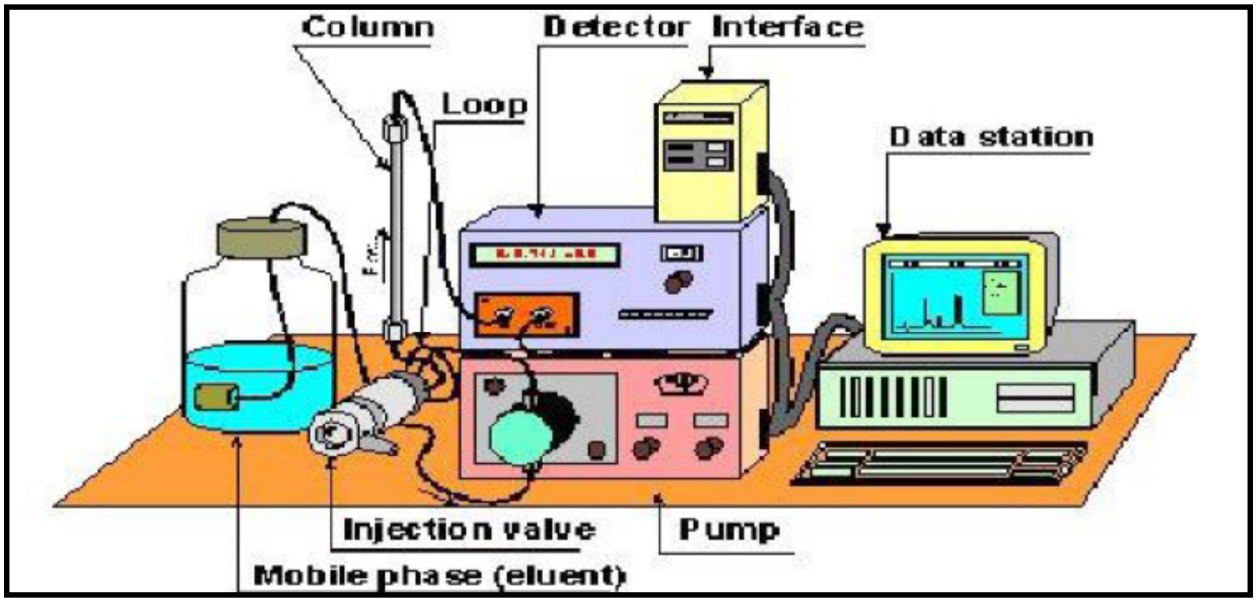

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Fig: HPLC Basic Instrument.

Solvent system*:*

The mobile phases used in Reversed-Phase Chromatography are based on a polar solvent, typically water to which a less polar solvent such as acetonitrile or methanol is added. Solvent selectivity is controlled by the nature of the added solvent in the same way as was described for Normal-Phase Chromatography. Solvents with large dipole moments such as methylene chloride and 1,2-dichloroethane interacts preferentially with solutes that have large dipole moments such as nitro-compounds, nitriles, amines and sulfoxides. Solvents that are good proton donors such as

chloroform, m-cresol and water interact preferentially with basic solutes such as amines and sulfoxides and solvents that are good proton acceptors such as alcohols, ethers, and amines tend to interact best with hydroxylated molecules such as acids and phenols.

Solvent degassing system:

The constituents of the mobile phase should be degassed and filtered before use. Several methods are employed to remove the dissolved gases in the mobile phase. They include heating and vacuum degassing with an aspirator, filtration through 0.45 filters, vacuum degassing with an air-soluble membrane, helium purging ultra sonification or purging or combination of these methods. HPLC systems are also provided an online degassing system which continuously removes the dissolved gases from the mobile phase.

Gradient Elution:

Steady changes of the mobile phase composition during the chromatographic run is called gradient elution. It may be considered as an analogy to the temperature programming in gas chromatography.
The main purpose of gradient elution is to move strongly retained components of the mixture faster, but having the least retained component well resolved.
Starting with the low content of the organic component in the eluent we allow the least retained components to be separated. Strongly retained components will sit on the adsorbent surface on the top of the column or will move very slowly.

When we start to increase an amount of organic component in the eluent (acetonitrile) then strongly retained components will move faster and faster because of the steady increase of the competition for the adsorption sites.

Gradient elution also increase quasi-efficiency of the column. In the isocratic elution, the longer a component is retained the wider its peak. In gradient elution especially with the smooth gradient shape without a flat regions the tail of the peak is always under the influence of the stronger mobile phase when compared to the peak front. Thus molecules on the tail of the chromatographic

zone (peak) will move faster. This will tend to compress zone and narrow the resultant peak. Performance of the gradient elution is strongly dependent on the instrumentation.

Isocratic Elution :

In this technique, the same mobile phase combination is used throughout the process of separation. The same polarity or elute strength is maintained throughout the process. In isocratic separation, mobile phase is prepared by using pure solvent or mixture of solvent i.e. , solvent of same eluting power or polarity is used.

2. PUMPS:

Pumps are used to flow mobile phase at high pressure and at controlled flow rates. The pumps must be capable of generating pressure of up to 5000 psi at flow rates up to 3ml/min for analytical purpose. Pumps used in preparative scale hplc may be required to pump at flow rates of upto20ml/min. The pumping systems used in HPLC can be categorized in three different types.

a. Reciprocating Pump

-single Piston

-Dual Piston

b. Syringe pumps

c. Constant pressure pumps

- Pneumatic Pump
- Amplifier Pump

d. Displacement Pump

The first classification is according to the eluent flow rate that the pump is capable of delivering.

a). RECIPROCATING PUMP: Reciprocating pumps are by far the most widely used and practically 100% of the pumps used in commercial HPLC equipment are of the reciprocating

type. In reciprocating pumps, a motor driven reciprocating piston controls the flow of mobile phase with the help of two ball check valves that opens and closes with then piston movement. The flow is thus not continuous and damping of flow is necessary. This is accomplished using pulse dampers which are a long coiled capillary tube.

Single Piston Reciprocating Pump:

The pump takes the form of a large metal syringe the piston being propelled by an electric motor and driven by a worm gear. The speed of the motor determines the pump delivery. Another motor actuates the piston by a different system of gearing to refill the syringe rapidly when required. The solvent is sucked into the cylinder through a hole in the center of the piston and between the piston and the outlet there is a coil that acts as a dampener. This type of pump is still occasionally used for the mobile phase supply to microbore columns that require small volumes of mobile phase to develop the separation. It is also sometimes used for reagent delivery in post column derivatization as it can be made to deliver a very constant reagent supply at very low flow rates. The single piston reciprocating pump was the first of its type to be used with high efficiency LC columns (columns packed with small particles) and is still very popular today. It is simple in design and relatively inexpensive.

Dual piston Reciprocating Pump:

A pump for producing a substantially smooth and continuous outflow of liquid at relatively high pressure has two piston assemblies flow connected in series. The first piston assembly includes a pressure piston having a long suction stroke and a relatively short and abrupt expulsion stroke. A valve at the inlet of the pressurization piston allows flow to enter (but not exit) and a valve at the outlet of the pressurization piston allows flow to exit (but not enter). The second piston assembly includes a damper piston which functions as a mechanically driven damper to smooth the outflow from the pressure piston. This smoothing is accomplished by storing of the liquid displaced by the expulsion stroke of the pressure piston and then delivering the stored pressurized liquid to the pump outlet during the suction stroke of the pressure piston. The drive for the pistons is constructed to produce an increased outflow of pressurized liquid for a short interval at the

beginning of the expulsion stroke of the pressure piston to compensate for compressibility of the liquid at high pressure. At low pressure the stepper motor drive is slowed down in response to the sensing of the increase of the outflow during this short interval to maintain the outflow smooth and continuous during this part of the cycle of operation.

b) SYRINGE PUMPS:

The first pumps to be used were syringe pumps which were in effect large stainless steel motor driven hypodermic syringes. They provided a very constant flow rate which was virtually pulse less but required a rather frequent, lengthy and involved refilling process. In addition unless some form of marine engineering was employed the pumps could not operate at very high pressures. Due to these disadvantages the syringe pump was soon replaced by piston pumps which, although far from being pulse less, were much easier to operate. Today syringe pumps are rarely used in mobile phase supplies but they are still used very effectively in both pre-column and post-column reactors. Their very constant flow rate provides accurate reagent addition and in post-column reactors does not introduce detector noise.

C) CONSTANT PRESSURE PUMP:

Pneumatic Pump: The pneumatic pump is double piston pump one piston having a relatively large diameter and the other a relatively small diameter. The two pistons are connected together and fit inside two connected cylinders. The smaller cylinder is fitted with inlet and outlet non-return valves. The large piston is driven by compressed air (the gas alternately driving the piston in one direction and then the other) and actuates the smaller piston which pumps the liquid. The system acts as a pressure amplifier as the output pressure from the pump with the smaller piston will be equal to the pressure applied to the larger piston times the ratio of the cross-sectional area of the larger piston to that of the smaller piston. This type of piston was originally used for normal liquid chromatography separations but was found to be noisy and produced strong flow pulses that destabilized the detector. It is now used almost exclusively for slurry packing liquid chromatography columns. It is the simplest type of pump that can be designed to provide exceedingly high pressures common use they were the pneumatic pump where the necessary high

pressures were achieved by pneumatic amplification and the syringe pump which was simply a large strongly constructed syringe with a plunger that was driven by a motor. Today the majority of modern chromatographs are fitted with reciprocating pumps fitted with either pistons or diaphragms. For more information on HPLC pump requirements see the pump section in the HPLC supplement. The pneumatic pump has a much larger flow capacity than the piston type pumps but nowadays is largely used for column packing and not for general analysis. The pneumatic pump can provide extremely high pressures and is relatively inexpensive, but the high pressure models are a little cumbersome and at high flow rates can consume considerable quantities of compressed air.

The second classification is according to the construction materials and the final classification Constant pressure pump not constant flow can deliver high pressures.The Construction of materials to be used for Pumps should be inert towards the solvents to be used .

3. INJECTOR:

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors. In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase but frequently it is judiciously chosen to avoid detector interference, column/component interference, loss inefficiency or all of these. It is always best to remove particles from the sample by filtering over a 5 μm filter or centrifuging, since continuous injections of particulate material will eventually cause blockages in injection devices or columns. Sample sizes may vary widely. The availability of highly sensitive detectors frequently allows use of the small samples which yield the highest column performance.

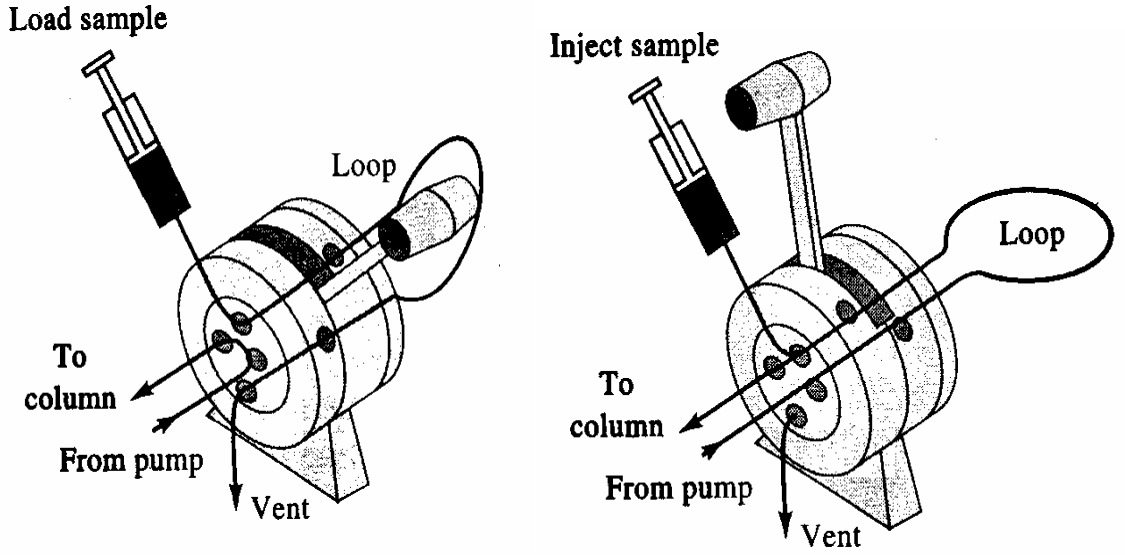
TYPES OF INJECTORS :

a) Septum Injectors : For injecting the sample through a rubber septum. This is not common, since the septum has to withstand high pressure.

b) Stop flow(on line) Injectors : In which the flow of mobile phase is stopped for a while and the sample is injected through a valve device.

c) Rheodyne Injector (Loop valve type) : It is the most popular injector. This has a fixed volume loop like 20µl or 50µl or more. Injector has two modes, i.e. , **load position** when the sample is loaded in the loop and **inject mode,** the sample is injected **.**

Examples of injectors



Insertion of a sample onto the column must be as a narrow plug so that the peak broadening attributable to this step is negligible. The injection system itself has no dead volume.

4. COLUMN:

The column is the heart of the chromatograph providing the means for separating a mixture into components. The selectivity, capacity and efficiency of the column are all affected by the nature of the packing material or the materials of construction.

Requirements for an Ideal HPLC Column:

1. Particles should be spherical and available in particle diameters ranging from 3 to10 µm.
2. Particles should withstand typical pressures encountered during HPLC {(900-3000 psi (6.1-20.5 MPa) but ideally up to 4000 psi (27.2 MPa)} and should not swell or shrink with the nature of the eluent.
3. Particles should have porosity in the range 50-70 %, extending to 80 % for Size-Exclusion Chromatography.
4. Particles should contain no pores smaller than ~60 A^0 in diameter and should have a uniform pore size distribution.
5. Particles should be available with a range of mean pore diameters of 60-1000 A^0 .
6. The internal surface of the material should be homogeneous.
7. The internal surface should be capable of modification to provide a range of surface functionalities.
8. Packing materials should be chemically inert under all conditions of pH and eluent composition.
9. The physico-chemical characteristics of the material should be reproducible from batch to batch and from manufacturer to manufacturer.
10. The material should be readily available and relatively inexpensive and its chemical behavior should be well understood.

There are different columns are available

A. Guard Columns: These columns are placed anterior to the separating column. This serve as a protective factor that prolongs the life and usefulness of the separation column. They are dependable columns designed to filter or remove

1) Particles that clog the separation column.

2)Compounds and ions that could ultimately cause "baseline drift", decreased resolution, decreased sensitivity, and create false peaks.

3) Compounds that may cause precipitation upon contact with the stationary or mobile phase.

B. Derivatizing Columns: Pre- or post-primary column derivatization can be an important aspect of the sample analysis. Reducing or altering the parent compound to a chemically related daughter molecule or fragment elicits potentially tangible data which may complement other results or prior analysis.

C. Capillary Columns: Advances in HPLC led to smaller analytical columns. Also known as micro columns, capillary columns have a diameter much less than a millimeter and there are three types: open-tubular, partially packed, and tightly packed. They allow the user to work with nanoliter sample volumes, decreased flow rate, and decreased solvent volume usage which may lead to cost effectiveness.

D. **Fast Columns**: One of the primary reasons for using these columns is to obtain improved sample throughput (amount of compound per unit time). For many columns, increasing the flow or migration rate through the stationary phase will adversely affect the resolution and separation. Therefore, fast columns are designed to decrease time of the chromatographic analysis without forsaking significant deviations in results.

E. Preparatory Columns: These columns are utilized when the objective is to prepare bulk (milligrams) of sample for laboratory preparatory applications. A preparatory column usually has a large column diameter which is designed to facilitate large volume injections into the HPLC system.

The heart of the system is the column. The choice of common packing material and mobile phases depend on the physical properties of the drug. The column is constructed from smooth bore stainless steel tubing or heavy walled glass tubing to withstand high pressure.

F. Analytical Columns : Analytical columns is the most important part of the HPLC technique which decides the efficiency of separation. There are several stationary phases available depending upon the technique or mode of separation used.

COLUMN PACKING:

The packing used in modern HPLC consist of small rigid particles having a narrow particle size distribution. There are three main type of column packing in HPLC.

- ✓ Porous, Polymeric Beads
- ✓ Porous Layer Beds
- ✓ Totally Porous Silica Particles (dia. <10 μm)

5. DETECTOR:

The detector converts a change in the column effluent into an electrical signal that is recorded by the data system. There are different types of detectors used in HPLC. Liquid chromatographic detectors are of two basic types. **Bulk property** detectors respond to a mobile-phase bulk property, such as refractive index, dielectric constant, or density. In contrast, **solute property** detectors respond to some property of solutes, such as UV absorbance, fluorescence, or diffusion current, that is not possessed by the mobile phase.

A) Refractive Index Detector: The detection principle involves measuring of the change in refractive index of the column effluent passing through the flow-cell. The greater the RI difference between sample and mobile phase, the larger the imbalance will become. Thus, the sensitivity will be higher for the higher difference in RI between sample and mobile phase. On the other hand, in complex mixtures, sample components may cover a wide range of refractive index values and some may closely match that of the mobile phase, becoming invisible to the detector.

B) UV Detector: In these systems detection depends on absorption of UV ray energy by the sample. They are capable to detect very wide range of compounds. The sensitivity ranges till microgram quantity of estimation.

C) PDA detector: These are detectors which follow principle similar to UV detectors but the only advantages are higher sensitivity and measure the entire absorption range i.e. It gives scan of entire spectrum.

D) Evaporative Light Scattering Detector (ELSD): In the ELSD, the mobile phase enters the detector is evaporated in a heated device and the remaining solute is finally detected by the way it scatters light. The intensity of the light scattered from solid suspended particles depends on their particle size. Therefore, the response is dependent on the solute particle size produced. This, in

turn, depends on the size of droplets generated by the nebulizer and the concentration of solute in the droplets. The droplet size produced in the instrument nebulizer depends on the physical properties of the liquid and the relative velocity and flow-rates of the gas and liquid stream. The importance of all these parameters emphasizes the need for careful design and rigorous optimization of the instrument parts.

E) Electro Chemical Detector: This detector is specially suitable to estimate oxidisable & reducible compounds .The principle is that when compound is either oxidized or reduced, the chemical reaction produces electron flow. This flow is measured as current which is the function of type and quantity of compound

F) Conductivity Detector: conductivity detector measures the conductivity of the mobile phase. There is usually background conductivity which must be backed-off by suitable electronic adjustments. If the mobile phase contains buffers the detector gives a base signal that completely overwhelms that from any solute usually making detection impossible. Thus the electrical conductivity detector is a bulk property detector. And senses all ions whether they are from a solute or from the mobile phase.

G) Fluorescence detectors: Fluorescence detectors are probably the most sensitive among the existing modern HPLC detectors. It is possible to detect even a presence of a single analyte molecule in the flow cell. Typically, fluorescence sensitivity is 10 -1000 times higher than that of the UV detector for strong UV absorbing materials. Fluorescence detectors are very specific and selective among the others optical detectors. This is normally used as an advantage in the measurement of specific fluorescent species in samples.

H) Mass Spectrometric Detection: The use of mass spectrometer for hplc detection is becoming common place, despite the high cost of such detector and need for a skilled operator. A mass spectrometer can facilitate hlpc method development and avoid common problem by

- Tracking and identifying individual peaks in the chromatogram between experiments
- Distinguishing compounds of interest from minor compounds or interferences.

- Recognizing unexpected and overlapping interference peaks to avoid a premature finish to method development.

The function of the detector in HPLC is to monitor the mobile phase as it merges from the column.

Detectors are of usually four types:

- ✓ Bulk Property Detectors
- ✓ Solute Property Detectors
- ✓ Desolvation detectors (flame ionization etc.)
- ✓ LC-MS detectors.

MOST COMMONLY USED METHODS IN HPLC:

The four main types of HPLC techniques are

1. Normal-Phase Chromatography.
2. Reversed-Phase Chromatography.
3. Ion-Exchange Chromatography.
4. Size-Exclusion Chromatography.

Normal-Phase Chromatography :

The term "normal phase" is used to denote a chromatographic system in which a polar stationary phase is employed and a less polar mobile phase is used for elution of the analytes. In the normal-phase mode, neutral solutes in solution are separated on the basis of their polarity the more polar the solute, the greater is its retention on the column. Since the mobile phase is less polar than the stationary phase, increasing the polarity of the mobile phase results in decreased solute retention. Normal-Phase chromatography is most commonly applied to the analysis of samples that are soluble in non-polar solvents and it is particularly well suited to the separation of isomers and to class separations.

Although the separation mode has occasionally been misidentified as reversed phase it is normal phase by virtue of the fact that increased aqueous levels of the mobile phase reduce carbohydrate retention and elution order follows carbohydrate polarity.

Normal-phase separations have occasionally been combined off-line with Reversed-phase chromatography to separate a wider range of species than could be accomplished by either technique alone. The feasibility of such a system however is contingent on the compatibility of the normal-phase eluent with that of the reversed-phase column.

Reversed-Phase Chromatography*:*

Reversed-Phase Chromatography, the most widely used chromatographic mode is used to separate neutral molecules in solution on the basis of their hydrophobicity. As the name suggests, Reversed-Phase Chromatography is the reverse of Normal-Phase Chromatography in the sense that it involves the use of a non-polar stationary phase and a polar mobile phase. As a result, a decrease in the polarity of the mobile phase results in a decrease in solute retention. Modern Reversed-Phase Chromatography typically refers to the use of chemically bonded stationary phases where a functional group is bonded to silica for this reason Reversed-Phase Chromatography is often referred to in the literature as Bonded-Phase Chromatography. Occasionally, however polymeric stationary phases such as polymathacrylate or polystyrene or solid stationary phases such as porous graphitic carbon are used. Weak acids and weak bases for which ionization can be suppressed, may be separated on reversed-phase columns by the technique known as ion suppression. In this technique a buffer of appropriate pH is added to the mobile phase to render the analyte neutral or only partially charged. Acidic buffers such as acetic acid are used for the separation of weak acids and alkaline buffers are used for the separation of weak bases.

The analysis of strong acids or strong bases using reversed-phase columns is typically accomplished by the technique known as ion-pair chromatography (also commonly called paired-ion or ion-interaction chromatography). In this technique, the pH of the eluent is adjusted in order to encourage ionization of the sample for acids pH 7.5 is used and for bases pH 3.5 is common.

Reversed-Phase Chromatography is the most popular mode for the separation of low molecular weight (<3000) neutral species that are soluble in water or other polar solvents. It is widely used in the pharmaceutical industry for separation of species such as steroids, vitamins and β-blockers. Because of the mobile phase in Reversed-Phase Chromatography is polar, Reversed-Phase Chromatography is suited to the separation of polar molecules that either are insoluble in organic solvents or bind too strongly to the polar normal-phase materials.

Ion-Exchange Chromatography:

In Ion-Exchange Chromatography (IEC) species are separated on the basis of differences in electric charge. The primary mechanism of retention is the electrostatic attraction of ionic solutes in solution to "fixed ions" of opposite charge on the stationary phase support. The stationary phase or ion exchanger is classified as an anion-exchange material when the fixed ion carries a positive charge and as a cation exchanger when it carries a negative charge.

A specialized form of IEC is ion chromatography (IC) which is the name applied to the analysis of inorganic anions, cations and low molecular weight, water-soluble organic acids and bases. Although any HPLC technique used to separate the above species can be termed ion chromatography in general IC involves the use of ion-exchange columns and a conductivity detector. Ion chromatography itself can be sub classified. Suppressed IC involves the use of a membrane device known as a suppressor between the column and the detector to lower the response of the eluent and thereby enhance the signal from the solute; nonsuppressed or "single-column" IC does not contain a suppressor.

Size-Exclusion Chromatography:

Size-Exclusion Chromatography (SEC) is a convenient and highly predictable method for separating simple mixtures whose components are sufficiently different in molecular weight. For small molecules, a size difference of more than about 10% is required for acceptable resolution; for macromolecules a twofold difference in molecular weight is necessary. Size-Exclusion Chromatography can be used to indicate the complexity of a sample mixture and to provide

approximate molecular weight values for the components. It is an easy technique to understand, and SEC can be applied to the separation of delicate bio macromolecules as well as to the separation of synthetic organic polymers.

Because SEC is a gentle technique, rarely resulting in loss of sample or reaction, it has become a popular choice for the separation of biologically active molecules. Each solute is retained as a relatively narrow band, which facilitates solute detection with detectors of only moderate sensitivity. One of the major applications of SEC is polymer characterization.

Applications of HPLC

1. **Preparative HPLC** refers to the process of isolation and purification of compounds. This differs from analytical HPLC, where the focus is to obtain includes identifications, quantification, and resolution of a compound.
2. **Chemical separations** can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. Thus the chromatography can separate compounds from each other using HPLC the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.
3. **Purification refers** to the process of separating or extracting the target compound from other (possibly structurally related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic condition. The migration of the compounds and contaminants through the column need to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound.
4. **Identification of the compounds by HPLC** is a crucial part of any HPLC assay. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels, in which the assay would be performed.
5. **Quantification of compounds by HPLC** is the process of determining the unknown concentration of a compound in a solution. It involves injecting a series of known concentration of the standard compound solution onto the HPLC for detection.

6. The chromatograph of these known concentrations will give a series of peaks that correlate to the concentration of the compound injected.

ADVANTAGES:

HPLC separations can be accomplished in a matter of minutes in some cases even in seconds. High resolution of complex sample mixture into individual components can be obtained.

- Rapid growth of HPLC is also because of its ability to analyse substances that are unsuitable for gas liquid chromatographic (GLC) analysis due to non-volatility or thermal-instability.
- Quantitative analysis is easily and accurately performed and errors of less than 1 % are common to most HPLC methods.
- Depending on sample type and detector used it is frequently possible to measure 10^{-9} g or 1 ng of sample. With special detectors analysis down to 10^{12ng} has been reported.

DISADVANTAGES:

- HPLC instrumentation is expensive and represents a major investment for many laboratories.
- It requires a proficient operator to handle the instrument.
- HPLC cannot handle gas samples.
- HPLC is poor identifier. It provides superior resolution but it does not provide the information that identifies each peak.
- Sample preparation is often required.
- Only one sample can be analyzed at a time.
- Finally there is at present time no universal and sensitive detector.

ANALYTICAL METHOD DEVELOPMENT

Method development is done for

1) New products

2) Existing products

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated.

When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available.

Selection of analytical method

First stage in the selection or development of method is to establish what is to be measured and how accurately it should be measured. The following analytical techniques are usually employed for estimations of different components in formulations:-

- ✓ Titrimetric and gravimetric
- ✓ Ultraviolet and visible spectrophotometry.
- ✓ Thin layer chromatography
- ✓ High performance liquid chromatography (HPLC)
- ✓ Gas Chromatography (GC)
- ✓ Atomic absorption spectrometry (AAS)
- ✓ Infra-Red absorption spectrophotometry.

Steps of method development

Documentation starts at the very beginning of the development process a system for full documentation of the development studies must be established. All data relating to these studies must be recorded in laboratory notebook or an electronic database.

1. Analyte standard characterization

a) All known information about the analyte and its structure is collected i.e., physical and chemical properties, toxicity, purity, hygroscopic nature, solubility and stability.

b) The standard analyte (100% purity) is obtained. Necessary arrangement is made for the proper storage (refrigerator, desiccators and freezer).

c) When multiple components are to be analyzed in the sample matrix the number of components is noted data is assembled and the availability of standards for each one is determined.

d) Only those methods (MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

2. Method requirements

The goals or requirements of the analytical method that need to be developed are considered and the analytical figures of merit are defined. The required detection limits, selectivity, linearity, range, accuracy and precision are defined.

3. Literature search and prior methodology

The literature for all types of information related to the analyte is surveyed. For synthesis, physical and chemical properties solubility and relevant analytical methods. Books, periodicals, chemical manufacturers and regulatory agency compendia such as USP / NF, AOAC and ASTM publications are reviewed. Chemical Abstracts Service (CAS) automated computerized literature searches are convenient.

4. Choosing a method

a) Using the information in the literatures and prints, methodology is adopted. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in house analytes and samples.

b) If there are no prior methods for the analyte in the literature from analogy the compounds that are similar in structure and chemical properties are investigated and are worked out. There is usually one compound for which analytical method already exist that is similar to the analyte of interest.

5. Instrumental setup and initial studies

a) The required instrumentation is setup. Installation, operational and performance qualification of instrumentation using laboratory standard operating procedures (SOP's) are verified.

b) Always new consumables (e.g. solvents, filters and gases) are used for example method development is never started on a HPLC column that has been used earlier.

c) The analyte standard in a suitable injection / introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (e.g., bulk drug) then it is possible to start work with the actual sample.

d) Analysis is done using analytical conditions described in the existing literature.

6. Optimization

During optimization one parameter is changed at a time, and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan, and every step is documented (in a lab notebook) in case of dead ends.

7. Documentation of analytical figures of merit

The originally determined analytical figures of merit limit of quantitation (LOQ), limit of detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

8. Evaluation of method development with actual samples

The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

9. Determination of percent recovery of actual sample and demonstration of Quantitative sample analysis:

Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average +/- standard deviation) from sample to sample and whether recovery has been optimized has been shown. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with a high degree of certainty. The validity of analytical method can be verified only by laboratory studies.

Guidelines for analytical Method validation.

For pharmaceutical method guidelines are prescribed by

- **United State Pharmacopoeia (USP)**
- **Food and Drug Administration (FDA)**
- **World Health Organization (WHO)**
- **International Conference on Harmonization (ICH)**

These Guidelines provide a framework for performing validation. In general, methods for routine analysis, standardization or regulatory submission must include studies on specificity, linearity, accuracy, precision, range detection limit, quantitation limit and robustness.

United State Pharmacopoeia (USP):

USP defines analytical method validation as "The process by which it is established; by laboratory studies that performance characteristics of method meet the requirement for intended analytical application".

Food and Drug Administration (FDA): FDA defines validation as "Establishing documented evidence, which provides a high degree of assurance that a specific process will consistently produce meeting its pre- determined specification and quality attributes".

World Health Organization (WHO):

WHO defines validation as "Process of providing documented evidences that a system / procedure dose what it is supposed to do precisely and reliably".

International Conference on Harmonization (ICH):

ICH is tripartite agreement between European community, USA and Japan. Its purpose is to provide a forum for constructive dialogue between regulatory authorities and Pharmacy industry on real and perceived differences in technical requirements for product registration in European community, USA and Japan.

Objective is lying down of minimum standards applicable uniformly, irrespective of where the product is manufactured or marketed in the three regions.

The ICH documents give guidance on the necessity for revalidation in the following circumstances.

- ✓ Changes in the synthesis of the drug substances and
- ✓ Changes in the composition of the drug product

Validation:

It presents a discussion of the characteristics for consideration during the validation of the analytical procedures included as part of registration applications submitted within the EC, Japan and USA. This document does not necessarily seek to cover the testing that may be required for registration in or export to other areas of the world. Furthermore this text presentation serves as a collection of terms and their definitions and is not intended to provide direction on how to accomplish validation. These terms and definitions are meant to bridge the differences that often exist between various compendia and regulators of the EC, Japan and USA.

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. A tabular summation of the characteristics applicable to identification, control of impurities and assay procedures is included. Other analytical procedures may be considered in future additions to this document

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Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

***Types of Validation*:**

Prospective validation: This is performed for all new equipments, products and processes. It is a proactive approach of documenting the design

specifications and performance before the system is operational. This is the most defendable type of validation.

Concurrent Validation: This is performed in two instances i.e., for existing Equipment verification of proper installation along with specific Operational tests is done. In case of an existing, infrequently made Product data is gathered from at least three successful trials.

Retrospective validation:

This is establishing documented evidence that the Process is performed satisfactory and consistently over time, based on review and analysis of historical data. The source of such data is production and QA/QC records. The issues to be addressed here are changes to equipment, process, specifications and other relevant changes in the past

Analytical methods need to be validated or revalidated before their introduction into routine use whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix) and whenever the method is changed and the change is outside the original scope of the method.

The validity of a specific method should be demonstrated in laboratory experiments using samples or standards that are similar to unknown samples analyzed routinely. The preparation and execution should follow a validation protocol, preferably written in a step by- step instruction format. This proposed procedure assumes that the instrument has been selected and the method has been developed. It meets criteria such as ease of use ability to be automated and to be controlled by computer systems, costs per analysis, sample throughput, turnaround time and environmental, health and safety requirements.

The validation characteristics

1. Specificity
2. Accuracy
3. Linearity
4. Precision

5. Limit of detection(LOD) & Limit of Quantification(LOQ)

6. Range

7. Robustness

8. Systen suitability Parameters

1. SPECIFICITY

a) **Identification:** Discrimination between compounds of closely related structures which are likely to be present.

b) Assay and Impurity test: For chromatographic procedures, representative chromatogram. Resolution of the two compounds which elute closest together. In case of non- specific assay is used, a combination can be applied. Titration for assay, suitable test for impurities.

c) Impurities are available: Assay: Spiking pure substance (drug substance or drug product) with appropriate levels of impurities and/or excipients. Assay result unaffected. Impurity test spiking drug substance or drug product with appropriate levels of impurities and demonstrating separation.

d) Impurities not available: Samples stored under relevant stress conditions assay: the two results are compared impurity test: impurity profiles are compared, Peak purity test, diode array, mass spectrometry.

2. ACCURACY

General 15 determinations over 3 concentrations covering specified range, upper and lower concentrations 6 replicates middle one 3 replicates.

Reporting;

- ✓ % Recovery or
- ✓ Difference between mean and accepted true value
- ✓ Confidence interval
- ✓ Drug substance
- ✓ Application of analytical procedure to analyte of known purity(reference material).
- ✓ Drug product
- ✓ Placebo + drug substance
- ✓ adding known quantities of drug substance to drug product

- ✓ Impurities(Quantification)
- ✓ Adding known quantities of impurities to drug product
- ✓ Placebo + impurities
- ✓ The individual or total impurities are determined e.g. weight/weight or area percent, in all cases with respect to the major analyte

3. LINEARITY

Linearity should be established across the range.

Minimum 5 concentrations:

- ✓ dilution standard stock solution
- ✓ separate weighing of synthetic mixtures

Linear relationship, regression analysis

- ✓ Correlation coefficient
- ✓ y-intercept
- ✓ Slope of regression line
- ✓ Residual sum of squares.

4. PRECISION

- ✓ Repeatability:
- ✓ 6 determinations at 100% of test concentration
- ✓ Intermediate precision
- ✓ Different days
- ✓ Analysts
- ✓ Equipment
- ✓ Not necessary to study these effects individually (2 x 6 determinations at 100 % of test concentration).
- ✓ Recommended data
- ✓ Standard deviation
- ✓ Relative standard deviation
- ✓ Confidence

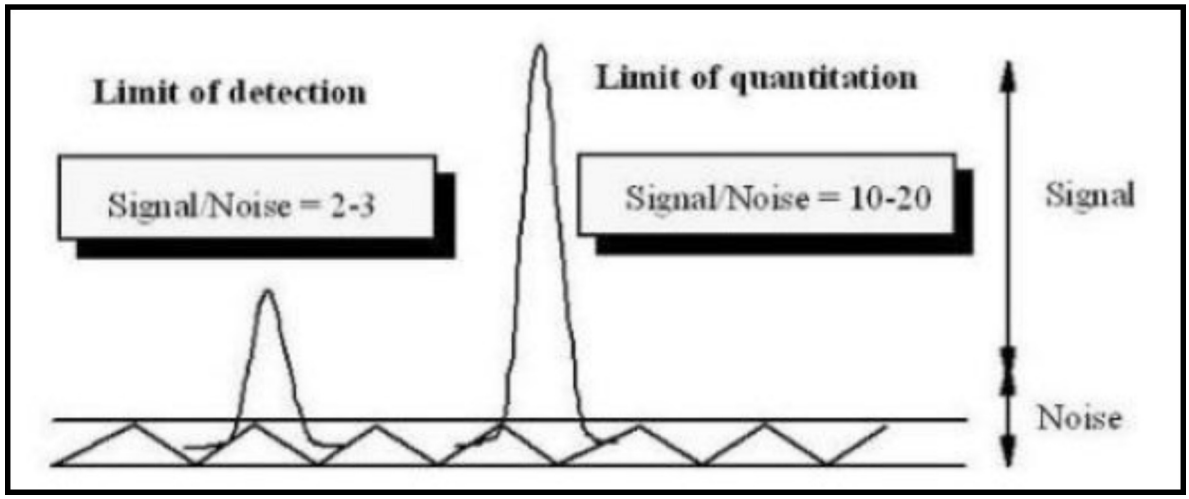
5 .LIMIT OF DETECTION(LOD) & LIMIT OF QUANTIFICATION(LOQ)

- ✓ Limit of detection of an individual analytical method is the lowest concentration/amount of analyte in a sample that the method can detect but not necessarily quantify under the

stated experimental conditions. The LOD will not only depend on the procedure of analysis but also on the type of instrument.

(DL) =3.3×σ\ S mcg/ml

- ✓ Where, σ = standard deviation of the response.
  S = slope of the calibration curve.
- ✓ The slope S may be estimated from the calibration curve of the analyte.
- ✓ The estimate of σ may be carried out in a variety of ways.



- ✓ Limit of quantification of an individual analytical method is the lowest concentration/ amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy under stated experimental conditions. The quantification limit is used particularly for the determination of impurities and/ or degradation products. The LOQ will not only depend on the procedure of analysis but also on the type of instrument**.**

- ✓ (QL) =10×σ\ S mcg/ml
  Where, σ = standard deviation of the response
  S = slope of the calibration curve

6. RANGE

Assay of drug substance or finished product; 80 - 120 % of test solution.

- ✓ Impurity (quantification); reporting threshold to 120% of acceptance criteria.
- ✓ Assay and impurity; One test with 100 % standard

- ✓ Linearity; Reporting threshold to 120 % assay acceptance criterion.
- ✓ Content uniformity; 70 - 130 % of test concentration
- ✓ Dissolution testing; ± 20% over specified range
- ✓ Drug release testing; 20% after 1 hour up to 90% after 24 hours 0-110 % of label claim

7. ROBUSTNESS:

- ❖ **Variations: ·**
- ✓ Stability of analytical solutions ·
- ✓ Different equipment ·
- ✓ Different analysts
- ❖ **HPLC:**
- ✓ Influence of pH in mobile phase
- ✓ Variations in mobile phase
- ✓ Different column
- ✓ Temperature
- ✓ Rate Flow

8. SYSTEM SUITABILITY PARAMETERS

System suitability tests are most often applied to analytical instrumentation. They are designed to evaluate the components of the analytical system in order to show that the performance of the system meets the standard required by the method. After the method has been validated an overall system suitability tests should be routinely run to determine if the operating system is performing properly.

System suitability tests are integral part of the gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for analysis

.The following information furnishes the parameters used to calculate the system performance values for the separation of two chromatographic components.

- ❖ **Relative rétention (selectivity)**:

$\alpha = (t2\text{-}ta) / (t1\text{-}ta)$

Where,

α = Relative retention.

t1 = Retention time of the first peak measured from point of injection.

t2 = Retention time of the second peak measured from point of injection.

ta = Retention time of an inert peak not retained by the column, measured from point of injection.

- **Theoretical plates:**

n = 16 (t / w) 2

Where, n = Theoretical plates.

t = Retention time of the component.

w = Width of the base of the component peak using tangent method.

- **Capacity factor:**

K1 = (t2 / ta) – 1

Where K1 = Capacity factor.

- **Resolution:**

R = 2 (t2 - t1) / (w2 + w1)

Where, R = Resolution between a peak of interest (peak 2) and the peak preceding it (Peak 1).

W2 = Width of the base of component peak 2.

W1 = Width of the base of component peak 1.

- **Peak asymmetry:**

T = W0.05 / 2f

Where, T = Peak asymmetry or tailing factor.

W0.05 = Distance from the leading edge to the tailing edge of the peak, measured at a point 5 % of the peak height from the baseline.

f = Distance from the peak maximum to the leading edge of the peak.

- **Plates per meter:**

N = n/ L

Where, N = Plates per meter.

L = Column length in meters.

HETP = L / n

- **Linear fit:**

A linear calibration fit determines the best line (linear regression) for a series of calibration points.

A minimum of two calibration points is required to determine a linear fit.

The equation for calibrating the uncorrected amount is:

[Y = a X + b]

Where, Y = Component area or height.

a = Slop of the calibration line.

X = Uncorrected amount.

b = Y- axis intercept of the calibration line.

STATISTICAL ANALYSIS:

Statistical procedures and representative calculations:

The consistency and suitability of the developed method are substantiated through the statistical analysis like standard deviation, relative standard deviation and theoretical plates per meter.

For Accuracy:

Standar deviation =σ=

$$\sqrt{\frac{\sum (x - x_i)^2}{n-1}}$$

Where, **x** = sample, **xi** = mean value of samples, **n** = number of samples

Relative Standard Deviation = σ/xi × 100

Molar extinction coefficient (mol-1 cm-1) = A/C × L

Where, **A**= Absorbance of drug

C= concentration of drug

L= Path length

Sandell's sensitivity (µg/ cm2/0.001 absorbance units) = C/A×0.001

Where, C= concentration of drug

A= Absorbance of drug

Unit- (mcg/cm2) =0.001 absorbance)

Coefficient of variance (σ) = $\Sigma(x-\bar{x})$ 2/ n-1

Regression Equation y = a+ b x

Slope = y/x

Where, x = Concentration, y= Absorbance, a= Intercept

LITERATURE REVIEW

Gefitinib is an orally administered synthetic anticancer drug and few analytical methods found in literature for the quantitative estimation in bulk drug and pharmaceutical formulations.

- **K. Ravi Shankar, *et al.*[32],** has reported the development and validation of sensitive reverse phase HPLC method for the determination of gefitinib in bulk and in its pharmaceutical formulation. A rapid and sensitive RP-HPLC method with UV detection at 205 nm for routine analysis of Gefitinib in bulk and pharmaceutical formulations was developed. Chromatography was performed with mobile phase containing a mixture of acetonitrile and 0.5% M ammonium dihydrogen phosphate buffer in the ratio of 30 : 70 v/v with flow rate 1.0 ml/min. The calibration curve of Gefitinib was found to be linear over the range of 0.05 to 0.15 mg/ml with correlation coefficient of 0.99. Sensitivity, accuracy, range, precision, robustness, ruggedness, stability, specificity, LOD, LOQ and system suitability parameters were validated for the developed method.

- **Subhash Chandra Bose, *et al* [33],** have reported the quanlitative analysis of gefitinib by quadrupole – time of flight (LCMS) coupled with PDA and ELSD detector, using dual electro spray ionization (dual ESI) multistage tandem mass spectrometry (LC-MS) were used to identify of Gefitinib in tablet dosage form for validated and estimation. Efficient chromatographic separation of analyte was achieved on Agilent XDB-C18 column (4.6 × 50 mm, 1.8 µm) using an isocratic elution mode with a mobile phase comprised acetonitrile and 0.1 % formic acid in water (75:25, v/v). The flow rate was 500µl / min . The developed LC-MS method is validated with a respect to linearity, accuracy, precision, specificity, limit of detection, limit of quantification and robustness. The method was observed to be limit of detection and limit of quantification was found to be 5.56 ppm and 11.12 ppm respectively. The method was successfully used for applied the quantitative determination of Gefitinib in tablet formulation.

- **P.V.V .Satyanarayana, *et al* [34].,** has reported the development and validation of liquid chromatography method for the estimation of gefitinib in pharmaceutical dosage form.
  A simple, specific, accurate and precise reverse phase high performance liquid chromatographic method was developed and validated for the estimation of Gefitinib in tablet dosage form. An Inertsil ODS C-18, 5µm column having 250 x 4.6mm internal diameter in isocratic mode with mobile phase containing acetonitrile: methanol: tetrahydrofuran in the ratio of 20:70:10 (v/v/v) was used. The flow rate was 1.0ml/min and effluents were monitored at 251nm. The retention time for Gefitinib was 4.282min. The method was validated for linearity, accuracy, precision, specificity, limit of detection, limit of quantification and robustness. Limit of detection and limit of quantification were found to be 0.09ppm and 0.29ppm respectively and recovery of Gefitinib from tablet formulation was found to be 99.16%. The proposed method was successfully applied for the quantitative determination of Gefitinib in tablet formulation
- **Lionel Faivre, *et al.*[35],**has a simple HPLC-UV method for the simultaneous quantification of gefitinib and erlotinib in human plasma. Gefitinib and erlotinib are two tyrosine kinase inhibitors (TKI) approved for the treatment of advanced non-small cell lung cancer (NSCLC). Published methods for simultaneous analysis of erlotinib and Gefitinib in plasma are exclusively based on mass spectrometry. The purpose of this study was to develop a simple and sensitive HPLC-UV method to simultaneously quantify these two TKI in plasma. Samples were eluted at a flow rate of 0.4 ml / min throughout the 15-min run. Dual UV wavelength mode was used, with gefitinib and erlotinib at 331 nm . The calibration was linear in the range 20-1000 ng / ml and 80-4000 ng / ml for gefitinib and erlotinib respectively. This simple, sensitive, accurate and cost-effective method can be used in routine clinical practice to monitor Gefitinib or erlotinib concentrations in plasma from NSCLC patients.
- **V. kalyana Chakravarthy, *et al.*[36],** have reported the development and validation of RP – HPLC method for the estimation of gefitinib in bulk and its pharmaceutical formulation.
  Separation was achieved with a Kromasil 100-5, C18 (150 mmx4.6 mm I.D; particle size 5 µm) and Potassium dihydrogen phosphate Buffer pH 2.4: Acetonitrile: Methanol

(65:21:14) as eluent at flow rate 1.5 ml/min. UV detection was performed at 250 nm . The method is simple, rapid and selective. The described method of gefitinib is linear over a range of 75.02 to 225.06 µg/ml . The method precision for the determination of assay was below 2.0 %RSD. The percentage recoveries of active pharmaceutical ingredient (API) from dosage forms ranged from 100.5 to 101.1. The method is useful in the quality control of Bulk and pharmaceutical formulations.

- **Stephane Bouchet, *et al* [37],** has reported the simultaneous determination of nine tyrosine kinase inhibitors by 96- well solid phase extraction and ultra performance LC/MS-MS.
- **Ling- Zhi Wing, *et al.*[38],** has reported a novel, rapid and specific liquid chromatography-tandem mass spectrometric (LC-MS / MS) method was developed and validated for the simultaneous quantification of Gefitinib and its predominant metabolite, o-desmethyl gefitinib in human plasma. Chromatographic separation of analyte was achieved on an Alltima C18 anlytical HPLC column (150 mm × 2.1 mm, 5µm) using an isocratic elution mode with a mobile phase comprised acetonitrile and 0.1 % formic acid in water (30:70 v/v). The flow rate was 300 µl / min.) . The chromatographic run time was 3 min. The column effluents were detected by API 4000 triple quadrupole mass spectrometer using electro spay ionization (ESI). Linearity was in the range of 5-1000 ng / ml for Gefitinib and 5-500 ng / ml for o-desmethyl Gefitinib and accuracies range from 89.7 to 104.7 % for Gefitinib and 100.4 to 106.0 % for o-desmethyl Gefitinib. The results of this study enabled clinicians to acertain the safety of the combination therapy of hydroxychloroquine and Gefitinib in patients with advanced non-small cell lung cancer (NSCLC).
- **P. Pravalika Reddy, *et al.*[39],** has reported new spectrophotometric methods for the estimation of gefitinib in bulk drug and formulations. Two simple, precise and accurate spectrophotometric methods were developed for the estimation of Gefitinib in bulk drug and in pharmaceutical formulations. Method A and B is based on oxidation of followed by coupling of 3- metyl-1,2-benzothiazolinone hydrazone (MBTH) with Gefitinib using Ferric chloride and Cerric ammonium nitrate(CAN) and measuring the chromogen at the λmax of 438 and 675 respectively. Both the methods follow beer's law in the concentration range of 100-700 µg/ml with MBTH and FeCl3 and 50-250 µg/ml with

MBTH and CAN. The accuracy of the methods was determined by recovery studies. The methods showed good reproducibility and recovery with relative standard deviation (in %) less than 2. The methods were found to be simple, economical, accurate and reproducible and can be used for routine analysis of Gefitinib in bulk drug and in pharmaceutical formulations.

- **R. Honey Well, *et al.*[40],** has reported simple and selective method for determination of various tyrokinase inhibitors used in the clinical setting by liquid chromatography tandem mass spectrometry.
- **Sandra Roche, *et al.*[41],** has reported the development and application of novel analytical methods for molecularly targeted cancer therapeutics. Based on LC-MS/MS techniques, novel sensitive analytical methods were developed for the quantification of 1) tyrosine kinase inhibitor anti-cancer drugs to examine the interaction of these agents with drug resistance mechanisms and 2) the multiple myeloma drug thalidomide as a pilot study to identify potential correlations between serum drug levels and toxicity/efficacy. Acquired resistance to chemotherapeutics through the over-expression of ABC transport proteins has presented a significant clinical challenge. The up-regulation of ABC transporters such as P-glycoprotein (P-gp), Breast Cancer Resistant Protein (BCRP) and Multidrug Resistance Protein (MRP-1) is an important cellular mechanism of resistance in vivo and in vitro. Using cell-line models and the developed LC-MS/MS quantification method it was established that dasatinib an agent used in treatment of chronic myelogenous leukemia is a substrate of ABC transporters P-gp and BCRP but not of MRP-1, and that dasatinib does not inhibit these transporters at clinically relevant concentrations. Another Tyrosine Kinase Inhibitor (TKI) being used in breast cancer, Lapatinib was shown to be an inhibitor of P-gp but not a substrate of this resistance mechanism. Levels of other TKIs were also found to vary in their uptake in primary models of brain cancer. Using LC-MS/MS, the circulating serum levels of thalidomide in multiple myeloma patients were studied. A clear correlation between dosing regime and serum level was seen however the research also unearthed evidence of a significant incidence of non- compliance which could have far reaching consequences for patient treatment. Overall, LC-MS/MS methods

for the quantification of chemotherapeutic agents in complex biological matrices produced sensitive and accurate data which we successfully exploited to characterize resistance to new drugs and examine correlations between serum levels and treatment variables.

- **Yoichi Nakamura, *et al.*[42],** has reported pharmacokinetics of Gefitinib predicts anti tumor activity for advanced non- small cell lung cancer. Plasma trough levels of Gefitinib were measured on days 3 (D3) and 8 (D8) by high performance liquid chromatography in 44 patients with advanced NSCLC treated with 250 mg Gefitinib daily. The median plasma Gefitinib values were 662 ng / ml on D3 and 1064 ng / ml on D8 and the D8/D3 ratio was 1.587. The median progression-free survival (PFS) was 71 days and the median overall survival was 224 days. Adenocarcinoma never smoking and high D8 / D3 ratio were associated with better PFS. Overall survival was not associated with D8 / D3 ratio. A high D8 / D3 ratio was independently associated with better PFS in patients with NSCLC treated with gefitinib. Our finding suggest that the pharmacokinetics of Gefitinib may be involed in its anti-tumor activity.
- **N. Appalaraju, *et al* [43].,** A simple, precise, rapid and accurate RP- HPLC method developed for the estimation of Gefitinib in tablet dosage form. An Hypersil BDS RP C18, 250x4.6 mm, 5 m particle size, with mobile phase consisting of 0.02 M Dipotassium Hydrogen orthophosphate and Methanol in the ratio of 10:90 v/v was used. The flow rate was 1.0 ml/min and the effluents were monitored at 246 nm. The retention time was 3.7 min. The detector response was linear in the concentration of 25-300g/ml. The respective linear regression equation being Y= 94342.26x+77672.7. The limit of detection and limit of quantification was 0.125g/ml and 0.15g/ml respectively. The percentage assay of Gefitinib was 99.5 %. The method was validated by determining its accuracy, precision and system suitability. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the routine determination of Gefitinib in bulk drug and in its pharmaceutical dosage form
- **N.A.G .Lankheet, *et al.*[44],** has developed a fast and accurate method for determination of anticancer tyrosine kinase inhibitors (TKIs) Gefitinib in human plasma was developed

using high performance liquid chromatography and detection with tandem mass spectrometry (HPLC-MS/MS). Plasma proteins were precipitated and a aliquot of supernatant was directly injected onto a reversed phase chromatography system consisting of a Gemini C_{18} column (50×2.0 mm, 5.0µm particle size) and then compounds were eluted with a gradient.This method was validated over a linear range from 20.0 to 10,000 ng / ml for Gefitinib. Results from the validation study demonstrated good intra and inter assay accuracy (<13.1 %) and precision (10.0 %) for all analyte. This method was successfully applied for routine therapeutic drug monitoring purposes in patients treated with the investigated TKIs.

- **Sano Kazumi, *et al.*[45],** has reported the quantitation of major metabolite of Gefitinib using HPLC and LC –MS. Our objective is measuring the plasma concentrations of Gefitinib and its major metabolite. The metabolites were generated in vitro by enzyme reaction using CYP2D6 or 3A4 produced using baculovirus. Reaction mixtures were analyzed by HPLC and LC-MS. Major metabolites, O-desmethyl-gefitinib (M1) and minor metabolites (M2, M3) were determined based on molecular weight using LC-MS. In this study we have got effective findings to analyze Gefitinib metabolism.
- **Ratna Kumari,*et al* [46].** has reported a UV Spectrophotometric method was developed for the quantitative estimation of Gefitinib in bulk drug and tablet dosage forms. Gefitinib is a drug that is used to treat several types of lung cancer and in particular, it is used alone for the treatment of patients with a specific type of lung cancer termed non - small cell lung cancer (NSCLC) that has not responded to chemotherapy. It works by preventing lung cancer cells from growing & multiplying. The drug exhibits absorption maximum at 252nm in 0.1N HCL and obeys Beer's law in the concentration range of 10-35µg/ml. The method was extended to pharmaceutical preparations and there is no interference from any common pharmaceutical additives

- **Ming Zhao, *et al.*[47],** has reported a specific method for the determination of gefitinib using HPLC coupled to tandem mass spectrometry.

- **Goukanapalli Chandra Sekara Reddy, *et al.*[48],** have reported the convergent approach for commercial synthesis of gefitinib. An efficient and large scale convergent synthesis of epidermal geowth factor receptor-tyrosine kinase inhibitors Gefitinib approved by U.S. FDA for the treatment of non- cell lung cancer. The formation of 4-anilinoquinazolines are achieved in a simple one- pot reaction of suitable formamide intermediates and substituted anilines involving Dimroth rearrangement, thereby avoiding the need to make quinazolin-4(3 H)- one intermediates which require a large experimental inputs. Using this process we have produced drug candidates 1 with overall yield of 66% from 4-methoxy-5-[3-(4- morpholinyl) propoxyl]-2- nitrobenzonitrite and 2 with 63% from 4,5-bis(2-methoxyethoxy)-2-nitrobenzonitrite on a multigram scale.
- **Madireddy Venkataramana, *et al.*[49],** Degradation pathway for gefitinib is established as per ICH recommendations by validated and stability indicating reverse phase liquid chromatographic method. Gefitinib is subjected to stress conditions of acid, base, oxidation, thermal and photolysis. Significant degradation is observed in acid and base stress conditions. Two impurities are studied among which one impurity is found prominent degradant. The stress samples are assayed against a qualified reference standard and the mass balance is found close to 99.5%. Efficient chromatographic separation is achieved on a Agilent make XDB-C18, 50 × 4.6 mm with 1.8 µm particles stationary phase with simple mobile phase combination delivered in gradient mode and quantification is carried at 250 nm at a flow rate of 0.5 ml /·min. In the developed RPLC method the resolution between gefitinib and the potential impurities is found to be greater than 5.0. Regression analysis shows an r value (correlation coefficient) of greater than 0.998 for gefitinib and the two potential impurities. This method is capable to detect the impurities of gefitinib at a level of 0.01% with respect to test concentration of 0.5 mg /·ml for a 4-µl injection volume. The developed RPLC method is validated with respect to specificity, linearity & range, accuracy, precision and robustness for impurities determination and assay determination.

OBJECTIVES

The present study is to make an attempt to establish sensitive, economic and accurate methods for the estimation of Gefitinib in pure and pharmaceutical dosage form. The proposed methods will be validated as per ICH guidelines.

Literature survey reveals that only few analytical methods have been reported for the estimation of Gefitinib in bulk drug and pharmaceutical formulations.

Hence an attempt has been made to develop simple, accurate, sensitive, rapid and economic method for the estimation of Gefitinib in pharmaceutical dosage forms using High Performance Liquid Chromatography techniques. These methods can also be applied for estimation of pure drug.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

To develop and validate Reversed phase high performance liquid chromatography method for estimation of Gefitinib in bulk drug and pharmaceutical dosage forms.

An attempt has been made to develop and validate the above mentioned methods to ensure their accuracy, precision, robustness and other analytical method validation parameters as mentioned in the ICH guidelines.

PLAN OF WORK

Based on the objective, the plan of the work is as follows. Plan of work was designed into following ways

1. Selection of analytical techniques.
2. Selection of analytical concentration ranges.
3. Selection of analytical method.
4. Selection of mobile phase.
5. Method development and optimized chromatographic condition.
   - Selection of wavelength
   - Choice of chromatographic method
6. Validation of developed HPLC method as per ICH guidelines.

INSTRUMENT AND CHEMICAL REAGENTS :

CHEMICALS AND REAGENTS

S. NO.	CHEMICALS/STANDARDS AND REAGENTS	GRADE
1	Ortho-phosphoric acid	AR
2	Triethylamine	AR
3	Trifluoro aceticacid	AR
4	Methanol	HPLC
5	Water	Milli Q

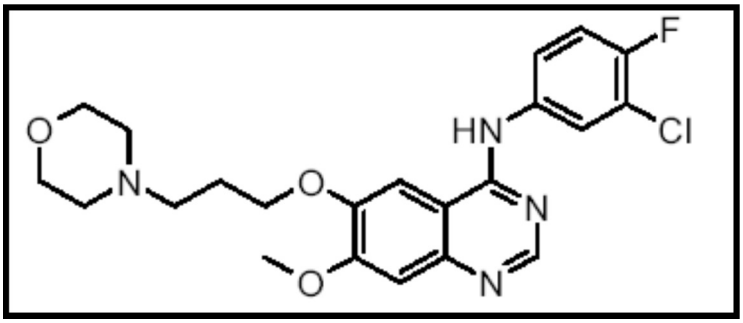
INSTRUMENTS AND EQUIPMENTS:

S.NO.	INSTRUMENTS AND EQUIPMENTS	SOFTWARE	MODEL	COMPANY
1	HPLC	EZ Chrome	1120	AGILENT
2	HPLC	Clarity	LC10ATvp	SHIMADZU
3	Weighing Balance	N/A	AUX 220	SHIMADZU
4	Sonicator	N/A	N/A	EQUITRON
5	pH Meter	N/A	LP139S	POLMON

Drug Profile

GEFITINIB:

Structure:



Molecular Formula: C $_{22}$H $_{24}$ CIFN $_{4}$ O $_{3}$

Molecular Weight: 446.902 g/mol

Chemical Name: N-(3-chloro-4-fluorophenyl)-7-methoxy-6-[3-(morpholin-4yl) propoxy] quinazolin-4-amine.

Description: A white to yellow colour powder

Melting Point: 192-194 ^{0}C

Category: Antineoplastic Agents, Protein Kinase Inhibitors

Solubility: sparingly soluble in aqueous media but readily soluble in organic solvents

Dosage: 250mg per day with meal

Metabolism: Hepatic

Bioavailability: 59% (oral)

Toxicity: The acute toxicity of gefitinib up to 500 mg in clinical studies has been low. In non-clinical studies, a single dose of 12,000 mg/m^2 (about 80 times the recommended clinical dose on a mg/m^2 basis) was lethal to rats. Half this dose caused no mortality in mice. Symptoms of overdose include diarrhea and skin rash.

Absorption: Absorbed slowly with oral administration.

Mechanism of Action:

Gefitinib inhibits the epidermal growth factor receptor (EGFR) tyrosine kinase by binding to the adenosine triphosphate (ATP)-binding site of the enzyme. Thus the function of the EGFR tyrosine kinase in activating the R as signal transduction cascade is inhibited; and malignant cells are inhibited. Gefitinib is the first selective inhibitor of the EGFR tyrosine kinase which is also referred to as Her1 or ErbB-1. EGFR is over expressed in the cells of certain types of human carcinomas - for example in lung and breast cancers. Over expression leads to inappropriate activation of the apoptotic Ras signal transduction cascade, eventually leading to uncontrolled cell proliferation.

ANTICANCER

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems. Anticancer drugs either kill the cancer cells or modify their growth. Validation of new analytical methods for estimation of anticancer drug is required. In view of the need for a suitable method for routine analysis in combined formulations, attempts are made to develop simple, precise and accurate analytical methods for simultaneous estimations of ingredients and extend it for their determination in formulation. Analytical validation is the corner stone of the process validation. Without a proven measurement system it is impossible to confirm whether the manufacturing process has done what it supports to do. Hence there is a need to validate the new methods developed. Various classes of drugs available are

CLASSIFICATION:

1. Alkylating Agents
   - ✓ Nitrogen mustards: Melphalan, Cyclophosphamide, Ifosfamide
   - ✓ Nitrosoureas
   - ✓ Alkylsulfonates
2. Antimetabolites
   - ✓ Folate Antagonists: Methotrexate
   - ✓ Purine antagonists:Fludarabine,mercaptopurine
   - ✓ Pyrimidine antagonists: 5-Fluorouracil, Cytarabibe
3. Natural Products

a. Plant Products

- ✓ Vinca Alkaloids: Vincristine, Vinblastine
- ✓ Taxanes: P
- ✓ aclitaxel
- ✓ Camptothecins: Irinotecan

b. Microorganism Products

- ✓ Antibiotics: Doxorubicin, Bleomycin
- ✓ Enzymes: L-Asparaginase

Miscellaneous

- ✓ Hydroxyurea
- ✓ Imatinib Mesylate

Hormones and Antagonists

- ✓ Corticosteroids: Prednisone, Dexamethasone
- ✓ Estrogens: Ethinyloestradiol
- ✓ Antiestrogens: Tamoxifen
- ✓ Progesteron derivative: Megestrol Acetate

Pharmacokinetics:

Gefitinib is absorbed slowly after oral administration with mean bioavailability of 60%. Elimination is by metabolism and excretion in feces. The elimination half life is about 48 hours. Daily oral administered of gefitinib to cancer patients resulted in a 2-fold accumulation compared to single dose administered. Steady state plasma concentrations are achieved within 10 days.

Absorption and Distribution: Gefitinib is slowly absorbed, with peak plasma levels occurring 3-7 hours after dosing and mean oral bioavailability of 60%. Bioavailability is not significantly altered by food. Gefitinib is extensively distributed throughout the body with a mean steady state volume of distribution of 1400 L following intravenous administration. *In vitro* binding of gefitinib to human plasma proteins (serum albumin α l-acid glycoprotein) is 90% and is independent of drug concentrations.

Metabolism and Elimination: Gefitinib undergoes extensive hepatic metabolism in humans, predominantly by CYP3A4. Three sites of biotransformation have been identified: metabolism of

the N-propoxymorpholino-group, demethylation of the methoxy- substitution on the quinazoline, and oxidative defluorination of the halogenated phenyl group.

Gefitinib is cleared primarily by the liver, with total plasma clearance and elimination half life values of 595 ml/min and 48 hours, respectively, after intravenous administration. Excretion is predominantly via feces (86%), with renal elimination of drug and metabolites accounting for less than 4% of the administered dose.

Pharmacology

Adverse Effects:

As gefitinib is a selective chemotherapeutic agent, its tolerability profile is better than previous cytotoxic agents. Adverse drug reactions (ADRs) are acceptable for a potentially fatal disease. Acne is reported very commonly. Other common adverse effects (≥1% of patients) include: diarrhoea, nausea, vomiting, anorexia, stomatitis, dehydration, skin reactions, paronychia, asymptomatic elevations of liver enzymes, asthenia, conjunctivitis, blepharitis Infrequent adverse effects (0.1–1% of patients) include: interstitial lung disease, corneal erosion, aberrant eyelash and hair growth.

Pharmcodynamics:

Gefitinib inhibits the intracellular phosphorylation of numerous tyrosine kinases associated with transmembrane cell surface receptors, including the tyrosine kinases associated with the epidermal growth factor receptor (EGFR-TK). EGFR is expressed on the cell surface of many normal cells and cancer cells

Drug Interactions:

In human liver microsome studies, gefitinib had no inhibitory effect on CYP1A2, CYP2C9, and CYP3A4 activities at concentrations ranging from 2-5000 ng/ml. At the highest concentration studied (5000 ng/ml), gefitinib inhibited CYP2C19 by 24% and CYP2D6 by 43%. Exposure to metoprolol, a substrate of CYP2D6, was increased by 30% when it was given in combination with gefitinib (500 mg daily for 28 days) in patients with solid tumors.

Rifampicin, an inducer of CYP3A4, reduced mean AUC of gefitinib by 85% in healthy male volunteers Concomitant administration of itraconazole (200 mg QD for 12 days),

an inhibitor of CYP3A4, with gefitinib (250 mg single dose) to healthy male volunteers, increased mean gefitinib AUC by 88%.

Co-administration of high doses of ranitidine with sodium bicarbonate (to maintain the gastric pH above pH 5.0) reduced mean gefitinib AUC by 44 %.

International Normalized Ratio (INR) elevations and/or bleeding events have been reported in some patients taking warfarin while on IRESSA therapy. Patients taking warfarin should be monitored regularly for changes in prothrombin time or INR.

Therapeutic uses

- ✓ It is used in treating non-small cell lung cancer
- ✓ Effective in treating non cancer related TNF-α mediated inflammatory diseases including autoimmune and non- autoimmune diseases.
- ✓ Used in the treatment of certain types of cancer.

Estimation of Gefitinib by RP-HPLC method.

Apparatus and software

The Agilent 1120 Compact LC HPLC system consisting of gradient pump (4MPa or 40barr), Rheodyne injector, UV variable detector, Standard cell and Agilent syringe (50µl) and also HPLC LC-10 ATvp instrument with clarity software was used. The separations were achieved on a Agilent column 5µm 4.6x250mm with UV detection at 246nm. Analytical weighing balance (Shimadzu AUX 220) was used for weighing, sonicator (EQUITRON-230VAC, 50Hz) vacuum pump (SUPER FIT 110336), Millipore filtration kit (TARSONS) with PALL membrane for solvents and sample filtration were used throughout the experiment. The EZ Chrome Elite software was used for acquisition, evaluation and storage of chromatographic data.

Reagents:

Analytically pure sample of Gefitinib procured as gift sample by celon laboratories (Hyderabad). The drug was used without further purification. HPLC grade Methanol (Merck), Pharmaceutical formulation gefitinib (Iressa) tablets (label claim 250mg) Manufactured by Astrazeneca Pharmaceuticals Limited was used in the HPLC. HPLC grade water obtained in-house by using Direct-Q3 with pump (Elec. Ratings: 100-230V of 50-60Hz 100VA) water purification system (made in France) were used in HPLC study.

Mobile phase:

A number of trials were made to find out the ideal solvent system (mobile phase) for eluting the drug. The mobile phase containing Methanol: Water (60:40), Potassium dihydrogen phosphate buffer: Methanol (50:50), Methanol: Glacial acetic acid buffer pH 3.0 (60:40) and Methanol: Di sodium hydrogen phosphate (80:20) was tried. Better peak and adequate retention time were obtained with the ratio of 0.1% triflouroacetic acid of pH 3.0(adjusted with Triethylamine): Methanol (HPLC grade) (35:65).

Preparation of buffer Solution (0.1% Triflouro acetic acid):

1750µl of Ortho-phosphoric acid was diluted to 1000mL using water and pH was adjusted to 3.0 by using $C_6H_{15}N$ (Triethylamine). This solution was filtered through a 0.45µm nylon filter paper (PALL) and degassed by ultrasonicator.

Preparation of Mobile Phase:

Transferred 1000 ml of above solution and 1000 ml of methanol to the mobile phase bottles separately. HPLC experiments were carried out using binary pump A containing triflouroacetic acid(0.1%) and pump B containing Methanol in the ratio of 35:65.

Diluent preparation:

Mixture of triflouroacetic acid and Methanol was used as diluent. Transferred 35 ml of triflouroacetic acid buffer to a 100 ml beaker and add 65 ml of Methanol.

Preparation of Standard Stock Solution of Gefitinib:

50 mg of gefitinib standard was accurately weighed and transferred to a 50ml volumetric flask dissolved in 10 ml of methanol and sonicated for 10mins and made up with methanol to give a solution containing 1000µg/ml. (stock solution 'A') From this stock solution, pipette out 5ml placed in to 50ml volumetric flask and volume was made up to mark with diluent to give a solution containing 100µg/ml (stock solution 'B'). From the above 0.5ml, 1ml, 1.5ml, 2ml, 2.5ml and 3mL of solutions were pipetted out into two separate 10 mL volumetric flasks and volume was made up to the mark with the diluent used. This gave the concentration of 5, 10, 15, 20, 25 and 30µg / ml. These six dilutions of **Gefitinib** were prepared and are estimated in HPLC.

After several trials with different combination of solvents, 0.1% triflouro acetic acid: methanol in the ratio 35:65 (v/v) by adjusting final pH 3.0 was selected because it gave a peak with retention time (R_t) of Gefitinib (3.8mins). Wavelength was selected by scanning the standard drug over a wide range of wavelength 200nm to 400 nm.

Table no: 1 Optimized Chromatographic conditions of Gefitinib

S. No	Parameters	Gefitinib
1	Mobile phase optimized	Triflouroaceticacid (0.1%) : Methanol (35:65)
2	Stationary phase	C_{18} 5µm 250 X 4.6 mm (Agilent)
3	Flow rate (ml/min)	1
4	Run time(min)	10
5	Column Temperature ^{O}C	25±1
6	Volume of Injection (µl)	20
7	Detection Wavelength (nm) 23232	246 nm
8	Retention time Rt	3.8min

Table no:2. Characteristic parameters of calibration equation for the proposed HPLC method for Estimation of Gefitinib

Parameters	Gefitinib	Acceptance criteria
Calibration range (µg mL^{-1})	5-30	----
Detection limit (µg mL^{-1})	1.481	----
Quantification limit (µg mL^{-1})	4.489	----
Slope (m)	759565	-----
Intercept (c)	100006	----
Correlation coefficient	0.998	NLT O.995
Theoretical plates	3799	NLT 2000
Tailing factor	1.20992	NMT 2

$Y = m\,x + c$, where C is the concentration of compound in µg ml^{-1} and Y is the peak area.

Assay of Gefitinib in Tablets:

Twenty tablets were weighed and finely powdered. An accurately weighed quantity of the powder equivalent to 100 mg of Gefitinib was transferred to 100 ml volumetric flask containing 40 ml of mobile phase and the contents of the flask were sonicated for 15 min, to ensure the complete solubility of the drug, then the mixture was made up to 100ml with mobile phase.

The resulting solution was thoroughly mixed and filtered through a 0.45 µm membrane filter. From this solution, required dilutions for HPLC method were prepared within the linearity range using mobile phase as solvent.

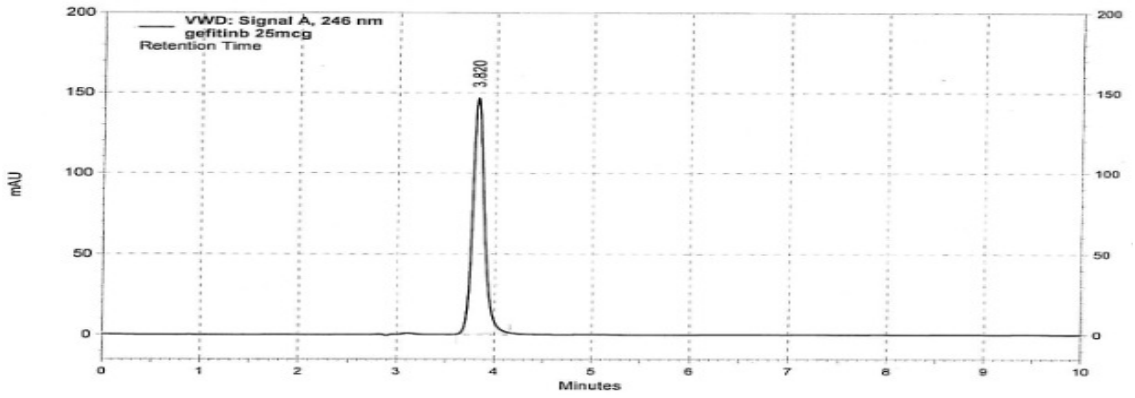


Fig. 1 STANDARD

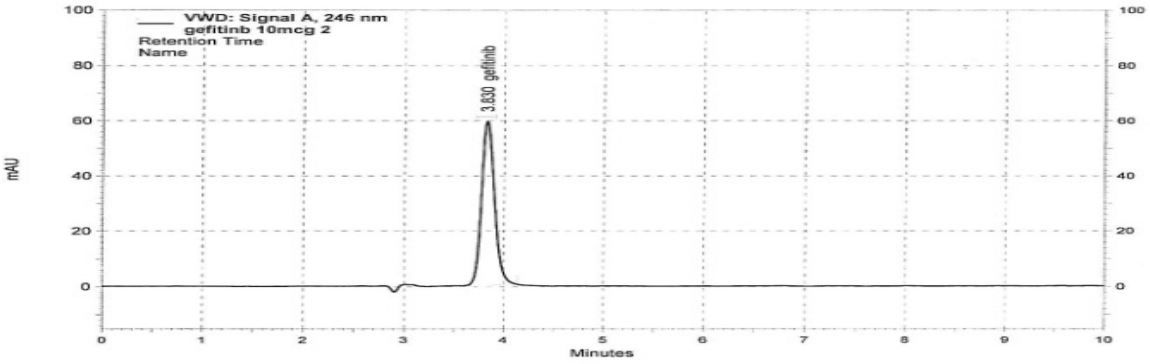


Fig. 2 SAMPLE

Table no: 3 Calculation of Percentage Purity

STANDARD	**AREA** 20417132	**ASSAY PERCENTAGE**
SAMPLE	**AREA** 20474693	100.28 % w/w

Standard Area = 20417132

Sample Area = 20474693

Percentage Purity = Sample Area / Standard Area × 100

= 20474693 / 20417132 × 100

= 100.28 % w/w

VALIDATION OF ANALYTICAL METHOD: Validation of an analytical method is the process to establish by laboratory studies that the performance characteristic of the method meets the requirements for the intended analytical application.

1. SPECIFICITY:

The specificity of the method was evaluated with regard to interference due to presence of any other excipients. The figure no: 3 shows that drug was clearly separated from its excipients. Thus, the HPLC method presented in this study is selective.

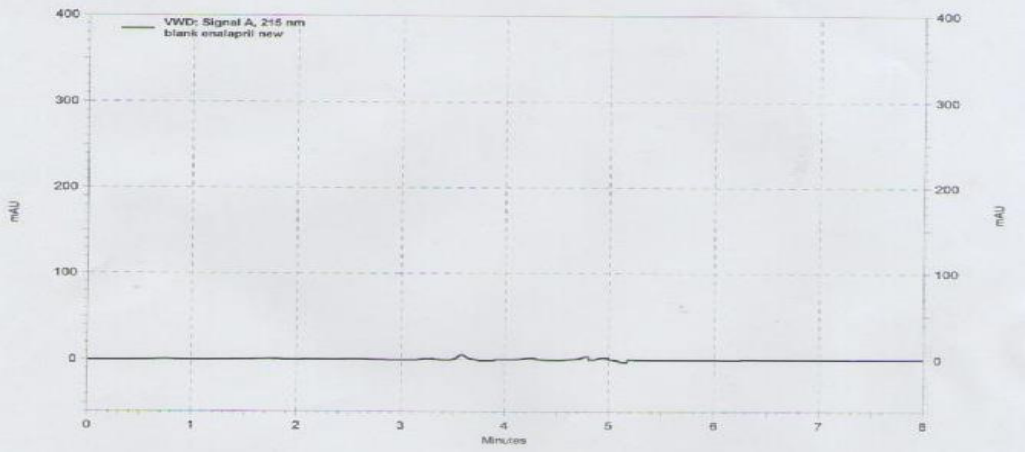


Fig. 3 Chromatogram of blank

Blank interference:

A study to establish the interference of blank was conducted. Mobile phase was injected as per the test method.

Chromatogram of blank not showed any peak at the retention time of analyte peak.

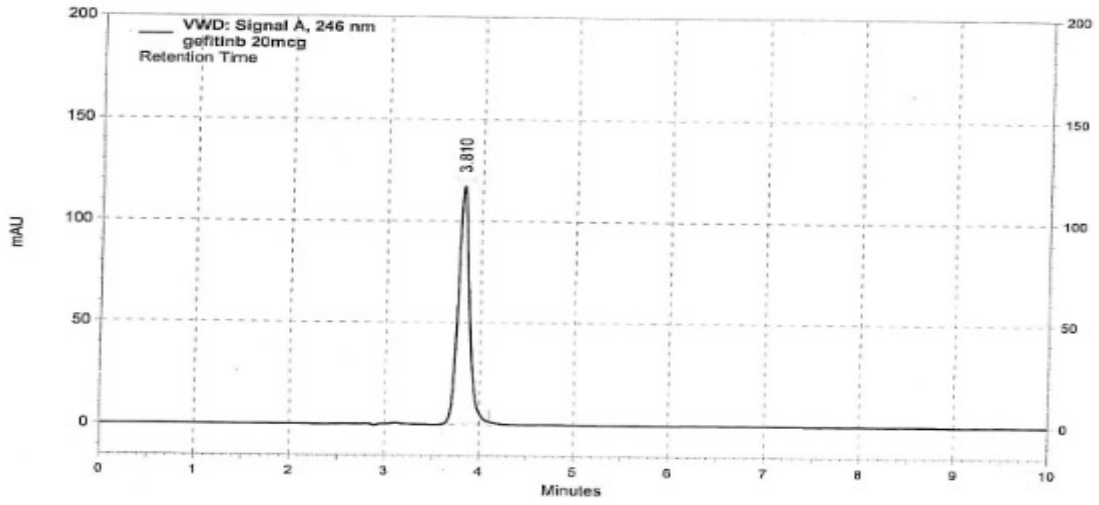


Fig. 4 Chromatogram of Gefitinib showing retention time (R_t)

2. ACCURACY

Accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. Accuracy is performed in three different levels, each level in triplicate for Gefitinib using standards at 50%, 100% and 150% are shown in Fig. 5 ,6 ,7 . Each sample is analyzed in triplicate for each level. From the results, % recovery is calculated are shown in Table no: 4 and 5.

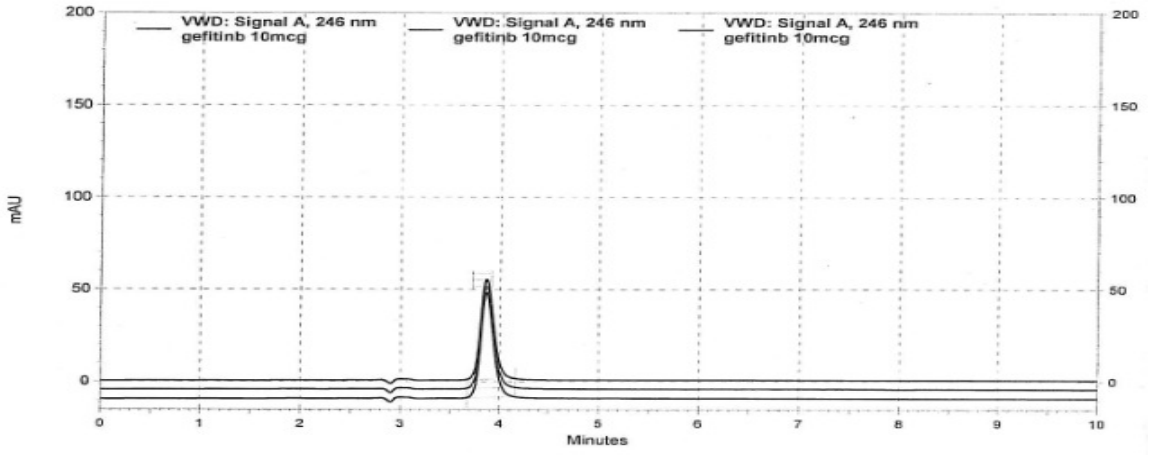


Fig. 5 Chromatogram of Gefitinib showing 50% accuracy

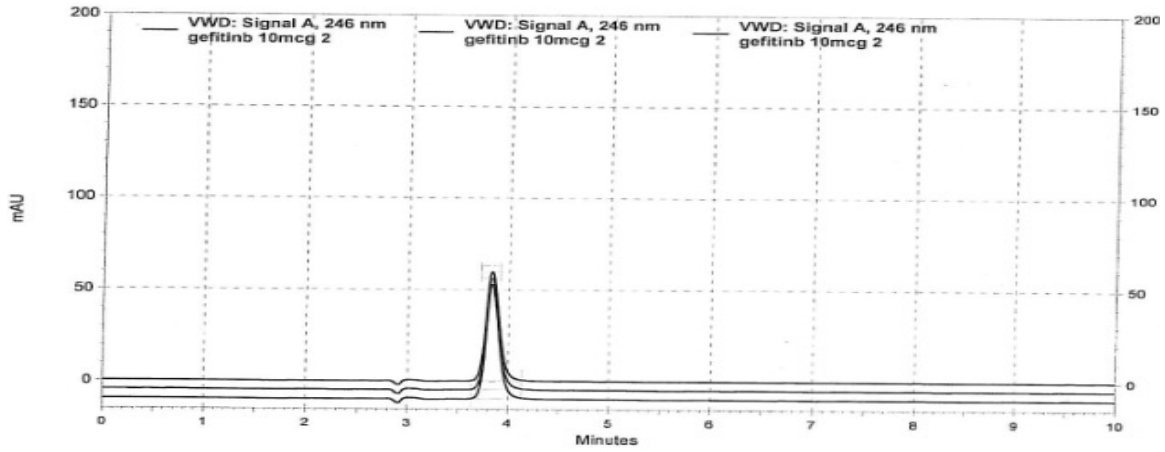


Fig. 6 Chromatogram of Gefitinib showing 100% accuracy

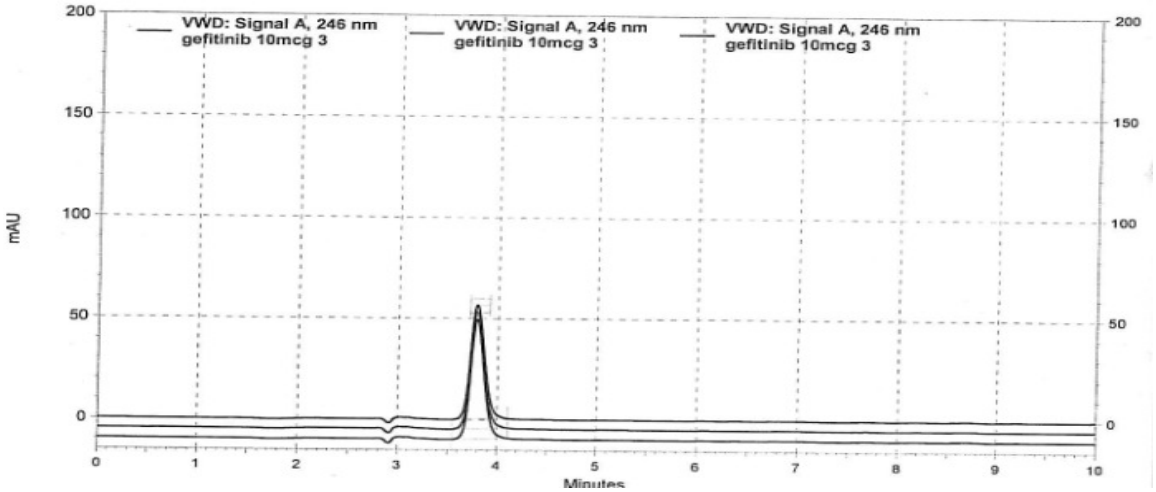


Fig. 7 Chromatogram of Gefitinib showing 150% accuracy.

Table no: 4. Accuracy of Gefitinib

S.NO	Recovery Level (%)	RP-HPLC		
		Standard Area	Area of Gefitinib	% Recovery
1	50	8231162	8214874	99.8
2	100	8231162	8291399	100.7
3	150	8231162	8240493	100.1

Mean of three determinations

Table no: 5. Results of recovery of Gefitinib

S.NO	Recovery Level in%	Area	Mean % Recovery ±RSD
1	50	8242161	8214874±0.29
2	50	8198334	
3	50	8204121	
4	100	8236132	8291399±0.63
5	100	8189411	
6	100	8293009	
7	150	8213212	8240493±0.27
8	150	8198428	
9	150	8242523	

3. LINEARITY

Linearity of the proposed HPLC method for determination of Geftinib was evaluated by analyzing a series of different concentrations of standard drug. In this study five concentrations were chosen ranging between 5-30µg/ml. By using the stock solution 'B', aliquots of 5, 10, 15, 20, 25 and 30µg/ml were prepared with diluent six dilutions of each of the above mentioned concentrations were prepared separately and from these six dilutions, 20 µl each concentration was injected three times and obtained information on variation in the peak area response of pure analytes was plotted against corresponding concentrations and result was shown in Table no: 6.

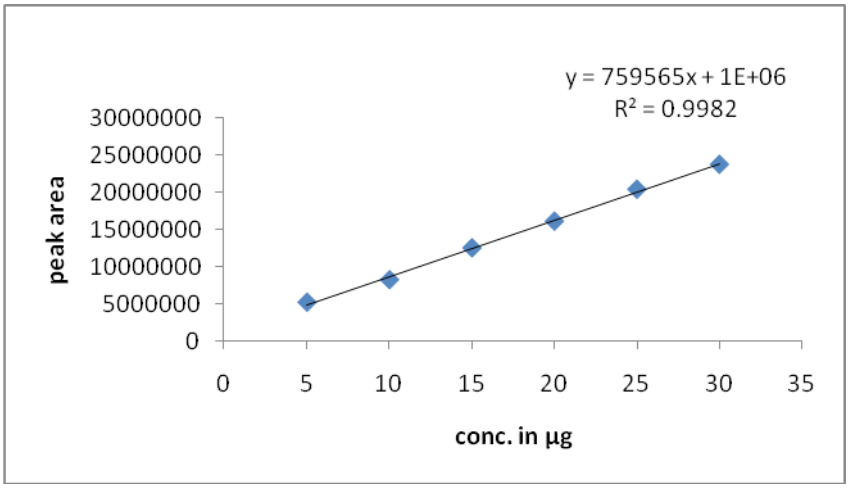


Fig. 8 Linearity curve of Gefitinib

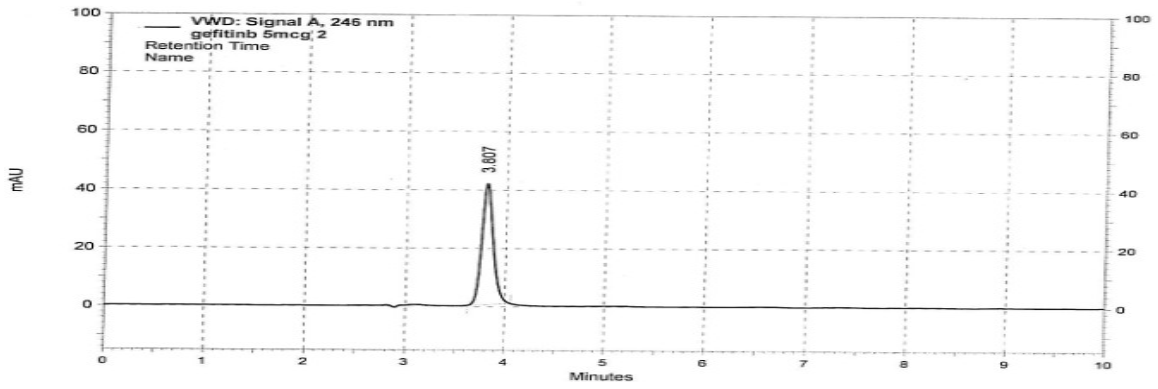


Fig. 9 Chromatogram of Gefitinib showing a concentration of 5µg/ml

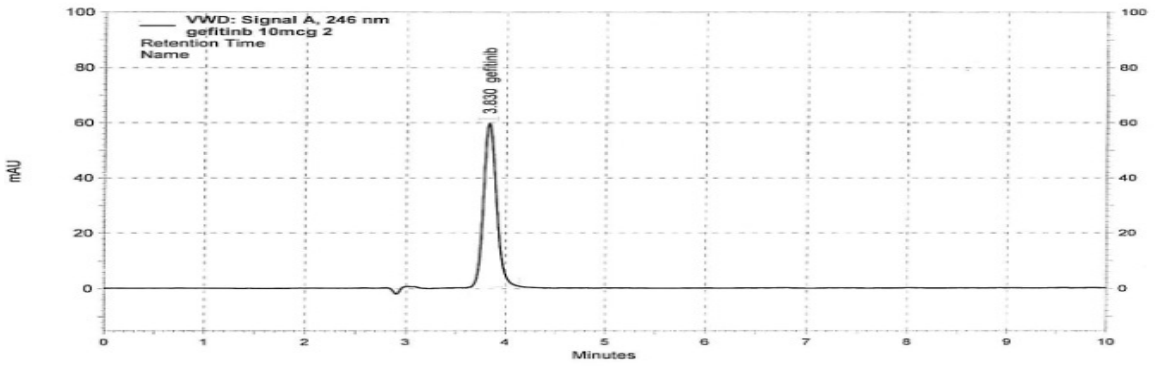


Fig. 10 Chromatogram of Gefitinib showing a concentration of 10µg/ml

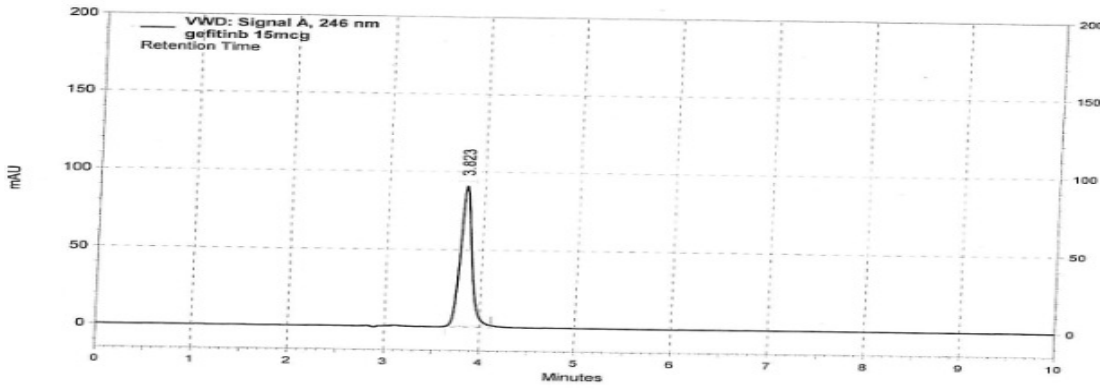


Fig. 11 Chromatogram of Gefitinib showing a concentration of 15µg/ml

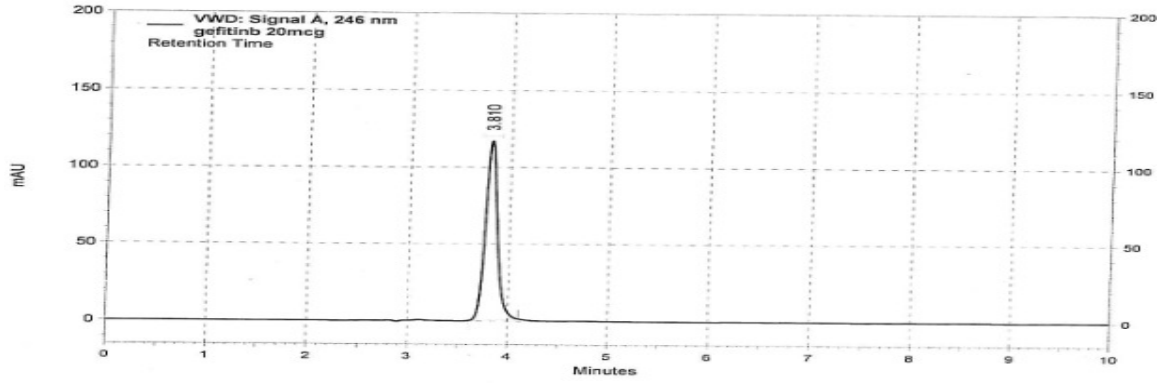


Fig. 12 Chromatogram of Gefitinib showing a concentration of 20µg/ml

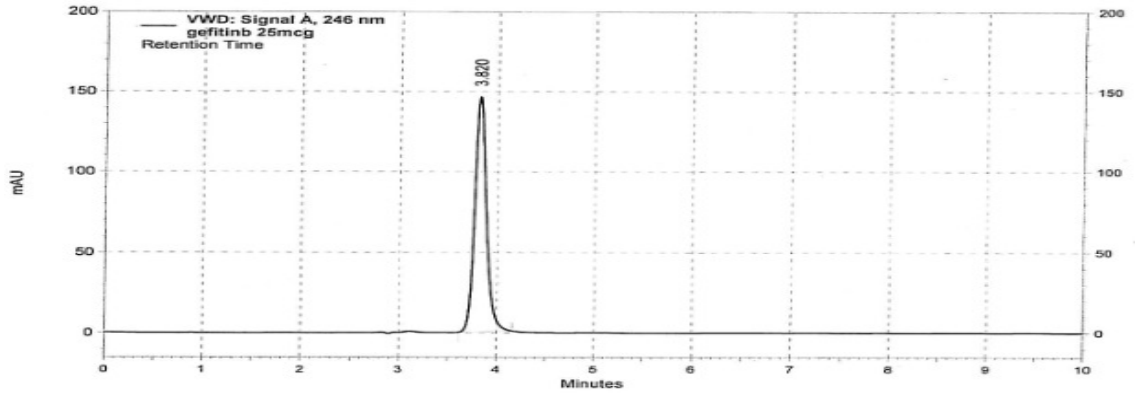


Fig. 13 Chromatogram of Gefitinib showing a concentration of 25µg/ml

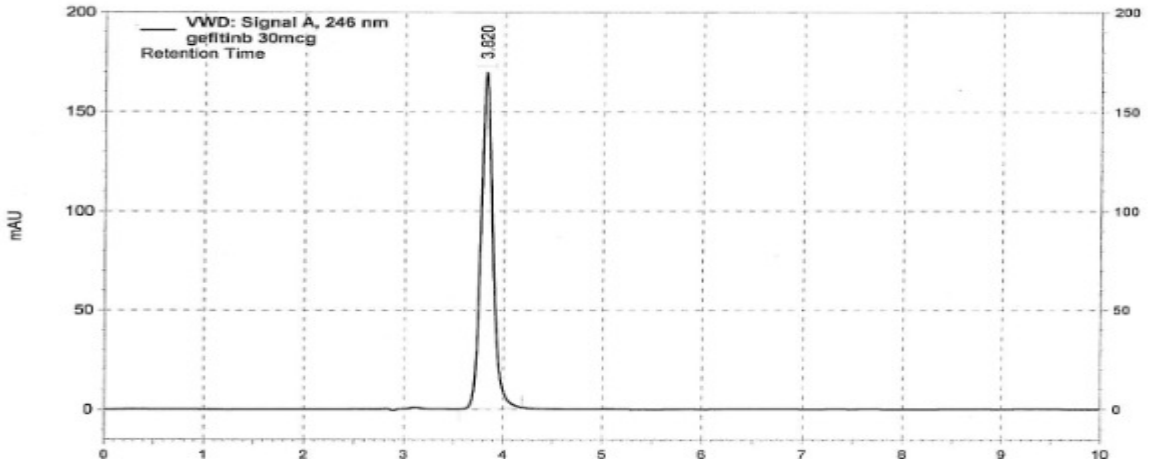


Fig. 14 Chromatogram of Gefitinib showing a concentration of 30µg/ml

Table no: 6. Linearity data for Gefitinib

S.No	**Gefitinib**	
	Concentration in µg/ml	**Peak area**
1	**5**	**5203009**
2	**10**	**8231162**
3	**15**	**12515723**
4	**20**	**16107083**
5	**25**	**20417132**
6	**30**	**23757913**

avg of 6 determinations

4. PRECISION

Precision of the analytical method was studied by analysis of multiple sampling of homogeneous sample. The precision expressed as % RSD were given in Table no. 7.

Method reproducibility was demonstrated by repeatability and intermediate precision measurements of peak area, retention time and peak symmetry parameters of HPLC method for each title ingredients.

Repeatability (of results of measurements) - the closeness of the agreement between the results of successive measurements of the same substance carried out under the same conditions (same measurement procedure, the same observer, the same measuring instrument, used under the same conditions, the same location, and repetition over a short period of time) of measurement.

The repeatability and intermediate precision were carried out at 100% concentration for Gefitinib. The obtained results within and between the days of trials were in acceptable range indicating good precision of the proposed methods were given in Table no 7 and 8

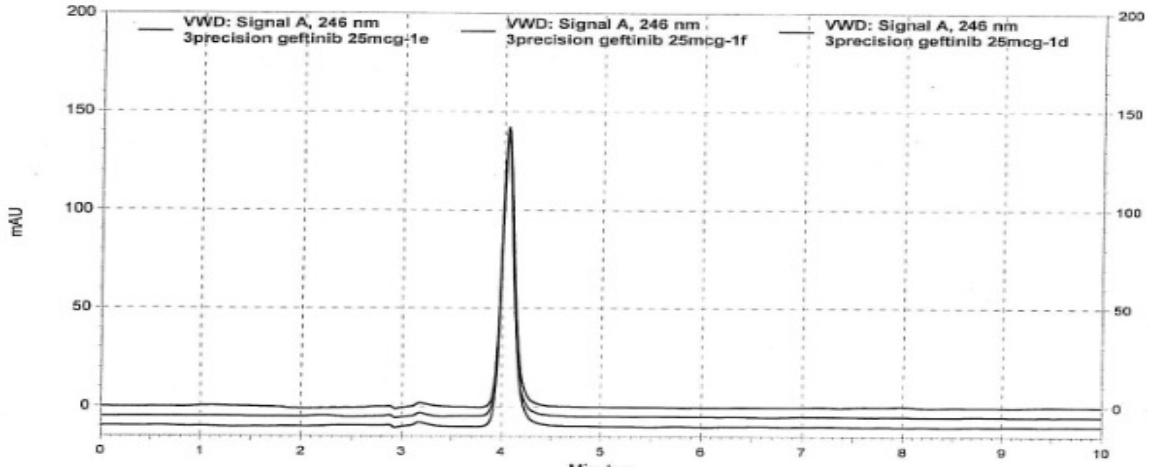


Fig. 15 Chromatogram of Gefitinib showing intraday (Forenoon) precision

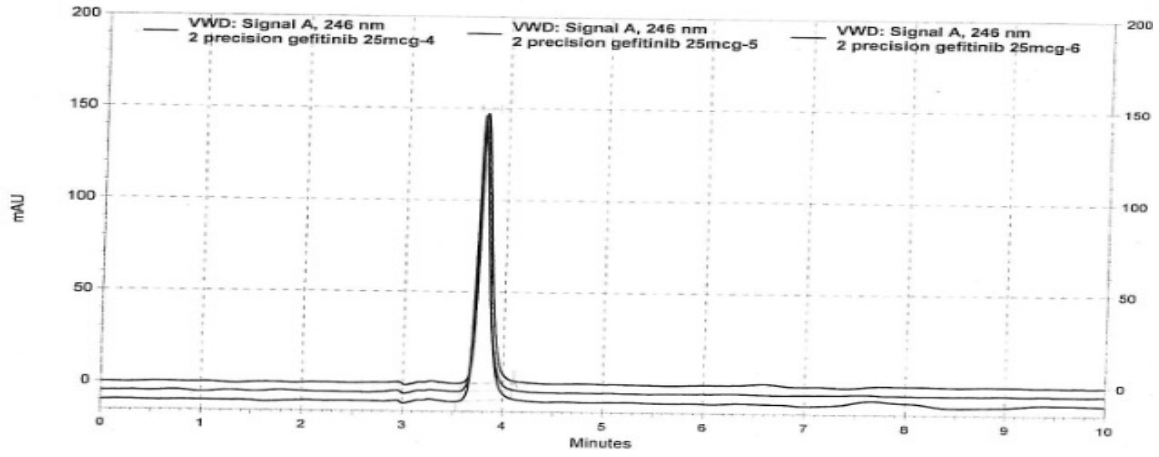


Fig. 16 Chromatogram of Gefitinib showing intraday (Afternoon) precision

Table no: 7. Intraday (Repeatability) study results by HPLC

S.NO	FORENOON		AFTERNOON	
	INJECTION	AREA	INJECTION	AREA
1	Injection 1	19761211	Injection 1	20685542
2	Injection 2	20390438	Injection 2	20664991
3	Injection 3	20332497	Injection 3	20716784
4	Injection 4	20547346	Injection 4	20397066
5	Injection 5	20474693	Injection 5	20412069
6	Injection 6	20444825	Injection 6	20210929
	Average	**20325168**	**Average**	**20719141**
	SD	**282797.1**	**SD**	**204577.6**
	%RSD	**1.406**	**%RSD**	**0.987**

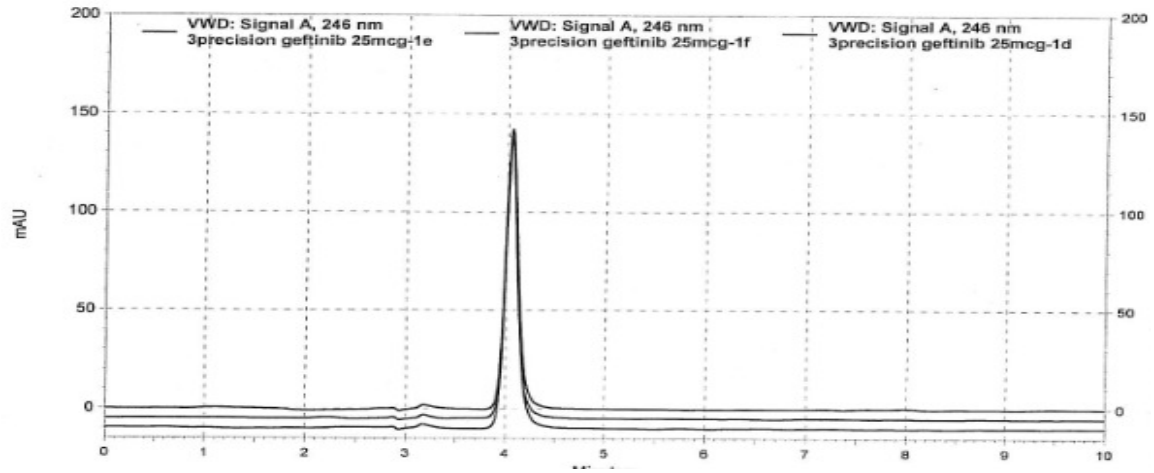


Fig. 17 Chromatogram of Gefitinib showing interday (day1) precision

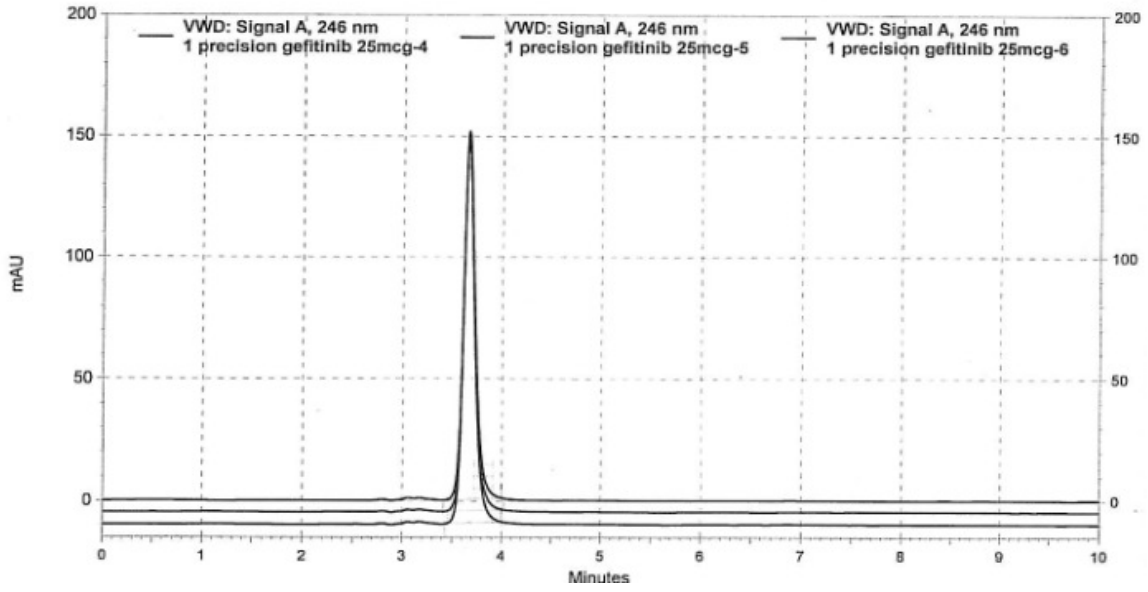


Fig. 18 Chromatogram of Gefitinib showing interday (day2) precision

Table no: 8. Inter day (intermediate precision) study results by HPLC

S.NO	DAY 1		DAY 2	
	INJECTION	AREA	INJECTION	AREA
1	Injection 1	19761211	Injection 1	20456575
2	Injection 2	20390438	Injection 2	20934578
3	Injection 3	20332497	Injection 3	20582365
4	Injection 4	20547346	Injection 4	20222589
5	Injection 5	2474693	Injection 5	20456238
6	Injection 6	20444825	Injection 6	20589632
Average		**20325168**	**Average**	**20540329**
SD		**285797.1**	**SD**	**234382**
%RSD		**1.407**	**%RSD**	**1.141**

5. LIMIT OF DETECTION(LOD) AND LIMIT OF QUANTITATION(LOQ)

Table no: 9. LOD & LOQ of Gefitinib

PARAMETERS	µg/ml
LOD	1.481
LOQ	4.489

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LOD and LOQ were calculated according to ICH recommendations where the approach is based on the signal-to-noise ratio. Chromatogram signals obtained with known low concentrations of analyte was compared with the signals of blank samples. A signal-to-noise ratio 3:1 and 10:1 was considered for calculating LOD and LOQ respectively. The values of LOD and LOQ were given in Table no. 9

6. SYSTEM SUITABILITY:

Table no. 10. Results of system suitability parameters

Parameters	Data obtained for Gefitinib
Theoretical plates per meter	**15196**
Theoretical plates per column	**3799**
Symmetry factor/Tailing factor	**1.2099**

Theoretical plates per meter were calculated from the data obtained from the peak using the following expression

$$n = (5.54Vr^2)/LW_{h2}$$

Theoretical plates per column were calculated from the data obtained from the peak.

$$n = (5.54Vr^2)/W_{h2}$$

Where, "n" is the number of theoretical plates per meter. "Vr" is the distance along the base line between the point of injection and perpendicular dropped from the maximum of the peak of interest and "W_h" is the width of the peak of interest at half peak height Table No.10

Tailing factor is also known as symmetry factor of peak and it was calculated from the following expression

Symmetry factor = *a/b*

Where, **a** = ½ width of the peak at one twentieth of the peak height

b= ½ width of the peak at one twentieth of the peak height

7. RUGGEDNESS OF TEST METHOD:

System to system/ Analyst to Analyst/column to Column variability:

System to system/ Analyst to Analyst/column to column variability study was conducted on different HPLC systems, different columns and different analysts under similar conditions at different times. Six samples were prepared and each was analyzed as per test method. The relative standard deviation for Gefitinib were found to be below 2 % on the columns, systems and analysts. Comparison of both the results obtained on two different HPLC systems/Analyst to Analyst/ column to column variability.

i) Shimadzu system:

Table No:11

Tablet ID	% Assay of Gefitinib	Mean	SD	%RSD
1	99.24	**98.59**	**0.56**	**0.56**
2	98.19			
3	98.34			

ii) Agilent System:

Table No:12

Tablet ID	% Assay of Gefitinib	Mean	SD	%RSD
1	99.32	**98.94**	**0.806**	**0.81**
2	98.02			
3	99.5			

ACCEPTANCE CRITERIA:

The % RSD of Gefitinib from the six sample preparations should be not more than 2.0 %.

The percentage recovery of Gefitinib should be between 95.0 % to 105 %.

OBSERVATION:

The %RSD was found within the limits

INTERMEDIATE PRECISION:

Day 1:

Table No:13

Tablet ID	% Assay of Gefitinib	Mean	SD	%RSD
1	99.87	**99.26**	**0.69**	**0.69**
2	99.43			
3	98.5			

Day-2
Table No:14

Tablet ID	% Assay of Gefitinib	Mean	SD	%RSD
1	99.14	**98.71**	**1.41**	**1.42**
2	98.24			
3	98.77			

8. ROBUSTNESS:

i) Effect of variation of flow rate;

A study was conducted to determine the effect of variation in flow rate. Standard solution prepared as per the test method was injected into the HPLC system using rates 0.8 ml/min and 1.2 ml/min flow.

Gefitinib were resolved from all other peaks and the retention times were comparable with those obtained for mobile phase having flow rates 1.0 ml/min.

ACCEPTANCE CRITERIA:

The Tailing Factor of Gefitinib standards should be NMT 2.0 for variation in flow.

OBSERVATION:

The Tailing Factor for Gefitinib are found to be within the limits.

ii) Effect of Variation of temperature:

A study was conducted to determine the effect of variation in temperature. Standard solution prepared as per the test method was injected into the HPLC system at 25 ^{0}C temperature.
The system suitability parameters were evaluated and found to be within the limits for temperature change of 25 ^{0}C.
Gefitinib were resolved from all other peaks and the retention times were comparable with those obtained for mobile phase having ambient temperature.

ACCEPTANCE CRITERIA:
The Tailing Factor of Gefitinib standard and sample solutions should be NMT 2.0 for Variation in temperature.

iii) Effect of variation in pH

Gefitinib standard should be NMT 2.0 for Variation in pH. A study was conducted to determine the effect of variation in pH. Standard and sample solutions were prepared as per the test method and injected into the HPLC system using pH 2.7 and 3.5. The system suitability parameters were evaluated and found to be within the limits for pH 2.7 and 3.5.
Gefitinib were solved from all other peaks and the retention times were comparable with those obtained for mobile phase having pH 3.0 .
Form the above study it was established that the allowable variation in pH 2.7 and 3.5.

ACCEPTANCE CRITERIA:
The %RSD Factor Gefitinib were found to be within the limits.

Table No:15

Parameters	Optimum range	Conditions in procedure	Remarks

Flow rate ml/min	0.8-1.2	1.0	Increase in flow rate resulted in early elution of all two active ingredients. Decrease in flow rate in late elution of all two actives but still within the proposed run time.
Temperature	20-35 ^{0}C	25 ^{0}C	Increase in temperature resulted in early elution of all two active ingredients. Decrease in temperature resulted in late elution of all two actives but still within the proposed run time
pH	2.7 -3.5	3.0	Gefitinib were solved from all other peaks and the retention times were comparable with those obtained for mobile phase having pH 3.0 .

CONCLUSION: From the above results it is concluded that the method was robust.

RESULTS AND DISCUSSION

SYSTEM SUITABILITY:

The system suitability parameters were evaluated. The %RSD for six replicate injections of Gefitinib is with in the limits. The tailing factors for Gefitinib peaks were 1.20. The theoretical plates for Gefitinib are 3799 were found to be with in the limits. The system suitability parameters were evaluated and found to be within the limits.

PRECISION:

The percentage assay of six replicate injections of the drugs were performed and the percentage RSD of individual drugs were calculated and found to be which are found to be with in the limits the %RSD is less than 2.

ACCURACY:

Accuracy of the method was performed with concentration level of 50%, 100% and 150% and the percentage recovery was calculated and found to be with in the limits of 95% to 105% and the %RSD is also less than 2.

LINEARITY:

The linearity of the method was tested for Gefitinib at 6 concentration levels and the correlation coefficient was calculated for the drugs and found to be with in limits (not less than 0.995).

SPECIFICITY:

There was no absolute interference from placebo preparations at retention time of Gefitinib. This shows that the method is specific.

Placebo interference:

It was concluded that there is no interference with placebo as no peaks were observed at the retention time of Gefitinib peaks.

Interference from the degradation products:

Purity angle was found to be less than threshold angle in all forced degradation studies without having signs of purity flags. All degradant peaks were resolved from Gefitinib peaks in the chromatograms of all samples and found that there was no interference with the main drug peaks.

RUGGEDNESS:

System to system/ Analyst to Analyst/column to column variability study was conducted on different HPLC systems. Six samples were prepared and each was analyzed as per test method. The relative standard deviation for Gefitinib were found to be below 2% on the columns, systems and analysts.

ROBUSTNESS:

The robustness of the method was tested with the variation in Flow rates, Temperature and Buffer pH. The %RSD of the varied parameters is calculated for both the drugs which are found to be with in the limits. The method was found to be robust.

CONCLUSION

In the present investigation we have validated a simple, sensitive, precise and accurate RP-HPLC method for the quantitative estimation of Gefitinib in pharmaceutical formulations. The results expressed in Tables for RP- HPLC method are promising. The RP- HPLC method is more sensitive, precise and accurate compared to the spectrophotometric methods. This method can be used for the routine determination of Gefitinib in bulk drug and in pharmaceutical formulations as per ICH guidelines.

Chromatographic conditions :

Mobile phase	: Triflouroaceticacid (0.1%) : Methanol (35:65)
Column	: C_{18} 5µm 250 × 4.6 mm (Agilent)
Flow rate	: 1ml / min
Detector wavelength	: 246 nm
Injection volume	: 20µl
Column oven temperature	: 25±1°c
Runtime	: 10 minutes

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