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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE ESTIMATION OF ERLOTINIB IN ITS PHARMACEUTICAL DOSAGE FORM

Dissertation

Submitted in partial fulfilment of the requirement for the award of the degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

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

CHENNAI

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

K. M. COLLEGE OF PHARMACY

UTHANGUDI, MADURAI – 625107

APRIL-2012

CERTIFICATE

This is to certify that the all project entitled "**DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE ESTIMATION OF ERLOTINIBIN ITS PHARMACEUTICAL DOSAGE FORM"** submitted by **D.JYOTHI** (**Reg. No.261017201**) in part fulfilment of the degree of Master of Pharmacy in Pharmaceutical Analysis **K.M.COLLEGE OF PHARMACY,** MADURAI-625107 under To The Tamilnadu Dr. M.G.R. Medical University, Chennai, carried out in **NATCO PHARMAPVT. LTD, HYD***,* is a bonafide work carried out by her under my guidance and supervision during the academic year **2011– 2012**.This dissertation partially or fully has not been submitted for any other degree or diploma of this University or any other Universities.

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ABBREVIATIONS

%	**: Percentage**
nm	**: Nanometer**
v/v	**: Volume by volume**
Min	**: Minute**
mg	**: Milligram**
µg	**: Microgram**
ng	**: Nanogram**
LC	**: Liquid chromatography**
HPLC	**: High-performance liquid chromatography**
GC	**: Gas chromatography**
UV	**: Ultraviolet**
HPTLC	**: High-performance thin layer chromatography**
TLC	**: Thin layer chromatography**
LC-MS	**: Liquid chromatography-Mass spectroscopy**
mm	**: Millimeter**
RSD	**: Relative standard deviation**
µ mol	**: Micromole**
RP-HPLC	**: Reversed-phase high-performance liquid chromatography**
RI	**: Refractive index**
USP	**: United States Pharmacopoeia**
HETP	**: Height equivalent to theoretical plate**
LOD	**: Limit of detection**
LOQ	**: Limit of quantification**
CV	**: Coefficient of variance**
S/N	**: Signal to noise ratio**
PDA	**: Photo diode array detector**
USFDA	**: United state Food and Drug Administration**
ODS	**: Octa decyl silane**

INTRODUCTION

GENERAL INTRODUCTION:

Analytical chemistry is a branch of chemistry that determines the nature and identity of a substance and its composition. In the early twentieth century there were only four accepted branches of chemistry, organic chemistry, inorganic chemistry, physical chemistry, biochemistry. At that time, analysis was considered to be a service to the other four branches. Its importance grew and in the process, absorbed techniques and skills from all other four branches. So by the 1950's, analytical chemistry was finally accepted as a branch of chemistry in its own right. There are basically two types of analysis, qualitative analysis and quantitative analysis. The former identifies the nature of substance and if it is mixture, the nature of the components present, where as the latter determines the elemental composition of the substance and/ or the quantitative distribution of each component.

Pharmaceutical analysis deals with the analysis of a pharmaceutical(s) substance. It is generally known that pharmaceutical is a chemical entity of therapeutic interest. A more appropriate term for pharmaceutical is active pharmaceutical ingredient (API) or active ingredient.

Pharmaceutical analysts in research and development(R&D) of Pharma industry plays a very comprehensive role in new drug development and follow up activities to assure that, a new drug product meets the established standards, its stability and continued to meet the purported quality throughout its shelf-life.

The different activity of R&D includes drug development (synthesis and manufacture) formulation, clinical trials, evaluation and finally launching i.e. finished products. Closely associated with these processes are regulatory and quality assurance functions.

Before submitting the drug product for approval to the regulatory authorities, assuring that all batches of drug products comply with specific standards, utilization of approved ingredients and production methods. It becomes the responsibility of pharmaceutical analysts in quality control (QC), quality assurance (QA) department. The methods are generally developed in an analytical R&D and transferred to QC or other department.

Quality Assurance and Quality Control plays a central role in determining the safety and efficacy of medicines. A highly specific and sensitive analytical technique holds the key to design, development, standardization and quality control of medicinal products.[1,2]

Modern pharmaceutical analysis entails much more than the analysis of active pharmaceutical ingredients or the formulated product. There are physicochemical properties of pharmaceutical compounds through the use of advanced instrumental methods. There is a need for quality assurance of pharmaceutical products throughout their shelf life. This requires that interactions of the drug substances with the excipients in the presence of residual solvents, as well as other potential degradation reactions that may occur in the formulated product.

The pharmaceutical industry is under increased scrutiny from the government and public interest groups to contain costs and yet consistently deliver to market safe, efficacious products that fulfill unmet medical needs. The industry has streamlined its operations with respect to drug discovery, development and manufacturing.

Traditionally viewed as a service organization, the analytical department has become a significant partner in the drug development process. Analytical data has become a critical path activity for the selection of candidate molecules for full development. Working under sample-limited conditions and in full compliances of current good manufacturing practice (cGMP), Pharmaceutical analysts are called on to generate accurate and precise data – almost on demand.

Novel delivery systems pose special analytical challenges. The commonly used tests of pharmaceutical analysis generally entail compendial testing; these methods, method development, setting specifications, and method validation. The drug product remains with in specifications established to ensure its identity, strength, quality and purity. It is necessary to conduct stability studies to predict, evaluate and ensure drug product safety[1].

Analytical data are the foundation and backbone for pharmaceutical development, leading to approval and production of new drugs for market. Potential areas in pharmaceutical analysis are highlighted, based on the successful demonstration made with analysis of proteins, peptides, DNA, and small molecules including chiral separations.

MODERN PHARMACEUTICAL ANALYSIS:

The activities that are highlighted are

- Discovery of NCE and high- throughput screening.
- Solid-state analysis of drug substances.
- Degradation and impurity analysis of drug substances.
- Preformulation analysis.
- Analysis of solid oral dosage forms.
- Analysis of injectable dosage forms.
- Compendial testing.
- Method development.
- Setting specifications.
- Method validation.
- Stability studies.
- Analytical methodology transfer.
- Documentation and inspections.
- Innovative analytical platforms.

THE ANALYTICAL LITERATURE:

Selection of optimum conditions, possible interferences, sensitivity and selectivity, accuracy and precision are all factors pertinent to the performance of an assay that should be known to the analyst before experimental operations are begun. Vast amounts of information are available in the literature, and the skilled analyst will learn to find it and profit by it[3].

The discipline involves qualitative analysis and quantitative analysis.

QUALITATIVE ANALYSIS: deals with identification of the substance[4].

QUANTITATIVE ANALYSIS: deals with the determination of how much of the constituent are present[4].

The applied science of analytical chemistry is instrumental analysis, which involves the study of theoretical principle of various instrumental methods.

In this the physical property of a substance is measured to determine its chemical composition. These methods save the time and avoid chemical separation and give increased accurate results.

They are equally important in pharmacokinetics and drug metabolism studies both of which are fundamental to the assessment of bioavailability and the duration of clinical response.

The pharmaceutical analysts play a major role in assuring the identity, safety, efficacy and quality of drug product. Safety and efficacy studies require that drug substance and drug product meet two critical requirement.

- Established identity and purity
- Established bioavailability and dissolution

Until 1920 all the methods were based upon volume and mass like volumetric and gravimetric methods have come to be known as classical or chemical method of analysis.

After that there is a drastic change in the field of pharmaceutical analysis due to the introduction of highly sensitive instrumental methods. In instrumental methods a physical property of a substance is measured to determine its chemical composition. These methods may be used by the analytical chemist to save time with increased accuracy in the method. The following are the important instrumental techniques used[5,6,7].

A. **Electroanalytical Methods**: These methods involve the measurement of current, voltage or resistance in relation to the concentration of a certain species in solution. Techniques are :
   - (i) Coulomety
   - (ii) Voltametry
   - (iii) Potentiometry
   - (iv) Conductimetry

B. **Spectroscopic Methods:**Spectroscopic methods of analysis depend on: Measurement of the amount of radiant energy of a particular wavelength absorbed or emitted by the sample.

(i) Absorption Methods
- Atomic absorption spectroscopy (AAS)

(ii) Emission Methods
- Flame Photometry
- Emission Spectroscopy
- Fluorimetry

(iii) Magnetic Resonance Spectroscopy

- Electron spin resonance (ESR) spectroscopy

(iv) Photoelectron Spectroscopy (PES)

(v) Scattering Methods

C. Chromatographic and Electrophoretic Methods: These are essentially separative processes for mixtures of substances but equipped with modern detector systems, they are also adapted to identify components of mixtures.

CHROMATOGRAPHY

Russian botanist Michael Tswett invented chromatography as a separation technique. He described in detail the separation of pigments, the colored substances by percolation through the column, followed by development with pure solvents. The first paper of Tswett, was published in 1903, contains a study of more than 100 absorbents used in conjunction with several different solvents.

Types of Chromatography

Chromatography characterized as a separation method based on the differential migration of solute through a system of two phases, one is mobile phase another one is stationary phase. Chromatography is a technique by which the components in a sample, carried by the liquid or gaseous phase, are resolved by sorption, desorption steps on the stationary phase.

Chromatography is mainly divided into two categories:

1. Adsorption Chromatography[8]:

Separation is mainly due to the interaction between solute and surface on the adsorbent. In this, stationary phase is solid and mobile phase is liquid.
e.g: TLC and HPTLC

2. Partition Chromatography:

Separation is based on the partition between two phases. In this mode, both stationary phase and mobile phase are liquids
e.g: HPLC, GLC, and PC.

TABLE-1

DIFFERENT CHROMOTOGRAPHIC METHODS

Name	Mechanism	Stationary phase	Mobile phase
Paper chromatography	Partition	Liquid	Liquid
Thin layer Chromatography	Adsorption	Solid	Liquid
Gas Chromatography	Adsorption/ Partition	Liquid/ Solid	Gas
Column Chromatography	Adsorption/ Partition	Liquid/ Solid	Liquid

A) High performance liquid chromatography (HPLC)

It is analytical chromatographic technique that is useful for separation of ions or molecules that are dissolved in a solvent.

TYPES OF HPLC:

a) Normal phase chromatography[9]

Normal phase chromatography is chromatographic technique that uses organic solvents for mobile phase and a polar stationary phase. Here, the less polar compound elutes faster than the more polar compound.

b) Reverse phase chromatography

Reverse phase chromatography - a bonded phase chromatography technique, uses water as base solvent. Separation is based on solvent strength and selectivity. Separation is also affected by column temperature and pH. In general, the more polar compounds elute faster than the less polar compounds. UV detection is the most common detection technique used.

MECHANISM:

The separation mechanism in reverse phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilized hydrophobic ligand, i.e. the stationary phase. Reverse phase chromatography is an adsorptive process by experimental design, which relies on a partitioning mechanism to effect separation. The solute molecules partition (i.e. an equilibrium is established) between the mobile phase and the stationary phase.

Stationary phase:

The most popular column is a octadecyl carbon chain (C18) bonded silica. This is followed by C8 bonded silica, pure silica, cyano bonded silica and phenyl bonded silica. C18, C8 and phenyl are dedicated reversed phase packing while cyano columns can be used in a reverse phase mode depending on analyte and mobile phase conditions.

Mobile phase:

Mixture of water or aqueous buffers and organic solvents are used to elute analytes from a reversed phase column. The solvent have to be miscible with water and the most common organic solvents used are acetonitrile, methanol or tetrahydrofuran (THF). Other solvents can be used such as ethanol, 2-propanol (isopropyl alcohol). Elution can be performed isocratic (the water-solvent composition does not change during the separation process) or by using a gradient (the water-solvent composition does change during the separation process). The pH of the mobile phase can have an important role on the retention of an analyte and can change the selectivity of certain analytes.

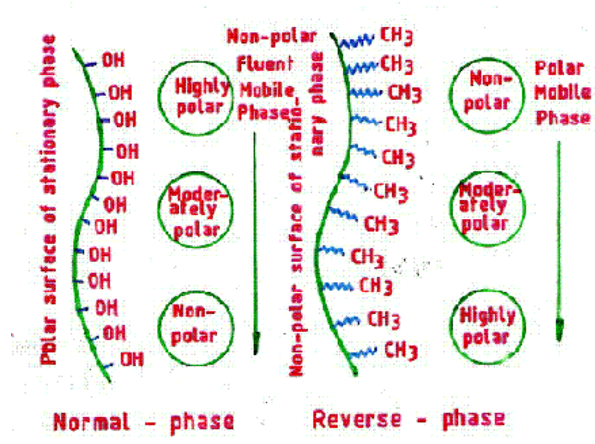


Fig. 1 Skeleton of Normal and Reverse phases

Certain limitations of RP-HPLC are:

- Compounds much more polar than the compound of interest may be masked (eluted together) in the solvent front / void volume.
- Compounds very less polar than the analyte may elute either late during the chromatographic run or are retained in the column.
- The compounds with lower UV extinction coefficients or different wavelengths maxima may not be detectable at the low level.

Reverse phase chromatography is widely in use due to the following advantages[10]

- Many compounds such as biologically active substance, have limited solubility in non-polar solvent that are employed in normal phase chromatography.
- Ionic or high polar compounds have high heats of adsorption on straight silica or alumina columns and therefore can elute as a tailing peaks.
- Column deactivation from polar modifiers is a problem in liquid – solid chromatography.

B) Size exclusion chromatography

It is also known as gel permeation or filtration chromatography. Here, separation is based on the molecular size or hydrodynamic volume of the components.

C) Ion Exchange Chromatography

The stationary phase is an ion exchange resin, and separations are governed by the strength of the interactions between solute ions and the exchange sites on the resin.

D) Ion pair /Affinity chromatography

Separation is based on chemical interaction, specific to the target species. The more popular reverse phase mode uses a buffer and an added counter ion of opposite charge to the sample with separation being influenced by pH, ion strength, temperature, concentration and organic modifier.

E) Chiral Chromatography

Chirality plays an important role in pharmaceutical industry. It is mainly because of the enantiomers exhibit different pharmacological and toxicological properties in living systems.

TABLE-2

COMPARISION OF NORMAL PHASE AND REVERSE PHASE HPLC

PROPERTIES	NORMAL PHASE	REVERSE PHASE
Polarity of stationary phase	High	Low
Polarity of mobile phase	Low to medium	Low to high
Sample elution order	Non polar first	Most polar first
Retention will increase by	Increasing surface area of stationary phase, Decreasing polarity of mobile phase Increasing polarity of sample molecules	Increasing surface of stationary phase Increasing polarity of mobile phase Decreasing polarity of sample molecules

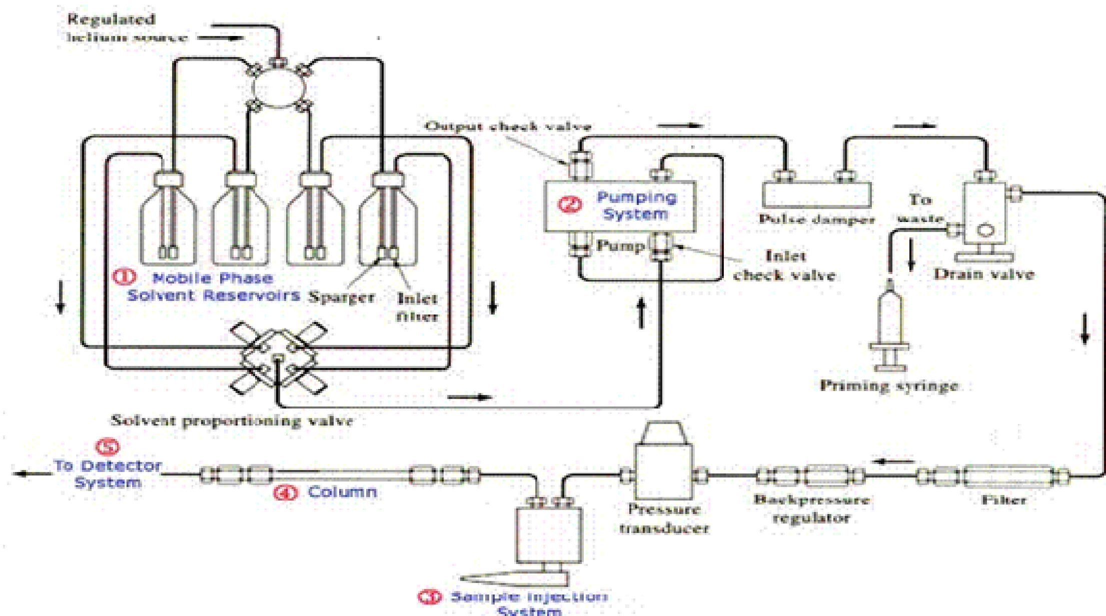


Fig. 2 Schematic diagram of an apparatus for HPLC.

INSTRUMENTATION

The essential parts of apparatus for the High Performance Liquid Chromatography are:

1) Solvent reservior
2) Mobile phase
3) Pump system
4) Sample Injection System
5) Column
6) Detector

1. Solvent reservoir

A modern HPLC apparatus is equipped with one or more glass or stainless steel reservoirs. The reservoir is often equipped with an online degasser which removes the dissolved gasses usually oxygen and nitrogen, which interfere by forming bubbles. Degasser may consist of vacuum pumping system, distillation system, system devices for heating, and solvent stirrer.

2. Mobile phase

One of the greatest advantages of HPLC is versatility afforded by liquid mobile phase. Sufficient solubility of solute molecules in the mobile phase must be ensured in order to prevent precipitation.

For the mobile phase, first variable to be decided is whether an organic or aqueous eluent should be used. With RP-HPLC analysis, either an aqueous eluent or variety of organic solvents such as methanol or acetonitrile is tried first. If the k values are too large with an aqueous solvent, then the separation should be attempted by using mixture, in various proportions. Many simple analyses can be carried out with isocratic elution using an aqueous eluent to which an organic modifier is added. If the sample to be analyzed contains a very complex mixtures or mixture of compounds of diverse structure and retention behavior, then either a ternary mixture of solvents can be used isocratically or gradient elution may be necessary.

3. Pumping system[11]

The function of the pump in HPLC is to pass mobile phase through the column at a controlled flow rate. Features of an ideal pumping system include:

- Generating pressure upto 6000 psi.
- Pulse free output.
- Flow rates ranging from 0.1 to 10 ml/min.
- Flow control and reproducibility of 0.5% relative or better.
- Corrosion resistant components.

There are three types of pumps commonly used

- Reciprocating pumps
- Displacement pumps
- Pneumatic pumps

4. Sample injection system

The limiting factor in the precision of LC measurements lie in reproducibility with which samples are introduced into the column packing. The earliest and simple means of sample introduction was syringe injection through a self-sealing elastomeric septum. In stop flow injections, the flow of solvent is stopped momentarily, and fitting at column head is removed and the sample is injected directly into the head of column packing. After replacing the fitting the system is again pressurized.

Commercial chromatographs use valves for sample injection. With these devices, sample is first transferred at atmospheric pressure from a syringe into a sample loop. Turning the valve from load to inject position connects the sample loop into the high-pressure mobile phase stream, whereby the contents of the sample loop are transferred on to the column.

In Rheodyne 7125 valve, sample from a microlitre syringe is loaded into the needle port, filling the sample loop, which is a small piece of stainless steel tube connected between ports. Any excess goes to waste from another port. On turning to ‘inject’, the loop contents are flushed on to the column. A variety of loop volumes is available, commonly 10-50 µl.

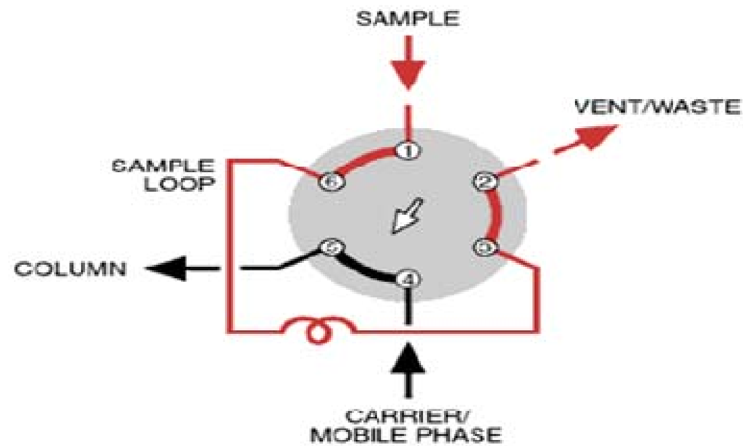


Fig.3 Load the sample

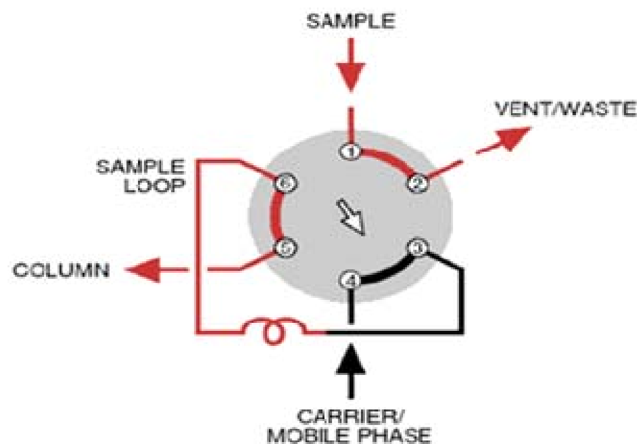


Fig.4 Inject the sample

5. Columns[11]

The columns most commonly used are made with 316-grade stainless steel (a Cr-Ni-Mo steel, relatively inert to chemical corrosion). The inside of the stainless steel tube should be as smooth as possible, so the tubes are precision drilled or electro-polished after manufacture. Common dimensions are 6.35 mm external diameter, 4.6 mm internal diameter and up to 25 cm long. The columns can be packed with 10, 5, 4 or 3 µm diameter particles.

At the top of the column, there is a distributor for directing the injected sample to the center of the column and then a stainless steel gauze or frit on top of the

packing. At the lower end there is another frit to retain the packing and then, for the 4.6 mm type, a reducing union and a short length of 0.25 mm (0.01 in.) i.d. tubing to connect the column to the detector. Materials other than stainless steel that are used for columns include glass, glass lined steel tube and polyethene or other inert plastics.

Most chromatography suppliers now offer a range of plastic fittings that can be tightened to be leak free, by hand. These are commonly made of Kel-F (PCFE) or Peek (a polyketone). Peek has excellent chemical resistance to most organic and inorganic liquids (except H_2SO_4, HNO_3 and tetrahydrofuran) and the tubing can be used at pressures up to 6000 psi for the smaller diameters.

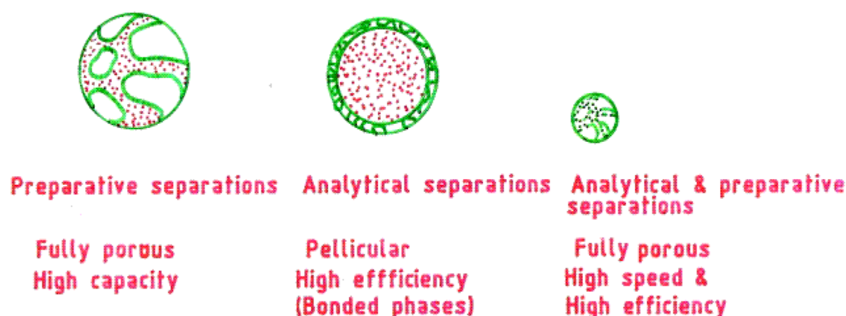


Fig.5 Properties of particles in various types of columns

5.1 Analytical columns

The majority of LC columns range in length from 5 to 30 cm. The inner diameter of columns is often 4 to 5 mm. Most common particle size of packing is 5 to 10 µm. Columns of this type contain 40000 - 60000 plates per meter.

5.2 Preparative columns

Preparative columns are typically 2-5 cm in diameter and 25 cm long with packings of 15-100-µm diameter. Columns for large-scale work can be 20-30 cm in diameter and 60 cm long, using flow rates up to 1000 cc min^{-1}. The commercial systems can be used isocratically or with gradients and allow small-scale development and preparative separation to be done using the same system.

5.3 Guard columns

Usually, a short guard column is introduced before the analytical column to increase its life. It removes particulate matter, contaminants from the solvents and also sample components that bind irreversibly to stationary phase. The composition of the guard column packing should be closely similar to that of analytical column.

Table-3

COLUMN DIMENSIONS

TYPE	INTERNAL DIAMETER (CM)	LENGTH (CM)	PARTICLE SIZE(CM)
Analytical	0.3-0.46	3-28	3-10
Semi micro	0.1-0.21	10-25	3-18
Semi preparative	0.8-1.0	10-25	5-10
Preparative	2.0-5.0	10-25	10-20

6. DETECTORS[12]

The function of the detector in HPLC is to monitor the mobile phase emerging from the column. The output of the detector is an electrical signal that is proportional to some property of the mobile phase and/or the solutes.

LC detectors are basically of two types.

Bulk property detectors respond to mobile phase bulk property such as refractive index, dielectric constant or density. Solute property detectors respond to some property of solutes, such as UV absorbing, fluorescence, diffusion current, which are not possessed by the mobile phase.

Most common HPLC detectors

- UV-Visible absorbance detector (UV-VIS)
- Photo-diode array detector (PDA)
- Fluorescence detector
- Electrochemical (ECD)
- Refractive Index (RI)
- Mass detectors (MS)
- Conductometric detector
- Chiral detector (Polarimetric & circular dichrosim)
- Evaporative Light scattering detector (ELSD)
- Radiochemical detector

QUANTIFICATION

Quantitative analysis using chromatography is based on calibration curves obtained from each of the substances analyzed. Calibration is needed in all those cases in which a signal related to mass or concentration of a component in mixture, is obtained. Chromatographic test methods use either external or internal standards for quantification.

1. External standard method

An external standard method is used when the standard is analyzed on a separated chromatogram from the sample. Quantification is based on a comparison of the peak area / height (HPLC or GC) or spot intensity (TLC) of the sample to that of a reference standard of the analyte of interest.

The external standard method is more appropriate for samples as follows:

- Samples with a single target concentration and narrow concentration range, e.g., acceptance and release tests. Simple sample preparation procedure.
- Increased baseline time for detection of potential extraneous peaks, e.g., impurities test.

2. Internal standard method

With an internal standard method, compound of known purity that does not cause interference in the analysis is added to the sample mixture. Quantification is based on the response ratio of compound of interest to the internal standard vs. the

response ratio of a similar preparation of the reference standard (HPLC or GC). This technique is rarely used for TLC methods.

The internal standard method is more appropriate for samples as follows:

1. Complex sample preparation procedures, e.g., multiple extractions.
2. Low concentration sample (sensitivity being an issue), e.g., pharmacokinetic studies.
3. Wide range of concentrations expected in the sample for analysis, e.g., pharmacokinetic studies.

Selection of Internal Standard

A compound added to a sample in known concentration to facilitate the qualitative identification or quantitative determination of the sample components. Internal standards (IS) - substance used as reference in quantitative analysis, the internal standard is first mixed with standard solutions, later it is added to the unknown, and the ratio of peak heights (or areas) of internal standard and analyte is used for quantitative analysis.

3. Standard addition method

Standard addition method are particularly useful for analyzing complex sample in which the likelihood of matrix effects is substantial. A standard addition method can take several forms, one of the most common forms involves adding one or more increments of a standard solution to sample aliquots of the same size. This process is often called spiking the sample. Each solution is then diluted to a fixed volume before measurement[11].

VALIDATION

GUIDELINES FOR ANALYTICAL METHOD VALIDATION

Validation[13]

Validation of an analytical method is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications

Validation is defined as follows by different agencies

Food and Drug administration (FDA)

Establishing documentation evidence, which provides a high degree of assurance that specific process, will consistently produce a product meeting its predetermined specification and quality attributes.

World Health Organization (WHO)

Action of providing that any procedure, process, equipment, material, activity, or system actually leads to the expected results

European Committee

Action of providing in accordance with the principles of good manufacturing practice that any procedure, process, equipment, material, activity or system actually leads to the expected results. In brief validation is a key process for effective quality assurance.

Reasons for Validation

There are two important reasons for validating assays in the pharmaceutical industry. The first, and by for the most important, is that assay validation is an integral part of the quality-control system. The second is that current good manufacturing practice regulation requires assay validation.

Steps followed for validation procedures

1. Proposed protocols or parameters for validations are established.
2. Experimental studies are conducted.
3. Analytical results are evaluated.
4. Statistical evaluation is carried out.
5. Report is prepared documenting all the results.

Objective and Parameters of Analytical Method Validation

The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the products have the identity, strength, quality and purity they purport or are represented to possess quality, safety and efficacy must be designed to build into the product. Each step of the manufacturing process must be controlled to maximize the probability that the finished products meet all quality and design specification.

ANALYTICAL METHOD DEVELOPMENT

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-Pharmacopoeia) 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 and demerits are made available[15].

Steps of method development[16,17]

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.
b. The standard analyte (≈100% purity) is obtained. Necessary arrangement is made for its proper storage (refrigerator, desicators 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 made.
d. Only those methods (spectroscopic, MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

2. Method requirement

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. Such as 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 adapted. 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

The required instrumentation is setup. Installation, operational and performance qualification of instrumentation using laboratory Standard Operating Procedures (SOP's) are verified. Always new consumables (e.g. chemicals, solvents and gases) are used.

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.

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. Therefore documentation of the successful completion of such studies is a basic requirement for determining whether a method is suitable for its intended application.

METHOD VALIDATION[14]

This process consists of establishments of the performance characteristics and the limitation of the method.

METHOD PERFORMANCE PARAMETERS ARE DETERMINED USING EQUIPMENT THAT IS:

1. Within specification
2.Working correctly
3. Adequately calibrated

METHOD VALIDATION IS REQUIRED WHEN:

1. A new method is being developed
2. Revision of the established method
3. When established method are used in different laboratories and by different analysts etc.

4. Comparision of method
5. When quality control indicates method changes

PERFORMANCE CHARACTERISTICS EXAMINED WHEN CARRYING OUT METHOD VALIDATION ARE[18]:

1. Accuracy
2. Precision
3. Specificity
4. Selectivity
5. Sensitivity
6. Limit of detection.
7. Limit of quantification
8. Linearity and Range
9. Ruggedness
10. Robustness
11. System suitability

1. Accuracy

The accuracy is the closeness of the measured value to the true value for the sample.

The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentrations levels the specified range (i.e, three concentrations and three replicates of each concentration).

Accuracy was tested (% Recovery and % RSD of individual measurements) by analyzing samples at least in triplicate, at each level (80,100 and 120 % of label claim) is recommended. For each determination fresh samples were prepared and assay value is calculated. Recovery was calculated from regression equation obtained in linearity study. Accuracy was determined from the mean relative error for a set of replicate analysis (i.e. the difference between measured and nominal concentration) for spiked samples.

2. Precision

The precision of an analytical procedure expresses the closeness of agreement between series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

Precision of an analytical method is usually expressed as the standard deviation, relative standard deviation or coefficient of variations of a series of measurements. The ICH documents recommend the repeatability should be assessed using a minimum of nine determinations covering specified range of procedure. Precision may be measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating conditions.

Repeatability:

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra –assay precision.

Intermediate Precision:

Intermediate precision expresses with in laboratories variations: different days, different analyst and different equipment.

Reproducibility:

When the procedure is carried out by different analyst in different laboratories using different equipment, regents and laboratories setting. Reproducibility was determined by measuring repeatability and intermediate precision. Reproducibility is assessed by means of an inter-laboratory trial.

3. Specificity

An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities. An ICH document defines specificity as the ability to assess unequivocally the analyte in the presence compounds that may be expected to products and matrix components.

The definition has the following implications:

Identification test:

Suitable identification tests should be able to discriminate compounds of closely related structure which are likely to be present .Ensure identity of an analyte, the analyte should have no interference from other extraneous components and be well resolved from them.

Purity Test:

To ensure that all the analytical procedures performed allow an accurate statement of the content of impurity of the content of impurity of an analyte i.e. related substances test, heavy metals, residual solvents etc.

Assay:

To provide an exact result, this allows an accurate statement on the content or potency of the analyte in a sample.

4. Selectivity:

It is a procedure to detect qualitatively the analyte in the presence of compounds that may be expected to be present in the sample matrix or the ability of a separative method to resolve different compounds. It is the measure of the relative method location or two peaks.

Determination of selectivity:

Selectivity is determined be comparing the test results obtained on the analyte with or without addition of potentially interfering material. When such components are either unidentified or unavailable a measure of selectivity can be obtained by determining the recovery of a standard addition of pure analyte to a material containing a constant level of the other compounds.

5. Sentivity:

Sensitivity is the capacity of the test procedure to record small variation in concentration. It is the slope of the calibration curve.

6. Limit of detection (LOD):

It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantities as an exact value, under the stated experimental conditions. The detection limit is usually expressed as the concentration of analyte (percentage parts per million) in the sample.

Determination of detection limit

For instrumental and non- instrumental methods detection limit is generally determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be reliably detected.

LOD = 3 x SD / slope of calibration curve

SD = Standard deviation of intercepts

7. Limit of quantification (LOQ):

It is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Quantification limit is expressed as the concentration of analyte (e.g: % ppm) in the sample.

Determination of quantification limit

For instrumental and non- instrumental methods, the quantitation limit is generally determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision.

LOQ = 10 x SD / slope of calibration curve

SD = Standard deviation of intercepts

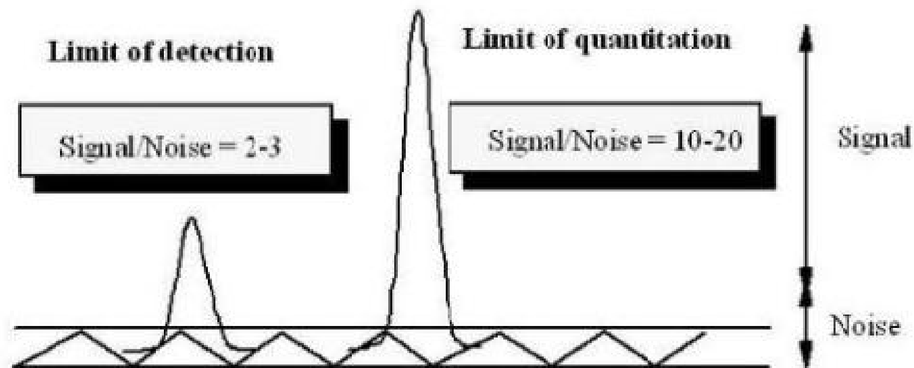


Fig. 6 Limit of detection and Limit of quantification via signal to noise

8. Linearity and Range

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to concentration of analyte in samples. The range of an analytical is the intervals between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision accuracy and linearity.

Determination of linearity and range:

These characteristics are determined by application of the procedure to a series of samples having analyte concentration spanning the claimed range of procedure. When the relationship between response and concentration is not linear, standardization may be providing by means of a calibration curve. The ICH recommends that for the establishment of linearity a minimum of five concentrations normally used.

9. Ruggedness

Degree of reproducibility of test results obtained by the analysis of the same samples under a variety of condition such as different laboratories, different analysts, different instruments etc., normally expressed as the lack of influence on test results of operational and environmental variable of the analytical method.

Ruggedness is a measurement of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst. Degree of representative of test results is then determined as a function of the assay variable.

By analysis of aliquots from homogenous lots in different laboratories, by different analyst, using operational and environmental conditions that may differs but is still with in the specified parameter of the assay variable.

10. Robustness

Robustness of an analytical method is measure of its capacity to remain unaffected small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Testing varying some or all condition:

- Column temperature
- pH of buffer in mobile phase
- Flow rate
- Wave length

11. System Suitability[19]

System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole.

According to USP system suitability are an integral part of chromatographic methods. These tests verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. One consequence of the evaluation of robustness and ruggedness should be that a series of system suitability parameters is established to ensure that the validity of the analytical method is maintained whenever used.

SYSTEM SUITABILITY SPECIFICATIONS:

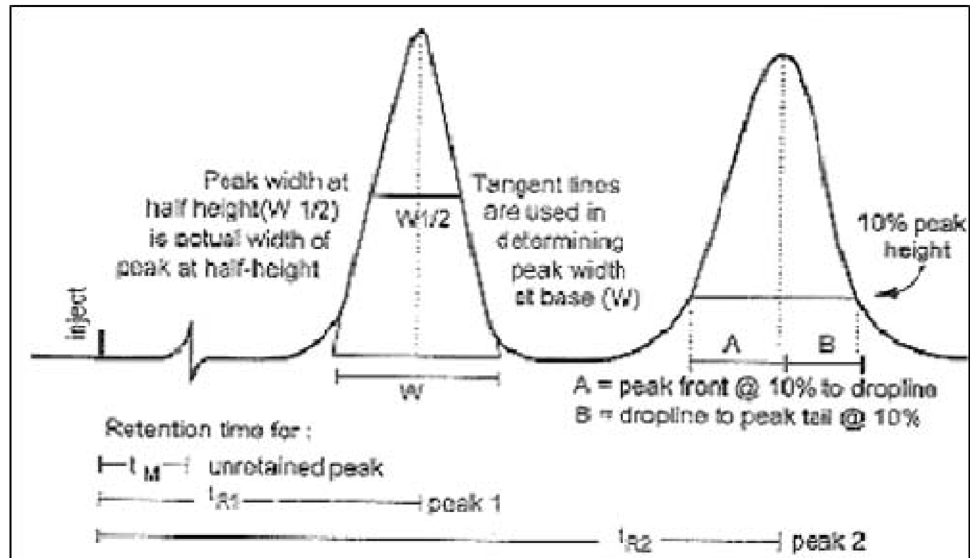


Fig.7 Typical chromatogram with examples of retention measurements

W_x = Width of the peak determined at either 5% or 10% above baseline

f = Distance between peak maximum and peak front at Wx

t_o = Elution time of void volume or non-retained components

t_r = Retention time of the analyte, R

t_w = Peak width measured at baseline of the extrapolated straight sides to baseline.

The parameters that are affected by the changes in chromatographic conditions are,

- Capacity factor (k')
- Peak asymmetry / tailing factor (As)
- Column efficiency (N)
- Selectivity (α)
- Dead volume
- Retention time R_t
- Retention volume R_v

a) Capacity factor (k')

The capacity factor, K' is related to the retention time is a reflection of the proportion time of a particular solute residues in the stationary phase as opposed to the mobile phase. Long retention times results in large valves of K'. The capacity factor K' can be calculated for every peak defined in a chromatogram, using the following equations.

$$\text{Capacity factor} \quad K' = \frac{\text{Moles of solute in stationary phase}}{\text{Moles of solute in mobile phase}}$$

$$K' = (t_R - t_0) / t_0$$

The capacity factor is a measure of the degree of retention of an analyte relative to an unretained peak, where t_R is the retention time for the sample peak and t_o is the retention time for an unretained peak.

Recommendations

The peak should be well-resolved from other peaks and the void volume. Generally the value of k' is > 2.

Precision / Injection repeatability (RSD) of < 1% for 'n' > 5 is desirable.

b) Tailing factor (T)

A measure of the symmetry of a peak, given by the following equation where $W_{0.05}$ is the peak width at 5% height and f is the distance from peak front to apex point at 5% height. Ideally, peaks should be Gaussian in shape or totally symmetrical.

$$T = W_{0.05} / 2f$$

The accuracy of quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where/when the peak ends and hence the calculation of the area under the peak. Integrator variables are preset by the analyst for optimum calculation of the area for the peak of interest.

Recommendations

T of </= 2 is preferred

c) Theoretical plate number / Efficiency (N)

A measure of peak band spreading determined by various methods, some of which are sensitive to peak asymmetry. The most common are shown here, with the ones most sensitive to peak shape shown first:

- 4-sigma / tangential

$$N = 16\,(t_R / W)^2 = L / H$$

- Half height

$$N = 5.54\,(t_R / W)^2 = L / H$$

Theoretical plate number is a measure of column efficiency .Theoretical plate number is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatogram, where t_R is the retention time for the sample peak and W is the peak width.

N is fairly constant for each peak on a chromatogram with a fixed set of operating conditions H, or HETP, the height equivalent of a theoretical plate, measures the column efficiency per unit length (L) of the column. Parameters which can affect N or H include peak position, particle size in column, flow-rate of mobile phase, column temperature, viscosity of mobile phase, and molecular weight of the analyte.

Recommendations

The theoretical plate number depends on elution time but in general should be > 2000.

d) Resolution (R_s)

Ability of a column to separate chromatographic peaks, Resolution can be improved by increasing column length, decreasing particle size, increasing temperature, changing the eluent or stationary phase. It can also be expressed in terms of the separation of the apex of two peaks divided by the tangential width average of the peaks.

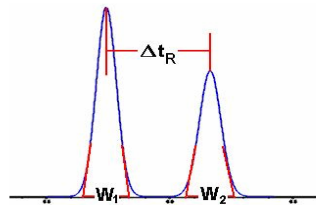


Fig.8 Resolution Chromatogram

$$R_S = (t_{R2}-t_{R1}) / 0.5(w_1+w_2)$$

Where t_{R1} & t_{R2} are the Retention time of the two compounds and w_1 & w_2 are the width of the two compounds. For reliable quantitation, well-separated peaks are essential for quantitation.

Recommendations

Rs of> 2 between the peak of interest and the closest potential interfering peak (impurity, excipient, degradation product, internal standard, etc.) are desirable.

e) Dead Volume

Dead Volume means any empty space or unoccupied volume, the presence of which can lead to disastrous losses in efficiency. There will be dead volume in the column itself, which will be the space that is not occupied by the stationary phase. The other sources of dead volume are the injection unit, the tubing and fittings at each end of the column and the detector cell.

f) Retention time (Rt)

Retention time is the difference in time between the point of injection and appearance of peak maxima. Retention time is the time required for 50% of a component to be eluted from a column. Retention time is measured in minutes of seconds. Retention time is also proportional to the distance moved on a chart paper, which can be measured in cm or mm.

g) Retention volume (R_v)

Retention volume is the mobile required to elute 50% of the component from the column. It is the product of retention time and flow rate.

Retention Volume = Retention time x flow rate

STATISTICAL PARAMETERS

Statistics consists of a set of methods and rules for organizing and interpreting observations. The precision or reproducibility of the analytical method was determined by repeating the analysis six times and the following statistical parameters were calculated.

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.

Mean :

Best estimation of the population mean mcg/ml for random samples from a population.

$$\boldsymbol{X} = \frac{\mathbf{x_1 + x_2 + x_3 + x_4 + x_5}}{\boldsymbol{n}}$$

Where

X = Mean

x = Individual observed valve

n = Number of observation

STANDARD DEVIATION:

Standard 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

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

Where,

C= concentration of drug,

A= Absorbance of drug

Standard error

It provides a measure of how well a sample mean approximates the population mean. Less value is more accurate

SE = SD/v n

Correlation: (Fit of regression line)

Purpose:

Measurement of the relation between two or more variables / measures how close the points are to the regression line.

Correlation co-efficient can range from -1.00 + 1.00

Correlation value denotes with the letter r

$$r = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{(n\sum x^2 - (\sum x)^2 (n\sum y^2 - (\sum y)^2}}$$

Regression:

Purpose:

1. When the concentration range is so wide that the errors, both random and systematic, are not independent (which is assumption).
2. When pairing is inappropriate for other reason, notably a long time span between two analysis (sample aging, change in laboratory conditions etc.)

Regression line

$$Y = mx + b$$

Where,

b = intercept of the line with the Y axis

m = Slope (tangent)

Slope m

$$m = \frac{n(\sum xy) - (\sum x)(\sum y)}{n(\sum(x^2)) - (\sum x)^2}$$

Intercept b

$$b = \frac{(\sum y)(\sum(x^2) - (\sum x)(\sum xy)}{n(\sum(x^2)) - (\sum x)^2}$$

HPLC method validation

Everyday many chromatographers face the need to develop a HPLC separation whereas individual approaches may exhibit considerable diversity; method development often follows the series of steps summarized in the following fig.

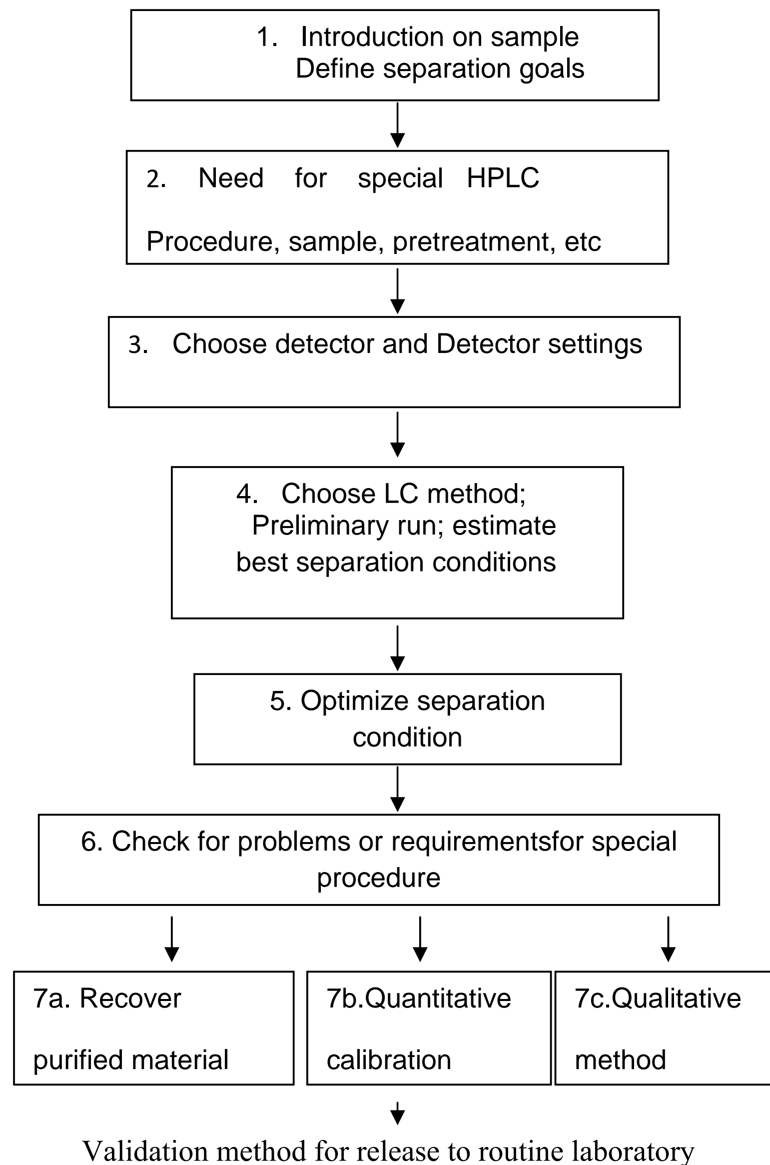


Fig.9 Steps involved in HPLC method validation

METHODS USED FOR THE EXAMINATION OF PHARMACEUTICAL MATERIAL MAY BE BROADLY CLASSIFIED AS FOLLOWS[20,21]

Class A:

Tests designed to establish identity, whether of bulk drug substances or have a particular ingredient in a finished dosage form.

Class B:

Methods designed to detect and quantitate impurities in a bulk drug substances or finished dosage form.

Class C:

Methods used to determine quantitatively the concentration of bulk drug substance or of a major ingredient in a finished dosage form.

Class D:

Methods used assess the characteristics of finished dosage forms such as dissolution profile and content uniformity.

TABLE-4

CHARACTERISTIC THAT SHOULD BE CONSIDERED FOR DIFFERENT TYPES OF ANALYTICAL PROCEDURE

(As per WHO guidelines)

S.No	Parameters	Class A	Class B		Class C	Class D
			Quantitative Tests	Limit Tests		
1.	Accuracy	-	Yes	-	Yes	Yes
2.	Precision	-	Yes	-	Yes	Yes
3.	Robustness	-	Yes	Yes	Yes	Yes
4.	Linearity and range	-	Yes	-	Yes	Yes
5.	Selectivity	Yes	Yes	Yes	Yes	Yes
6.	Limit of detection	Yes	-	Yes	-	-
7.	Limit of quantification	-	Yes	-	-	-

TABLE-5

PARAMETERS

S.No	Goal	Comment
1.	Resolution	Precise and rugged quantitative analysis requires Resolution >1.5
2.	Separation	<5-10 min
3.	Quantitation	RSD <1.0% for assays; <5% for trace analysis
4.	Pressure	<150kgf/cm^2 is desirable, <200kgf/cm^2 is usually essential
5.	Peak Shape	Narrow peaks
6.	Solvent consumption	Minimum mobile phase usage per run is desirable

LEARN ABOUT CANCER[22]

What is cancer?

Cancer is the general name for a group of more than 100 diseases in which cells in a part of the body begin to grow out of control. Although there are many kinds of cancer, they all start because abnormal cells grow out of control. Untreated cancers can cause serious illness and even death.

Normal cells in body

The body is made up of trillions of living cells. Normally body cells grow, divide, and die in an orderly fashion. During the early years of a person's life, normal cells divide faster to allow the person to grow. After the person becomes an adult, most cells divide only to replace worn-out or dying cells or to repair injuries.

How cancer starts:-

Cancer starts when cells in a part of the body start to grow out of control. Cancer cell growth is different from normal cell growth. Instead of dying, cancer cells continue to grow and form new, abnormal cells. Cancer cells can also invade other tissues, something that normal cells cannot do. Growing out of control and invading other tissues are what makes a cell a cancer cell. Cells become cancer cells because of damage to DNA.

Tumors that are not cancer

Not all tumors are cancerous. Tumors that aren't cancer are called benign. Benign tumors can cause problems-they can grow very large and press on health organs and tissues. But they cannot grow into (invade) other tissues. Because they can't invade, they also can't spread to other parts of the body (metastasize).These tumors are almost never life threatening.

Cancer types

- Adrenal cortical cancer
- Anal cancer
- Bladder cancer
- Bone cancer

- Breast cancer
- Cervical cancer
- Endometrial cancer
- Eye cancer
- Gallbladder cancer
- Kidney cancer
- Liver cancer
- Lung cancer- non-small cell
- Lung cancer- small cell

Erlotinib[23]

(er-lot-tin-nib)

Trade/other name: Tarceva, OSI-774

How does this drug work?

Erlotinib is a type of targeted therapy known as a tyrosine kinase inhibitor. Its target is a tyrosine kinase protein called epidermal growth factor receptor (EGFR), which is located on the surface of certain cells in the body. Some cancers have higher than normal number of these receptors on their surfaces. Erlotinib blocks the receptor so that it can't signal the cell to divide and grow.

Interactions with other drugs

The following drugs can cause Erlotinib to build up in the body, raising the risk of serious side effects:

- The antidepressant nefazodone (serzone), fluvoxamine (luvox)
- HIV drugs such as indinavir, ritonavir, fosamprenavir, nelfinavir, atazanavir, and others
- Anti-seizure drugs carbamazepine (Tegretol). Phenobarbital (luminal) and phenytoin (dilantin).
- TB drug rifampin (Rifadin, Rimactance;also in Rifamate And Rifater), andrifabutin (mycobutin)
- The steroid drug dexamethasone (decadron).

- Any H2 blocker such as cimetidine (tagamet), ranitidine (zantac), famotidine (pepcid) or nizatidine (axid).

Interactions with foods

Taking this drug with food can raise the levels of the drug in the body, so it should be taken on an empty stomach.

How is this drug taken or given?

Erlotinib is in pill form, and is taken by mouth, once a day. The dose depends on a number of factors, including the type of cancer being treated.

Possible side effects

Common

- Skin rash on face, neck, and trunk, diarrhea.
- Feeling tired

Less common

- Shortness of breath
- Cough

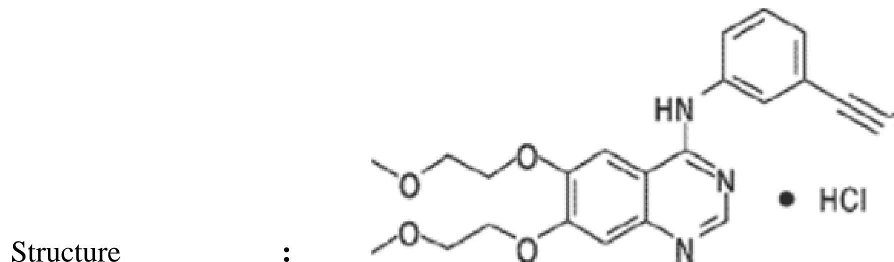
Rare:

- Eye irritation
- Abdominal pain

DRUG PROFILE[24]

Name : ERLOTINIB

Structure :



Chemistry:

Chemical Name : N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)4-quinazolinamine hydrochloride.

Molecular Formula : $C_{22}H_{23}N_3O_4.HCl$

Molecular Weight : 429.90

Description : white to cream coloured , amorphous powder.

PKa : 5.42 at 25°c

Solubility : Very slightly soluble in water, slightly soluble in methanol,practically insoluble in acetonitrile,acetone, ethyl acetate and hexane.

Mechanism of action : Selective epidermalgrowth factor receptor tyrosine kinase inhibitor.

Contraindication : Monotherapy for the treatment of patients with locally advanced or metastatic non-small cell lung cancer.

Bulk density : 0.1899 g/ml

Tapped density : 0.3331g/ml

PHARMACOKINETICS

Oral absorption	:	Erlotinib is about 60% absorbed after oral administration and its bioavailability is substantially increased by food to almost 100%.
Distribution	:	Peak plasma levels is 3 to 4 hrs after oral administration Steady state achieved in 7-8 days. Crosses blood brain barrier. Plasma protein binding 93% to albumin and alpha-1 acid glycoprotein (AAG)
Metabolism	:	Primarily hepatic via CYP3A4,lesser extent by CYP1A2 and extra hepatic isoform CYP1A1
Excreation	:	Excreation predominantly via feces(83%),urine 8%. Elemination half life 36.2 hrs.
Adverse effects	:	Rash/acne,diarrohea.
Dosing	:	Daily dose of erlotinib is 150mg atleast one hour before or 2 hrs after ingestion of food. When dose reduction is necessary, should be reduced in 50mg. Elderly no dose adjustment is required.
Interaction	:	CYP3A4 inhibitors (phenytoin, carbamazepine) CYP3A4 inducers (rifampicin)

LITERATURE REVIEW

Chahbouni A[25]et.al:developed and validated liquid chromatography (LC)-mass spectrometry (MS)/MS method in human plasma for the tyrosine kinase inhibitors erlotinib, gefitinib, and imatinib in human plasma. Pre-treatment of the samples was achieved by using liquid-liquid extraction and imatinib as internal standard. Separation was performed on a Waters Alliance 2795 LC system using an XBridge RP18 column. The mass spectrometer Micromass was equipped with an electro spray ionization probe, operating in the positive mode. The calibration curves in plasma were linear for erlotinib, gefitinib, and imatinib over the concentration range of 5 to 3,000; 5 to 3,000, and 5 to 5,000 ng/mL, respectively. The intraday and interday accuracy ranged from 90% to 110% and the intraday and interday precision of the method was within 5%.

R. Honeywell[26]et.al:developed a simple and selective method for the determination of various tyrosine kinase inhibitors by liquid chromatography tandem mass spectrometry. Utilizing a simple protein precipitation with acetonitrile a 20 µl sample volume of biological matrixes can be extracted at 4 °C with minimal effort. After centrifugation the sample extract is introduced directly onto the LC–MS/MS system without further clean-up and assayed across a linear range of 1–4000 ng/ml. Chromatography was performed using a Dionex Ultimate 3000 with a Phenomenex prodigy ODS3 (2.0 mm × 100 mm, 3 µm) column and eluted at 200 µl/min with a tertiary mobile phase consisting of 20 mM ammonium acetate: acetonitrile: methanol (2.5:6.7:8.3%). Injection volume varied from 0.1 µl to 1 µl depending on the concentration of the drug. Samples were observed to be stable for a maximum of 48 h after extraction when kept at 4 °C. Detection was performed using a turbo-spray ionization source and mass spectrometric positive multi-reaction-monitoring-mode (+MRM) for Gefitinib (447.1 m/z; 127.9 m/z), Erlotinib (393.9 m/z; 278.2 m/z), Sunitinib (399.1 m/z; 283.1 m/z) and Sorafenib (465.0 m/z; 251.9 m/z) at an ion voltage of +3500 V. The accuracy, precision and limit-of-quantification (LOQ) from cell culture medium were as follows: Gefitinib: 100.2 ± 3.8%, 11.2 nM; Erlotinib:

101.6 ± 3.7%, 12.7 nM; Sunitinib: 100.8 ± 4.3%, 12.6 nM; Sorafenib: 93.9 ± 3.0%, 10.8 nM, respectively. This was reproducible for plasma, whole blood, and serum. The method was observed to be linear between the LOQ and 4000 ng/ml for each analyte.

V.Rajesh[27]et.al:developed a simple, specific and precise high performance thin layer chromatographic method for estimation of Erlotinib hydrochloride as bulk drug. The chromatographic development was carried out on precoated silica gel 60 F254 aluminium plates using mixture of Methanol: Ammonia (8:0.2 v/v) as mobile phase and densitometric evaluation of band was carried out at 250 nm using Camag TLC Scanner-3 with win CAT 1.4.3 version software. The RF value of drug was found to be 0.52 ± 0.01. The method was validated with respect to linearity, accuracy, precision and robustness. The calibration curve was found to be linear over a range of 200- 1200 ng/ band. The % assay (Mean ± S.D.) was found to be 101.3 ± 1.02. The proposed HPTLC method was found to provide a faster and cost effective quantitative control for routine analysis of Erlotinib hydrochloride as bulk drug.

Faivre L, Gomo C[28]et.al:developed a simple HPLC-UV method for the simultaneous quantification of gefitinib and erlotinib in human plasma. Gefitinib and erlotinib are two oral tyrosine kinase inhibitors (TKI). Following liquid-liquid extraction, gefitinib, erlotinib and sorafenib (internal standard), were separated with gradient elution using C8 column and mobile phase of acetonitrile/20mM ammonium acetate pH 4.5. 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 monitored at 331 nm, and sorafenib at 249 nm. The calibration was linear in the range 20-1000 ng/ml and 80-4000 ng/ml for gefitinib and erlotinib, respectively. Inter- and intra-day precision were less than 7.2% and 7.6% for gefitinib and erlotinib, respectively.

G.Usha Rani[29]et.al:developed and validated extractive colorimetric method for estimation of erlotinib in bulk and tablet dosage form**.** Two simple, rapid sensitive, precise and economic spectrophotometric methods for the estimation of erlotinib. The solution of the drug formed colored ion-pair complexes with Bromocresol Green (BCG) and Methyl Orange (MO) in phosphate buffer pH 2.5, and extracted in chloroform.The complex of etoricoxib with BCG and MO showed λ max at 418.5nm and 424.4nm respectively. The complex was stable up to 22 hrs and obeyed Beer's law over the concentration ranges of 10-1000 ug/ml. Correlation coefficient was found to be 0.9985.

M.Padmalatha[30]et.al:developed a spectrophotometric method for the determination of erlotinib in pure and pharmaceutical dosage form. The developed methods were based on reaction of erlotinib with Phenol red, BromoCresolGreen and Erichrome black T. They are quantified spectrophotometrically at their absorption maximum at 418nm (MethodA), 424nm (MethodB) and 333nm (MethodC). Beer's law was obeyed in the concentration range of 40-80ug/ml, 20-40ug/ml and 10-50ug/ml for the three method respectively. The colors were found to be stable for more than 4 hrs.

V.Kalyana Chakravarthy[31]et.al:development and validated of RP HPLC method for estimation of erlotinib in bulk and its pharmaceutical formulation**.** A RP-LC method used a kromasil 100-5,C18(150mm×4.6mmi.d;particle size 5um) and potassium dihydrogen phosphate buffer pH 2.4: acetonitrile : methanol(65:21:14) as eluent at flow rate 1.5ml/min with UV detection at 250 nm. The erlotinib content was linear over a range of 75.02 to 225.06 ug/ml. The method precision for the determination of assay was below 2.0 %RSD. The percentage recoveries of active pharmaceutical ingredients from dosage forms ranged from 100.5 to 101.1.

G.Vidya Sagar[32]et.at:developed and validated a simple, accurate and cost efficient spectrophotometric method, for the estimation of erlotinib in tablet dosage form. The optimum conditions for the analysis of the drug were established. The maximum wave length (λmax) was found to be 247 nm. The percentage recovery of erlotinib was in the range of 99.7±0.12.Beer's law was obeyed in the concentration range of 2-10ug/ml. Calibration curves showed a linear relationship between the absorbance and concentration.

M.Padmalatha[33]et.al:developed and validated High Performance Liquid Chromatographic Method for the determination of Erlotinib. They used 250x4.6mm, 5μ particle, IntersilODS-3V C18column with 0.03M potassium dihydrogen orthophosphate in water pH 3.2, orthophosphoric acid acetonitrile (55:45),as mobile phase at a flow rate of 0.8 ml/min. PDA detection was performed at 246.0nm.Injection volume was20µl.HPLC grade water, Acetonitrile(50:50v/v)was used as diluents. The method was validated for accuracy, precision, linearity, specificity and sensitivity. Total run time was 20min, erlotinib eluted with retention time of 4.75min.Calibration plots were linear over the concentration range 5-40µg/ml. Intra and inter day relative standard deviation for erlotinib was less than 3.3 and 4.1% respectively.

Luca Signor[34]et.al:reported analysis of erlotinib and its metabolites in rat tissue sections by MALDI quadrupole time-of- flight mass spectrometry. The analysis was carried out on rat tissue sections from liver, spleen and muscle. Following oral administration at a dose of 5mg/kg, Samples were analyzed by matrix assisted laser desorption ionization (MALDI) with mass spectrometry (MS) using a orthogonal quadrupole time of flight instrument. The presence of the parent compound and of its o-demethylated metabolites was confirmed in all tissues types and their absolute amounts calculated. In liver the intact drug was found to be 3.76ng/mg tissue, while in spleen and muscle 6-30 folds lower values. These results were compared with drug quantitation obtained by whole-body autoradiography, which was found to be similar.

Lutz Gotze[35]et.al:development and clinical application of a LC/MS/MS method for simultaneous determination of various tyrosine kinase inhibitors in human plasma**.** Developed and validated a specific, simple and rapid quantification method for various TKI's in human plasma. A simultaneous test for six TKI's (erlotinib, imatinib,lapatinib, nilotinib, sorafenib, sunitinib) was developed using liquid chromatography tandem mass spectrometry in a multiple reaction monitoring mode. After protein precipitation the specimens were applied to the HPLC system and separated using a gradient of acetonitrile containing 1% formic acid with 10mM ammonium formate on an analytic RP C18 column. The calibration range was 10-1000ng/ml for sunitinib and 50-5000ng/ml for the other TKI's with coefficient of determination ≤15% and the chromatographic run time was 12 min. Plasma specimens were stable for measurement for atleast 1 week at 4°c.

S.S Pujeri[36]et.al:developed and validated stability – indicating chromatographic method for the assay of erlotinib active pharmaceutical ingredient in the presence of its degradation products on a C18 column using a mobile phase of 0.01M ammoniumformate-acetonitrile-containing formic acid with a flow rate of 1.0ml/min. Selectivity was validated by subjecting the stock solution of erlotinib to acidic, basic, photolysis, oxidative and thermal degradation. The linearity range and values for limit of detection (LOD) and quantification (LOQ) were found to be 1-198, 0.33, and 1.1ug/ml, respectively. The analysis of the tablet containing erlotinib was quite precise (relative standard deviation<1%).

ErrinR.Lepper[37]et.al:developed and validated a high- performance Liquid Chromatographic (HPLC) assay with U.V detection for the quantitative determination of erlotinib in human plasma. Quantitative extraction was achieved by a single- solvent extraction involving a mixture of acetonitrile and n-butyl chloride(1:4v/v). Erlotinib and the internal standard hydrochloride salt (OSI-597) were seperated on a column packed with NOVA-PAK C18 material and a mobile phase composed of acetonitrile and water, pH 2.0 (60:40,v/v).The column effluent

was monitored with dual U.V detection at wavelengths of 348nm erlotinib and 383nm erlotinib hydrochloride. The calibration graph was linear in the range of 100-4500ng/ml, with values for accuracy and precision ranging from 87.9 to 96.2% and 2.13 to 5.10% respectively, for three different sets of quality control samples.

Rasoulzadeh F[38]et.al:studied the mutual interaction of anticancer drug erlotinib hydrochloride with bovine serum albumin (BSA) using fluorescence and U.V /VIS spectroscopy. The BSA solution(0.1Mm) was prepared daily in tris buffer (0.05mol-1,ph=7.4) and treated at final concentration of $1.67x10^{-5}$M with different amount of erlotinib hydrochloride to obtain final concentration of 0,0.2,0.4,0.8,1,2,4,6,8,20 and 42µm respectively. The mixture was allowed to stand for 5 min and the fluorescence quenching spectra were recorded at 298,303, 308 and 313k. It was found that erlotinib hydrochloride caused the fluorescence quenching of BSA by the formation of a BSA –ERLOTINIB HYDROCHLORIDE complex. The mechanism of the complex formation was then analysed by determination of the number of binding sites the apparent binding constant Ka, and calculation of the corresponding thermodynamic parameters. Such as the free energy (ΔG), enthalpy (ΔH) and entropy changes (ΔS) at different temperatures. Results showed that binding of erlotinib hydrochloride to BSA was spontaneous and the hydrophobic forces played a major role in the complex formation. The distance r between donar (BSA) and the acceptor (ERLOTINIB HYDROCHLORIDE) was found to be less than 8nm. Non radioactive energy transferring and static quenching between these two molecules. The presence of single binding site on BSA and Ka values for the association of BSA with ERLOTINIB HYDROCHLORIDE increased by the increase in temperature.

Jiongwei Pan[39]et.al:developed a novel bioanalytical method and validated for the quantitative determination of erlotinib in human plasma by using the supported liquid extraction (SLE), sample cleanup coupled with hydrophilic interaction liquid chromatography and tandemmass spectrometric detection (HILIC-MS/MS). The SLE extract could be directly injected into the HILIC-MS/MS system for analysis without

the solvent evaporation and reconstitution steps. Erlotinib was used as the internal standard. The SLE extraction recovery was 101.3%.The validated linear curve range was 2 to 2,000 ng/mL based on a sample volume of 0.100-mL, with a linear correlation coefficient of > 0.999. The validation results demonstrated that the present method gave a satisfactory precision and accuracy: intra-day CV < 5.9% (<8.4% for the lower limit of quantitation, LLOQ) with n = 6 and the accuracy of 98.0–106.0%; inter-day CV < 3.2% (<1.5% for LLOQ) with n = 18 and the accuracy of 100.0–103.2%. A dilution factor of 10 with blank plasma was validated for partial volume analysis. The stability tests indicated that the erlotinib in human plasma is stable for three freeze-thaw cycles (100.0–104.5% of the nominal values), or 24-h ambient storage (100.0–\104.8% of the nominal values), or 227-day frozen storage at both -20 ºC (91.5–94.5% of the nominal values) and -70 ºC (93.3–93.8% of the nominal values). The results also showed no significant matrix effect (<6.3%) even with direct injection of organic extract into the LC-MS/MS system.

Fouad Chiadmi[40]et.al:developed and validated an isocratic high-performance liquid chromatographic method for the determination of erlotinib in human plasma with detection at 348 nm. Quinine was used as internal standard. A reversed-phase symmetry C18 column (250 mm x 4.6 mm, 5 µm), was equilibrated with a mobile phase composed of potassium dihydrogen phosphate 0.05M and acetonitrile (60:40, v/v) with a final pH of 4.8 and having a flow rate of 1 mL/minute. The elution time for erlotinib and internal standard was approximately 7.4 and 2.6 minutes, respectively. Calibration curves of erlotinib in human plasma were linear in the concentration range of 50-1,000 ng/mL. Limits of detection and quantification in plasma were 6.3 and 21 ng/mL, respectively. Intra- and inter-day relative standard deviation for erlotinib in plasma was less than 3.3 and 4.1%, respectively.

Hanqing Li[41] et.al: developed a new synthetic and differential antiproliferative activity of two active isomeric metabolites of Erlotinib were investigated. This synthetic process had demonstrated to avoid the unstable 4- chloro-quinazoline

intermediates and long procedures. New intermediates and final compounds were identified by ^{1}H NMR, ^{13}C NMR and their purities were determined by HPLC. Invitro proliferative assay indicates that these two metabolites possessed antiproliferative activity against some conventional tumor cell lines and EGFR tyrosine kinase over-expression tumor cell lines as compared to Erlotinib control and their antitumor activity in cellular level was reported.

Han-Qing Li[42]et.al: developed and validated a new HPLC-UV method for the quantitative determination of epidermal growth facto rreceptor inhibitor erlotinib in the plasma of tumor bearing BALB/c nude mice. Erlotinib and its internal standard 1-(3-((6,7-bis (2-methoxyethoxy) quinazolin-4-yl) amino) phenyl) ethanone were extracted from mice plasma samples using liquid-liquid extraction with a mixed solvent of methyl t-butyl ether and ethyl acetate (9:1, v/v). Luna C18 column (4.6 mm×250 mm, 5 μm) with acetonitrile: 5 mM potassium phosphate buffer pH = 5.2 (41:59, v/v) as the mobile phase. UV detector was set at the wavelength of 345 nm, and the flow rate was 1.0 mL/min. The calibration curve was linear over the range of 20–10 000 ng/mL with acceptable intra- and inter-day precision and accuracy. The intra-day and inter-day precisions were within the range of 1.69%–5.66%, and the accuracies of intra- and inter-day assays were within the range of 105%–113%.The mean recoveries were 85.2% and 96.1% for erlotinib and internal standard, respectively.

V.RAJESH[43]et.al: developed a simple and sensitive spectrofluorimetric method for the estimation of erlotinib hydrochloride in pure and pharmaceutical dosage forms. Erlotinib hydrochloride exhibits maximum fluorescence intensity in methanol and the Beer's law was obeyed in the range of 1-5 μg/mL at an excitation wavelength (λex) of 295 nm and an emission wavelength (λem) of 339 nm. Stability studies with respect to time and temperature were also carried out. The results obtained were in good agreement with the labelled amounts of the marketed formulations. This method has been statistically evaluated and found to be accurate and precise.

OBJECTIVE OF WORK

The literature survey revealed that spectrophotometric method, HPTLC method and Reverse phase HPLC method were used for the determination of drug in the tablet dosage form and also for its determination in biological specimens.

In the present study the aim was to develop a new RP HPLC method for the determination of the drug in tablet formulation and its validation. The plan of the work can be represented as follows.

Plan of Work

- To obtain through knowledge in practical HPLC method development.
- To implement a step-by-step procedure for method development and to set initial chromatographic conditions for the assay of Erlotinib tablets.
- To conduct trails for the initial chromatographic conditions and to find optimum conditions.
- To validate the developed RP-HPLC method.

EXPERIMENTAL DETAILS

A simple and sensitive reverse phase HPLC method has been developed for the analysis of Erlotinib. The method utilizes sample preparation followed by separation on a Develosil ODS HG-5, column 150mm length, 4.6mm inner diameter, with 5µm particle size. The analyte was monitored by UV detection at 246nm using an isocratic mode with buffer and methanol in the ratio 520:480v/v as mobile phase. The flow rate was set at 1.0ml/min. The retention time for the drug was at 4.540min. Calibration curves for Erlotinib was recorded.

Equipment and Apparatus used:

- Analytical balance (Metller Toledo AG-245)
- Waters LC system equipped with 2695 pump and 2996 photo diode array detector.
- Chromatographic data software : EMPOWER
- Develosil ODS HG-5, 150×4.6mm. 5µ column
- Vacuum filter pump
- Ultrasonicator (sonarex)
- Membrane filter(0.45 and 0.2microns)
- pH-Meter (Lab India)

Chemicals and Reagents used:

a) Acetonitrile (HPLC grade)

b) Ortophosphoric acid (HPLC grade).

c) Triethylamine (HPLC grade)

d) Water (HPLC grade)

e) Methanol (HPLC grade)

Reference standards:

Erlotinib Hydrochloride **--** Natco Chemical Division at Mekaguda (HYD)

% purity -- 99.5%

The reference standard was obtained as gift sample and the authenticity and purity of the sample was certified.

Tablet used: Tarceva (Erlotinib- 25 mg)

METHOD DEVELOPMENT

The objective of this experiment was to optimize the assay method for the estimation of Erlotinib.The trials were done to optimize the chromatographic conditions.

Preparation of mobile phase and standard solution of the drug for trails:-

The mobile phase was prepared with a composition according as indicated in the table no 6 for the purpose of different trails. The mobile phase was filtered through 0.4µ filter and sonicated. The mobile phase was used as a diluent to prepare the standard solution.

Standard solution was prepared for each trail in the respective mobile phase.

Working standard solution of Erlotinib Hydrochloride:

About 50 mg ofworking standard of Erlotinib Hydrochloride was weighed and transferred into a clean and dry 50 ml standard flask. The sample was dissolved in a small volume of mobile phase by sonication for about 10 min and the volume was made up with the mobile phase. (1000µg/ml).0.5 ml of the stock solution was pipetted into a10 ml standard flask and diluted to mark with mobile phase (concentration-50 mcg/ml).

The standard solution was injected into the column in each trail. The Retention time at each trail was determined. The column, mobile phase and results obtained in the trails have been indicated in the table 6. The table also reveals the result of the chromatograms in term of retention time. The trail no 5 employed was found to be satisfactory in which column Develosil ODS HG-5, mobile phase orthophosphoric acid, and triethylamine buffer: methanol (520:480) were used to obtain adequate results.

Table: 6

DIFFERENT COMBINATIONS OF BUFFERED SOLVENT SYSTEM TRIED WITH DIFFERENT COLUMN IN TRAILS

Sl.no/Trail.no	Stationary phase	Mobile phase	Flow rate	Temperature	Wave length	Retention time	Remarks
1.	OEM Column, C_{18}(250X4.6X5µ)	Water :Methanol 65 : 35	1ml/min	40ºC	246 nm	16.043	Symmetric peak, More retention time
2.	Inertsil ODS C_{18} (250X4.6X5µ)	Water :Acetonitrile 60: 40	1ml/min	40ºC	246 nm	11.870	Small tailing
3.	Inertsil ODS C_{18} (150X4.6X5µ)	0.1M Ammonium Phosphate buffer: Acetonitrile 40 : 60	1ml/min	40ºC	246 nm	5.587	Less retention time
4.	Kromasil C_{18} (150X4.6X5µ)	Phosphate buffer : Acetonitrile : Methanol 650 : 210 : 140	1.5ml/min	Ambient	250 nm	8.302	Peak broadening
5.	Develosil ODS HG- 5 (250X4.6X5µ)	Ortho phosphoric acid, Triethylamine buffer : Methanol 520 : 480	1ml/min	40ºC	246 nm	4.543	Well resolved

Peak of Erlotinib was well resolved with the column Develosil ODS HG- 5 (250X4.6X5µ), with the solvent system of orthphosphoric acid, triethylamine buffer : methanol in the ratio of 520 : 480 as shown in following Fig 10.

Optimized method

Preparation of Mobile phase:

Preparation of buffer:

The buffer solution was prepared by mixing 2 ml of triethylamine and 2 ml of orthophosphoric acid in water and the volume was madeup to 1000 ml.

Preparation of mobile phase:

The mobile phase was prepared by mixing buffer and methanol in the ratio 520:480v/v respectively and filtered through 0.45µ filter. The mobile phase was then sonicated using Ultra-sonicator to remove the dissolved gases.

Preparation of Diluent:

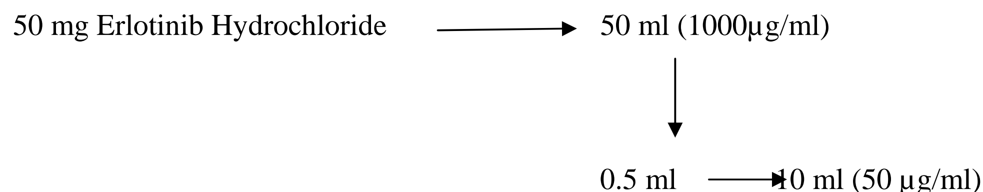
Mobile phase was used as the diluent.

Determination of Retention time:

Preparation of Erlotinib Hydrochloride standard stocksolution:

Working standard solution of Erlotinib Hydrochloride:

About 50 mg ofworking standard of Erlotinib Hydrochloride was weighed and transferred into a clean and dry 50 ml standard flask, the sample was dissolved in a small volume of mobile phase by sonication for about 10 min and the volume was made up with the mobile phase filtered through 0.45µ filter. (1000µg/ml).0.5 ml of the stock solution was pipetted into a10 ml standard flask and diluted to mark with mobile phase (concentration-50 mcg/ml).



The retention time of Erlotinib Hydrochloride was found to be 4.540 min when injected and chromatograms are shown in the Fig.11 and Fig.12.

Application of standard method for the sample:

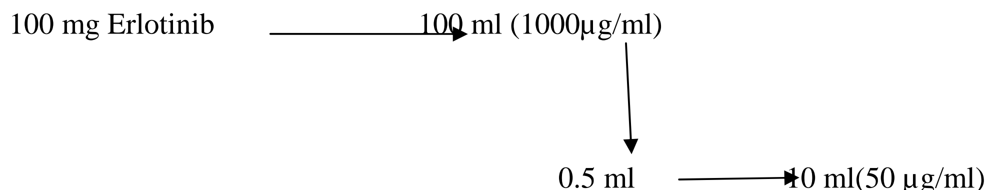
Sample : Erlotinib tablet.

Label claim : Erlotinib 25 mg.

Mfg. by : Natco Pharma Pvt Limited.

Preparation of working sample solution:

Average weight of the tablet was computed from the weight of 20 tablets. The tablets were powdered. The tablet powder equivalent to 100 mg of Erlotinib was accurately weighed and transferred into a clean and dry 100 ml standard flask. The sample was dissolved in a small volume of mobile phase by sonication for about 10 min and the volume was made up with the mobile phase. The solution was filtered by using Whatmann filter paper. (Concentration 1000µg/ml).0.5 ml of the stock solution was pipetted into a10 ml standard flask and diluted to mark with mobile phase. It was filtered through 0.45µ filter (Concentration-50 mcg/ml).



The sample was injected and chromatograms were recorded and shown in the Fig.13

The amount of Erlotinib present in each tablet formulation was calculated by comparing the peak area of the test with that of the standard.

Assay:

Assay of formulation available in the market was carried by preparing the sample solution as indicated above procedure injected into HPLC system. The percentage purity was found out by using following formula. Recovery studies were also carried out. The results were discussed.

The content of Erlotinib present in the tabletof average weight:

$$= \frac{AT}{AS} \, x \, \frac{WS}{25} \, x \, \frac{0.5}{10} \, x \, \frac{25}{TW} \, x \, \frac{10}{0.5} \, x \, \frac{393.3}{429.9} \, x \, \frac{P}{100} \, x \, \frac{Avg.Wt.}{LC} \, x \, 100$$

Where,

AT = average area counts of sample preparation.

AS = average area counts of standard preparation.

WS = weight of working standard taken in mg.

P = Percentage purity of working standard

LC = Label Claim

TW = weight of sample taken

Avgwt = average weight of tablet in mg

393.9 = Molecular weight of Erlotinib

429.9 = Molecular weight of Erlotinib hydrochloride

Tablet average weight	45.8	mg
Weight of standard	25.4	mg
Weight of sample	42.6	mg
Label claim	25	mg
Stdpurity	99.5	%
Factor for calculation:	393.9/429.9mg	
Avg.standard Area	2015.879	
Avg.sample area	2014.965	

$$= \frac{2014.965}{2015.879} x \frac{25.4}{25} x \frac{0.5}{10} x \frac{25}{42.6} x \frac{10}{0.5} x \frac{393.3}{429.9} x \frac{99.5}{100} x \frac{45.8.}{25} x\, 100$$

= 99.5%.

The amount of Erlotinib present in the tablet of average weight

= 99.5 / 100 X 25

= 24.875 mg.

VALIDATION

Validation of analytical method for the assay of Erlotinib:

Validation of analytical method is a process to establish that the performance characteristics of the developed method meet the requirement of the intended analytical application.

Design of the experiment:

Typical analytical parameters used in assay validation are:

METHOD VALIDATION

1. **SYSTEM SUITABILITY STUDIES**
2. **SPECIFICITY**
3. **LINEARITY AND RANGE**
4. **PRECISION**
    - **a) SYSTEM PRECISION**
    - **b) METHOD PRECISION**
5. **RUGGEDNESS**
6. **ACCURACY**
7. **ROBUSTNESS**
8. **LOD**
9. **LOQ**

1. SYSTEM SUITABILITY

Preparation of standard stock solution:

About 50 mg of working standard of Erlotinib Hydrochloride was weighed and transferred into a clean and dry 50 ml standard flask, the sample was dissolved in a small volume of mobile phase by sonication for about 10 min and the volume was made up with the mobile phase. (1000µg/ml)

0.5 ml of the stock solution was pipetted into a10 ml standard flask and diluted to mark with mobile phase (concentration-50 mcg/ml) and filtered through 0.45µ filter.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits. The Chromatograms were shown in Fig.14. The results were discussed in the following table 7.

Table No. 7

Data for System Suitability

Injection	t_R	Peak Area	USP Plate count	USP Tailing
1	4.547	2036.567	6470	1.147
2	4.553	2032.964	6865	1.65
3	4.53	2036.427	6911	1.69
4	4.55	2028.420	6932	1.69
5	4.547	2037.567	6890	1.65
Mean	4.5454	2034.389	6906.6	1.67
SD	0.00896	3.7635222	46.418	0.02
% RSD	0.20	0.18	0.672	1.43

2. Specificity

The specificity of the method was evaluated by analyzing the sample solution spiked with the excipients at appropriate levels. The assay result was unaffected by the presence of extraneous materials.

Preparation of placebo:

Placebo is prepared by mixing all excipients with out active ingredients.

Determination:

About 100mg of placebo was weighed accurately and transferred in to 100 ml of volumetric flask, mixed thoroughly with sufficient mobile phase and the volume was made up to 100 ml with diluent. The solution was filtered. 0.5 ml of this solution was diluted to 10 ml with mobile phase. The solution was again filtered through millipore filter and 10 µl of this solution was injected and chromatogram was recorded shown in the Fig.15.

About 50 mg of Erlotinib working standard was weighed accurately and transferred into 100 ml standard flask, dissolved in small volume of the mobile phase. 100 mg of placebo was mixed with above solution and made up the volume with mobile phase, filtered through millipore filter, 10µl of this solution was injected and chromatogram was recorded shown in Fig.16, 17 and reports were shown in table 8.

Table No. 8

Specificity for Erlotinib

S.No	Sample	Area obtained	%Content of drug w/v
1	Standard	2037.567	99.81%w/v
2	Standard + placebo	2028.420	99.43%w/v
3	Placebo	0	0

3. LINEARITY AND RANGE

Linearity was assessed by performing measurement at several analyte concentrations. A minimum five concentrations were recommended for linearity studies.

The linearity of an analytical method is its ability to show test results that is directly proportional to the concentration of analyte in sample with in a given range. The linearity of an analytical method was determined by mathematical treatment of test result obtained by analysis of samples with analyte concentration across claimed range of peak area Vs concentration is plotted and percentage curve fitting is calculated.

Acceptance criteria	:	Percentage curve fitting should not be less than 99.7%

Preparation of working standard solution

Erlotinib was weighed accurately and stock solution was prepared. Different volumes of stock solution were diluted to get a concentration range of 10 to 60 µg/ml.

Table No. 9

S. No	Volume of stock solution taken (ml)	Volumetric flask taken (ml)	Concentration of Solution (mcg/ml)
1	0.1	10	10
2	0.2	10	20
3	0.3	10	30
4	0.4	10	40
5	0.5	10	50
6	0.6	10	60

Procedure:

10µl of working standard solution were injected in duplicate and the chromatograms were recorded and shown in Fig.18 to 23.

The correlation co-efficient and percentage curve fitting were calculated from the following formula.

$$\boldsymbol{R = \frac{3\,(x-\bar{x})^{\,2}(y-\bar{y})^{\,2}}{(n-1)S_x\,S_y}}$$

Where

x = concentration

y = instrumental response

S_x=standard deviation of x

S_Y = standard deviation of y

Percentage curve fitting = 100 x correlation coefficient

Acceptance criteria	:	Correlation coefficient should not be less than 0.99%
	:	Curve fitting should not be less than 99.7%

The linearity data and analytical performance parameters of Erlotinib was shown in table and calibration curve of Erlotinib was shown in Fig.24.

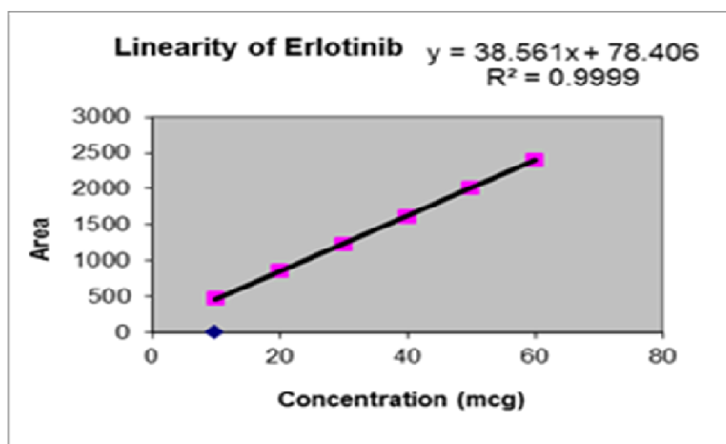


Fig.24 Calibration curve of Erlotinib.

Table No. 10

Data for Linearity

Solution No.	Conc. (µg / ml)	Avg Area
1	10	464.112
2	20	860.935
3	30	1222.921
4	40	1612.944
5	50	2011.541
6	60	2395.321

Table No. 11

Linearity results for Erlotinib

Conc.(µg/ml)	10	20	30	40	50	60
Peak area	464.112	861.433	1222.921	1612.944	2011.541	2395.321
Correlation	0.999					

Table No. 12

Calibration parameters for Erlotinib

Parameter	**Results**
Slope	38.56
Intercept	78.40
Correlation co-efficient	0.999
Percentage curve fitting	99.9 %

4. PRECISION

Precision of an analytical method is the degree of agreement among individual test result when the procedure is applied repeatedly to multiple samplings of a homogenous sample. Precision of analytical method is usually expressed as the standard deviation or relative standard deviation.

Determination:

The precision of an analytical method was determined by assaying sufficient number of sample and relative standard deviation is calculated.

The precision of the instrument is determined by assaying the samples consecutively number of times and relative standard deviation is calculated.

Acceptance criteria	:	The relative standard deviation should be with in 2%

A. SYSTEM PRECISION

Preparation of standard solution:

About 50 mg of working standard of Erlotinib Hydrochloride was weighed and transferred into a clean and dry 50 ml standard flask, the sample was dissolved in a small volume of mobile phase by sonication for about 10 min and the volume was made up with the mobile phase. (1000µg/ml).0.5 ml of the stock solution was pipetted into a10 ml standard flask and diluted to mark with diluent and filtered through 0.45µ filter (concentration-50 mcg/ml).

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits. The Chromatograms were shown in Fig 25. The results were discussed in the Table 13, 14.

B.METHOD PRECISION

Preparation of working sample solution

Average weight of the tablet was computed from the weight of 20 tablets. The tablets were powdered. The tablet powder equivalent to 100 mg of Erlotinib was accurately weighed and transferred into a clean and dry 100 ml standard flask. The sample was dissolved in a small volume of mobile phase by sonication for about 10 min and the volume was made up with the mobile phase. The solution was filtered by using Whatmann filter paper (Concentration 1000µg/ml).0.5 ml of the stock solution was pipetted into a10 ml standard flask and diluted to mark with mobile phase and filtered through 0.45 µ filter (concentration-50 mcg/ml).

Procedure:

The sample solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits. The Chromatograms were shown in the Fig.26. The results were discussed in the Table 15,16.

The standard deviation and relative standard deviation were calculated from statistical formula.

Standard 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

Table No. 13

Precision data of the system

Injection No	Peak Area	% Recovery
1	2036.567	100.7
2	2032.964	100.5
3	2036.427	100.7
4	2028.420	100.3
5	2037.567	100.7
Mean	2034.389	100.58
SD	3.7635222	0.1789
% RSD	0.18	0.18

Table No. 14

System precision report for Erlotinib

Relative standard deviation	Erlotinib	Acceptance criteria
	0.18	**< 2.0%**

Table No. 15

Method precision of Erlotinib

Injection no	Peak Area	% Recovery
1	2018.593	100.1
2	2014.965	99.96
3	2013.985	99.92
4	2015.879	100.0
5	2011.118	100.3
Mean	2017.031	100.056
SD	3.140105	0.151912
% RSD	0.16	0.15

Table No. 16

Method precision report for Erlotinib

	Erlotinib	**Acceptance criteria**
Relative standard deviation	**0.16**	**< 2.0%**

5. RUGGEDNESS

The ruggedness of an analytical method is degree of reproducibility of test result obtained by the analyst under a variety of normal test condition. Such as different laboratories, different analysts, different instruments, lots of reagents, different elapsed assay times, different temperature, different days etc.

The ruggedness of an analytical method is determined by aliquots from homogenous lots by different analyst using operational and environmental conditions that may differ but are also with in the specified parameters of the assay. The degree of reproducibility of test results is then determined as function of the assay variables. This reproducibility may be compared with the precision of the assay under normal condition to obtain a measure of the ruggedness of the analytical method. The assay of Erlotinib was performed in different days.

Procedure:

Working standard solution and working sample solution of Erlotinib were prepared by different analyst and on different days and 10 µl of working sample solution was injected and chromatograms were recorded shown in Fig.27,28 and ruggedness of the method and report of Erlotinib was shown in Table 17,18.

Table No. 17

Ruggedness results for Erlotinib: (Day-1, Analyst-1)

Parameter	Peak Area	% Assay
Avg	2054.018	99.68
% RSD	0.04	0.43

Table No. 18

Ruggedness results for Erlotinib: (Day-2, Analyst-2)

Parameter	Peak Area	% Assay
Avg	2056.393	100.865
% RSD	0.03	0.26

6. ACCURACY

The Accuracy of an analytical method is the closeness of the test result obtained by that method to the true value

Accuracy is measured as the percentage of the analytes recovered by the assay. Spiked samples were prepared in triplicate at three intervals a range of 100-150% of the target concentration, and injected into the HPLC system.

Acceptance criteria	:	Percentage recovery should be within 90-110%w/w

Preparation of Sample Stock Solution:

Average weight of the tablet was computed from the weight of 20 tablets. The tablets were powdered. The tablet powder equivalent to 100 mg of Erlotinib was accurately weighed and transferred into a clean and dry 100 ml standard flask The sample was dissolved in a small volume of mobile phase by sonication for about 10 min and the volume was made up with the mobile phase. The solution was filtered by using whatmann filter paper. (Concentration 1000µg/ml).0.5 ml of the stock solution was pipetted into a10 ml standard flask and diluted to mark with mobile phase and filtered through 0.45 µ filter. (Concentration-50 mcg/ml)

The stock solution was diluted with mobile phase. Further to obtain a concentration ranging from 45mcg to 65 mcg/ml. The dilution made was shown in the Table 19.

Table No. 19

S.NO	From stock sample solution volume taken (ml)	50 mcg/ml solution taken (ml)	Volumetric flask taken (ml)	Concentration of solution (mcg/ml)
1	0.4	1	10	45
2	0.5	1	10	55
3	0.6	1	10	65

Procedure:

The standard solution, 45 mcg/ml, 55 mcg/ml and 65 mcg/ml solutions were separately injected into the HPLC.T he individual recovery and mean recovery values were calculated. The chromatograms were shown in Fig.29 to 32. The results were discussed in the Table 20.

Table No. 20

Percentage Recovery data for Erlotinib

S.No	Spike Level	Amount (µg / ml) added	Amount (µg / ml) found	% Recovery	Mean % Recovery
1	100 %	45	45.85	101.88	101.78
	100 %	45	45.77	101.77	
	100 %	45	45.78	101.75	
2	125 %	55	54.8	99.68	99.77
	125 %	55	54.84	99.72	
	125 %	55	54.87	99.77	
3	150 %	65	64.05	98.55	99.86
	150 %	65	64.23	98.81	
	150 %	65	64.12	98.70	

7. ROBUSTNESS

Robustness of an analytical method is measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides on indication of its reliability during normal usage.

Determination:

The robustness of an analytical method is determined by analysis of aliquots from homogenous lots by differing physical parameters that may differ but are still with in the specified parameters of the assay. For example change in physical parameters like flow rate and wavelength.

a) Effect of variation of Flow rate:

A study was conducted to determine the effect of variation in flow rate by injecting 0.9 ml/min and 1.1ml/min. Sample solution was prepared and injected into the HPLC system. The retention time values were measured. The chromatograms were shown in the Fig. 33 to 35.The results were discussed in the Table 22 to 24.

b) Effect of variation of wavelength:

A study was conducted to determine the effect of variation in wavelength. Standard solution was prepared and injected into the HPLC system at 248nm and 244nm. The effects of variation in wavelength were measured. The chromatograms were shown in the Fig.36 & 37.The results were discussed in the Table 25 to 28.

Table No. 21

Chromatographic condition for Robustness

Change in flow rate 0.9 ml/min

Change in flow	0.9 ml/min
Instrument	HPLC Shimadzu Separation Module LC - 20 AT Prominence Liquid
Column	Develosil ODS HG-5 250X4.6,5µm
Wavelength	246nm
Injection volume	10µl
Column oven	40°c
Run time	10min

Table No. 22

Report of Robustness

Drug	Average R_t in 0.9ml/min	Average Rt in 1.0ml/min	Average Asymmetry in 0.9ml/min	%RSD
Erlotinib	5.180	4.540	1.105	0.019

Table No. 23

Chromatographic condition for Robustness

Change in flow rate 1.1 ml/min

Change in flow	**1.1 ml/min**
Instrument	HPLC Shimadzu Separation Module LC -20 AT Prominence Liquid
Column	Develosil ODS HG-5 250X4.6,5µm
Wavelength	246nm
Injection volume	10µl
Column oven	40°c
Run time	10min

Table No. 24

Report of Robustness

Drug	**Average R_t in 1.1ml/min**	**Average Rt in 1.0ml/min**	**Average Asymmetry in 1.1ml/min**	**%RSD**
Erlotinib	4.113	4.540	1.161	0.21

Table No. 25

Chromatographic condition for Robustness

Change in wavelength 244 nm

Change in Wavelength	**244 nm**
Instrument	HPLC Shimadzu Separation Module LC -20 AT Prominence Liquid
Column	Develosil ODS HG-5 250X4.6,5µm
Flow rate	1.0 ml/min
Injection volume	10µl
Column oven	40°c
Run time	10min

Table No. 26

Report of Robustness

Drug	**Average R_t in 244 nm**	**Average Rt in 246 nm**	**Average Asymmetry in 244 nm**	**%RSD**
Erlotinib	4.543	4.547	1.167	0.043

Table No. 27

Chromatographic condition for Robustness

Change in wavelength 248 nm

Change in Wavelength	**248 nm**
Instrument	HPLC Shimadzu Separation Module LC -20 AT Prominence Liquid
Column	Develosil ODS HG-5 250X4.6,5µm
Flow rate	1.0 ml/min
Injection volume	10µl
Column oven	40°c
Run time	10min

Table No. 28

Report of Robustness

Drug	**Average R_t in 248 nm**	**Average Rt in 246 nm**	**Average Asymmetry in 248 nm**	**%RSD**
Erlotinib	4.543	4.547	1.114	0.015

Limit of Detection (LOD)

It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantities as an exact value, under the stated experimental conditions. The detection limit is usually expressed as the concentration of analyte (percentage parts per million) in the sample.

It is determined by based on the standard deviation of response and the slope. The detection limit may be expressed as

The LOD was determined by the formula:

$$\mathbf{LOD = 3.3\ \sigma / S}$$

Where

σ = standard deviation of the response

S = slope of calibration curve

LOD = 3.3 (0.00896/38)

= 0.000766 µg/ml

From the formula limit of detection was found to be = 0.000766 µg/ml

8. Limit of Quantification (LOQ)

It is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Quantification limit is expressed as the concentration of analyte (e.g: % ppm) in the sample. Which can be quantitated with suitable precision and accuracy.

Based on the deviation of the response and the slope.

Quantitation limit (QL) may be expressed as:

$$\mathbf{LOQ = 10\ \sigma / S}$$

Where

σ = Standard deviation of the response

S = slope of calibration curve

LOQ = 10 (0.00896/38.56)

= 0.002323 µg / ml.

From the formula limit of quantitation was found to be = 0.002323 µg / ml.

RESULTS AND DISCUSSION

Validation of analytical method for determination of assay of Erlotinib 25 mg tablets was performed for the parameters including – Specificity, Linearity and Range, Precision (System precision, Method precision), Intermediate precision (Ruggedness), Accuracy and Robustness. The summary of results obtained isappended below.

Parameter	Acceptance Criteria	Results
Specificity	There should not be any interference from placebo, blank and main peak. (Active)	There is no interference from blank, placebo and sample peak.
Linearity and Range	Correlation coefficient should be not less than 0.995 over working range.	Correlation coefficient = 0.9999.
Precision Repeatability System precision	%RSD should not be more than 2.0%	SD=0.1789 %RSD=0.18
Repeatability Method precision	%RSD should not be more than 2.0%	SD=0.1519 %RSD=0.15
Intermediate precision Ruggedness	%RSD should not be more than 2.0% The difference between assay of method precision and intermediate precision should not be more than 2.0%	Day 1 and Analyst 1 % Assay =99.68 % RSD =0.43 Day 2 and Analyst 2 % Assay =100.865 % RSD =0.26

Accuracy	Recovery at each level and % mean recovery should be between 100% to 150% with % RSD should not be more than 2.0%	Recovery at each level 98.55 to 101.88. Mean Recovery 99.86 to 101.78. % RSD = 0.18
System suitability	% RSD should not be more than 2.0%	SD= 3.76352 %RSD=0.18
Robustness :	**By change in flow rate**	
a)0.9 ml/min	%RSD should not be more than 2.0% Asymmetry factor should not be more than 2.0%.	%RSD = 0.019 Asymmetry factor =1.105
b)1.1 ml/min	%RSD should not be more than 2.0%. Asymmetry factor should not be more than 2.0%	%RSD = 0.21 Asymmetry factor =1.161
	By change in wavelength	
a)248 nm	% RSD should not be more than 2.0% A symmetry factor should not be more than 2.0%	% RSD =0.015 Asymmetry factor = 1.114.
b)244 nm	% RSD should not be more than 2.0% A symmetry factor should not be more than 2.0%	% RSD =0.043 Asymmetry factor = 1.167.

DISCUSSION:

The observations and results obtained for each parameter including Specificity, Linearity and Range, Precision (System precision, Method precision), Intermediate precision (Ruggedness), Accuracy and Robustness lie well within the acceptance criteria.

CONCLUSION:

Since the results are with in acceptance criteria for all validation parameters, therefore, the method is considered as validated and suitable for intended use.

CONCLUSION

For routine analytical purpose it is desirable to establish methods capable of analyzing huge number of samples in a short time period with good robustness, accuracy and precision without any prior separation step. HPLC method generates large amount of quality data, which serve as highly powerful and convenient analytical tool.

Erlotinib was slightly soluble in methanol and very slightly soluble in water. Methanol and Mixture of Buffer was chosen as the mobile phase. The run time of the HPLC procedure was 10 minutes.

The method was validated for system suitability, linearity, precision, accuracy, specificity, ruggedness robustness, LOD and LOQ. The system suitability parameters were within limit, hence it was concluded that the system was suitable to perform the assay. The method shows linearity between the concentration range of 10-60 µg / ml. The % recovery of Erlotinib was found to be in the range of 99.86 % - 101.78 %. As there was no interference due to excipients and mobile phase, the method was found to be specific. The method was robust and rugged as observed from insignificant variation in the results of analysis by changes in Flow rate and wave length separately and analysis being performed by different analysts.

Good agreement was seen in the assay results of pharmaceutical formulation by developed method. Hence it can be concluded that the proposed method was a good approach for obtaining reliable results and found to be suitable for the routine analysis of Erlotinib in the pharmaceutical formulation.

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