INTRODUCTION:

The term drug refers to a chemical or plant-derived substance that is used in the diagnosis, prevention, treatment or cure of a disease or used to otherwise enhance physical or mental well-being leading to varying degrees of dependence or addiction. The World Health Organization (WHO) defines drug as “A substance, material or product used or intended to be used to modify or explore the physiological processes or pathological states for the benefit of the recipient.”

Ancient people believed drug to be both a medicine and a poison depending upon its function. With drugs many people would live as long as they do or enjoy life from diseases and pains. Thus a drug when put into the body can change the way the body works and can be referred as soft or hard drug. Soft drug is considered to be less harmful and addictive to the body whereas hard drug is highly addictive and more harmful to the body and to the society also. So an ideal drug needs to satisfy the following requirements if and when administered to any ailing living being:

- It should be very potent and effective
- It should be highly selective for its target site, having little or no effect on other body systems
- It should have minimal or no side effects
- It should not injure host tissues or physiological processes
- It should not develop tolerance by the tissues even when administered for longer duration

However drugs with these capabilities are rare and needs to be developed substantially.

Drugs may be classified based on the structure of the active chemical ingredient or by the pharmacological action mainly as chemotherapeutic agents and pharmacodynamic agents.¹-⁵
The general features of a drug are the variability in molecular size, shape, chemical nature, lipid/water partition coefficient, degree of ionization and physical properties.

Usually drugs have three names i.e. a chemical name, a common or generic name and a brand or trade name. The chemical name is assigned according to rules of nomenclature of chemical compounds. The generic name refers to a common established name irrespective of its manufacturer. The brand name is always capitalized and is selected by the manufacturer.

In most cases, a generic name and a brand name of a drug may be equivalent. However, this equivalency may not always be true. Although drugs are chemically the same, different manufacturing processes may cause differences in pharmacological action. Several differences may be crystal size or form, isomers, crystal hydration, purity, vehicles, binders, coatings, dissolution rate and storage stability.

Drugs can be naturally occurring, semi-synthetic or synthetically prepared. A majority of the drugs are synthetic in origin. These are prepared in bulk forms and used for their therapeutic effects in pharmaceutical formulations as tablets, powders, capsules, creams or ointments, dry syrups, liquid orals, parenterals, lotions, aerosols, metered or dry powder dose inhalers etc. In these forms the drug substance is delivered in a stable, non-toxic and acceptable form ensuring its therapeutic activity and bio availability. In tablets one or more of the diluents such as lactose monohydrate, hydroxy propyl cellulose, sodium starch glycolate, microcrystalline cellulose, magnesium stearate, sodium stearyl fumarate, calcium phosphate, sodium benzoate, mannitol, sorbitol, sucrose, aerosol, acacia, gelatin, crospovidone, alginic acid, tragacanth, talc, waxes, methyl paraben, meglumine, permitted flavours and colours are added. In capsules one or more among the excipients, plasticizers, starch, lactose, certified dyes, gelatin, talc and preservatives are added. In dry syrups and liquid orals, sucrose, sorbitol, preservatives, certified colours and flavours are added. In creams and ointments,
waxes, carbopol, petroleum jelly, surfactants, preservatives, permitted colours and perfumes are added. In parenterals, water, vegetable oils, mineral oils, simulated oils, propylene glycol, dioxalamines, dimethyl acetamide are used as carriers. Any one or more among stabilizers, anti oxidants, buffering agents like citrate, acetate, phosphate, co solvents, wetting, suspending and emulsifying agents like tween-80, sorbitol oleate, and preservatives are added. In lotions, dusting powders and aerosols, talc, silica derivatives, alcohol, preservatives are added.

Analysis of pharmaceutical dosage forms deals with both the precursors and their medicaments. Analytical techniques can be used in analyzing the chemical composition of substances both qualitatively and quantitatively. Modern analytical methods make it possible to identify hundreds of components in a single sample and to detect specific substances present in less than one part per million. There are a wide variety of techniques used for analysis from simple weighing, titrations and to very advanced techniques using highly specialized instrumentation. The most common and major techniques used in analytical chemistry include titrimetry (based on the quantity of reagent needed to react with the analyte), Electro analytical methods including potentiometry and voltammetry, Spectroscopy (based on the interaction of the analyte with electromagnetic radiation), Chromatography (in which the analyte is separated from the rest of the sample so that it may be measured without interference from other compounds) Microscopy, Bioanalysis, Radioanalytical chemistry, etc.

There are many more techniques that have specialized applications and within each major analytical technique there are many applications and variations of the general techniques of which microanalysis is a prominent one. Microanalysis is the chemical identification and quantitative analysis of very small amounts of chemical substances (generally less than 10 mg or 1 ml) or very small surfaces of material (generally less than 1 cm$^2$). They have the advantage of involving less amount of sample, solvent and also producing less waste compared
to normal analysis methods. Moreover most of them are economical, fast, reliable and accurate.

These methods involve spectroscopy, chromatography, electrophoresis, X-ray diffraction, field flow fractionation, thermal analysis, etc. Of these the chromatographic method is well acclaimed as it provides scope for both separation and analysis of a compound in a myriad of individual compounds. Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction. It is based on the concept of partition coefficient.

Chromatographic methods can be differentiated based on the physical means of bringing the stationary and mobile phases into contact. Of this planar Chromatography including Paper chromatography, Thin layer chromatography (TLC) is where the stationary phase is supported on a flat plate or in the fibers of a paper. Here the mobile phase moves through the stationary phase by capillary action or by gravity. The other known as Column Chromatography is one where the stationary phase is held in a narrow tube through which the mobile phase is forced either by pressure or by gravity. It broadly includes Liquid chromatography (simple column and HPLC), Gel chromatography and Gas chromatography (GC). This broader classification is based on the types of stationary and mobile phases and the kinds of equilibria involved in solute transfer between the phases. Liquid Chromatography (LC) includes a number of specific methods like Affinity, Partition, Chemically bonded-phase like reverse phase, Adsorption, Ion exchange, Size exclusion chromatographic techniques. Gel chromatography includes Filtration and Permeation chromatography. Gas Chromatography includes methods like Gas-liquid, Gas-bonded phase, Gas-solid techniques. Gas Chromatography is used for the analysis of volatile samples, while gel chromatography is used for non-volatile samples with a molecular weight greater
than 2000 whereas liquid chromatography is used for analysis of non-volatile samples with a molecular weight less than 2000.

Of the above techniques, High performance Liquid Chromatography is one of the powerful separation methods that can resolve mixtures with a large number of analytes. HPLC has been the main technique used for the analysis of impurities in drugs. Most workers use the reversed phase mode with UV absorbance detection as it is more reliable, repeatable and sensitive with a shorter analysis time. Non-volatile, thermally unstable or polar/ionic compounds can also be analyzed by this technique. It is a versatile tool for the qualitative and quantitative analysis of drugs and pharmaceuticals, chemical and biological samples and has become indispensable in pharmacokinetic studies. Though HPLC technique involves heavy cost and maintenance, it is yet regarded the best among various analytical techniques.

The main components of an HPLC system are a high pressure pump, a column, an injector system and a detector. A high pressure pump is required to force the mobile phase through the column at typical flow rates of 0.5-2 ml/min. The sample to be separated is introduced into the mobile phase by injection device, manual or automatic, prior to the column. The detector usually contains low volume cell through which the mobile phase passes carrying the sample components eluting from the column. In addition to these a data handling device and a microprocessor control are also present in HPLC equipment. The practicality of HPLC operation can be understood based on the basic performance of various types of components.

1) **Pumps:** A pump is a precision instrument that needs to be treated with care. It directly affects the retention time, reproducibility and detector sensitivity. Hence it is considered one of the most important components of HPLC. The various types of pumps used in HPLC are:
i. **Syringe type pumps:** This pump delivers only a finite volume of mobile phase before it has to be refilled and hence most suitable for small bore columns. They have a volume between 250 to 500 ml that operates by a motorized lead screw that delivers mobile phase to the column at a constant rate. The rate of solvent delivery is controlled by changing the voltage on the motor.

ii. **Reciprocating piston pumps:** This consists of a small motor driven piston which moves rapidly back and forth in a hydraulic chamber that may vary from 35-400 µl in volume. On the backstroke, the separation column valve is closed and the piston pulls in solvent from the mobile phase reservoir. On the forward stroke, the pump pushes solvent out to the column from the reservoir. This type of a system is apparently smoother because one pump is filling while the other is in the delivery cycle.

iii. **Constant pressure pumps:** Here the mobile phase is driven through the column with the use of low pressure from a gas cylinder to generate high liquid pressures. The arrangement of the valve allows the rapid refill of the solvent chamber whose capacity is about 70 ml. This provides continuous mobile phase flow rates.

2) **Injector:** For injecting sample various possibilities are there such as:

   a) With syringe and septum injector
   b) With a loop valve
   c) With an automated injection system (autosampler)

The septum injector is not suitable for HPLC as it is only applicable at pressures lower than 100 bar. While using the loop valves the loop is filled with sample solution and the internal channeled rotor seal is then turned to bring the loop within the eluent flux. The rotor seal is usually made from vespel which has the best mechanical properties useful in the pH range of 0-10. Loops can be used in two different ways either by complete filling or by
partial filling. Loops are available in sizes ranging from 5 to 2000 µl. For micro columns an internal loop of capacity between 0.5 and 5 µl is also available. An auto sampler\textsuperscript{12} has different working principles such as pull loop injection, push loop injection and integral loop injection.

3) **Column:** Pre columns positioned prior to sample injector can condition the mobile phase by playing the role of a scavenger column that can protect the separation column from dissolution when silica or silica based bonded phase is used as stationary phase. Likewise guard columns placed between injector and column also protect the column from contamination. Most of the High Performance Liquid Chromatography columns are made up of 316-grade stainless steel typically of length 5-30 cm and 2-5 mm inner diameter. In addition to this glass tube other materials like tantalum, peek and flexible polythene are also used but rarely or in special cases. The small diameter columns have the advantage of less solvent consumption and better signal height-to-sample mass ratio. The stationary phase or packing is retained at each end by thin stainless steel frits with a mesh of 2 µm or less. A number of stationary phases like silica\textsuperscript{13}, styrene-divinyl benzene\textsuperscript{14}, Alumina\textsuperscript{15}, magnesium silicate, Hydroxyalkylmethacrylate gel, Agarose\textsuperscript{16}, Hydroxyl apatite\textsuperscript{17}, Porous graphite carbon\textsuperscript{18}, Titania, Zirconia\textsuperscript{19} and some restricted surface access phases\textsuperscript{20} are available.

The conventional HPLC column packings are based on silica. They are mechanically strong and can withstand high pressures encountered in HPLC without considerable breakage or compression. Silica is an adsorbent with outstanding properties that can be used as chemically bonded stationary phase. Its lattice is saturated at the surface with silanol groups (≡Si-OH) and siloxane bridges (≡Si-O-Si=). Though all the functional groups at the surface are active, the various types have different properties:

a) Free silanols being slightly acidic, will preferably be adsorbed by the basic compounds giving rise to chemical tailing.
b) Geminal silanols are not acidic.

c) Vicinal silanols are not acidic and compounds with OH groups tend to adsorb here.

d) Silanols near metal cations are strongly acidic and less suitable for separation of basic compounds due to surface heterogeneity.

e) Siloxanes in which more amount of siloxane and less concentration of silanol is present.

Silica can be chemically modified\textsuperscript{21-22} to give stationary phases with specific properties. In majority of the bonded phases, the bonded entity is simply a hydrocarbon chain which is attached to silica producing a non-polar surface suitable for reversed phase chromatography where mixtures of water and organic solvents are used as eluents. Octadecyl silane (ODS) is the most widely used of the chemically modified products. It contains C\textsubscript{18} chains, but materials with C\textsubscript{2}, C\textsubscript{6}, C\textsubscript{8} and C\textsubscript{22} chains are also available. Silica can be functionalized by various methods\textsuperscript{23} and then it is treated with trimethyl chlorosilane to reduce the number of silanol groups that remain unreacted for steric reasons. This is known as end capping. There is a vast range of materials which have intermediate surface polarities arising from the bonding to silica of other organic compounds which contains groups such as phenyl, nitro, nitrile, amino, fluoroalkyl, hydroxyl, etc., as shown in Table 1. Strong ion exchangers are also available in which sulphonic acid groups or quarternary ammonium groups are bonded to silica.
Table 1: Stationary phases and their structure for HPLC

<table>
<thead>
<tr>
<th>Phase</th>
<th>Description</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si</td>
<td>Silica&lt;br&gt;Classical normal phase material</td>
<td>Si-&lt;br&gt;OH</td>
</tr>
<tr>
<td>C1</td>
<td>TMS, SAS, Trimethyl&lt;br&gt;Reversed phase material. Unique selectivity for polar and multifunctional compounds</td>
<td>Si-&lt;br&gt;CH₃</td>
</tr>
<tr>
<td>C2</td>
<td>RP-2, Dimethyl&lt;br&gt;Reversed phase material</td>
<td>Si-&lt;br&gt;C₆H₅</td>
</tr>
<tr>
<td>C3</td>
<td>Propyl&lt;br&gt;Reversed phase material used in hydrophobic interaction chromatography of proteins and peptides</td>
<td>Si-&lt;br&gt;C₃H₇</td>
</tr>
<tr>
<td>C4</td>
<td>Butyl&lt;br&gt;Reversed phase material useful for ion-pairing chromatography. Offers less retention than C8 phase for non-polar solutes. It is an ideal phase for analyzing large proteins and hydrophobic peptides when bonded to 300Å silica</td>
<td>Si-&lt;br&gt;C₄H₉</td>
</tr>
<tr>
<td>C5</td>
<td>Pentyl&lt;br&gt;Reversed phase material useful for separation of hydrophobic proteins and oligonucleotides when bonded to 300Å silica. More hydrostatically stable than C4.</td>
<td>Si-&lt;br&gt;C₅H₁₁</td>
</tr>
<tr>
<td>C6</td>
<td>Hexyl&lt;br&gt;Reversed phase material useful for ion-pairing chromatography.</td>
<td>Si-&lt;br&gt;C₆H₁₃</td>
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<tr>
<td><strong>C8</strong></td>
<td>MOS, RP-8, LC8, Octyl</td>
<td>Reversed phase material. Similar selectivity to C18 but less retentive. Has wide applicability in pharmaceuticals, nucleosides, steroids, etc.</td>
</tr>
<tr>
<td></td>
<td></td>
<td><img src="si-c8h17" alt="" /></td>
</tr>
<tr>
<td><strong>C12</strong></td>
<td>Reversed phase material. Shorter chain length provides higher bonded phase coverage than C18 columns for sharp peak shapes of non-polar and moderately polar compounds.</td>
<td><img src="si-c12h25" alt="" /></td>
</tr>
<tr>
<td><strong>C18</strong></td>
<td>ODS, RP-18, LC18, Octadecyl</td>
<td>Classical reversed phase material that is more retentive for non-polar solutes and is excellent for ion-pair chromatography. It has wide applicability for the assay of nucleosides, nucleotides, pharmaceuticals, steroids, vitamins, fatty acids and environmental compounds when bonded to 300 Å silica and is perfect for separating large proteins and macromolecules.</td>
</tr>
<tr>
<td><strong>CN</strong></td>
<td>CPS, PCN, Cyano, Cyanopropyl, Nitrile</td>
<td>Normal or reversed phase material. It is slightly polar and has unique selectivity for polar compounds in both the modes. It equilibrates very rapidly and is suitable for gradient separations. It has many pharmaceutical applications (e.g. tricyclic antidepressants).</td>
</tr>
</tbody>
</table>
| NH₂ | APS, Amino, Amino Propyl Silyl  
Reversed phase, normal phase or weak anion exchange material, useful for separating carbohydrates. Normal phase: alternative selectivity to silica, not deactivated by small amounts of water. Ion-Exchange: weak anion exchanger when used with buffers separates anions and organic acids. |
|---|---|
| NO₂ | Nitro  
Normal phase material. Separates aromatic compounds and compounds with double bonds. |
| OH | Diol, Glycerol  
Normal or reversed phase material.  
Normal phase: Similar selectivity to silica, not deactivated by small amounts of water.  
Reversed phase: Used for gel filtration chromatography of proteins and peptides. |
| C₆H₅ | Phenyl  
Reversed phase material. Useful for analyzing organic compounds. When bonded to 300Å silica this phase is useful for HIC |
| C₆H₅ (C₃H₆O linker) | Phenyl ether  
Reversed phase material. Used to separate extremely polar organic compounds.  
Alternative selectivity to phenyl hexyl and classic phenyl systems. |
<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
<th>Structure</th>
</tr>
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<tbody>
<tr>
<td>C\textsubscript{6}H\textsubscript{5} (C\textsubscript{6}H\textsubscript{12} linker)</td>
<td>Phenyl Hexyl Reversed phase material. Uses a patented linker as opposed to traditional propyl. Offers selectivity of a phenyl phase with greatly enhanced stability.</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>SAX</td>
<td>SB, Quarternary amine, strong base Ion-exchange material. Strong anion exchanger (basic) that is useful for separating nucleotides, nucleosides and organic acids.</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>SCX</td>
<td>SA, Strong acid Ion-exchange material. Strong cation exchanger (acidic) that is useful for separating organic bases.</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>WAX</td>
<td>DEAE, Weak base, PEI Ion-exchange material. Weak anion exchanger (basic) that is useful for analyzing and purifying basic proteins and peptides.</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>WCX</td>
<td>CM, Weak acid Ion-exchange material. Weak cation exchanger (acidic) that is useful for analyzing and purifying basic proteins and peptides.</td>
<td><img src="image" alt="Structure" /></td>
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</table>
4) **Detectors:** A detector in HPLC should be able to recognize when a substance zone is eluted from the column. It converts the monitored change in the mobile phase composition into electric signal which is transmitted to the display or recorder that is shown as a deviation from the baseline. Thus a signal as a peak on the chromatogram is obtained. An ideal detector should:

- a) Be able to perform even trace analysis.
- b) Be equally sensitive to all eluted peaks or record only those of interest.
- c) Not be affected by changes in temperature or in mobile phase composition.
- d) Not contribute to band broadening.
- e) React quickly to pick up correctly narrow peaks which pass rapidly through the cell.
- f) Easy to manipulate, robust and cheap.

Various types of detectors that can be used with HPLC are:

I. UV detectors
II. Refractive index detectors
III. Fluorescence detectors
IV. Electrochemical or Amperometric detectors
V. Light scattering detectors
VI. Conductivity detectors
VII. IR detectors
VIII. Photoconductivity detectors
IX. Radioactivity detectors

HPLC can also be combined with other analytical techniques mainly spectroscopy which sometimes use other detectors and prominent of them are HPLC-UV, HPLC-FTIR, HPLC-Mass and HPLC-NMR.
Properties of Solvent used in HPLC:

A mobile phase chosen for the chromatographic process should be capable of interacting with the stationary phase to separate a mixture as fast and as efficiently as possible. The major criteria for selection of the solvent are:

1) UV transparent: If the detector is UV based then the mobile phase must be completely transparent at the required wavelength.

2) Viscosity: A solvent with a lower pressure drop is preferable for a specific flow rate. Hence low viscosity solvents are preferred rather than high viscosity ones.

3) Strength: The elution strength of the solvent as a mobile phase is important which can be known from the eluotropic series.\(^1\)

4) Boiling point: If the eluate is to be recovered and further processed low boiling solvents are required but not too low as vapour bubbles may be formed.

5) Inert with respect to sample constituents: The mobile phase should not react at all with the sample mixture.

6) Purity: Generally pure mobile phase is preferred, as the impurities present may interfere with the chosen mode of detection.

7) Corrosion resistant: Composition of mobile phase that may give rise to chances of corrosion need to be avoided.

8) Refractive index: It is considered only when a refractive index detector is used and in such cases the difference between that of solvent and sample should be high.

9) Toxicity and Price: These should be as low as possible for the solvent to be highly recommendable.

In addition to these, there are solvatochromic parameters like dipole character, acidity, basicity that are useful for the characterization of selectivity properties of the solvent. These are very important as a mixture of solvents when selected as mobile phases bring in large differences in the elution pattern. The miscibility
properties of the common HPLC solvents at ambient temperature are to be thoroughly considered for this selection.

In some cases, like ion-exchange or reverse phase chromatography, buffers are to be added along with solvents to maintain the pH. It is required to check the total miscibility of the chosen buffer with the chosen organic solvent over the full range of the mixing ratios. A buffer solution must not remain with the HPLC system and column when not in use.

**Modes of HPLC**

There are various modes of operation of HPLC. Of these the Reversed phase HPLC is an important mode as it is compatible with aqueous and organic solutions as well as with different detection systems and it has high consistency and repeatability. The classification of the mode of liquid chromatography depends on the mechanism of interaction of the solutes with the stationary phase as shown below in Table 2.
Reversed Phase Chromatography:

Sensitive and accurate RPLC analysis necessitates the use of stationary phases which give symmetrical and efficient peaks. Hence continuous developments of this phase are being carried out to scale up new heights in the field of pharmaceutical or bio analysis.

The stationary phase in the Reversed Phase chromatographic columns is hydrophobic in nature that is made up of mainly porous particles of silica gel in various shapes having various pore sizes. The surface of these particles is chemically bonded with various hydrocarbons (C1, C4, C6, C8, C18, etc) as shown in figure 1.

Figure 1: Reversed phase-Stationary phase surface

In RPLC, the stationary phase is less polar than the mobile phase. The most frequently used stationary phase is chemically bonded Octadecyl silane (ODS). The retention increases with increasing contact area between sample and stationary phase.

In Reversed Phase systems the following affect the retention:

1. The chemical nature of the stationary phase.
2. The composition of the mobile phase.
3. The ionic strength, pH and additives of the mobile phase.
The chemical nature of the stationary phase

The chemical nature is determined by the size and chemistry of hydrocarbon bonded on the silica gel surface, its bonding density, and the purity and quality of the silica gel support. The more carbons in a bonded hydrocarbon the more it retains organic solutes. The higher the bonding density of the organic solutes the longer is its retention. Surface-active substances such as tri-ethylamine or tetrabutylamine or hexyl, heptyl, octyl sulfonate can be added as modifiers that get distributed between mobile phase and hydrophobic surface thereby charging the surface for better retention.

Composition of the mobile phase

The weakest mobile phase of all in RPHPLC is water and its amount when greater in the eluent leads to longer retention time. Sample compounds that are less water soluble (more non-polar) are better retained by the RP surface.

In case of non-polar analytes, non-aqueous eluents are required for RPHPLC. In the remaining cases, the mobile phase generally consist of mixtures of water or aqueous buffer solutions with various water miscible solvents. The order of increasing elution power with decreasing polarity is shown below:

<table>
<thead>
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<th>elution power</th>
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<tr>
<td>Methanol</td>
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</table>

For the optimization of solvent selectivity, usually methanol, acetonitrile and tetrahydrofuran are a good choice.

Ionic strength and pH of the mobile phase

When the samples are made up of solutes of ionizable functional groups, the ionization degree can be controlled with the help of buffers in the mobile phase. In general, the change of an ionizable molecule to an ion makes it more polar and less available to the stationary phase. In most of the traditional silica-gel
based stationary phases it is not possible to increase the mobile phase’s pH above 8 due to hydrolysis of the silica gel.

**Applications of HPLC:**

1) Chemical Separation: This can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. The extent or degree of separation is mostly determined by the choice of the stationary phase and mobile phase.

2) Identification: It is to identify the peaks on the chromatogram or a specific component in the eluate. It is compared with a reference compound and confirmed by comparing with other detection methods. Selection of the column mobile phase and flow rate is considerable to certain level in this process.

3) Quantification: It can be done by various methods like area normalization method, internal standard method and external standard method by quantification of known and unknown areas with respect to the principal peak.

4) Preparative HPLC: It involves the process of separating and purifying substances for further use.

**Chromatographic Method Development:**

While developing a chromatographic method, the following are to be considered carefully:

**(1) Literature survey:**

Proper survey of the literature available is the first step towards carrying out a project of this magnitude. This may include information regarding the analytical profile (physico-chemical properties like pKa, melting point, etc.), solubility profile (solubility of drug in different solvents and at different
conditions of pH, temperature, etc.) and stability profile (sensitivity of the drug towards light, heat, moisture, etc.)

(2) **Chemical Structure:**

The structure of the molecule and the impurities that may be present is to be collected. Likewise that of the starting materials, intermediates, byproducts and degradation products is also to be collected. The closely related structures are identified and the design of the method is done to get a good resolution between them. The structure of the impurities, starting material, intermediates, by-product and degradation products is compared with the structure of drug substances and the polarity at where they are less or more polar than the compound of interest is arrived at.

(3) **Selection of the diluent:**

A diluent in which the analyte, impurities, starting material, intermediates, by-product and degradation products are soluble is selected. The diluent selected should be compatible with the mobile phase to obtain a good peak. So it should be checked with the mobile phase initially. All the analytes should be completely soluble and the solution should be clear in case of dosage forms where the analyte should be extracted at least 95%.

(4) **Selection of stationary phase:**

The bonding phase is chosen based on the polarity of the molecule. For reverse phase chromatography, a wide variety of columns are available covering a wide range of polarity by cross linking the Si-OH groups with alkyl chains like C4, C6, C8, C18, nitrile groups (CN), amino groups (NH₂) and phenyl groups (C₆H₅).

Silica based columns with different crosslinkings in the increasing order of polarity are as follows:

```
<--------------Non-polar--------------Moderately polar--------------Polar-------------->
C18<C8<C6/C4<Phenyl<Amino<Cyano<Silica
```
(5) Selection of detector:

Photodiode array (PDA) detector is useful for initial method development based on the chromophores present in the compounds to be separated. The initial wavelength for analyzing the UV spectra of the compounds is selected by using the UV-Visible spectrophotometer. If the compounds do not have chromophores, other detectors like RI, ELSD/CCAD is chosen.

(6) Selection of mobile phase:

Depending upon the analyte’s pKa, which depends upon the structure of the molecule, the pH of the mobile phase is decided and also if at all any buffer is required. Initially the nature of the compound is assessed whether it is acidic, basic or neutral. If the compound is acidic an acidic mobile phase is selected. Likewise a neutral mobile phase is selected for a neutral compound. If the compound is basic, a basic mobile phase or that of low pH is selected. Based on the functional groups, the pH value is taken as ± 1 from the pKa values. Usually a buffer of strength 10-25mM is advisable for initial experiments.

Solvents like acetonitrile, methanol and THF are widely used to control selectivity and separations (of the same order) because of favourable UV transmittance and low viscosity. Though THF has slower column equilibration, higher UV absorbance and reactivity with oxygen, it sometimes gives unique selectivity for closely eluting peaks. In case of intermediate selectivity, if required, these solvents can be blended with one another.

In case of acids and bases, if any tailing is observed, the salt concentration can be increased to reduce the same. However increase beyond 50mM is not recommended due to possible solubility problems of the salt in organic portion of the mobile phase. 1% acetic acid can be added to mobile phase to reduce the solute-silanol interactions by acting as a competent acid. A 0.1% TFA, as aqueous modifier, can be a better choice as it results in a more transparent mobile phase.
and reduces tailing. Triethyl amine (TEA) or diethyl amine (DEA) can be added to control the peak tailing for bases by acting as competing bases and to minimize solute-silanol interactions. These can reduce retention, complicate the mobile phase or modify the column. Hence, if forced to be used for reducing tailing, column with very low levels of silanols is to be used.

In case of separation of a mixture of ionic and non-ionic analytes, the method has to be optimized starting with the non-ionic ones. The selectivity is then provided by selection of suitable ion-pair reagent. These are used generally when separation of closely related compounds, whose pKa values of the analytes are similar, is required. Moreover the HPLC column needs to be dedicated for that specific analysis only or an ion-pair needs to be used for cleaning every time.

Alkyl or aryl sulphonates are preferred ion-pair reagents for basic compounds in the concentration range of 1-10 mM. Likewise quarternary amines or sodium perchlorate are preferred for acidic compounds.

**Elution pattern:**

When the initial chromatogram indicates a retention range of 0.5<k<20, isocratic elution is possible. If this range is exceeded, gradient elution is necessary. For an ionic sample, when a wide retention range is indicated (0.5>k>20), the use of ion-pair reagents often permits isocratic separation with 0.5<k<20. The retention range change can also be achieved by a change in pH to ionize late eluting compounds for reduced retention or by reducing the ionization of early eluting sample components for late elution.

**Flow rate and column temperature:**

Initial flow rate between 0.5 and 1.5 ml/min and column temperature as 25\(^\circ\)C-30\(^\circ\)C (ambient) is preferable.
**Validation of a proposed method:**

Whenever an analytical method is established it should subsequently be validated. 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 application. The need for validation is that the proposed method should be capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. But the type of validation required depends entirely on the particular method and the evidence that it does what it is proposed or intended to do.

The typical analytical parameters used in assay validation include

1. Precision
2. Accuracy
3. Linearity
4. Range
5. Ruggedness
6. Robustness
7. Limit of detection
8. Limit of quantitation
9. Selectivity
10. Specificity

**Precision**

The precision of an analytical method expresses the degree of agreement between the true value and the value obtained. It is a measure of the reproducibility of the whole analytical method i.e. sampling, sample preparation and analysis, under normal operating circumstances. Precision should be investigated with homogenous and authentic samples. In case a homogenous
sample is not obtained, then an artificially prepared sample or its solution can be
used. The precision is then expressed as the relative standard deviation.

\[
\%\text{RSD} = \text{std dev} \times 100\% \\
\text{Mean}
\]

It can also be expressed as variance or coefficient of variation of a series of
measurements. Precision may be based on any of these levels: Repeatability,
Reproducibility and Intermediate precision.

(i) **Repeatability**: It is intra-assay precision that expresses the precision under
the same operating conditions over a short interval of time.

(ii) **Reproducibility**: It expresses the precision between laboratories.

(iii) **Intermediate precision**: It expresses the precision within a laboratory.

**Accuracy**

"Accuracy is a measure of the closeness of the test results obtained by a
method to the true value." This is sometimes termed trueness. A method can have
good precision and yet may not be accurate. Several methods are available for
determining accuracy.

(i) Application of the analytical procedure to synthetic mixture of the drug
product components to which known quantities of the drug substance to
be analysed have been added.

(ii) In cases where it is not possible to obtain drug product components, it
may be acceptable either to add known quantities of the analyte to the
drug product or to compare the results obtained from a second, well
characterized procedure, the accuracy of which is stated and/or defined.

**Linearity**

This is the method's ability to obtain results which are either directly or
after mathematical transformation proportional to the concentration of the analyte
within a given range.
Linearity is evaluated by proper statistical methods say by calculation of the regression line using mathematical treatment of the results (ie by least mean squares). This is done after a visual inspection of a plot of signals as a function of analyte concentration and arriving at the degree of linear relationship between the two.

For establishing linearity, a minimum of five concentrations is required. From this plot, the correlation co-efficient, y-intercept, slope of the regression line and residual sum of the squares should be arrived at in order to evaluate linearity. An analysis of the deviation of the actual data points from the regression line may also be helpful for evaluation of linearity.

**Range**

The range of the method is the interval between the upper and lower levels of an analyte that have been determined with acceptable precision, accuracy and linearity. It is determined on either a linear or nonlinear response curve and is normally expressed in the same units as the test results.

**Ruggedness**

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions (ie) different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc.

**Robustness**

It is a measure of the method’s capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

**Limit of Detection**

This is the lowest concentration in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The limit of
detection is important for impurity tests and the assays of dosages containing low drug levels and placebos.

The limit of detection is generally quoted as the concentration yielding a signal-to-noise ratio of 2:1 and is confirmed by analyzing a number of samples near this value using the following equation. The signal-to-noise ratio is determined by:

$$s = \frac{H}{h}$$

Where $H$ = height of the peak corresponding to the component

$h$ = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

Since the limit of detection is dependent on the signal-to-noise ratio, it can be improved by enhancing the analyte signal and reducing the detector noise. The signal (ie peak height) can be increased by selecting the optimum monitoring wavelength, increasing the injection volume or mass, increasing the peak sharpness with high efficiency columns and by optimizing the mobile phase. For absorbance detectors, a longer path length in the flow cell enhances sensitivity though often to the detriment of post column dispersion.

Noise can be reduced by using high sensitivity detectors with low noise and drift characteristics, slower detector response time, mobile phases with low absorbance and pumps with low pulsation.

**Limit of Quantification**

This is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.

It is quoted as the concentration yielding a signal-to-noise ratio of 10:1 and is confirmed by analyzing a number of samples near this value.

**Selectivity and Specificity**

Selectivity is the ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix.
Specificity is the ability to assess unequivocally the analyte in the presence of excipient and/or degradation products and/or impurities. Determination of this can be carried out by assessing the peak identity and purity.

Based on this introductory to the different areas of chromatographic analysis, especially reverse phase HPLC, some drugs shown in Table 3 were selected which have a fair and good biological activity for the estimation of the same in their bulk and pharmaceutical dosage forms. Some of them were determined in single dosage forms while the others were determined in combined dosage forms.

Table 3: Drugs selected for investigation

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gefitinib</td>
</tr>
<tr>
<td>2</td>
<td>Letrozole</td>
</tr>
<tr>
<td>3</td>
<td>Ritonavir</td>
</tr>
<tr>
<td>4</td>
<td>Tolperisone</td>
</tr>
<tr>
<td>5</td>
<td>Tretinoin</td>
</tr>
<tr>
<td>6</td>
<td>Vandetanib</td>
</tr>
<tr>
<td>7</td>
<td>Terbutaline</td>
</tr>
<tr>
<td>8</td>
<td>Bromhexine</td>
</tr>
<tr>
<td>9</td>
<td>Silymarin</td>
</tr>
<tr>
<td>10</td>
<td>Thiamine</td>
</tr>
</tbody>
</table>
REFERENCES:
<table>
<thead>
<tr>
<th>Mode</th>
<th>Normal Phase</th>
<th>Reverse Phase</th>
<th>Ion Exchange</th>
<th>Chiral</th>
<th>Affinity</th>
<th>Size Exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nature of Stationary phase</td>
<td>Polar Hydrophilic</td>
<td>Non polar Lipophilic</td>
<td>Ion bonding</td>
<td>Chiral recognisers</td>
<td>Bioaffinity</td>
<td>Sieving by size</td>
</tr>
<tr>
<td>Common Stationary Phases</td>
<td>Silica</td>
<td>Alkylated silica</td>
<td>Ionic functional groups on silica</td>
<td>Chiral groups on Silica surface</td>
<td>Biomolecules or Substrates</td>
<td>Gel type polymers</td>
</tr>
<tr>
<td>Common mobile phase</td>
<td>Hexane Isopropanol Methylene chloride</td>
<td>Water Methanol Acetonitrile Buffers</td>
<td>Water Buffers Acid Base</td>
<td>Aqueous and non-aqueous modes</td>
<td>Water Buffers</td>
<td>Aqueous and non-aqueous modes</td>
</tr>
<tr>
<td>Types of solutes</td>
<td>Fatty and oily</td>
<td>Most of the organic compounds</td>
<td>Ionic charged compounds</td>
<td>Enantiomers Both small and large molecules</td>
<td>Biomolecules or their Substrates</td>
<td>Synthetic or biological polymers</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Gefitinib is used in the treatment of certain cancers by selective inhibition of the epidermal growth factor receptor (EGFR)\textsuperscript{1,2}. This is done by binding it to the adenosine triphosphate (ATP) binding site of the enzyme. Even though it does not destroy the tumour cells, yet it interferes with the growth of new cancer cells by inhibiting the phosphorylation of several tyrosine kinases inside of the cell. Thus it belongs to a class of anti-cancer medications. As Gefitinib is a selective cancer chemotherapeutic agent, it has a superior tolerability profile as a novel antiemetic agent. Gefitinib is also used for the treatment of patients with a certain type of non-small cell lung cancer that has not responded to chemotherapy. It can be used in treatment of glioblastoma, a primary malignant brain tumor.

The adverse effects of Gefitinib usage commonly include acne, stomatitis, diarrhoea, nausea, vomiting, anorexia, asthenia, skin reactions, asymptomatic elevations of liver enzymes, dehydration and conjunctivitis. It should not be used by pregnant women. The structure of Gefitinib is shown in Figure 1.

**Figure 1: STRUCTURE OF GEFITINIB**
Physical properties of Gefitinib:

Molecular formula : C\textsubscript{22}H\textsubscript{24}ClF\textsubscript{4}N\textsubscript{4}O\textsubscript{3}

Molecular mass : 446.9

IUPAC name : \textit{N}-(3-chloro-4-fluoro-phenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy) quinazolin-4-amine

Appearance : White powder

Nature : Basic

pK\textsubscript{a} : 5.4 and 7.2

Solubility : Sparingly soluble in water, Soluble in glacial acetic acid, dimethyl sulphoxide and pyridine, sparingly soluble in methanol, ethanol, ethyl acetate, tetrahydrofuran, 2-propanol and acetonitrile.

Available HPLC methods:

Various analytical methods have been reported for the determination of gefitinib in pure drug, pharmaceutical dosage forms and in biological samples using Spectrophotometry, Liquid Chromatography, Electrokinetic Chromatography, High Performance Thin Layer Chromatography and Mass Spectrometry either in single or in combined forms. The chromatographic methods are as follows:

\textbf{F. Bai et al}\textsuperscript{3} developed a highly sensitive LC-tandem electrospray MS method for the measurement of gefitinib in human plasma. Chromatographic separation was achieved on a Phenomenex Synergi 4 \textmu m MAX-RP 80 C12 column with an isocratic mobile phase of acetonitrile and 1.0% formic acid in the ratio of 30:70. Deuterated gefitinib was used as the internal standard. Using 6 ml butyl methyl
ether, the compounds were extracted from 500 µl of sodium heparin plasma and after drying the residue it was reconstituted with 20% acetonitrile and 1% formic acid which was detected with a Perkin-Elmer SCIEX API 365 triple quadrupole mass spectrometer using a turbo ion spray source with positive ionization. The method was validated over a linear range of 0.5-1000 ng/ml with LOQ of 0.30 ng/ml and accuracy range of 91.0-97.7%.

S. D. Baker et al developed a rapid, sensitive and specific method using LC/MS/MS for determination of gefitinib in human plasma, mouse plasma and tissue. The sample was prepared by addition of acetonitrile to these plasmas. The separation of the compounds was done by an isocratic flow at 0.15 ml/min for 3 min of the mobile phase consisting acetonitrile and water (70:30 v/v) along with 0.1% formic acid. Linearity over the range of 1–1000 ng/ml for the human plasma samples and 5–1000 ng/ml for mouse plasma and tissue samples with values for the coefficient of determination of >0.99 was observed. The intra and inter day precision and accuracy were found to be within acceptance levels.

G. Guetens et al developed an on-column focusing gradient capillary LC method coupled to tandem mass spectrometry for the quantitative determination of Gefitinib in blood plasma. Plasma samples were extracted with methyl tert-butyl ether. The analytes of interest were eluted on a capillary ODS Hypersil column using an aqueous ammonium acetate gradient at 40 µl/min. Mass spectrometric detection was performed by a Q-Trap tandem mass spectrometer with electrospray positive ionisation, and monitored in the multiple reaction monitoring transitions 447 > 128 and 455 > 136, respectively. The limit of quantification was 0.1 ng/ml. The method proved to be robust allowing quantification of Gefitinib with sufficient precision, accuracy and sensitivity.

W. Franklin Smyth et al used an Electrospray ionisation-quadrupole ion trap mass spectrometry to generate tandem mass spectrometric data of the drugs studied and structural assignments of product ions were supported by quadrupole time-of-flight mass spectrometry (QToF-MS/MS). These fragmentation studies
were then utilised in the development and validation of a specific and sensitive liquid chromatographic method (LC–ESI-MS) to identify and determine these drugs at therapeutic concentration levels in serum after a single protein precipitation procedure with acetonitrile. In addition, this method was compared to the application of gas liquid chromatography-flame ionisation detection (GLC-FID) and differential pulse polarography (DPP) for the analysis of these NMEs in serum.

D. Gowri Sankar et al\textsuperscript{7} developed a RPHPLC method for the estimation of gefitinib in its pharmaceutical formulations using C-18 column. The mobile phase consisted of buffer which was prepared by dissolving 1.5 g of potassium dihydrogen phosphate in 550 ml of milli Q water (0.02 M) and the pH was adjusted to 6.5 with triethylamine and acetonitrile in the ratio of 55:45 (v/v). The detection was carried out at 220 nm and the linearity was found to be in the range of 10-60 µg/ml.

V. Kiran Kumar et al\textsuperscript{8} developed a simple, precise, rapid and accurate reverse phase HPLC method for the estimation of Gefitinib in pharmaceutical dosage form. A Hypersil BDS RP C18, column with mobile phase consisting of 0.02 M dipotassium hydrogen ortho phosphate and methanol in the ratio of 10:90 v/v was used. The flow rate was 1.0 ml/min and the effluents were monitored at 246 nm. The detector response was linear in the concentration of 25-300µg/ml with a retention time of 3.7 min. The limit of detection and limit of quantification was 0.125µg/ml and 0.15µg/ml respectively. The percentage assay of Gefitinib was 99.5 %. The method was validated by determining its accuracy, precision and system suitability.

G. J. Peters et al\textsuperscript{9} proposed a fast, sensitive and accurate method for the determination of gefitinib out of four different tyrosine kinase inhibitors from biological material using LC–MS/MS techniques. Simple protein precipitation of biological matrixes with acetonitrile at 4 °C was done with ease. Linearity was observed in the 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 was varied from 0.1 µl to 1 µl depending on the concentration of the drug observed. 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 for Gefitinib (447.1 m/z; 127.9 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. 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. Effectiveness of the method was illustrated with the analysis of samples from a cellular accumulation investigation and from determination of steady state concentrations in clinically treated patients.

Benoit Blanchet et al\textsuperscript{10} developed a simple and sensitive HPLC-UV method to simultaneously quantify Gefitinib and erlotinib in plasma. Following liquid–liquid extraction, gefitinib, erlotinib and sorafenib (internal standard), were separated with gradient elution on a C8+ Satisfaction using a mobile phase of acetonitrile/20 mM 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 imprecision were less than 7.2% and 7.6% for gefitinib and erlotinib, respectively. This analytical method was successfully applied to assess the steady state plasma exposure to Gefitinib and erlotinib in non-small cell lung cancer patients. This simple, sensitive, accurate and cost-effective method can be used in routine clinical practice to monitor gefitinib or erlotinib concentrations in plasma.
Xin Zheng et al\textsuperscript{11} developed a specific, sensitive, and rapid LC–MS–MS method for determination of gefitinib in human serum and cerebrospinal fluid. The analyte was detected by tandem mass spectrometry operating in positive electrospray ionization mode with multiple reaction monitoring. Gefitinib was extracted from serum or CSF samples with ethyl acetate using icotinib as internal standard. The method was validated over the concentration range of 1.00–1,000 ng/ml in human serum and 0.05–50.0 ng/ml in CSF. For both matrices, inter- and intraday precision (CV\%) were less than 15\% and accuracy was within 85–115\%. Average extraction recoveries were 78.9 and 61.8\% in human serum and CSF, respectively. Linearity, recovery, matrix effects, and stability were validated in the two matrices. The method was successfully used for analysis of clinical samples from lung cancer patients with brain metastases treated with gefitinib in the dosage range of 250–500 mg/day\textsuperscript{1}.

Ling-Zhi Wang et al\textsuperscript{12} developed a novel, rapid and specific LC–MS/MS method for the simultaneous quantification of gefitinib and its predominant metabolite O-desmethyl gefitinib in human plasma. Chromatographic separation of analytes was achieved on an Alltima C18 analytical HPLC column (150 mm × 2.1 mm, 5 \(\mu\) m) using an isocratic elution mode with a mobile phase comprised acetonitrile and 0.1\% formic acid in water (30:70 v/v). The flow rate was 300 \(\mu\)L/min. The chromatographic run time was 3 min. The column effluents were detected by API 4000 triple quadrupole mass spectrometer using electrospray ionization (ESI) in positive mode. Linearity was demonstrated in the range of 5–1000 ng/ml for gefitinib and 5–500 ng/ml for O-desmethyl gefitinib. The intra- and inter-day precisions for gefitinib and O-desmethyl gefitinib were \(\leq10.8\%\) and the accuracies ranged from 89.7 to 104.7\% for gefitinib and 100.4 to 106.0\% for O-desmethyl gefitinib. This method was used as a bioanalytical tool in a phase I clinical trial to investigate the possible effect of hydroxychloroquine on the pharmacokinetics of gefitinib.
The list of available brands of this drug is shown in Table 1.1.

**Table 1.1: List of available brand names of Gefitinib**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Brand name</th>
<th>Formulation</th>
<th>Available strength</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IRESSA</td>
<td>Tablet</td>
<td>250 mg</td>
<td>ASTRA ZENECA</td>
</tr>
<tr>
<td>2</td>
<td>GEFTINAT</td>
<td>Tablet</td>
<td>250 mg</td>
<td>NATCO PHARMA</td>
</tr>
<tr>
<td>3</td>
<td>CHEMOFIT</td>
<td>Tablet</td>
<td>250 mg</td>
<td>RPG-LS</td>
</tr>
<tr>
<td>4</td>
<td>GEFTIB</td>
<td>Tablet</td>
<td>250 mg</td>
<td>GLENMARK</td>
</tr>
<tr>
<td>5</td>
<td>GEFONIB</td>
<td>Tablet</td>
<td>250 mg</td>
<td>MIRACALUS</td>
</tr>
<tr>
<td>6</td>
<td>GEFTILON</td>
<td>Tablet</td>
<td>250 mg</td>
<td>CELON</td>
</tr>
<tr>
<td>7</td>
<td>GEFITERO</td>
<td>Tablet</td>
<td>250 mg</td>
<td>HETERO HC</td>
</tr>
<tr>
<td>8</td>
<td>GEFITEC</td>
<td>Tablet</td>
<td>250 mg</td>
<td>UNITED BIOTECH</td>
</tr>
<tr>
<td>9</td>
<td>CHEMOGEF</td>
<td>Tablet</td>
<td>250 mg</td>
<td>NEON LABORATORIES</td>
</tr>
<tr>
<td>10</td>
<td>GEFIRES</td>
<td>Tablet</td>
<td>250 mg</td>
<td>RESONANCE LABORATORIES</td>
</tr>
<tr>
<td>11</td>
<td>GEFFY</td>
<td>Tablet</td>
<td>250 mg</td>
<td>INTAS BIO-PHARMA</td>
</tr>
</tbody>
</table>
2. EXPERIMENTAL

2.1. Instrumentation
A Shimadzu electronic balance (AX-200) was used to weigh the drug and then for wavelength checking UV-2306 spectrophotometer was used. An isocratic Shimadzu HPLC model (VP series) instrument with Inertsil ODS C18 column (250 mm x 4.6 mm, 5µm) was used to develop a High Pressure Liquid Chromatographic method for the quantitative estimation. The instrument was equipped with a LC 20AT pump for solvent delivery and variable wavelength programmable SPD-10AVP detector. Degassing of the mobile phase was done using a Loba ultrasonic bath sonicator. A 20µL Rheodyne inject port (7725i) was used for injecting the samples. Data was analyzed by using PEAK software.

2.2. Chemicals and Solvents
Methanol, acetonitrile and tetrahydrofuran of HPLC grade were purchased from E.Merck, Mumbai, India. Gefitinib, as a pharmaceutical form, in the brand name of Gefonib was purchased from the local market.

2.3. The Mobile phase
The mobile phase containing acetonitrile, methanol and tetrahydrofuran in the ratio of 20:70:10 (v/v/v) was used for the elution.

2.4. Standard solution of the drug
Initially a stock solution was prepared by dissolving 10 mg of the drug in the solvent, made up to 100 ml in a volumetric flask and appropriate dilutions were done using the solvent chosen. A standard solution of 25 ppm was obtained by this process for subsequent analysis.

2.5. Sample solution
The tablet forms of Gefitinib (Gefonib) were powdered to a fine form and then powder equivalent to 10 mg of the drug was dissolved in 5 ml of the mobile phase.
taken in 10 ml volumetric flask. After dissolution the solution was filtered through Ultipor Nylon 6, 6 membrane sample filter paper and the filtrate was adjusted to the mark with the same solvent to obtain a concentration of 20 ppm.

3. METHOD DEVELOPMENT

Development of a suitable RP HPLC method involves selection of the appropriate wavelength, solvent, stationary and mobile phases. In order to establish these requirements, a systematic study on the effect of various factors involved was undertaken by varying each of them keeping all other conditions constant as follows:

3.1. Detection of wavelength
The wavelength of maximum absorbance was recorded on an UV spectrophotometer using a solution of Gefitinib and found to be 251 nm. Hence it was selected for detection of the drug.

3.2. Choice of stationary phase
A number of trials using different octadecyl columns of various types and configurations from different manufacturers were performed and finally an Inertsil ODS C-18 5µm column having 250 x 4.6mm internal diameter was chosen for the method development as it gave the expected separation with good peak shapes.

3.3. Selection of the Mobile phase
A number of solvents were analyzed, mixed in various proportions and tested under isocratic conditions with varied flow rates to separate the drug on the ODS C-18 column with various combinations. An ideal separation was achieved with mobile phase containing acetonitrile, methanol and tetrahydrofuran in the ratio of 20:70:10 (v/v/v). This was finally selected as it gave a well defined chromatographic peak with better resolution, base line separation and low tailing factor.
3.4. Flow rate
An effective flow rate is one that is minimum with a short run time which can minimize the usage of solvents. The optimum flow rate of 1.0 ml/min was attained by varying it between 0.5–1.5 ml/min. This was ideal for the successful elution of the analyte.

3.5. Optimized chromatographic conditions
Optimization of mobile phase was performed based on chromatographic separation, peak shape and peak area obtained. The composition, pH and flow rate of the mobile phase were changed to optimize the separation conditions. Based on the above proceedings, the Chromatographic conditions thus optimized are shown in Table 1.2.

These optimized conditions were maintained for the determination of Gefitinib in bulk and pharmaceutical forms. When blank solution containing only the mobile phase without the drug was injected, no peak was obtained. The chromatograms of standard, blank, tablet sample are shown in Figure 2, 3 and 4 respectively.

Figure 2: Chromatogram of standard solution
Figure 3: Chromatogram of blank (No Peak)

Figure 4: Chromatogram of formulation
Table 1.2: Optimized chromatographic conditions for estimation of Gefitinib

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameter</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mobile phase</td>
<td>Methanol : Acetonitrile : THF (70:20:10)</td>
</tr>
<tr>
<td>2</td>
<td>Pump mode</td>
<td>Isocratic</td>
</tr>
<tr>
<td>3</td>
<td>Mobile phase pH</td>
<td>5.8</td>
</tr>
<tr>
<td>4</td>
<td>Diluent</td>
<td>Mobile phase</td>
</tr>
<tr>
<td>5</td>
<td>Column</td>
<td>Inertsil ODS C-18, 5µm, 250 x 4.6mm</td>
</tr>
<tr>
<td>6</td>
<td>Column Temp</td>
<td>Ambient</td>
</tr>
<tr>
<td>7</td>
<td>Wavelength</td>
<td>251 nm</td>
</tr>
<tr>
<td>8</td>
<td>Injection Volume</td>
<td>20 µL</td>
</tr>
<tr>
<td>9</td>
<td>Flow rate</td>
<td>1.0 ml/min</td>
</tr>
<tr>
<td>10</td>
<td>Run time</td>
<td>7 min</td>
</tr>
<tr>
<td>11</td>
<td>Retention Time</td>
<td>4.282 min</td>
</tr>
</tbody>
</table>
4. RESULTS AND DISCUSSION

The experimental method developed above was employed for its subsequent validation and determination of Gefitinib in bulk and pharmaceutical forms. The following results were obtained correspondingly.

Validation of a proposed analytical method to determine the assay should meet the requirements for the intended analytical application as per ICH guidelines. The typical analytical parameters used in validation of the assay include Precision, Accuracy, Linearity, Robustness, Limit of detection, Limit of Quantification, Selectivity or Specificity.

4.1 Linearity

Linearity is the method's ability to obtain peak area results that are proportional to the concentration of the analyte within a given range. Linearity was performed by preparing standard solutions of Gefitinib at different concentration levels including working concentration mentioned in experimental condition i.e. 25 ppm. Twenty micro liters of each concentration was injected in duplicate into the HPLC system. The peak responses were read at 251 nm and the corresponding chromatograms were recorded. From these chromatograms, the mean peak areas were calculated and linearity plots of concentration over the mean peak areas were constructed individually. The calibration plot is shown in Figure 5. The regressions of the plots were computed by least square regression method. Linearity results obtained are presented in Table 1.3.
Table 1.3: Linearity studies of Gefitinib

<table>
<thead>
<tr>
<th>Level</th>
<th>Concentration of Gefitinib (in ppm)</th>
<th>Mean peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>115765.9</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>233031.0</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>344495.7</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>451641.1</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>566470.4</td>
</tr>
</tbody>
</table>

Range: 5 ppm to 25 ppm

Slope: 22400.38
Intercept: 6275.09
Correlation coefficient: 0.9998

Figure 5: Calibration plot for Gefitinib

The results obtained indicate a linear relationship between peak response and concentration of Gefitinib in the range of 5-25 ppm.
4.2 Precision

Precision is the degree of reproducibility of an analytical method under normal operational conditions. Precision is determined by using the method to assay a sample for a sufficient number of times to obtain statistically valid results. Precision of the method was performed as Intraday precision and Interday precision. The precision is then expressed as the relative standard deviation.

4.2.1. Intraday precision

The Intraday precision was studied by preparing and injecting six replicate standard solutions of Gefitinib (25 ppm) using the proposed method. The percent relative standard deviation (% RSD) was calculated for the peak areas and it was found to be 0.935%, which is well within the acceptance criteria of not more than 2.0%. Results of intraday system precision studies are shown in Table 1.4.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc.(in ppm)</th>
<th>Injection No.</th>
<th>Peak Area</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gefitinib</td>
<td>25</td>
<td>1</td>
<td>561526.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>559239.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>553074.0</td>
<td>0.935</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>558136.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>564357.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>568237.1</td>
<td></td>
</tr>
</tbody>
</table>

4.2.2. Interday precision

The interday precision was studied by preparing and injecting six replicates of standard solutions of Gefitinib (25 ppm) on three different days over a period of
one week. The percent relative standard deviation (% RSD) was calculated and it was found to be 0.819%, which is well within the acceptance criteria of not more than 2.0%. Results of interday system precision studies are shown in Table 1.5. The precision results obtained above indicate a very good agreement of the method with respect to its reproducibility at any period of time.

**Table 1.5: Interday Precision Results for Gefitinib**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc. (in ppm)</th>
<th>Injection No.</th>
<th>Peak Area</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gefitinib</td>
<td>25</td>
<td>1</td>
<td>566489.6</td>
<td>0.819</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>563568.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>562560.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>559324.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>553867.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>565149.3</td>
<td></td>
</tr>
</tbody>
</table>

**4.3. Selectivity**

Selectivity of an analytical method is its ability to measure accurately an analyte in the presence of possible interfering substances such as synthetic precursors, excipients, etc. The selectivity of method was confirmed by comparing the chromatograms of blank, standard and tablet sample. It was found that there is no interference due to excipients in the tablet formulation and also found good correlation between the retention times of standard and sample. The results are shown in Table 1.6.
Table 1.6: Selectivity Study

<table>
<thead>
<tr>
<th>Name of the solution</th>
<th>Retention Time (in min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>No peak</td>
</tr>
<tr>
<td>Standard</td>
<td>4.28</td>
</tr>
<tr>
<td>Sample</td>
<td>4.56</td>
</tr>
</tbody>
</table>

4.4. Accuracy

Accuracy of an analytical method is the extent to which test results are close to their true value. It is measured from the result of a quantitative determination of a well characterized known sample. The amount measured is compared to the known amount. The accuracy of the method was determined by standard addition method. A known amount of standard drug was added to the fixed amount of pre-analyzed tablet solution. Peak area was compared before and after the addition of the drug. The standard addition method was performed at 50%, 100% and 150% level of 10 ppm. The solutions were analyzed at each level as per the proposed method. The percent recovery and % RSD was calculated and results are presented in Table 1.7. This indicates that the proposed method is accurate.

Table 1.7: Accuracy results

<table>
<thead>
<tr>
<th>% Level</th>
<th>Conc. (in ppm)</th>
<th>Area</th>
<th>% Recovery</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>15</td>
<td>340391</td>
<td>98.80869</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>450264</td>
<td>99.69511</td>
<td>0.56</td>
</tr>
<tr>
<td>150</td>
<td>25</td>
<td>558933</td>
<td>98.66948</td>
<td></td>
</tr>
</tbody>
</table>
4.5. Robustness

Robustness is a measure of the method’s capacity to remain unaffected by slight variations in the parameters of the method which consequently indicates its reliability during normal usage. This was carried out by varying three parameters from the optimized chromatographic conditions. The results are shown in Table 1.8.

<table>
<thead>
<tr>
<th>Parameter changed</th>
<th>Change</th>
<th>Area</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>-</td>
<td>566470</td>
<td>-</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>80:10:10</td>
<td>560841</td>
<td>99.0063</td>
</tr>
<tr>
<td></td>
<td>60:30:10</td>
<td>566711</td>
<td>100.0425</td>
</tr>
<tr>
<td>Wavelength</td>
<td>253</td>
<td>560595</td>
<td>98.96288</td>
</tr>
<tr>
<td></td>
<td>249</td>
<td>560719</td>
<td>98.98477</td>
</tr>
<tr>
<td>pH</td>
<td>5.6</td>
<td>560207</td>
<td>98.89438</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>556499</td>
<td>98.2398</td>
</tr>
</tbody>
</table>

4.6. Limit of detection and Limit of quantification

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. In chromatography the detection limit is the injected amount that results in a peak height of at least twice or three times as high as the baseline noise level.

Limit of Quantification (LOQ) is the minimum injected amount that gives precise measurements. In chromatography it typically requires peak heights of 10 to 20 times higher than baseline noise at precision of <10-15% RSD between results.
The sample was dissolved by using the mobile phase and injected until the peak disappeared. After 0.29 ppm dilution, peak was not observed clearly. So it confirms that 0.29 ppm is the Limit of Detection and Limit of Quantification was found to be 0.09 ppm. The LOD and LOQ of Gefitinib are given in Table 1.9.

Table 1.9: Limit of Detection and Limit of Quantification for Gefitinib

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Measured volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit of Quantification</td>
<td>0.29 ppm</td>
</tr>
<tr>
<td>Limit of Detection</td>
<td>0.09 ppm</td>
</tr>
</tbody>
</table>

4.7. Formulation

The validated method was applied for the assay of commercial tablets containing Gefitinib. The formulation tablets of Gefitinib were crushed to give finely powdered material. Powder equivalent to 10 mg of drug was taken in 10 ml of volumetric flask containing 5 ml of mobile phase and was shaken to dissolve the drug and then filtered through Ultipor N_{66} Nylon 6.6 membrane sample filter paper. Volume of the filtrate was adjusted to the mark with the same solvent to obtain concentration of 25 ppm. An aliquot of this solution was injected into HPLC system. Peak area of Gefitinib was measured and compared against the peak area of the standard solution. The proposed method was able to estimate Gefitinib in the tablet formulation with an accuracy of 97.84%. The results presented good agreement with the labeled content as shown in Table 1.10.
Table 1.10: Formulation results

<table>
<thead>
<tr>
<th>Brand</th>
<th>Dose (mg)</th>
<th>Sample Conc.</th>
<th>Standard area</th>
<th>Sample area</th>
<th>Amount found</th>
<th>% assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gefonib</td>
<td>250</td>
<td>20 ppm</td>
<td>115765.9</td>
<td>31655.2</td>
<td>19.568 ppm</td>
<td>97.84</td>
</tr>
</tbody>
</table>

5. CONCLUSION

The statistical evaluation of the proposed method revealed its good linearity, reproducibility and its validation for different parameters made us to conclude that the current RP-HPLC method can successfully used for rapid and reliable determination of Gefitinib in tablet formulation and also in bulk drugs. Unlike the earlier methods this method having a chromatographic run time of 7 minutes allows the analysis of a large number of samples in short period of time, making it suitable for the routine analysis of Gefitinib and also quantification of Gefitinib in pharmaceutical dosage forms.
REFERENCES:


