CHAPTER - 1

General introduction to chromatography & Oncology compounds
1.1. INTRODUCTION

It is defined as a procedure by which solutes are separated by a dynamic differential migration process in a system consisting of two or more phases, one of which moves continuously in a given direction and in which the individual substances exhibit different motilities by reason of differences in adsorption partition solubility, vapor pressure, molecular size or ionic charge density. The individual substances thus separated can be identified or determined by various analytical procedures like UPLC, HPLC, LC-MS, GC, GC-MS, GC-HS, TLC and HP-TLC etc.

1.2. PHARMACEUTICAL INDUSTRY

The pharmaceutical industry develops, produces, and markets drugs licensed for use as medications. Pharmaceutical companies are allowed to deal in generic or brand medications and medical devices. They are subject to a variety of laws and regulations regarding the patenting, testing and ensuring safety and efficacy and marketing of drugs.

Drug discovery is the process by which potential drugs are discovered or designed. In the past, most drugs have been discovered either by isolating the active ingredient from traditional remedies (or) by serendipitous discovery. Modern biotechnology often focuses on understanding the metabolic pathways related to a disease state or pathogen, and manipulating these pathways using molecular biology (or) biochemistry. A great deal of early-stage drug discovery has traditionally been carried out by universities and research institutions.

Drug development refers to activities undertaken after a compound is identified as a potential drug in order to establish its suitability as a medication. Objectives of drug development are to determine appropriate research in these areas generally includes a combination of in-vitro studies, in-vivo studies, and clinical trials. The amount of capital required for late stage development has made it a historical strength of the larger pharmaceutical companies.
Suggested citation: Tufts Center for the Study of drug development, annual impact report. Often, large multinational corporations exhibit vertical integration, participating in a broad range of drug discovery and development, manufacturing and quality control, marketing, sales, and distribution. Smaller organizations, on the other hand, often focus on a specific aspect such as discovering drug candidates or developing formulations. Often, collaborative agreements between research organizations and large pharmaceutical companies are formed to explore the potential of new drug substances.

1.3. ANALYTICAL CHEMISTRY

Analytical chemistry is the study of the separation, identification and quantification of the chemical components of natural and artificial materials. Qualitative analysis gives an indication of the identity of the chemical species in the sample and quantitative analysis determines the amount of one (or) more of these components. The separation of components is often performed prior to analysis.

Analytical methods can be separated into classical and instrumental. Classical methods use separations such as precipitation, extraction, and distillation and qualitative analysis by color, odor, or melting point. Quantitative analysis is achieved by measurement of weight or volume. Instrumental methods use an apparatus to measure physical quantities of the analyte such as light absorption, fluorescence (or) conductivity. The separation of materials is accomplished using chromatography (or) electrophoresis methods.

Analytical chemistry is also focused on improvements in experimental design, chemometrics, and the creation of new measurement tools to provide better chemical information. Analytical chemistry has applications in forensics, bioanalysis, clinical analysis, environmental analysis and materials analysis.

Although modern analytical chemistry is dominated by sophisticated instrumentation, the roots of analytical chemistry and some of the principles used in modern instruments are from traditional techniques many of which are still used
today. These techniques also tend to form the backbone of most undergraduate analytical chemistry educational labs.

**Qualitative analysis**

A qualitative analysis determines the presence or absence of a particular compound, but not the mass or concentration. That is, it is not related to quantity.

**Chemical tests**

There are numerous qualitative chemical tests, for example, the acid test for gold and the Kastle-Meyer test for the presence of blood.

**Flame test**

Inorganic qualitative analysis generally refers to a systematic scheme to confirm the presence of certain, usually aqueous ions or elements by performing a series of reactions that eliminate ranges of possibilities and then confirms suspected ions with a confirming test. Sometimes small carbon containing ions are included in such schemes. With modern instrumentation these tests are rarely used but can be useful for educational purposes and in field work or other situations where access to state-of-the-art instruments is not available or expedient.

**Gravimetric analysis**

Gravimetric analysis involves determining the amount of material present by weighing the sample before and/or after some transformation. A common example used in undergraduate education is the determination of the amount of water in a hydrate by heating the sample to remove the water such that the difference in weight is due to the loss of water.

**Volumetric analysis**

Titration involves the addition of a reactant to a solution being analyzed until some equivalence point is reached. Often, the amount of material in the solution being analyzed may be determined. Most familiar to those who have taken college chemistry is the acid-base titration involving a color changing indicator. There are many other types of titrations, for example potentiometric titrations. These titrations may use different types of indicators to reach some equivalence point.
Spectroscopy

Spectroscopy measures the interaction of the molecules with electromagnetic radiation. Spectroscopy consists of many different applications such as atomic absorption spectroscopy, atomic emission spectroscopy, UV-visible spectroscopy, X-ray fluorescence spectroscopy, IR-spectroscopy, Raman-spectroscopy, dual polarisation-interferometry, NMR-spectroscopy, photo emission spectroscopy, Mössbauer spectroscopy and so on.

Mass spectrometry

Mass spectrometry measures mass-to-charge ratio of molecules using electric and magnetic fields. There are several ionization methods: electron impact, chemical ionization, electrospray, fast atom bombardment, matrix assisted laser desorption ionization, and others. Also, mass spectrometry is categorized by approaches of mass analyzers: magnetic-sector, quadrupole mass analyzer, quadrupole ion trap, time-of-flight, Fourier transform ion cyclotron resonance, and so on.

Electrochemical analysis

Electro analytical methods measure the potential (volts) and/or current (amps) in an electrochemical cell containing the analyte. These methods can be categorized according to which aspects of the cell are controlled and which are measured. The three main categories are potentiometry (the difference in electrode potentials is measured), coulometry (the cell's current is measured over time), and voltammetry (the cell's current is measured while actively altering the cell's potential).

Thermal analysis

Calorimetry and thermogravimetric analysis measure the interaction of a material and heat.

Separation

Separation processes are used to decrease the complexity of material mixtures. Chromatography and electrophoresis is representative of this field.
Hybrid techniques

Combinations of the above techniques produce a "hybrid" (or) "hyphenated" technique. Several examples are in popular use today and new hybrid techniques are under development. For example, gas chromatography/mass spectrometry, gas chromatography-IR spectroscopy, liquid chromatography-mass spectrometry, LC-NMR-spectroscopy. LC-IR spectroscopy and capillary electrophoresis-mass spectrometry.

Hyphenated separation techniques refer to a combination of two (or more) techniques to detect and separate chemicals from solutions. Most often the other technique is some form of chromatography. Hyphenated techniques are widely used in chemistry and biochemistry. A slash is sometimes used instead of hyphen, especially if the name of one of the methods contains a hyphen itself.

Microscopy

The visualization of single molecules, single cells, biological tissues and nanomaterials is an important and attractive approach in analytical science. Also, hybridization with other traditional analytical tools is revolutionizing analytical science. Microscopy can be categorized into three different fields: optical microscopy, electron microscopy and scanning probe microscopy. Recently, this field is rapidly progressing because of the rapid development of the computer and camera industries.

Analytical chemistry research is largely driven by performance (sensitivity, selectivity, robustness, linear range, accuracy, precision, and speed), and cost (purchase, operation, training, time, and space). Among the main branches of contemporary analytical atomic spectrometry, the most widespread and universal are optical and mass spectrometry. In the direct elemental analysis of solid samples, the new leaders are laser-induced breakdown and laser-ablation mass spectrometry, and the related techniques with transfer of the laser-ablation products into inductively coupled plasma. Advances in design of diode lasers and optical parametric oscillators promote developments in fluorescence and ionization spectrometry and also in absorption techniques where uses of optical cavities for
increased effective absorption pathlength are expected to expand. Steady progress and growth in applications of plasma- and laser-based methods are noticeable. An interest towards the absolute (standardless) analysis has revived, particularly in the emission spectrometry.

1.4. CHROMATOGRAPHY

Chromatography involves a sample (or sample extract) being dissolved in a *mobile phase* (which may be a gas, a liquid or a supercritical fluid). The mobile phase is then forced through an immobile, immiscible *stationary phase*. The phases are chosen such that components of the sample have differing solubilities in each phase. A component which is quite soluble in the stationary phase will take longer to travel through it than a component which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase.

Techniques such as HPLC (High Performance Liquid Chromatography) and GC (Gas Chromatography) use *columns* - narrow tubes packed with stationary phase, through which the mobile phase is forced. The sample is transported through the column by continuous addition of mobile phase. This process is called *elution*. The average rate at which an analyte moves through the column is determined by the time it spends in the mobile phase.

1.5. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC (High-Performance Liquid Chromatography) is a form of column chromatography used frequently in biochemistry and analytical chemistry. It is also sometimes referred to as high pressure liquid chromatography. HPLC is used to separate components of a mixture by using a variety of chemical interactions between the substance being analyzed in the form of analyte and the stationary phase of column.
Types of HPLC:

There are many ways to classify liquid column chromatography. If this classification is based on the nature of the stationary phase and the separation process, three modes can be specified.

a. Adsorption chromatography:

The stationary phase is an adsorbent (like silica gel or any other silica based packing) and the separation is based on repeated adsorption-desorption steps.

b. Ion-exchange chromatography:

The stationary bed has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

c. Size exclusion chromatography:

The column is filled with material having precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later. This technique is also called gel filtration or gel permeation chromatography.

Concerning the first type, two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography. In normal phase chromatography, the stationary bed is strongly polar in nature (e.g. silica gel), and the mobile phase is non-polar (such as n-hexane). Polar samples are thus retained on the polar surface of the column packing for longer than less polar materials.

Reversed-phase chromatography is the inverse of this. The stationary bed is (non-polar) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained.
Reversed-phase chromatography is used for almost 90% of all chromatographic applications. Eluent polarity plays the major role in all types of HPLC. There are two elution types: isocratic and gradient. In the first type, constant eluent composition is pumped through the column during the whole analysis. In the second type, eluent composition (and strength) is steadily changed during the run.

HPLC as compared with the classical LC technique is characterised by high resolution small diameter (4.6 mm), stainless steel, glass or titanium columns; column packing with very small (3, 5 and 10µ) particles; relatively high inlet pressures and controlled flow of the mobile phase; continuous flow detectors capable of handling small flow rates and detecting very small amounts; rapid analysis; Initially, pressure was selected as the principal criterion of modern liquid chromatography and thus the name was "high pressure liquid chromatography" or HPLC. This was, however, an unfortunate term because it seems to indicate that the improved performance is primarily due to the high pressure. This is, however, not true. In fact, high performance is the result of many factors: very small particles of narrow distribution range and uniform pore size and distribution, high pressure column slurry packing techniques, accurate low volume sample injectors, and sensitive low volume detectors and, of course, good pumping systems. Naturally, pressure is needed to permit a given flow rate of the mobile phase.

**HPLC instrument:**

Function of the HPLC instrument is involves the following items. The basic operating principle of HPLC is to force the analyte through a column of the stationary phase, usually a tube packed with small round particles with a certain surface chemistry by pumping a liquid mobile phase at high pressure through the column. The sample is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase as it traverses the length of the column. Retardation depends on the nature of the analyte, stationary phase and mobile phase composition.
Components in a liquid chromatography system:
A Solvent delivery D Detector
B Flow mixing valve E Flow mixing valve
C Auto-Sampler F Solvent waste

**Fig-1.1:** Schematic diagram of HPLC instrument

The time at which a specific analyte elutes comes out of the end of the column is called the retention time and is considered a reasonably unique identifying characteristic of a given analyte. The use of pressure increases the linear velocity giving the components less time to diffuse within the column, leading to improved resolution in the resulting chromatogram. Common solvents used like water or various organic liquids like methanol, acetonitrile, etc. Water may contain buffers or salts to assist in the separation of the analyte components.
**Fig-1.2:** Agilent make HPLC instrument.

**Mobile phase reservoir:**

The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with special caps, Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air. Helium purging and storage of the solvent under helium is not sufficient for degassing aqueous solvents. It is useful to apply a vacuum for 5-10 min. and then keep the solvent under a helium atmosphere.
High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations.

The most important advantages are: higher resolution, faster analyses, and increased sample load capacity. However, only the most demanding separations require these advances in significant amounts. Many separation problems can be resolved with larger particle packings that require less pressure.
Injector

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of autosamplers and microprocessors. In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference, and loss in efficiency or all of these. It is always best to remove particles from the sample by filtering over a 5μ filter, or centrifusing. Since, continuous injections of particulate material will eventually cause blockages in injection devices or columns.
Typical HPLC columns are 5, 10, 15 and 25 cm in length and are filled with small diameter (3, 5 or 10 µ) particles. The internal diameter of the columns is usually 4.6 mm; this is considered the best compromise for sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale), then larger diameter columns may be needed. Packing the column tubing with small diameter particles requires high skill and specialized equipment. For this reason, it is generally recommended that all but the most experienced chromatographers purchase prepacked columns, since it is difficult to match the high performance of professionally packed LC columns without a large investment in time and equipment.
Fig-1.6. HPLC columns.

Fig-1.7: HPLC column internal view
Detector

Today, optical detectors are used most frequently in liquid chromatographic systems. These detectors pass a beam of light through the flowing column effluent as it passes through a low volume (~10μL) flow cell. The variations in light intensity caused by UV absorption, fluorescence emission or change in refractive index, from the sample components passing through the cell, are monitored as changes in the output voltage. These voltage changes are recorded on a strip chart recorder and frequently are fed into a computer to provide retention time and peak area data. The most commonly used detector in LC is the ultraviolet absorption detector (fig-1.8). A variable wavelength detector of this type, capable of monitoring from 190 to 400 nm, will be found suitable for the detection of the majority samples. Other detectors in common use include: Photo diode array UV detector (PDA), refractive index (RI), fluorescence (FLU), electrochemical (EC). The RI detector is universal but also the less sensitive one. FLU and EC detectors are quite sensitive but also quite selective.

a. Photo diode array UV detector (PDA):

A photodiode array (PDA) is a linear array of discrete photodiodes on an integrated circuit (IC) chip. For spectroscopy it is placed at the image plane of a spectrometer to allow a range of wavelengths to be detected simultaneously. In this regard it can be thought of as an electronic version of photographic film. Array detectors are especially useful for recording the full UV-Visible absorption spectra of samples that are rapidly passing through a sample flow cell, such as in an HPLC detector.

Light creates electron-hole pairs and the electrons migrate to the nearest PIN junction. After a fixed integration time the charge at each element is sequentially read with solid-state circuitry to generate the detector response as a function of linear distance along the array. PDAs are available with 512, 1024, or 2048 elements with typical dimensions of ~ 25 μm wide and 1-2 mm high.
b. **Refractive index (RI):**

The detection principle involves measuring of the change in refractive index of the column effluent passing through the flow-cell. The greater the RI difference between sample and mobile phase, the larger the imbalance will become. Thus, the sensitivity will be higher for the higher difference in RI between sample and mobile phase. On the other hand, in complex mixtures, sample components may cover a wide range of refractive index values and some may closely match that of the mobile phase, becoming invisible to the detector. RI detector is a pure differential instrument, and any changes in the eluent composition require the rebalancing of the detector. This factor is severely limiting RI detector application in the analyses requiring the gradient elution, where mobile phase composition is changed during the analysis to effect the separation.
c. **Fluorescence (FLU):**

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

When compounds having specific functional groups are excited by shorter wavelength energy and emit higher wavelength radiation which called fluorescence. Usually, the emission is measured at right angles to the excitation.
d. **Electrochemical (EC):**

The electrochemical detector responds to substances that are either oxidizable or reducible and the electrical output is an electron flow generated by a reaction that takes place at the surface of the electrodes. If the reaction proceeds to completion (exhausting all the reactant) the current becomes zero and the total charge generated will be proportional to the total mass of material that has been reacted. This process is called *coulometric* detection. If, however, the mobile phase is flowing past the electrodes, the reacting solute will be continuously replaced as the peak passes through the detector. All the time there is solute present between the electrodes, a current will be maintained, albeit varying in magnitude. Until relatively recently, this procedure was that most common employed in electrochemical detection and is called *amperometric* detection.

The electrochemical detector requires three electrodes, the working electrode (where the oxidation or reduction takes place), the auxiliary electrode and the reference electrode (which compensates for any changes in the background conductivity of the mobile phase).

The processes taking place at the electrode surface can be very complex; nevertheless, the dominant reaction can be broadly described as follows.
At the actual electrode surface the reaction is extremely rapid and proceeds almost to completion. This results in the layer close to the electrode being virtually depleted of reactant. As a consequence, a concentration gradient is established between the electrode surface and the bulk of the solution.

![Diagram of Fluorescence detector](image)

**Fig-1.11**: Schematic diagram of Fluoroscence detector.

e. **Evaporative light scattering detector (ELSD):**

The evaporative light detection system has revolutionized the analysis of lipids by HPLC since its introduction around 1980. This type of detector works by measuring the light scattered from the solid solute particles remaining after nebulization and evaporation of the mobile phase. For native lipids (not derivatized), the light-scattering detector (ELSD) is far more useful for on-line lipid quantification than the commonly used UV-detector.

More recently, an alternative instrument derived from the light scattering detector was proposed. This new detection device, charged aerosol detector, is based on an unique technology, in which the HPLC column eluent is first nebulized with nitrogen and the droplets are dried to remove mobile phase, producing analyte particles. Then, a secondary stream of nitrogen becomes positively charged as it...
passes a high-voltage, platinum corona wire. This charge transfers to the opposing stream of analyte particles. The charge is transferred to a collector where it is measured by a highly sensitive electrometer, generating a signal in direct proportion to the quantity of analyte present. This device shows consistent inter-analyte response independent of chemical structure. This means that the Corona detector can be used routinely for quantitation. This technology is said to be superior to light scattering for quantitative measurements.

**Fig-1.12:** Schematic diagram of ELS detector.

**Data system**

Since the detector signal is electronic, using modern data collection techniques can aid the signal analysis. In addition, some systems can store data in a retrievable form for highly sophisticated computer analysis at a later time. The
main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operator attention. There are several types of data systems, each differing in terms of available features. In routine analysis, where no automation (in terms of data management or process control) is needed, a pre-programmed computing integrator may be sufficient.

1.6. HPLC METHOD DEVELOPMENT

Reverse phase chromatography is the most popular analytical technique in the pharmaceutical industry. It is widely used for assay and impurity profiling of pharmaceutical substances. The quality of HPLC methods has become increasingly important. The requirements for methods usually depend on the stage of development of the drug. In early phases, the focus is mainly on high throughput and rapid turn-round time, while methods for late phase pharmaceutical development need to be simple and technically straightforward, moreover robust and rugged.

Literature review

Literature of selected and similar molecules may be collected from USP, EP, JP, IP, chromatography journals and patents. If method is available check the suitability of the method to meet the requirements or modify the method to suit the requirements such as resolution of possible impurities as per the synthetic process. Impurities to be considered are intermediates, process impurities and degradants. Collect samples, standards and all possible impurities in each stage and also collect information on physico-chemical properties.

General properties

Compare the structure of impurities, starting materials, byproducts, intermediates and degradation products with the structure of drug substances and arrive at the polarity whether they are less polar or more polar than the compound of interest. By observing the molecule based on the functional groups it can be determined whether the molecule is acid, basic or neutral. Based on the nature of the compound, pH of the mobile phase can be selected. If compound is acidic, acidic mobile phase is preferable, for basic compounds low pH and basic mobile phases are
preferable. For a neutral compound neutral mobile phase is suitable. Elution of the compounds is based on polarity. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, therefore, its retention decreases.

**pH and pKa value**

Based on pH or pKa values the nature of the compound and polarity of the compound can be assumed. When pH is equivalent to pKa, the compound is half ionized. Almost all the pH related change occurs within ± 1.5 units of the pKa value. Outside this range the compound is either ionized or non-ionized, and its retention does not change much with pH.

**Solubility**

All the components of the drug substance to be checked in solutions like mobile phase, mobile phase organic mixtures and water-organic mixtures. Mobile phase is best choice for sample diluent, as it eliminates ghost peaks, base line noise and negative peaks. All the components should be completely soluble to obtain clear solution.

**Column Selection**

In the commercial market, there are so many types of columns are available. Before choosing the column we need to consider the quality of the column by verifying batch to batch and lot to lot reproducibility. Column parameters like internal diameter, particle size, surface area, carbon load to be checked to verify system suitability criteria. In reversed phase analysis the mobile phase is polar and column is non-polar.
Fig-1.13: Schematic diagram of column selection

C₆, C₁₈, cyano, phenyl and amino columns can be used against a more polar mobile phase. Similar way in normal phase analysis the column is more polar when
compared to mobile phase. Cyano, phenyl, silica and chiral columns are used in normal phase mode.

**Selection of detector**

A detector is an important part of the liquid chromatography, it should be chosen very carefully for selective separation and accurate determination. The single most crucial factor is continuous detection based on the progress of separation of a component which may be immediately displayed and then recorded. However, a good detector should have linear response to solutes that extends over several orders of magnitude. It must have good stability, reproducibility and reliability. It should have low dead volume to minimize extra-column band broadening.

<table>
<thead>
<tr>
<th>Type of detector</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-detectors</td>
<td>Used for compound having chromophore.</td>
</tr>
<tr>
<td>Fluorescence detectors</td>
<td>Used for compounds exhibits fluorescence properties.</td>
</tr>
<tr>
<td>Electrochemical detectors:</td>
<td>For easily oxidizable compounds.</td>
</tr>
<tr>
<td>Refractive index detectors:</td>
<td>These are universal, but cannot be used with Gradient elution. Used for the compounds which are not having chromophores and where high sensitivity is not required.</td>
</tr>
<tr>
<td>Evaporative light scattering detectors(ELSD):</td>
<td>These are superior to RI detectors can be used for higher sensitivity with gradient elution.</td>
</tr>
</tbody>
</table>

Based on the structure and nature of the molecule suitable detection technique is to be selected. Detectors are of two basic types, bulk property detectors and solute property detectors.

Type of detector suitable for analysis is shown in table-1.1. If the compounds are not having chromophores choose other detectors like RI, ELSD or gas chromatography.

Table 1.1: Detector selection based on application of compound
If selected detection technique is UV-Visible spectrophotometer or photo diode array detector record spectrum of all the components of molecule. Select absorption maximum for product by considering all components in the product. Components to be considered are degradants, intermediates, process related impurities and raw materials. Also check the data at different scanning range if any component behaves differently, analyze in dual wavelength. Ensure peak purity of all the components, purity angle should be less than purity threshold. Peak purity test measures whether the compound is spectrally pure or not.

**Selection of Buffer**

In reversed phase HPLC, the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, therefore, its retention decreases. Acids lose a proton and become ionized when pH increases and bases gain a proton and become ionized when pH decreases. Therefore, when separating mixtures containing acids and/or bases by reversed phase HPLC, it is necessary to control the pH of the mobile phase using an appropriate buffer in order to achieve reproducible results.

Ideally the buffer should transmit light below 220nm. All buffers are most soluble in methanol and least soluble in tetrahydrofuran. Ammonium acetate is the most soluble buffer salt, ammonium phosphate is less soluble, and potassium phosphate is the least soluble buffer salt in each aqueous-organic mixture. Phosphate buffers at pH 3.0 are more soluble than phosphate buffers at pH 7.0. Commonly used HPLC buffers are showed in table-1.2. Optimum buffering capacity occurs at a pH equal to the pKa of the buffer. In general, most buffers may provide adequate buffering capacity for controlling mobile phase pH only within ±1 unit of their pKa, beyond that, buffering capacity will be inadequate. A buffer concentration in the range of 25 to 50 mM is adequate for most reversed phase applications.
Buffer | pK\(_\text{a}\) (25°C) | Useful pH Range
--- | --- | ---
TFA | 0.5 | <1.5
Sulfonate | 1.8 | <1-2.8
Phosphate | 2.1 | 1.1-3.1
Chloroacetate | 2.9 | 1.9-3.9
Formate | 3.8 | 2.8-4.8
Acetate | 4.8 | 3.8-5.8
Sulfonate | 6.9 | 5.9-7.9
Phosphate | 7.2 | 6.2-8.2
Ammonia | 9.2 | 8.2-10.2
Phosphate | 12.3 | 11.3-13.3

Table 1.2: commonly used HPLC buffers for reverse phase HPLC

1.7. HPLC METHOD VALIDATION

The efficacy and safety of a pharmaceutical product can be assured by analytical monitoring of its quality. Therefore the overall purity of a medicine must be assessed throughout its storage, distribution and use. This objective can possibly be achieved if the specifications to be applied are based on a validated procedure. It is therefore imperative that, any analytical procedure proposed for analysis of a particular active ingredient or its dosage form, be systematically evaluated so as to demonstrate that the method is scientifically sound under the conditions in which it is to be applied. The purpose of analytical validation is to ensure that the proposed analytical procedure under consideration is capable of giving reproducible and reliable results. The following parameters are checked in method validation.

Specificity and interference

While carrying out the particular test procedure in the prescribed manner, the analyst assumes that the results of the test refer only to the active ingredients in the sample matrix under analysis. Sample matrix may contain impurities arising from manufacturer or degradation, related chemical components or placebo ingredients. Specificity is a measure of the degree of interference in the analysis of complex sample mixtures.
Precision

The precision of the analytical methods refers to the degree of agreement among individually test result and how individual results are scattered from the mean value, usually expressed as, relative standard deviation. Precision is a measure of the degree of reproducibility of the analytical method under normal circumstances.

Accuracy

Accuracy of the procedure relates to the closeness of the results obtained by the procedure to the true values. The accuracy of a test procedure can usually be determined by applying it to the quantitatively prepared samples of the material to be analysed and expressed as percent recovery by the known amount of analyte.

Reproducibility

When the procedure is carried out many times using the samples from same homogeneous batch, the analytical data provide information about the reproducibility of the test procedure under validation.

Linearity and range

The Linearity of an analytical procedure is its ability to produce results that are directly or indirectly proportional to the concentration of analytes in the samples within a given range. The range of the procedure is an expression of the lowest and highest levels of analytes that the method can determine with reasonable accuracy and precision.

Limit of detection

The limit of detection is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitative, under the stated experimental conditions. The detection limit (DL) may be expressed as

\[ DL = 3.3\sigma / S \]

Where \( \sigma \) = the residual standard deviation of the regression line
\( S \) = the slope of calibration curve

The slope \( S \) may be estimated from the calibration curve of the analyte. The estimate of \( \sigma \) may be carried out based on the calibration curve. A specific calibration curve
should be studied using samples containing an analyte in the range of detection limit.

Limit of quantitation

The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The quantitation limit (QL) may be expressed as

\[
QL = 10 \sigma / S
\]

Where \( \sigma \) = the residual standard deviation of the regression line

\( S \) = the slope of calibration curve.

The slope \( S \) may be estimated from the calibration curve of the analyte. The estimation of \( \sigma \) may be carried out based on the calibration curve. A specific calibration curve should be studied using samples containing an analyte in the range of quantitation limit.

Ruggedness

Method ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analyst, laboratories, columns and instruments. Method ruggedness may not be known when a method is first developed. The strategy for determining method ruggedness will vary depending on the type and complexity of the method and the time available for validation. Determining method ruggedness may be limited to a few critical experiments, such as checking effects of different columns of same manufacturer and type and the effects of running the method in a different laboratory. In this case all other factors are kept constant, including mobile phases and reagents and the same sample is used and the final results are compared to assess equivalence.

Robustness

A measure of its capacity to remain unaffected by small, deliberate variations in method parameters by changing flow rate ±10%, buffer pH ± 0.2 units column oven ± 2°C, wavelength ± 2nm.
1.8. ONCOLOGY

An area of medicine that deals with the study and treatment of cancer. About Boehringer Ingelheim in Oncology building on scientific expertise and excellence in the fields of pulmonary and cardiovascular medicine, metabolic disease, neurology, virology and immunology, Boehringer Ingelheim has embarked on a major research programme to develop innovative cancer drugs. Working in close collaboration with the international scientific community and a number of the world’s leading cancer centres, Boehringer Ingelheim is committed to discovering and developing novel cancer treatments. This commitment is underpinned by using advances in science to develop a range of targeted therapies in areas of medical need, including various solid tumours and hematological cancers.

The current focus of research includes compounds in three areas: angiogenesis inhibition, signal transduction inhibition and cell-cycle kinase inhibition. Afatinib is currently in phase-III clinical development in NSCLC (Non-small cell lung cancer). In addition, the LUME-Lung phase-III clinical trial program, which is investigating in combination with standard second-line chemotherapy treatments for patients with advanced NSCLC, is ongoing. In the area of cell-cycle kinase inhibition, Boehringer Ingelheim is developing inhibitors of polo-like kinase-1 (Plk-1), a protein that is involved in the processes of cell division. These molecules are in the earlier stages of clinical development.

Cancer is not a single illness but a collection of many diseases that share common features. Cancer is widely viewed as a disease of genetic origin caused by mutations of DNA that make a cell multiply uncontrollably. The description and definitions of cancer, however, vary depending on the perspective as described below.

**Epidemiological perspective**

Cancer is a major cause of morbidity in the UK, with around 267000 new cases diagnosed in 1999. There are more than 200 different types of cancer, but four of them—breast, lung, colorectal and prostate—account for over half of all new cases. Overall, it is estimated that one in three people will develop some form of cancer during their lifetime. In the 10-year period 1989–1998, the overall age
standardized incidence rates for cancer increased by 1.6% in men and 6.3% in women. The fastest-growing cancers in men were malignant melanoma and prostate cancer, while in women, they were kidney cancer, non-Hodgkin's lymphoma and breast cancer.

Cancer incidence refers to the number of new cancer cases arising in a specified period of time. Prevalence refers to the number of people who have received a diagnosis of cancer who are alive at any given time, some of whom will be cured and others will not. Therefore, prevalence reflects both the incidence of cancer and its associated survival pattern. Overall, it is estimated that approximately 2% of the population of the UK (around 1.2 million people) are alive, having received a diagnosis of cancer. The single cancer that contributes most to this is breast cancer, with an estimated 172,000 women alive who have had a diagnosis of breast cancer.

**Sociological perspective:**

Patients with cancer adopt a medically sanctioned form of deviant behaviour described in the 1950s by Talcott Parsons as 'the sick role'. In order to be excused their usual duties and to be considered not to be responsible for their condition, patients are expected to seek professional advice and to adhere to treatments in order to get well. Medical practitioners are empowered to sanction their temporary absence from the workforce and family duties, as well as to absolve them of blame. This behavioural model minimizes the impact of illness on society and reduces secondary gain that the patient benefits from as a consequence of their illness. As Ivan Illich pointed out, however, this sets up physicians as agents of social control by medicalizing health and contributing to iatrogenic illness — 'a medical nemesis'. Of all the common medical diagnoses, cancer probably carries the greatest stigma and is associated with the most fear. The many different ways in which cancer affects people have been explored.

**Types of Cancers:**

1. Anaplastic Thyroid Cancer
2. Bladder Cancer
3. Bone cancer
4. Brain cancer
5. Breast cancer
6. Cervical cancer
7. Colon cancer
8. Esophageal cancer
9. Gastric cancer
10. Head & Neck cancer
11. Hodgkin's lymphoma leukemia liver cancer
12. Lung cancer
13. Melanoma
14. Mesothelioma
15. Multiple myeloma
16. Myelodysplastic syndrome
17. Non-Hodgkin's lymphoma
18. Ovarian cancer
19. Pancreatic cancer
20. Prostate cancer
21. Thyroid cancer
22. Rectal cancer
23. Renal cancer
24. Sarcoma
25. Uterine cancer
26. Skin cancer
27. Testicular cancer

1. **Anaplastic thyroid cancer**

Anaplastic thyroid cancer is a rare and aggressive form of cancer. It cannot be cured by surgery, and of all other treatment options, only radiation therapy combined with chemotherapy can provide any significant benefit.
Most people do not survive longer than 6 months due to the aggressive nature of this disease and lack of effective treatment options. Because this disease makes up less than 1% of all thyroid cancers, there is very little research funding for it.

2. Bladder cancer

Radiation therapy may be an integral part of the treatment of bladder cancer. However, since cancer of the bladder is not exclusively treated with radiation therapy, it may be important for patients to be treated at a medical center that can offer multi-modality treatment involving medical oncologists, radiation oncologists, and surgeons.

3. Bone cancer

Osteosarcoma is the most common type of cancer of the bone. It is the third most common malignancy in children and adolescents, accounting for approximately 5% of all cancers in these age groups. In children and adolescents, 50% of osteosarcomas arise from the bones around the knee. The cause of most cases of osteosarcoma is unknown although a genetic predisposition is suspected. The main known cause of osteosarcoma is radiation therapy. Osteosarcoma is a relatively frequent complication in survivors of childhood cancers treated with radiation therapy with a latency period of 15-20 years.

4. Brain cancer

Surgery is the primary treatment for brain tumors that can be removed without causing severe damage. Many benign (non-cancerous) tumors are treated only by surgery. Most malignant (cancerous) tumors, however, require treatment in addition to the surgery, such as radiation therapy and/or chemotherapy.

The goals of surgical treatment for brain tumors are multiple and may include one or more of the following:

- Confirm diagnosis by obtaining tissue that is examined under a microscope.
- Remove all or as much of the tumor as possible.
- Reduce symptoms and improve quality of life by relieving intracranial pressure caused by the cancer.
• Provide access for implantation of internal chemotherapy or radiation.
• Provide access for delivering intra-surgical treatments, including hypertherpay or laser surgery.

5. Breast cancer

Despite an increased global effort to end breast cancer, it continues to be the most common cancer and the second leading cause of cancer deaths in women in the United States. In 2011, an estimated 230,480 new cases of breast cancer are expected among women in the United States. The number of victims of this deadly cancer can reach 40,000 or more each year.

These troubling numbers are a constant reminder that new therapeutic approaches that will improve patient survival are still desperately needed. We cannot forget that making advances in clinical care for this devastating disease requires continuous support of cutting-edge research -- research that will lead to more effective strategies for breast cancer treatment and prevention.

That's why NFCR (National foundation for cancer research) funds cutting-edge breast cancer research projects pioneered by leaders in the field. Read below to learn how our scientists have mounted a solid attack on breast cancer.

6. Cervical cancer

Information about the prevention of cancer and the science of screening appropriate individuals at high-risk of developing cancer is gaining interest. Physicians and individuals alike recognize that the best "treatment" of cancer is preventing its occurrence in the first place or detecting it early when it may be most treatable. Each year in the United States, there are an estimated 9710 new cases of cervical cancer and 3700 deaths due to the disease. Widespread use of a screening test called the Pap smear has led to a decline in the number of deaths resulting from cervical cancer. Continued progress and education about screening may allow for earlier detection and higher cure rates.

7. Colorectal cancer

Radiation therapy is not a common way to treat colon cancer, though it may be used in certain circumstances. Radiation therapy, often with chemotherapy, is
frequently used in the adjuvant or neoadjuvant setting for the treatment of rectal cancers, whereas chemotherapy alone is more common for the adjuvant and neoadjuvant treatment of colon cancers.

Doctors who specialize in treating cancers with radiation are known as radiation oncologists. During radiation therapy, high-energy X-rays are used to kill cancer cells. In advanced stages of colon cancer, radiation therapy is often given instead of surgery when an operation cannot be performed. Radiation therapy is also commonly given in combination with chemotherapy. Chemotherapy drugs have the ability to kill cancer cells directly and help make radiation therapy more effective in killing cancer cells.

8. Esophageal cancer

Radiation therapy can be an integral part of the treatment of esophageal cancer. However, since esophageal cancer is not exclusively treated with radiation therapy, it is important for patients to be treated in an environment that can offer multi-modality treatment involving radiation oncologists, surgeons, gastroenterologists, medical oncologists and nutritionists.

The objective of radiation therapy to the esophagus is to kill cancer cells that could otherwise persist after therapy and cause the cancer to relapse locally. Radiation therapy uses high energy x-rays to kill cancer cells that remain in or near the esophagus and surrounding lymph nodes. Radiation therapy can be externally or internally delivered to the esophagus and surrounding lymph nodes. External beam radiation therapy (EBRT) delivers radiation from a machine outside the body, called a linear accelerator. EBRT treatments are typically delivered 5 days a week, for 2-6 weeks, depending on the overall goals of treatment and each treatment lasts between 10-15 minutes. The internal delivery of radiation therapy (brachytherapy) involves the placement of a radioactive isotope, such as iridium 192, within the esophagus.

9. Gastric cancer

Information about the prevention of cancer and the science of screening appropriate individuals at high risk of developing cancer is gaining interest. Physicians
and individuals alike recognize that the best "treatment" of cancer is preventing its occurrence in the first place or detecting it early when it may be most treatable.

Gastric cancer is characterized by the presence of cancer cells in the tissues of the stomach, which is located in the upper abdomen. Worldwide, gastric cancer is the third leading cause of cancer death in men and the fifth leading cause of cancer death in women.

The chance of an individual developing cancer depends on both genetic and non-genetic factors. A genetic factor is an inherited, unchangeable trait, while a non-genetic factor is a variable in a person's environment, which can often be changed. Non-genetic factors may include diet, exercise, or exposure to other substances present in our surroundings. These non-genetic factors are often referred to as environmental factors. Some non-genetic factors play a role in facilitating the process of healthy cells turning cancerous (e.g. the correlation between smoking and lung cancer) while other cancers have no known environmental correlation but are known to have a genetic predisposition. A genetic predisposition means that a person may be at higher risk for a certain cancer if a family member has that type of cancer.

10. Head and neck cancers

The larynx is a short passageway shaped like a triangle that is just below the pharynx in the neck. The pharynx is a hollow tube about five inches long that starts behind the nose and goes down to the neck to become part of the esophagus. Food passes through the pharynx on the way to the esophagus. Air passes through the pharynx and then the larynx on the way to the windpipe (trachea) and into the lungs. The larynx has a small piece of tissue over it called the epiglottis to keep food from going into it or the air passages.

11. Hodgkin's lymphoma leukemia liver cancer

Patients classified as having stage-III or IV disease with "A" or "B" symptoms, stage-II disease and "B" symptoms, or bulky disease (site of disease greater than 10 centimeters) are all considered to have advanced stage Hodgkin's lymphoma.
12. Lung cancer

Lung cancer is the leading killer among all types of cancer in the United States. It is estimated that lung cancer accounts for about 14% of all new cancer cases in 2011, but will cause nearly 27% of all cancer deaths. Over the past three decades, little improvement has been achieved in extending the lives of lung cancer patients. In the late 1970's, about 37% of people survived one year or longer after initial diagnosis; now, three decades later, this number has only improved to 42%.

But there is hope. NFCR funds numerous leading researchers who are committed to finding more effective strategies for preventing, diagnosing and treating lung cancer. Through their dedicated efforts to build risk prediction models, identify cancer genes in early-stage lung cancer, design cutting-edge devices for monitoring drug response, and seek new strategies to overcome tumor drug resistance, NFCR scientists are leading the battle against the deadliest cancer.

13. Melanoma

Patients with recurrent or refractory metastatic melanoma may be divided into 2 groups: patients who have failed initial systemic therapy (chemotherapy and/or biologic therapy) and experience progression or recurrence after an initial response to treatment or patients who have local recurrences (skin and/or regional lymph nodes) after initial surgery or surgery and adjuvant therapy.

A variety of factors ultimately influence a patient's decision to receive treatment of cancer. The purpose of receiving cancer treatment may be to improve symptoms through local control of the cancer, increase a patient's chance of cure, or prolong a patient's survival. The potential benefits of receiving cancer treatment must be carefully balanced with the potential risks of receiving cancer treatment.

14. Mesothelioma

Radiation therapy uses high-energy rays to damage or kill cancer cells by preventing them from growing and dividing. Similar to surgery, radiation therapy is a local treatment used to eliminate or eradicate cancer in a defined area. Radiation therapy is not typically useful in eradicating cancer cells that have already spread to other parts of the body. Radiation may be used to cure or control cancer, or to ease
some of the symptoms caused by cancer. Visit the radiation therapy center for in-depth information on how radiation therapy is delivered, common side effects and answers to frequently asked questions. The following information discusses the role of radiation therapy in the management of mesothelioma.

15. Multiple myeloma

The treatment of multiple myeloma is focused on treating the underlying disease (the increased number of abnormal plasma cells). Managing the symptoms and other medical problems resulting from the increased numbers of plasma cells and abnormal (monoclonal) proteins is equally important. Several complications that result from multiple myeloma have specific treatments available.

16. Myelodysplastic syndrome

A targeted therapy is one that is designed to treat the cancer cells and minimize damage to normal, healthy cells. Cancer treatments that "target" cancer cells may offer the advantage of reduced treatment-related side effects and improved outcomes.

Conventional cancer treatments cannot distinguish between cancer cells and healthy cells. Consequently, healthy cells are commonly damaged in the process of treating the cancer, which results in side effects. Chemotherapy damages rapidly dividing cells, a hallmark trait of cancer cells. In the process, healthy cells that are also rapidly dividing (such as blood cells and the cells lining the mouth and GI tract) are also damaged. Treatment-related damage to healthy cells leads to complications of treatment, or side effects. These side effects may be severe, reducing a patient's quality of life, compromising their ability to receive their full, prescribed treatment, and sometimes, limiting their chance for an optimal outcome from treatment.

17. Non-Hodgkin's lymphoma

Follicular non-Hodgkin's lymphoma (NHL) are considered low-grade cancers because they are slow growing compared to the more common, aggressive forms of NHL. Follicular lymphoma affects the B-cells, a type of lymphoma cell. It occurs more commonly in elderly patients; the average age at diagnosis is 60 years. The majority
of patients have advanced disease at the time of diagnosis, and patients commonly have cancer cells that have spread outside the lymph system.

Follicular lymphoma cells are characterized by how they look under the microscope; most cases are associated with a specific abnormality in the cell’s DNA. DNA is organized into 23 paired chromosomes. In the case of follicular lymphoma, genetic material from chromosome 14 is moved to chromosome 18, which is called a translocation (t14:18).

18. Ovarian cancer

Ovarian cancer is the most deadly cancer of the female reproductive system. It is estimated that this cancer alone will claim 15,460 American women’s lives in 2011. Often known as "the silent killer," ovarian cancer is difficult to detect early because the ovaries are deep within the pelvis and initial symptoms are often ambiguous. Too often the cancer goes undiagnosed until after the disease is far advanced and has spread throughout the abdomen or to distant organs. After the cancer has metastasized, survival rates plummet because the current treatments are largely ineffective in fighting late stage ovarian cancer. More effective treatments and better early detection tools must be developed to meet the unmet needs of ovarian cancer patients and save their lives.

19. Pancreatic cancer

In 2011, an estimated 44,030 individuals will be diagnosed with pancreatic cancer, and approximately 37,660 people will lose their battle with this disease. Pancreatic cancer has the lowest survival rate of all types of malignancies; for all stages combined, the one-year relative survival rate is 26% and the five-year survival rate is only about 6%.

There are two main reasons that account for the extremely low survival rate of this disease. First, pancreatic cancer patients seldom exhibit disease-specific symptoms until later stages, and in 80-90% of patients the tumor is already at an advanced stage upon diagnosis. Second, the treatment options currently available for pancreatic cancer are limited and ineffective. Thus, NFCR has been focusing on projects that improve early detection and treatment of pancreatic cancer.
20. Prostate cancer

Prostate cancer is the most common male malignancy in the USA: it is estimated that 240,890 new cases of prostate cancer will be diagnosed in 2011 alone. Over the past 25 years, dramatic improvements have been made in patient survival of this disease; in fact, the 5-year survival rate has increased from 69% to 99.6%. However, once the cancer has spread (or) "metastasized," the disease is fatal. Currently, no effective treatment is available.

That's why prostate cancer remains the second leading cause of cancer death in American males, and an estimated 33,720 patients will lose their battle to the disease in 2011, dying predominantly from metastatic prostate cancer.

21. Thyroid cancer

Thyroid cancer that has returned after treatment is called recurrent disease. Most cases of recurrent thyroid cancer occur in the neck region, but some also have distant metastases, or cancer that has spread to distant locations in the body. The most common site of distant metastasis is the lung.

The following is a general overview of treatment for recurrent thyroid cancer. Cancer treatment may consist of radioactive iodine treatment, surgery, radiation, chemotherapy (or) a combination of these treatment techniques. Combining two or more of these treatment techniques has become an important approach for increasing a patient's chance of cure and prolonging survival.

22. Rectal cancer

Surgery is a common treatment for rectal cancer. The type of operation used to remove the rectal cancer depends on the extent and location of the cancer. If the rectal cancer is located well above the anus, a low anterior resection (LAR) can be performed. This operation allows the patient to keep anal function and pass stools in a normal manner. If the rectal cancer is located close to the anus, sometimes the anus must be removed with the cancer in an operation called an abdominoperineal resection (APR). The patient must then use a colostomy bag. A colostomy is an opening where the large intestine is attached to the abdominal wall. A replaceable bag that encloses the colostomy is worn by the patient to collect stool.
23. Renal cancer

Radiation therapy uses high-energy radiation to kill cancer cells. External beam radiation therapy uses radiation delivered from outside the body that is focused on the cancer. Radiation therapy is sometimes used as the main treatment for kidney cancer for patients whose general health is too poor to undergo surgery. Radiation therapy can also be used to temporarily palliate or ease symptoms of kidney cancer such as pain, bleeding or problems caused by metastasis. Unfortunately, renal cell cancer is not very sensitive to radiation and while the growth of cancer can be slowed, it cannot be entirely eliminated. Currently, the use of radiation therapy before or after removing the cancer is not routinely recommended because clinical studies have not shown any improvement in patient outcomes.

24. Sarcoma

Soft tissue sarcomas comprise a relatively rare group of sarcomas which occur in both children and adults and account for approximately one percent of all cancers. Most of the literature on this subject deals with the treatment of adults. Soft tissue sarcomas can occur on any part of the body, but the majority occur in a limb or in the abdomen. The soft tissues involved include fat and fibrous tissue. These cancers are usually treated with surgery and radiation therapy and are relatively insensitive to chemotherapy.

25. Uterine cancer:

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Uterine (endometrial) cancer is the most common invasive gynecologic cancer in women, with 36,100 new cases each year. This incidence would be higher if it weren’t for the relatively large number of hysterectomies performed for non-cancerous reasons. It is estimated that approximately 6,500 women will die of
uterine cancer in the United States each year. The lifetime risk of developing uterine cancer for an American woman is 2%.

26. Skin cancer:

More than 2 million people are diagnosed with skin cancer every year in the United States, including non-melanoma and melanoma. Melanoma is the most serious form of skin cancer that can be fatal if it spreads to distant sites in the body. Among those skin cancer patients, it is estimated that more than 70,000 will be diagnosed with melanoma this year.

Although most skin cancers are curable, melanoma is estimated to claim more than 8,700 American people's lives in 2011 alone, accounting for more than 70% of all skin cancer deaths. Melanoma is more difficult to prevent because, unlike in other types of skin cancer, heredity plays a major role in melanoma development. It is also more aggressive in spreading (metastasizing) to distant body parts, and treatment is often ineffective once metastasis occurs. Studies show that only 15 to 20% of patients with metastatic melanoma could survive for 5 years or longer. Better treatment strategies are in high demand for this lethal skin cancer.

27. Testicular cancer:

The objective of radiation therapy is to kill testicular cancer cells for a maximum probability of cure with a minimum of side effects. The role of radiation in the treatment for testicular cancer depends predominantly on the histologic classification and the stage of the cancer. Radiation is generally given in the form of high-energy beams that deposit the radiation dose in the body where the risk of cancer cells is greatest. Radiation therapy, unlike chemotherapy, is considered a local treatment. Cancer cells can only be killed where the actual radiation is delivered to the body. If cancer exists outside of the radiation field, the cancer cells are not destroyed by the radiation. Therefore, radiation therapy is typically used as primary treatment for early stage cancers confined to a single location (field) in the body.
1.9. LENALIDOMIDE

Lenalidomide is used to treat a certain type of myelodysplastic syndrome (a group of conditions in which the bone marrow produces blood cells that are misshapen and does not produce enough healthy blood cells). Lenalidomide is also used along with dexamethasone to treat people with multiple myeloma (a type of cancer of the bone marrow) who have already been treated with at least one other medication. Lenalidomide is in a class of medications called immunomodulatory agents. It works by helping the bone marrow to produce normal blood cells and by killing abnormal cells in the bone marrow.

![Fig-1.14: The structure of Lenalidomide](image)

Lenalidomide has been used to successfully treat both inflammatory disorders and cancers in the past 10 years. There are multiple mechanisms of action, and they can be simplified by organizing them as mechanisms of action in vitro and in-vivo. In-vitro, Lenalidomide has three main activities: direct anti-tumor effect, inhibition of the microenvironment support for tumor cells, and immunomodulatory role. In-vivo, Lenalidomide induces tumor cell apoptosis directly and indirectly by inhibition of bone marrow stromal cell support by anti-angiogenic and anti-osteoclastogenic effects and by immunomodulatory activity. Lenalidomide has a broad range of activities that can be exploited to treat many hematologic and solid cancers.

Multiple myeloma is a rare cancer of the blood, characterized by accumulation of a plasma cell clone in the bone marrow. Lenalidomide is one of the novel drug agents used to treat multiple myeloma. It is a small molecular analog of
thalidomide that was originally found based on its ability to effectively inhibit tumor necrosis factor production. Lenalidomide is 50,000 times more potent than that Lenalidomide in inhibiting tumor necrosis factor-alpha, and has less severe adverse drug reactions. In a phase-III clinical study, Weber-et-al found that Lenalidomide plus dexamethasone in patients with relapsed or refractory multiple myeloma was superior to the old treatment of multiple myeloma consisting of high-dose dexamethasone alone.

1.10. PEMETREXED

Pemetrexed \(^{1-6}\) is used with another chemotherapy (anti-cancer) medication to treat malignant pleural mesothelioma (a type of cancer that affects the inside lining of the chest cavity). Pemetrexed is also used to treat non-small cell lung cancer. Pemetrexed is in a class of medications called antifolate antineoplastic agents. It works by blocking the action of a certain substance in the body that may help cancer cells multiply.

![Fig-1.15: The structure of Pemetrexed](image)

Pemetrexed is chemically similar to folic acid and is in the class of chemotherapy drugs called folate anti-metabolites. It works by inhibiting three enzymes used in purine and pyrimidine synthesis—thymidylate synthase (TS), dihy drofolate reductase (DHFR), and glycinamide ribonucleotide formyl transferase (GARFT). By inhibiting the formation of pre-cursor purine and pyrimidine nucleotides, pemetrexed prevents the formation of DNA and RNA, which are required for the growth and survival of both normal cells and cancer cells.
Pemetrexed comes as a solution (liquid) to be injected into a vein. Pemetrexed is administered by a doctor or nurse in a medical office or infusion center. It is usually given once every 21 days.

Side effects:
• ale skin, easy bruising or bleeding, unusual weakness;
• fever, chills, body aches, flu symptoms;
• white patches or sores inside your mouth or on your lips;
• urinating less than usual, or not at all;
• chest pain, trouble breathing;
• swelling,

1.11. ZOLEDRONIC ACID

Zoledronic acid ([1,5]) is used to prevent or treat osteoporosis (condition in which the bones become thin and weak and break easily) in women who have undergone menopause (change of life, end of regular menstrual periods). Zoledronic acid (Reclast) is also used to treat osteoporosis in men, and to prevent or treat osteoporosis in men and women who are taking glucocorticoids (a type of corticosteroid medication that may cause osteoporosis). Zoledronic acid (Reclast) is also used to treat Paget's disease of bone (a condition in which the bones are soft and weak and may be deformed, painful, or easily broken). Zoledronic acid (Zometa) is used to treat high levels of calcium in the blood that may be caused by certain types of cancer. Zoledronic acid (Zometa) is also used along with cancer chemotherapy to treat bone damage caused by multiple myeloma [cancer that begins in the plasma cells (white blood cells that produce substances needed to fight infection)] or by cancer that began in another part of the body but has spread to the bones. Zoledronic acid (Zometa) is not cancer chemotherapy, and it will not slow or stop the spread of cancer. However, it can be used to treat bone disease in patients who have cancer. Zoledronic acid is in a class of medications called bisphosphonates. It works by slowing bone breakdown, increasing bone density (thickness), and decreasing the amount of calcium released from the bones into the blood.
Zoledronic acid comes as a solution (liquid) to inject into a vein over at least 15 minutes. It is usually injected by a healthcare provider in a doctor's office, hospital, or clinic. When zoledronic acid injection is used to treat high blood levels of calcium caused by cancer it is usually given as a single dose. A second dose may be given at least 7 days after the first dose if blood calcium does not drop to normal levels or does not remain at normal levels. When zoledronic acid injection is used to treat bone damage caused by multiple myeloma or cancer that has spread to the bones, it is usually given once every 3 to 4 weeks. When zoledronic acid injection is used to treat osteoporosis in women who have undergone menopause, or in men, or to treat or prevent osteoporosis in people who are taking glucocorticoids, it is usually given once a year. When zoledronic acid is used to prevent osteoporosis in women who have undergone menopause, it is usually given once every 2 years. When zoledronic acid is used to treat Paget's disease of bone, it is usually given as a single dose, but additional doses may be given after some time has passed.

Side effects can include fatigue, anemia, muscle aches, fever, and swelling in the feet or legs. Flu-like symptoms are commonly experienced after the first zoledronate infusion, although not subsequent infusions, and are thought to occur because of its potential to activate human γ/δ T-cells (gamma/delta T-cells).

Zoledronate is rapidly processed via the kidneys; consequently its administration is not recommended for patients with reduced renal function or kidney disease. Some cases of acute renal failure requiring dialysis or having a fatal
outcome, following Reclast use, have been reported to the US Food and Drug Administration (FDA).

A rare complication that has been recently observed in cancer patients being treated with bisphosphonates is osteonecrosis of the jaw. This has mainly been seen in patients with multiple myeloma treated with zoledronate who have had dental extractions.

1.12. GEMCITABINE

Gemcitabine(1-7) is a nucleoside analog used as chemotherapy. Cancerous tumors are characterized by cell division, which is no longer controlled as it is in normal tissue. Normal cells stop dividing when they come into contact with like cells, a mechanism known as contact inhibition. Cancerous cells lose this ability. Cancer cells no longer have the normal checks and balances in place that control and limit cell division. The process of cell division, whether normal or cancerous cells, is through the cell cycle. The cell cycle goes from the resting phase, through active growing phased and then to mitosis.

![Structure of Gemcitabine](image)

**Fig-1.17:** The structure of Gemcitabine

The ability of chemotherapy to kill cancer cells depends on its ability to halt cell division. Usually, the drugs work by damaging the RNA or DNA that tells the cell how to copy itself in division. If the cells are unable to divide, they die. The faster the cells are dividing, the more likely it is that chemotherapy will kill the cells causing the tumor to shrink.

Chemically Gemcitabine is a nucleoside analogue in which the hydrogen atoms on the 2' carbon of deoxycytidine are replaced by fluorine atoms.
As with fluorouracil and other analogues of pyrimidines, the triphosphate analogue of gemcitabine replaces one of the building blocks of nucleic acids, in this case cytidine, during DNA replication. The process arrests tumor growth, as only one additional nucleoside can be attached to the "faulty" nucleoside, resulting in apoptosis.

Another target of gemcitabine is the enzyme ribonucleotide-reductase (RNR). The diphosphate analogue binds to RNR active site and inactivates the enzyme irreversibly. Once RNR is inhibited, the cell cannot produce the deoxyribonucleotides required for DNA replication and repair, and cell apoptosis is induced.

The following side effects are common (occurring in more than 30%) for patients taking Gemcitabine:

- Flu-like symptoms such as muscle pain, fever, headache, chills, and fatigue
- Fever (within 6–12 hours of first dose)
- Fatigue
- Nausea (mild)
- Vomiting
- Poor appetite
- Skin rash

Low blood counts. White and red blood cells and platelets may temporarily decrease, increasing risk of infection, anemia, and bleeding.

Nadir (low point), the time between chemotherapy cycles at which blood counts are at their lowest.

Onset: none noted Nadir: 10–14 days recovery: day 21 temporary increases in liver enzymes. Blood (or) protein in the urine. These are less common side effects (occurring in 10-29%) for patients receiving Gemcitabine:

- Diarrhea
- Weakness
- Hair loss
- Mouth sores
- Difficulty sleeping
1.13. OBJECTIVE

The main objective of this study is to develop a novel and stability indicating RP-HPLC methods for oncology drug products. The qualitative and quantitative analysis of drug substance and its related compounds estimation was carried out by high performance liquid chromatography. In the present study developed and validated for the following oncology drug products.

1. Lenalidomide formulations
2. Pemetrexed solid dosage forms
3. Zoledronic acid drug products
4. Gemcitabine formulations

Developed and validated the assay and impurities methods as per ICH and USFDA guidelines.
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