1. CHROMATOGRAPHY

1.1. History:

Chromatography is one of the essential techniques today. The mixture of two or more component can easily separate out using this technique. The concept of chromatography is not too old, in his paper" On the the new form of adsorption phenomena and its application in biochemical analysis", In 1903, Russian botanist Tswett [1] find out a new concept for the separation of 'pigments. He observed a colourful band of pigment so he gave name of this technique "Chromatography". After development of such a beautiful technique no one works for the further development. A very few similar type of work published L.S. Palmer[2] and C. Dhere in 10 years after innovation of new technique. After a long time martin and synge[3-4], in around 1930-1940 used concept of tswett for the separation of amino acid and introduced a new technique "liquid-solid chromatography" just by supporting silica solid stationary phase in water, for which they were awarded the Noble Prize in 1952. First time james and martin[5] introduced a new technique "Gas chromatography" by using gas as a mobile phase insted of liquid mobile phase. When worked on paper chromatography, they observed faster separation with gas phase compare to liquid phase with higher efficiency. In 1960s prof. Horvath developed a technique of continues flow of mobile phase through a column and introduce HPLC as a new name of separation technique. The development of mordent chromatography more on depends on the theory of Giddings described in his well known book "Dynamics of chromatography"[6].

1.2. Introduction:

Chromatography is a separation technique in which one phase is stationary and another one is mobile phase. Theory of separation well explain by Martin[3] , according to that the Component have different affinity towards stationary phase. The compounds which have lower affinity, retained less than the component have higher affinity and were separate out according to their affinity. Mobile phase is continuously passed through the stationary phase to elute out the retained component. Component mixture separated by using selective stationary phase and mobile phase. Different stationary phase have variable selectivity for particular component, so component retention be differ on altered stationary phase.

In current days chromatography is used as a versatile technique for development and analysis of complex mixtures. Chromatography is one of the powerful techniques available for analyst for separation, identification, isolation of sample in small quantity. Sample may be a chemical complex, mixture of two isomer, multi component mixture and mixture of natural species, it was easily analysed as well as quantified using latest chromatographic technique.

1.3. Types of Chromatography:

Chromatography can be classified by various ways (I) On the basis of interaction of solute to the stationary phase [7], (II) On the basis of chromatographic bed shape[8], (III) Techniques by physical state of mobile phase and (V) Few Special Chromatographic Techniques.

1.3.1. Based on interaction of solute with stationary phase:

Adsorption chromatography:

Adsorption chromatography is probably one of the oldest types of chromatography around. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes.

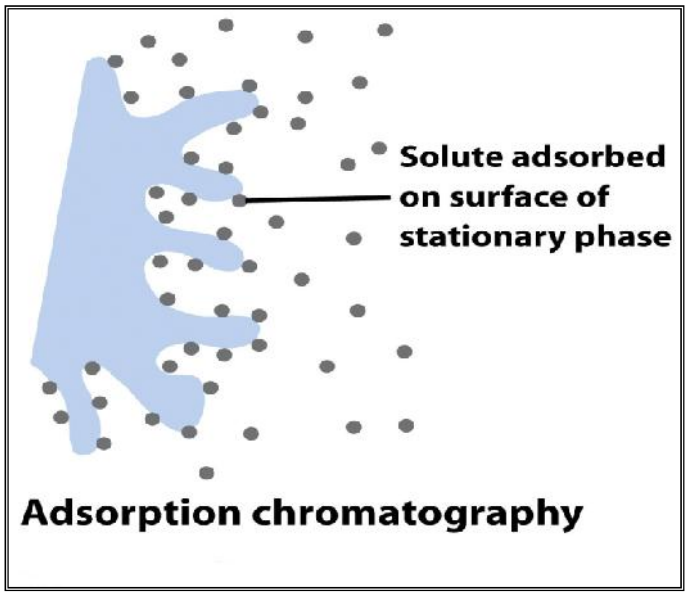

Figure 1: adsorption phenomenon in chromatography

Partition chromatography:

This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid.

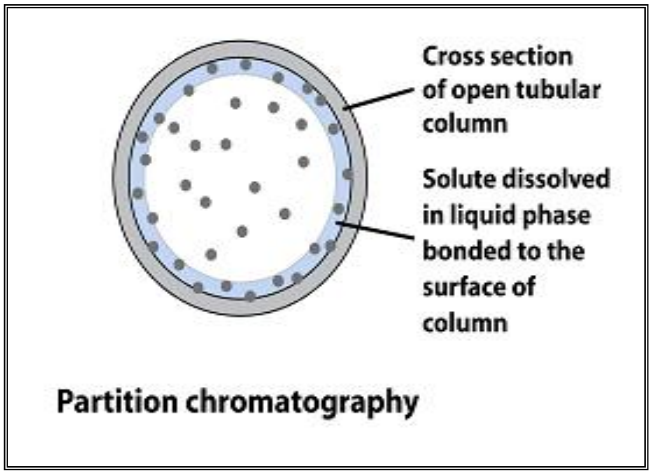

Figure 2: Partition phenomenon in chromatography

Ion exchange chromatography:

In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations onto it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.

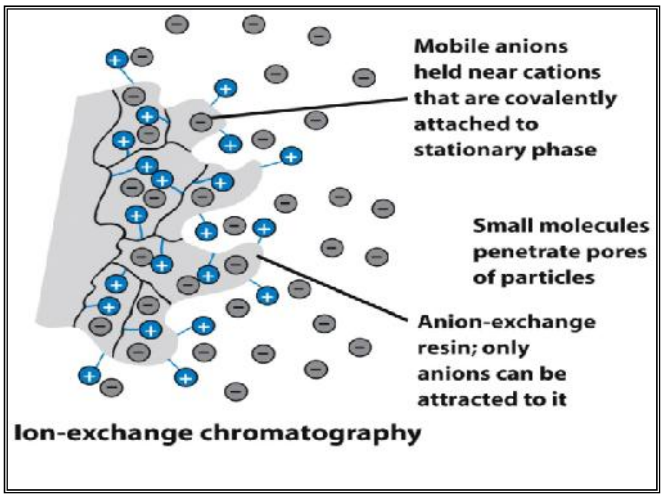

Figure 3: Ion Exchange phenomenon in chromatography

Molecular exclusion chromatography:

Also known as gel permeation or gel filtration, this type of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size. The pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

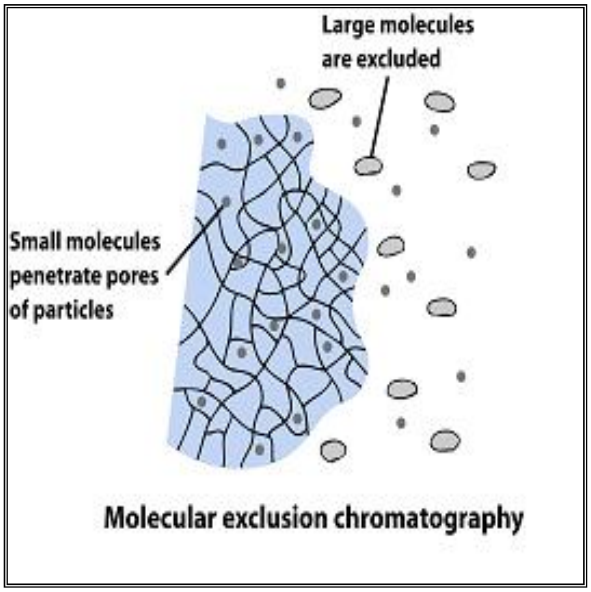

Figure 4 : Molecular Exchange phenomenon in chromatography

1.3.2. Based on nature of stationary phase :

Column chromatography:

Column chromatography is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column). Differences in rates of movement through the medium are calculated to different retention times of the sample [9].

In 1978, W.C.Still introduced a modified version of column chromatography called flash column chromatography [10-11]. The technique is very similar to the traditional column chromatography, except for that the solvent is driven through the column by applying positive pressure. This allowed most separations to be performed in less than 20 minutes, with improved separations compared to the old method. Modern flash chromatography systems are sold as pre-packed plastic cartridges, and the solvent is pumped through the cartridge. Systems may also be linked with detectors and fraction collectors providing automation. The introduction of gradient pumps resulted in quicker separations and less solvent usage.

In expanded bed adsorption, a fluidized bed is used, rather than a solid phase made by a packed bed. This allows omission of initial clearing steps such as centrifugation and filtration, for culture broths or slurries of broken cells.

Planer chromatography:

Planar chromatography is a separation technique in which the stationary phase is present as or on a plane. The plane can be a paper, serving as such or impregnated by a substance as the stationary bed (paper chromatography) or a layer of solid particles spread on a support such as a glass plate (thin layer chromatography). Different compounds in the sample mixture travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase. The specific Retention factor (Rf) of each chemical can be used to aid in the identification of an unknown substance.

Paper chromatography*:*

Paper chromatography is a technique that involves placing a small dot or line of sample solution onto a strip of chromatography paper. The paper is placed in a jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture which starts to travel up the paper with the solvent. This paper is made of cellulose, a polar substance, and the compounds within the mixture travel farther if they are non-polar. More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far.

Thin layer chromatography:

Thin layer chromatography (TLC) is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents. For even better resolution and to allow for quantification, high-performance TLC can be used.

Displacement chromatography**:**

The basic principle of displacement chromatography is, “A molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser affinities” [12]. There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably

to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds, and thereby be resolved, there must be substantial differences in some interaction between the biomolecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with gradient elution and low column loadings. Thus, two drawbacks to elution mode chromatography, especially at the preparative scale, are operational complexity, due to gradient solvent pumping, and low throughput, due to low column loadings.

Displacement chromatography has advantages over elution chromatography in that components are resolved into consecutive zones of pure substances rather than “peaks”. Because the process takes advantage of the nonlinearity of the isotherms, a larger column feed can be separated on a given column with the purified components recovered at significantly higher concentrations.

1.3.3. Based on nature of mobile phase:

Gas chromatography:

Gas chromatography (GC), also sometimes known as Gas-Liquid chromatography, (GLC), is a separation technique in which the mobile phase is a gas. Gas chromatography is always carried out in a column, which is typically "packed" or "capillary". Gas chromatography (GC) is based on a partition equilibrium of analyte between a solid stationary phase (often a liquid silicone-based material) and a mobile gas (most often Helium). The stationary phase is adhered to the inside of a small-diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column). It is widely used in analytical chemistry; though the high temperatures used in GC make it unsuitable for high molecular weight biopolymers or proteins (heat will denature them), frequently encountered in biochemistry, it is well suited for use in the petrochemical, environmental monitoring, and industrial chemical fields. It is also used extensively in chemistry research.

Liquid chromatography:

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Present day liquid chromatography that generally utilizes very small packing particles and a

relatively high pressure is referred as high performance liquid chromatography (HPLC). In the HPLC technique, the sample is forced through a column that is packed with irregularly or spherically shaped particles or a porous monolithic layer (stationary phase) by a liquid (mobile phase) at high pressure. HPLC is historically divided into two different sub-classes based on the polarity of the mobile and stationary phases. Technique in which the stationary phase is more polar than the mobile phase (e.g. toluene as the mobile phase, silica as the stationary phase) is called normal phase liquid chromatography (NPLC) and the opposite (e.g. water-methanol mixture as the mobile phase and C18 = octadecylsilyl as the stationary phase) is called reversed phase liquid chromatography (RPLC). Ironically the "normal phase" has fewer applications and RPLC is therefore used considerably more.

Specific techniques which come under this broad heading are listed below. It should also be noted that the following techniques can also be considered fast protein liquid chromatography if no pressure is used to drive the mobile phase through the stationary phase.

Affinity chromatography:

Affinity chromatography [13] is based on selective non-covalent interaction between an analyte and specific molecules. It is very specific, but not very robust. It is often used in biochemistry in the purification of proteins bound to tags. These fusion proteins are labeled with compounds such as His-tags, biotin or antigens, which bind to the stationary phase specifically. After purification, some of these tags are usually removed and the pure protein is obtained. Affinity chromatography often utilizes a biomolecule's affinity for a metal (Zn, Cu, Fe, etc.). Columns are often manually prepared. Traditional affinity columns are used as a preparative step to flush out unwanted biomolecules. However, HPLC techniques exist that do utilize affinity chromatography properties. Immobilized Metal Affinity Chromatography (IMAC) is useful to separate aforementioned molecules based on the relative affinity for the metal (I.e. Dionex IMAC). Often these columns can be loaded with different metals to create a column with a targeted affinity.

Supercritical fluid chromatography:

Supercritical fluid chromatography is a separation technique in which the mobile phase is a fluid above and relatively close to its critical temperature and pressure.

1.3.4. Some special techniques:

Two - dimensional chromatography:

In some cases, the chemistry within a given column can be insufficient to separate some analytes. It is possible to direct a series of unresolved peaks onto a second column with different physico-chemical (Chemical classification) properties. Since the mechanism of retention on this new solid support is different from the first dimensional separation, it can be possible to separate compounds that are indistinguishable by one-dimensional chromatography. The sample is spotted at one corner of a square plate, developed, air-dried, then rotated by 90° and usually redeveloped in a second solvent system.

Simulated moving-bed chromatography:

In chromatography, the simulated moving bed (SMB) technique is a variant of high Performance liquid chromatography; it is used to separate particles and/or chemical compounds that would be difficult or impossible to resolve otherwise. This increased separation is brought about by a valve-and-column arrangement that is used to lengthen the stationary phase indefinitely. In the moving bed technique of preparative chromatography the feed entry and the analyte recovery are simultaneous and continuous, but because of practical difficulties with a continuously moving bed in the simulated moving bed technique instead of moving the bed the sample inlet and the analyte exit positions are moved continuously, giving the impression of a moving bed.[14-15].

True moving bed chromatography (MBC) is only a theoretical concept. Its simulation, SMBC is achieved by the use of a multiplicity of columns in series and a complex valve arrangement, which provides for sample and solvent feed, analyte and waste take off at appropriate locations of any column, whereby it allows switching at regular intervals the sample entry in one direction the solvent entry in the opposite direction, whilst changing the analyte and waste takeoff positions appropriately as wells. The advantage of the SMBC is high speed, because a system could be near continuous, whilst its disadvantage is that it only separates binary mixtures. It does not say, but perhaps it can be assumed that this is equivalent with the separation of a single component from a group of compounds. With regard to efficiency it compares with simple chromatography technique like continuous distillation does with batch distillation.

Pyrolysis gas chromatography:

Pyrolysis is the thermal dissociation of materials in an inert atmosphere or a vacuum. The sample is put into direct contact with a platinum wire, or placed in a quartz boat inside a platinum coil, and rapidly heated to 600 – 800 C. Large molecules cleave at their weakest points and produce smaller, more volatile fragments. Various methylating reagents, which increase the volatility of polar fragments, can be added to a sample before pyrolysis. These fragments can then it can be easily separated by gas chromatograph (GC).

Not as well-known is the pyrolysis inside a Programmable Temperature Vaporizer (PTV) injector. The reason this technique is relatively unknown is that PTV injectors in general are of yet not often used. With some of the latest versions it is now possible to heat the sample very quickly until an end temperature of 600°C is reached. Some PTV's have a ramp rate of 30°C/sec. The fast ramp rate and the end temperature of 600°C are enough to make good pyrolysis. There are many advantages to using the injector as a pyrolyser. There is a price advantage, and switching from pyrolysing to normal GC injections can be done without changing the configuration of the hardware. Both liquid as well solid samples can be used when a PTV injector with a relative big liner is used. Real quantitative data can be acquired, and good results of derivatization inside the PTV injector are published.

Fast protein liquid chromatography:

Fast protein liquid chromatography (FPLC) is a term applied to several chromatography techniques which are used to purify proteins. Many of these techniques are identical to those carried out under high performance liquid chromatography, however use of FPLC techniques are typically for preparing large scale batches of a purified product.

Counter current chromatography:

Countercurrent chromatography (CCC) is a type of liquid-liquid chromatography, where both the stationary and mobile phases are liquids. It involves mixing a solution of liquids, allowing them to settle into layers and then separating the layers.

Chiral chromatography:

Chiral chromatography involves the separation of stereoisomers. In the case of enantiomers, these have no chemical or physical differences apart from being three-dimensional mirror images. Conventional chromatography or other separation processes

are incapable of separating them. To enable chiral separations to take place, either the mobile phase or the stationary phase must themselves be made chiral, giving differing affinities between the analytes. Chiral chromatography HPLC columns (with a chiral stationary phase) in both normal and reversed phase are commercially available.

Pyrolysis gas chromatography is very useful for the identification of synthetic polymeric media, such as acrylics or alkyds, and synthetic varnishes [16]. It can also be used for environmental samples [17], including fossils [18]. Most Known Chromatographic equipments are [19],

- High Performance Liquid Chromatography(HPLC)
- Ultra Performance Liquid Chromatography(UPLC, upgraded version of HPLC)
- High Performance Thin Layer Chromatography(HPTLC)
- Ion Chromatography(IC)
- Gas Chromatography(GC)
- Flash Chromatography
- Preparative High Performance Liquid Chromatography (Prep. HPLC)
- Capillary Chromatography.

1.4. High performance chromatography:

The highly sophisticated reliable and fast liquid chromatographic (LC) separation techniques are become a requirement in many industries like pharmaceuticals, agrochemicals, dyes, petrochemicals, natural products and others. Early LC used gravity fed open tubular columns with particles 100s of microns in size; the human eye was used for a detector and separations often took hours to develop.

High performance liquid chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as parts per trillion (ppt) may easily be identified. HPLC can be, and has been, applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals [18].The components of a basic high-performance liquid chromatography (HPLC) system are shown in the simple diagram in Figure-5.

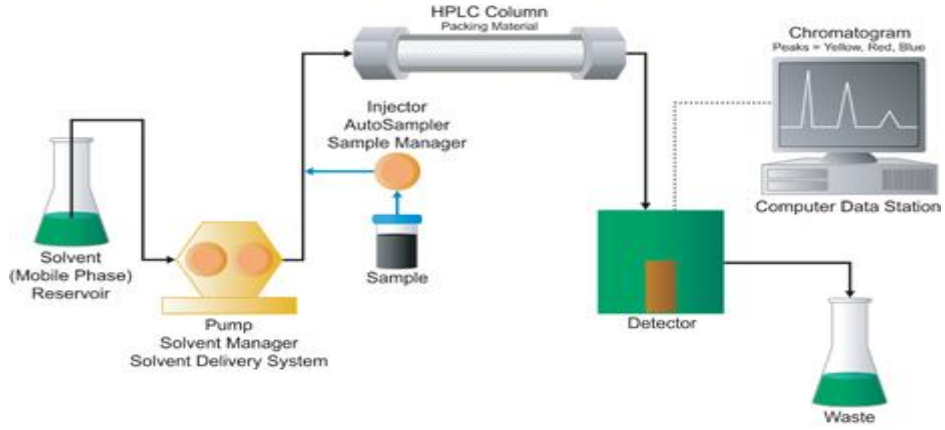

Figure 5: Schematic Diagram of HPLC system

A reservoir (Solvent Delivery) holds the solvent (called the mobile phase, because it moves). A high-pressure pump solvent manager is used to generate and meter a specified flow rate of mobile phase, typically milliliters per minute.

An injector (sample manager or auto sampler) is able to introduce (inject) the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column. The column contains the chromatographic packing material needed to effect the separation. This packing material is called the stationary phase because it is held in place by the column hardware. A detector is needed to see the separated compound bands as they elute from the HPLC column (most compounds have no color, so we cannot see them with our eyes). The mobile phase exits the detector and can be sent to waste, or collected, as desired. When the mobile phase contains a separated compound band, HPLC provides the ability to collect this fraction of the eluate containing that purified compound for further study. This is called preparative chromatography. The high-pressure tubing and fittings are used to interconnect the pump, injector, column, and detector components to form the conduit for the mobile phase, sample, and separated compound bands.

The detector is wired to the computer data station, the HPLC system component that records the electrical signal needed to generate the chromatogram on its display and to identify and quantitate the concentration of the sample constituents. Since sample compound characteristics can be very different, several types of detectors have been developed. A molecule's structure, activity, and physicochemical characteristics are determined by the arrangement of its constituent atoms and the bonds between them. Within a molecule, a specific arrangement of certain atoms that is responsible for special properties and predictable chemical reactions is called a functional group. This structure

often determines whether the molecule is polar or non-polar. Organic molecules are sorted into classes according to the principal functional group(s) each contains. Using a separation mode based on polarity, the relative chromatographic retention of different kinds of molecules is largely determined by the nature and location of these functional groups. As shown in Figure 6, classes of molecules can be ordered by their relative retention into a range or spectrum of chromatographic polarity from highly polar to highly non-polar.

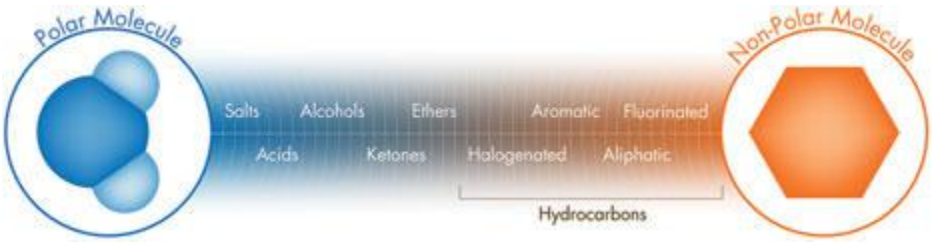

Figure 6: Chromatographic Polarity Spectrum by Analyte Functional Group

Water (a small molecule with a high dipole moment) is a polar compound. Benzene (an aromatic hydrocarbon) is a non-polar compound. Molecules with similar chromatographic polarity tend to be attracted to each other; those with dissimilar polarity exhibit much weaker attraction, if any, and may even repel one another. This becomes the basis for chromatographic separation modes based on polarity.

Another way to think of this is by the familiar analogy: oil (non-polar) and water (polar). Unlike in magnetism where opposite poles attract each other, chromatographic separations based on polarity depend upon the stronger attraction between likes and the weaker attraction between opposites. Remember, ***"Like attracts like"*** in polarity-based chromatography.

Figure 7, 8, and 9 display typical chromatographic polarity ranges for mobile phases, stationary phases, and sample analytes, respectively. Let's consider each in turn to see how a chromatographer chooses the appropriate phases to develop the attraction competition needed to achieve a polarity-based HPLC separation.

Figure 7: Mobile Phase Chromatographic Polarity Spectrum

A scale, such as that shown in Figure 7, upon which some common solvents are placed in order of relative chromatographic polarity is called an eluotropic series. Mobile phase molecules that compete effectively with analyte molecules for the attractive stationary phase sites displace these analytes, causing them to move faster through the column (weakly retained). Water is at the polar end of mobile-phase-solvent scale, while hexane, an aliphatic hydrocarbon, is at the non-polar end. In between, single solvents, as well as miscible-solvent mixtures (blended in proportions appropriate to meet specific separation requirements), can be placed in order of elution strength. Which end of the scale represents the 'strongest' mobile phase depends upon the nature of the stationary phase surface where the competition for the analyte molecules occurs.

Silica has an active, hydrophilic (water-loving) surface containing acidic silanol (silicon-containing analog of alcohol) functional groups. Consequently, it falls at the polar end of the stationary-phase scale shown in Figure 8. The activity or polarity of the silica surface may be modified selectively by chemically bonding to it less polar functional groups (bonded phase). Examples shown here include, in order of decreasing polarity, cyanopropylsilyl- (CN), n-octylsilyl- (C8), and n-octadecylsilyl- (C18, ODS)moieties on silica. The latter is a hydrophobic (water-hating), very non-polar packing.

Figure 8: Stationary Phase Particle Chromatographic Polarity Spectrum

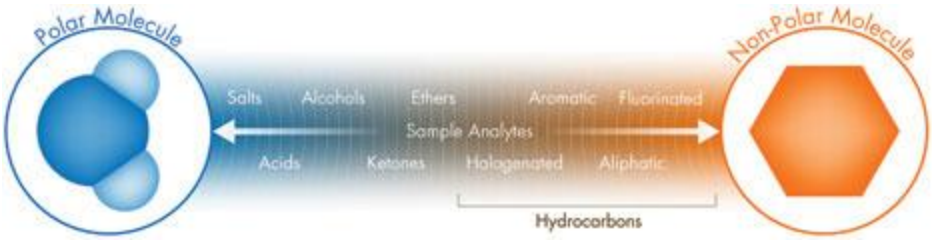

Figure 9: Compound/Analyte Chromatographic Polarity Spectrum

Figure 9 repeats the chromatographic polarity spectrum of the sample. After considering the polarity of both phases, then, for a given stationary phase, a chromatographer must choose a mobile phase in which the analytes of interest are retained, but not so strongly that they cannot be eluted. Among solvents of similar strength, the chromatographer considers which phase combination may best exploit the more subtle differences in analyte polarity and solubility to maximize the selectivity of the chromatographic system. Like attracts like, but, as you probably can imagine from the discussion so far, creating a separation based upon polarity involves knowledge of the sample and experience with various kinds of analytes and retention modes.

To summarize, the chromatographer will choose the best combination of a mobile phase and particle stationary phase with appropriately opposite polarities. Then, as the sample analytes move through the column, the rule like attracts like will determine which analytes slow down and which proceed at a faster speed.

1.5. Ultra performance liquid chromatography (UPLC):

Advances in instrumentation and column technology were made to achieve very significant increases in resolution, speed, and sensitivity in liquid chromatography. Columns with smaller particles (1.7 micron) and instrumentation with specialized capabilities designed to deliver mobile phase at 15,000 psi (1,000 bars) were needed to achieve a new level of performance. A new system had to be holistically created to perform ultra-performance liquid chromatography, now known as UPLC technology.

Basic research is being conducted today by scientists working with columns containing even smaller 1-micron-diameter particles and instrumentation capable of performing at 100,000 psi. This provides a glimpse of what we may expect in the future. UPLC refers to

Ultra Performance Liquid Chromatography. It improves in three areas: chromatographic resolution, speed and sensitivity analysis. It uses fine particles and saves time and reduces solvent consumption [20-24]. UPLC is comes from HPLC. HPLC has been the evolution of the packing materials used to effect the separation. An underlying principle of HPLC dictates that as column packing particle size decreases, efficiency and thus resolution also increases. As particle size decreases to less than 2.5µm, there is a significant gain in efficiency and it's doesn't diminish at increased linear velocities or flow rates according to the common Van Deemter equation [25]. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time) can be extended to new limits which is known as Ultra Performance.

The classic separation method is of HPLC (High Performance Liquid Chromatography) with many advantages like robustness, ease of use, good selectivity and adjustable sensitivity. Its main limitation is the lack of efficiency compared to gas chromatography or the capillary electrophoresis [26, 27] due to low diffusion coefficients in liquid phase, involving slow diffusion of analytes in the stationary phase. The Van Deemter equation shows that efficiency increases with the use of smaller size particles but this leads to a rapid increase in back pressure, while most of the HPLC system can operate only up to 400 bar. That is why short columns filled with particles of about 2µm are used with these systems, to accelerate the analysis without loss of efficiency, while maintaining an acceptable loss of load.

Resolution is increased in a 1.7 µm particle packed column because efficiency is better. Separation of the components of a sample requires a bonded phase that provides both retention and selectivity. Four bonded phases are available for UPLC separations: ACQUITY UPLC^{TM} BEH C18 and C8 (straight chain alkyl columns), ACQUITY UPLC BEH Shield RP18 (embedded polar group column) and ACQUITY UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C6 alkyl) [28]. Column chemistry provides a different combination of hydrophobicity, silanol activity, hydrolytic stability and chemical interaction with analytes.

ACQUITY UPLC BEH C18 and C8 columns are considered the universal columns of choice for most UPLC separations by providing the widest pH range. They incorporate trifunctional ligand bonding chemistries which produce superior low pH stability. This low pH stability is combined with the high pH stability of the 1.7 µm BEH particle to

deliver the widest usable pH operating range. ACQUITY UPLC BEH Shield RP18 columns are designed to provide selectivities that complement the ACQUITY UPLC BEH C18 and C8 phases.

ACQUITY UPLC BEH Phenyl columns utilize a trifunctional C6 alkyl tether between the phenyl ring and the silyl functionality. This ligand, combined with the same proprietary endcapping processes as the ACQUITY UPLC BEH C18 and C8 columns, provides long column lifetimes and excellent peak shape. This unique combination of ligand and endcapping on the 1.7 µm BEH particle creates a new dimension in selectivity allowing a quick match to the existing HPLC column. An internal dimension (ID) of 2.1 mm column is used. For maximum resolution, choose a 100 mm length and for faster analysis, and higher sample throughput, choose 50 mm column.

Half-height peak widths of less than one second are obtained with 1.7µm particles, which gives significant challenges for the detector. In order to integrate an analyte peak accurately and reproducibly, the detector sampling rate must be high enough to capture enough data points across the peak

The detector cell must have minimal dispersion (volume) to preserve separation efficiency. Conceptually, the sensitivity increase for UPLC detection should be 2-3 times higher than HPLC separations, depending on the detection technique.

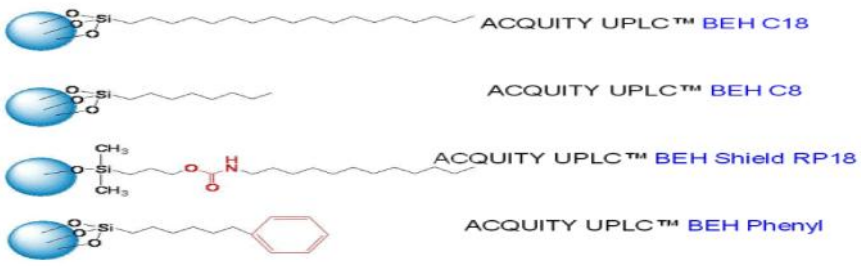

Figure 10: Different column chemistry available for UPLC

MS detection is significantly enhanced by UPLC; increased peak concentrations with reduced chromatographic dispersion at lower flow rates promote increased source ionization efficiencies.

The ACQUITY UPLC System consists of a binary solvent manager, sample manager including the column heater, detector, and optional sample organizer. The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient. There

are built-in solvent select valves to choose from up to four solvents. There is a 15,000-psi pressure limit (about 1000 bar) to take full advantage of the sub-2µm particles. The sample manager also incorporates several technology advancements. Using pressure assisted sample introduction, low dispersion is maintained through the injection process, and a series of pressures transducers facilitate self-monitoring and diagnostics. It uses needle-in-needle sampling for improved ruggedness and a needle calibration sensor increases accuracy. Injection cycle time is 25 seconds without a wash and 60 sec with a dual wash used to further decrease carry over. A variety of microtiter plate formats (deep well, mid height, or vials) can also be accommodated in a thermostatically controlled environment. Using the optional sample organizer, the sample manager can inject from up to 22 microtiter plates. The sample manager also controls the column heater. Column temperatures up to 65°C can be attained. To minimize sample dispersion, a "pivot out" design allows the column outlet to be placed in closer proximity to the source inlet of an MS detector [29].

For UPLC detection, the tunable UV/Visible detector is used which includes new electronics and firmware to support Ethernet communications at the high data rates. Conventional absorbance-based optical detectors are concentration sensitive detectors, and for UPLC use, the flow cell volume would have to be reduced in standard UV/Visible detectors to maintain concentration and signal. According to Beer's Law, smaller volume conventional flow cells would also reduce the path length upon which the signal strength depends. A reduction in cross-section means the light path is reduced, and transmission drops with increasing noise. Therefore, if a conventional HPLC flow cell were used, UPLC sensitivity would be compromised. The ACQUITY Tunable UV/Visible detector cell consists of a light guided flow cell equivalent to an optical fiber. Light is efficiently transferred down the flow cell in an internal reflectance mode that still maintains a 10mm flow cell path length with a volume of only 500mL. Tubing and connections in the system are efficiently routed to maintain low dispersion and to take advantage of leak detectors that interact with the software to alert the user to potential problems [30].

Advantage:

- Decreases run time and increases sensitivity.
- Provides the selectivity, sensitivity, and dynamic range of LC analysis.
- Maintaining resolution performance.
- Expands scope of Multiresidue Methods.

- UPLC's fast resolving power quickly quantifies related and unrelated compounds.
- Faster analysis through the use of a novel separation material of small particle size.
- Operation cost is reduced.
- Less solvent consumption.
- Reduces process cycle times, so that more product can be produced with existing resources.
- Increases sample throughput and enables manufacturers to produce more material that consistently meet or exceeds the product specifications, potentially eliminating variability, failed batches, or the need to re-work material [31, 32].
- Delivers real-time analysis in step with manufacturing processes.
- Assures end-product quality, including final release testing.

Disadvantages:

Due to increased pressure requires more maintenance and reduces the life of the columns of this type. So far performance similar or even higher has been demonstrated by using stationary phases of size around 2 μm without the adverse effects of high pressure. In addition, the phases of less than 2 μm are generally non-regenerable and thus have limited use [33, 34].

1.6. Application of chromatography:

In recent times chromatography is most widely used analytical tool for various applications. Hence it could not be possible for include all of it but some of very important and recently developed applications are discussed here.

1.6.1 Affinity chromatography: Clinical application

Affinity chromatography is a type of liquid chromatography that makes use of biological-like interactions for the separation and specific analysis of sample components. This work describes the basic principles of affinity chromatography and examines its use in the testing of clinical samples, with an emphasis on HPLC-based methods. Some traditional applications of this approach include the use of boronate, lectin, protein A or protein G, and immunoaffinity supports for the direct quantification of solutes. Newer techniques that use antibody-based columns for on- or off-line sample extraction are examined in detail, as are methods that use affinity chromatography in combination with other analytical methods, such as reversed-phase liquid chromatography, gas chromatography,

and capillary electrophoresis. Indirect analyte detection methods are also described in which immunoaffinity chromatography is used to perform flow-based immunoassays. Other applications that are include affinity-based chiral separations and the use of affinity chromatography for the study of drug or hormone interactions with binding proteins. Some areas of possible future developments are then considered, such as tandem affinity methods and the use of synthetic dyes, immobilized metal ions, molecular imprints, or aptamers as affinity ligands for clinical analyte [35].

1.6.2 Protein purification: Membrane chromatography

Adsorption chromatography is increasingly used for protein purification and medical applications. Synthetic membranes have advantages as support matrices in comparison to conventional bead supports because they are not compressible and they eliminate diffusion limitations. As a result, higher throughput and faster processing times are possible in membrane systems. In this paper, there are various applications of membrane chromatography by focusing on affinity, ion exchange, hydrophobic interaction, reversed-phase and multistage chromatography. The prospects of further development of membrane chromatography are massive [36].

1.6.3 Chromatographic and electrophoretic methodology to the speciation of organ mercury compounds in food analysis.

Trace metals such as mercury, especially its organic compounds, are an important risk to the environment and to man due to their accumulation in the food chain. For this reason, the routine determination of the very toxic methylmercury and of other organic and inorganic mercury compounds in marine and land animals, vegetables, fruits and fresh water is of increasing importance in health and environmental control programmes throughout the world. The majority of speciation methods for organomercurials involve a series of fundamental steps for the identification and quantification of samples of biological origin: extraction or isolation from the matrix; derivatisation and concentration; detection; separation of different species of interest and of interference. Each of these steps, as part of the chromatographic analysis of MeHg and of other organomercurials is revised using food samples [37].

1.6.4 Enantioselective chromatography as a alternative for the preparative of drug enentiomers:

The preparative separation of enantiomers by chromatography on chiral stationary phases (CSPs) has been recognized as being a useful alternative to the more conventional approaches such as enantioselective synthesis and enzymatically catalyzed transformations. The possible contribution of enantioselective chromatography with respect to the preparation of enantiomerically pure compounds is reviewed in the context of the competitive approaches and depending on the application scale, with a special emphasis on the recent progresses achieved in this particular field of separation [38].

1.6.5 Micro emulsion electro kinetic chromatography :

Compared to MEKC, the presence of a water-immiscible oil phase in the microemulsion droplets of microemulsion EKC (MEEKC) gives rise to some special properties, such as enhanced solubilization capacity and enlarged migration window, which could allow for the improved separation of various hydrophobic and hydrophilic compounds, with reduced sample pretreatment steps, unique selectivities and/or higher efficiencies. Typically, stable and optically clear oil-in-water microemulsions containing a surfactant (SDS), oil (octane or heptane), and cosurfactant (1-butanol) in phosphate buffer are employed as separation media in conventional MEEKC. However, in recent years, the applicability of reverse MEEKC (water-in-oil microemulsions) has also been demonstrated, such as for the enhanced separation of highly hydrophobic substances. Also, during the past few years, the development and application of MEEKC for the separation of chiral molecules has been expanded, based on the use of enantioselective microemulsions that contained a chiral surfactant or chiral alcohol.

On the other hand, the application of MEEKC for the characterization of the lipophilicity of chemical substances remains an active and important area of research, such as the use of multiplex MEEKC for the high-throughput determination of partition coefficients (log P values) of pharmaceutical compounds. Emphases are placed on the discussion of MEEKC in the separation of chiral molecules and highly hydrophobic substances, as well as in the determination of partition coefficients, followed by a survey of recent applications of MEEKC in the analysis of pharmaceuticals, cosmetics and health-care products, biological and environmental compounds, plant materials, and foods [39].

1.6.6 LC–MS in forensic & clinical toxicology In forensic & clinical toxicology:

Liquid chromatographic–mass spectrometric (LC–MS) procedures are available for the identification and/or quantification of drugs of abuse, therapeutic drugs, poisons and/or their metabolites in biosamples (whole blood, plasma, serum, urine, cerebrospinal fluid, vitreous humor, liver or hair) of humans or animals (cattle, dog, horse, mouse, pig or rat). This is relevant to clinical toxicology, forensic toxicology, doping control or drug metabolism and pharmacokinetics[40].

1.6.7 LC–MS to the characterization of food proteins and derived peptides :

This review describes the development of mass spectrometry off-line and on-line coupled with liquid chromatography to the analysis of food proteins. It includes the significant results recently obtained in the field of milk, egg and cereal proteins. This paper also outlines the research carried out in the area of food protein hydrolysates, which are important components in foodstuffs due to their functional properties. Liquid chromatography and mass spectrometry have been particularly used for the characterization of food peptides and especially in dairy products[41].

1.6.8 Affinity chromatography in proteomics :

Affinity chromatography is a powerful protein separation method that is based on the specific interaction between immobilized ligands and target proteins. Peptides can also be separated effectively by affinity chromatography through the use of peptide-specific ligands. Both two-dimensional electrophoresis (2-DE) - and non-2-DE-based proteomic approaches benefit from the application of affinity chromatography. Before protein separation by 2-DE, affinity separation is used primarily for preconcentration and pretreatment of samples. Those applications entail the removal of one protein or a class of proteins that might interfere with 2-DE resolution, the concentration of low-abundance proteins to enable them to be visualized in the gel, and the classification of total protein into two or more groups for further separation by gel electrophoresis. Non-2-DE-based approaches have extensively employed affinity chromatography to reduce the complexity of protein and peptide mixtures. Prior to mass spectrometry (MS), preconcentration and capture of specific proteins or peptides to enhance sensitivity can be accomplished by using affinity adsorption. Affinity purification of protein complexes followed by identification of proteins by MS serves as a powerful tool for generating a map of protein–protein interactions and cellular locations of complexes. Affinity chromatography

of peptide mixtures, coupled with mass spectrometry, provides a tool for the study of protein posttranslational modification (PTM) sites and quantitative proteomics.

Quantitation of proteomes is possible via the use of isotope-coded affinity tags and isolation of proteolytic peptides by affinity chromatography. An emerging area of proteomics technology development is miniaturization. Affinity chromatography is becoming more widely used for exploring PTM and protein–protein interactions, especially with a view toward developing new general tag systems and strategies of chemical derivatization on peptides for affinity selection. More applications of affinity-based purification can be expected, including increasing the resolution in 2-DE, improving the sensitivity of MS quantification, and incorporating purification as part of multidimensional liquid chromatography [42].

1.6.9 Clinical application of gas chromatography and gas chromatography-mass spectrometry of steroids:

This review article underlines the importance of gas chromatography–mass spectrometry (GC–MS) for determination of steroids in man. The use of steroids labelled with stable isotopes as internal standard and subsequent analysis by GC–MS yields up to now the only reliable measurement of steroids in serum. Isotope dilution GC–MS is the reference method for evaluation of routine analysis of serum steroid hormones. GC–MS is an important tool for detection of steroid hormone doping and combined with a combustion furnace and an isotope ratio mass spectrometer the misuse of testosterone by athletes can be discovered. Finally the so called urinary steroid profile by GC and GC–MS is the method of choice for detection of steroid metabolites in health and disease [43].

1.6.10 LC-MS/MS for the analysis of pharmaceutical residues in environmental samples:

Pharmaceutical residues are environmental contaminants of recent concern and the requirements for analytical methods are mainly dictated by low concentrations found in aqueous and solid environmental samples. This can be determined by LC-MS/MS. Pharmaceuticals included are antibiotics, non-steroidal anti-inflammatory drugs, β-blockers, lipid regulating agents and psychiatric drugs. This can deal by advanced aspects of current LC–MS/MS methodology [44].

1.6.11 Chromatography as a tool for selected antibiotic classes: Pharmacokinetic application:

The first antibiotic discovered, penicillin, appeared on the market just after the Second World War. Intensive research in subsequent years led to the discovery and development of cephalosporins, aminoglycosides, tetracyclines and rifamycin. The chemotherapeutic quinolones and the more recently discovered fluoroquinolones have added promising new therapeutic weapons to fight the microbial challenge.

The major role pharmacokinetics has played in developing these compounds should be highlighted. Plasma concentration–time profiles and the therapeutic activity evoked by these compounds allow the therapeutic window, doses and dose turnovers to be appropriately defined, as well as possible dose adjustment to be made in renal failure. The pharmacokinetics of antimicrobial agents was initially explored by using microbiological methods, but these lack specificity. The HPLC technique with UV, fluorimetric, electrochemical and, in some cases, mass spectrometry detection has satisfactorily solved the problem of antimicrobial agent assay for pharmacokinetic, bioavailability and bioequivalence purposes alike. Indeed, in these studies, plasma concentrations of the given analyte must be followed up for a period ≥3 times the half-life, which calls for specific sensitive assays [45].

1.6.12 LC-MASS Spectrometry in analytical toxicology:

Liquid chromatography-mass spectrometry (LC-MS), after long-term development that has introduced seven major interfacing techniques, is finally suitable for application in the field of analytical toxicology. Various compound classes can be analyzed, and sensitivities for more or less polar analytes that are as good as or better than those of gas chromatography-mass spectrometry can be obtained with modern interfaces. In addition, because ionization is often softer than classical electron impact, some LC-MS interfaces are able to handle fragile species that are otherwise not amenable to MS [46].

1.6.13 Determination of pesticide residue in bio sample:

The use of hyphenated technique, liquid chromatography/mass spectrometry (LC–MS) for the identification and quantification of pesticides and their metabolites in human biosamples are very well known. The first applications of LC–MS in this field began in the early 1990s. Since then, increasing interest has been shown in applying this powerful technique, with most applications dealing with the determination of a variety of

chemically diverse metabolites in urine. The use of different LC–MS interfaces and mass spectral detection modes are discussed. Special attention is given to tandem mass spectrometry (MS/MS) due to its inherent advantages of increased sensitivity and selectivity, as well as its advantages for identification and confirmation of analytes in samples [47].

1.6.14 Micellar electro kinetic capillary chromatography to the analysis of illicit drug seizures:

Micellar electrokinetic capillary chromatography (MECC) is used for the analysis of illicit drug seizures. Quantitative analysis of various drugs, including heroin, opium, cocaine, amphetamines, LSD and anabolic steroids are available. Due to its high efficiency, high selectivity and general applicability, MECC is well suited for forensic drug analyses [48].

1.6.15 Environmental analysis using multidimensional gas chromatography and comprehensive GC:

Developments in coupled-column gas chromatography methods for qualitative analysis of selected environmental toxicants such as dioxin, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), etc are easily available. In particular, the new technique of GC×GC will be introduced, and its role, and the promise it offers to this field is discussed. The benefits of enhanced separation to quantitative analysis are considered. In order to perform an accurate risk assessment, both the dose and biological effects of environmental toxicants have to be determined with a high degree of certainty. This is most often achieved by using chromatographic methods. Given the complexity of most environmental sample extracts, single-column gas chromatography is unable to fully resolve all the components of interest frequently leading to a positive bias in the reported concentrations. Advanced separation tools, such as multidimensional gas chromatography (MDGC), were investigated quite early and demonstrated improvements in separation[49].

1.6.16 The determination of amines by gas chromatography and their applications in environmental analysis:

The environmental analysis of amines is important to preserve human health because these compounds often have toxic effects. Gas chromatography (GC) of free amines is generally unsatisfactory owing to the adsorption and decomposition of the solutes on the

column. Derivatization of amines is employed to reduce the polarity and to improve the GC properties. Derivatization reactions for the determination of amines by GC could give the best results. Many other applications of chromatography as an important analytical tool are also available in literature. Only few of them are presented here, scientists are more concentrated on the unfolded area of chromatography and its applications. The vast area of chromatography is now the target for the analytical scientist to resolve the critical problems and also for the cost effective and hazardless analysis of different chemical entities [50].

1.7. Method Development:

Method development should be based on several considerations. It is preferable to have maximum sample information to make development fast and desired for intended analytical method application, physical and chemical properties are most preferable as primary information. Moreover, separation goal needs to define at beginning so; appropriate method can be developed for the purpose. An LC method development is very huge area for even pharmaceuticals with regulatory requirement of international standards. So, prior to method validation and usage at quality control many aspects need to focus as per ICH guideline. Method development can be based on sample and goal as well as available resources for chromatography but few basic steps for method development are can be discussed as below.

Steps in method development:

- Sample information, define separation goals
- Sample pretreatment, need of special HPLC procedure
- Selection of detector and detector settings
- Selection of LC method; preliminary run; estimate best separation conditions
- Optimize separation conditions
- Check for problems or requirement for special procedure
- Method validation

Sample information:

- Number of compounds present
- Chemical structure of compounds

- Chemical nature
- Molecular weight of compounds
- pKa Value(s) of compounds
- Sample solubility
- Sample stability and storage
- Concentration range of compounds in sample
- UV spectra of compounds or properties for detection of compounds

1.8. Method Validation:

A developed analytical method for routine use needs to be validated as per ICH guideline for following validation parameters based on its intended application [10, 11]:

Parameters of method development as per ICH Guide lines

- Specificity
- Linearity & Range
- Precision
    a. Method precision / Repeatability
    b. Intermediate precision / Ruggedness
- Accuracy / Recovery
- Robustness
- Solution stability
- System suitability

1.8.1 Specificity:

An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and the assay. The procedures used to demonstrate specificity will depend on the intended objective of the analytical procedure. It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte (complete discrimination). In this case a combination of two or more analytical procedures is recommended to achieve the necessary level of discrimination.

Identification:

Suitable identification tests should be able to discriminate between compounds of closely related structures which are likely to be present. The discrimination of a procedure may be confirmed by obtaining positive results (perhaps by comparison with a known

reference material) from samples containing the analyte, coupled with negative results from samples which do not contain the analyte. In addition, the identification test may be applied to materials structurally similar to or closely related to the analyte to confirm that a positive response is not obtained. The choice of such potentially interfering materials should be based on sound scientific judgment with a consideration of the interferences that could occur.

Assay and Impurity Test(s):

For chromatographic procedures, representative chromatograms should be used to demonstrate specificity and individual components should be appropriately labelled. Similar considerations should be given to other separation techniques. Critical separations in chromatography should be investigated at an appropriate level. For critical separations, specificity can be demonstrated by the resolution of the two components which elute closest to each other. In cases where a non-specific assay is used, other supporting analytical procedures should be used to demonstrate overall specificity. For example, where a titration is adopted to assay the drug substance for release, the combination of the assay and a suitable test for impurities can be used. The approach is similar for both assay and impurity tests:

Impurities are available:

For the assay, this should involve demonstration of the discrimination of the analyte in the presence of impurities and/or excipients; practically, this can be done by spiking pure substances (drug substance or drug product) with appropriate levels of impurities and/or excipients and demonstrating that the assay result is unaffected by the presence of these materials (by comparison with the assay result obtained on unspiked samples). For the impurity test, the discrimination may be established by spiking drug substance or drug product with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix.

Impurities are not available:

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure e.g.: Pharmacopeial method or other validated analytical procedure (independent procedure). As appropriate,

this should include samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis and oxidation.

- For the assay, the two results should be compared.
- For the impurity tests, the impurity profiles should be compared.

Peak purity tests may be useful to show that the analyte chromatographic peak is not attributable to more than one component (e.g., diode array, mass spectrometry).

1.8.2 Linearity:

A linear relationship should be evaluated across the range (see section 3) of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighings of synthetic mixtures of the drug product components, using the proposed procedure. The latter aspect can be studied during investigation of the range.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity. Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample. For the establishment of linearity, a minimum of 5 concentrations is recommended. Other approaches should be justified.

1.8.3 Range:

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the

specified range of the analytical procedure. The following minimum specified ranges should be considered:

- For the assay of a drug substance or a finished (drug) product: normally from 80 to 120 percent of the test concentration;
- For content uniformity, covering a minimum of 70 to 130 percent of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers), is justified;
- For dissolution testing: +/-20 % over the specified range;
  e.g., if the specifications for a controlled released product cover a region from 20%, after 1 hour, up to 90%, after 24 hours, the validated range would be 0- 110% of the label claim.
- For the determination of an impurity: from the reporting level of an impurity1 to 120% of the specification.

For impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the detection/quantitation limit should be commensurate with the level at which the impurities must be controlled.

Note: for validation of impurity test procedures carried out during development, it may be necessary to consider the range around a suggested (probable) limit; if assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities to 120% of the assay specification.

1.8.4 Accuracy:

Accuracy should be established across the specified range of the analytical procedure.

Assay of Drug Substance:

Several methods of determining accuracy are available:

- Application of an analytical procedure to an analyte of known purity (e.g. reference material).
- Comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined (independent procedure, see 1.2.).
- Accuracy may be inferred once precision, linearity and specificity have been established.

Assay of Drug Product:

Several methods for determining accuracy are available:

- Application of the analytical procedure to synthetic mixtures of the drug product components to which known quantities of the drug substance to be analyzed have been added.
- In cases where it is impossible to obtain samples of all 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 (independent procedure, see 1.2.).
- Accuracy may be inferred once precision, linearity and specificity have been established.

Impurities (Quantitation):

Accuracy should be assessed on samples (drug substance/drug product) spiked with known amounts of impurities. In cases where it is impossible to obtain samples of certain impurities and/or degradation products, it is considered acceptable to compare results obtained by an independent procedure (see 1.2.). The response factor of the drug substance can be used. It should be clear how the individual or total impurities are to be determined e.g., weight/weight or area percent, in all cases with respect to the major analyte.

Recommended Data:

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations/3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

1.8.5 Precision:

Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.

Repeatability

Repeatability should be assessed using:

- A minimum of 9 determinations covering the specified range for the procedure (e.g. 3 concentrations/3 replicates each) or
- A minimum of 6 determinations at 100% of the test concentration.

Intermediate Precision:

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. The use of an experimental design (matrix) is encouraged.

Reproducibility:

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. These data are not part of the marketing authorization dossier.

Recommended Data:

The standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision investigated.

1.8.6 Detection limit:

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

Based on Visual Evaluation:

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Based on Signal-to-Noise:

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and

establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Based on the Standard Deviation of the Response and the Slope:

The detection limit (DL) may be expressed as:

DL = 3.3 óS

where ó = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of s may be carried out in a variety of ways, for example:

Based on the Standard Deviation of the Blank:

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve:

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommended Data:

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification. In cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

1.8.7 Quantitation limit:

Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

Approaches other than those listed below may be acceptable.

Based on Visual Evaluation:

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on Signal-to-Noise Approach:

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Based on the Standard Deviation of the Response and the Slope:

The quantitation limit (QL) may be expressed as:

QL = 10 óS

Where ó = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of s may be carried out in a variety of ways for example:

Based on Standard Deviation of the Blank:

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve:

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommended Data:

The quantitation limit and the method used for determining the quantitation limit should be presented. The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantitation limit.

1.8.8 Robustness:

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variations are:

- stability of analytical solutions,
- extraction time

In the case of liquid chromatography, examples of typical variations are

- Influence of variations of pH in a mobile phase,
- Influence of variations in mobile phase composition,
- Different columns (different lots and/or suppliers),
- Temperature,
- Flow rate.

In the case of gas-chromatography, examples of typical variations are

- Different columns (different lots and/or suppliers),
- Temperature,
- Flow rate.

1.8.9 System suitability testing:

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. See Pharmacopoeias for additional information.

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