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A Review on High Performance Liquid Chromatography

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A B S T R A C T

The analytical technique of HPLC is used extensively throughout the pharmaceutical Industry. It is used to provide the information on the composition of drug related samples. It is widely used for the quantitative and qualitative analysis. HPLC is used at all the different stages in the creation of new drug, and also used during drug manufacture. The aim of the analysis will depend on both the nature of the sample and the stage of the development. HPLC is a chromatographic technique; therefore it is necessary to have a basic understanding of chromatography to understand how it works.

Keywords: HPLC, Quantitative, Qualitative analysis, Chromatographic technique, Development

A R T I C L E I N F O

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1. Introduction

High Performance Liquid Chromatography (HPLC)

In the modern pharmaceutical industry, high performance liquid chromatography is the major and integral analytical tool applied in all stages of drug discovery, development and production. Effective and fast method development is of paramount importance throughout this drug development life cycle. This requires a thorough understanding of HPLC principles and theory which lay a solid foundation for appreciating the many variables that are optimized during fast and effective HPLC method development and optimization[1]. Chromatographic separations are based on a forced transport of the liquid (mobile phase) carrying the

analyte mixture through the porous media and the differences in the interactions at analytes with the surface of this porous media resulting in different migration times for a mixture of components. High surface area of the interface between mobile and stationary phases is essential for space discrimination of different components in the mixture. Analyte molecules undergo multiple phase transitions between mobile phase and adsorbent surface. Average residence time of the molecule on the stationary phase surface is dependent on the interaction energy. For different molecules with very small interaction energy difference the presence of significant surface is critical since the higher the number of phase transitions that analyte molecules undergo while moving through the chromatographic column, the higher the difference in their retention.

1.1 Advantages and Limitations

Table 1 highlights the advantages and limitations of HPLC. HPLC is a premier separation technique capable of multi component analysis of real-life samples and complex mixtures. Few techniques can match its versatility and precision of 0.5% relative standard deviation (RSD). HPLC is highly automated, using sophisticated auto samplers and data systems for unattended analysis and report generation. A host of highly sensitive and specific detectors extend detection limits to nanogram, picogram, and even femtogram levels. As a preparative technique, it provides quantitative recovery of many labile components in milligram to kilogram quantities.

Advantages and Limitations of HPLC

Advantages

- Rapid and precise quantitative analysis
- Automated operation
- High-sensitivity detection
- Quantitative sample recovery
- Amenable to diverse samples

Limitations

- No universal detector
- Less separation efficiency than capillary GC
- More difficult for novices

1.2. Types of HPLC Techniques

Based on modes of chromatography

- Normal phase chromatography
- Reverse phase chromatography

Based on principle of separation

- Adsorption chromatography
- Ion exchange chromatography
- Size exclusion chromatography
- Affinity chromatography
- Chiral phase chromatography

Based on elution technique

- Isocratic separation
- Gradient separation

Based on the scale of operation

- Analytical HPLC
- Preparative HPLC

Table 2: Various types and applications of HPLC

Type	**Sample Polarity**	**Molecular weight range**	**Stationary Phase**	**Mobile Phase**
Adsorption	non-polar to somewhat polar	10^0 - 10^4	silica or alumina	non-polar to polar
Partition (reversed-phase)	non-polar to somewhat polar	10^0 - 10^4	non-polar liquid adsorbed or chemically bonded to the packing material	relatively polar
Partition (normal phase)	somewhat polar to highly polar	10^0 - 10^4	highly polar liquid adsorbed or chemically bonded to the packing material	relatively non-polar
Ion Exchange	highly polar to ionic	10^0 - 10^4	ion-exchange resins made of insoluble, high-molecular weight solids functionalized typically with sulfonic acid (cationic exchange) or amine (anionic exchange) groups	aqueous buffers with added organic solvents to moderate solvent strength
Size-Exclusion	non-polar to ionic	10^3 – 10^6	small, porous, silica or polymeric particles	polar to non-polar

1.3 Modes of Separation in HPLC

Principle

The principle of separation in normal phase mode and reverse phase mode is adsorption. When mixtures of components are introduced in to a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent travels slower. The component which has less affinity towards the stationary phase travels faster. Since no two components have the same affinity towards the stationary phase, the components are separated[2].

Normal Phase Chromatography

In normal phase chromatography, the stationary phase is a polar adsorbent and the mobile phase is generally a mixture of non-aqueous solvents. The silica structure is saturated with silanol groups at the end. These OH groups are statistically disturbed over the whole of the surface. The silanol groups represent the active sites (very polar) in the stationary phase. This forms a weak type of bond with any molecule in the vicinity when any of the following interactions are present.

- Dipole-induced Dipole
- Dipole-Dipole
- Hydrogen bonding
- π-Complex bonding

These situations arise when the molecule has one or several atoms with lone pair electron or a double bond. The absorption strengths and hence k' values (elution series) increase in the following order. Saturated hydrocarbon < olefins < aromatics < organic halogen compounds < sulphides< ethers < esters < aldehydes and ketones < amines < sulphones < amides < carboxylic acids. The strength of interactions depends not only on the functional groups in the sample molecule but also on steric factors. If a molecule has several functional groups, then the most polar one determines the reaction properties. Chemically modified silica, such as the aminopropyl, cyanopropyl and diol phases are useful alternatives to silica gel as stationary phase in normal phase chromatography. The aminopropyl and cyanopropyl phases provide opportunities for specific interactions between the analyte and the stationary phases and thus offer additional options for the optimizations of separations. Other advantages of bonded phases lie in their increased homogeneity of the phase surface[3]. Resolution with water in weak mobile phase may be most conveniently achieved by drying the solvents and then adding a constant concentration of water or some very polar modifier such as acetic acid or trietylamine (TEA) to the mobile phase. The addition of such polar modifiers serves to deactivate the more polar shape as well as the reproducibility of the retention times.

2. Reversed Phase Chromatography

In 1960's chromatographers started modifying the polar nature of silanol group by chemically reacting silica with organic silanes. The objective was to make less polar or non polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica is now reversed i.e. it is non-polar or the nature of the phase is reversed. The chromatographic separation carried out with such silica is referred to as reversed- phase chromatography[4].

1.4 Instrumentation

A large number of chemically bonded stationary phases based on silica are available commercially. Table 1 lists some of the functional groups bonded in chemically modified silica. Silica based stationary phases are still most popular in reversed phase chromatography however other absorbents based on polymer (styrene-divinyl benzene copolymer) are slowly gaining ground. Simple compounds are better retained by the reversed phase surface, the less water- soluble (i.e. the more non-polar) they are.

The retention decreases in the following order: aliphatics > induced dipoles (i.e. CCl_4) > permanent dipoles (e.g.CHC_{l3}) > weak lewis bases (ethers, aldehydes, ketones) > strong lewis bases (amines) > weak lewis acids (alcohols, phenols) > strong lewis acids (carboxylic acids). The retention also increases as the number of carbon atoms increases. As a general rule, the retention increases with increasing contact area between sample molecule and stationary phase i.e. with increasing number of water molecules, which are released during the adsorption of a compound. Branched chain compounds are eluted more rapidly than their corresponding normal isomers.

In reversed phase systems the strong attractive forces between water molecules arising from the 3-dimensional inter molecular hydrogen bonded network, from a structure of water that must be distorted or disrupted when a solute is dissolved. Only higher polar or ionic solutes can interact with the water structure. Non- polar solutes are squeezed out of the mobile phase and are relatively insoluble in it but with the hydrocarbon moieties of the stationary phase.

Chemically bonded octadecyl silane (ODS) an alkaline with 18 carbon atoms it is the most popular stationary phase used in pharmaceutical industry. Since most pharmaceutical compounds are polar and water soluble, the majority of HPLC methods used for quality assurance, decomposition studies, quantitative analysis of both bulk drugs and their formulations use ODS HPLC columns.

The solvent strength in reversed phase chromatography is reversed from that of adsorption chromatography (silica gel) as stated earlier. Water interacts strongly with silanol groups, so that, adsorption of sample molecules become highly restricted and they are rapidly eluted as a result. Exactly opposite applies in reversed phase system; water cannot wet the non-polar (hydrophobic) alkyl groups such as C_{18} of ODS phase and therefore does not interact with the bonded moiety. Hence water is the weakest solvent of all and gives slowest elution rate. The elution time (retention time) in reversed phase chromatography increases with increasing amount of water in the mobile phase[5].

Minimum requirement for HPLC Temperature

Room temperature is the first choice. Elevated temperatures are sometimes used to reduce column pressure to enhance selectivity. Typically, temperatures in excess of 60^{o}C are not used.

Retention time mechanism

In general, HPLC is a dynamic adsorption process. Analyte molecules, while moving through the porous packing bed, tend to interact with the surface adsorption sites. Depending on the HPLC mode, the different types of the adsorption forces may be included in the retention process

- Hydrophobic (non-specific) interactions are the main ones in Reversed-Phase separations.
- Dipole-dipole (polar) interactions are dominated in normal phase mode.
- Ionic interactions are responsible for the retention in ion-exchange Chromatography.

All these interactions are competitive. Analyte molecules are competing with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface and the weaker the eluent interaction, the longer analyte will be retained on the surface [6].

The instrument consists of

a. Reservoir or eluent containers for the mobile phase
b. A Pump to move the eluent and sample through the system
c. Solvent Degassing system
d. An Injection device to allow sample introduction
e. A Column to provide solute separation
f. A Detector to visualize the separated components
g. A waste container for the used solvent and
h. A data collection device to assist in interpretation and storage of results.

The Pump, Injector, Column, and Detector connected with tubing of narrow inner diameter. The inner diameter of the tubing that are used between the column and the detector must be as narrow as possible (0.010 inch or less for analytical work) to minimize band broadening. The choice of detector is based on intrinsic properties of the solute. Often more than one detector can be used to minimize sample information and confirm peak identities. For example, an absorbance detector could be placed in series in a conductivity detector for the visualization of charged, chromatographic solute[7]. Basic components of the HPLC are shown in the schematic diagram and presented in Fig: 2.

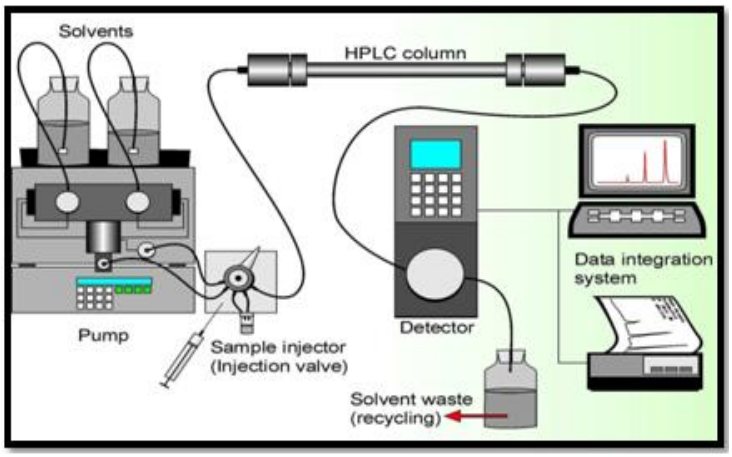

Figure 2: Schematic diagram of HPLC

H. Reservoirs:

The solvent reservoirs are glass or stainless steel containers capable of holding out one to five liters of mobile phase. The reservoir has special caps, Teflon tubing and filters. In HPLC the mobile phase can be an aqueous- organic mixture or buffer solution or a mixture of organic solvents. The mobile phase is pumped under pressure from one or several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a high- pressure drop across a chromatography column. Eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase separations eluting power increases with increasing polarity of solvent but for reversed phase separations, eluting power decreases with increasing solvent polarity. Optimum separating conditions can be achieved by making use of mixture of two solvents. Some other properties of the solvents, which need to be considered for a successful separation, are boiling point, viscosity, detector compatibility, flammability and toxicity[8].

I Pumps:

The pumping systems used in HPLC can be categorized in three different ways. The first classification is according to the eluent flow rate that the pump is capable of delivering. The second classification is according to the construction materials, and the final classification is according to the mechanism by which the pump delivers the eluent. Each of these classifications is depicted below.

Table. 3: List of mobile phase solvents

Solvent	MW	BP (°C)	RI (25° C)	UV[a] cut off (nm)	Density g/ml (25°C)	Viscosity (25° C)	Dielectric Constant
ACN	41.0	82	1.342	190	0.787	0.358	38.8
Dioxane	88.1	101	1.420	215	1.034	1.26	2.21
Ethanol	46.1	78	1.359	205	0.789	1.19	24.5
Ethylacetate	88.1	77	1.372	256	0.901	0.45	6.02
Methanol	32.0	65	1.326	205	0.792	0.584	32.7
CH_2Cl_2	84.9	40	1.424	233	1.326	0.44	8.93
Isopropanol	60.1	82	1.375	205	0.785	2.39	19.9
n-propanol	60.1	97	1.383	205	0.804	2.20	20.3
THF	72.1	66	1.404	210	0.889	0.51	7.58
Water	18.0	100	1.333	170	0.998	1.00	78.5

Pump Classification According to Flow Rate

Figure 3: Classification of pumps according to flow rate

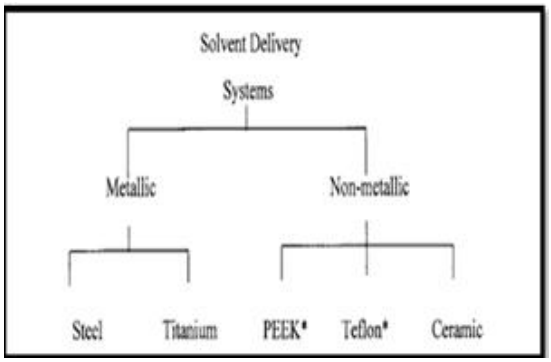

Figure 4: Classification of pumps according to materials of construction

Pump Classification According to Materials of Construction:

Pump Classification according to Mechanism of Eluent Displacement:

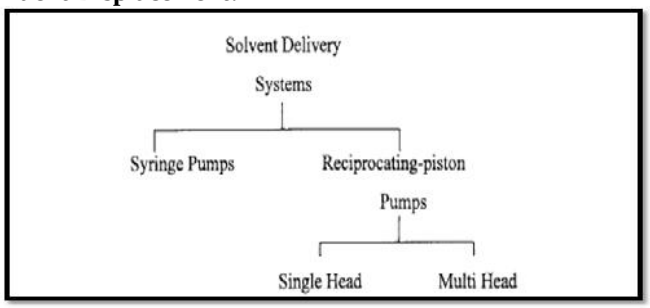

Figure 5: Classification of pumps according to mechanism of eluent displacement

Figure 6: Syringe-Type pumps

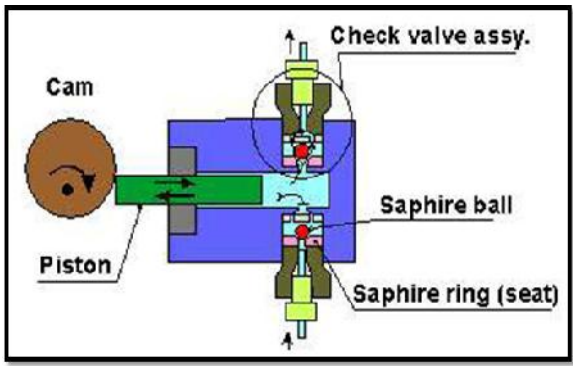

Figure 7: Reciprocating-piston pump

There are two types of elution: Isocratic and Gradient

Isocratic: In this, constant eluent composition is pumped through the column during the whole analysis.

Gradient: In this, eluent composition (and strength) is steadily changed during the run.

iii. Solvent Degassing System

The constituents of the mobile phase should be degassed and filtered before use. Several methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45µ filters, vacuum degassing with an air- soluble membrane, helium purging, ultra-sonication or purging or combination of these methods. HPLC systems are also provided an online degassing system, which continuously removes the dissolved gases from the mobile phase[9].

iv. Sample Injector:

Two means for analyte introduction in the column are injection into a flowing stream and a stop flow injection. These techniques use a syringe or an injection valve. Automatic injector is a microprocessor- controlled version of a manual universal injector. Usually, up to 100 samples can be loaded into the auto injector tray. The system parameters such as flow rates, gradient, run time, volume to be injected etc., are chosen, stored in memory and sequentially executed on consecutive injections.

Figure 8: Auto Sampler for Injection

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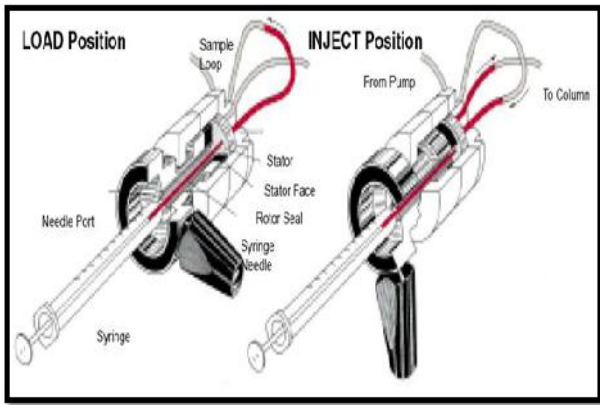


Figure 9: Manual Injector Valve

v. Columns:

The heart of the system is the column. Many different reverse phase columns will provide excellent specificity for any particular separation. It is therefore best to routinely attempt separations with a standard C-8 or C-18 column (e.g. Zorbax SB C-8, YMC ODS Pack AQ - C_8) and determine if it provides good separations. If this column does not provide good separation or the mobile phase is unsatisfactory, alternate methods or columns should be explored. Reverse phase column differ by the carbon chain lengths, degree of end capping and percent carbon loading. Diol, Cyano and amino groups can be used for Reverse Phase Chromatography.

- Guard column
- Analytical column
- Micro column
- Open tubular micro columns.

vi. Detector:

The purpose of the detector is to monitor the eluent coming out of the column. Generally two types of detectors are used in HPLC there are bulk property and solute property detectors.

- **Bulk property detectors**
  These detectors are based on differential measurement of a property, which is common to both the sample and the mobile phase. Examples of such detectors are refractive index, conductivity and dielectric constant detectors.
- **Solute property detectors**
  Solute property detectors respond to a physical property of the solute, which is not exhibited by the pure mobile phase.

These detectors measure a property, which is specific to the sample, either with or without the removal of the mobile phase prior to the detection. Solute property detectors which do not require the removal of the mobile phase before detection include spectrophotometric (UV and UV-VIS) detector, fluorescence detectors, polarographic, electro-chemical and radioactivity detectors, while the moving wire flame ionization detector and electron capture detector, UV-VIS and fluorescent detectors are suitable for gradient elution, because many solvents used in HPLC do not absorb to any significant extent[10].

Role of the Column

The HPLC column is the heart of the method, which plays a critical role in the separation. The column must possess the selectivity, efficiency, and reproducibility to provide a good separation. All of these characteristic are dependent on the column manufacture, production of good quality columns and packing materials.

Role of Flow Rate

The slower flow rate will also decrease the column back pressure. The disadvantage is that when flow rate is decreased to increase the resolution slightly, there is a corresponding increase in the run time.

Role of Temperature

While temperature is a variable that can affect selectivity (a), its effect is relatively small. Also, the *k* ' generally decreases with an increase in temperature for neutral

compounds but less dramatically for partially ionized analytes. An increase of 1º C will decrease the *k* ' by 1 to 2 %, and both ionic and neutral samples.

Role of pH

pH is another factor in the resolution equation that will affect the selectivity of the separation. Thus when an acid (HA) or base (B) is ionized (converted from the unionized free acid or base) it becomes more hydrophilic (less hydrophobic, more soluble in the aqueous phase) and less interactive with the column's binding sites.

$HA \quad « \quad H^+ + A^-$

$B + H^+ \quad « \quad BH^+$

Hydrophobic Hydrophilic

(More retained on column) (Less retained on column)

vii. Recorder

Recorders are used to record the responses obtained from detector after amplification. They record the base line and all the peaks obtained with respect to time.

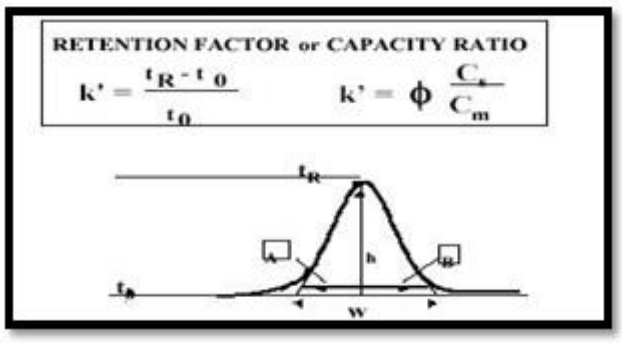


Figure 10: Capacity Factor

1.5 HPLC Instrumental Parameters

- Resolution (R_s)
- Capacity factor (k')
- Selectivity (a)
- Column efficiency (N)
- Peak asymmetry factor (A_f)

Resolution (R_s)

Resolution is the parameter describing the separation power of the complete chromatographic system relative to the particular components of the mixture. The resolution (R_s), of two neighboring peaks is defined as the ratio of the distance between two peak maxima (Fig. 10). It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_s is 1.5. It is calculated by using the formula,

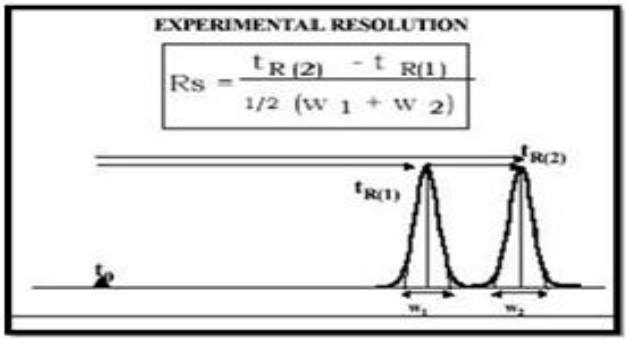


Figure 11: Resolution between two peaks

Where,

$t_{R(1)}$ and $t_{R(2)}$ are the retention times of components 1 and 2 and

W_1 and W_2 are peak width of components 1 and 2.

(b) Capacity Factor (k')

Capacity factor is the ratio of the reduced retention volume to the dead volume (Fig. 11). Capacity factor (k'), is Where,

t_R = retention volume at the apex of the peak (solute) and

t_0 = void volume of the system.

(c) Selectivity (a)

The selectivity (or separation factor, a) is a measure of relative retention of two components in a mixture. Selectivity is the ratio of the capacity factors of both peaks, and the ratio of its adjusted retention times (Fig. 12). Selectivity represents the separation power of particular adsorbent to the mixture of these particular components. This parameter is independent of the column efficiency; it only depends on the nature of the components, eluent type, and eluent composition, and adsorbent surface chemistry. In general, if the selectivity of two components is equal to 1, then there is no way to separate them by improving the column efficiency.

The ideal value of 'a' is 2. It can be calculated by using formula,

$$a = V_2 - V_1 / V_1 - V_0 = k_1' / k_2'$$

defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factor is a measure of how well the sample molecule is retained by a column during an isocratic separation. The ideal value of k' ranges from 2-10. Capacity factor can be determined by using the formula,

Where,

V_0 is the void volume of the column,

V_1 and V_2 are the retention volumes of the first and the second peak respectively.

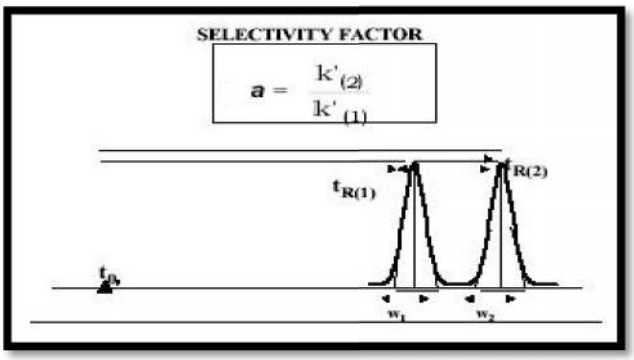


Figure12: Selectivity

(d) Column Efficiency / Band broadening (N)

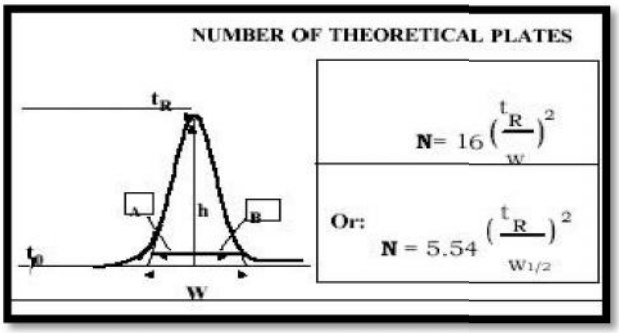


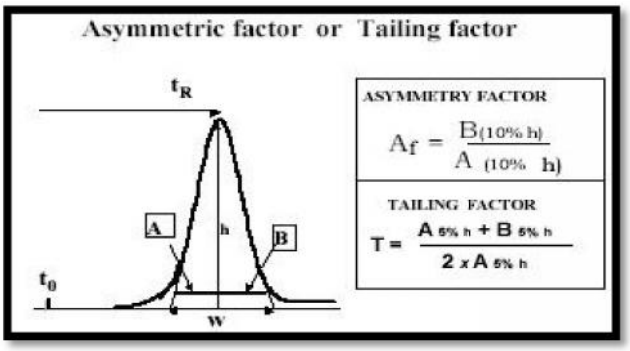
Figure 13: Number of Theoretical Plates

Efficiency (N), of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Similar the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 5,000 to 100,000 plates / meter are ideal for a good system. Efficiency is calculated by using the formula.

Where,

t_R is the retention time and W is the peak width.

(e) Peak asymmetry factor (A_f): Peak asymmetry factor (A_f), can be used as a criterion of column performance. The peak half width, b, of a peak at 10 % of the peak height, divided by the corresponding front half width, a, gives the asymmetry factor (Fig. 14).



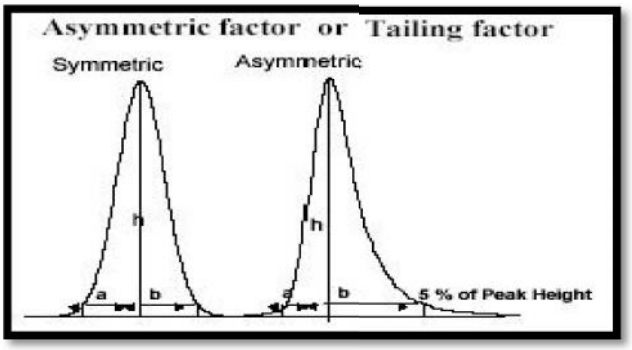


Figure 14: Asymmetric Factor

For a well packed column, an asymmetry factor of 0.9 to 1.1 should be achievable.

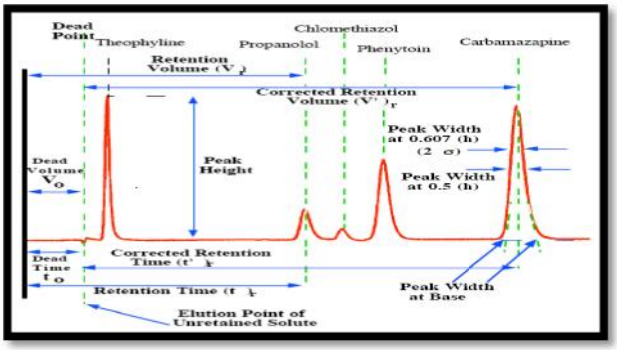


Figure 15: Nomenclature of Chromatogram

1.6. Applications of HPLC[11]

1) Preparative HPLC

For this purpose a clean peak of known sample assay has to be observed from the chromatogram. Selection of column, mobile phase and flow rate matter to certain level in this process by comparing with reference compound does identification and it can be assured by combining two or more detection methods.

5) Quantification

It is the analyte confirmation by using the known reference standards. Quantification of known and unknown areas with respect to the principal peak by various methods like

- Area normalization method.
- Internal standard method

It refers to the process of isolation and purification of compounds. Importance is the degree of solute purity and the throughput, which is the amount of compound produced per unit time.

2) Analytical HPLC

Here the focus is to obtain information about the sample compound, which includes relative comparison, quantification and resolution of a compound.

3) Chemical Seperation

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

4) Identification

- External standard method.

a) HPLC is used to estimate the concentration (potency) of standard as well as dosage formulation method.

b) Half peak height method

Diffusion of sample drugs, to and from interaction sites (adsorbents) in column is minimized by 2 criteria.

1. Finely divided spherical particles in packed column allow optimum homogeneity and packing density.
2. Stationary liquid phase should be in thin film with no stagnant pools.

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