CHAPTER-1

PREAMBLE

1.1. INTRODUCTION

Now a day’s many pharmaceutical products are came into market for existences since the quality of the product is the major concern, How ever the Quality is major factors which contribute the quality mainly of the products which are responsible for the directly or indirectly to the safety, efficacy or acceptability of a products. But now a days the Pharmaceutical industries were highly scrutiny under the government regulatory bodies and public groups to truncate the costs and to persistently deliver the safe and efficacious products in market. However quality is major concern for the marketed drug products and become a major focus for both industry and regulatory authorities.

Pharmaceutical companies are most scientifically regulated because the drugs and their products has direct impact on lives. To maintain the quality is major concern because it used to treat patients, In this regard they have to undergo some strict concern measurements in order to maintain quality of products. Hence the product is tested in laboratories which ensure the quality of products. By this it cannot be certify the quality of product else it have to meet certain standard regarding the analytical/ testing labs. Which are recognized in world such as International Standards Organization (ISO) and also in within country National Good Laboratory Practices Compliance Monitoring Authority (NGCMA), National Accreditation Board for Laboratories (NABL).

Analysis of drugs is done which is obtained either from synthetic or natural source is to evaluate purity of drug, and also to determine the composition is it’s concerned with their chemicals characterization of samples are done by both the quantitatively and qualitatively. In the recents years a many formulation came into existences either in single or multi–component formulations (Frederick William field, 2000)

In order to maintain quality control there is an tremendously increasing the load on analytical department. Mean while an significance improvement occurred in analytical techniques.

Analytical chemistry deals with separation, quantification and identification of the chemical components either natural or artificial materials.
In general analysis is divided into two major parts:
Qualitative analysis is an important step in spectroscopic analysis used to determine the presence or absence of components of mixture. Mostly used tool for separation, is used comparison either on height or area of the analytical peak with the one or more standard. It also used to determine by using analysis based on peak height, peak areas, calibration internal standard method and area normalization method (Skoog, Holler, Crouch, 2012)
Qualitative analysis is used to the determination of their identifications of elementes, specie and/or compouends present in the sample. Qualitative inorganic analysis determines the element present in a sample. Qualitative organic analysis is to determines their presences of their functional group in sample. Is the used to determination of the total or reltaive amouents of elements, specie either in their compound detected in its given sample.
Types of pharmaceutical analysis (David C, 2003)

Qualitative analysis
- Identification test
- Limit test

Quantitative analysis

Chemical methods
- Spectroscopic methods
- Volumetric or Titrimetric methods
- Gravimetric method
- Gasometric analysis

Microbiological methods
- Identification test
- Limit test

Instrumental methods
- Chromatographic methods
  - GC
  - HPLC
- Hyphenated methods
  - GC-MS
  - ICP-MS
  - GC-IR
- Spectroscopic methods
  - Ultraviolet and visible spectrophotometry
  - Infrared spectrophotometry
  - Fluorescence
  - Atomic spectroscopy
  - X-ray spectroscopy
  - Radiochemical techniques
  - NMR spectroscopy
- Electrochemical methods
  - Voltammetry
  - Electrogravimetry
  - Amperometric techniques
  - Potentiometry
  - Conductometry

Miscellaneous methods
- Thermal analysis
- MS
- Kinetic analysis
1.1.2 INTRODUCTION TO CHROMATOGRAPHY (A.H Beckett, J.B Stenlake, 2007)

The chromatography first used by a Russian Chemist and botanist Michele Tswett (1872-1919) The term derived from Greek for colour – Chroma, and write – graphein for separation of coloured plant pigments into colour bands passing through a column of chalk and other material such as polysaccharides, sucrose and insulin (Anand SK, Chatwal GR, 2002)

CHROMATOGRAPHY PRINCIPLE (Andrea Weston and Phyllis R. Brown, 1997)

This is a technique which is used to separate the component are distributed in across the two equivalents phases i.e. stationary phases is of solid or liquid supported on a solid or a gel which is packed into a column while sample moves in along with mobile phase is liquid or gas.

Showing flow chart for classification of chromatography (Yuri Kazakevich, 2007)

---

**Principles and classification of chromatography**

---

METHODS IN CHROMATOGRAPHY

---
1. According to nature of stationary and mobile phase
   - Solid- Liquid chromatography
   - Liquid-Liquid chromatography
   - Solid-gas chromatography
   - Liquid-gas chromatography

2. According to principle of separation
   A. Adsorption chromatography
      - Solid- Gas chromatography
      - Thin layer chromatography
      - Column chromatography
      - High performance liquid chromatography
      - Affinity phase chromatography
      - Hydrophobic Interaction chromatography (HIC)
   B. Partition chromatography
      - Gas liquids chromatography
      - Paper partition chromatography
      - Column partition chromatography

3. Based on modes of the chromatography
   - Normal phase chromatography
   - Reversed phase chromatography

4. Other types of chromatography
   - Size exclusion chromatography (SEC)
   - Gel permeation chromatography
   - Gel chromatography
   - Gel Filtration
   - Gel permeation chromatography
   - Ion exchange chromatography
   - Chiral chromatography

Table-1
**ADSORPTION CHROMATOGRAPHY**
This method involves separation of samples based on the adsorption affinities of components on active solid. In which the analyte interact with solid surface elute on active sites on surface.

**PARTITION CHROMATOGRAPHY**
This method based on distribution of analytes between two liquid phases. Based on polarities of stationares and mobile phase, it is of divided in to normal phase and reverse phase chromatography. In normal’s phasa chromatography the stationery phasa is strongly polar (ex-. Silica gel) and the mobiel phasa is non-pollar (ex.g. n-hexane). Polar sample retained on column. Revere phase chromatography, and stationery phases is non-poler, the moblie phasa is poler. The non poler compounds will retain.

**SIZE-EXCLUSION CHROMATOGRAPHY**
In this the stationery phase is solid constists of definite pore size. The solid samples move accordingly to their molecular size, the larger molecule will retain on surface, which elute first.

**ION’S- EXCHANGE CHROMOTOGRAPHY**
It includes a solid stationery phase with contains either of the anion or cation groupes on the surfaces to separation, HPLC and HPTLC methods have widely been exploited in pharmaceutical analysis because of its simplicity, precision, accuracy and reproducibility of result.

The acronym HPLC, coined by the Late Prof. Csaba Horvath for his 1970 Pittcon paper, originating the fact about that high force of pressure is required for flow of liquid chromatography in a packed columns. Initially at the beginning pump has only have a pressure capability of 500 psi [35 bars], therefore it is called high pressure liquid chromatography, or HPLC. The early 1970s which it was saw an tremendous leap in technology. The new HPLC instruments could develop up to 6,000 psi [400 bars] of pressure, which is incorporated imporved injectors, detectars, and columnes. Which upon streaming continues advancedes in its performance during this time [smallar particles, even with highar pressure] the acronym HPLC remained the same, but the name hase been changed to high performance liquid chromatography. And to the this coloum the solids stationery phase is filled in a coloum and at the end which is fixed to the pressure liquid mobile phase usually stationary phase is of (3-50µm) of particle size attached to a coloum of a very fine pore(2-5mm) and the other end is fix to the a source of a liquid pressure elutes as moblie phase.

High Performance Liumid Chromotography is the most widely used in analyetical chemistry. Its used to saparate, identeify, quantitative determination of the compouneds. Even the sample in a trace amount i.e. concentrations low amount of parts per treillion (ppt) could be easily identified. With using HPLC we could identify any sample, such as pharmaceutikals, foods, nutraceutickals, cosmetices, environmenttal matrices, forenic samples, and industrial chemicals. To characterize metabolites and also in assay active ingredients, impurities, degradation products and in dissolution assays. In pharmacodynamics and pharmacokinetic studies.
High-Performance Liquid Chromatography

ADVANTAGES:
It can perform more analysis at a time.
It shows broad sensitivity hence we can use various types of detectors.
It process wide resolution which make use of variety of stationary phases
Columns can be Reusable; even though it is expensive can be used for several times of analysis.
It can even use for substances at low viscosity.
Sample recovery can be done easily and easy to maintain, handling.
It shows Precise and reproducible
It can calculate even in integrator also.

DISADVANTAGES:
It is highly expensive which have a major effort on laboratories investments.
It requires expert to handle the instrument.
Gas samples cannot be handle.
Sample preparation is often required.

Figure-1

1 = eluent reservoir
2 = filter
3 = high pressure pump with pulse dampener
4 = pressure gauge
5 = sample injection valve with syringe
6 = column oven
7 = guard column
8 = column
9 = detector
10 = recorder (integrator, PC etc.)
Different HPLC techniques (Sahajwalla CG, 2004)

**Base on modes of disjunction**
- Normal phase chromatography
- Reverse phase chromatography

**Base on principal of disjunction**
- Adsorption chromatography
- Partition chromatography
- Ion exchange chromatography
- Ion pair chromatography
- Size exclusion or Gel permeation chromatography
- Affinity chromatography

**Base on its elution technique**
- Isocratic elution
- Gradient elution

**Base on their scale of operation**
- Analytical’s HPLC
- Preparative’s HPLC

**Based on types of analysis**
- Qualitative analysis
- Quantitative analysis.

**Normal-Phase Chromatography (NP-HPLC) (G. Vidya Sagar. 2005)**

In this it shows the strength of interaction of polar compounds of in which the analytes of the combinations of the stationery phase. The stronger the polar analyte in stationery phases interaction it shows their longer its analyte retention, as analyte molecules which is consists of the mobile-phase of molecules at the adsorption sites on to the surface of the stationary phase. If at all the mobile phase shows their covalent interactions with’s the stationary phase then its minimizes the analyte retention.

Mobile phases here are nonpolar solvents like (hexane, heptane, etc.) which upon addition with small portion its modifier the polar (i.e., methanol, ethanol). And the Packing material which are usually used are porous oxides such as silica and the alumina. If at all OH functional group is used its make to modify the
which surfaces to be high polar. And also we can use some modified chemical stationary phases such as Silica modified with trimethoxy glycidoxypropyl silanes is an packaging material with decreased surface polarity. Generally this method is choice for highly hydrophobic molecules those which are practically poorly soluble in polar or aqueous solvents.

Normal-Phase Chromatography

Reversed Phase Chromatography (RP-HPLC) (Sandie Lindsay, 1992)

In 1960s, then after they began to change or modified the chemical natures of the polar natures of the silanols groups making reacting with silicon with organics silanes.

The main aim of these are modifying the silica group to lesser polar or non polar by using polar solvents which enhances the separations of water solvable polar molecules.

In such conditions if the nature of silica is chemically reverted to its phases then the chromatographic separation is done by using silica which is know as reversed-phase chromatography.

The mostly wide used are octadecyl silane (ODS) and alkane its c18 which is most widely used as the stationary phase in the pharma industries.

Since the many of the compounds which are using in the pharmaceutical have been polar in nature and water soluble.

Since the water makes to interacts with the silanol groups, such that the adsorption of the sample it will be inherent to elute rapidly. Precisely happen reverse is applied in reversed phase system, such that water will not come into contact with the non-polar (hydrophobic) alkyl group is C18 of ODS phase and in this conditions there will not occurs a interaction with molecules which raises the slow elution. The time of elution the reversed phase chromatography will be enhances with increasing amount of water in the mobile phase.
High-Pressure-Isocratic System

In this condition in which the mobile phases remain unchanged through over the disunion of solution is known as isocratic flow.

Diagrammatic representation of isocratic elution

A condition in which the separation is occurring by using a gradient type at 20% methanol and finally with of 80% methanol then after a 20 min we see a two different typical peaks which are known as "A" and "B", in which A referred as weak solvents which makes to elute the solute slowly, while B referred as strong solvents in which it makes to elute fast from the corresponding columns. In reverse-phase chromatography the solvent A contain mainly aqueous or water or and aqueous buffer, and in the B which possesses the organic solvent which are pure miscible to the water, such as acetonitrile, methanol, THF, or isopropanol.

In the above diagram of figure -5 its shows the working components of HPLC. It mainly contains a reservoir in which it can store the solvents, and usually a pump is used with high pressure which can able to delivery the solvents with specified flow rates. And it also posses a injectors in which the mobile phase that carries the sample into the HPLC column.

The column is packed with chromatographic packing material which is needed for separation. This packing material is know as stationarey phase. By using detector the separated compounds bands which are come from column. The detector is connected to the computer which records the peaks which are developed in the chromatogram and which are display which helps in the identify the quantitative concentration of the sample.

As the different sample are emerged from columns hence need a different type of detectors are develop are, If an sample can able to absorb UV light, then UV-absorbence detector is used. If the compound is universal type then the detector used are Evaporative-Light-Scattaring Detectar [ELSD]. The most hybrid type detect used are, if suppose a UV and ELSD detector if used in combination with a Mass Spectrometer [MS] to see the results of the chromatographic separation.

The practice of coupling a mass spectrometer to an HPLC system is called LC/MS.
Components of HPLC

Solvent, Solvent Delivery System (Pumps),
Injector
Column
Detectors
Recorder (Data Collection)

Solvent & Mobile Phase Reservoir (Agilent Technologies, 2001)

Mobile phase
The most commonly used HPLC Mobile phases are mixtures of the organic solvents and water and aqueous buffers. In Table 1.3 given shows the lists of organic solvents their physical properties. Isocratic methods are preferable over gradient methods. In Gradient methods due its different partitioning properties the molecules being separated have become vastly. While using gradient elution methods ensure that all solvents are miscible.
The mentioned belows points to be administered during the selection of the mobile phase:

The drug molecules should possess enough stability during its analysis.

The presences of high concentrations of salt’s which leads to formation of precipitation as a result it may causes the destroying the materials of the equipmanets.

And in case of pH of the mobile phase it should be in between pH 2.5 to pH 7.0 so that it will causes increases the lifetime of the column.

As the acetonitrile is the costly compare to methanol and also regarding the toxicity also, hence it is prefered to used methanol whenever it is possible.

When we used the buffer it may leads to minimes the absorbencences, since trifluoroacetic acid, acetic acid or farnic acid will get absorbs at the short wavelengths, which may leads to stop’s the detection of the products without chromophores above 220 nm. Carboxyelic acid which changes the frequently which are replaced by phosphorric acid, which will doesn’t absorb above 200 nm.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>MW</th>
<th>BP (25°C)</th>
<th>RI</th>
<th>UV Cut-off (nm)</th>
<th>Density g/ml(25°C)</th>
<th>Viscosity (25°C)</th>
<th>Dielectric Constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>41.0</td>
<td>82</td>
<td>1.342</td>
<td>190</td>
<td>0.787</td>
<td>0.358</td>
<td>38.8</td>
</tr>
<tr>
<td>Dioxane</td>
<td>88.1</td>
<td>101</td>
<td>1.420</td>
<td>215</td>
<td>1.034</td>
<td>1.26</td>
<td>2.21</td>
</tr>
<tr>
<td>Ethanol</td>
<td>46.1</td>
<td>78</td>
<td>1.359</td>
<td>205</td>
<td>0.789</td>
<td>1.19</td>
<td>24.5</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>88.1</td>
<td>77</td>
<td>1.372</td>
<td>256</td>
<td>0.901</td>
<td>0.450</td>
<td>6.02</td>
</tr>
<tr>
<td>Methanol</td>
<td>32.0</td>
<td>65</td>
<td>1.326</td>
<td>205</td>
<td>0.792</td>
<td>0.584</td>
<td>32.7</td>
</tr>
<tr>
<td>CH₂Cl₂</td>
<td>84.9</td>
<td>40</td>
<td>1.424</td>
<td>233</td>
<td>1.326</td>
<td>0.44</td>
<td>8.93</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>60.1</td>
<td>82</td>
<td>1.375</td>
<td>205</td>
<td>0.785</td>
<td>2.39</td>
<td>19.9</td>
</tr>
<tr>
<td>n-propanol</td>
<td>60.1</td>
<td>97</td>
<td>1.383</td>
<td>205</td>
<td>0.804</td>
<td>2.20</td>
<td>20.3</td>
</tr>
<tr>
<td>THF</td>
<td>72.1</td>
<td>66</td>
<td>1.404</td>
<td>210</td>
<td>0.889</td>
<td>0.51</td>
<td>7.58</td>
</tr>
<tr>
<td>Water</td>
<td>18.0</td>
<td>100</td>
<td>1.333</td>
<td>170</td>
<td>0.998</td>
<td>1.00</td>
<td>78.5</td>
</tr>
</tbody>
</table>

The wavelength at which the absorbance of 1cm cell is 1.0

Table- 2 Physical Properties of Common HPLC Solvents
During analyzing by reverse phase chromatography some ionable compounds may causes problems. By Two modifications of the mobile phase it can be minimise. i.e. ion suppression, ion pairing chromatography. In both techniques, a buffer is used to ensure that the pH of the solution is constant and usually at least 1.5 pH units from a pKa of the drug to ensure that one form predominates. If pH is approximately equal to pKa, peak broadening can occur. In ion suppression the pH of the aqueous portion of mobile phase is calibrated to allow the neutral forms of drug to predominate. It shows that drug may be present only in one form which shows the improvement of peak shape and consistency of retention times. In this the pH of mobile phase is adjusted in such a ways the drug is completely ionized. If necessary to improve peak shape or lengthen retention time, an alkyl sulfonic acid salt or bulky anion such as trifluoroacetic acid is added to the ion pair to cationic drugs or a quaternary alkyl ammonium salt is added to ion-pair to anionic drugs. Ion pairing chromatography also allows the simultaneous analysis of both neutral and charged compounds.

**STATIONARY PHASES**

In liquid–liuqid chromatography the stationery phase is an liuqid fim which is coated on the above of packeing material which consists of 3-10mms of poroues silica particles. Stationery phases which is lightly soloble in the moblie phase by which causing the “bleed”from the coloum overtime.

In orerder to stop the loss of a stationery phases, it must be covalently bonded to silica particals .and the bonded particales will be reacting with the silica particles with an organochlorasilane of a form such as \( \text{Si(CH3)}_2\text{RCl} \), where R is an alkyl or substituted alkyl group.

The nature of the stationery phases have been decided based on their nature of organosillane’s alkyl group.

In such cases if the R is the functional group then ultimately the stationery phase will become polar.

Usually the modern HPLC consists of one or more glass or stainless steel. In generally usually stored in a glass containers reservoirs of capacity of 500 ml or up to 2lit of solvent. Plastic containers are not been used because it may leach into mobile phase The reservoirs are equipped in such a way by taking off the dissloved gass such as \( \text{O}_2\)and\( \text{N}_2 \) which generally causes the disturbances by
formation of bubbles in the columns and detector systems. These bubbles interfere with the performance of the detector.

There are four main methods used to degas solvents:

1) Sparing with a less soluble gas like helium (on-line degassing method)
2) Reducing pressure by vacuum (off-line degassing method)
3) Heating (off-line degassing method)
4) Sonication (off-line degassing method)

Requirements for a solvent reservoir are:

The reservoir is attached to the pump; the material should not contaminate the mobile phase: Teflon, glass, or stainless steel. It should be compatible with the instrument i.e., pumps, seals, fittings, and detector etc.

It should be compatible with the stationary phase.

Should be readily available and should possess adequate purity.

Not too compressible (causes pump/flow problems).

It should be Free of gases, it may cause compressibility problems.

By measuring the polarity index the relative polarity is measured by which it is used to identifying suitable mobile phase solvents.

The sample and analyte(s) must be compatible with mobile phase and stationary phase.

**Solvent Delivery Systems**

It plays a crucial role because it affects the directly the

For ideal HPLC it should possess

- Output at least 5000psi
- Pulse free output
- Flow rate specifying from 0.1 to 0.1ml/min. the out flow is control and reproduceibility is of 0.5% or better
- Corrosion resistant components (seals of stainless steel or Teflon)

**PUMPS** *(Settle F.A, 2004) (Cazes J, 2001)*

The two major functions of the pump in a modern HPLC are namely

(i) To deliver the mobile-phase via column at high pressures, and

(ii) At a constant a controlled flow rate.

**TYPES OF PUMPS**

Syringe pumps
Reciprocating pumps

Pneumatic pumps

The mobile phase is carry from reservoir to the column high pressure and at controlled flow rate which may alter effects the RT, reproduceibility and detectar sensitivity.

Therefore fine and small particles are oftenly used in modern HPLC Because of this modern pumps are used to operat authenic and most accurate at pressures of 10,000 psi. or at last 6,000 psi. The flow rates should be in renge 0 to 10 mL/minuts for analytical purposes and for preparative it should not be exceed 100 ml per minu.

Flow is control and flow is kept under reproduceibility of ±0.5%

HPLC pump are usual have saphire pistons, stainlees steel cylindars and return valvas fitted with saphehire balls and stainlees steel saets.

Structure of pump

Displacement pumps

In a large chamber like a syringe in which a plunger is initated by stepping motor screw n drive mechaneism.

Reciprocating pumps

This pump is well connected to the hydraulically pumped which is transfer to the diaphragam .it contained a small chamber in that solvent is pushed forcibly back with help of a motor driven pistion.

Schematic of the dual-headed reciprocating pump
It is very often explain how its works by without involving the any effect of solvent compress. as the moment if the outlet valves of the cyindered hase been close then at moment the entrance valve will be open, in such case the piston A will moves backs and sucks the eluent at the inlet as the chamber will be fill. Simultaneous the B cylinder will be opens in such cases the pistions moves towards forwards as a results the mobile phas will be deliverd to the coloum. the total volume resulted by the B is half about available in the A. as the A valves begains to close and the outer valve will be opened.

Now the pistion gets ready to push the contents from the chamber, such that the total half of its volumes will be come in contact directly to the coloum. and the remaining half will retain at the B.

**Pneumatic pumps**

In generally these are arranged in a vessel containing a mobile phase such that it can be passed out by pressurely with a compress gas.

---

**Types of Pumps used in HPLC**

<table>
<thead>
<tr>
<th>HPLC Type of pumps</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>

---

Figure no-9
**INJECTORS**

In the injection port the sample is inject directly into it

General requirements for an injector are:

When a liquid sample is injected range - 0.1 to 10.0 mL it should have able to provide higher reproducibility and under a high pressure (up to the 4000 psi) which can able to produce at least band broadening, and its lowers the flow disturbances.

There are three types of injectors, they are

Septum injectors
Stop flow injectors
Rheodyne injectors

There are two modes of sample injection in LC, they are

Load position
Inject position

---

<table>
<thead>
<tr>
<th>Reciprocating</th>
<th>Constant flow rate independent of viscosity of solvents. Suitable for continuous operations.</th>
<th>Detection noise due to pulsating output.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumatic</td>
<td>Rugged, inexpensive, easy to operate. Pulse-free.</td>
<td>Flow rate dependent on the viscosity of the mobile phase. Change of solvent is inconvenient.</td>
</tr>
<tr>
<td>Syringe</td>
<td>Pulse-free delivery at high pressure. Flow rate independent of viscosity of solvents.</td>
<td>Limited solvent capacity. Change of solvent is inconvenient</td>
</tr>
</tbody>
</table>

Table-3

Injector’s port used in HPLC.
Columns

Columns are the heart of HPLC system. Based upon the packing material, mobile phase and physical properties of the drug the column are chosen. Many different reverse phase columns which provides a good specificity for anyone of the particular separation. For best separation and for routine C8 or C18 can be used. In case the column is not enough separating or the method used is not satisfactory then an alternate method is used. In generally the reverse phase columns are generally shows different in their carbon chain length, end capping and also by the total amount of carbon loading. Such as Diols, cyans and aminos groups are those which are use for reverse phase chromatography.

Hichrom RPB column

Hichrom RPB (Settle F.A, 2004) is specially made for the Reversed-Phase separation of the basic molecules. By which the organic changer uses are be reduced.

the octyl- and octadecylsilane are well attached to the a material that attached to the robustness of a C18 phase with the high coverage of the C8 phase. In figure below a selected mixture of basic drugs is chromatographed.
Phase | Functional Group | Carboxyl load | Provided column size | Surface Area
---|---|---|---|---
RPB | C8--C18 multy-alkyl | Yes | 3.5.0 5, 10µm | 110Å 340m2/g

Length: 1, 3, 5, 15, 25cm, guards
(The sample loop) (Move the sample loop in the mobile phase)

Table-4

Figure No-11
- The column plays crucial role because the leads to separate the compounds is achieved when it passed by columns along with mobile phase.
- The Liuqid Chromotography apparatus is made upon of stainless steel tubes with a diametar of 3 to 5mm and a length renging from 10 to 30cm.

Figure No-12
Guard columns is fix on the anterior of the separating column which acts as a
protective and also enhances its lifetime which is useful in separation of the column.

The columns are designed to filter or remove Particles may interfere the separation column which my “baseliny drift”, it decrease the resalution, decreases its sensitivity which creates pseudo peaks. The compounds that’s which come upon touch with stationery phase leads to. The compound may elute simultaneous which may interfere with detection and/or quantification.

The columns must be change upon regularly to optimize their protection and also enhances the performances.

**Types of columns**

1. Guard column
2. Analytical column

**Guard column**

It is placed between injector and analytical column and which does not contributes any separation but eliminates the particulate and impurities.

The material used here are of same as that of analytical column.

It protects the system component and extends the life time of analytical column

**Analytical column (Brain L., et al, 1998)**

Most important part of HPLC technique which decides efficiency of separation.

**Column material**

Generally made of Stainless steel, Glass, Polyethylene and Polyether ether ketone (PEEK)

**Column dimensions**

The Column length is of 10-30 cm long and the internal diameter is 5-10 mm

**Packing techniques**

They are two types of packing technique are

- Dry packing
- Wet / slurry packing

  **Column packing material**

  Micro porous supports

  - It is of 3-10 μm in diameter, Composed of silica, alumina, ion exchange resin.
Pellicular supports
- It is of 40 µm in diameter, the Porous particles are coated onto an inert solid core such as glass bed.

**Bonded phases**
In this Stationary phase is chemically bonded onto inert support. It is used in Liq uid- liq uid partition system. Stationery phase is coated on the inert support. It is used in both micro porous and pellicular supports are used for supporting liquid Phase.

**For adsorption chromatography**
- Adsorbents such as silica or alumina are available as micro porous forms.
- Pellicular systems generally have a high efficiency but low sample capacity and so micro porous supports are preferred.
- Spherical shape gives good efficiency and flow properties.

**Stationary phase**
It may be polar and non polar

**Polar**- it is used in normal phase such as Silica, alumina, cyano, amino or diol on the bonded phase.

**Non-polar**- it is used in reversed phase of C$_{18}$ to C$_{8}$ on the bonded phase such as Phenyl and cyano.

![Different Types of Analytical Columns](image)

**Figure No-13**

**DIFFERENT TYPES OF ANALYTICAL COLUMNS**
General HPLC column care (Sethi P.D, 2010)
The columns play a major role in the separation of compounds. The lifetime of HPLC columns depends upon the correct use which benefits for analysis. By taking some precaution during cleaning, storage, and also by nature of the chromatographic support (silica, polymers or others) stationary phase we can extend the lifetime.

**Silica based columns**

Silica is mainly used in HPLC columns. Which provides a big mechanical stability with physicochemical surface properties and it is compatible with organic solvents. However, some points to be considered during its working with silica based columns are:

- **pH stability**
  
  Normally, HPLC columns which are highly stable within the pH range of 2- to pH 8. For measuring the pH value initially, it must have to done for aqueous mediae before combining the eluent with the organic solvent. Exceptionally, the modern HPLC columns can also be used in the outside of the pH range. The newly bonding chemistry allows using below the pH 1 for some stationary phases. For better life times it must be used between pH 2.0 and pH 6.8. In some columns, the stationary phases are ultra-pure silica gel which are been used at the high pH range up to the pH 11, there are wide variety of modifiers which are been used which depends upon the chemical nature of mobile phase that which are been used. At higher value of pH the large bases are generally used because they cannot attack the silica surface. In such cases, if it is using at small pH of above 8 with small bases modifiers the hence the extremely recommend using stationary phase base on the Polymer or Zirconium dioxide.

- **Mechanical stability**

  Stationary phases is silica which provide high mechanically stable. The columns are tightly packed used at pressure limit more than that of 40 MPa (6000 ps) without any problem. It may be noted that to inherit the pressure shocks to the column which may lead to channeling in the column, as its leads to peak dividing in the corresponding chromatogram.

**Mobile phases (Eluents)**
As the Silica based stationary phases columns are compatible with all organic solvents in the above mentioned pH range. Always it should be used only HPLC grade solvents. Before using it should filter the prepared buffer passed through a 0.5µm filter. In any circumstance by using of impure solvents it may causes irreversible adsorption of impurities on the column. Which blocks the impurities at adsorption sites, as a result its alter its selectivity of a column and which shows the peaks splitting in the chromatogram? Gradiant elution, impurity which leads to “Ghost Peaks”. Generally the Ghosts peak that will forever shows at the similar positions of the chromatogram. By using pre-column the irreversible adsorption at the column heads can be avoided and it also enhanced its life-time of column. By a result a pre-column can filter solid are fix to filter directly such that it is directly attached which resut inher the adsorption of organic impurites.

Coloumns storage
If it is for short time storage that for a overnight the it can be stored and use it for last analysis.
If it for middle storage that for a two days then the coloums need to flush with a water so that it inhibit the growth of microbial
In such case if at all need to sore for longer period then should be store in a aprotic solvents, in which the water contens must not be about 50percent.
The most better solvents used for storieng the coloums are acetonitrile.
Make sure that before using the coloums all the buffers need to washed properly and also acetonitrile need to washed, the buffer salts are insolouble in that solvents.
Even some time the acetonitrile may leads to blockage of coloums capillarys.

Factors that affecting column efficiency are
Dimensions of column, Particle size of adsorbent, Nature of solvent, Temperature of column and Pressure.

Equilibration time
The equilibration time may changes which are summarized in below Table. No.5.
By increasing the flow rate it Shorter equilibration times although 20 coloumn content volume is essential to for 100 percent equilibration.
### Table-5

<table>
<thead>
<tr>
<th>Column dimension</th>
<th>Column volume</th>
<th>Flow rate [ml/min]</th>
<th>Equilibration time [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>250.0 x 0.460 mn</td>
<td>2.91</td>
<td>1.00</td>
<td>58</td>
</tr>
<tr>
<td>150.0 x 0.460 mn</td>
<td>1.74</td>
<td>1.00</td>
<td>35</td>
</tr>
<tr>
<td>100.0 x 0.460 mn</td>
<td>1.16</td>
<td>1.00</td>
<td>23</td>
</tr>
<tr>
<td>50.0 x 0.460 mn</td>
<td>0.58</td>
<td>1.00</td>
<td>12</td>
</tr>
<tr>
<td>250.0 x 0.400 mn</td>
<td>2.20</td>
<td>1.00</td>
<td>44</td>
</tr>
<tr>
<td>125.0 x 0.400 mn</td>
<td>1.10</td>
<td>1.00</td>
<td>22</td>
</tr>
<tr>
<td>250.0 x 0.200 mn</td>
<td>0.33</td>
<td>0.25</td>
<td>26</td>
</tr>
<tr>
<td>150.0 x 0.200 mn</td>
<td>0.11</td>
<td>0.25</td>
<td>9</td>
</tr>
<tr>
<td>50.0 x 0.200 mn</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Regeneration of a column

Irreversible adsorption of impurities in column head it result cause change in selectivity or peak splitting, these are called as “dirty columns” it can be regenerated by follows.

**Regeneration of RP (Reverse phase) packing’s**

- RP- packing’s are C18,- C8, and- C4, -C1, -C30, -CN or Phenyl stationery phase.
- Allow to sluice the column by 20 column voloumes of water
- Allow to sluice the column by 20 column voloumes of acetonitrile
- Allow to sluice the column by 20 column voloumes of isopropanol
- Allow to sluice the column by 20 column voloumes of heptane
- Allow to sluice the column by 20 column voloumes of isopropanol

**Regeneration of NP (Normal Phase) packing's**
NP-packing’s are Silica,- Diolo, Nitroa and Amino- stationery phases.

- Allow to sluice the column by 20 column volumes of Heptane.
- Allow to sluice the column by 20 column volumes of Isopropanol.
- Allow to sluice the column by 20 column volumes of Acetonitrile.
- Allow to sluice the column by 20 column volumes of Water.
- Allow to sluice the column by 20 column volumes of Acetonitrile.
- Allow to sluice the column by 20 column volumes of Isopropanol.
- Allow to sluice the column by 20 column volumes of Heptane.

**Regeneration of Ion Exchange Pickings**

In case of the ion exchange are either of the cation or anion exchangers Allow to Flush the column with 20 column volumes of of the same eluents but increase the buffer conce.

<table>
<thead>
<tr>
<th>Type</th>
<th>Internal diameter(cm)</th>
<th>Length(cm)</th>
<th>Particle Size(µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical</td>
<td>0.3- 0.46</td>
<td>3-28</td>
<td>3-10</td>
</tr>
<tr>
<td>Semimicro</td>
<td>0.1– 0.21</td>
<td>10 – 25</td>
<td>3 – 18</td>
</tr>
<tr>
<td>Semipreparative</td>
<td>0.8-1.0</td>
<td>10 – 25</td>
<td>5 – 10</td>
</tr>
<tr>
<td>Preparative</td>
<td>2.0-5.0</td>
<td>10 – 25</td>
<td>10 – 20</td>
</tr>
</tbody>
</table>

Table-6

**1.12 Detectors of HPLC (Dong M.W, 2005) (Michael W. Dong, 2006)**

The primary role of detector are to visualize the compounds mixture that which are separated from columns.

In generally they are wo types of detectors are used they are destructive and non-destructive.

The destructive detectors are able to perform continuous transformation of the column effluent (such as burning, evaporation or mixing with reagents) and also some subsequent measurement of some physical property of the resulting material (plasma, aerosol or reaction mixture).

The non-destructive detectors mainly directly measuring some property of the column effluent (for example UV absorption) and for affords the further analyte recovery.
Detector are present at posterior side of the stationary phase or column as the sample elute off the immediately signal peaks are detected on chromatogram by detector.

**The following characteristic should have for LC detector.**

- It should process low drift and noise level which is very important in trace analysis.
- It should have high sensitivity and fast response.
- It should have broad linear dynaemic range which lowers the quantitation).
- Minimies the dead volume (minimal peak broadaning).

However they are many types of detectors which can be used with HPLC. Out of which the more common detectors are --

- Refractive Indexe (RI)
- Ultra-Violett (UV)
- Fluoraescent
- Radeiochemical
- Electrochemical
- Near-Infra Red (Near-IR)
- Mass Spectrometry (MS)
- Nuclear Magneti Resonce (NMR)
- Light Scattering (LS)

Different types of detectors and their specifications

<table>
<thead>
<tr>
<th>DETECTOR</th>
<th>ANALYSIS</th>
<th>SOLVENT REQUIREMENT</th>
<th>COMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVi-VS</td>
<td>Chromophore</td>
<td>UVi grade, non-UVi absorbing solvents</td>
<td>Usefull for the manyof HPLC applications</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Flouresscent Compeunds</td>
<td>UVi grade, and also the none- UVi absorbing</td>
<td>Which is used for the hugely selective and uses</td>
</tr>
</tbody>
</table>
solvents for the determination for compounds

<table>
<thead>
<tr>
<th>Refractive Index (RI)</th>
<th>A mobile phase containing with varies RI</th>
<th>Different mobile phase gradients are not used.</th>
<th>It is an universal detector enough shows limit sensitivity.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass Spectroemeter (MS)</td>
<td>Broad ranges of compounds</td>
<td>It have to possess volatility properties of solvents and buffers</td>
<td>Highly sensitive and is a strong two scientific tool</td>
</tr>
</tbody>
</table>

Table-7

**Recorders**

A data collection devices such as a computer, recorder devices recives the detector output and prints out chromatograms. The complexity ranges from these providing a printout of peaks area to those providing chromatograms complete with peaks heights, peak area, sample identification and method variables. As the components elute from the column which passes thoughts into a detector where analyte process answer from the detector. This response is modified which is plotted towards time impart to rise a ‘chromatogram’ (Figure 14). Ingredients which are injected to the solvent which are not preserve in the column elute in the ‘deads times’ or ‘holds up’s times’ $t_0$. Which compounds which are persist to elute as approximataly ‘Gaussian’ shape peak later in the chromatogram. Data handling is done in two ways i.e. qualitative as well as quantitative.
System suitability specifications and tests for HPLC (Lloyd R. Snyder, 1997)

The system suitability specifications and tests parameters which providing the assistance in achieving accuracy and precision of HPLC data.

**Capacity factor (k')**

\[
k' = \frac{t_R - t_0}{t_0}
\]

It measure the degree of the retention of analyte with respect to the unretained peak,

Where as the \( t_R \) is the retention times of a sample peaks and where as \( t_0 \) is the retention time for an unretained peaks.

Generally the value is \( k' \) is > 2.

**Resolution (R_s)**

It is an process in which the column are able to separate chromatographic peaks is known as Resolutions. Which can be enhanced by increasing length of column, and by allowing to minimize the particle size and also by allowing increasing the temperature, altering the eluent or stationery phase. And even which may also expressed in words of separation the apex of 2 peaks which divides the tangential widths mean of its peaks.
R_s = \Delta t_R / 0.5 (W_1 + W_2)
Where \Delta t_R = t_2 - t_1
For better quantization, well-separated peaks are essential.

**Recommendations**

It should be > 2 between the two peaks shows the interest and also the closest potential of the interfering peak such as impurity, excipient, degradation product, interanal standard, etc) are desirable.

**Tailing factor (T)**

It is used to measure the symmetry of a peak which an calculation where w0.050 represents a peaks area an 5% of its heights where as (f) denotes the gap of peaks from anterior to point of 5 percents of their heights. and their peak s must be possess the Gaussian in its shape or enterily symetrical.

\[ T = W_{0.05} / 2f \]

Recommendations

\[ T \leq 2 \]

**Theoretical plate number / Efficiency (N)**

There are various methods to determine the peaks bands and even some are most even sensitiev to peaks shapes which are represents earlier.

4-sigma / tangential

\[ N = 16 \left( \frac{t_R}{W} \right)^2 = \frac{L}{H} \]

Half height

\[ N = 5.54 \left( \frac{t_R}{W} \right)^2 = \frac{L}{H} \]
Column efficiency can be measured based on the number of Theoretical plate i,e that how many peak are present per time/ run of the chromatogram, where \( r_t \) is the retention time of a sample peak where \( W \) as know as peak width.

![Diagram of chromatogram](image)

Figure No-16

Here the \( N \) represents the fairly constants of each peaks on chromatogram which as a following fix set of standard operating condition .

HETP which represents the height equivalent of an therorectial plates it measures the efficencay of coloum per unit length-L of the coloum.

The parametareas can effects the \( N \) or \( H \) of the peak positian,size of the particles in a coloum, effect of flow rate of a mobile phase,temperatore of coloum, molecular weight of analyte and finally the viscosity of moblie phase.

Recommendations

The number of theotircel plates will depends upon the based on eluction time ,which is offen should be > 2000.

Chromatographic Parameters (Srivastava, VK, 1991) (Sethi PD, 2010)

- Retention time (Rt)
- Efficiency (N)
- Retention volume (Vr)
- Resolution factor (Rs)
- Column Efficiency (N)
- HETP (High Equivalent Theoretical Plates)
- Capacity factor (mass distribution ratio, Dm)
- Symmetry factor (As)
- Tailing Factor (T)

**Retention time (RT)**

It is difference between the time points of injection and eluted from a column after injection of sample in which it show the peak maximum.

It is measured in minutes or seconds.
**Retention volume (RV)**
Which is represents the total volume of the carrier gas which is existed from one half of its compounds of a column at the maximum peak, and which is represented as:
Retention volume (RV) = Retention time (RT) x flow rate.

**Height equivalent of the theoretical plates (HETP’s)**
The column is make of with huge counts of opposite layers or ‘theoretical plates’ in which the mobile phases stream below to the column it distribute between the stationary and mobile phases.

A theoretical plate determines the efficiency of columns it can be of any height. In case if HETP’s is least then the column will be extra efficient. If HETP’s is huge, then the column is least efficient. Heights equivalent to the theoretical plate (HETP’s) is given by:

\[ \text{HETP} = \frac{\text{Length of column (L)}}{N} \]

\( N = \) plates per meter.

**Capacity factor (Dm)**
This measure the retention time of solute from chromatograph can be calculated by operating this formula.

\[ D_m = \frac{(t_R - t_M)}{t_M} \]

where:
- \( t_R \) = retention times of a solute
- \( t_M \) = retention times of the un retained components

Lower the Dm values shows the peaks exist closer to the solvent fronts. A lesser the Dm values of 1 is shows for its peak of interest.

**Void volume**
Volume of an liquid phase which is present in an column is represents known as “-void volumes” (\( \omega \)). It may also called as “dead volume,” “hold-up volume.”

\[ t_R = \frac{V_R}{F}, \quad t_0 = \frac{V_0}{F} \]

Void time is can measure by the analyte total present at the time of retention times and the total time of which the analyte spend during in a mobile phase while passing via column.
Retention factor
The proportion of the reduce retention portion to their void volume which are oftenly commonly use as dimensionaless parameter which is represents as retention factor - k.

\[ k = \frac{V_R - V_0}{V_0} = \frac{V_R'}{V_0} = \frac{t_R - t_0}{t_0} \]

In generally the retention factor is very often convuent chromatographic factors for descriptive because it is an dimensionless ans also it is indepents on amount of the mobile phase its dimension of the columns. generally if need to measure the retention time of the analyte of same on the two different instrumentans with well equipped columns possessing different dimensions with same stationery phase and similar mobile phase theoretically and values of retention time will be same and identical on the both system.

Resolution
It denotes the peak maxima of the two peaks distances between them which shows the selectivity the system..
Higher the selectivity which represents the greater the distances of a peaks. The effiency is represented by by the peak width of chromatographic.
It is also define the proportion of distance of 2 peaks and their averages peak areas from at baselines.

\[ R = 2 \frac{t_{R,2} - t_{R,1}}{W_{R,2} + W_{R,1}} \]
**Efficiency (N)**

It measures the peak broadening and the total number of theoretical plates of the column, which are calculated using the following equation:

\[ N = 16 \left( \frac{t_r}{t_w} \right)^2 \]

TR is the analyte retention time and \( T_w \) represents the width of a peak.

The efficiency of the column depends upon the kinetic factors of the chromatographic system, such as molecular diffusions, flow of mass particles, based on column packing bed, and also on flow rate. The tiny size particles show more even uniform packing in the columns which possess the higher efficiency. As long as the flow rate is faster, which results in showing lesser time of an analyte molecule for the diffusional band-broadening. There should be an optimum flow rate for efficiency for a given column.

The column which possesses a higher efficiency results in showing very narrow chromatographic bands and which leads to separate the analyte with low selectivity.

**Figure No-18**

The column which possesses a higher efficiency will result in showing very narrow chromatographic bands and which leads to separate the analyte with low selectivity.
Tailing factor (T)

It is used to measure the symmetry of a peak, in which the value will be increase as the peak tailing becomes more. But in some exceptions cases the value is to observes as less than 1 is observe. As asymmetry peaks increases the integration hence its precision will becomes less reliable.

\[
T = \frac{W_{0.05}}{2f}
\]

And,

\( W_{0.05} = \) diameter of peak at 5percents of its heights

\( f = \) represents the interval of an peak maxima to the boundary of an crest peak which is calculate the distances at 5percent of crest peak their Limit: ≤2
HPLC METHOD DEVELOPMENT (Snyder, LR, 1997)

For HPLC method development it should have systematic knowledge in chromatograph, the experimental runs are important. Based upon these results a good method is achieve for final result.

Flow chart for Method development

1. Collecting the Information of sample for separation

2. HPLC procedure sample Pre treatment etc.

3. Choosing of a detector.

4. Choosing of method which preliminary runs shows the Best separation

5. Optimizing the separation conditions.

6. Method in Separation Procedure
7a. Recovery of purified material  
7b. Quantitative method &  
7c. Qualitative method  

8. Validate the method for labs, released for routine use. 

In order to achieve the better and efficient resolution the operating parameters should be selected which are follows, columns Dimensions (length, ID)

<table>
<thead>
<tr>
<th>SNO</th>
<th>Separation Variable</th>
<th>Preferred</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Column</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dimenions (lengths, internal diameter)</td>
<td>15.0 X 0.460 cm</td>
</tr>
<tr>
<td></td>
<td>Particale size</td>
<td>5.0 µm&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Stationary phases</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;</td>
</tr>
<tr>
<td>2</td>
<td><strong>Mobile phases</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Solvents A.&amp;B.</td>
<td>Buffer- acetonitrile</td>
</tr>
<tr>
<td></td>
<td>Percent of B.</td>
<td>80.0-100.0%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Buffer (compound, PH, concentration)</td>
<td>25mopotassiumphosphate, more than two and less than three should be its ph</td>
</tr>
<tr>
<td></td>
<td>Additives (amines modifiers, ion's-pairs)</td>
<td>Will not used early</td>
</tr>
<tr>
<td></td>
<td>Flow of rate is</td>
<td>1.05-2 ml per min</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>Thirtyfive to forty five °C</td>
</tr>
<tr>
<td>3</td>
<td><strong>Sample- Size</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Volume&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; 25µL</td>
</tr>
<tr>
<td></td>
<td>Weight</td>
<td>&lt; 100.00 µg</td>
</tr>
</tbody>
</table>

<sup>a</sup> is of 3.50 µm these are in sequential, utilizing a 7.50 cm column.
b is of starting isocratic runs; and at the starting gradient flow is prefer.

c is of total number of bufer necessary for its neutral samples and for the greater ph of 2.5 which is highly prefer as the coloum will be stable.

d is of lesser value essential for their least-voloume coloumns (e.g.-- 7.5 x 0.46-cm, 3.5-µm column).

Validation provides the documented evidence whether the method is fit for intended purpose. Validation is done for new process, new equipment, the process validation includes,

Analytical test procedures, Instrument calibration, critical support systems, Raw materials,

Equipment, Effect of Stability.

The validation parameters are

- Accuracy,
- Precision, (repeatability and reproducibility)
- Linearity and range,
- Limit of detection, Limit of quantitation
- System suitability studies,
- Ruggedness,
- Robustness,
- Specificity.

OBJECTIVES OF THE VALIDATION

The preliminary intentions of validation enhances to provide the documented evidence for process control which are done to drug products to assure the identity, quality, and purity of possess.

IMPORTANCE OF VALIDATION

The quality of the products will not be assured by testing of statically insignificant number of sample, thus the validation should provide adequacy and reliability for a product to meet the criteria or attributes to provide high degree of confidence that builds the same level of consistently of finished product

Retrospective Validation which is useful to compare the results of cGMP to cGLP.
Prospective validation              analytical validation
 ↑                          ↑
 Reterospective validation ←VALIDATION→ instrumental validation
 ↓                          ↓
 Revalidation                process validation

**Types of Validation**
ANALYTICAL METHOD VALIDATION

By Method validation we can establish the performance of analytical method for intended use. Initially the chromatographic method is to be validate for first routine use in order to achieve accurate result of all variables such as sampling procedure, sample preparation, chromatographic separation, detection and data evaluation.

The validation of method can done only in laboratory studies which are provide the document proof.

The method is to be validated are and also for Identification for test of impurities,
Quantitative test for impurities,
Limit test control of impurities,
And also for quantitative test for drug substances of the active molecules of a sample Dissolution testing.

The analytical method development parameters are

ACCURACY

The accuracy may be define as degree to which is determining the value of the analyte of sample at response to the true value of sample.

There are many methods to determine accuracy and the method used should relevant to matrix.

The accuracy may determine in any one of the following method.

- By analyzing the know concentration of sample by comparing the measured value to that of true value, and reference value should be used.
- In the case of Spiked – placebo recovery method the measured constitute the active constituent is mixe with the blank sample the mixture ,which is assayed the which obtained are compared with results.
- Incase of Standard addition method in which known amount pure of sample is assayed and
- Again sample is assayed, then the differences in the results of a two assays are compare with the expect result.
- The accuracy range is determine at different levels .it should at least covers three different concentrations of 80%, 100 %and 120% are in the expected range.
• The results which obtained by test method is compare to validated method, by this accuracy determined.

Acceptaence criteria: based on the sample matrix the recovery will occur. The sample processaing procedure and on the analyte concentrretion. The mean % recovery should be within the following range

Mean Recovery Ranges

<table>
<thead>
<tr>
<th>% Active and impurity content</th>
<th>Acceptable mean recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 10</td>
<td>98 – 102%</td>
</tr>
<tr>
<td>≥ 1</td>
<td>90 – 110%</td>
</tr>
<tr>
<td>0.1 – 1</td>
<td>80 – 120%</td>
</tr>
<tr>
<td>&lt; 0.1</td>
<td>75 – 125%</td>
</tr>
</tbody>
</table>

Table-9

PRECISION

Precision is a measure by the multiple samples obtain from homogenous sample under prescribe condition. It is a closeness degree of samples. As per ICH precision is performed at 3 levels are of repeatability, intermeadiate precision and reproducibaility. The analytical procedures of precision is commonly expressed as varieance, standard deviations or co efficieant of vareation and the sequences of computation. Atleat the five duplicates are be pocess with including percent relative standard deviation. The prescribed precission levels will be recommended
SPECIFICITY:
It measures specifically and accurately of other component which is present in a sample. Due to ingredients, excipients, impurities and degradation products the peak shows interferences, hence it is also measures and ensures peak response will be is due to of single component.
It is an measurements and documented of data by various parameters like resolution, plates counts and also by tailing factors. Specificity is also used to determine the total separation of peaks and their efficiency & tailing factors.
Specificity is used for identification which distinguishes between the compounds or by comparing to now reference sample.
Based on eluting nature of compounds it is used for assay and impurity tests.
If any impurity are presence it should to be demonstrate that by performing an assay is unaffected by in the present of these spiked materials. However in practically by spiking the appropriate levels of impurities the assay will not be affected by the present of the extraneous materials. If the impurities standards are unavailable then result is compared the sample containing impurities follows the second procedure. The comparison is done for sample store under the appropriate pressure atmospheres so far as like light, heat, humidity, ascid and basic hydrolysiss reaction oxidation.

<table>
<thead>
<tr>
<th>Component to be measured in sample is to be</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 10.0%</td>
<td>≤ 2%</td>
</tr>
<tr>
<td>1.0 Up to 10.0%</td>
<td>≤ 5%</td>
</tr>
<tr>
<td>0.1 Up to 1.0%</td>
<td>≤ 10%</td>
</tr>
<tr>
<td>&lt;0.1%</td>
<td>≤ 20%</td>
</tr>
</tbody>
</table>

Table-10
THE LIMIT OF DETECTION (LOD)

The limits of detection is define by in which of least amount of concentraction of the component of a sample which can be illustrated but notting is quantieated. These limeit tests determine the analyte whether it is present above or below a particular value. Therefore it is commonly expressed as concentraction of signal-to-the noise ratio, preferably two-or-3-to-one. The ICH’s is been recoganized their signal- noise ratio conventaion, and there are other 2 options to determin LOD are visual non-instrumental methods is to calculating LOD. Visual non- instrumental methods is of as thin layer chromatography it is summarized base on the standard deveiation of the response such as (SD) slopes an of calibretion curves(-S-) at the levals approx the lod is the according to the formule

\[ \text{LOD} = \frac{3.3}{(SD/\times S)} \]

And standerd deavation shows respons cn will determne base staendard deavation and the blank,which is above resuidal standerd deavation of the regression line or the standerd deavation of y-/intercept of the regression lines.

In such cases if LOD has been determine base visual evaluaetion or either base on signal to nosie ratio then the projection of the corresponding chromatography is considerable acceptable for justifaction.

QUANTITATION LIMIT

It determines lowest amount of the analite in a sample which can be determine quantitave with precise under the experimental condition.

It determines the quantitivetive assays even fore low level of compouennds in a sample for determination of impuerities and degradeation products of active constituent.

Signal-to the -noise ratio is done by compaering and measuring the signal of the samples even at low concentration of analyte with the blanks sample, it establishes a relation at which minimum concentration of analyte is quantiffied.
LINEARITY
In this method it will elicit the result which is directly concerned to concentration of analyte of a sample within the range. Linearity is used on test substances i.e. dilution of standard stock solution and by weighting of mixture and also for proposed method. It is determined by a series of the injection of 3-6, and the response is directly proportional to concentration of analyte. This is applicable if regression equation having intercept of zero value. And it has to shows that it doesn’t have any effect on accuracy method.

RANGE
This is the interval between lower and upper levels which determines precision, accuracy and linearity. Generally range can be expressed as similar units of the test results (e.g. percentages, parts per million) obtained by the analytical methods. As per ICH’s to perform assay the minimum specified range is to be 80.0 to 120.0-% percent of the test concentration, and for determination of impurities range to extend to 50%- percent of the each specification of the each impurity.

RUGGEDNESS
ICH doesn’t address the Ruggedness in its documents (4,5) which is replaced as reproducibility having the same meaning of Ruggedness. As per USP it defines that the degree of reproducibility result obtaineds under variety condition such as different laboratories, analysts, instruments, environmental conditions, operators and materials. It can be expressed as % RSD. Ruggedness is determined by the analysis of aliquots from homogeneous lots in different laboratories.
ROBOUSTNESS

According to the ICH’s guidelines, it have to consider before in any new method development. It determines variable parameter of analysis result. For an method robustness the parameters to be examine like pH, flows rate, coloumn temperature, injection volume, detectaion wavelength or moboile phase composeition, are varies within a realistics range which are quanteatative influences. If so results of influences variable is within the tolerances of previous then the method is robustness within the range. By obtaining data which help us as whether method yet to revalidated, if one or morethan parameters are changed.
1.2 PROBLEMS ON HAND

- Now a days numerous methods are been developed for newly marked drugs so many efforts need to present studies to initiate a new process in such a way that it should be most economical and accurate and precise.
- It is highly expensive which have a major effort on laboratories investments.
- It requires expert to handle the instrument.
- Gas samples cannot be handle.
- Sample preparation is often required.
- As it is highly sophisticated method and also very expensive so it cannot used for normal routines analysis.
- Compare to spectrophotometric process it is expansive. And there is no universal dectatar is used in it.
- For the selection of mobile and stationery phases it have undergoes many of the trails.
1.3 RESEARCH OBJECTIVES

A new HPLC method development and validations studies can be performed by should have systematic knowledge in chromatograph, the experimental runs are important. Based upon these results a good method is achieved for final result.

Validation provides the documented evidence whether the method is fit for intended purpose. Validation is done for new process, new equipment, the process validation includes,

The preliminary intentions of validation enhances to provide the documented evidence for process control which are done to drug products to assure the identity, quality, and purity of possess.

Importance of validation is quality of the products will not be assured by testing of statically insignificant number of sample, thus the validation should provide adequacy and reliability for a product to meet the criteria or attributes to provide high degree of confidence that builds the same level of consistently of finished product.

Retrospective Validation which is useful to compare the results of cGMP to cGLP.

It can also be done by Method validation we can establish the performance of analytical method for intended use. Initially the chromatographic method is to be validated for first routine use in order to achieve accurate result of all variables such as sampling procedure, sample preparation, chromatographic separation, detection and data evaluation.

The validation of method can be done only in laboratory studies which are provide the document proof.

The method is to be validated are and also for Identification for test of impurities, And also for quantitative test for drug substances of the active molecules of a sample Dissolution testing.
1.4 SCOPE OF RESEARCH WORK

- Developed a new scientific and analytical process for the drugs which are been studied are well applicable to it.
- The sensitivity selectivity of detector which are used here will helpfull in the analysis.
- The used of mobile phases and the stationary phases here help in the resolution.
- The saperated samples can be easy collected.
- Here HPLC used which has many application and advantages over the many of the components of classes such as ionic, polar & nonpolar where Gas chromatography is not used.
- Where we have a plenty of choices to use mobile phases by using of gradient techniques.
- And also by using of sensitivity of dectar use compare to gas chromatography we can collecte the sample by at the colour as they elutes which cannot done in gc.
- A new scientific process is reinforces upon investigations of various parameters. Which can determine the maxima absorbences at different nm of drugs which produces a good peakas. And by injecting the selected voloume. For this study column used is at moderate temperatre is selected. the rate of flows is adjusted at ml per minute which shows the satisfy retention time and superior resolution. After studied this a mobile phase is maintained throught out the process as. and the recovery studies are been determined which should be accurate over its scale. The limit of detacation is determine. And further linearity for the drug. This process is proceeds for robustness and ruggedness. And the corelation coefection are at in premiscible levels.
1.5 ORGANISATION.

Where the work is carried out: This work is carried out at SURA LABS, Hyderabad, Telangana under the guidences of DR. Kumara swamy.

The instruments mainly used are:

- HPLC WATERS, software: Empower 2, Alliance 2695 separation module. 996 PDA detectors.
- pH meter Lab India,
- Weighing machine Sartorius,
- Volumetric flasks Borosil,
- Pipettes and Burettes Borosil,
- Beakers Borosil,
- Digital ultra sonicator Labman.

- solvents are used are HPLC grades and analytical grades are used for method development and validations. Different pharmaceutical dosages forms are procured at markets, and pure drugs are available at labs.
**DRUG PROFILE**

Selected Formulation for Studies-

<table>
<thead>
<tr>
<th>S NO</th>
<th>BRAND NAMES</th>
<th>AMOUNT PRESEN(MG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TRIUMEQ</td>
<td>ABACAVIR 50MG + DOLUTEGRAVIR 600MG + LAMIVUDINE 300MG</td>
</tr>
<tr>
<td>2</td>
<td>PREZCOBIX</td>
<td>DARUNAVIR ETHANOLATE 800MG + COBICISTAT 500MG</td>
</tr>
<tr>
<td>3</td>
<td>LORCHEK MR</td>
<td>LORNOXICAM 8MG + THIOCOLCHICOSIDE 8MG</td>
</tr>
<tr>
<td>4</td>
<td>AC SERA</td>
<td>ACECLOFENAC 15MG + SERRATIOPEPTIDASE 100MG</td>
</tr>
<tr>
<td>5</td>
<td>SAQUIN</td>
<td>500MG SAQUINAVIR</td>
</tr>
<tr>
<td>6</td>
<td>LIPAGLYN</td>
<td>4MG SAROGLITAZAR</td>
</tr>
<tr>
<td>7</td>
<td>CRIXIVAN</td>
<td>800MG INDINAVIR</td>
</tr>
</tbody>
</table>

**SAROGLITAZAR**

Chemical structure-

IUPAC name. 2 ethoxy -(2s) -[4-[2-[2-methyl-5-(4-methylsulfenylphenyl)pyrrol-1- yl]3 propanoic acid.-phenyl

Chemical formula- C_{25}H_{29}NO_{4}S.

Molecular weight- 439.56 g/mol.

Category – Type 2 diabetes mellitus.

Solubility-shows miscible in dimethyl sulfoxide, dichloromethane & partial miscible in methanol-1, immiscible in water.

Brand names- LIPAGLYN, ZYDUS MEDICA.

**SAQUINAVIR**

IUPAC name  N-[3R,2S)-[(3S)4-3(tertbutaylcarbamoyl)- (2S)-decahydroisoquinoline-2-yl]3(1-hydroxy-phenyl2butan-)yl-butanediamide - 2(quinolin-formamido)

Chemical formula- C_{38}H_{50}N_{6}O_{5}.  

![Chemical structure of SAROGLITAZAR](image)
Molecular weight- 766.96 g/mol.
Category – HIV- protease inhibitor.
Solubility- Soluble in methanol, water.
Brand names - SAQUIN.

**LORNOXICAM**
Chemical structure-
Chemical formula- C13H10ClN3O4S2
Molecular weight- 371.8 g/mol.
Category – Non steroidal anti-inflammatory drug.
Solubility- soluble in 0.1 N Sodium hydroxide, methanol and dimethyl sulphate

**THIOCOLCHICOSIDE**
Chemical structure-
Chemical formula- C27H33NO10S.
Molecular weight-563.6 g/mol.
Category – skeletal muscle relaxants.
Solubility- soluble in water, ethanol.
Dosages- 8 mg once or twice daily.
Brand names - LORCHEK-MR, LORNOXI-T.

**INDINAVIR**
Chemical structure-
Chemical formula- C36H47N5O4.
Molecular weight- 613.7 g/mol.
Category – antiretroviral.
Solubility- water, ethanol, dimethyl sulphate.
Brand names - Crixivan.

**DARUNAVIR**
Chemical structure-
Chemical formula-C27H37N5O7S.
Molecular weight- 547.66 g/mol.
Category –antiretroviral.
Solubility-in water.
**COBICISTAT**

Chemical structure-
IUPAC name- \( N-[1\text{-benzyl}4\text{-}[2\text{-}[[2\text{-isopropylthiazol}4\text{-yl}4\text{-morpholino}4\text{-butanoyl}][\text{amino}]\text{-methyl-methyl-carbamoyl}][\text{amino}]\text{-5-phenyl-pentyl}][\text{carbamate- Thiazol-5-ylmethyl}} \)

Chemical formula- \( C_{40}H_{53}N_{7}O_{5}S_{2} \).

Molecular weight- 776.02 g/mol.

Category – Human immune deficiency (HIV).

Solubility- In DMSO

Brand names - Prezcobix.

**ACECLOFENAC**

Chemical structure-
Chemical formula- \( C_{16}H_{13}Cl_{2}NO_{4} \).

Molecular weight- 354.1 g/mol.

Category - non-steroidal anti-inflammatory drug.

Solubility- soluable in acetone, alcohol.

**SERRATIOPEPTIDASE.**

It is a proteolytic enzymes which produced by the enterobacterium Serratia sps

Brand name - AC Sera

**ABACAVIR**

Chemical structure-
Chemical formula- \( C_{14}H_{18}N_{6}O \).

Molecular weight- 286.332 g/mol.

Category – anti-retroviral agent.

Solubility- soluable in water.
DOLUTEGRAVIR
Chemical structure-
Chemical formula- \( C_{20}H_{19}F_2N_3O_5 \).
Molecular weight- 419.38 g/mol.
Category – antiretroviral agent.
Solubility- soluble in water.

LAMIVUDINE
Chemical structure-
IUPAC name- 1,2-dihydropyrimidin-2,one 4,amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl].
Chemical formula- C8H11N3O3S.
Molecular weight- 229.26 g/mol.
Category – antiretroviral agent.
Solubility- soluble in water.
Brand names - Triumeq.