8. High-performance liquid chromatography (HPLC)

Introduction

- Type of chromatography that employe liquid mobile phase and a very finely divided stationary phase packed in a column
- Since the particles of the stationary phase are very small (between 2 & 5 µm) , there is a need to pump the mobile phase through a column under a high pressure
- High-performance liquid chromatography (HPLC) is the technique most commonly used for the quantitation of drugs in formulations

Basic principle of HPLC

- Sample is injected into a HPLC system
- Interactions happen between the samples with the mobile phase and stationary phase (column) which results in separation of samples which is detected through the detector and converted into a chromatogram
- The main principle of separation is adsorption
- They travel according to their relative affinities towards the stationary phase.
- The component which has more affinity towards the SP, 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

Introduction……

- Based on the relative polarities of the mobile phase and stationary phases, HPLC can be classified:

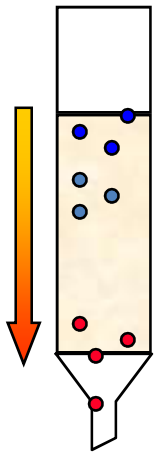
a. Normal phase chromatography

- Stationary phase is highly polar e.g. silica gel
- The mobile phase is relatively non polar solvent e.g. hexane
- The least polar component is eluted first
- Polar compounds travels slower & eluted slowly due to higher affinity to stationary phase
- Non-polar compounds travels faster & eluted first due to lower affinity to stationary phase

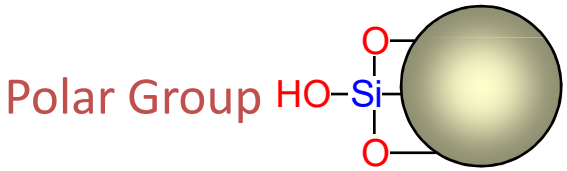
STATIONARY PHASES

(NORMAL POLARITY)

- Silica or alumina possess polar sites that interact with polar molecules

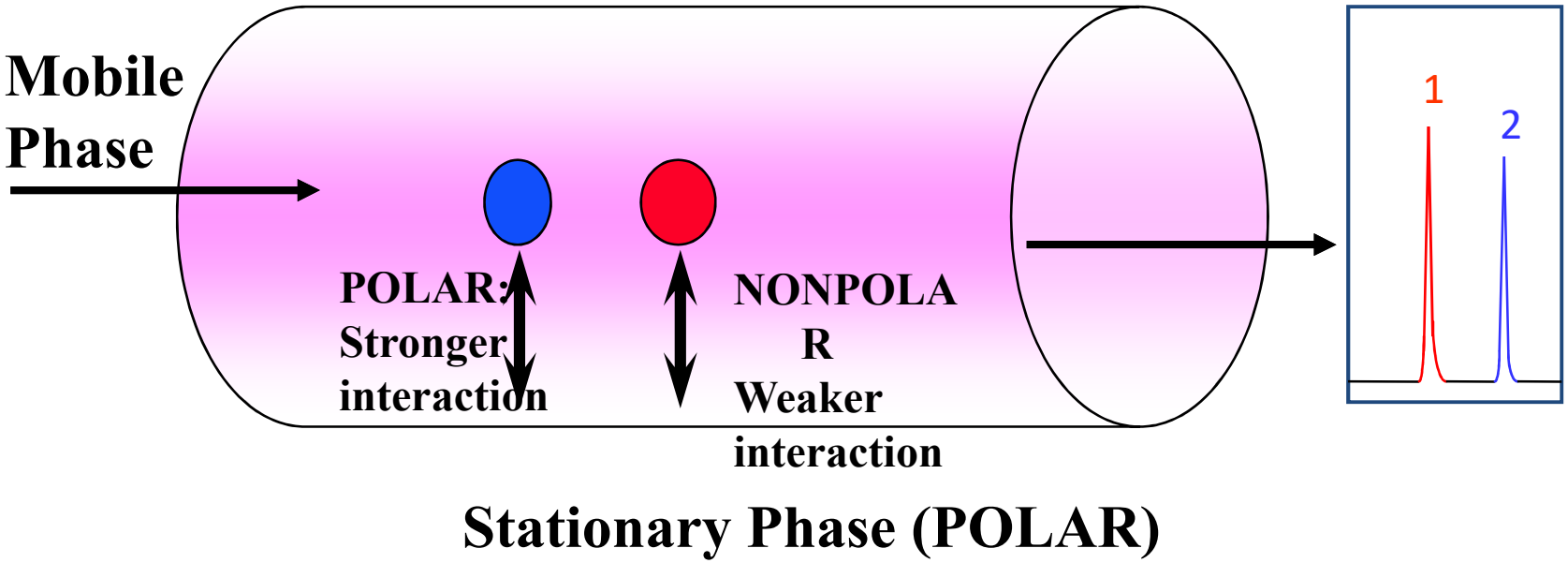


silica



Components elute in increasing order of polarity.

- Normal HPLC


Mobile Phase
POLAR: Stronger interaction
NONPOLAR Weaker interaction
1
2
Stationary Phase (POLAR)

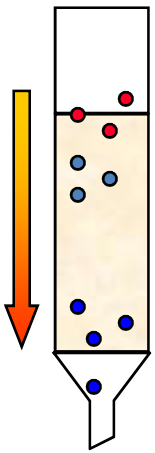
Introduction

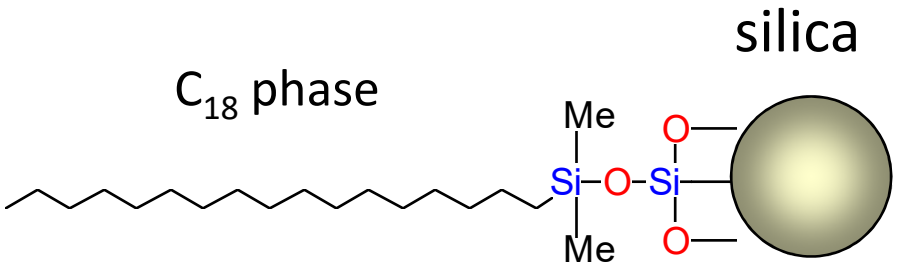
b. Reversed phase chromatography

- The stationary phase is non polar
- The stationary phase is usually Octyl- or Octyl Decyl Siloxane packing (ODS) (C18, C8, etc)
- The mobile phase is relatively polar solvent
  - Eg: methanol- water or acetonitrile- water
- Polar compounds travels faster & eluted 1st due to lesser affinity to stationary phase
- Non-Polar compounds travels slower & eluted slowly due to higher affinity to stationary phase

STATIONARY PHASES
(REVERSE POLARITY)

- If the polar sites on silica or alumina are capped with non-polar groups, they interact strongly with non-polar molecules





Components elute in decreasing order of polarity.

Normal vs. Reverse phase HPLC

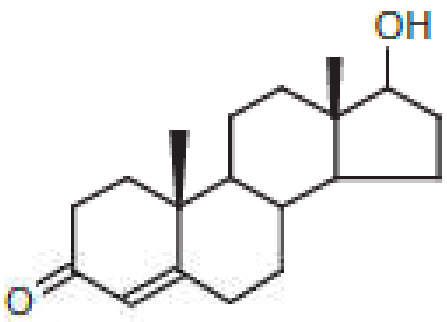
	Normal Phase	Reversed Phase
Stationary phase	Polar (silica gel)	Non-polar (C18)
Mobile phase	Non-polar (organic solvents)	Polar (aqueous/organic)
Sample movement	Non-polar fastest	Polar **fastest**

Structural factors which govern rate of elution of compounds from HPLC columns

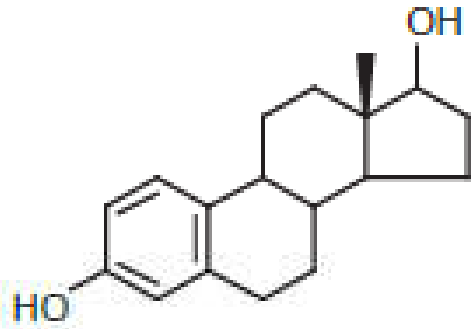
- In a reverse-phase column, the more lipophilic a compound is the more it will be retained
- For a polar column such as a silica gel column, the more polar a compound is the more it will be retained
- Polarity can often be related to the number of the hydroxyl groups present in the molecule

exercise

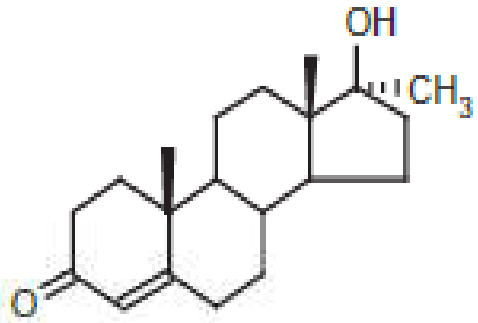
Predict the order of elution, from first to last, of the following steroids from an ODS column with methanol/water (70:30) as the mobile phase



Testosterone



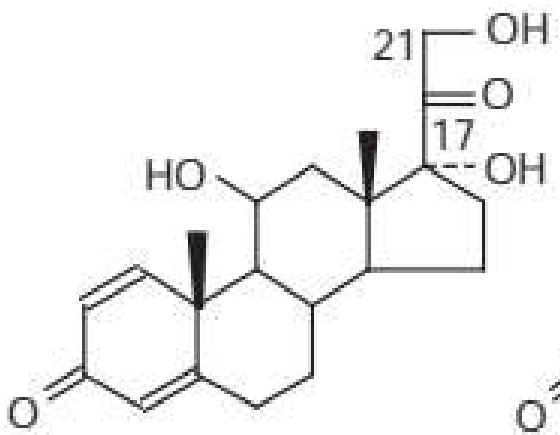
Estradiol



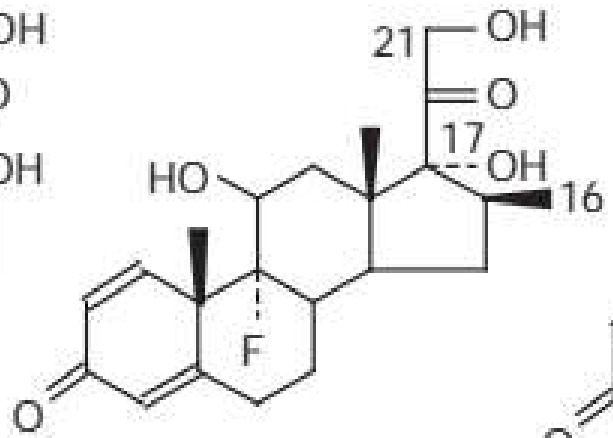
Methyltestosterone

Exercise

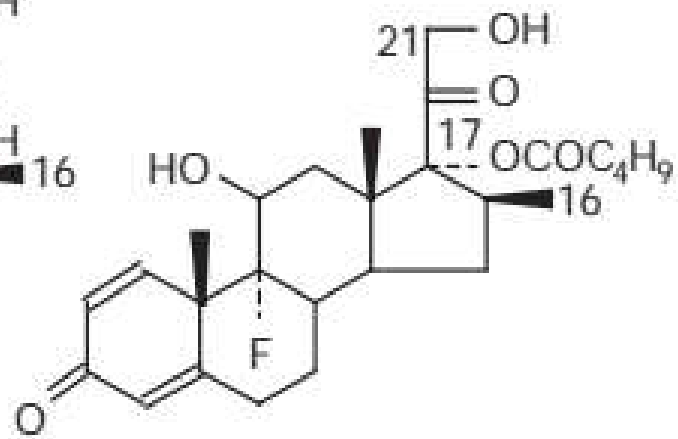
Predict the expected order of elution from a reverse-phase column using a mobile phase containing methanol/water (75:25),



Prednisolone



Betamethasone



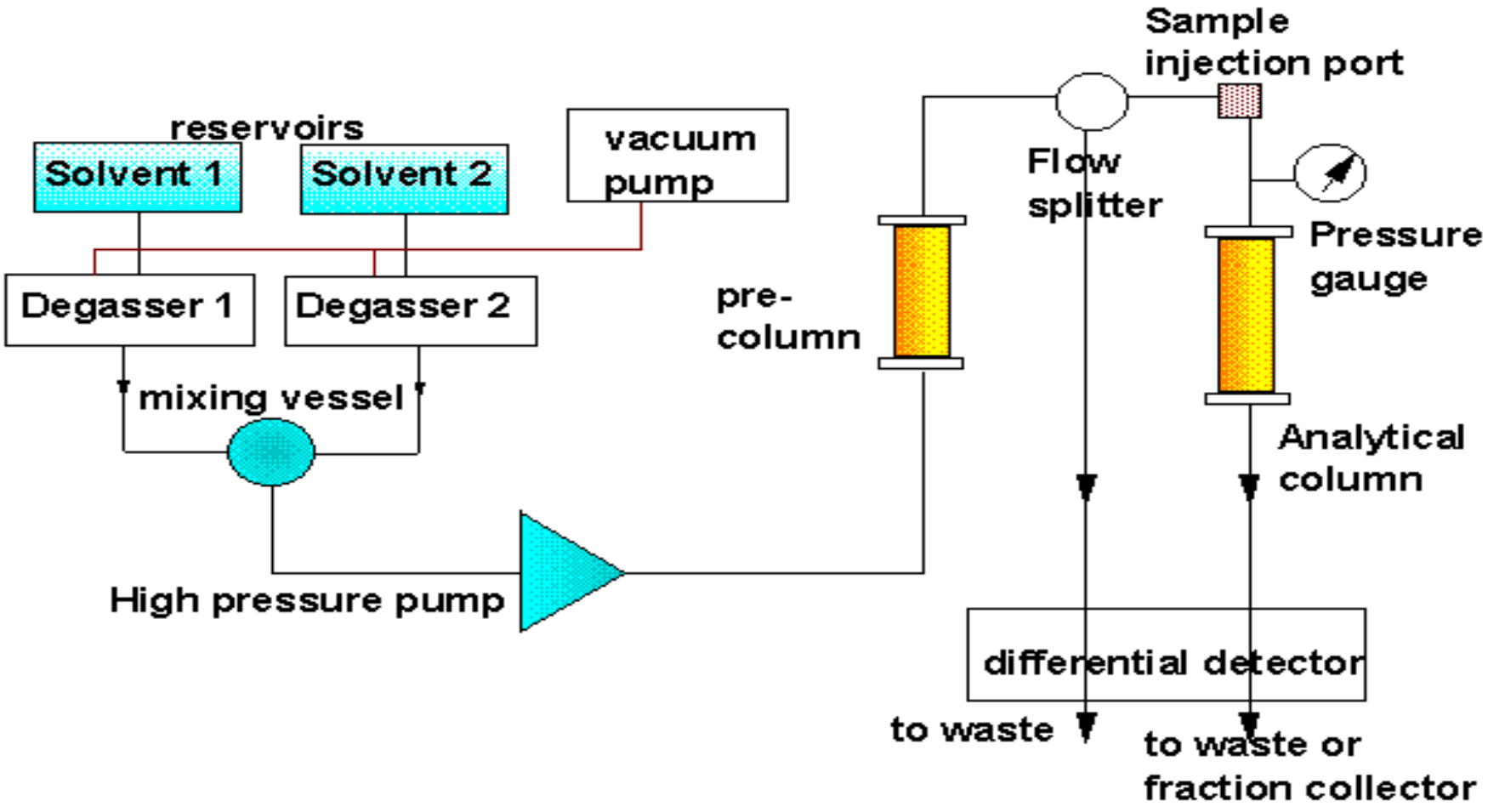
Betamethasone 17-valerate

Instrumentation

The main components of modern LC (HPLC) are:

1. mobile phase reservoir
2. High pressure pump
3. An injection port
4. column
5. detectors
6. recording systems

Schematic diagram of HPLC instrument



Outline of LC-2010

1. Mobile phase reservoirs and Solvents

- Solvent Reservoirs are used to store mobile phase
- A modern HPLC apparatus is equipped with one or more glass or stainless steel reservoir, each of which contains 500 ml or more of a solvent
- Multiple solvents are necessary for performing gradient elution's
- It can be transparent or can be amber colored.
- Solvent reservoirs are placed above HPLC system (at higher level) in a tray

Mobile phase reservoirs……..

- Gases like O2 always present in solvents that form the mobile phase
- This affect separation by modifying the compressibility of the mobile phase and eventual formation of bubbles
- Particularly, oxygen can interfere with electrochemical detection and shortens the life time of a column
- Hence, de-gassing is very important and it can be done by various ways

(i) Vacuum filtration: applying a partial vacuum to the solvent container

(ii) Ultrasonication: ultra sonicator converts ultra high frequency to mechanical vibrations

There are two types of solvent delivery system:

1. **Isocratic elution**

   - is one in which the composition of the solvent remains constant
   - A separation that employs a single solvent or solvent mixture of constant composition with single pump

2. **Gradient elution**

   - composition of the mobile phase is continuously varied
   - Incorporated to achieve a better or/and faster separation

2. Pumping system

- High pressure pumps are needed to force solvents through packed stationary phase beds
- HPLC Pump is very important component of the system
- The Pump delivers the constant flow of the Mobile Phase
- The pumps provide a steady high pressure with no pulsating, and can be programmed to vary the composition of the solvent during the course of the separation

- The role of the pump is to force the mobile phase through the LC system

Pumping system….cont’d

Requirements:

- Ability to generate pressures up to 6000 psi
- Pulse free out put (control flow rate)
- Typical flow rates ranging from 0.1 to 10 mL/minute
- Flow reproducibility (0.5% relative or better)
- Resistance to corrosion by solvent
- the pump must be inert

Types of pump

Two basic pumping system:

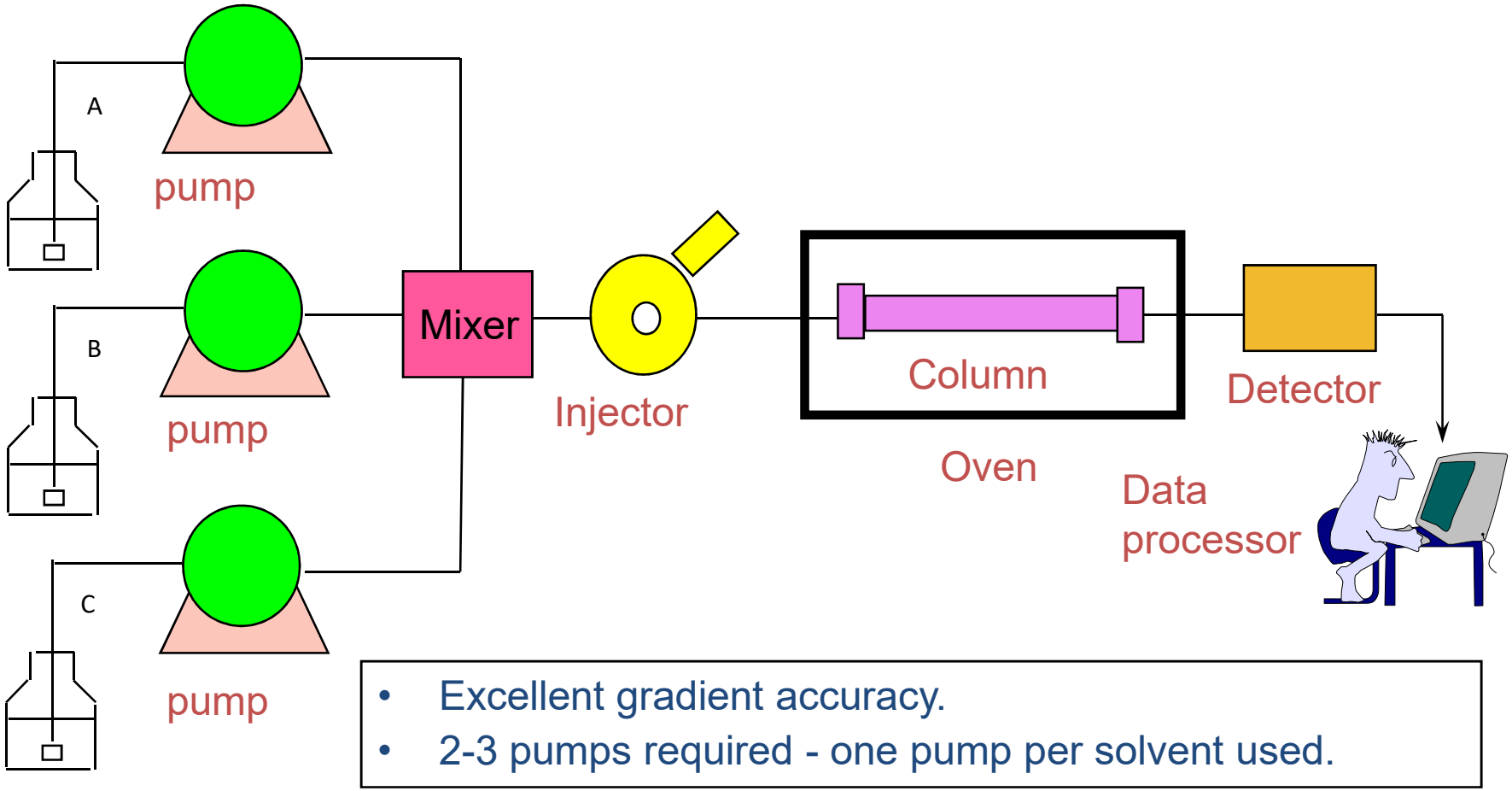
i. Screw driven syringe type pump

- Produce Pulse free delivery whose flow rate is readily controlled
- Inconvenient when solvent must be changed
- Readily adaptable for isocratic elusion

ii. Reciprocating pump

- Most widely used
- Pulsed flow(due to back &forth motion of piston)
- High out put pressure (up to 10,000 psi)
- Readily adaptablle for gradient elusion
- Not dependent on solvent viscosity &back pressure

High-pressure Gradient System



- Excellent gradient accuracy.
- 2-3 pumps required - one pump per solvent used.

3. Sample injection system

- Introduces the liquid sample into the flow stream of the mobile phase
- Typical sample volumes: 20 µl

Common requirements:

- Must allow delivery of very constant smaller sample size
- Reproducibility of the sample into the column must be ensured
- There should not be adsorption and desorption of samples
- Must tolerate pressure up to 10,000 psi
- Sample volume can varies 5 to 500µl

3. Sample Injector

a. Manual sample Injector:

- Manually load sample into the injector using a syringe
- and then injected sample → into the flowing mobile phase →which transports the sample into the beginning (head) of the column, which is at high pressure

b. Auto sampler injector:

- User loads vials filled with sample solution into the auto sampler tray
- Auto sampler automatically
  - Takes appropriate sample volume
  - injects the sample
  - then flushes the injector to be ready for the next sample

4. HPLC column

- It is the back bone of the system where separation is takes place
- It may be steel, glass or plastic tube with 5-30 cm in length and 1-5 mm internal diameter
- Packing materials can be silica, alumina
- Stationary phase is packed with 5 to 10 µm
- Columns are expensive and easily degraded by dust or particles in the sample or solvent
- A pre column or guard column is introduced in front of the main column to increase the life of the main column by removing particulate matter & contaminants from the solvent

Guard column

- A short guard column placed between the injector and analytical column
- Contain the same stationary phase as analytical column
- Used to remove particulate matter and contaminants from the solvent

  - Increase the life of analytical column

5. Detectors

- The elution of a compound from the column is detected as a peak in the chromatogram

Ideal HPLC Detector Properties:

1. Adequate sensitivity (typical range: 10^{-8} – 10^{-15} g solutes/s);
2. Good stability and reproducibility
3. A linear response to solutes
4. A short response time

HPLC Instrumentation

5. Detectors…cont'd

- Optical detectors are most frequently used.
- These detectors pass a beam of light through the flowing column effluent as it passes through a low volume (~ 10 ml) flow cell.
- The most commonly used detector in LC is the ultraviolet absorption detector

a. UV/visible absorbance detector

- Measure the analyte absorption at one or many wave lengths in the UV or visible region
- Important for unsaturated and aromatic cpds
- Principle: beer's law
- Sensitivity: 100 pg to 1 ng

UV-Visible detectors could be

- Fixed wave length
  - Measures at one wave length
- Variable wave length
  - Measures at one wave length at a time, but can detect over a wide range of wave length
- Diode array detectors
  - Simultaneously record the absorbance simultaneously

b. Fluorescence detector

- Based on measurement of fluorescence
- Important for substances that exhibit fluorescence properties
- Compared to UV-Vis detectors , fluorescence detectors offer a higher sensitivity and selectivity (trace level analysis).
- Detection limit: 1-10 pg
- Limitation: only fluorescent analytes measured.

Other types of detectors

- Refractive index (RI) indicators

6. Recorder / Data System

- For higher control levels, a more intelligent device is necessary, such as a data station or minicomputer
- Electronic signals generated by detectors are recorded in the form of chromatographic peak at varied function of time
- Peak Area, height, retention time, base width of chromatographic peak is measured to compute analyte concentration of each peak

Applications of HPLC in pharmaceutical analysis

1. Qualitative Analysis

- Identification of compound identity
- Require a known standard
- Identified by comparing retention time

2. Quantitative Analysis

- The majority of applications of HPLC in pharmaceutical analysis are to the quantitative determinations of drugs in formulations
- Require a standard with known amount of concentrations
- Identified by interpolating the area of unknown into a set of standards with known concentration

Exercise

- Assay of Paracetamol was done by HPLC and Calculate the percentage of stated content in paracetamol tablets using the calibration curve given and the following data:

Data

- Weight of 20 tablets=12.2243 g
- Weight of tablet powder taken=152.5 mg
- Stated content per tablet=500 mg
- Initial extraction volume=200 ml

Dilution steps

- 20 ml into 100 ml
- 10 ml into 100 ml
- Calibration curve for paracetamol: Y= 35656X + 80 where X is in mg/100 ml
- Area of peak obtained for paracetamol in diluted sample extract =44 519