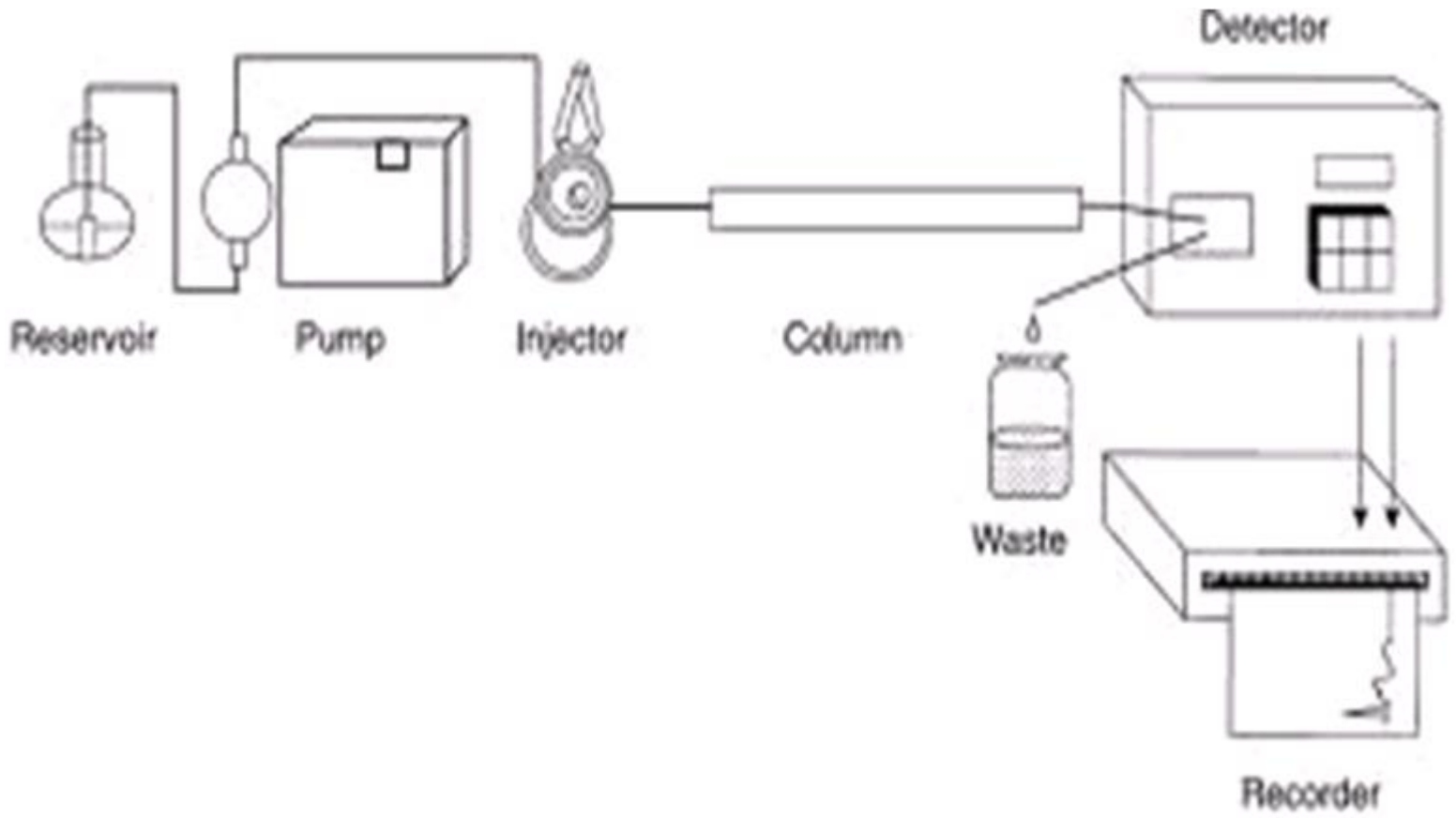
Schematic diagram and basic components of HPLC system

- Most of the HPLC systems are modular. One can just add a new component or accessory to change or extend the capabilities

Schematic diagram of HPLC system



Mobile phase reservoir and its Functions

- Is a tray having glass bottles fitted a lid and 1/8 inch diameter PTFE tubing to carry mobile phase to pump
- Liquid entering in pump should not contain air, dust or any particulate matter which can interfere with pumping action or cause damage to seals and valves- need filteration
- Air bubble can also effect behavior of detector, hence, need to degas the mobile phase

- Filtered solvents extend the life of pump and reduce column plugging
- Stainless steel filtering element (filter size 2µm) at one end of PTFE tubing serves as an in-line filter
- All solvents should be HPLC grade

Explain HPLC Pump

- Function: Passes mobile phase through the column at a high pressure and constant flow rate
- Characteristics:

1-Inetrior of the pump is made up of inert material so that it could not be corroded with solvents

2-May allow to change a range of flow rates

3-Non pulsating solvent flow. Pulsation may cause baseline noise especially if detector is flow sensitive. Pulse effect should be minimized using pulse damper

4-Large dead volume between pump and injector should be avoided

5-Easy to change from one mobile phase to another

6- Easy to dismantle and repair

Types of pumps

- Constant pressure pump

1-Apply a constant pressure to the mobile phase

2- Flow rate is determined by the resistance of the column and restrictions between pump and outlet. Hence flow rate will change if flow resistance change

3- It is not suitable for HPLC analysis

4- Only suitable for packing of column whereby small changes in flow are not important

- Constant flow pump

1-Generates a given flow of liquid so that the pressure developed depends on flow resistance

2-Changes in flow resistance compensated for by a change of pressure

3-Two types of constant flow pumps used for HPLC are

A-Motor-driven syringe pump

B- Reciprocating piston pump (mostly used)

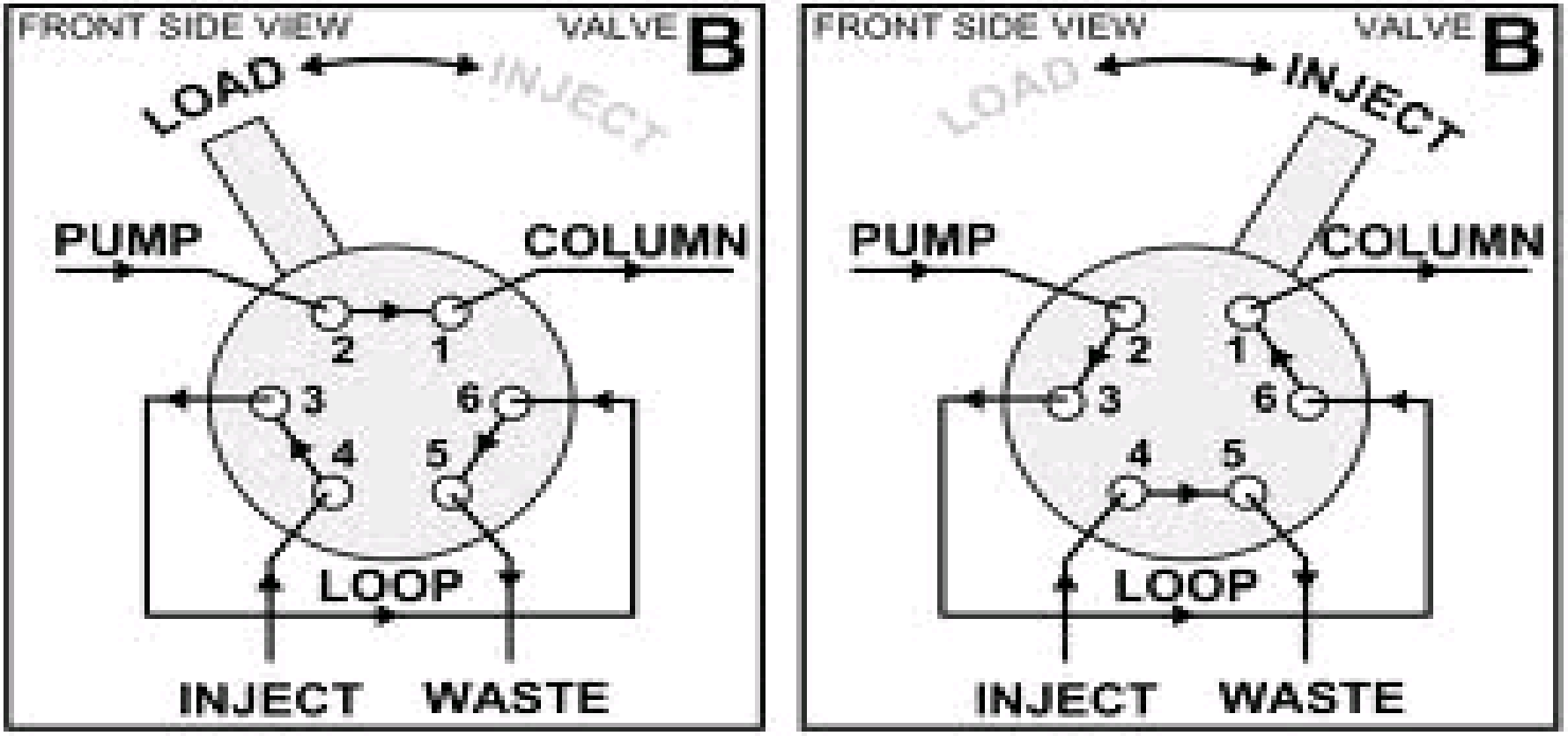
Gradient controller and function in HPLC system

- It is a device that allows one to create a gradient program so as to alter the nature or polarity of the solvent
- Gradient may be linear, concave, convex or even stepped in terms of percentage of the stronger solvent
- Must form a homogenous mixture before it reaches the column

Sample injector

- To introduce sample solution into the column via a sampling loop
- It must have zero dead volume valve
- Manual and automated valve systems are available
- Change sample loop 5-500 µl or larger volumes to alter sample size

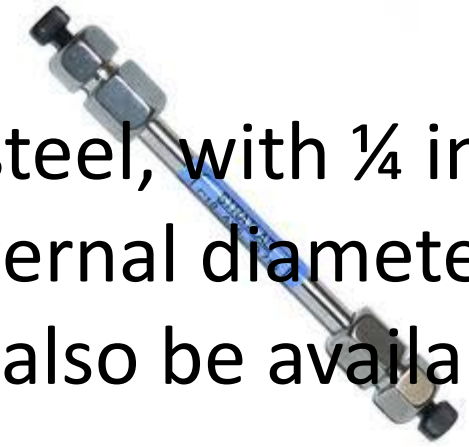
Sample solution loading and injection



Analytical columns of HPLC

- Column: is a heart of chromatography system
- It is a place where separation of components takes place
- It is usually made up of stainless steel, with ¼ inch external diameter and 4.6 mm internal diameter and up to 25 cm long. These may also be available in other dimensions
- Has stainless steel gauze/frit

  at the end of the column to retain packing



- Small bore HPLC columns are also available for low mobile phase flow rates for higher efficiency and small sample size
- Guard column: small column placed between injector and column
- It extends column life by preventing entry of materials from sample or solvent into column
- It also functions as saturator column to avoid dissolution of the stationery phase in the column
- It should have the same packing as of analytical column
- Ratio of guard column: analytical column (1:15 or 1:25)

Column packing

- Three types of packings are used in HPLC

1-Fully porous

- Originally used silica or alumina
- Has porous channels through the packing
- Give low efficiency because solute takes a long time to diffuse from porous structure
- No longer used in analytical HPLC
- However, still used in preparative columns because of high sample capacity

2-Superficially porous layer beads

- Rough surface
- Consist of inert solid core of glass or plastic with a thin outer coating of silica or modified silica
- Fast mass transfer
- High efficiency
- Rapid re-equilibriation
- For analytical separation
- More efficient than porous packing

3-Micro-particulate

- Small diameter 3, 5, 10 µm
- Fully porous
- Spherical or irregular
- Analytical or preparative
- Combines the best features of fully porous and superficially porous beads

Sample preparation

- Goal of sample preparation: obtain a sample with the components of interest free from interfering constituents of the matrix at a suitable concentration for detection and measurement in a suitable solvent
- Sample preparation: liquid-liquid extraction and solid phase extraction
- Sample should be dissolved in same solvent or mixture like mobile phase wherever possible

Detectors

- Monitor mobile phase emerging from the column
- Its output is an electrical signal which is proportional to some property of the mobile phase/solute or both e.g.

1-Refractive index-property of solutes and mobile phase (bulk property)

2- Absorbance (UV/Vis, fluorescence, electrochemical activity)- solute property

- Required characteristics of detectors

1- Sensitivity

2-Linearity

3-Universal or selective response

4-Predictable response, unaffected by changes in conditions

5- Low dead volume (cell volume, length and bore of tubing)

6- Non-destructive

7- Cheap, reliable and easy to use

Types of detectors

- Bulk property

1- Principle: Sense the difference in refractive index between column eluent and reference beam of pure mobile phase

2- Universal, not sensitive (limit of detection 1 μg)

3-Difficult to do gradient elution work

4- Must have good control of temperature of the instrument and composition of mobile phase

- Solute property

1-UV/Vis spectrophotometer

- Most popular
- Only detects solute that absorb UV/Vis radiation
- Mobile phase should not absorb radiation
- Absorption of radiation by solute as a function of concentration is according to Beer Lambert's law
- Limit of detection is sub ng

2-Spectrofluorometric detectors

- Absorb UV radiation and subsequently emit radiation of longer wavelength, either instantly (fluorescence) or after a time of delay (phosphorescence)
- For compounds that are inherently fluorescent, otherwise compound has to be made fluorescent by derivatization using suitable reagent
- Limit of detection 1pg

4-Electrochemical detector

- Measures conductance of eluent (analyte must be ionic)
- Measures current associated with oxidation and reduction of solutes, may be coulometric or amperometric

1- Amperometric detector-most commonly used- a known potential is applied across a set of electrodes typically glassy carbon as a working electrode. It requires conducting mobile phase. Limit of detection pg

Problem-1

- A capsule of 500 mg containing drug paracetamol is dissolved in 200 ml methanol. The solution exhibits absorbance of 0.75 at 300 nm in 10 mm cell. A 10 mg of standard of the drug paracetamol is dissolved in 1000 ml methanol, exhibits absorbance of 0.25 at 300 nm in the same cell. What is the %age of paracetamol in capsule?

Solution

- Concentration of standard = 10 mg/1000ml
  =0.01g/liter
- Absorbance of standard (A) = 0.25
- Cell length = 10 mm =1 cm
- A=abc
- 0.25=aX 1X 0.01
- a=25

- A of the sample= 0.75
- (a)=25
- (b)=1 cm
- (c)= ?
- A=abc and c=A/ab=0.75/25(1)

  = 0.03g/liter
- Concentration = 500 mg/200ml

  =2.5 g/liter

- % age drug=(0.03/2.5)x100=1.2%

Problem 2

- A solution of trimethoprim ($1.00X10^{-3}M$) in methanol exhibits absorbance of 0.6 and 0.009 at 271 nm and 350 nm, respectively, using 9.8 mm cell. A quinine solution of $8 x10^{-4}$ M in same solvent exhibits absorbance 0.04 at 271nm and 0.64 at 350 nm. A tablet containing trimethoprim and quinine is dissolved in 250 ml methanol. The absorbance of this solution, determined in the same cell gave absorbance of 0.75 at 271nm and 0.900 at 350 nm. Calculate the concentration (mg) of each ingredient in the tablet (MW of trimethoprim is 290 and quinine 378)