2.0 INSTRUMENTATION

2.1 High Performance Liquid Chromatography

Chromatographic separations can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. Thus, the chromatographer can separate compounds (more on chiral separations) from each other using HPLC; the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.

Identification of compounds by HPLC is a crucial part of any HPLC assay. In order to identify any compound by HPLC a detector [13] must first be selected. Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels that the assay will be performed. To alter the retention time of a compound, several parameters can be manipulated. The first is the choice of column [14], another is the choice of mobile phase [15], and last is the choice in flow rate.

Identifying a compound by HPLC is accomplished by researching the literature and by trial and error. A sample of a known compound must be utilized in order to assure identification of the unknown compound. Identification of compounds can be assured by combining two or more detection methods.

2.1.1 Instruments

Pumping pressures of several hundred atmospheres are required to achieve reasonable flow rates with packing in the 3 to 10 μm size range, which are common in modern liquid chromatography. Figure 6 is a diagram showing the important components of a typical high – performance chromatographic instrument.

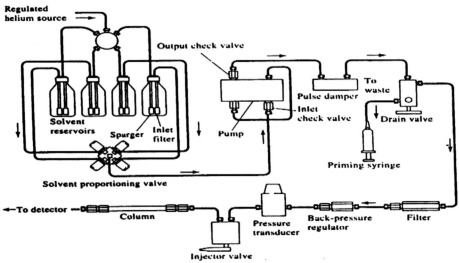

Figure 6. Schematic of an apparatus for High Performance Liquid Chromatography

2.1.2 Mobile Phase Reservoirs And Solvent Treating Systems

A HPLC apparatus is equipped with one or more glass or stainless steel reservoirs, each of which contains 500mL or more of a solvent. Provisions are often included to remove dissolved gases and dust from the liquids. The former produce bubbles in the column and thereby cause band spreading; in addition, both bubbles and dust interface with the performance of detectors. Degassers may consist of a system for sparging, in which the dissolved gases are swept out of solution by fine bubbles of an inert gas that is not soluble in the mobile phase.

2.1.3 Pumping Systems

The function of the pump in HPLC is to pass mobile phase through the column at a controlled flow rate.

In addition to being able to pump solvent at high pressure and constant flow, the pump should also have the following characteristics:

(a) The interior of the pump should not be attacked by any of the solvents that are to be used;

(b) A range of flow rates should be available, and it should be easy to change flow rate;

(c) The solvent flow should be non – pulsing;

(d) It should be easy to change from one solvent to another;

(e) The pump should be easy to dismantle and repair.

A reciprocating pump is employed. Reciprocating pumps usually consist of a small cylindrical chamber that is filled and then emptied by the back – and – forth motion of a piston. The pumping motion produces a pulsed flow that must be subsequently damped. Advantages of reciprocating pumps include small internal volume. High output pressure (up to 10,000psi), ready adaptability to gradient elution, and constant flow rates, which are largely independent of column back – pressure and solvent viscosity.

2.1.4 Sample-Injection System

The most widely used method of sample introduction in liquid chromatography is based upon sampling loops. With these devices, sample is first transferred at atmospheric pressure from a syringe into a sample loop (sample container). Turning the valve from load to inject position connects the sample loop into the high-pressure mobile phase stream, whereby the contents of the sample loop are transferred on to the column. The reproducibility of injections with a typical sampling loop is a few tenths of a percent relative.

2.1.5 Columns for High-Performance Liquid Chromatography

The HPLC column is usually a stainless steel tube packed with the stationary phase. The column is of importance because this is where the separation takes place. Most columns range in length from 10 to 30 cm and have inside diameters of 4 to 10 mm. Column packing typically have particle sizes of 5 to 10 μm.

The stationary phase in HPLC refers to the solid support contained within the column over which the mobile phase continuously flows. The sample solution is injected into the mobile phase of the assay through the injector port. As the sample solution flows with the mobile through the stationary phase, the components of that solution will migrate according to the non–covalent interactions of the compounds with the stationary phase. The chemical interactions of the stationary phase and the sample with the mobile phase, determines the degree of migration and separation of the components contained in the sample. For example, those samples which have stronger interactions with the stationary phase than with the mobile phase elute from the column less quickly, and thus have a longer retention time, while the reverse is also true. The column containing a reversed phase was used in this study. In a reversed–phase column, the stationary phase is nonpolar, often a hydrocarbon, and the mobile phase is a relatively polar solvent. A reversed phase operates on the basis of hydrophilicity and lipophilicity. The stationary phase consists of silica based packing with n–alkyl chains covalently bound. For example, C–18 an octadecyl ligand in the matrix. The more hydrophobic the matrix on each ligand, the greater is the tendency of the column to retain hydrophobic moieties. Thus hydrophilic compounds elute more quickly than do hydrophobic compounds.

The mobile phase used with these packing is often an aqueous solution containing various concentrations of such solvents as methanol, acetonitrile, or tetrahydrofuran. As a rule, most chromatographic separations are achieved by matching the polarity of the analyte to that of the stationary phase; a mobile phase of considerably different polarity is then used. This procedure is generally more successful than one in which the polarities of

the analyte and the mobile phase are matched but are different from that of the stationary phase. Here, the stationary phase often cannot compete successfully for the sample components; retention times then become too short for practical application. At the other extreme is the situation where the polarities of the analyte and stationary phase are too much alike; here, retention times become inordinately long.

Guard columns are placed anterior to the separating column. This serves as a protective factor that prolongs the life and usefulness of the separation column. They are dependable columns designed to filter or remove: 1) particles that clog the separation column; 2) compounds and ions that could ultimately cause "baseline drift", decreased resolution, decreased sensitivity, and create false peaks; 3) compounds that may cause precipitation upon contact with the stationary or mobile phase; and 4) compounds that might co – eluted and cause extraneous peaks and interfere with detection and/or quantification. These columns must be changed on a regular basis in order to optimize their protective function. Size of the packing varies with the type of protection needed.

2.1.6 Detectors

UV Detector

UV absorbance detectors so far are the most popular detectors in HPLC. The principle is that the mobile phase from the column is passed through a small flow cell held in the radiation beam of a UV/visible photometer or spectrophotometer. These detectors are selective in the sense that they will detect only those solutes that absorb UV (or visible) radiation. Such solutes include alkenes, aromatics and compounds having multiple bonds between C and O, N or S. the mobile phase, on the hand, should absorb little or no

radiation. Absorption of radiation by solutes as a function of concentration, c, is described by the Beer – Lambert law:

$$A = ecb$$

Where A = absorbance, b = path length of the cell and e= molar absorptivity, which is a constant for a given solute and wavelength. It is common to measure c in mol dm^{-3} and b in cm, when e will be in $dm^3\ mol^{-1}\ cm^{-1}$.

Strictly, the Beer – Lambert law applies only to monochromatic radiation. However, the detector system does not provide truly monochromatic radiation, but rather a narrow band of wavelengths centered on the selected wavelength.

Both fixed and variable wavelength UV/visible detectors are available. The variable types use a deuterium and/or a tungsten filament lamp as the radiation source, and can operate between about 190 and 700nm. They will have a number of switched sensitivities (absorbance ranges) measured in 'a.u.f.s.' which means absorbance units corresponding to full scale deflection on the recorder. Fixed wavelength detectors normally operate at 254nm or 280nm, but other wavelengths are possible [16].

Detector	Radiation source	Wavelength range, nm	Bandwidth, nm	Absorbance ranges a.u.f.s.	Noise a.u.
Waters 484	Deuterium lamp	190 – 600	8	0 – 2	1.5×10^{-5}

Photodiode Array Detector

Photodiode array detectors can be used to measure and detect samples over the entire UV to visible (UV-Vis, wavelength range 190-650nm) spectrum. They are highly beneficial tools in identification and analysis of sample compounds. To detect over an entire spectrum, the detector must proceed in one of two ways. The first is to scan across the entire spectral region, which may be accomplished by a scanning monochromator spectrometer: A standard scanning monochromator spectrometer uses a tungsten or deuterium lamp that emits a continuous light source. The light is then directed across a grating or prism that reflects the light through an exit slit to the sample cell. The sample is then detected by a photomultiplier tube. The wavelength of the light can be adjusted by rotating the grating or prism, but only one region can be scanned at a time. Subsequently, data points are obtained at different times, which may hinder efficiency and accuracy [17]. The second method involves monitoring the entire UV-Vis region simultaneously.

2.2 Thin – Layer Chromatography

At present, TLC is the simplest, needs minimum instrumentation, minimum laboratory space and provides excellent resolution of components. With the TLC method it is possible to detect simultaneously a wide variety of drugs in a single run. Furthermore, an unequivocal specificity can be achieved by the careful design of the developing solvent and the subsequent selection of the visualization techniques. The results are qualitative and not quantitative, i.e., they provide only "yes/no" or "positive/negative" result.

The two stages which are fundamental to all TLC procedures: (1) separation of drugs on the TLC plate, and (2) the detection and read – out of the separated drugs.

First step. The first step in chromatography is separation of the drugs. This step first involves the spotting of the residue on a plate precoated with a thin layer of solid support phase, usually silica gel. In TLC, the transfer of the residue from the test tube on to the plate is the most critical step. In fact the state of the art of TLC lies in quantitative spotting of the residue. The plate is then placed in a developing solvent and by capillary action the solvent slowly moves upward on the plate in a uniform manner. The solvent is allowed to rise to a certain distance to the extent the resolution of the components is desired. The plate is then taken out and allowed to air dry. The separation of various drugs and their metabolites is achieved because different drugs migrate or travel different distances from the starting point. It must be pointed out that the efficacy of a TLC technique primarily depends on the proper design of the developing solvent; a poorly designed developing solvent such as too polar solvent mixture could result in false positives for various drugs of abuse.

Second step. The second step involves the detection of the separated drugs and their interpretation. Detection of various drugs and their metabolites is achieved by spraying the plate with chemicals which produce characteristic colored spots with various drugs. By the combination of specific spraying reagents and the use of UV light, different drugs can be specifically identified.

2.3 Gas Chromatography – Mass Spectrometry

Gas Chromatography–Mass Spectrometry (GC–MS) allows for the on–line separation of complex mixtures of volatile species (nonvolatile if derivatized) followed immediately by mass selective detection.

Chromatography is widely used for separation, identification, and determination of chemical components in complex mixture. Chromatography is a technique in which the components of a mixture are separated based upon the rates of at which they are carried through a stationary phase by a gaseous or liquid mobile phase. GC contains a mobile phase that is carrier gas and stationary phase, which is surface active in the column. The major component of GC is shown in Figure 7.

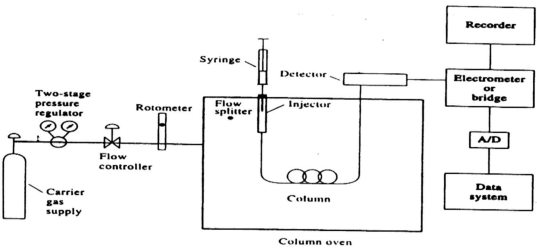

Figure 7. Schematic of Gas Chromatography System

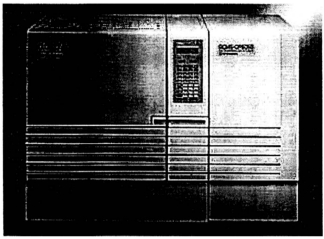

Figure 8. Picture of Gas Chromatography – Mass Spectrometry

In Mass Spectrometry the compound under investigation is ionized and fragmented ion source. The ions are then selected by mass/charge ratio by passing them through electrostatic sectors in order to generate a spectrum.

The spectrum typically contains peaks for the molecular ion and all of the fragments ions. Analysis of the fragmentation pattern can give important information about molecular ion.

Most GC/MS analyses are performed using electron impact (EI), but GC/MS using CI is more difficult but will be considered on an individual basis. This electron impact source produces high electrons which bombard the sample ionizing and fragmenting it.

This mass spectrometry has the capacity to observe ions up to a mass/charge ratio of m/z = 6 focusing instrument is capable of highly accurate mass measurement (1ppm). The molecular ion, determined with such accuracy, can enable the composition of the component established.

Basic Components of a GC – MS

2.3.1 Gas Supply

Gas chromatographs require a supply of carrier gas of sufficient quality and pressure to achieve the desired separations. Carrier gases, usually nitrogen, helium or hydrogen are normally supplied from compressed gas cylinders. Carrier gas should be inert, dry and free of oxygen to prevent degradation of the column. For capillary column work, helium is the preferred gas and provided that the system is free of leaks, it is not very expensive since a cylinder should last in excess of six months. If running with gas chromatograph with a mass spectrometer then helium gas must be used.

High – purity gases should be used because apart from the problems with oxygen and moisture in the carrier gas, it is important that gas supplies should be free of other contaminants that might be detected by the detector. Organic impurities in the carrier or in the hydrogen and air to the flame ionization detector may give rise to a significant base current response which may reduce the sensitivity of the detector.

2.3.2 Column Oven

This is normally an accurately temperature controlled fan – blown oven with adequate space to accommodate columns and with an even temperature distribution throughout the oven. Oven can normally be temperature programmed at a variety of rates with isothermal periods set as desired. Larger ovens are generally easier to work in, particularly when installing columns.

2.3.3 Injection System

The injection system provides a means of putting the sample or the sample solution onto the column. The injection device also provides a means of maintaining the pressure and flow through the analytical column during the injection process and at the same time prevents the ingress of air into the column system. The principle capillary injection devices are the 'split/splitless' injector and the 'on – column' injector.

The most common method of sample injection involves the use of a microsyringe to inject a liquid or gaseous sample through a silicone – rubber diaphragm or septum into a flash vaporizer port located at the head of the column. The sample port is ordinarily about 50° C above the boiling point of the least volatile component in the sample.

2.3.4 Splitless Injections

In splitless injection, the splitter vent is closed so that the entire sample flows onto the head of column. After a specific time called the purge activation time, the splitter vent is open to purge solvent from the injector and any low – boiling component in the samples that are not absorbed by the column. Splitless injector, therefore, concentrated the sample onto the head of the cool column and purges most of the volatile solvent. For this reason, and because large amounts of samples can be injected, splitless injector is used for trace analysis. The splitless method is not recommended for wide – boiling range sample if quantitation is required. For best result, the solvent boiling point should be at least 20° C below the lowest boiling component of the sample. Although splitless injection is the preferred method for trace analysis, it does require optimization of such parameters as column temperature as column temperature and purge time.

2.3.5 Sample Inlet Systems

The introduction of a sample into GC is the first stage in the chromatographic process and its determine efficiency of the separation procedure and the accuracy and precision of the qualitative and quantitative results. The sample may be introduced as a liquid introduced into chromatography via an injected port. Liquid sample injectors contain a self – sealing septum to retain the high – pressure carrier gas. The resealing capability of the septum depends on temperature, flexibility of the rubber, sharpness of the syringe needle and design of the injector. The septum holder usually incorporates a needle to guide to reduce the mechanical damage. High injection block temperatures can gradually lead to loss of flexibility of the silicone septum due to degradation, particularly at temperature over 300°C. This is accompanied by septum bleed of low molecular weight and depolymerised material which results in ghost peaks and baseline drift. Another phenomena with similar results is the memory effect, a consequence of the ability of silicon rubber to absorb compounds which are later desorbed. These problems can be reduced by the use of PTFE – coated septa.

2.3.6 Capillary Column Gas Chromatography

For gas – chromatographic columns, a major growth in the use of open tubular has occurred in the present day. Fused silica capillaries are drawn from specially purified silica that contains minimal amounts of metal oxides. These capillaries have much thinner walls than their glass counterparts. The tubes are given added strength by an outside protective polyimide coating, which is applied as the capillary tubing is being

drawn. The resulting columns are quite flexible and can be bent into coils having diameters of a few inches. Silica open tubular columns offer several important advantages such as physical strength, much lower reactivity toward sample components, and flexibility[18].The most widely used silica open tubular columns have inside diameters of 0.32 and 0.25 mm such as J & W Scientific Products DB –1 is a simple methylsilicone phase column[19]; DB – 5 is a 5% phenyl, methylpolysiloxane phase column, for non – polar separations[20].

2.3.7 Column Temperature

Column temperature can be progressively increased whole the flow rate is kept constant to avoid problems of the least volatile components in a mixture taking too long to elute and hence forming broad tailing peaks. The rate of temperature increase is preset and typically involves as initial – hold stage when starting temperature is held, perhaps long enough for the solvent to elute, a temperature ramp stage where the temperature is increased at a selected rate, usually between 1 to 40°C min^{-1}, and a final stage where the upper temperature is held for a pre – selected time. At the end of the cycle the oven is cooled before a new analysis is carried out. Again, microcomputer controlled instrument functions enable temperature increase profiles other than a linear increase profiles other than a linear increase to be programmed and also many more stages can be included in the overall cycle.

2.3.8 GC Interface

For capillary columns, the usual practice is to insert the exit end of the column into the ion source. This is possible because under normal operating conditions the mass spectrometer pumping system can handle entire effluent from the column. It is then only necessary to heat the capillary column between the GC and MS ion source, taking care to eliminate cold spots where analyte could be condense. The interface must be heated above the boiling point of the highest – boiling component of the sample (250 – 320 °C).

2.3.9 Mass Spectrometer

Mass spectrometer work on the principle that a charged ion being propelled through a curved magnetic field will be deflected inversely proportional to its molecular mass and proportionally to its charge. The lighter the mass the more deflection that will occur at a given charge. The higher the charge the more deflection that will occur at a given mass.

There are several operation modes in which GC – MS may be employed. For identification of a few components present at 0.1 – 1 mg/ml, the spectrometer may be scanned over the entire mass range to give individual spectra as each component emerges, using the total ion current (TIC) as a monitor. Background spectra can be subtracted from the sample spectra with a data system to remove noise due to column bleed and other contaminants. The TIC is a nonselective monitor, which produces a chromatogram similar to a FID. This may result in difficulties when attempting to match peaks with those observed in gas chromatogram from selective detector.

Another mode of acquiring mass – spectra data is by ion monitoring (SIM) where the ion current is measured at selected masses. The SIM methods are use in the quantitative determination of certain specific compounds in a complex mixture, especially when the compounds are present at low levels (up to ppb level). The advantage of this method is that both high sensitivity and high specificity are achieved. Although SIM is sensitive to picograms of material, this sensitivity is highly dependent on the matrix containing the compounds of interest and the interference that are produced. Frequently, it is only possible to detect nanogram levels of the compound because of the chemical interference. It is unusual to obtain erratic results when using small amounts of material because interfering ions are within the mass window of the selected ion monitoring experiment even when internal standards are employed.

2.4 Solid – Phase Extraction (SPE)

Solid – phase extraction (SPE) is an extraction method that uses a solid phase and a liquid phase to isolate one, or one type, of analyte from a solution. It is usually used to clean up a sample before using a chromatographic or other analytical method to quantitate the amount of analyte(s) in the sample.

Solid – phase extractions use the same type of stationary phases as are used in liquid chromatography columns. The stationary phase is contained in a glass or plastic column above a frit or glass wool. The column might have a frit on top of the stationary phase and might also have a stopcock to control the flow of solvent through the column. The picture below shows a cartridge on a vacuum manifold, which increases the solvent floe rate through the cartridge. A collection tube is placed beneath the SPE cartridge (inside

the vacuum manifold for the example in the picture) to collect the liquid that passes through the column [21].

Sep–Pak C18 cartridges:

The packing in these columns is based on 5μm spherical silica modified to a hydrophilic external surface but with the internal surface (the pores) having a selective reverse phase bonding. They are used for the determination of drugs or other analytes in serum. Proteins in the serum are not retained by the outer surface of the packing and are too large to get into the pores of the structure. They are therefore unretained and pass straight through the column. Drugs and other metabolites, being smaller, can penetrated the inner surface of the packing and undergo a reverse phase separation.

In order for the proper 'phase interface' to exist between the sorbent (C18) and the sample, the column must be solvated prior to loading the sample. For the non – polar sorbents (C 18), it is important that the residual solvation solvent (typically methanol) that remains in the bonded phase after the solvation step is not removed by sucking air through the column prior to loading the sample.

When the components of interest are retained on the cartridge they can subsequently be removed by eluting with a solvent of a different polarity. The principle of the method[22] :

(i) Load sample

(ii) Elute with polar solvent (e.g. methanol) to remove polar material

(iii) Elute with less polar solvent (e.g. petroleum ether) to remove non – polar material.