CHAPTER-III
Instrumentation of High Performance Liquid Chromatography
Figure 3.1 shows a schematic representation of an HPLC chromatograph. The main components of the HPLC chromatography are a high-pressure pump (1) a column (2), injector system (3) and a detector (4). In this technique, eluent from the solvent reservoir (5), is filtered through a filter (6), pressurized and pumped through the chromatographic column. A mixture of solutes injected at the top of the column is separated in the column while traveling down and are detected individually by the detector, if proper separation takes place. The concentrations are expressed as peak heights and / or as peak areas. In addition, components such as solvent reservoir, on – line filters, pressure gauges (7), integrators (8), printers and minor components are also required. The main components may be individually purchased and assembled or bought as a whole. Line filters, flow calls and pump components such as non-return valves or high pressure seals must be removed from time to time, cleaned, and replaced. A full description is given in references 1-5.

Pump

There are several types of pumping systems available, viz., direct gas pressure, pneumatic (and hydraulic) intensifier pumps, reciprocating and screw driven pumps. In the direct gas pressure pump gas under pressure exerts pressure directly into the column. Even though this type is cheapest and most reliable, as no moving parts are involved, is not suited for research. A pneumatic intensifier pump is operated under gas pressure.

Hydraulic intensifier pumps work on the same principle as pneumatic pumps, but pressure is exerted by liquid and not by gas. An electrical current usually runs a reciprocation piston pump. The driven piston either pressurizes the eluent directly or hydraulic oil, acting via a diaphragm, pressurizes the liquid from the solvent reservoir. While pneumatic intensifier pumps work at constant pressure, reciprocating pumps work
Figure 3.1: Schematic Diagram of complete HPLC Chromatography
on constant flow. In syringe pumps an electrically driven lead-screw moves a piston, which pressurizes a given volume of solvent and delivers a pulse less constant flow of solvent to the system. These pumps are expensive, but reliable. A combination of two syringe pumps allows the generation of multi-shaped gradient profiles and use of flow programming.

Column and Injection System

The most efficient chromatogram can be expected, if the solute, introduced at the top of the column as a point injection, does not reach the walls of the column as it travels down. The relationship between column length and diameter and particle diameter for these so-called infinite “diameter” column packed with porous particles and taking into account the distributed wall regions has been given by Knox et. al. as

\[
\frac{(d_c - 60 \, d_p)^2}{L \, d_p} > 16 \left( \frac{1.8}{v} + 0.060 \right) \tag{1.36}
\]

Where \( d_c \) is the column diameter and \( L \) is the column length. For difficult separation two or more columns can be connected in series and may have a higher efficiency. Efficiencies as high as 2,50,000 have been reported using such coupled columns. Recycle chromatography in which the column effluents are sent back to the pump and then continuously rechromatographed is also a cure for poorly resolved components.

Injection Valves

Injection valves operate by loading a sample loop while bypass stream into the column pumps the mobile phase. Valves may be either a fixed volume loop or an external replaceable loop, the flow is switched through the loop, carrying with the sample solution onto the column. It is possible to introduce reproducible volumes into the column with
this system, even though it is somewhat wasteful, as the loop has to be filled with more than the injection volume. External loop injections are advantageous since it is possible to change the injection volumes.

**Automated Injection Devices**

Devices are commercially available which will automatically inject up to 384 samples. These devices are very useful in the clinical and industrial fields where a large number of samples are to be analysed.

**Detection in HPLC**

Several types of detectors are used in HPLC and brief reviews are available. By far the most widely used detector is the UV detector, which measures the change in absorption as the solute passes through a flow cell (usually 5 – 50μL volume) in a UV transparent solvent. UV detectors are concentration sensitive and have the advantage that they do not destroy the solute. Not all molecules possess a sufficiently strong chromopore for satisfactory detection. Here the possibility of derivatisation has to be explored. In recent times photodiode array detectors are available which give a three dimensional picture of the absorption profile as a function of time and wavelength from 190 – 800 nm. Refractive index detectors function by measuring the change in refractive index in the eluent as the solute flows through the sample cell. In fluorimetric detectors, the solute is excited by UV radiation of a given wavelength (the excitation wavelength) and the fluorescent energy which is emitted at a longer wavelength (the emission wavelength) is measured. Fluorimetric detection has been successfully employed with compounds which are naturally fluorescent or which have been rendered fluorescent by derivatisation.
The latest generation fluorimetric detectors are several times more sensitive than the UV detectors. The electron capture detector\textsuperscript{17} is useful for detecting halogenated molecules. Column effluent, in this case, is volatalized in an oven purged with nitrogen and the halogenated solutes are determined. A potentially useful and selective range of detection system can be envisaged involving reactions of the solute after elution from the column. Several post column reaction detectors include the well-known ninhydrin reaction for amino acids\textsuperscript{18}, the 2,4-dinitrophenyl hydrazine reaction for the carbonyl compounds\textsuperscript{19} and the cerium reaction detector for carbohydrate analysis\textsuperscript{20}. In the transport detector, column effluent is picked up on a wire or a porous disc, the solvent evaporated and the solute samples pyrolysed in a hydrogen/air atmosphere and detected using a flame ionization detector\textsuperscript{21-22}. HPLC is interphased on a mass spectrometer by transporting the eluent into a chamber where solvent is evaporated and the solutes are ionized and detected in a mass spectrometer\textsuperscript{23-30}. Various other detectors for HPLC have been described, including a commercially available microwave plasma detector\textsuperscript{31-32}, infrared detector\textsuperscript{33-34}, atomic absorption\textsuperscript{35-36} and flame photometric detector\textsuperscript{37}. Detectors based on the heat of adsorption\textsuperscript{38}, spray impact\textsuperscript{39}, phase molecular fluorescence\textsuperscript{40}, dual UV–fluorescence\textsuperscript{41} and radiometric detection\textsuperscript{42-43} have also been reported.

**UV spectrophotometric detection**

**Sensitivity and Selectivity**

In most cases HPLC method development is carried out using ultraviolet (UV) detector with either a variable wavelength (spectrophotometer) or a photo diode array detector (PDA). In HPLC alternate methods of detection are selected only when (1) samples have little or no UV absorbance (2) analyte concentrations are too low for UV detection (3) matrix of samples interfere and (4) qualitative structural information is
required. Detector type is selected based on potential interference in three interrelated ways, viz, sensitivity, selectivity and baseline noise.

**General considerations**

In UV detection the light source is typically a deuterium lamp, which provides acceptable high intensity from 180 to 400 nm. When measurements at higher wavelengths (> 400 nm) are required a higher energy tungsten-halide lamp is often used. However most HPLC measurements are done at wavelength below 400 nm. Light from the lamp passes through the solution contained in a microcell and impinges on a diode that measures the light intensity, $I$. Usually the light from the lamp is also directed against another diode to measure the intensity of the incident light $I_0$. The detection electronics converts the signal from the two diodes into absorbance $A$ and transmits to the data system.

$$A = \log \frac{I_0}{I} \quad \text{(1.37)}$$

Analyte concentration $C$ in the flow cell is related to absorbance $A$, analyte molar absorptivity and the flow cell length, $L$ by Beer's law.

$$A = CEL \quad \text{(1.38)}$$

A general goal in selecting experimental conditions that affect the determination is to maximize the signal $S$ (equal to $A$ at peak maximum) of the sample components of interest.

**Choice of wavelength**

For many samples good analytical results are obtained only by careful selection of the wavelength used for detection. This choice requires knowledge of the UV spectra of
the individual sample components. If analyte standards are available, its UV spectra can be measured prior to HPLC development. Alternatively a PDA (Photo diode Array Detector) permits the acquisition of the UV spectra for all sample concentrations during method development.

**Sample absorbance**

The wavelength of the UV radiation must be chosen such that the absorption is maximum for analytes and is negligible for the mobile phase. This problem is not acute for a single analyte determination, but when several analytes are to be determined in a solution the problem assumes importance. A balance wavelength has to be chosen in such circumstances. If sample interference (e.g. near $t_o$) complicates the separation and quantitation of the compounds, a choice of wavelength is absolutely necessary.

The detector signal $A$ is proportional to the molar absorptivity $€$ of the compound of interest. For UV detection to provide adequate sensitivity for the analysis of the major sample components, $€$ should be greater than 10 at some wavelength above 185 nm. For trace analysis $€$ should be $> 1000$. The trace analysis of substances below the value of 100 for $€$ is usually not possible. The only organic compounds for which UV detection is completely unsuitable are saturated hydrocarbons, amino acids and nitrile derivatives. Saturated hydrocarbons substituted by ether (-O-), hydroxy (-OH), chloro (-Cl), carboxy (-COOH) or ester (-COOR) groups have marginal $€$ ($€ < 100$) and may require detection at lower wavelengths (185 to 210 nm). When the detection wavelengths are less than 210 nm, sample interferences generally absorb strongly and the choice of mobile phase solvents and additives is somewhat restricted.
Wavelengths < 200nm, even though available in some instruments, are not convenient as the detection is less rugged. Compound types other than those mentioned above generally have larger values of $\epsilon$ and can be detected at higher wavelength (> 210nm). Aromatic compounds have small values of $\epsilon$ (<1000) at wavelength above 210 nm. The mobile phase must transmit sufficiently at the wavelength used for detection. As the light intensity reaching detector probe decreases baseline noise increases and detection sensitivity decreases. Base line noise increases significantly when $A > 0.7$ for the mobile phase. This suggests that the mobile phase absorbance should be < 0.5 at the wavelength of detection. When absorbance $A$ of the mobile phase exceeds a value of about 1.0, the detector becomes unstable. Water is effectively non-absorbing above 180 nm, so can be ignored. As far as acetonitrile is concerned, detection at 200nm or higher is possible. Normal phase chromatography uses solvents that are generally more strongly absorbing, so that detection at higher wavelengths is normally required.

**Signal, Noise and Assay Precision**

Precise results are of prime importance in carrying out quantitative analysis by HPLC. Detection affects precision via the signal / noise ratio (S/N). Signal $S$ refers to the base line corrected absorbance of the analyte peak and noise ($N$) refers to the width of the base line. Base line noise has normally two components – a short term (high frequency) contribution from strong light and the detector electronics and a long term contribution from temperature fluctuations, pump noise and / or a dirty column. High frequency noise is more important for a noise time of 0.15 s and long term noise is more important for noise time of 55s. A rough estimate of assay precision as a fraction of $S / N$ is possible. The coefficient of variation due to low values of $S / N$ can be shown to be
It can be derived that

\[ \text{Minimum mass (µg)} = \frac{1.25 \times 10^5 M V_m (1 + K) N}{(CV) N^{1/2} L C} \]  

(1.40)

Here \( M \) is the analyte molecular weight (Daltons Da), \( V_m \) is the column dead volume (ml), \( N' \) the base line noise (in terms of A), \( CV \) is the desired precision (in %), \( N \) is the plate number, \( L_{fe} \) is the path length of the flow cell and \( C \), the analyte molar absorptionity. The minimum concentration of the analyte (µg/ml) can be calculated as follows

\[ \text{Minimum analyte concentration (µg/ml)} = \frac{\text{minimum mass (µg)}}{\text{sample volume (ml)}} \]  

(1.41)

**Better Assay Precision**

When assay precision varies with analyte concentration, better precision can be obtained by increasing the S/N ratio. This can be achieved by an increase in signal \( S \) or a decrease in noise \( N \). An increase in the signal \( S \) can be achieved either by increasing the analyte concentration in the flow cell or by an increase in the flow path length. The concentration \( C \) in the flow of flow cell is given by

\[ C = \frac{0.4 C_0 V_s (N)^{0.5}}{V_m (1 + k)} \]  

(1.42)

Increase in peak signal can be achieved in the following ways:

1. Increase in the analyte concentration \( C_0 \).
2. Increase in the injected volume sample \( V_s \).
(3) Increase in column efficiency $N$.

(4) Decrease in the column volume $V_m$.

(5) Decrease in analyte retention $k$.

Noise may be reduced in several ways, depending upon whether high frequency or long term noise is most prevalent. Noise increases as light intensity decreases due to ageing of the lamp. Assay precision is degraded significantly by detector noise which can increase with lamp age. Therefore the precision of the method can vary with the time with the same detector or when different detectors are used. The optimized value of rise time $T$ is 2.0s. The optimum rise time or time constant depends upon whether peak height or peak area is used for quantization. Peak heights are used for trace analysis. Pump pulsations can contribute to long term noise. Base line noise type is characterized by a regular rise and fall of base line, which parallels cycle times of the pump. The use of pump dampers has definitely reduced this type of noise. Another kind of long term noise is caused by contamination of the column with substances of the prior injection. Base-line noises of this kind are fairly common when “dirty” samples are used, e.g. samples of biological samples like plasma, serum etc., and environmental samples of water or soil, organic reaction mixtures etc.

**Detection Linearity**

The equation

$$A = C \cdot C \cdot L \quad \text{(1.38)}$$

is obeyed over a wide range of absorbance values, typically of $A$ up to 1.0. Assuming a minimum noise of $2 \times 10^{-5} \text{ A}$ and a minimum quantifiable signal ($\text{CV} = 20\%$, $S/N= 2.5$) a dynamic range of $2 \times 10^{-2}$ to 1.0 is therefore available. A wide dynamic range is one of the many reasons for the popularity of UV detection. When several analytes are present in
the same sample, it may not be possible to measure each at their peak maxima. This does not present a problem as linearity is usually observed for \( A < 0.1 \) even when measurements are observed at both sides of the peak absorption bands.

**The Systematic Approach for the Detection:**

For accurate detection of the signal, the following steps are to be taken in chronological order.

1. Selection of wavelength for maximum of \( \varepsilon \) (S)
2. Injection of the largest possible sample volume (V)
3. Concentration of the sample for increase in mass injected (m)
4. Reduction of k value to minimum possible (M)
5. Increasing detector time constant (T)
6. Ensuring that aged lamp is replaced with new lamp (S, N)
7. Use of pulse dampers to eliminate pump noise (P)
8. Minimizing late elutes with sample clean-up or column switching (C)
References


31. Wilks Scientific Corp. Box 449, S. Norwalk, Ct 06856 USA.


