HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1.1 Introduction to HPLC

In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development and production. It is ideal for the analysis of many drugs in both dosage forms and biological fluids due to its simplicity, high specificity and good sensitivity.

High Performance Liquid Chromatography (HPLC) is a technique that has arisen from the application to liquid chromatography the use of an instrumentation that was originally developed for gas chromatography. High Pressure Liquid Chromatography was developed in the mid-1970 and was improved with the development of column packing material and the additional convenience of on-line detectors. The various components of HPLC are pumps (solvent delivery system), mixing unit, gradient controller and solvent degasser, injector (manual or automatic), guard column, analytical columns, detectors, recorders and/or integrators. Recent models are equipped with computers and software for data acquisition and processing. The mobile phase in HPLC refers to the solvent being continuously applied to the column or stationary phase at a flow rate of 1-5 cm³/min. The mobile phase acts as a carrier for the sample solution. The chemical interactions of the mobile phase and sample with the column determine the degree of migration and separation of components contained in the sample. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase.

1.1.1 Types of Chromatography

1. Normal-phase chromatography

Mechanism: Retention by interaction with the polar surface of the stationary phase with polar parts of the sample molecules.

Stationary phase: SiO2, Al2O3, -NH2, -CN, -Diol, -NO2, etc.

Mobile phase: Heptane, hexane, cyclohexane, CHCl3, CH2Cl2, dioxane, methanol, etc.
Application: Separation of non-ionic, non-polar to medium polar substances. Disadvantage: Lack of reproducibility of retention times as water or protic organic solvents change the hydration state of the silica or alumina chromatographic media.

2. Reversed-phase chromatography

Mechanism: Retention by interaction of the stationary phase’s non-polar hydrocarbon chain with non-polar parts of the sample molecules.

Stationary phase: n-octadecyl (RP-18), n-octyl (RP-8), ethyl (RP-2), phenyl, (CH2)n-CN, (CH2)n-diol, etc.

Mobile phase: Methanol, acetonitrile, water, buffer (sometimes with additives of THF or Dioxane), etc.

Application: Separation of non-ionic and ion forming non-polar to medium polar substances (carboxylic acids, hydrocarbons). If ion forming substances (as carboxylic acids) are to be separated, a pH control by buffers is necessary.

3. Reversed-phase ion-pair chromatography

Mechanism: Ionic sample molecules are ionically bound to an ion-pair reagent. The ion-pair reagent contains an unpolar part suitable for interaction with the unpolar hydrocarbon chain of the stationary phase.

Stationary phase: Reversed phase materials (RP-18, RP-8, CN), etc.

Mobile phase: Methanol, acetonitrile, buffer with added ion-pair reagent in the concentration range of 0.001 to 0.01 M, etc.

Application: Ionic substances often show very poor retention in reversed phase chromatography. To overcome this difficulty an ion-pair reagent is added to the eluent.

4. Ion-exchange chromatography

Mechanism: Retention of reversible ionic bonds on charged groups of the stationary phase
Stationary phase:

<table>
<thead>
<tr>
<th></th>
<th>Strong</th>
<th>Weak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cation exchanger</td>
<td>$\text{SO}_3^-$</td>
<td>$\text{COO}^-$</td>
</tr>
<tr>
<td>Anion exchanger</td>
<td>$\text{NR}_3^-$</td>
<td>$\text{NHR}_2^-$</td>
</tr>
</tbody>
</table>

Mobile phase: Aqueous buffer systems.

Application: Separation of substances which can form ions such as inorganic ions, organic acids, organic bases, proteins, nucleic acids.

### 1.1.2 Advantages of HPLC

1) It provides specific, sensitive and precise method for analysis of the different complicated sample.

2) There is ease of sample preparation and sample introduction.

3) There is speed of analysis.

4) The analysis by HPLC is specific, accurate and precise.

5) It offers advantage over gas chromatography in analysis of many polar, ionic substances, high molecular weight substances, metabolic products and thermolabile as well as nonvolatile substances.

### 1.1.3 Applications of HPLC

a) Natural Products: HPLC is an ideal method for the estimation of various components in plant extracts which resemble in structure and thus demand a specific and very sensitive method e.g., analysis of digitalis, cinchona, liquorice, and ergot extracts.

b) Stability studies: HPLC is now used for ascertaining the stability of various pharmaceuticals. With HPLC the analysis of the various degradation products can be done and thus stability indicating HPLC systems have been developed.

c) Bioassays and its complementation: Complex molecules as antibiotics and peptide hormones are mainly analysed by bioassay which suffer from high cost, necessity replicates,
poor precision and length of time required. Also bioassay gives an overall estimate of potency and gives no guidance about the composition. Thus HPLC can be used to complement bioassays and give an activity profile. It has been used for analysis of chloramphenicol, penicillins, clotrimoxazole, sulfas and peptides hormones.

d) HPLC has also been used in the cosmetic industry for quality control of various cosmetics.

1.1.4 Instrumentation

The basic components of HPLC are:

1. Pumping System
2. Sample Introduction Device
3. Chromatographic Column
4. Detector
5. Data handling Device

1. **Pumping System:** The HPLC pump is very important component of the system. It delivers the constant flow of the mobile phase or phases so that the separation of the components of the mixture occur in a reasonable time. Its performance directly affects retention time, reproducibility and detector sensitivity. Three main types of pumps are used in HPLC to propel the liquid mobile phase through the system are as under:
a. **Displacement pump**: It produces a flow that tends to independent of viscosity and backpressure and also output is pulse free. But it possesses limited capacity (250 ml).

b. **Reciprocating pump**: It has small internal volume (35 to 400 μl). It has high output pressure (up to 10,000 psi) and constant flow rates. But it produces a pulsed flow.

c. **Pneumatic or constant pressure pump**: They are pulse free, suffer from limited capacity as well as a dependence of flow rate on solvent viscosity and column back pressure. They are limited to pressure less than 2000 psi.

There are two type of elution process, i.e. isocratic and gradient

**Isocratic**: In this system, the things are kept constant throughout the run. In the case of pumping of mobile phase, the mobile phase composition is kept constant throughout the run. The nominal flow rate accuracy required is ±1% of the set flow

**Gradient**: There is some change purposely incorporated during the particular sample run to achieve a better or/and faster separation. In case of pumping mobile phase, the composition of mobile phase is continuously varied during the particular run. The gradient accuracy of ±1% of the step gradient composition is typical.

2. **Sample Introducing Device**

It is not possible to use direct syringe injection on column like GC, as the inlet pressure in LC is too high. Insertion of the sample onto the pressurized column must be as a narrow plug so that the peak broadening attributable to this step is negligible. The injection system itself should have no dead (void) volume. There are three important ways of introducing the sample into injection port.

a. **Loop injection**: In which, a fixed amount of volume is introduced by making use of fixed volume loop injector.

b. **Valve injection**: In which, a variable volume is introduced by making use of an injection valve.

c. **On column injection**: In which, a variable volume is introduced by means of a syringe through a septum.
3. Chromatographic Column

Column is a heart of chromatography. The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure. The columns are usually 10-30 cm long and 4-10 mm inside diameter containing stationary phase at particle diameter of 25 μm or less. Columns with an internal diameter of 5 mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

![Different Types of Columns](image)

**Figure 2. Different Types of Columns**

**Column packing:**

The packing used in modern HPLC consists of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC.

**a. Porous, polymeric beds:** Porous, polymeric beds based on styrene divinyl benzene co-polymers used for ion exchange and size exclusion chromatography. For analytical purpose these have now been replaced by silica based, packing which are more efficient and more stable.

**b. Porous layer beds:** Consisting of a thin shell (1-3 μm) of silica or modified silica on an spherical inert core (e.g. Glass). After the development of totally porous micro particulate packings, these have not been used in HPLC.

**c. Totally Porous silica particles (dia. < 10 μm):** These packing have widely been used for analytical HPLC in recent years. Particles of diameter > 20 μm are usually dry packed. While particles of diameter < 20 μm are slurry packed in which particles are suspended on a suitable solvent and the slurry so obtained is driven into the column under pressure.
4. **Detector**

The function of the detector in HPLC is to monitor the mobile phase as it merges from the column. There are several detectors available in the market. However UV Visible detector, photo diode array detector, fluorescence detector, conductometric and coulometric detector are more commonly used. The new ELSD detector is proving to be important detector, while the MS detector is outstanding. Detectors are usually of two types:

**a. Bulk property detectors:** It compares overall changes in a physical property of the mobile phase with and without an eluting solute e.g. refractive index, dielectric constant or density.

**b. Solute property detectors:** It responds to a physical property of the solute, which is not exhibited by the pure mobile phase e.g. UV absorbance, fluorescence or diffusion current.

5. **Data handling Device**

Computer-based system that controls all components of HPLC instrument (eluent composition (mixing of different solvents); temperature, injection sequence, etc.) and acquires data from the detector and monitors system performance (continuous monitoring of the mobile phase composition, temperature, back pressure, etc.)

1.1.5 **Methods for Quantitative Analysis**

Normalized peak area

External standard calibration

Internal standard calculation method

Standard addition calculation method

1. **Area normalization method**

After integrating all significant peaks in a chromatogram, total peak area may be calculated. Area (%) of any individual peak is called normalized peak area. This technique is widely used particularly in preliminary method development.

\[
\% A = \frac{\text{Area of peak } A}{\text{Total area of peaks } (A + B + C + D)} \times 100
\]
2. External standard calibration

The external standard is the same substance as that being analyzed in the sample. This method of calculation is most commonly used. The external standard must be pure or its composition known through prior analysis. Many times, the standard is costly or is in short supply so a secondary standard can be employed.

By injecting standard solution in different concentrations, peak response is plotted vs concentration. Unknown samples are analyzed in similar manner and their concentrations determined from the calibration curve. The calibration curve must cover the range of unknown sample.

\[
RF = \frac{\text{Standard peak area}}{\text{Concentration of the sample}}
\]

\[
\text{Sample concentration} = \frac{\text{Sample peak area}}{RF}
\]

Accuracy and precision of the results can further be enhanced if the external standard also contains sample matrix.

3. Internal standard calculation method

It is the addition of known quantity of a foreign substance (internal standard-IS) to the analyzed sample, the response coefficient of which is known or arbitrarily fixed. Addition of internal standard is essential for the sample requiring significant pre-treatment such as derivatisation, extraction to reduce chances of error due to these steps as it is expected to mimic the behavior of analyte in such re-treatment steps. A calibration curve is produced by analyzing different concentrations of the pure drug with constant amount of internal standard and from the chromatogram. Calculate the ratio (Rs) for each concentration of the analyte.

\[
Rs = \frac{\text{Area of drug}}{\text{Area of the internal standard}}
\]

Plot this ratio against concentration of the pure drug. The slope of this plot is the response factor.
The requirements for internal standards are that; it must completely resolve peak with no interferences. It should elute close to the compound of interest and behave equivalent to the compound of interest for analysis like pretreatments and derivative formations. It should also be stable, unreactive with sample components, column packing and the mobile phase and commercially available in high purity. This technique gives reliable, accurate, and precise results. If the internal standard is truly inert, the method is useful for determining the rate of analyte conversion in a chemical reaction.

4. Standard addition calculation method

The principle involved is that, the analytical signal is proportional to concentration, the initial analyte content is determined through measurements of this signal before and after the addition of known amount of the analyte to the analyzed sample. The method of standard addition, also denoted as “spiking,” is used when an analyte is to be quantified inside the matrix, the effect of which are likely to affect the chromatographic peak behavior.

In peak area method standard solution containing known quantities of all component of the mixture is chromatographed several times. The mean of peak area of each component is calculated and used to calibrate the detector response. Samples are also chromatographed several times; area for each sample is co-related with peak area of the standard.

1.1.6 Parameters Used in Chromatogram Characterization

1.1.6.1 Van Deemter Equation

Chromatography relates the variance per unit length of a separation column to the linear mobile phase velocity by considering physical, kinetic and thermodynamic properties of a separation. These properties include pathways within the column, diffusion (axial and longitudinal) and mass transfer kinetics between stationary and mobile phases. In liquid chromatography, the mobile phase velocity is taken as the exit velocity that is the ratio of the flow rate in ml/sec to the cross-sectional area of the ‘column-exit flow path.’ For a packed column, the cross-sectional area of the column exit flow path is usually taken as 0.6 times the cross-sectional area of the column. Alternatively, the linear velocity can be taken as the ratio of the column length to the dead time. The Van Deemter equation is:
Where, $H = \text{Height equivalent of a theoretical plate (HETP)}$

$u = \text{Velocity (flow rate) of the mobile phase}$

$A = \text{Multipath term}$

$B = \text{Longitudinal diffusion term}$

$C = \text{Mass transfer term for mobile and stationary phases}$

The lower the resulting value of $H$, greater is the efficiency of the procedure. So, ideally a scientist will want to minimize all three terms in order to minimize $H$. The other three terms refer to factors that come into play while the chromatography is performed.

**A - Multipath Term (Eddy Diffusion Factor)**

It is concerned with different paths travelled by molecules in a particular column. This leads to band broadening coefficient $A$.

The length of these pathways may differ significantly thus residence time in column for molecules of the same species is also variable.

The solute molecule then reached at the end of the column over a time interval which leads to band broadening.

Due to:

- Packing density irregularities (which creates void space)
- Differences in the shapes & sizes of the particles
- Packing is more dense in the middle than at the walls of the column
- Too large diameter columns or too small diameter columns are difficult to pack evenly (1-6 mm used)
Minimize Eddy Diffusion by:

- Selecting well packed columns
- Using smaller stationary phase particles

**B - Longitudinal Diffusion Term**

Molecules from solution of high concentration tend to move to low concentration by diffusion. This phenomenon of broadening by diffusion occurs during the travel of band of solute on column.

The diffusion by concentration gradient occurs ahead of and behind the center of the zone.

Due to:

- Tubing length too long
- Tubing that is too wide

Minimize Longitudinal Diffusion by:

- Using higher mobile phase flow rates

![Figure 3. Van Deemter Plot](image)
C - Mass Transfer Coefficients (CS and CM)

This term arises largely due to the fact that the stationary phase material is porous and the mobile phase within the pores is stagnant. The packing material is porous to allow a very large surface area for separation to occur.

As the analyte molecules move through the stagnant mobile phase to reach the surface of the packing material, they do so by diffusion only. Analyte molecules entering the pore, those that don’t enter the pore and those that penetrate more deeply into the pore will all be held up at that point to different extents causing a broadening of the band.

Minimise Mass Transfer Effects by:

- Using smaller stationary phase particles
- Using lower mobile phase flow rates

1.2.6.2. Retention Time (Rt)

The time taken for a particular compound to travel through the column to the detector is known as its ‘retention time’.

Retention time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.

1.2.6.3. Capacity Factor (K’)

A column must have the capacity to retain sample & the ability to separate sample components efficiently. Generally the value of K’ is > 2.

Capacity factor is the ratio of the reduced retention volume to the dead volume. Capacity factor (k’) is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factor is a measure of how well the sample molecule is retained by a column during an isocratic separation. The ideal value of k’ ranges from 2-10. Capacity factor can be determined by using the formula,

\[
K' = \frac{V_r - V_0}{V_0}
\]

Where, \( V_r \) = retention volume at the apex of the peak (solute) and \( V_0 \) = void volume of the system.
**Capacity Factor (k’) changes are typically due to:**

- Variations in mobile phase composition
- Changes in column surface chemistry (due to aging)
- Changes in operating temperature.

In most chromatography modes, capacity factor (k’) changes by 10 percent for a temperature change of 5°C.

**Adjusting Capacity Factor (k’)**

Good isocratic methods usually have a capacity factor (k’) in the range of 2 to 10 (typically between 2 and 5). Lower values may give inadequate resolution. Higher values are usually associated with excessively broad peaks and unacceptably long run times.

If the analytes fall outside their specified windows run the initial column test protocol to compare the results obtained with a new column.

**Capacity Factor (k’) values are sensitive to:**

- Solvent strength
- Composition
- Purity
- Temperature
- Column chemistry
- Sample

If the shift in capacity Factor (k’) value is observed with both analytes and the column test solution, the problem is most likely due to change in the column, temperature or mobile phase composition. This is particularly true if the shift occurred gradually over a series of runs. If, however the test mixture runs as expected, the problem is most likely sample related.

1.1.6.4. Column Resolution

The resolution (Rs) of a column provides a quantitative measure of its ability to separate two analytes. The resolution for a given stationary phase can be improved by lengthening the column, thus increasing the number of plates.

\[
T = \frac{W_x}{2F}
\]

Where, \( T = \) Tailing factor

\( W_x = \) Width of the peak determined at 5% from the baseline of the peak

\( F = \) distance between peak maximum and peak front. The accuracy of quantitation
decrease with increase in peak tailing. \((0.5 \leq T \leq 2)\)

Resolution is the parameter describing the separation power of the complete chromatographic system relative to the particular components of the mixture.

The resolution \((R_s)\), of two neighboring peaks is defined as the ratio of the distance between two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of \(R_s\) is 1.5. It is calculated by using the formula given in the (Fig 4),

\[
R_s = \frac{t_{R(2)} - t_{R(1)}}{1/2(W_1 + W_2)}
\]

![Experimental Resolution](image)

**Figure 4. Resolution between two peaks [20]**

Where, \(t_{R(1)}\) and \(t_{R(2)}\) are the retention times of components 1 and 2 and \(W_1\) and \(W_2\) are peak width of components 1 and 2.

There are three fundamental parameters that influence the resolution of a chromatographic separation:

- Capacity Factor (k’)
- Selectivity (\(\alpha\))
- Column Efficiency (N)

These parameters provide you with different means to achieve better resolution, as well as defining different problem sources.

**Table 2. System Suitability Parameters and Recommendations**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacity Factor (k’)</td>
<td>The peak should be well-resolved from other peaks generally k’&gt;2.0</td>
</tr>
<tr>
<td>Repeatability</td>
<td>RSD (\leq 1%) for (n \geq 5) is desirable.</td>
</tr>
<tr>
<td>Relative retention</td>
<td>Not essential as long as the resolution is stated.</td>
</tr>
<tr>
<td>Resolution (Rs)</td>
<td>Rs of ≥ 1.5 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard) etc.</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Tailing Factor (T)</td>
<td>T of ≤ 2</td>
</tr>
<tr>
<td>Theoretical Plates (N)</td>
<td>In general should be &gt; 2000</td>
</tr>
</tbody>
</table>

1.1.6.5 Theoretical Plate Number

It is a measure of column efficiency that is how many peaks can be located per unit run time of the chromatogram.

\[ N = \frac{L}{H} \]

Parameters which can affect N or H include peak position, particle size in column, flow rate of mobile phase, column temperature and viscosity of mobile phase and molecular weight of analyte.

1.1.6.6 Selectivity (\(\alpha\))

The selectivity (or separation factor) is a measure of relative retention of two components in a mixture. Selectivity is the ratio of the capacity factors of both peaks, and the ratio of its adjusted retention times. Selectivity represents the separation power of particular adsorbent to the mixture of these particular components.

This parameter is independent of the column efficiency; it only depends on the nature of the components, eluent type, eluent composition and adsorbent surface chemistry. In general, if the selectivity of two components is equal to 1, then there is no way to separate them by improving the column efficiency.

The ideal value is 2. It can be calculated by using formula,

\[ \alpha = \frac{V_2 - V_1}{V_1 - V_0} = \frac{k_1'}{k_2'} \]

Where, \(V_0 = \) Void volume of the column
\(V_1\) and \(V_2 = \) Retention volumes of the second and first peak respectively

Adjusting selectivity (\(\alpha\))

When troubleshooting changes in selectivity (\(\alpha\)), the approach is similar to the approach used to troubleshoot changes in capacity factor (\(k'\)). When selectivity (\(\alpha\)) is affected, the corrective action depends on whether the problem is mobile phase or column related. Be sure
to compare results obtained with the test solution to those observed when the column was new. Use these results to distinguish column changes from problems with mobile phase or other operating parameters.

Selectivity (α) values are sensitive to:
- Changes in mobile phase composition (pH ionic strength)
- Temperature and Purity

1.1.6.7 Column Efficiency/ Band broadening

Efficiency (N) of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Similar the band spread, higher theoretical plates number, indicates good column and system performance. Columns with N ranging from 5,000 to 1,00,000 plates/meter are ideal for a good system. Efficiency is calculated by using the formula given in the (Fig 5),

\[
N = 16 \left( \frac{t_R}{W} \right)^2 \quad \text{OR} \quad N = 5.54 \left( \frac{t_R}{W_{1/2}} \right)^2
\]

Figure 5. Numbers of Theoretical Plates

Where, 
- \( t_R \) = Retention time
- \( W \) = Peak width

A decline in measured efficiency may be due to:
- Age and history of the column
- Extra column band broadening (such as due to malfunctioning injector or improper tubing ID)
- Inappropriate detector settings (for example, time constant)
- Change in flow rate and solvent viscosity

We can recognize problems in separation due to a loss of column efficiency when the
width and/or shape of all peaks are affected. If the measured efficiency has degraded, either the column has degraded, or system band broadening has increased. At this point, check system band spreading against established benchmarks.

**Methods of measuring column efficiency (N)**

Figure 5 above illustrates the use of the different peak widths of a Gaussian peak for the calculation of column efficiency (N). When measuring column efficiency, use test conditions identical to those used in the established benchmark performance (such as test sample, flow rate, mobile phase composition and so on). Measure the column efficiency against the established performance.

**1.2.6.8 Peak asymmetry factor (Tf)**

Peak asymmetry factor (Tf) can be used as a criterion of column performance. The peak half width (B) of a peak at 10% of the peak height, divided by the corresponding front half width (A) gives the asymmetry factor.

\[
A_f = \frac{B_{10\%h}}{A_{10\%h}}
\]

\[
T = \frac{A_{5\%h} + B_{5\%h}}{2 \times A_{5\%h}}
\]

**Figure 6. Asymmetric Factors**

For a well packed column, an asymmetry factor of 0.9 to 1.1 should be achievable.

**Figure 7. Asymmetric Factors**
1.2.7 Parameter Used in Method Development

“Best column, best mobile phase, best detection wavelength, efforts in separation can make a world of difference while developing HPLC method for routine analysis. Determining the ideal combination of these factors assures faster delivery of desired results.”

1.2.7.1 The Best Mobile Phase

In reverse-phase chromatography, the mobile phase is more polar than the stationary phase. Mobile phase in these systems is usually mixtures of two or more individual solvents with or without additives or organic solvent modifiers. The usual approach is to choose what appears to be the most appropriate column and then to design a mobile phase that will optimize the retention and selectivity of the system.

Separations in these systems are considered to be due to different degrees of hydrophobicity of the solutes. The polarity of organic modifier and its proportion control the rate of elution of the components in the mobile phase. The rate of elution is increased by reducing the polarity.

The simple alteration of composition of the mobile phase or of the flow rate allows the rate of the elution of the solutes to be adjusted to an optimum value and permits the separation of a wide range of the chemical types. First isocratic run followed by gradient run is preferred.

Since the mobile phase governs solute stationary phase interaction, its choice is critical.

- Practical considerations dictate that it should not degrade the equipment or the column packing. For this reason, strong acids, bases and halide solutions should be avoided.
- Chemical purity of solvents is an important factor. Since large volumes of solvent are pumped through the column, trace impurities can easily concentrate in column and eventually be detrimental to the results. HPLC grade solvents are recommended.
- Volatility should be considered if sample recovery is required.
- Viscosity should be less than 0.5 centipoises, otherwise higher pump pressures are required and mass transfer between solvent and stationary phase will be reduced.

1.2.7.2 The Best Detector

The next consideration should be the choice of detector. There is little use in running a separation if detector one uses cannot “see” all the components of interest or conversely, if it “sees” too much. UV-visible detectors are the most popular as they can detect a broad range of compounds and have a fair degree of selectivity for some analytes.

Unfortunately UV-visible detectors are not universal detectors so it is worthwhile to look at the chemical structure of the analyte to see if it has suitable chromospheres, such as aromatic rings for UV-visible detection.
### Table 3. Detector options

<table>
<thead>
<tr>
<th>Detector</th>
<th>Analytes</th>
<th>Solvent Requirements</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-visible</td>
<td>Any with chromophores</td>
<td>UV-grade non-UV absorbing solvents</td>
<td>Has a degree of selectivity and is useful for many HPLC applications</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Fluorescent compounds</td>
<td>UV-grade non-UV absorbing solvents</td>
<td>Highly selective and sensitive. Often used to analyze derivatized compounds</td>
</tr>
<tr>
<td>Refractive Index (RI)</td>
<td>Compounds with a different RI to the mobile phase</td>
<td>Cannot run mobile phase gradients</td>
<td>Virtually a universal detector but has limited sensitivity</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>Readily oxidized or reduced compounds, especially biological samples</td>
<td>Mobile phase must be conducting</td>
<td>Very selective and sensitive</td>
</tr>
<tr>
<td>Evaporative Light Scattering (ELSD)</td>
<td>Virtually all compounds</td>
<td>Must use volatile solvents and volatile buffers</td>
<td>A universal detector which is highly sensitive. Not selective</td>
</tr>
<tr>
<td>Mass Spectrometer (MS)</td>
<td>Broad range of compounds</td>
<td>Must use volatile solvents and volatile buffers</td>
<td>Highly sensitive and is a powerful 2nd dimensional analytical tool. Many modes available. Needs trained operators</td>
</tr>
</tbody>
</table>

#### 1.2.7.3 The Best Column Length

Many chromatographers make the mistake of simply using what is available. Often this is a 250 × 4.6mm C18 column. These columns are able to resolve a wide variety of compounds (due to their selectivity and high plate counts) and are common to most laboratories. While many reverse phase separations can be carried out on such column, its high resolving capabilities are often unnecessary, as illustrated in Fig 8. Method development can be streamlined by starting with shorter columns; 150, 100 or even 50mm long. This is simply because they have proportionally shorter run times.
1.1.7.4 The Best Stationary Phase

Selecting an appropriate stationary phase can also help to improve the efficiency of method development. For example, a C8 phase (reversed phase) can provide a further time saving over a C18, as it does not retain analytes as strongly as the C18 phase. For normal phase applications cyano (nitrile) phases are most versatile.

1.1.7.5 The Best Internal Diameter

By selecting a shorter column with an appropriate phase, run times can be minimized so that an elution order and an optimum mobile phase can be quickly determined. It can also be advantageous to consider the column internal diameter. Many laboratories use 4.6mm ID columns as a standard.

1.1.7.6 Gradient Programming

The fastest and easiest way to develop a method is to use a mobile phase gradient. Always start with a weak solvent strength and move to a higher solvent strength. To begin, use a very fast gradient (e.g., 10 minutes) and then modify the starting and finishing mobile phases to achieve a suitable separation.

The choice of solvents and buffers may need to be modified during method development. Different HPLC instruments will give different results for the same gradient, so if a method is to be validated for use by several different laboratories, isocratic methods are recommended. Optimizing the mobile phase for an analysis will help to improve the separation. A number of factors depend upon the solvents chosen.
1.1.7.7 Retention

Analytes may be too strongly retained (producing long run times). If this occurs, the solvent strength should be increased. In reverse phase analysis this means a higher % of organic solvent in the mobile phase.

1.1.7.8 Peak Shape

This is often a problem, especially for basic compounds analyzed by reversed phase HPLC. To minimize any potential problems always use a high purity silica phase. These modern phases are very highly deactivated so secondary interactions with the support are minimal. Buffers can be used effectively to give sharp peaks.

If peak shape remains a problem, use an organic modifier such as triethylamine. To maximize the reproducibility of a method, it is best to use a column heater to control the temperature of the separation. A temperature of 35 – 40 °C is recommended.

1.1.7.9 Buffer selection

In reverse phase HPLC, the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and therefore, its retention decreases. When separating mixtures containing acid and/or bases by reversed phase HPLC, it is necessary to control the pH of mobile phase using appropriate buffer in order to achieve reproducible results.

For separating acids and bases, a buffered mobile phase is recommended to maintain consistent retention and selectivity. A buffered mobile phase, by definition, resists changes in pH so that the analytes and silica will be consistently ionized resulting in reproducible chromatography. If the sample is neutral, buffers or additives are generally not required in the mobile phase.

Acids or bases usually require the addition of a buffer to the mobile phase. For basic or cationic samples, “less acidic” reverse phase columns are recommended and amine additives for the mobile phase may be beneficial. Optimum buffering capacity occurs at a pH equal to the pKa of the buffer. Beyond that buffering capacity will be inadequate.
Buffers play an additional role in the reproducibility of a separation. The buffer salts reduce peak tailing for basic compounds by effectively masking silanols. They also reduce potential ion-exchange interactions with unprotonated silanols. To be most effective, a buffer concentration range of 10 - 50 mM is recommended for most basic compounds.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pKα (25°C)</th>
<th>Maximum Buffer Range</th>
<th>UV Cut-off (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA</td>
<td>0.3</td>
<td>-</td>
<td>210</td>
</tr>
<tr>
<td>Phosphate, pKα₂, HPO₄²⁻</td>
<td>7.2</td>
<td>6.2-8.2</td>
<td>&lt; 200</td>
</tr>
<tr>
<td>Phosphate, pKα₃, PO₄³⁻</td>
<td>12.3</td>
<td>11.3-13.3</td>
<td>&lt; 200</td>
</tr>
<tr>
<td>Citrate, pKα₁, C₅H₇O(COOH)₂(COO)⁻¹</td>
<td>3.1</td>
<td>2.1-4.1</td>
<td>230</td>
</tr>
<tr>
<td>Citrate, pKα₂, C₅H₇O(COOH)₃(COO)⁻²</td>
<td>4.7</td>
<td>3.7-5.7</td>
<td>230</td>
</tr>
<tr>
<td>Citrate, pKα₃, C₅H₇O(COO)⁻³</td>
<td>6.4</td>
<td>4.4-6.4</td>
<td>230</td>
</tr>
<tr>
<td>Carbonate, pKα₁, HCO₃⁻¹</td>
<td>6.1</td>
<td>5.1-7.1</td>
<td>&lt; 200</td>
</tr>
<tr>
<td>Carbonate, pKα₂, CO₃²⁻</td>
<td>10.3</td>
<td>9.3-11.3</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Formate</td>
<td>3.8</td>
<td>2.8-4.8</td>
<td>210</td>
</tr>
<tr>
<td>Acetate</td>
<td>4.8</td>
<td>3.8-5.8</td>
<td>210</td>
</tr>
<tr>
<td>Ammonia</td>
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<td>8.3-10.3</td>
<td>200</td>
</tr>
<tr>
<td>Borate</td>
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<td>8.2-10.2</td>
<td>N/A</td>
</tr>
<tr>
<td>TEA</td>
<td>10.8</td>
<td>9.8-11.8</td>
<td>&lt; 200</td>
</tr>
</tbody>
</table>

### 1.1.7.10 Selection of pH

The pH range most often used for reversed phase HPLC is 1-8 and can be divided into low pH (1-4) and intermediate pH (4-8) ranges. Each range has a number of advantages. Low pH has the advantage of creating an environment in which peak tailing is minimized and method ruggedness is maximized. For this reason operating at low pH is recommended.

At a mobile phase pH greater than 7, dissolution of silica can severely shorten the lifetime of columns packed with silica base stationary phases.

The pKa value (acid dissociation [ionization] constant) for a compound is the pH at which equal concentrations of the acidic and basic forms of the molecule are present in aqueous
solutions. Analytes may sometimes appear as broad or tailing peaks when the mobile phase pH is at or near their pKa values.

Dramatic changes in the retention and selectivity (peak spacing) of basic and acidic compounds can occur when the pH of the mobile phase is changed. This is often a result of different interactions between the column and the analytes when the ionization of these compounds changes. It is important to evaluate these changes when a method is developed in order to select the mobile phase pH that provides the most reproducible results.