BASIC SKILLS TRAINING GUIDE - HPLC method development and validation - an overview

Dr Sarma Krishna pathy, YLN Murthy, Sunithasarma, Atchuutha ramaiah

Introduction
This paper covers the basic practical aspects of High Performance Liquid Chromatography (HPLC) and is aimed at the inexperienced analyst who may have no or very little knowledge of this technique. It includes basic tips, identifies key skills, raises awareness and gives guidance on good practice of the basic aspects of HPLC. It provides information to help analysts in their understanding of the important issues to consider during analysis and to develop further skills.

HPLC method development and validation play an important role in the discovery, development and manufacture of agro chemicals, pharmaceutical products. This article mainly focuses on the optimization of HPLC conditions and other important perspectives during method development and validation. Various critical steps related to analytical method development and validation are discussed. A sequence of events required for method development and analytical validation are described. The steps involved in developing a stability-indicating HPLC method influences the analysis of degradation products/impurities in stability study and its validation demonstrate the suitability for its intended purpose. High Performance Liquid Chromatography (HPLC) is one of the most widely used analytical techniques in industry. It is used to separate and analyse compounds through the mass-transfer of analytes between stationary and mobile phases. The technique is employed in a broad range of activities, such as the analysis of foods, drugs and agrochemicals. The technique of HPLC utilises a liquid mobile phase to separate the components of a mixture. The components themselves are first dissolved in a solvent and then forced to flow (via the mobile phase) through a column (stationary phase) under high pressure. The mixture is resolved into its components within the column and the amount of resolution is dependent upon the interaction between the solute components and the column stationary phase (immobile packing within the column) and liquid phase. The interaction of the solute with the mobile and stationary phases can be manipulated through different choices of both solvent and stationary phases. The individual units which form an HPLC chromatograph is shown in Figure 1.
HPLC can be divided into two broad categories, normal phase and reversed phase. For normal phase, a polar stationary phase (usually silica) is used to retain analytes, which are polar, whilst reversed phase separations are based upon forces between non-polar compounds and non-polar functional groups, which are bonded to the silica support. The majority of applications today are based on reversed phase separations. For a more in-depth explanation of the principles and categories of HPLC, the analyst should consult references 1 – 3 and explore some of the websites listed in Part 2.

2.2 Key parameters
This section describes some of the basic parameters that govern the effectiveness of a separation. The analyst should learn, by reading this section, which are the key separation parameters involved, their relative importance and how they can be calculated manually from a chromatographic trace. This is an important skill to master, despite the fact that with modern data handling software many of these parameters will be calculated automatically. For a more detailed explanation of those and other theoretical equations, it is advisable to consult references 1–4.

2.2.1 The retention factor \((k)\)
The retention factor, \(k\), is used to describe the migration rate of an analyte on a column and can be defined as shown in Equation 1:

\[
k = \frac{t_R - t_M}{t_M}
\]

\(t_R\) = retention time of a component

\(t_M\) = dead time (time required for the mobile phase to pass through the column)

Ideal separations are performed under conditions in which \(k\) is between 1 – 5

• Components which take a long time to elute from the column compared to the mobile phase will have a large retention factor \((k > 20)\)

• \(k\) can be manipulated by varying the mobile and stationary phases

2.2.2 The selectivity factor \((\alpha)\)
The selectivity factor, \(\alpha\), for two analytes within a column provides a measure of how well two components will separate on a column. The factor for a column with analytes A and B can be expressed as shown in Equation 2:

\[
\alpha = \frac{(t_R)_B - t_M}{((t_R)_A - t_M)}
\]

\((t_R)_B\) = retention time of a component B which is more strongly retained

\((t_R)_A\) = retention time of a component A which is less strongly held

\(t_M\) = dead time (time required for the mobile phase to pass through the column)
• When $\alpha = 1$, it is not possible to separate the two components using the given system.

Analytic method development and validation are key elements of any pharmaceutical development program. HPLC analysis methods are developed to identify, quantify or purify compounds of interest. This technical brief will focus on development and validation activities as applied to drug products. Various steps for HPLC method development are given in Fig. 1. Effective method development ensures that laboratory resources are optimized, while methods meet the objectives required at each stage of drug development.

Method validation, required by regulatory agencies at certain stages of the drug approval process, is defined as the “process of demonstrating that analytical procedures are suitable for their intended use” 1,2. To know information concerning your compound or analyte is worth. Understand its physical and chemical characteristics allow you to select the most appropriate HPCL method development from your vast literature.

Information concerning sample, for example, molecular mass, structure and functionality, pKa values and UV spectra, solubility of compound(s) should be compiled. The requirement of removal of insoluble impurities by filtration, centrifugation, dilution or concentration to control the concentration, extraction (liquid or solid phase), derivatization for detection etc. should be checked.

For pure compound, determine sample solubility whether

### Steps for HPLC method development

1. Information on sample
2. Define separation goals
3. Special procedure requirement, sample pretreatment, if any.
4. Detector selection and setting
5. Separation conditions optimization
6. Check for problems or special procedure requirements
7. Recovery of purified material,
8. Quantitative calibration/ Qualitative method
9. Method validation for release to laboratories

it’s organic soluble or water soluble, as this helps to select the best mobile phase and column to be used in your HPLC Method development. Various detectors include: UV/Visible, photodiode array detector, fluorescence detector, conductivity detector, refractive index detector, electrochemical detector, Mass spectrometer detector, evaporative light scattering detector.

UV-Vis detectors are typical in many laboratories as they HPLC method development and validation- an overview
HPLC method development and validation play important role in the discovery, development and manufacture of agro chemical/pharmaceutical products. This article mainly focuses on the optimization of HPLC conditions and other important perspectives during method development and validation. Various critical steps related to analytical method development and validation is discussed. A sequence of events required for method development and analytical validation are described. The steps involved in developing a stability-indicating HPLC method influences the analysis of degradation products/impurities in stability study and its validation demonstrate the suitability for its intended purpose.

Methods are developed to support drug testing against specifications during manufacturing and quality release operations, as well as during longterm stability studies. Methods may also support safety and characterization studies or evaluations of drug performance. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients. The validation of an analytic method demonstrates the scientific soundness of the measurement or characterization. It is required to varying extents throughout the regulatory submission process. The validation practice demonstrates that an analytic method measures the correct substance, in the correct amount, and in the appropriate range for the intended samples. It allows the analyst to understand the behavior of the method and to establish the performance limits of the method. The goal is to identify the critical parameters and to establish acceptance criteria for method system suitability. The HPLC system with its different components is shown in Figure 2 (organic, pH, flow rate, temperature, wavelength, and column age) and standardization (integration, wavelength, standard concentration, and response factor correction). During the preliminary method development stage, all individual components should be investigated before the final method optimization. This gives the scientist a chance to critically evaluate the method performance in each component and streamline the final method optimization. The percentage of time spent on each stage is proposed to ensure the scientist will allocate sufficient time to different steps. In this approach, the three critical components for a HPLC method (sample preparation, HPLC analysis and standardization) will first be investigated individually.

2.3 Quantitative measures of column efficiency
The plate model supposes that the chromatographic column contains a large number of theoretical plates. The sample equilibrates between the mobile and stationary phase in these plates, moving down the column by transfer from one plate to the next as shown in Figure 3.

![Figure 3. Column as a series of theoretical plates](image)

In reality, plates do not exist but the theory provides a model that serves as a way of measuring column efficiency. The terms plate height ($H$) and number of theoretical plates ($N$) are commonly used as quantitative measures to describe column efficiency. The relationship between the two is shown in Equation 3.

**Equation 3**

$$N = \frac{L}{H}$$

$L$ is the length of the column

An efficient new column producing sharp component peaks would be expected to exhibit a large number of theoretical plates ($N$). As the column ages and the peaks become broader, the
efficiency of the column will drop accordingly. By measuring the number of theoretical plates for the column each time it is used, it is possible to monitor this deterioration over time. This can help the analyst determine whether the column is still ‘fit for purpose’, whether it needs reconditioning or replacing. The value of $N$ can be calculated from Equation 4.

![Diagram of HPLC system](image)

**Fig 2: HPLC system with its different components**

The degraded drug samples obtained are subjected to preliminary chromatographic separation to study the number and types of degradation products formed under various conditions. Scouting experiments are run and then conditions are chosen for further optimization. Resolving power, specificity, and speed are key chromatographic method attributes to keep in mind during method development. Selectivity can be manipulated by combination of different factors like solvent composition, type of stationary phase, mobile phase, buffers and pH. Changing solvents and stationary phases are the most comfortable approaches to achieve the separation.

The proper range of pH is an important tool for separation of ionizable compounds. Acidic compounds are retained at low pH while basic compounds are more retained at higher pH. The neutral compounds remain unaffected. The pH range 4-8 is not generally employed because slight change in pH in this range would result in a dramatic shift in retention (up-slope or down-slope of curve) (Figure 3). However, by operating at pH extremes (2-4 or 8-10), not only is there a 10-30 fold difference in retention that can
Figure 3: Reversed-phase retention behavior as pH is varied

be exploited in method development but also the method can be made more robust which is a desirable outcome with validation in min 15,16. Method development within the different pH range from 1 to 12 for better chromatographic resolution between two or more peak of an analyte depends upon three main factors that are - Column efficiency, Selectivity, Retention time. The ionizable analytes are either bases or acids and it effects above three factors dramatically with change in pH. Retention time can be improved by changing the pH that will lead to easy separation of ionizable analytes from non-ionized form. By changing the mobile phase pH can also improve column efficiency because it altered both the ionization of the analyte and the residual silanols and it also minimizes secondary interactions between analytes and the silica surface that will lead to poor peak shape. To achieve optimum resolution, it require change in the pH of mobile phase. Method development can proceeds by investigating parameters of chromatographic separations first at low pH and then at higher pH until optimum results are achieved 17.

Mobile phase composition (or solvent strength) plays an important role in RP-HPLC separation. Acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) are commonly used solvents in RP-HPLC having low UV cut-off of 190, 205 and 212nm respectively. These solvents are miscible with water. Mixture of acetonitrile and water is the best initial choice for the mobile phase during method development. An HPLC column packed with stationary phase of C18-bonded silica (C18 Column) and C8-bonded silica (C8 Column) is used in RP-HPLC separation of a wide range of organic compounds. The separation selectivity for certain components vary between the columns of different manufacturer as well as between column production batches from the same manufacturer. Column dimensions, silica substrate properties and bonded stationary phase characteristics are the main ones. The use of silica-based packing is favoured in most of the present HPLC columns due to several physical characteristics.

Silica substrates are available in spherical or irregular shapes and can be prepared with different surface areas, pore sizes and particle sizes, which make them suitable for most HPLC applications. Totally porous silica particles with 5 μm diameter provide the desired characteristics for most HPLC separations 14,16,18.

Zirconia-based columns are revolutionary HPLC phases. Zirconia particles are mechanically stable, and have a porous structure similar to that of silica. However, zirconia’s main advantage over silica is that it is very stable in a wide range of eluent pH; indeed the ZirChrom®-EZ and ZirChrom®-MS phases are stable over the pH range of 1-10. Separation of many samples can be enhanced by selecting the right column temperature. Higher column temperature reduces system backpressure by decreasing mobile phase viscosity, which in turn allows use of longer columns with higher separation efficiency. However, an
overall loss of resolution between mixture components in many samples occurs by increasing column temperature. The optimum temperature is dependent upon nature of the mixture components. The overall separation can be improved by simultaneous changes in column temperature and mobile phase composition. Recently, normal phase HPLC is back popular with the birth of HILIC technology that proved to improve reproducibility in separating polar and hydrophilic compounds such as peptides, carbohydrate, vitamins, polar drugs and metabolites. In order to develop a HPLC method effectively, most of the effort should be spent in method development and optimization as that will improve the final method performance. Method validation is important to complete method development.

**Equation 4**

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

- \( t_R \): retention time
- \( W_{1/2} \): the width of the peak at 50% of the peak height
- \( W \): width of the peak at the baseline
- 5.54 and 16 are constants

Chromatograms of samples often contain several peaks and it is not always possible to accurately measure the width of the peak at the baseline. An alternative solution is to measure the width of the peak at 50% of the peak height.

**2.4 Column resolution**

The resolution (\( R \)) of a column provides a quantitative measure of its ability to separate two components within a mixture. For a mixture with two compounds A & B, the resolution can be defined by equation 5.

**Equation 5**

\[ R = 2 \times \frac{[(t_R)_B - (t_R)_A]}{(W_A + W_B)} \]

- \( (t_R)_B \): retention time of a component B which is more strongly retained
- \( (t_R)_A \): retention time of a component A which is less strongly held
- \( W_B \): width of peak for component B at the base line
- \( W_A \): width of peak for component A at the base line
- If \( R \) is less than 1, the components are overlapping
- If \( R \) is equal to or greater than 1, this indicates good separation

**3 HPLC components**

**3.1 The mobile phase**
Selecting the correct composition and type of mobile phase is important because it is a variable that governs separation. However, choice is restricted because of the column used, i.e., the type of stationary phase employed. The main distinction is between reversed phase and normal phase chromatography. In normal phase systems, nonpolar solvents such as hexane or iso-octane are used whereas reversed phase requires polar solvents such as water, acetonitrile or methanol.

The choice of mobile phase is governed by the physical properties of the solvent. Factors to consider are, polarity, miscibility with other solvents, chemical inertness, UV cut off wavelength and toxicity. The polarity index gives an indication of the ability of a solvent to elute a compound from the column Table 1 a summary of the typical solvents that are used for mobile phases and some of the important parameters that govern choice.

**Table 1. Typical solvents for HPLC mobile phases**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Polarity index</th>
<th>UV cut off (nm)</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal phase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexane</td>
<td>0.1</td>
<td>210</td>
<td>Chronic neurotoxic</td>
</tr>
<tr>
<td>Isooctane</td>
<td>0.1</td>
<td>205</td>
<td>Low</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>2.8</td>
<td>218</td>
<td>Low</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>3.1</td>
<td>245</td>
<td>Chronic carcinogen</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>3.9</td>
<td>205</td>
<td>Low</td>
</tr>
<tr>
<td><strong>Reversed phase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>10.2</td>
<td>200</td>
<td>None</td>
</tr>
<tr>
<td>Methanol</td>
<td>5.1</td>
<td>210</td>
<td>Mildly toxic</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>5.8</td>
<td>210</td>
<td>Toxic by inhalation</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>4.0</td>
<td>280</td>
<td>Toxic by inhalation</td>
</tr>
</tbody>
</table>

The HPLC system can be set up either for isocratic or gradient elution.
- Isocratic elution is where the mobile phase composition remains constant during the whole analysis.
- Gradient elution is where the mobile phase composition is steadily changed during the analysis, e.g., to obtain better resolution and / or decrease analysis time.

**Practical tips for handling mobile phase**

a) Consider the purity of solvents and only use HPLC grade materials:
- impurities give rise to noisy baselines, e.g., UV detection at low wavelengths;
- de-mineralised water should be employed for mobile phases.

b) Ensure that the mobile phase is free from dust; connect a stainless steel filter element to the end of the tube leading from the reservoir to the pump.

c) Remove dissolved air, because this can cause irregular pumping action and fluctuating signals from the detector, by performing one or more of the following:
- degas the mobile phase with helium;
• place the mobile phase under vacuum
• agitate the mobile phase in an ultrasonic bath.
d) When mixing solvents to form mobile phases:
• the analyst must understand the terminology which is used to describe the constituents of the mobile phase - a common expression is for example, 75/25 v/v methanol / water which indicates a volume measurement, e.g., 75 mL of methanol + 25 mL of water);
• most systems have a facility to mix the solvents from different reservoir bottles - the analyst only has to set the percentages (e.g. 75% methanol, 25% water).
• if a mobile phase of mixed composition is to be prepared manually, the volume of each solvent should be measured separately before they are mixed together.
e) Be aware that volatile components in a mobile phase of mixed composition may evaporate. This can be minimised by:
• keeping the solution cool during the degassing procedure;
• keeping the reservoir bottle stoppered at all times.
f) Ensure that the sample to be analysed is soluble in the mobile phase.
g) When using UV detectors you must consider the UV absorption of the mobile phase. This is indicated by the UV cut-off value. For example, tetrahydrofuran has a UV cut-off of 280nm (Table 1), therefore it cannot be used for analysis of samples for pyridine as the peak maximum for pyridine is ca 260nm (Figure 14).
h) Ensure that the mobile phase does not react with the stationary phase. Buffers and pH modifiers may contain, for example, ammonia which can replace one of the NR groups of an amide on an amino propyl stationary phase.
i) It is also important to monitor the levels of the mobile phases and ensure that they are constantly topped up and therefore the system is never allowed to run dry.
j) Whenever there is a change in the mobile phase, ensure that the labels on the bottles are also changed and labelled correctly with the following information:

![Mobile phase constituents](image)

3.2 The pump
The main purpose of the pump in HPLC is to pass a constant flow of mobile phase through the chromatographic column. There are two main types of pump used in HPLC incorporating either a syringe or reciprocating piston element in their design.

3.2.1 Syringe pump
Syringe type pumps are attractive because they operate pulse free. However, the total volume of mobile phase that the pump can deliver is limited by the capacity of the syringe. The pumps are expensive in comparison with the more commonly used reciprocating piston type.

3.2.2 Reciprocating piston

A schematic of this type of pump is shown below. A rotating cardioid cam causes the two pistons alternatively to draw mobile phase from the reservoir and then to force the liquid in the direction of the column.

Figure 5

![Figure 5 - Dual piston reciprocating pump](attachment:image.png)

The check valves are opened or shut in synchrony with the piston movement to ensure that flow of mobile phase only occurs in one direction. The volume delivered by each piston per cam revolution is typically 0.1 cm$^3$. The design is relatively simple and has the advantage that a very large reservoir of mobile phase can be accommodated to allow almost continuous use of the equipment. Flow rate can be altered simply by changing the speed of the motor driving the cam. This type of pump has the disadvantage that it causes pulses in the flow of mobile phase. Other considerations concerning the pump are:-

a) The pump must be able to deliver the mobile phase at high pressures in order to overcome the flow resistance associated with HPLC columns. High working pressures are particularly important when microbore columns, smaller particle size packings, high flow rates and viscous mobile phases are used in the analysis.
b) The components of the pump must be resistant to corrosive chemicals and solvents. Acids, bases and aggressive organic solvents are commonly used in mobile phase formulations so the analyst should be aware that these chemicals can cause damage to the equipment.

c) Flow rate should be easy to set with a possibility of settings between ~0.1 and 10 mL min⁻¹.

d) Pumps should be robust and should be able to function routinely with only a minimum requirement for maintenance and servicing.

e) Flow should be pulse free and stable. Pulses, fluctuations in the flow of mobile phase, are always undesirable and can lead to poor precision for component retention times. If the detector attached to the column is ‘flow sensitive’ the chromatogram baselines may exhibit peaks and troughs which mirror the changing flow rate. This increased noise leads to raised detection limits for the sample components.

f) The pump should be a ‘constant flow’ device. Constant pressure pumps do not give satisfactory performance because the flow rate will vary as the resistance to flow offered by the column changes, e.g. when internal sinters are blocked by particulates and columns become overloaded with sample residues.

**Tips for pump use**

- After use, always flush water, buffered solutions, acids, bases and aggressive organic solvents out of the pump using an appropriate solvent. This prevents damage to seals, valves and pump heads.
- Always de-gas mobile phases to prevent bubble formation in the pump.
- Check the pump is delivering the mobile phase at the desired flow rate – measure the actual flow rate by measuring the time it takes for a fixed volume of mobile phase to exit the column.
- Always ensure, when using gradient elution, that the components of the mobile phase are miscible for all compositions employed in the method. Buffer salts have a tendency to precipitate from solution as the content of the organic solvent in the mobile phase is increased.
- Prime the pump before use to confirm that there are no bubbles in the cylinders.
- If the mobile phase is to be changed, ensure that the new phase is miscible with the previous phase; if not, employ an intermediate solvent to thoroughly flush out the old mobile phase. It is also advisable to purge the system to remove any air bubbles that may have been introduced during the change.

**3.2.3 Other considerations and rules for using HPLC pumps**

a) Never operate a pump without a solvent reservoir filter on the inlet tubing. Unfiltered solvent may block pumps and/or shorten seal lifetime which could adversely affect the check valve.

b) Always check the pump for leaks before and during analysis, this is especially important when the system will be unattended for long periods of time.

![Diagram of Loop Injector](image)
is allowed to exit the loop via the vents. The injector is then switched to the ‘inject’ position where the loop ends are connected to the pump and column. The sample slug (occupying the loop) is then carried by the flow of mobile phase to the top of the column. This design allows the injector loop to be loaded at ambient pressure so the problems associated with high column back pressures and leaking injectors and septa are eliminated. Because the loop volume is fixed the injection process is highly reproducible allowing the HPLC to be used for quantitative analysis. Loops are interchangeable allowing the injection volume to be selected according to the analysis required.

**Tips for use of injector**

- Never use syringes with sharp tips because these will damage the injector.
- Check the injector for leaks prior to the analysis.
- Never allow the loop to syphon during the loading sequence, this can allow air to enter the loop. Long lengths of vent tubing positioned vertically can cause syphoning.
- Ensure that the column is not overloaded with sample – select the loop volume that is appropriate for the size of the HPLC column used. The load for analytical columns can be in the range 0.5 to 100 µL but this has to be determined for the particular column being used.

### 3.4 The column

#### 3.4.1 Introduction

The column is the most important chromatographic component and it is crucial in determining the performance and resolution of the whole HPLC system. The choice of column is governed firstly the type of chromatography used, *i.e.* reversed phase or normal phase chromatography. Figure 7 summarises the types of stationary phases and their relative usage from a survey conducted by LCGC magazine (1998) and Table 2 shows some typical applications.

**Figure 7. HPLC Mode & Stationary Phase Usage**

For further details, the analyst should refer to their column manufacturers / suppliers. NB Many manufacturers now have their own web site. These contain useful information about the types of column that are available along with suitable applications.

### 3.4.2 Reversed phase columns

*Why are reversed phased columns so popular?*

- They are versatile and their stationary phase chemistry meets the needs of a wide range of samples.
- The stationary phase material can be manufactured to high quality and is reasonably stable under normal operating conditions.
- The elution order of the compounds being separated can be predicted easily and is based upon their hydrophobicity.
- Mobile phases are simple mixtures of aqueous solvents. Water, which is usually the predominant component, is inexpensive, safe and plentiful.

#### 3.4.3 Types of columns

There are many types of reversed phase columns, which are distinguished by the functional group that is bonded to the silica support. Figure 8 shows some of the common types of reversed phase columns. For a fuller understanding of the reasons behind the development of reversed phase packings, see reference 7. Increasing Polarity
Figure 8. Typical reversed phase columns and their relative polarity

Octadecylsilane (ODS) columns are the most commonly used and are usually the preferred choice to begin analyses of unknown non-polar compounds (see Figure 9). This is a very non-polar phase and polar compounds will therefore be eluted faster than their non-polar counterparts. A C(8) column might therefore be more useful than the C(18) analogue if the compounds to be analysed are polar.

Figure 9. Stationary phases used in reversed phase chromatography

Column selection is governed by a number of factors, these include:
- reviewing data from previous work on similar compounds;
- using the knowledge of the chemical structure of the compounds to be separated;
- consulting the literature, e.g. look at the information provided in the manufacturer’s catalogue;
- analysis of a trial sample to test if the selected column performs satisfactorily.

3.4.4 Column characteristics

Once you have decided which phase to use it is important to consider other column characteristics which may affect the analysis. Even if you are not required to choose a column,
you should at least know and understand some of the fundamental column properties that control separation.

3.4.4.1 Column length and internal diameter
- Columns are typically 15 cm long with an internal diameter of 4.6 mm but alternative lengths and internal diameters are available.
- Column dimensions determine the flow and sample injection volume used and greatly influence the length of time for an analysis.

<table>
<thead>
<tr>
<th>Column Type</th>
<th>Internal diameter/mm</th>
<th>Injection volume</th>
<th>Flowrate/ µL min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>4.6</td>
<td>5-20 µL</td>
<td>500-2000</td>
</tr>
<tr>
<td>Narrow bore</td>
<td>2</td>
<td>1-5 µL</td>
<td>200</td>
</tr>
<tr>
<td>Microbore</td>
<td>1</td>
<td>0.5-2 µL</td>
<td>50</td>
</tr>
<tr>
<td>Capillary</td>
<td>0.5</td>
<td>0.1 µL</td>
<td>5</td>
</tr>
<tr>
<td>Nanoflow</td>
<td>0.05</td>
<td>nL</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The current trend is to use shorter lengths (3 or 5 cm) and smaller internal diameters (1 or 2 mm) for quicker analysis.
- Capillary columns (with diameters ranging from 180 to 300 µm) are becoming more popular due to quicker analysis and savings because less solvent is consumed.

3.4.4.2 Column support material
Most support materials (to which the functional group is bonded) used in HPLC column manufacture are made from silica. Manufacturers of HPLC columns offer a wide range of porous silicas, which are characterised by pore size, particle size and surface area. The particles can be either spherical or irregular in shape. The latter is less efficient but usually cheaper and is used mainly for large scale preparative applications. The name of the column is usually derived from the type of silica used for packing and examples of commercially available materials include:
- spherical silica - Nucleosil, Spherisorb and Hypersil;
- irregular silica – LiChrosorb, Partisil and Sorbsil.

3.4.4.3 Choosing particle size
The particle size of spherical packing materials range from 3 µm to 20 µm in diameter. It affects column efficiency and back pressure. The efficiency of the column decreases as particle size increases so there is poorer peak separation. However, the flow resistance of the column also decreases with particle size which means that the back pressure decreases. The most popular particle size for analytical columns is 5 µm.

3.4.4.4 Pore size and surface area of support materials
Packings with a large pore size will have a small surface area whereas those with a small pore size have a large surface area. For most applications, analysts should select a column material with a small pore size (<10 nm) because the increase in surface area improves the capacity of the column. In practice column materials have a pore size distribution that spans about an order of magnitude. However when analysing samples containing high molecular weight materials, e.g. proteins, a column with a large pore size will be required (>>10 nm).
3.4.4.5 Column specification – a summary

<table>
<thead>
<tr>
<th>Stationary phase</th>
<th>Type</th>
<th>Application</th>
<th>Mobile phase</th>
<th>Typical analytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica</td>
<td></td>
<td>Normal phase (NP)</td>
<td>Hexane, alcohols</td>
<td>Pesticides and natural products</td>
</tr>
<tr>
<td>Octadecylsilyl (ODS)</td>
<td>C18 hydrocarbon chain</td>
<td>Reversed phase (RP)</td>
<td>Water, methanol, acetonitrile, buffers (pH 2 – 8)</td>
<td>Peptides and amino acids</td>
</tr>
<tr>
<td>C8</td>
<td>C8 hydrocarbon chain</td>
<td>RP</td>
<td>See ODS</td>
<td>Drugs and Pharmaceuticals</td>
</tr>
<tr>
<td>Cyanopropyl (CN)</td>
<td>Cyanopropyl group bonded to silica support</td>
<td>Both RP and NP</td>
<td>RP- water, alcohol NP- hexane, ether</td>
<td>Foods and fatty acids</td>
</tr>
<tr>
<td>Aminopropyl (NH₂)</td>
<td>Aminopropyl group bonded to silica support</td>
<td>Both NP and RP</td>
<td>RP- water, alcohol NP- hexane, ether</td>
<td>Surfactants</td>
</tr>
<tr>
<td>Pirkle</td>
<td>Phenylglycine enantiomer bonded to a silica support</td>
<td>Chiral ionic separations are more effective but less robust</td>
<td>Hexane, modifiers</td>
<td>Pesticides and herbicides</td>
</tr>
</tbody>
</table>

Table 2. HPLC stationary phases and their typical applications

Figure 10. Column specification

3.4.5 Guard Columns
Guard columns are employed as a protective factor and are installed between the injector and the main HPLC column. Essentially, they are designed to filter and remove unwanted particles that may clog up the main column. Use of a guard column often prolongs the life of the main column. The total internal volume of the guard column should be small to minimise peak broadening.

3.4.6 Column care – practical tips
3.4.6.1 Monitoring column performance
A new column is usually required when the column has either been damaged and/or degraded with time. Usual indicators of inefficient performance include:

- consistently poor peak shapes and noisy baselines on the chromatogram;
- a significant decrease in retention times and poor peak separation.

This can be confirmed by measuring the column efficiency (see Section 2.3), i.e., using a test sample mixture to determine the number of theoretical plates. This value can be compared with the original column efficiency when the column was purchased to determine if there has been a notable deterioration in performance. Many columns come with a certificate of analysis and a test sample chromatogram showing the original column’s performance. Performance in the short term may be improved by carrying out column cleaning procedures recommended by the manufacturer. If it is often necessary to change the column for a different method. The steps involved in the changeover are shown in Figure 11.

**Figure 11. Steps to follow when changing a column**

Switch off pump or put on standby mode.

Store column in a safe place, e.g., in its original transportation container. Keep a record of when it was removed from the system. Install the new column and check that the connections are consistent with those already in place, if not, use appropriate connectors. Connections need to be clean and aligned correctly to get a good seal. Once fitting has been completed, ensure that connections are adequately tightened so as to prevent leakage. Beware of over-tightening components because this may cause unnecessary stress to joints that may result in leaks within the system.

Check the new column is being fitted the correct way. An arrow is marked on the side of the column to indicate the direction of flow of the mobile phase. Flush the new column with the mobile phase intended for its use. Check for miscibility problems between the column storage solvent and the mobile phase. Typically pump through ten column volumes of mobile phase before use. Allow the new column to settle to the required operating temperature within the oven, prior to use. NB Flow rates are dependent on the diameter of the column. Typical flow rate for a 4.6 mm column is 1 mL min⁻¹.

Remove the column. The column ends must be immediately sealed with the appropriate caps (which should be kept safely in a drawer) to prevent the column from drying out and producing voids that will deteriorate its performance.

Flush out the column with the appropriate storage solvent (e.g., methanol or propan-2-ol for an ODS column) – have regard to miscibility of solvents when changing from the method mobile phase to the storage solvent. Allow sufficient time for the storage solution to replace the mobile phase in the column. A typical HPLC system with a 4.6 mm ×250 mm column and flow rate 1 mL min⁻¹ needs approximately 10 minutes to change the solvent.

**3.4.6.2 Optimise column performance**

To improve column performance, the following points must be considered.

a) Avoid extra connections between the injector and column, and between the column and detector. Use only one piece of capillary tubing between the injector and column, and between the column and detector.

b) pH stability is important because the operating conditions must be kept within the ranges specified by the manufacturer. Any deviation outside the limits will affect the column and undoubtedly, decrease its life. Most systems employ a buffer solution to control and maintain the
pH stability of mobile phase. For example, if the pH is not controlled when using ODS columns, the following might occur:
• at low pH (<2.5), the siloxane bonds can be destroyed by hydrolysis;
• at high pH (>8.5), silica dissolution can occur.
c) Maintain the column at constant temperature (typically 40°C) by housing it in an oven. Variation in temperature can cause inconsistencies such as problems in the identification of components from the chromatogram due to changing retention times. It is also necessary to check that the temperature reading corresponds to the actual temperature of the oven. Using a column oven is beneficial because:
• reproducibility of analysis is improved;
• consistent retention times are promoted;
• drift due to laboratory temperature and seasonal fluctuations is eliminated.
d) Testing - The performance of the column should be monitored on a regular basis. This is performed by running a standard test mixture through the HPLC system. NB Manufacturer’s columns are often supplied with a chromatogram of a standard test mixture. The column should be tested on receipt with the test mixture to identify if damage has occurred in transit. The test mixture should be run on a regular basis when the column is in use. Data such as retention times and peak areas of individual components can be monitored to determine if they have changed significantly with time. Any significant change means that there is a problem with the column or HPLC system and remedial action is required. The chromatograms of the test mixture obtained should be archived to facilitate easy checking of the column’s performance over time.
e) Cleaning - Column cleaning is recommended and details of solvents to use are usually included in manufacturers’ literature. The appearance of unexpected peaks and / or drifting baselines on your chromatogram is usually a good indicator of a dirty column. Most analysts will use components of the mobile phase to flush columns in an attempt to remove impurities that may have built up with time. For example, if using ODS columns, it may be possible to wash with a series of solvents such as water, tetrahydrofuran and acetonitrile. Column frits, which are porous elements located at the ends of the column (their purpose is to retain the column packing) can sometimes get blocked and may require cleaning and / or replacement. Additional guidance and tips on column washing can be found in reference
3.4.7 Column Lifetimes

Columns are (often) expensive and must therefore be treated with care. It is essential to use them in accordance with manufacturers' recommendations. For additional details on the care and maintenance of modern columns see reference 9. Tips of how to maximise the life of a column are indicated in Figure 12.

- Operate within the conditions specified on the manufacturers' certificate
  - Operate within pH range
  - Only use compatible solvents
- Never let the column run dry of mobile phase / solvent
  - Limit sudden pressure variations by slowly increasing to the required operating flow and gradually decreasing the flow at the end of analysis
  - Keep temperature constant
- Avoid sudden changes which might disturb the column packing
- Clean 'dirty' samples prior to introducing into the system
  - Filter sample to remove any unwanted solids, prior to injection onto the column
  - Employ an appropriate guard column to further remove unwanted particulates
- Do not overload the column with an excess of sample. The maximum volume is usually 100 μL but injection volumes are typically 25 μL for a 4.6 mm x 25 cm column
  - Clean column by flushing with solvent before removal
  - Use inert storage solvent as recommended by the manufacturer
  - Ensure column ends are fitted with end-caps
  - Place the column in a safe place where it is unlikely to be knocked or jarred, record the date of removal and note the storage solvent

Figure 12. Tips on how to maintain and extend column life.
3.5 The detector
3.5.1 Ultra-violet detectors (UV)
UV detectors are the most commonly used detectors because they can be used to analyse a range of organic compounds and are relatively simple to use. A cross section of a UV flow cell is shown in Figure 13.

Figure 3.5 The detector
3.5.1 Ultra-violet detectors (UV)
UV detectors are the most commonly used detectors because they can be used to analyse a range of organic compounds and are relatively simple to use. A crosssection of a UV flow cell is shown in Figure 13.
3.5.1.1 Types of UV detector

*Fixed wavelength* measures at one wavelength (typically 254 nm).

*Variable wavelength* measures at one wavelength at a time but can detect over a wide range, e.g., 190 to 400 nm. Such detectors offer the best sensitivity for any absorptive component by allowing the user to select an appropriate wavelength at which to monitor. Individual analytes may have high absorptivity at different wavelengths and thus, single wavelength detection would reduce the system’s sensitivity. *Diode array* measures a spectrum of wavelengths simultaneously and allows the user to perform spectroscopic scanning to determine precise absorbance readings at a variety of wavelengths, while the peak is passing through the flow cell.

3.5.1.2 Choice of wavelength for UV detectors

If working in accordance with established methods, wavelength settings are specified. Otherwise, they can be determined from the literature or by measuring the absorption spectrum of the analytes. For example, aromatic rings show strong absorption near 254 nm and this is a popular choice of wavelength for detecting compounds of this nature.

3.5.1.3 Maintenance awareness

- UV lamps must be replaced before they get too old and become inefficient.
- Monitoring of lamp performance is recommended and its intensity should be checked against manufacturer’s specification.

3.5.2 Refractive Index Detector (RI)

A schematic of the RI detector is shown in Figure 15.

![Figure 15. Schematic of the refractive index detector](image-url)
In this schematic the magnitude of the detector signal is a function of the refractive index of the liquid in the flow cell, i.e. the mobile phase. When the sample components enter the flow cell the refractive index of the liquid is altered and the detector response changes accordingly.

In the differential RI detector a reference flow cell is located next to the sample flow cell. This is filled with mobile phase and is illuminated by the same light source. Here the detector output corresponds to the difference in signal arising from the sample and reference beams. Detectability is increased using this design and the detector is less sensitive to changes in source intensity and detector temperature.

3.5.3 Other Detection Systems

There several types of detection systems and a summary of these detectors and their typical applications is shown in Table 3.

Table 3.

<table>
<thead>
<tr>
<th>Detector</th>
<th>Application</th>
<th>Comments</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence</td>
<td>Fluorescent compounds</td>
<td>High sensitivity and very specific</td>
<td>Moderate</td>
</tr>
<tr>
<td>Chemiluminescence</td>
<td>Chemiluminescent reactants or activators</td>
<td>Very sensitive and highly specific.</td>
<td>Cheap (often home made)</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>Acids and bases</td>
<td>High sensitivity and very specific</td>
<td>Cheap</td>
</tr>
<tr>
<td>Mass Spectroscopy</td>
<td>Universal</td>
<td>Reasonably sensitive but complex to use</td>
<td>Expensive</td>
</tr>
</tbody>
</table>

A more comprehensive review of detectors can be obtained from references 1-4.

4 HPLC system parameters and system suitability checks

4.1 System parameters

Apart from the column and mobile phase there are several other significant parameters within the HPLC system which, if altered, can affect the separation procedure during analysis.

4.1.1 Flow rates

The flow rate is invariably specified in established methods. It is important to check that the correct flow rate has been selected (in accordance with the written procedure) and that the value displayed agrees with the volume being delivered. Changes in the flow rate from the set values are usually identified from the chromatogram. For example, inconsistent peak retention times and drifting baselines are indicators of possible flow problems.

Flow rate fluctuation may be due to:
- problems with the pump;
- blockages within the system;
- leaks within the system.

Check that flow rate(s) are consistent with the programmed values by measuring the amount of mobile phase that comes out from the waste (into a measuring cylinder), over a specified period of time.

4.1.2 Temperature changes

The effect of temperature on the chromatographic processes in HPLC is quite small in comparison to that found with GC. Although there is a performance benefit
associated with an increase (above ambient) in the column temperature, in practice most analytical methods specify temperatures around 25 - 40°C. Column thermostats have become an essential feature in modern instrumentation, because it is important to control the temperature of the column. Large fluctuations in the temperature will alter peak retention times, which may result in mis-identification of peaks. Therefore, the temperature of the column should be kept within ± 2°C.

Check oven temperatures for consistency by inserting a calibrated temperature probe/thermometer into the oven and monitor over a specified period of time.

4.1.3 Detector sensitivity and wavelength (when using UV detectors)
Changes in sensitivity and wavelength will critically affect the size of peaks on the chromatogram. Any deviations from set values will therefore alter the results and hence, it is important to know the exact operating values and that these have been correctly set, prior to running samples. Before beginning an analysis, check by observation, that the settings are correct.

4.1.4 Integration parameters
Computer software is invariably used to determine the peak areas (or peak height) in the chromatogram. The software works on integration parameters that can be set by the analyst. Such parameters include:
• peak width;
• slope sensitivity;
• threshold;
• baseline setting (e.g. maintaining a horizontal baseline from the point where the signal was first recorded may not be appropriate).

The analyst must check that the integration parameters are appropriate for the sample chromatogram. For example, if the peak width has been set too narrow then noise might be interpreted as peaks whereas if it is set too wide then the software will not correctly integrate two peaks that are adjacent to each other in the chromatogram.

4.2 System suitability checks
System suitability testing should be conducted regularly and are essential when a new analysis is to be performed or changes have been made such as repair or replacement of parts.
The parameters which should be monitored are listed; they may be calculated from chromatograms using the equations listed in the theory section of this guide or via HPLC computer software.
• Column performance:
  – the number of theoretical plates (N);
  – the resolution of a column.
• Repeatability of injection:
  – repeat injections of a standard solution should provide a coefficient of variation which is no greater than ± 1.0% of the mean peak area.

4.3 Equipment Qualification tests (EQ)
EQ is a documented process which provides evidence that an instrument is fit for purpose and kept in a state of maintenance and calibration consistent with its use. Detailed information on the EQ procedure and suggestions of what tests should be conducted are described in reference 10.

5 HPLC test
5.1 Introduction
The following series of experiments will provide the analyst with an example of how changes to
the parameters of the HPLC can affect the results.
Use an HPLC chromatograph system with a UV/VIS detector and a reversed phase column, e.g.
an ODS column (25 cm x 4.6 mm i.d.).
Suggested initial operating conditions are:
Mobile phase 65% acetonitrile / 35% phosphate buffer pH 3.2
UV wavelength 254 nm
Temperature ambient
Injection volume 1 μL
Flow rate 1 mL min⁻¹
Prepare a mixed solution containing each of the following (in order of elution):
uracil, pyridine, phenol, dimethylaniline, 4-butyl benzoic acid, toluene.
The suggested volume is 100 mL as virtually all laboratories keep 100 mL volumetric
flasks, however 10 mL would be sufficient.
1. Prepare a set of 1 mg L⁻¹ solutions by weighing out 100 mg ± 5 mg of each of the
compounds, transferring to separate 100 mL labelled volumetric flask and making up to the mark with
acetonitrile. Stopper and shake gently to mix.
2. Prepare a mixed stock solution by pipetting, with an automatic pipette, 5 mL of each solution (2
mL in the case of Uracil) into a 100 mL labelled volumetric flask and make up to the mark with
mobile phase. Stopper and shake gently to mix.
3. Prepare a working solution by pipetting 10 mL of the mixed stock solution into a labelled 100
mL volumetric flask and make up to the mark with mobile phase. Stopper and shake gently to
mix.
The working solution contains 5 μg mL⁻¹ of each of the components (2 μg mL⁻¹ of Uracil).
N.B. It is possible to buy test mixtures to check column performance.
Perform the following experiments to determine the best operating conditions to achieve good
separation and reliable results.
5.1.1 Flow rate
Start the pump and allow the system to equilibrate then inject the sample. Record the retention
times and peak areas of the analyte peaks.
Then vary the flow rate by ± 25% and see what effect this has on retention times, peak areas and
peak separation (and back pressure).
Decide on which flow rate achieved good separation and short analysis time.
5.1.2 Mobile phase composition.
Repeat the experiment but this time vary the mobile phase composition (e.g. 55% acetonitrile /
45% phosphate buffer).
Decide on an optimum mobile phase composition for achieving good separation.
5.1.3 Detector wavelength
Repeat the experiment but this time use a different detector wavelength (e.g. 254 nm ± 15 nm).
Tabulate the effect this had on the peak areas of the various components. Decide how critical it
is to control wavelength (look at the spectrum in Figure 14 for a guide and if equipment is
available record the UV spectrum of the components).
5.1.4 Repeatability
Perform five replicate injections using the operating conditions that give the best separation and
a reasonable analysis time.
Calculate the mean and standard deviation of the retention times and peak areas for each component. Use these figures to estimate what the repeatability would be at the 95% confidence level (» 2 standard deviations) using this system.

6 Calibration
Calibration of the HPLC system is important to obtain accurate results. It is also recommended to run frequent checks to ensure that all instrumental parameters are not drifting. Standards and / or test mixes can be run as often as necessary as a part of Quality Control (QC). When components, such as the mobile phase, have been changed, calibrate the instrument immediately and ensure that the repeatability of results are within the specified tolerances, before it is to be used for analysis. You should keep all chromatograms from the standard runs and note changes in the operating parameters which have been made to overcome problems (if any). This not only allows you to frequently compare chromatograms (to identify any obvious problems) but also enables you to build up a profile on the history of the column. It is also advisable to plot the results from QC samples on a control chart, to monitor progress and to help identify any potential problems.

6.1 External standard
External standards are analysed with test samples to quantify the analyte concentrations in the sample (External Standardisation). The standards can be used to establish response factors for individual components within a sample. Generally a range of analyte concentrations is expected from the samples which require testing. Therefore, by preparing a series of calibration standards of known concentration and running these on the HPLC system, a calibration plot of detector response versus analyte concentration can be obtained. This series should at least cover 120% of the full range of concentrations of the samples to be tested and should also contain at least six calibration levels. From this plot, the concentration of unknown samples can be determined.

6.2 Internal standard
Internal standardisation involves the addition of a known amount of a compound to the sample prior to its injection onto the column. Although not widely used in HPLC (because of the predominant use of auto-injectors) the advantages of internal standards include:
- any loss of sample in the preparatory stages will also be accounted for by a similar loss on the amount of internal standard;
- the quantification procedure is less dependent on the injection technique, e.g., inconsistency in the amount of sample from manual injections.
However, the problems with using internal standards include:
- finding a suitable compound which is readily available;
- complications due to the presence of an additional peak on the chromatograph.

7 Problem-solving
7.1 Introduction
One of the tasks you will undoubtedly face during HPLC operations is the necessity to troubleshoot. Many problems, when first encountered, appear to be baffling but once identified, they can usually be resolved by checking and performing remedial actions, e.g., fix a leaking connection.

7.1.1 Daily checks and fault checking
If the problem has been identified by observations from the chromatogram:
- run the sample again and / or employ a standard to check if there is a fault;
- check solvents are filtered and degassed;
• check settings are correct for the method, e.g., flow rate, detector wavelength etc.;
• check the system flow is correctly set;
• observe pressure during analysis to check for abnormality.
Diagnose the fault (if possible) and seek advice where necessary. If a hardware component is to be changed, carefully dismantle and throw away defective items, reassemble with replacement of the damaged parts and record changes.

7.1.2 Troubleshooting
The following figures show some of the typical problems encountered and the tables provide possible solutions. Further detailed information can be obtained from the references indicated for specific problems.

7.1.3 Unusual peak shapes
Figure 16. Broad peaks 11,12
Table 4. Broad peaks

<table>
<thead>
<tr>
<th>Cause</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample is too concentrated</td>
<td>Dilute sample</td>
</tr>
<tr>
<td>Large injection volume</td>
<td>Reduce injection volume</td>
</tr>
<tr>
<td>Column deterioration</td>
<td>Wash column, replace column and use guard column</td>
</tr>
<tr>
<td>Dead space</td>
<td>Check fittings are secured properly</td>
</tr>
</tbody>
</table>

Figure 17. Ghost peaks 13-15

Table 4. Broad peaks
Figure 16. Broad peaks \textsuperscript{11,12}

Table 5. Ghost peaks

<table>
<thead>
<tr>
<th>Cause</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contamination</td>
<td>Flush column with solvents to remove contaminant</td>
</tr>
<tr>
<td>Compound from earlier injections</td>
<td>Flush column with strong solvent</td>
</tr>
<tr>
<td>Unknown</td>
<td>Apply sample clean-up or check purity of mobile phase</td>
</tr>
</tbody>
</table>
**Figure 18. Negative peaks**

**Table 6. Negative peaks**

<table>
<thead>
<tr>
<th>Cause</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase absorbance is larger than sample absorbance</td>
<td>Use mobile phase that does not absorb at the wavelength used</td>
</tr>
<tr>
<td>Recorder connections</td>
<td>Check polarity of recorder connections</td>
</tr>
</tbody>
</table>
Table 7. Peak doubling

<table>
<thead>
<tr>
<th>Cause</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-elution of interfering compound</td>
<td>Improve sample clean-up or use pre-fractionation</td>
</tr>
<tr>
<td></td>
<td>Adjust selectivity by changing mobile / stationary phase</td>
</tr>
<tr>
<td>Column overload</td>
<td>Use higher capacity stationary phase, increase column diameter or decrease sample load</td>
</tr>
<tr>
<td>Channeling in column</td>
<td>Replace column</td>
</tr>
</tbody>
</table>

Figure 20. Fronting and tailing peaks\textsuperscript{15}
### Table 8. Peak fronting and tailing

<table>
<thead>
<tr>
<th>Cause</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peak fronting</strong></td>
<td></td>
</tr>
<tr>
<td>Overloaded column</td>
<td>Decrease sample size, use higher capacity column</td>
</tr>
<tr>
<td><strong>Peak tailing</strong></td>
<td></td>
</tr>
<tr>
<td>Overloaded column</td>
<td>(see peak fronting)</td>
</tr>
<tr>
<td>Basic compounds which cause silanol interactions</td>
<td>Use competing base such as triethylamine or base deactivated silica reversed phase column</td>
</tr>
<tr>
<td>Column deterioration</td>
<td>Use guard column / apply less vigorous conditions</td>
</tr>
</tbody>
</table>

### 7.1.4 Baseline spikes

![Spikes](image)

**Figure 21. Baseline spikes**

### Table 9. Baseline spikes

<table>
<thead>
<tr>
<th>Cause</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air bubbles in the mobile phase and / or detector</td>
<td>Degas mobile phase</td>
</tr>
<tr>
<td></td>
<td>Ensure that all fittings are tight</td>
</tr>
<tr>
<td>Electrical interference</td>
<td>Identify and remove sources, e.g., thermostatted equipment.</td>
</tr>
<tr>
<td></td>
<td>Poor electrical configuration or bad connections</td>
</tr>
<tr>
<td>Column deterioration</td>
<td>Change column / use guard column</td>
</tr>
</tbody>
</table>
7.1.5 Clipped peaks

![Clipped peaks diagram](image_url)

**Figure 22 Clipped peaks**

Table 10. Clipped peaks

<table>
<thead>
<tr>
<th>Cause</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clipped at bottom</strong></td>
<td></td>
</tr>
<tr>
<td>Integrator zero set too low</td>
<td>Set zero correctly</td>
</tr>
<tr>
<td>Detector drifts below zero</td>
<td>Use auto-zero function</td>
</tr>
<tr>
<td><strong>Clipped at top</strong></td>
<td></td>
</tr>
<tr>
<td>Detector range is too sensitive</td>
<td>Set less sensitive range</td>
</tr>
<tr>
<td>Column overload</td>
<td>Dilute sample</td>
</tr>
</tbody>
</table>
7.1.6 Inconsistent retention times

Table 11. Fluctuating retention times

<table>
<thead>
<tr>
<th>Cause</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General</strong></td>
<td></td>
</tr>
<tr>
<td>Equilibration time of new mobile phase is</td>
<td>Pass 20 column volumes of new phase through the column</td>
</tr>
<tr>
<td>insufficient</td>
<td></td>
</tr>
<tr>
<td>Selective evaporation of mobile phase</td>
<td>Cover solvent reservoirs</td>
</tr>
<tr>
<td>component</td>
<td>Use less vigorous helium degassing</td>
</tr>
<tr>
<td>Temperature fluctuations</td>
<td>Use thermostatted column oven</td>
</tr>
<tr>
<td><strong>Decreasing retention times</strong></td>
<td></td>
</tr>
<tr>
<td>Increasing flow rate</td>
<td>Check and if necessary, reset pump</td>
</tr>
<tr>
<td>Degradation of silica stationary phase</td>
<td>Ensure that the mobile phase pH is within the column’s tolerance</td>
</tr>
<tr>
<td></td>
<td>limit of the column (typically between pH 2 and 8)</td>
</tr>
<tr>
<td>Temperature fluctuations</td>
<td>Use thermostatted column oven.</td>
</tr>
<tr>
<td><strong>Increasing retention times</strong></td>
<td></td>
</tr>
<tr>
<td>Decreasing flow rate</td>
<td>Check and if necessary, reset pump</td>
</tr>
<tr>
<td></td>
<td>Check for leaks within the system</td>
</tr>
<tr>
<td>Temperature fluctuations</td>
<td>Use thermostatted column oven.</td>
</tr>
</tbody>
</table>

7.1.7 Drifting baseline & noise

![Rising baseline](image)

![High noise](image)

Figure 23. Baseline problems

Table 12. Drifting baseline & noise

<table>
<thead>
<tr>
<th>Cause</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline drift</strong></td>
<td></td>
</tr>
<tr>
<td>Contamination build-up / column ageing</td>
<td>Flush column / use guard column</td>
</tr>
<tr>
<td></td>
<td>Change column</td>
</tr>
<tr>
<td>Temperature changes</td>
<td>Use a thermostatted column.</td>
</tr>
<tr>
<td><strong>Noise</strong></td>
<td></td>
</tr>
<tr>
<td>Continuous – detector lamp problem or dirty</td>
<td>Replace UV lamp (should last for about 2000 hours)</td>
</tr>
<tr>
<td>flow cell</td>
<td>Clean and flush flow cell</td>
</tr>
<tr>
<td>Noise</td>
<td>Action</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Continuous – detector lamp problem or dirty flow cell</td>
<td>Replace UV lamp (should last for about 2000 hours)</td>
</tr>
<tr>
<td></td>
<td>Clean and flush flow cell</td>
</tr>
<tr>
<td>Random contamination</td>
<td>Flush column with strong solvent / clean-up sample</td>
</tr>
</tbody>
</table>
### 7.1.8Leaks and pressure changes

**Table 13. Problems with leaks and fluctuating pressure**

<table>
<thead>
<tr>
<th>Cause</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General</strong></td>
<td></td>
</tr>
<tr>
<td>Column – loose fitting</td>
<td>Tighten or replace fitting, disassemble fitting, rinse and / or replace ferrules</td>
</tr>
<tr>
<td>Poor connection between components</td>
<td>Check fittings and connectors – clean, align or replace defective components</td>
</tr>
<tr>
<td><strong>Fluctuating pressure</strong></td>
<td></td>
</tr>
<tr>
<td>Air in pump</td>
<td>Degas solvent</td>
</tr>
<tr>
<td>Leaking pump</td>
<td>Replace or clean check valves, replace pump seals</td>
</tr>
<tr>
<td><strong>Decreasing pressure</strong></td>
<td></td>
</tr>
<tr>
<td>Insufficient flow from pump</td>
<td>Loosen cap on mobile phase reservoir</td>
</tr>
<tr>
<td>Leak in lines from pump to solvent reservoir.</td>
<td>Tighten or replace fittings</td>
</tr>
<tr>
<td>Leaking pump</td>
<td>Replace or clean check valves, replace pump seals</td>
</tr>
<tr>
<td><strong>Increasing pressure</strong></td>
<td></td>
</tr>
<tr>
<td>Blocked flow lines</td>
<td>Systematically disconnect components from detector end to column end to find blockage, replace or clean any blocked component(s)</td>
</tr>
<tr>
<td>Particulate build up at head of column</td>
<td>Replace or clean frit, install 0.5 μm porosity in-line filter between pump and injector to eliminate mobile phase contaminants or between injector and column to eliminate sample contaminants</td>
</tr>
<tr>
<td>Water/organic solvent systems – buffer precipitation</td>
<td>Ensure mobile phase compatibility with buffer concentration</td>
</tr>
<tr>
<td><strong>High back-pressure</strong></td>
<td></td>
</tr>
<tr>
<td>Column blocked with irreversibly adsorbed sample</td>
<td>Improve sample clean-up, use guard columns</td>
</tr>
<tr>
<td>Column particle size too small</td>
<td>Use larger particle size</td>
</tr>
<tr>
<td>Mobile phase viscosity too high</td>
<td>Use solvents with lower viscosity</td>
</tr>
<tr>
<td>Salt precipitation, e.g., in reversed phase systems with high concentration of organic solvent in mobile phase</td>
<td>Ensure mobile phase compatibility with buffer concentration, decrease ionic strength of the mobile phase, premix mobile phase</td>
</tr>
</tbody>
</table>
METHOD VALIDATION
Validation is defined by the International Organization for Standardization (ISO) as verification, where specified requirements are adequate for an intended use”, where the term verification is defined as “provision of objective evidence that a given item fulfills specified requirements” 24. The applicability and scope of an analytical method should be defined before starting the validation process. It includes defining the analytes, concentration range, description of equipment and procedures, validation level and criteria required. The validated range is defined by IUPAC as “the interval of analyte concentration within which the method can be regarded as validated” 25,26. This range does not have to be the highest and lowest possible levels of the analyte that can be determined by the method. Instead, it is defined on the basis of the intended purpose of the method 27,28. The method can be validated for use as a screening (qualitative), semi-quantitative (e.g. 5-10 ppm) or quantitative method. It can also be validated for use on single equipment, different equipments in the laboratory, different laboratories or even for international use at different climatic and environmental conditions. The criteria of each type of validation will of course be different with the validation level required 24,29. The various validation parameters include linearity, accuracy, precision, ruggedness, robustness, LOD, LOQ and selectivity or specificity.

Linearity
The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of analyte in the sample 30,31. It is essential to determine the useful range at which the instrumental response is proportional to the analyte concentration. Generally, a value of co-relation coefficient (r2) > 0.998 is considered as evidence of an acceptable fit of the data to the regression line 32. Significance of deviation of intercept of calibration line from the origin value of zero can be evaluated statistically by determining confidence limits for the intercept, generally at the 95 % level 33,34. Linearity is determined by a series of three to six injections of five or more standards. Peak areas (or heights) of the calibration standards are usually plotted in the Y-axis against the nominal standard concentration, and the linearity of the plotted curve is evaluated through the value of the co-relation coefficient (r2). Because deviations from linearity are sometimes difficult to detect, two additional graphical procedures can be used. The first one is to plot deviations from regression line versus concentration or versus logarithm of concentration. For linear ranges, the deviations should be equally distributed between positive and negative values. Another approach is to divide signal data by their respective concentrations yielding the relative responses 35. A graph is plotted with the relative responses on Y-axis and the corresponding concentrations on X-axis on a log scale. The obtained line should be horizontal over the full linear range. At higher concentrations, there will typically be a negative deviation from linearity. Parallel horizontal lines are drawn in the graph corresponding to, for example, 95 % and 105 % of the horizontal line. The method is linear up to the point where the plotted relative response line intersects the 95 % line. A comparison of the two graphical evaluations using HPLC is shown in Figure 4 where Rc = Line of constant response 36,37.
**Accuracy**

Accuracy is defined by ISO as “closeness of agreement between a measured quantity value and a true quantity value of a measurand”. It is a qualitative characteristic that cannot be expressed as a numerical value. It has an inverse relation to both random and systematic errors, where higher accuracy means lower errors. Accuracy is evaluated by analyzing test drug at different concentration levels. Typically, known amounts of related substances and the drug substance in placebo spiked to prepare an accuracy sample of known concentration of related substance. Samples are prepared in triplicate. ICH recommends accuracy evaluation using a minimum of nine determinations over a minimum of three concentration levels covering the range specified. It is determined by comparing the found concentration with the added concentration. The methods of determining accuracy include analysis of an analyte of known purity (i.e., reference material), comparisons of results of the proposed analytical procedure with those of a second well-characterized procedure and standard addition method. The accuracy may also be inferred once precision, linearity and specificity have been established. Accuracy expresses closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Repeatability is also referred to as intra-assay precision. It is a measure of precision of analysis in one laboratory by one operator using one piece of equipment over a relatively short time-span. It is degree of agreement of results when experimental conditions are maintained as
constant as possible, and expressed as RSD of replicate. ICH recommends a minimum of nine determinations covering the specified range for the procedure (e.g., three concentrations/three replicates as in the accuracy experiment), or a minimum of six determinations at 100% of the test concentration for evaluation of repeatability which should be reported as standard deviation, relative standard deviation (coefficient of variation) or confidence interval. ICH defines intermediate precision as long-term variability of the measurement process and is determined by comparing the results of a method run within a single laboratory over a number of weeks. It is also called as inter day precision 40. It reflects discrepancies in results obtained by different operators, from different instruments, with standards and reagents from different suppliers, with columns from different batches or a combination of these but in the same laboratory 29. The objective of intermediate precision validation is to verify that the method will provide same results in the same laboratory once the development phase is over. Reproducibility expresses precision of analysis of the same sample by different analysts in different laboratories using operational and environmental conditions that may differ but are still within the specified parameters of the method 24,42. The objective is to verify that the method will provide the same results despite differences in room temperature and humidity, variedly experienced operators, different characteristics of equipments (e.g., delay volume of an HPLC system), variations in material and instrument conditions (e.g. in HPLC, mobile phase composition, pH, flow rate of mobile phase), equipments and consumables of different ages, columns from different suppliers or different batches and solvents, reagents and other material with different quality 29,43.

Selectivity and Specificity
Selectivity and specificity are sometimes used interchangeably to describe the same concept in method validation. Selectivity of an analytical method is defined by the ISO as “property of a measuring system, used with a specified measurement procedure, whereby it provides measured quantity values for one or more measurands such that the values of each measurand are independent of other measurands or other quantities in the phenomenon, body, or substance being investigated” 24. Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. The specificity of a test method is determined by comparing test results from an analysis of samples containing impurities, degradation products, or placebo ingredients with those obtained from an analysis of samples without impurities, degradation products, or placebo ingredients. Specificity can best be demonstrated by resolution between the analyte peak and the other closely eluting peak(s) 29,44.

Detection limit (LOD) and Quantitation limit (LOQ)
LOD of an analytical procedure is the lowest concentration of an analyte in a sample which can be detected but not necessarily quantitated as an exact value where as LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. ICH guidelines describe three methods for determining LOD and LOQ that include:

Visual evaluation
It may be used for both non instrumental and instrumental methods. The LOD and LOQ is determined by analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected or quantified with acceptable accuracy and precision respectively.

Figure
S/N Ratio approach
This method can only be applied to analytical procedures which exhibit baseline noise. It is determined by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing minimum concentration at which the analyte can be reliably detected. A S/N ratio of 3:1 is considered acceptable for estimating LOD (with Relative Standard Deviation (RSD) ≤10%) whereas for whereas for LOQ S/N ratio of 10:1 is considered appropriate (with Relative Standard Deviation (RSD) ≤ 3%) described in Figure 5.

**Standard deviation of the response and slope**
The LOD and LOQ may be expressed as: LOD = 3.3 x σ/S and LOD = 10 x σ/S
where σ = the standard deviation of the response, S = the slope of the calibration curve of analyte. The slope S may be estimated from the calibration curve of the analyte. The value of σ may be taken from as standard deviation of analytical background responses of an appropriate number of blank samples. Alternatively, it can be taken as residual standard deviation of a regression line or standard deviation of y-intercepts if regression lines obtained after samples containing an analyte in the range of LOD and LOQ.

**Range**
The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity (Figure 6) 45. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The range of an analytical method varies with its intended purpose. It is generally 80-120% of the test concentration for assay of a drug substance or a finished (drug) product, 70-130% of the test concentration for content uniformity, ±20% over the specified range for dissolution testing, reporting level of an impurity to 120% of the specification for determination of an impurity and it should commensurate with LOD or LOQ (the control level of impurities), for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects 40.

**Robustness**
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For determination of method robustness, a number of chromatographic parameters, for example, flow rate, column temperature, injection volume, detection wavelength or mobile phase composition are varied within a realistic range and quantitative influence of the variables is determined. If influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method’s robustness range. Obtaining data on these effects will allow to judge whether a method needs to be revalidated when one or more of parameters are changed, for example to compensate for column performance over time 46,47.

Variation in method conditions for robustness should be small and reflect typical day-to-day variation. Critical parameters are identified during the method development process. Only these critical method parameters should be investigated for robustness. Common critical method parameters can be divided into two categories. The HPLC conditions include HPLC column (lot, age, and brand), Mobile-phase composition (pH ± 0.05 unit, organic content ± 2%) and HPLC instrument (dwell volume, detection wavelength ± 2 nm, column
temperature ± 5°C, flow rate). The sample preparation variations include sample solvent (pH ± 0.05 unit, organic content ± 2%), sample preparation procedure (shaking time, different membrane filters) and HPLC solution stability. The variations in chromatographic parameters for robustness study are given in the Table 29, 40.

**CONCLUSION**
The method development and validation are continuous and interrelated processes that are conducted throughout the drug development process. The analytical validation verifies that a given method measures a parameter as intended and establishes the performance limits of the measurement. Reproducible quality HPLC results can only be obtained if proper attention has been paid to the method development, validation and system’s suitability to carry out the analysis. The validated methods produce results within known uncertainties that are helpful to continuing drug development and provide emerging knowledge supporting the product. The time and effort that is devoted into developing scientifically sound and robust analytic methods should be aligned with the drug development stage. The resources that are constantly used during method development and validation must be balanced with regulatory requirements and the probability for product commercialization.
**Figure 5:** Limit of detection and limit of quantitation via signal to noise ratio (S/N)

**Fig. 6:** Range
Table 1: Variations in chromatographic parameters for robustness study

<table>
<thead>
<tr>
<th>S.No</th>
<th>Robustness parameter</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Detection wavelength</td>
<td>± 5 nm</td>
</tr>
<tr>
<td></td>
<td>Flow rate</td>
<td>± 0.05 ml/min</td>
</tr>
<tr>
<td></td>
<td>Buffer pH</td>
<td>± 0.1 unit</td>
</tr>
<tr>
<td></td>
<td>Mobile phase</td>
<td>± 2 ml</td>
</tr>
<tr>
<td></td>
<td>Column</td>
<td>Different brand or batch number</td>
</tr>
</tbody>
</table>

REFERENCES
8. FDA Guidance for Industry (2000) - Analytical Procedures and Method Validation, Chemistry, Manufacturing, and Controls Documentation, Center for Drug Evaluation and Research (CDER) and Center for Biologics Evaluation and Research (CBER)