Chapter: 2

A) Analytical Method Development
B) Validation
Chapter: 2

Analytical Method Development:

HPLC method development and validation are important for the analysis of drugs in any formulation. Whatever method is used for quantitation or identification of drug, the method should be a validated one. The method must be able to detect or quantitate the particular drug in presence of other components. Before starting any method development one has to have knowledge about the information of the nature of sample, definite separation goals, number of compounds present, structures, molecular weights, pKa values, solubility and UV spectrum of the compounds. Perhaps method development involves the trial and error procedures.

The most difficult problem usually is where to start, what type of column is worth trying and with what kind of mobile phase. While there are a number of HPLC methods available to the development chemist, perhaps the most commonly applied method is reversed phase chromatography. A typical pharmaceutical compound is considered to be an active pharmaceutical ingredient (API) of less than 1000 Daltons, either soluble in water or in an organic solvent.

The water soluble drug is further differentiated as ionic or nonionic which can be separated by reverse phase. Similarly, the organic soluble drugs can be classed as polar and non polar and are equally separated by reverse phase. In some cases the non polar API may have to be separated using adsorption or normal phase HPLC, in which mobile phase would be non polar organic solvent. The other chromatographic modes may be necessary for separation. These include ion exchange, chiral and size exclusion chromatography. In case of samples like proteins, peptides, nucleic acids and synthetic polymers analysed by using some special columns or ion pair reagents (i.e. 0.1% TFA).
General conditions to initiate HPLC method development

Either isocratic or gradient mode may be used to determine the initial conditions of the separation, following the suggested experimental conditions given in Table-1.

Table-1: Selection of initial HPLC chromatographic conditions

<table>
<thead>
<tr>
<th>Chromatographic variables</th>
<th>Neutral compounds</th>
<th>Ionic-acidic compounds (carboxylic acids)</th>
<th>Ionic-basic compound (amines)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column dimension (length, i.d.)</td>
<td>250 x 4.6 mm</td>
<td>250 x 4.6 mm</td>
<td>250 x 4.6 mm</td>
</tr>
<tr>
<td>Packing material</td>
<td>C&lt;sub&gt;18&lt;/sub&gt; or C&lt;sub&gt;8&lt;/sub&gt;</td>
<td>C&lt;sub&gt;18&lt;/sub&gt; or C&lt;sub&gt;8&lt;/sub&gt;</td>
<td>C&lt;sub&gt;18&lt;/sub&gt; or C&lt;sub&gt;8&lt;/sub&gt;</td>
</tr>
<tr>
<td>Particle size</td>
<td>5 μm</td>
<td>10μm or 5 μm</td>
<td>10μm or 5 μm</td>
</tr>
<tr>
<td>Mobile phase Solvents A and B</td>
<td>Water-Acetonitrile</td>
<td>Buffer-Acetonitrile</td>
<td>Buffer-Acetonitrile</td>
</tr>
<tr>
<td>Ratio</td>
<td>50:50</td>
<td>20:80</td>
<td>20:80</td>
</tr>
<tr>
<td>Buffer and pH</td>
<td>No buffer required</td>
<td>Phosphate 25 mM 3.0&amp;7.5</td>
<td>Phosphate 25 mM 3.0&amp;7.5</td>
</tr>
<tr>
<td>Peak modifier</td>
<td>Do not use initially</td>
<td>1% v/v acetic acid</td>
<td>0.1% v/v triethylamine</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 to 2.0 ml/min</td>
<td>1.0 to 2.0 ml/min</td>
<td>1.0 to 2.0 ml/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient</td>
<td>Ambient</td>
<td>Ambient</td>
</tr>
<tr>
<td>Injection volume</td>
<td>10μl to 25 μl</td>
<td>10μl to 25 μl</td>
<td>10μl to 25 μl</td>
</tr>
<tr>
<td>Sample concentration</td>
<td>&lt; 100 μg</td>
<td>&lt; 100 μg</td>
<td>&lt; 100 μg</td>
</tr>
</tbody>
</table>
Table-2: Column & mobile phase selection shall be done as per the table given below

<table>
<thead>
<tr>
<th>Method/Description/Column</th>
<th>When the method preferred</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reverse-Phase HPLC</strong></td>
<td></td>
</tr>
<tr>
<td>Water/organic mobile phase</td>
<td>For neutral and non ionized compounds that dissolve in water/organic mixtures.</td>
</tr>
<tr>
<td><strong>Column</strong>: C18, C8, Phenyl, Cyano, and Trimethylsilyls (TMS) columns.</td>
<td></td>
</tr>
<tr>
<td><strong>Normal Phase HPLC</strong></td>
<td></td>
</tr>
<tr>
<td>Mixture of organic solvents as mobile phase</td>
<td>For samples that do not dissolve in water/organic mixture.</td>
</tr>
<tr>
<td><strong>Column</strong>: Silica, Cyano and Amino columns.</td>
<td></td>
</tr>
</tbody>
</table>

In general, one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble. The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient can be started with 5-10% organic phase in the mobile phase and the organic phase concentration can be increased up to 100% within 20-30 min. Separation can be optimized by changing the initial mobile phase composition and the slope of gradient according to the chromatogram obtained from preliminary sample run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, namely at what mobile phase composition. Changing the polarity of a mobile phase can alter elution of drug molecules.

The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, higher is the elution. Ionic samples (acidic and basic) can be separated, if they are present in the undissociated form. Dissociation of ionic samples may be
suppressed by proper selection of pH. The buffer selected for a particular separation should be used to control pH over the range of \( \approx \text{pKa} \pm 2.0 \). The buffer should transmit light at or below 200 nm so as to allow low UV detection and pH of the buffer should be adjusted before adding organic solvent. Optimization can be started only after a reasonable analysis has been obtained. A reasonable chromatogram means that more or less symmetrical peaks on the chromatogram with detection of all the compounds. The optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time.

The peak resolution can be increased by using a more efficient column (column with higher theoretical plate number), which can be achieved by using a column of smaller particle size, or a longer column in length. These factors will increase the analysis time. Flow rate does not influence resolution, but it has a strong effect on the analysis time.

**The parameters that are affected by the changes in chromatographic conditions are,**

- Capacity factor (\( K' \)).
- Selectivity (\( \alpha \)).
- Column efficiency (N).
- Peak asymmetry factor or Tailing factor (As).

**Selection of mobile phase:**

The selection of the mobile phase is mainly based on the solubility and polarity of the compound. Usually, in RP-HPLC method water and organic solvents are used as the mobile phase. In NP-HPLC method non polar solvents like hexane and THF were used. If the sample contains ionic or ionizable compounds, then use of a buffered mobile phase is referred to ensure the reproducible results. Under
unfavorable circumstances, pH changes as little as 0.1 pH units can have a significant effect on the separation. On the other hand properly used buffer allows controlling the pH easily. Buffer works best at the pKa values of its acid. At this pH, the concentration of the acidic form and the basic form of the buffering species are equal, and the buffering capacity is maximum. Phosphate has three pKa values in the range of interest for silica based chromatography at pH 2, pH 7 and pH 12.32. The pKa of acidic buffer is 4.75. Citrate has three pKa values 3.08, 4.77 and 6.40. Between citrate and phosphate buffers, the entire pH range useful for silica chromatography can be covered.

In many cases, a silanophilic interaction causes tailing, mainly for the basic compounds due to ion-exchange interaction. This can usually be reduced or suppressed by the use of mobile phases modifiers (0.1% v/v triethylamine for basic analyte or 1% v/v glacial acetic acid for the acidic analyte), or a combination thereof. Whenever buffers or other mobile phase activities are used, check the solubility in mobile phase. This is especially true for gradient applications. Acetonitrile is the preferred organic modifier in reversed phase chromatography. Acetonitrile based mobile phases can give up to two fold lower pressure drop. This means that column efficiency is higher. The elution strength increases in the order methanol, Acetonitrile and tetrahydrofuran. The retention changes by roughly 10% for every 1% change in the concentration of organic modifier.

Mobile phase composition:

In reverse phase chromatography, the separation is mainly controlled by the hydrophobic interaction between drugs molecules and the alkyl chains on the column packing materials. Most chromatographic separation can be achieved by choosing the optimum mobile phase composition. This is due to the fact that a fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used
solvents in reverse phase chromatography are methanol and Acetonitrile. Tetrahydrofuran is also used but to a lesser extent.

Initially experiments shall be conducted with mobile phases having buffers with different pH and solution organic phases to check for the best separation between the impurities. A drug solution having all possible known impurities can be used for checking the extent of separation with different mobile phase ratios. Alternatively, solution of stressed drug substance can be used to check for separation of impurities. Silica based column with different crosslinkings in the increasing order of polarity are as follows.

<-----Non-polar-----Moderately polar-----Polar------>

$C_{18} < C_8 < C_6 < \text{Phenyl} < \text{Amino} < \text{Cyano} < \text{Silica}.$

Experiments are to be conducted using different columns with different mobile phase to achieve best separation in chromatography. A column which gives separation of all the individual impurities and degradants from each other and from API peak and which is rugged for variation in mobile phase shall be selected.

**Selection of pH:**

pH is another factor in the resolution that will affect the selectivity of the separation in reversed-phase HPLC. In reverse-phase chromatography sample retention ($K'$) increases when the analyte is more hydrophobic (nonpolar). Sample retention ($K'$) decreases when the analyte is more hydrophilic (polar). Thus when an acid or base undergoes ionization it becomes more hydrophilic and less interacting with column binding sites. When the pH value of the mobile phase equal to the pKa value of the analyte, it is said to be half ionized, i.e. the
concentration of the ionized and unionized species are equal. As mostly all of the pH caused changes in the retention occur within ± 2.0 pH unit of the pKa value, it is best to adjust the mobile phase to pH value at least ± 2.0 pH unit above or below the pKa to ensure practically 100% unionization of analyte for retention purpose. Generally at low pH peak tailing is minimized and method ruggedness is maximized. On the other hand, operating in the intermediate pH offers an advantage in increased analyte retention and selectivity.

pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics. Drug molecules retention time depending on the pKa value Eg: Acids shows an increase in retention as the pH is reduced, While base a show a decrease, Experiments shall be conducted using buffers having different pH to obtain the required separations. It is important to maintain the pH of the mobile phase in the range of 2.0 to 0.8 as most column does not withstand to the pH which are out of range. This is due to the fact that the siloxane linkages are cleaved below pH 2.0, While at values above 8.0 silica may dissolve. If a pH outside the range of 2.0 to 8.0 is found to be necessary, packing materials which can withstand to that ranges shall be chosen.

Selection of buffer:

In reversed phase chromatography mobile phase pH values are usually between 2.0 and 7.5. Buffers are needed when an analyte is ionizable under reversed phase conditions or the sample solution is outside this pH range. Analyte ionisable under reverse phase conditions often have amine or acid functional group with pKa between 1.0 and 11.0. A correctly chosen buffer pH will ensure that the ionisable functional group is in a single form, whether ionic or neutral. If the sample solution is at pH damaging to the column, the buffer will quickly bring the pH of the injected solution to a less harmful pH.
If the analyte contains only amine functional group buffer selection is easier. Most amine will be in cationic form at pH value less than 9.0, so any buffer effective at pH 7.0 or lower will work. Buffers at pH 7.0 are used, even though pH of water is 7.0, because amine retention and peak shapes are pH dependent. As pH is lowered amine retention time shortens and peak shape sharpens as the buffer protonates the acidic silanols on silica surface. Any buffer with pKa less than 7.0 is suitable, but we have found potassium phosphate at pH 3.0 is the best for amines. In both the condition (acidic and basic) potassium phosphate buffer of pH 3.0 works well in general. The potassium salt works better than the sodium salt for amines. It is important to use the buffers with suitable strength to cope up for the injection load on the column otherwise peak tailing may arise during chromatography. Therefore, strength of the buffer should be suitable enough to take injection load on the column so that peak tailing is avoided.

The retention time also depends on the molar strength is inversely proportional times. Ideally, the strength of the buffers shall be between 0.05M to 0.20M. The selection of buffer and its strength is done always in combination with selection of organic phase composition in mobile phase. The strength of the buffer can be increased, if necessary, to achieve the required separation. But it is to be ensured that the higher buffer strengths shall not result in precipitants and turbidities either in mobile phase or in standard and test solution which are allowed to stand in bench top or in refrigerator. Experiments shall be conducted using different strengths to obtain the required separations. The buffers having a particular strength which give separation of all individual impurities and degradants. The selected buffer strength and the effect of variation shall be studied.
Selection of column:

The HPLC column is the heart of the instrument and critical for performing the separation. The column must possess the selectivity, efficiency and reproducibility to provide good separation. Commonly used reversed phases are crosslinking the Si-OH groups with alkyl chains like, C8 (octylsilane), C18 (octadecyl silane) nitrile groups (CN), phenyl groups (-C6H6) and amino groups (-NH2). They are chemically different bounded phases and demonstrate significant changes in the selectivity using the same mobile phase. During method development selection of column can be streamlined by starting with shorter column (150, 100 or even 50 mm long). By selecting a shorter column with an appropriate phase run time 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 (i.d.), many laboratories use 4.6 mm internal diameter as standard. But it is worth considering use of 44.0 mm i.d. column as an alternative. This requires only 75% of the solvent flow that a 4.6 mm column used. Selecting an appropriate stationary phase can also help to improve the efficiency of the method development. For example, a C8 phase (reverse phase) can provide a further time saving over a C18 as it doesn’t retain analyte as strongly as the C18 phase.

The following are the parameters of a chromatographic column which are to be considered while choosing a column for separation of impurities and degradants.

1) Length and diameter of the column, 2) Packing material, 3) Shape of the particles. 4) Size of the particles, 5) % of carbon loading, 6) Pore volume, 7) Surface area and 8) End capping.

Selection of Column temperature:

Temperature variation over the course of a day has quite significant effect on HPLC separations. This can even occur in air conditioned rooms. While
temperature is a variable that can affect the selectivity, its effect is relatively small. Always it is preferable to optimize the chromatographic conditions with column temperature as ambient. However, if the peak is symmetrical is could not be achieved by any combination of column and mobile phase, then the column temperatures above ambient can be adopted. The increase in column temperature generally will result in reduction in peak asymmetry and peak retentions. When found necessary, the column temperatures between 30°C and 80°C shall be adopted. If a column temperature of above 80°C is found to be necessary, packing materials which can withstand to that temperature shall be chosen.

**Selection of flow rate:**

Flow rate, more for isocratic than gradient separation, can sometimes be useful and readily utilized to increase the resolution, although its effect is very modest. The slower flow rate will also decrease the column back pressure.

**Flow rate shall be selected based on the following data.**

1. Retention times.
2. Column back pressures
3. Separation of impurities.
4. Peak symmetries.

Preferably the flow rate shall be not more than 2.5 ml/min. check the ruggedness of the method by varying the flow rate by ± 0.2 ml from the selected flow rate. Select the flow rate which gives least retention times, good peak symmetries, least back pressures and better separation of impurities from each other and from API peak.
Selection of Solvent delivery system:

Chromatographic separation with a single eluent (isocratic elution) in this method all the constituents of the mobile phase are mixed and pumped together as single eluent is always preferable. Gradient elution is a powerful tool in achieving separation between closely eluting compounds or compounds having widely differing polarities. The important feature of the gradient elution which makes it a powerful tool is that the polarity and ionic strength of the mobile phase can be changed (can be increased or decreased) during the run. Conduct experiments using different mobile phase combinations and different gradient programmes to achieve separation of all the impurities and degradants from others and from API peak. In general, while running a gradient, two mobile phases having different compositions are kept in different channels.

The two mobile phases are then introduced into the column by two different ways

1. By Low -pressure gradient-i.e. the mobile phases are mixed at the predetermined ratios and then pumped using a single pump.
2. A mobile phase which gives separation of all the individual impurities and degradants from each other and from API peak and which is rugged for variation of both aqueous and organic phase by at least ±5% of the selected mobile phase composition.

The mobile phases are pumped at different flow rates so as to achieve the required composition and then mixed in a chamber and then introduced into the column. While optimizing the separation of impurities, it is to be decided whether low-pressure gradient [not more than 80% organic phase is to be pumped] or High-pressure gradient [more than 80% of the organic phase is to be pumped]. While optimizing the gradient programme, especially using low viscous solvents like
Acetonitrile and phosphate buffers, it is recommended to mix about 10% aqueous portion preferably the same buffer used in mobile phase to avoid pumping problems.

**Selection of detector wavelength:**

Selection of detector wavelength is a critical step in finalization of the analytical method for impurities and degradants. Inject the impurity and API standard solutions into the chromatographic system with photodiode array detector and collect the spectra. Also conduct forced degradation studies and collect the UV spectra of all the major degradation products. Overlay the spectra of all the compounds and select a wavelength which is most common and gives higher responses for all compounds.

**Selection of Diluents for Test preparation and Extraction procedures:**

Diluent for test preparation is selected initially based on solubility of the drug substances and known impurities. Finalization of diluent is based on its extraction efficiency, peak symmetries and resolution of impurities and diluent blank injection interference. Inject the diluent blank and test solution spiked with known impurities into the chromatographic system and establish the non-interference of blank in estimation of impurities and the effect of diluent on resolution of impurities and peak symmetries.

Conduct experiments to optimize the extraction of API in presence of recipients at different test concentrations using the diluent chosen based on solubility and select the test concentration at which the extraction is most efficient. Select a diluent in which all the known impurities/degradants and drug substances are soluble, in which the extraction is complete, due to which there is no blank interference, in which the peak symmetries and resolution between impurities is found to be satisfactory.
Selection of Test concentration, Injection volume:

The test concentration is generally chosen based upon the response of API peak and impurities at the selected detector wavelength. The test concentration shall be finalized after it is proved that API is completely extractable at the selected test concentration. Generally an injection volume of 10 to 20μl is recommended for estimation of impurities.

If the extractions are found to be difficult, then the test concentrations can be kept low and the injection volume can be increased up to 50 μl. But it is to be ensured that at the selected injection volume the column is not overloaded, resolution between individual impurities and the peak symmetries are not compromised. After the test concentration and the diluent is finalized, prepare a test solution and keep the filtered solution in closed condition in a stoppered flask on the bench top and observe for any precipitation or turbidity after 24 hours. The solution should not show any turbidity / precipitation.

Establishment of Relative Response factor for known impurities

After finalizing the test method, establish the relative response factor of all the known impurities with respect to drug substance and the procedure is to prepare individual solutions of impurities and drug substance having concentration equivalent to 0.3% and 0.6% of the test preparation concentration and inject them into chromatographic system. Perform the establishment of RRF in duplicate preferably by a different analyst and on a different day. Calculate the average RRF for each trail from the RRF’s obtained for both 0.3% and 0.6% levels. The RRF obtained for 0.3% and 0.6% levels of a single trail should not differ by more than 0.05. Conduct recovery studies to prove that the established RRF’s are correct. Prepare an unspiked test preparation, inject into the chromatographic system and record the chromatograms.
Prepare spiked test solutions by spiking the test preparation with impurities at 0.3% and at 0.6% level. Inject the 0.3% and 0.6% spiked test solutions into the chromatographic system and record the chromatograms. % recovery shall be within the range of 90% to 110% for each trail. If not investigate and repeat the experiment again. Calculate the Average RRF from the RRF's obtained from both trails. Calculate the % recovery using the Average RRF's obtained from both trails. If the % recovery is within the range of 90% to 110% when Average RRF of both trails is taken into calculation, then the RRF’s are considered acceptable and shall be incorporated while calculation of % of known individual impurities.

**ANALYTICAL METHOD VALIDATION**

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications.

Validation is the process of determining the performance characteristics of a method/procedure or process. It is a prerequisite for judgment of the suitability of produced analytical data for the intended use. This implies that a method may be valid in one situation and invalid in another. Consequently, the requirements for data may, or rather must, decide which method is to be used. When this is ill-considered, the analysis can be unnecessarily accurate (and expensive), inadequate if the method is less accurate than required, or useless if the accuracy is unknown.

**Types of Analytical Procedures to be validated**

The validation of analytical procedures is directed to the four most common types of analytical procedures.
- Identification tests
- Quantitative tests for impurities' content
- Limit tests for the control of impurities
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below.

- Accuracy
- Precision
- Repeatability
- Intermediate Precision
- Specificity
- Detection Limit
- Quantitation Limit
- Linearity
- Range

This list should be considered typical for the analytical procedures cited but occasional exceptions should be dealt with on a case-by-case basis. It should be noted that robustness is not listed but should be considered at an appropriate stage in the development of the analytical procedure.

Furthermore revalidation may be necessary in the following circumstances

- Changes in the synthesis of the drug substance.
- Changes in the composition of the finished product.
- Changes in the analytical procedure.

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.
Specificity
Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

This definition has the following implications:

**Identification**: to ensure the identity of an analyte.

**Purity Tests**: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

**Assay (content or potency)**: to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

Developing a separation method for HPLC involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The response of the analyte in test mixtures containing the analyte and all potential sample components (placebo formulation, synthesis intermediates, excipients, degradation products and process impurities) is compared with the response of a solution containing only the analyte. Other potential sample components are generated by exposing the analyte to stress conditions sufficient to degrade it to 80–90% purity. For bulk pharmaceuticals, stress conditions such as heat (50–60 °C), light (600 FC of UV), acid (0.1 M HCl), base (0.1 M NaOH) and oxidant (3% H2O2) are typical. For formulated products, heat, light and humidity (70-80% RH) are often used. The resulting mixtures are then analysed, and the analyte peak is evaluated for peak purity and resolution from the nearest eluting peak.

Once acceptable resolution is obtained for the analyte and potential sample components, the chromatographic parameters, such as column type, mobile phase composition, flow rate and detection mode, are considered set.
Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

A method is said to be accurate if it gives the correct numerical answer for the analyte. The method should be able to determine whether the material in question conforms to its specification (for example, it should be able to supply the exact amount of substance present). Furthermore, it is rare that the results of several replicate tests all give the same answer, so the mean or average value is taken as the estimate of the accurate answer.

Some analysts adopt a more practical attitude to accuracy, which is expressed in terms of error. The absolute error is the difference between the observed and the expected concentrations of the analyte. Percentage accuracy can be defined in terms of the percentage difference between the expected and the observed concentrations.

Percentage accuracy tends to be lower at the lower end of the calibration curve. The term accuracy is usually applied to quantitative methods but it may also be applied to methods such as limit tests. Accuracy is usually determined by measuring a known amount of standard material under a variety of conditions but preferably in the formulation, bulk material or intermediate product to ensure that other components do not interfere with the analytical method. For assay methods, spiked samples are prepared in triplicate at three levels across a range of 50-150% of the target concentration. The per cent recovery should then be calculated. The accuracy criterion for an assay method is that the mean recovery will be 100±2% at each concentration across the range of 80-120% of the target concentration. To
document accuracy, ICH guidelines regarding methodology recommend collecting
data from a minimum of nine determinations across a minimum of three
concentration levels covering the specified range (for example, three
concentrations, three replicates each).

**Precision**
The precision of an analytical procedure expresses the 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. Precision should be investigated using homogeneous,
authentic samples. However, if it is not possible to obtain a homogeneous sample
it may be investigated using artificially prepared samples or a sample solution.
The precision of an analytical procedure is usually expressed as the variance,
standard deviation or coefficient of variation of a series of measurements.

1. **Repeatability**: Repeatability expresses the precision under the same
   operating conditions over a short interval of time. Repeatability is also
termed intra-assay precision.

2. **Intermediate precision**: Intermediate precision expresses within-
laboratories variations: different days, different analysts, different
equipment, etc.

3. **Reproducibility**: Reproducibility expresses the precision between
   laboratories (collaborative studies, usually applied to standardization of
   methodology).

Precision means that all measurements of an analyte should be very close together.
All quantitative results should be of high precision - there should be no more than
a ±2% variation in the assay system. A useful criterion is the relative standard
deviation (RSD) or coefficient of variation (CV), which is an indication of the
imprecision of the system.
According to the ICH, precision should be performed at two different levels - repeatability and intermediate precision. Repeatability is an indication of how easy it is for an operator in a laboratory to obtain the same result for the same batch of material using the same method at different times using the same equipment and reagents. It should be determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each) or from a minimum of six determinations at 100% of the test or target concentration.

Intermediate precision results from variations such as different days, analysts and equipment. In determining intermediate precision, experimental design should be employed so that the effects (if any) of the individual variables can be monitored. Precision criteria for an assay method are that the instrument precision and the intra-assay precision (RSD) will be ≤2%.

**Detection limit**
The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

**Quantitation limit**
The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.
Limits of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. It is expressed as a concentration at a specified signal: noise ratio, 2 usually 3:1. The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The ICH has recommended a signal:noise ratio 10:1. LOD and LOQ may also be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve(s) at levels approximating the LOD according to the formulae: LOD = 3.3(SD/S) and LOQ = 10(SD/S).

The standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines. The method used to determine LOD and LOQ should be documented and supported, and an appropriate number of samples should be analysed at the limit to validate the level.

**Linearity**

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

The linearity of a test procedure is its ability (within a given range) to produce results that are directly proportional to the concentration of analyte in the sample. The range is the interval between the upper and lower levels of the analyte that have been determined with precision, accuracy and linearity using the method as written. ICH guidelines specify a minimum of five concentration levels, along with certain minimum specified ranges. For assay, the minimum specified range is 80–120% of the theoretical content of active. Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear
regression line for the response versus concentration plot. The regression coefficient (r^2) is 0.998 and is generally considered as evidence of acceptable fit of the data (Figure 3) to the regression line. The per cent relative standard deviation (RSD), intercept and slope should be calculated.

**Range**

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) 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.

**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.

Robustness measures the capacity of an analytical method to remain unaffected by small but deliberate variations in method parameters. It also provides some indication of the reliability of an analytical method during normal usage. Parameters that should be investigated are per cent organic content in the mobile phase or gradient ramp; pH of the mobile phase; buffer concentration; temperature; and injection volume. These parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment. The chromatography obtained for a sample containing representative impurities when using modified parameter(s) should be compared with the chromatography obtained using the target parameters.

Analytical solution stability Validation of sample and standard solution preparation may be divided into sections, each of which can be validated. These include extraction; recovery efficiency; dilution process when appropriate; and
addition of suitable internal standards. Although extraction processes do not actually affect the measuring stage they are of critical importance to the analytical test method as a whole. The extraction process must be able to recover the analyte from the product; it must not lose (for example, by oxidation or hydrolysis) any of the analyte in subsequent stages, and must produce extraction replicates with high precision. For example, during analysis of an ester prodrug the extraction process involves the use of strongly alkaline or acid solutions, it may cause some of the prodrug to be hydrolysed and, therefore, give false results.

Reference substances should be prepared so that they do not lose any of their potency. Thus it is necessary to validate that the method will give reliable reference solutions that have not been deactivated by weighing so little that an error is produced; adsorption onto containers; decomposition by light; and decomposition by the solvent. If the reference is to be made up from a stock solution then it must be validated that the stock solution does not degrade during storage. Reagent preparation should be validated to ensure that the method is reliable and will not give rise to incorrect solutions, concentrations and pH values.

Samples and standards should be tested during a period of at least 24 h (depending on intended use), and component quantitation should be determined by comparison with freshly prepared standards. For the assay method, the sample solutions, standard solutions and HPLC mobile phase should be stable for 24 h under defined storage conditions. Acceptable stability is ≤2% change in standard or sample response, relative to freshly prepared standards. The mobile phase is considered to have acceptable stability if aged mobile phase produces equivalent chromatography (capacity factors, resolution or tailing factor) and the assay results are within 2% of the value obtained with fresh mobile phase.