CHAPTER-2
METHOD DEVELOPMENT DESIGN AND ITS VALIDATION

2.1 ANALYTICAL METHOD DEVELOPMENT

First step in HPLC method development is to collect the maximum information about drug with literature review, check availability of drug in the pharmacopeia and journals article related to active substance. After literature search, solubility study should be performed (at different pH, media, water and solvent) of “components of interest” to understand the characteristic of the drug. All possible degradation products/related substance/ key starting materials and by products should be identified before initiation of related substances method development. Steps in Method development are as follows

2.1.1 Selection and optimization of mobile phase

Selection of mobile phase depends upon physio-chemical characteristics of active component and related substances. Mobile phase containing strong acids, halides and bases solution should not be used because they can spoil the stationary phase and chromatography system. The key objective of selection and optimization of mobile phase is to separate all the degradants peaks, individual impurities and placebo peaks from each other and active peak.

During mobile phase selection following factors should be considered:
2.1.1.1 Buffer, ion pair and its concentration:

Ionic strength of mobile phase is maintained by buffers. Buffer selection should be based on pH of mobile phase and UV cutoff of the buffer. A choice of buffers can be employed for achieving the desired separations. Molar strength of buffer is inversely proportional to retention time of analyte. The recommended buffers concentration for mobile phase is in between 0.01-0.05M. Following buffers are used for reversed phase chromatography.

<table>
<thead>
<tr>
<th>Buffer name</th>
<th>pH range of buffer</th>
<th>UV cut (nm) off</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>2.8-4.8</td>
<td>230</td>
</tr>
<tr>
<td>Formic acid</td>
<td>3.8-5.8</td>
<td>230</td>
</tr>
<tr>
<td>Phosphate pKa1</td>
<td>1.1-3.1</td>
<td>200</td>
</tr>
<tr>
<td>Phosphate pKa2</td>
<td>6.2-8.2</td>
<td>200</td>
</tr>
<tr>
<td>Phosphate pKa3</td>
<td>11.3-13.3</td>
<td>200</td>
</tr>
<tr>
<td>Citric acid pKa1</td>
<td>2.1-4.1</td>
<td>230</td>
</tr>
<tr>
<td>Citric acid pKa2</td>
<td>3.8-5.8</td>
<td>230</td>
</tr>
<tr>
<td>Citric acid pKa3</td>
<td>5.4-7.4</td>
<td>230</td>
</tr>
<tr>
<td>Tris</td>
<td>7.3-9.3</td>
<td>205</td>
</tr>
<tr>
<td>Ammonia</td>
<td>8.3-10.3</td>
<td>200</td>
</tr>
<tr>
<td>Triethyl amine</td>
<td>10.0-12.0</td>
<td>200</td>
</tr>
<tr>
<td>Pyrrolidine</td>
<td>10.3-12.3</td>
<td>200</td>
</tr>
</tbody>
</table>

When the separation of closely related components is required, various ion pair reagents can be used along with buffers or alone. For the separation and retention of acidic compounds, tetra butyl ammonium hydrogen sulphate (quaternary amines) and sodium perchlorate can be used. Alkyl chain sulphonic acid sodium salt is best choice for the separation and retention of basic compounds. Experiments shall be conducted using different buffers with the addition of ion-pair reagent at different concentration to obtain the desired
separation. The recommended concentration of ion-pair in mobile phase is from 0.005-0.02 M.

If peak tailing is observed during separation of basic compound on C8-C18 columns due to strong interaction between basic analyte and free silanol groups of silica, use triethylamine (TEA) or diethylamine (DEA) solvents in mobile phase, which act as competitive base and reduce solute free silanol interaction resulting in reduced peak tailing. For the analyte of acidic nature, 1% acetic acid or 0.1% trifluoro acetic acid (TFA) is used in mobile phase.

2.1.1.2 pH of buffer or pH of mobile phase

Selection of pH of buffer used in mobile phase is depends on pKa of analyte. Depending on the pKa, retention time of drug molecule varies with change in the pH of mobile phase. Eg. : An acidic molecule shows increase in retention time as the pH is reduced, while basic analyte shows a decrease in retention time. When the analyte/sample is ionized in diluents, it becomes polar and does not retain in the stationary phase for longer period, elutes too fast. The retention time of non-polar analyte in the column increases. When pH decreases basic analyte will be in an ionized form and acidic analyte in un-ionized form.

With the reference of [Henderson-Hasselback] equation \[ \text{pH}=\text{pKa}+\log(\frac{[A^-]}{[HA]}) \]. When pH of mobile phase and pKa of the analyte is equal, analyte will be in equilibrium between both states ie. half ionized and half un-ionized; this may lead to distortion of any peak.

Best choice of mobile phase pH is in between 2 to 8. The pH below 2 leads the cleavage of siloxane linkages and pH above 8 may dissolve silica particles. The pH of the buffer which gives separation of all individual impurities from each other and from API peak should be best choice. To establish the robustness of the method, variation in selected pH with ± 0.2 units shall be performed and the effect of variation on the separation of components shall be studied.
2.1.1.3 Composition of mobile phase

Separation of analytes in reverse phase chromatography depends upon hydrophobic interaction of drug molecules and alkyl chains of stationary phase. The solvents like acetonitrile having low viscosity and low UV cut off absorbance are the best choice for mobile phase. Solvent selection criteria are the polarity and its solvent strength.

Polarity of solvents in decreasing order (As per eluotropic series)

Acetonitrile > Methanol > Ethanol > THF > Propanol

Tetrahydrofuran (THF) is very less recommended solvent to use in mobile phase due to high UV cut off and reactivity with oxygen. It takes longer time in column equilibration.

Robustness should be performed with making change in the ratio of buffer and organic solvent by atleast ± 10% relative or ± 2% absolute (whichever is less) of minor component to the final mobile phase composition.

2.1.2 Selection of diluent

Selection of diluent based on the solubility of analyte/impurities and compatibility with mobile phase. If diluent and mobile phase are not compatible with each other, peak shape and sensitivity of analyte peak is diminished. Diluent should extract more than 98% active component from the matrix of pharmaceutical dosage forms. USP peak tailing (Symmetry) of all analytes should be less than 2.0 in selected diluent. To dissolve the polar compounds, selected pH buffer can be used individually or in combination with organic solvent. Select organic solvents to dissolve non polar analytes. For complete extraction of drug from sample matrix of dosage form sonication, magnetic stirring, rotator shaking can be used.
2.1.3 Selection of stationary phase

2.1.3.1 Column Dimensions

This reveals the internal diameter and length of cylindrical column tube filled with silica bed packing material inside.

2.1.3.2 Particle shape

Two types of particle shapes are available for silica: irregular and spherical shapes. The spherical particles of silica are more in use to get higher reproducibility as compared to irregular shape.

Figure 2.1 Shapes of irregular and sphere shaped particles size

Particle size specifies the average diameter of the stationary phase particles. For HPLC analysis, preferred size of particles are 3-10μm. Smaller particle size produces higher efficiency but also creates higher back pressure and lesser sample loading capacity.

2.1.3.3 Surface area

Column surface area is measured in “m²/g”; the total surface area of particle is the sum of inner pore surface and outer surface of the particle. As per column chemistry, pore size is inversely proportional to the surface area means smaller
pore size greater surface area which consequently increases the retention and capacity factor of analytes.

Select the column having silica pore sizes ranging from 60Å to 10000Å on the basis of molecular weight of analyte. Larger pores size allows larger solute molecules to be retained for longer time through maximum exposure to the surface area of particles. Silica with pore size 150Å or less should be selected for sample having molecular weight less than 2000.

2.1.3.4 Carbon loading

Carbon load describe the quantity of carbon chain length attached to the silica. The % carbon load is an excellent indicator of hydrophobic retention of analyte. Increased % carbon loads offers better resolution and longer analysis time.

2.1.3.5 End capping

End capping is the process of chemical bonding between small hydrocarbon chains like trimethylchlorosilane (Silating agent) and free silanol groups on a packing surface. End capping prevents direct interaction between basic analyte and acidic silanol groups resulting reduced peak tailing of polar compounds. The basic criteria for HPLC column selection is the polarity of molecule. Increasing polarity order of stationary phase is as follows:

C18<C8<C6/C4/C3<Phenyl<Amino<Cyano<Silica

Advantage of HPLC columns with shorter length (30-50 mm) is reduced run time, low column back pressure, lesser equilibration time and high sensitivity. While longer HPLC columns (250-300 nm) increase resolution, increase capacity to more sample load, but also increase analysis run time, column back pressure and solvent consumption.
2.1.4 Force degradation studies

Force degradation studies are conducted to explore the possible degradation products of the molecule which provides information about the degradation pathways and inherent stability of the drug molecule. Force degradation study is vital to establish the stability indicating power of the analytical method attaining base to base separation of all possible degradation products. The impurities produced during force degradation studies are termed as “potential degradation products”. Major forced degradation conditions are as follows:

- Acid degradation: Acidic stress
- Base degradation: Alkaline stress
- Oxidative degradation: Hydrogen peroxide stress
- Thermal degradation: Dry heat stress
- Photolytic degradation: Exposure to UV and visible light

2.2 ANALYTICAL METHOD VALIDATION

Once we have developed the methods suitable to quantify the amount of drug present in the formulation (assay), and quantification of impurities in the formulation (Related Substances), their suitability for their respective intended purposes is to be established by means of Validation. Analytical method validation is the process of demonstrating that an analytical procedure is suitable for its intended purpose.

Validation is defined as “Validation is the process of collecting documented evidence that the method performs according to its intended purpose”.

It is mandatory to submit the analytical method validation report to regulatory agencies with a proof that the method and instrument is suitable for its application in drug analysis. During analytical method validation following parameters need to be performed.
2.2.1 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.

2.2.2 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 as trueness.

2.2.3 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.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

2.2.4 Repeatability

“Repeatability expresses the precision under the same operating conditions over a short interval of time”.

Repeatability should be assessed using:

A minimum of 9 determinations covering the specified range for the procedure (e.g. 3 concentrations/3 replicates each) or

A minimum of 6 determinations at 100% of the test concentration.
2.2.5 Intermediate precision

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

2.2.6 Reproducibility

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

2.2.7 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”.

2.2.7.1 Based on visual evaluation

Visual evaluation may be used for non-instrumental and instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

2.2.7.2 Based on signal to noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio (S/N) is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio (S/N) between 3 or 2:1 is generally considered acceptable for estimating the detection limit.
2.2.7.3 Based on standard deviation of slope and response

The detection limit (DL) may be expressed as

\[ DL = \frac{3.3 \, s}{S} \]

Where,
- \( s \) = the standard deviation of the response
- \( S \) = the slope of the calibration curve

2.2.8 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.

2.2.8.1 Based on visual evaluation

Visual evaluation may be used for non-instrumental and instrumental methods. The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

2.2.8.2 Based on signal to noise approach

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio (S/N) is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio (S/N) is 10:1.
2.2.8.3 Based on standard deviation of the response and the slope

The quantitation limit (QL) may be expressed as:

\[ QL = \frac{10 \times s}{S} \]

Where \( s \) = the standard deviation of the response
\( S \) = the slope of the calibration curve.

2.2.9 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”.

2.2.10 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”.

2.2.11 Robustness

The robustness of an analytical procedure (liquid chromatography) is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters say variations of pH in mobile phase, variation in mobile phase composition, column temperature, mobile phase flow rate and wavelength.
2.3 REFERENCES


