Chapter-1

INTRODUCTION
# CHAPTER 1

## Chapter 1: Introduction

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CHAPTER 1

1.1 General introduction to Basic principles of “Liquid Chromatography”

1.1.1 Brief Biography and Definition

The biography of liquid chromatography distinguished that in the beginning of 1900’s it was defined by Mikhail S. Tswett who is Russian botanist. His innovative studies made spotlight and initiated the path by using a column packed with particles for separation of mixture of compounds present in the leaf pigments which were extracted from plant using a solvent.

Tswett prepared the stationary phase by filling a long glass column with the particles belongs two specific materials. Those are alumina and calcium carbonate known as powdered chalk. The sample prepared from the leaves extract Tswett poured on the top of the glass column to trespass between the particles present in the column. Further he continuously poured little by little the pure solvent which was used for extraction and due to gravity the solvent started trespassing through particle beads along with the extract matrix by resulting into disparate colored bands due to their varied movement. Based this he had concluded that the disparately colored bands are present as mixture originally in the extract. Thus he fashioned to create a separation procedure in the discipline of analysis and defined that separation was due varying chemical attractions between the plant extract and particles present in the column.
The components present in the plant extract having less chemical affinity towards the particle beads present in the column were moved firstly down the gravity. The components of plant extract with more chemical fondness towards the particle beads packed in glass column moved slowly down to the gravity. The whole process can be briefed as components present in the sample matrix will be separated based on their partition between the mobile phase and stationary phase which controls the movement or speed of compounds present in the sample matrix.

Tswett coined the above said separation methodology as chromatography. The terminology of chromatography was derived from Greek words Chroma which was known as color and graph which means writing. In the present day scenario thus defined liquid chromatography had undergone various technical stages and has became one of the most feasible and predominant analytical tool in the ocean of analytical chemistry.

![Plant Extract in Solvent](image)

**Fig. 1.1 - Tswett's Experiment**
1.1.2 Various LC (Liquid Chromatography) Procedures

The LC procedures can be defined broadly into three techniques. The first and second techniques purely related to planar chromatography which contains mobile phase as eluent and planar support as stationary phase. The third one is most popular, dominant and influential technique identified as column chromatography. All the three techniques require preparing the sample in solution by dissolving it in a suitable solvent or liquid. Further the prepared solution will loaded on to top of the stationary phase or into the device meant for chromatography.

The first technique was acknowledged as thin layer chromatography. In this the stationary phase is made of glass plate coated with supporting materials such as alumina and calcium carbonate etc. The pre-coated and dried glass plate bottom edge will be placed in a mobile phase which is mixture of solvents existing in chamber as represented in Fig. 1.2. The mobile phase will start moving on the glass plate through capillary action due to presence of dry particles coated on the glass plate. The sample was loaded on the glass plate as spots will also be in motion along with the solvent mixture so the compounds present in the sample spot which were having less likeness with the stationary phase had been separated on the top of the glass plate and which were more likeness had been eluted middle and just above the sample spots as shown in the same figure.
The second technique was recognized as paper chromatography. In this the stationary phase is made of paper such as whatman paper etc. The sample will be loaded as small spots on the edge of the paper and dried to evaporate the solvent present in the sample spot, will be hanged keeping the sample spots towards bottom edge in a mobile phase existing in chamber as represented in Fig. 1.3. The mobile phase will start moving on the paper through capillary action due to presence of pores present on the surface of paper. The sample was loaded on the paper as spots will also be in motion along with the solvent mixture so the compounds present in the sample spot which were having less affinity with the stationary phase, had been separated on the top of the paper and which were more affinity, had been eluted middle and just above the sample spots as shown in the same figure.

**Fig. 1.2 - Thin-Layer Chromatography**
The third technique was recognized as column chromatography. In this the stationary phase was made up of glass or stainless steel packed/filled with the appropriate particles which were well known as packing material for chromatography. As a consequence the column will be fixed in equipment. The sample dissolved in a solvent will be loaded on top of the column or into the device before column inlet. The solvent or solvent mixture (mobile phase) will be present as continuous flow as stream will carry the sample solution with its flow into the column and there the actual separation starts. Based on the attraction towards the packed material in the column the compounds present in the sample will vary their speed along with the eluent flow. The flow of eluent can be accomplished in numerous ways. The mobile phase flow will be maintained either gravity or vacuum columns which were not designed to withstand pressure. The particle size in this case will be large in size and the diameter will be greater than 50 µm. The
best example for these type experiments is open glass column typically used in Tswett’s experiment.

![Image of column chromatography process]

**Fig. 1.4 - Column Chromatography – Solid-Phase Extraction**

However, the mobile phase flow can be maintained through pneumatic pumps which will produce constant flow in isocratic and gradient manner where the columns filled with particles smaller in size (less than 10µm). These columns will give more resistance to the flow but provide good separation. The new techniques were designed to withstand the back pressure generated with the flow of mobile phase. Based on the pressure withstanding limit the techniques were designated as HPLC and UPLC. HPLC withstands the back pressure in the range 0-5000psi where as UPLC withstands in the range of 0-12000psi.
Fig. 1.5 - Overview of HPLC
1.2 General introduction on Analytical Procedures Development and Validation

1.2.1 Analytical Method Life Cycle

The analytical method development and validation/revalidation is unique study and will not be a one time. The concise life cycle of an analytical procedure was represented in the Fig. 1.6.

![Fig. 1.6 – Life Cycle of Analytical Procedure](image)

The development of an analytical procedure has to be initiated in the early stage of medicinally important compound synthesis i.e. API synthesis stage or starting stage for designing of new pharmaceutical dosage form. This will help to develop the procedure in parallel to the API synthesis or dosage form design. Consequently it will minimize the time and as well increases the better understanding on the analytical procedure with respect to the API as well as dosage form. Once the API or dosage form is ready to manufacture batches for regulatory submissions, the finalized analytical procedure has to be validated as per the current regulatory requirements and ICH Q (R1) methodology. Followed by successful completion of analytical procedure validation it can be transferred to regular quality control lab for their routine use.
for the quality checks to the respective API or pharmaceutical dosage form. In future if any change in the API synthesis or dosage form design the same method has to be verified against new API or dosage form for its compatibility and the analytical methodology has to be revalidated for its intended use in the quality control laboratories for regular quality checks.

1.2.2 Significance of Analytical Procedure Development and Validation

Medicinally important organic compounds are more in the market and gradually increasing in the world of pharmaceutical sector. These should be pure enough to have its therapeutic activity when it was got synthesized/extracted from natural source known as Active Pharmaceutical Ingredient (API) and the API converted into a pharmaceutical dosage form which is an acceptable form for Human consumption to cure a particular disease. In day to day life due quick relief benefit of those dosage form human beings habituated to use these medicines in their routine life, due to regular usage they are getting resistance to the older medicinally important compounds. Consequently the scenario leads to develop new medicinally important compounds or modified forms the same compounds.

The purity of API is dependent on various factors such as route of synthesis, purification process, manufacturing process, storage, transportation etc. API or pharmaceutical dosage forms are prone to get contaminated with toxic impurities (such as process impurities and degradation impurities) during the synthesis of
API/manufacturing of a pharmaceutical dosage form which will tremendously impact on its therapeutic activity. In this scenario the analytical method plays vital role to determine the purity of drug which ultimately helps to protect Human beings from furious drugs.

The major challenge of pharmaceutical industry in today’s scenario is to develop new drugs with high safety, efficacy and potentiality through advanced therapeutic techniques for the safe human health. However, competent and validated analytical methods are very indispensable for all these explorations. Most of the pharmaceutical manufacturing endeavours rely on both quality and quantitative chemical analysis to make sure that the substance used ought to meet required specifications and furthermore to ascertain the eminence quality of the finished dosage form.

1.2.3 Analytical Method Development

The basic criteria required to initiate the development of an analytical procedure might be briefed as followed,

- The medicinally important compound or pharmaceutical dosage form may not be official in all the pharmacopoeias
- May be unavailability of suitable analytical procedure for the respective medicinally important compound or pharmaceutical dosage form.
- Analytical procedures may not available which can determine the purity of medicinally important compounds without any interference from the contaminants or excipients.
- Unavailability of Analytical methodologies to estimate combination dosage forms of two or more medicinally important components.
- Tedious or costly analytical procedures existence or may require special reagents or troublesome detection techniques.

Numerous factors need to keep in mind before opting the development of new analytical procedure. Initially basic information has to be collected about the medicinally important component which needs to be determined with respect to its physicochemical properties such as solubility, chemical structure; pKa value etc. further proceeded to identify the detection technique/mode feasible for the analyte. In later stage the analytical technique has to be finalized by which the medicinally important compound has to be estimated for its content. Once the technique was finalized, eluent or mobile phase composition has to be opted based on compound polarity and solubility. Last but not least selection of stationary phase was also an important stage for the development an analytical procedure.

1.2.3.1 Typical Stages of HPLC Method Development

a. Information on medicinally important compound
b. Clarity on the requirement of special analytical technique
c. Selection of detection technique
d. Preliminary method development trial runs
e. Optimize method conditions
f. Optimize System suitability.
g. Validate method for routine use.
h. Method transfer to commercial purpose.
1.2.4 ANALYTICAL METHOD VALIDATION\textsuperscript{16-19}

Validation is a prime requirement of an analytical procedure to ensure its intended purpose of use. The data obtained during the analytical procedure validation will define and judge the reliability of the procedure, quality and reproducibility of procedure with respect to analytical results. The different characteristics of analytical method validation for different analytical methods were summarized in table 1.1.

Validation of analytical procedures is briefly defined into four most common types,

- Identification procedure
- Quantitative procedure for impurities
- Qualitative procedure (Limit test) for the control of impurities;
- Quantitative tests of the medicinally important compound in API or pharmaceutical dosage forms or other selected component(s) in the present in the dosage form.
Table 1.1 - Different Characteristics of Analytical Method Validation

<table>
<thead>
<tr>
<th>Validation Characteristics</th>
<th>Identification</th>
<th>Testing for impurities</th>
<th>Assay - dissolution (measurement only) - content/potency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Quantitative test</td>
<td>Limit test</td>
</tr>
<tr>
<td>Specificity (1)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Precision - Repeatability</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Precision - Intermediate Precision</td>
<td>No</td>
<td>Yes (2)</td>
<td>No</td>
</tr>
<tr>
<td>Detection Limit</td>
<td>No</td>
<td>No (3)</td>
<td>Yes</td>
</tr>
<tr>
<td>Quantitation Limit</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Linearity</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Accuracy</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Range</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

(1) An analytical procedure lack of specificity could be compensated by other supporting analytical procedure(s)

(2) It is not required to perform where the “Reproducibility” was proven between two laboratories

(3) Case specific – needed may be some cases
1.2.4.1 SPECIFICITY

Specificity is nothing but the selectivity of the analytical procedure capability to determine the selected component in the desired analytical methodology without any interference from the other components or contaminants or diluents or impurities etc. The inefficiency of analytical procedure with respect to specificity may be justified or compensated by means of other supporting procedures.

The above said definition for specificity will have the following implication,

- By ensuring the analyte identity in the identification procedure
- Quantitative procedure for impurities: Make sure that the analytical procedure has nil interference and capable to reproduce the results of impurities content.
- Potency estimating procedure (Assay): Ensure and grant the perfect results with respect to reproducibility and make confidence on the statement of result.

1.2.4.2 PRECISION

The precision of an analytical methodology can be demonstrated by measuring the closeness between the results obtained from the series of experiments performed by multiple sampling for the same homogenous sample under the predefined conditions. Precision will be generally prove in three levels; repeatability (System & Method Precision), Intermediate Precision and Reproducibility.
1.2.4.3 DETECTION LIMIT (LOD)

LOD value is the amount present in the sample matrix with respect to analyte and below this level the analyte may not be in detectable range. And is the lowest value of detection amount for an analyte.

1.2.4.4 QUANTITATION LIMIT (LOQ)

LOQ value is the amount present in the sample matrix with respect to analyte and below this value the analyte may not be possible to quantify but can be detected. And is the least value for the quantitation amount for an analyte.

The LOD and LOQ values were majorly useful for the analytical procedures which especially quantitatively measure the content of impurities and degradation products at low level in sample matrices.

1.2.4.5 LINEARITY

Statistically linearity can be defined as proportionality between the independent values and dependant values obtained with respect to the independent values. The linearity of an analytical methodology will be defined in a typical range where the test results are directly proportional to the concentration (amount) of analyte in the sample. The linearity can be measured in regression analysis with use of statistical formulas such regression coefficient, slope, intercept etc.

1.2.4.6 ACCURACY

An analytical procedure accuracy or recovery can be defined as an agreement for the closeness between the theoretical value and practically obtained value. The accuracy also expressed as trueness or recovery.
1.2.4.7 RANGE

The range of an analytical procedure can be inferred from linearity and accuracy. It is the interval between the upper and lower concentration (amounts) of analyte in the sample.

1.2.4.8 ROBUSTNESS

The robustness or ruggedness defines the ability of the analytical procedure to remain unchanged or constant even though a small deliberate changes done in the method parameters. However, robustness study gives an identity for the method reliability during the routine use.
Table 1.2 - Comparison of Analytical Parameters required for Validation

<table>
<thead>
<tr>
<th>USP General chapter &lt;1225&gt;</th>
<th>ICH Q2A Guidelines</th>
<th>FDA Reviewer Guidance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>Accuracy</td>
<td>Accuracy</td>
</tr>
<tr>
<td>Precision</td>
<td>Precision</td>
<td>Precision</td>
</tr>
<tr>
<td>No</td>
<td>Repeatability</td>
<td>Repeatability</td>
</tr>
<tr>
<td>No</td>
<td>Intermediate precision</td>
<td>Intermediate precision</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>Reproducibility</td>
</tr>
<tr>
<td>Specificity</td>
<td>Specificity</td>
<td>Specificity/selectivity</td>
</tr>
<tr>
<td>Detection limit</td>
<td>Detection limit</td>
<td>Detection limit</td>
</tr>
<tr>
<td>Quantitation limit</td>
<td>Quantitation limit</td>
<td>Quantitation limit</td>
</tr>
<tr>
<td>Linearity</td>
<td>Linearity</td>
<td>Linearity</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Robustness</td>
<td>Robustness</td>
<td>Robustness</td>
</tr>
<tr>
<td>System suitability</td>
<td>System suitability</td>
<td>System suitability</td>
</tr>
<tr>
<td>(discussed separately in USP 23 general chapter &lt;621&gt;)</td>
<td></td>
<td>Sample solution stability</td>
</tr>
</tbody>
</table>
1.3 General Test Procedure for validation parameters

One should make sure that the analytical instrument is ready for use with the completion all its qualification data along with the calibration data before initiating the task of methods validation.

1.3.1 Specificity

Test procedure

The specificity of the assay procedure will be investigated by injecting the extracted placebo to confirm the absence of interference with the eluted analyte.

Acceptance criteria

The excipient components should show nil interference with the targeted analyte.

1.3.2 Linearity

Test procedure

Standard solutions will be prepared at six concentrations, typically 25, 50, 75, 100, 150, and 200% of target concentration. Three individually prepared replicates at each concentration will be analyzed. The method of standard preparation and the number of injections will be same as used in the final procedure.

Acceptance criteria

Regression analysis will be done for the data generated during the linearity study by using the statistical formulas. The correlation coefficient for all concentration levels should be \(\geq 0.999\) for the range of 80 to 120% of the target concentration. The y-intercept should be \(\leq 2\%\) of the target concentration. A graph has to be plotted by considering the
concentration levels on the x-axis and the resulted response factor on the y-axis. In the literature it was stated that the coefficient of determination for active ingredients should be ≥ 0.997, for impurities 0.98 and for bio- logics 0.95.

1.3.3 Range

Test procedure

The data obtained during the linearity and accuracy studies will be used to assess the range of the method. The precision data used for this assessment is the precision of the three replicate samples analyzed at each level in the accuracy studies.

Acceptance criteria

The range will be acceptable when the concentration range inferred from the linearity and accuracy should meet individual acceptance criteria and also precision should be ≤3% RSD.

1.3.4 Accuracy

Test procedure

Spiked samples will be prepared at three concentrations over the range of 50 to 150% of the target concentration. Three individually prepared replicates at each concentration will be analyzed. When it is impossible or difficult to prepare known placebos, a low concentration of a known standard was used.

Acceptance criteria

The mean recovery will be within 90 to 110% of the theoretical value for non-regulated products. For the U.S. pharmaceutical industry, 100 ± 2% is typical for an assay of an active ingredient in a
drug product over the range of 80 to 120% of the target concentration. Lower percent recoveries may be acceptable based on the needs of the methods. HC states that the required accuracy is a bias of ≤ 2% for dosage forms and ≤ 1% for drug substance.

1.3.5 Precision – Repeatability

Test procedure

One sample solution containing the target level of analyte will be prepared. Ten replicates will be made from this sample solution according to the final method procedure. Record the peak area counts on the datasheet. Calculate the mean, standard deviation, and RSD.

Acceptance criteria

The FDA states that the typical RSD should be 1% for drug substances and drug products, ± 2% for bulk drugs and finished products. HC states that the RSD should be 1% for drug substances and 2% for drug products. For minor components, it should be ± 5% but may reach 10% at the limit of quantitation.

1.3.6 Intermediate Precision

Test procedure

Intermediate precision (within-laboratory variation) will be confirmed by two analysts, using different HPLC systems on different days. The measurement can be done by calculating the % RSD for the results across the two HPLC’s at precision level.

Acceptance criteria

The assay results obtained by two operators using two instruments on different days should have a statistical RSD ≤ 2%.
1.3.7 Limit of Detection

Test procedure

The lowest concentration of the standard solution will be determined by sequentially diluting the sample. Six replicates will be made from this sample solution.

Acceptance criteria

The ICH references a signal-to-noise ratio of 3:1. HC recommends a signal-to-noise ratio of 3:1. Some analysts calculate the standard deviation of the signal (or response) of a number of blank samples and then multiply this number by two to estimate the signal at the limit of detection.

1.3.8 Limit of Quantitation

Test procedure

Establish the lowest concentration at which an analyte present in the sample matrix can be estimated or determined with the accuracy and precision required for the method in question. This value may be the lowest concentration in the standard curve. Make six replicates from this solution.

Acceptance criteria

The limit of quantitation for chromatographic methods has been described as the concentration that gives signal-to-noise ratio (peak of interest height should be at least ten times the height of the baseline noise level) of 10:1.

1.3.9 System Suitability

Test procedure
This test will be performed on both HPLC systems to determine the accuracy and precision of the system will be proven with the six injections of a solution containing analyte at 100% of test concentration. The parameters of theoretical plate count, tailing factor, resolution, and % RSD of peak area counts for six injections.

**Acceptance criteria**

The void/dead volume (Retention factor-k) should be ≥2.0. Resolution (Rs): Rs should be ≥2 between the analyte peak/peak of interest and the closest eluted peak which is potentially interfering (impurity, excipient, and degradation product). Reproducibility: RSD for peak area count will be 1% for six injections. Tailing factor (T): T should be less than 2. Theoretical plates (N): ≥2000.

**1.3.10 Robustness**

As defined by the USP, robustness defines the measure of ability for small changes intentionally made in the methodology conditions and confirms the reliability on analytical methodology in routine usage. The method parameters generally used to undergo deliberate change to perform the robustness study are extraction time, variation in pH of buffer used in the composition of mobile phase, eluent composition ratio, different lot of column with same make/supplier, sample & column temperature and flow rate.