A drug may be defined as a substance meant for diagnosis, cure, mitigation, prevention or treatment of diseases in human beings or animals for alternating any structure or function of the body of human being or animals. Pharmaceutical chemistry is a science that makes use of general laws of chemistry to study drugs i.e., their preparation, chemical natures, composition, structure, influence on an organism and study the physical and chemical properties of drugs, the methods of quality control and the conditions of their storage etc. The family of drugs may be broadly classified as

1. Pharmacodynamic agents
2. Chemotherapeutic agents

Every country has legislation on bulk drugs and their pharmaceutical formulations that sets standards and obligatory quality indices for them. These regulations are presented in separate articles, journals and specific relating to individual drugs and are published in the form of a book called a pharmacopoeia (eg: IP, BP, USP etc.).

Modern pharmaceuticals for human use are required to meet exact standards, which relate to their quality, safety and efficacy. The evaluation of safety and efficacy and their maintenance in practice is dependent upon the existence of adequate methods for quality control of the product. The standard of purity is must, therefore it can be strictly defined in such a way as to ensure that successive batches are consistent in composition, irrespective of the same or different manufacturers.

Pharmaceutical Analysis and Quality assurance plays an important role in determining the safety and efficacy of medicines. Highly specific and sensitive
analytical techniques hold the key to the design, development, standardization and quality control of medicinal products. They are equally important in pharmacokinetic studies, which are fundamental to the assessment of bioavailability and the duration of clinical response.

Modern methods of estimation of drugs are divided into physical, chemical, physico-chemical and biological. Physical methods of analysis involve the studying of the physical properties of the substance. They include determination of the solubility, colour, specific gravity (for liquids), moisture content, melting, freezing and boiling points. Physico-chemical methods are used to study the physical phenomena that occur as a result of chemical reactions.

Among the physico-chemical methods the most important are optical (Refractometry, Polarimetry, Emission and Fluorescence methods of analysis. Photometry including photocolorimetry and spectrometry covering UV, Visible, IR regions, Nephelometry and Turbidimetry), electrochemical (Potentiometry, Amperometry, Coulometry and Polarography) and Chromatography (Column, Paper, Thin-layer, Gas-liquid, High performance liquid, High performance thin layer) methods.

Methods involving nuclear reactions such as nuclear magnetic resonance (NMR) and paramagnetic resonance (PMR) are becoming more and more popular. The combination of mass spectrometry with gas chromatography is one of the most powerful tools available.¹

To study the structure of atoms and molecules, the most powerful tool is spectroscopy, which is used in the analysis of a wide range of samples. Among
various spectrophotometric methods, UV-Visible spectrophotometery is frequently employed technique in pharmaceutical analysis.\textsuperscript{2}

Most of the formulations comprise of complex mixtures including in addition of the one or more medicinally active ingredients, a number of inert materials such as diluents, disintegrants, colours and flavours. In order to ensure quality and stability of the final product, these mixtures are to be separated into individual components prior to qualitative analysis.

**Nitric Oxide**

Nitric oxide, also known as nitrogen monoxide, is a molecule with chemical formula NO. In animals including humans, NO is an important cellular signalling involved in physiological, pharmacological and pathological processes\textsuperscript{3}. Nitric oxide is a strong vasodilator with a half-life of a few seconds in the blood. Nitric oxide production is important in protecting the liver from ischemic failure. Nitric oxide is also known as the 'endothelium-derived relaxing factor', or 'EDRF', is produced from L-arginine, oxygen, and NADPH by various nitric oxide synthase. Inorganic nitrate reduced to form nitric oxide.

The endothelium of blood vessels combine with nitric oxide to signal the smooth muscle to relax, which results in vasodilation and increased blood flow. Since, It has a short life span of seconds, it diffuses easily across biological membranes.\textsuperscript{4} Nitric oxide is deemed to be an antianginal agent which involves in dilatation of blood vessels, and helpful in relieving the symptoms of pain associated with angina. The dilation of veins by nitric oxide lower arterial pressure and left ventricular filling pressure. Nitrates may be beneficial for treatment of angina due to
reduced myocardial oxygen consumption both by decreasing preload and after load.\textsuperscript{5} Nitric oxide relaxes vascular smooth muscle by binding to the heme moiety of cytosolic guanylate cyclase, activating guanylate cyclase and increasing intracellular levels of cGMP leads to vasodilation.\textsuperscript{6} It increases the partial pressure of arterial oxygen (PaO\textsubscript{2}) by dilating pulmonary vessels of the lung, passing pulmonary blood flow away from lung segments.\textsuperscript{7} The efficacy of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA) inhibitors (statins such as atorvastatin, rosvastatin and simvastatin, etc) decreases mortality due to antioxidant properties and eNOS expression activities. Statins are involved in the down regulation of eNOS expression induced by hypoxia and improve the vascular activity of NO and useful for the primary and secondary treatment of coronary heart disease.\textsuperscript{8-14}

Nitric oxide (NO) is an important chemical messenger of many physiological and biochemical processes\textsuperscript{15}, including thrombosis, inflammation, immunity, vasodilation and neurotransmission. Several methods exist in the literature for measuring NO in biological systems,\textsuperscript{16} one of these simplest method involves the use of the diazotization reaction to spectrophotometrically detect nitrite formed by the oxidation of NO.\textsuperscript{17-19} The limit of detection for this method is 1.0 \(\mu\text{M}\) nitrite.\textsuperscript{20}

The proposed study involves the administration of 80 mg of Atorvastatin calcium and estimation of nitric oxide in the serum for a period of four weeks in the rats. Nitric oxide can be easily detected by treating the serum with Griess reagent.

Chromatography is the most powerful technique available for the resolution of these mixtures. The features that distinguish chromatography from most other chemical methods of separation are that two mutually immiscible phases are brought
into contact, one phase is stationary and the other is mobile. Among different chromatographic methods, high performance liquid chromatography (HPLC) offers a greater variety of stationary phases, which thereby allows selective interactions and more possibilities for separation. The separating column being the heart of chromatography provides separation of ionic compounds, organic compounds, liable naturally occurring products, polymeric and high molecular weight poly functional compounds.\textsuperscript{21}

**Bioanalytical method development and validation**

Bioanalytical assay validation is important in the characterization of stability of active ingredients in biological matrices collected during the clinical study programs in combination with indispensable assay reagents and standard stock solutions. It is mandatory to develop selective and sensitive analytical procedures, which are essential for the quantitative estimation of drugs and their metabolites for the successful outcome of preclinical and/or pharmacokinetic and pharmacological studies.

Bioanalytical method development and validation studies include reliable and reproducible determination of active molecule concentration in a bio-matrix such as plasma, serum or urine.\textsuperscript{22}

The essential parameters for this validation include accuracy, precision, selectivity, sensitivity, reproducibility and stability. Validation is the process of documentation, through the utilization of specific laboratory findings which are reliable and suitable for predetermined analytical applications.
The standard guidelines provide the information to sponsors of New Drug Applications (NDAs), Investigational New Drug (INDs), Abbreviated New Drug Applications (ANDAs) and in developing the validated analytical method used in pharmacology, bioavailability (BA) and bioequivalence (BE) studies requiring biopharmaceutical evaluation. 

These studies are even applicable for non-human pharmacotherapeutic/toxicological and preclinical studies.

Types of chromatographic techniques,

1. Gas Chromatography (GC)
2. High-pressure Liquid chromatography (HPLC)
3. Combined GC and LC mass spectrometric (MS)
4. LC-MS
5. LC-MS-MS
6. GC-MS and
7. GC-MS-MS

The bioanalytical method validation is essential not only for the quantitative estimation of drugs and metabolites in complex bio-matrices (serum, plasma or urine) but also for immunological, microbiological, brain and skin tissue preparations.

These guideline procedures can be used in quantitative estimation to support biopharmaceutical and clinical pharmacological parameters of the therapeutic agents.
For example, in the evaluations of Ligand Binding Assays (LBAs or Immunological assays), which require specificity and selectivity of therapeutic molecules against receptors and antibodies. The results obtained with these methods are indirectly correlated to the presence therapeutic at the site of action, i.e. the platform of identification is a radiochemical or the enzymatic response together with a variety of binding interactions.

The importance of bioanalytical method validation is employed by the regulated organizations for the quantitative estimation of active pharmaceutical ingredients and their metabolites in biological samples and plays a vital role in determination of bioavailability, bioequivalence, pharmacokinetic and clinical pharmacological studies. These data are essential in filing New Drug Approvals and Abbreviated New Drug Application procedures. Therefore, it is mandatory for the organizations in coming up with innovator and generic formulations to conduct bioanalytical method validation studies.25

**How to choose an analytical / Bioanalytical method to quantify the drug product?**

Identify the drug product parameters according to pharmacopoeia.

**Method is not found in pharmacopoeia, but recommended by regulatory agency**

Evaluate the actual drug product parameters in different media and conditions. It is mandatory to come up with tripartite guidelines.

**If the method is not in pharmacopoeia, and not recommended by regulatory agency**
Develop a suitable, sensitive, selective, robust and reproducible analytical method with good solubility profile in recommended solvents.

**Analytical method development**

There are four common types of analytical methods.

1. Identification tests
2. Quantitative tests for impurities
3. Limit tests for impurities
4. Quantitative tests for drug substance or other selected component(s) in the drug product.

**Objectives for method development**

- What sample should be used at each step?
- What should the scientists look for outcome in these experiments?
- What are the acceptance criteria for the mentioned methodology?

**Design of the method development**

Step 1: Define objectives and understand the molecular properties.

- Determine intended use of the method?

Step 2: Primary laboratory conditions.

- Develop preliminary parameters to get minimally acceptable values, so that the results could be used for all subsequent experiments.

Step 3: Sample preparation steps

- Develop a sample preparation protocol.
Step 4: Standardization

- Determine the suitable standardization procedure.

Step 5: Final method optimization

- Identify “weakness” of the procedures and optimize through experimental design.
- Expose the drug sample in different conditions, different instrument set ups and different laboratories.

Step 6: Method validation

- Accomplish method validation steps according to ICH guidelines.

Analytical method development

1. Analytes

According to current ICH guidelines, the presence of related substances (RS) together with analyte should be tested for the related substances.

During this step, related substances should be divided into two groups:

a) Significant (RS):

- Linearity, accuracy and response parameters should be ascertained during the method validation.
- Usually three or less significant RS should be selected.\(^\text{26}\)

b) Other related substances:

- The developed method has the ability to provide good resolution for the RS to ensure that they do not exist at significant levels.

2. Adaptability for automation
• It is essential to adopt the instrument set up for automation where the high sample volume application is required.
• The manual sampling procedure should be made easy for quantitative procedures.

3. Understanding the Chemical Properties
• Most sampling preparations utilize organic-aqueous and acid-base extraction techniques.
• Therefore it is essential to understand the drug solubility profile and pKa.
• Best suitable and sensitive solvent system should be selected to minimize the expenditure involved in the study.
• pKa of drug molecule that determines the pH, where analyte exist as a neutral or ionic species.

4. Potential degradation products
• Exposing the drug molecules to stress conditions helps in identifying the pathways of drug degradation process and the most common stress conditions include:
  a) Change in pH conditions
  b) Different temperatures (ambient and accelerated)
  c) Humidity conditions
  d) Oxidation-reduction
  e) Photo degradation.
• The above mentioned stress evaluations helps in estimation of significant RS during the process of method development.
• The General concept of organic chemistry will help in tracing the reactivity of attached functional groups.

• Computer assisted method development can be very helpful in developing the preliminary experimental conditions.

5. Reference standard

• Quantitation of drugs with their metabolites can be carried out by spiking reference along with quality control samples.

• The reference standard purity may affect actual estimation, which may not be exactly interpreted.

• Due to the above fact, standard analytical sample should be used to prepare concentrations.

• If possible, the reference standard should be identical to the analyte.

The General Reference standards

1. Certified compendial reference standards sample

2. Commercially supplied reference standard sample

3. Samples are prepared by an analytical laboratory.

6. Sample preparation

• Extraction procedures and the proper sample preparation steps.

• Determine the effect of solvents on variation in organic phase, pH and extraction procedure on accuracy, precision and selectivity.

7. Method optimization

• Execute the final optimization to improve the accuracy, precision and LOQ.
Developing experimental design helps to determine the factors that have a significant role in the method.

**Bioanalytical Method Validation** \(^{27, 28}\)

Bioanalytical method validation includes determination of,

1. Selectivity
2. Accuracy
3. Precision
4. Recovery
5. Calibration curve
6. Stability of analyte in spiked samples. \(^{29}\)

**1. Selectivity**

It is the ability of the method to differentiate and quantify the drug in the presence of sample matrix. Blank samples of the biological matrices (plasma or serum) should be procured from six sources. Each blank should be analyzed for interference.

**2. Accuracy**

It describes the closeness of average test values to the true value (concentration) of the analyte. Accuracy is determined by replication of samples containing known amounts of the drug substance. Accuracy should be measured using a minimum of five determinations per concentration. The average value should be within 15% of the actual value except at LLOQ, where it should not deviate more than 20%. The deviation of the average from true value helps on the estimation of accuracy. \(^{30}\)
3. Precision

It is the closeness of individual drug response when the method is applied repeatedly to multiple aliquots of a single uniform volume of biological matrix. Precision should be determined using a minimum of five determinations per concentration. A minimum of three determinations in the range of expected concentrations is allowed. The precision at each concentration level should not be more than 15% of the coefficient of variation (CV) except for the LLOQ, where it should not be more than 20% of the CV. Precision is further classified into

i. Intra-batch precision or repeatability: This measures precision during a single determination,

ii. Between-run, inter-batch precision or repeatability: This involves different analysts, equipment, reagents, and laboratories.31,32

4. Recovery

The recovery is the detector response obtained from the biological matrix, compared to the true concentration of the pure standard. Recovery is the extraction efficiency of the determination within the limits of variability. Recovery experiments should be performed by comparing the analytical responses for extracted samples at three determinations (low, medium and high) with unextracted standards that represent 100% recovery.33

5. Calibration/Standard Curve
A calibration curve is the relation between response and known concentrations of the drug sample and be generated for each concentration. A calibration curve should consist of

i. A blank sample

ii. Internal standard

iii. Nine non-zero samples covering the expected range, including LLOQ.\textsuperscript{34}

a) Lower Limit of Quantitation (LLOQ)

The lowest concentration should be accepted as the limit of quantitation such that,

- The analyte response should be at least five times the response compared to blank.
- Analyte should be reproducible with a precision of 20% and accuracy of 80-120%.

b) Standard Curve – Concentration vs. Response

The following conditions should be met in developing a calibration curve:

- 20% deviation of the LLOQ from nominal concentration.
- 15% deviation of standards other than LLOQ from nominal concentration.

---

**Bioanalytical Method Validation**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Development</th>
<th>Assay Objectives</th>
<th>Validation</th>
</tr>
</thead>
</table>

14
<table>
<thead>
<tr>
<th>objectives</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Discovery</td>
<td>Comparison of related compounds</td>
</tr>
<tr>
<td></td>
<td>Similar selectivity towards all compounds examined</td>
</tr>
<tr>
<td>2. Pre-Clinical</td>
<td>Toxicology/metabolism support regulatory standard</td>
</tr>
<tr>
<td></td>
<td>Reliable selective; for blood/plasma</td>
</tr>
<tr>
<td>3. Healthy volunteers</td>
<td>Definitive clinical studies</td>
</tr>
<tr>
<td></td>
<td>Excellent selectivity in human bio-fluids</td>
</tr>
<tr>
<td>4. Clinical Patients</td>
<td>Routine monitoring of patients varying in medication and dietary background</td>
</tr>
<tr>
<td></td>
<td>Selectivity in the presence of numerous substances</td>
</tr>
</tbody>
</table>

**Pre-Validation**

Before the actual validation process, Pre-validation is done. In Pre-validation, samples are analyzed in a single batch before a method validation is started. Following validation criteria is studied in Pre-validation and Validation steps.

**Validation criteria to be studied in Pre-validation and validation steps**

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Pre – validation step</th>
<th>Validation step</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selectivity</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Response function</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Linearity</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Accuracy</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Precision</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Limit of detection</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Limit of quantitation Range</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+): estimation of the criteria; +: validation of the criteria

**Full Validation**

- Full validation is implementing a bioanalytical method for the first time.
- It is important for a new drug entity.

**6. Stability of drug in a Biological Fluid**
It is a function of the storage conditions, the chemistry of the drug, the matrix and the container. Stability studies should evaluate the sustainability of drugs during sample collection and handling, after long-term (frozen) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles.

Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.\textsuperscript{33}

\textbf{a) Freeze and Thaw Stability}

At least three standards at each of the low and high concentrations should be stored for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze-thaw cycle should be repeated two more times, and then analyzed on the third cycle. If an analyte is unstable at storage temperature, the stability sample should be frozen at $-7^\circ$C during the three freeze and thaw cycles.\textsuperscript{35}

\textbf{b) Short-Term Temperature Stability}

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

c) \textbf{Long-Term Stability}
Long-term stability should be determined by storing at least three standards of the low and high concentrations. The concentrations of samples should be compared to the average of back-calculated responses for the standards at the appropriate concentrations from the first day of long-term stability testing.

d) Stock Solution Stability

The stability of stock solutions of the drug and the internal standard should be evaluated at room temperature for at least 6 hours. After completion of the storage time, the stability should be tested by comparing the instrument response to that of original responses.

e) Post-Preparative Stability

The stability of the drug and the internal standard should be examined for the expected run time for the batch size in samples by estimating concentrations on the basis of original analytical standards. SOPs should clearly describe the statistical method and rules used. Additional validation may include investigation of samples from dosed subjects.36

Selectivity Issues

1. Interference from substances should be analyzed such that

   ✓ Cross-reaction of metabolites or endogenous compound should be evaluated individually and in combination with drug.

   ✓ The dilution linearity to the reference standard should be examined using study (incurred) samples.

2. Matrix effects unrelated to the analyte
The standard curve in biological samples should be compared with standard in buffer.

Parallel study samples should be evaluated with diluted samples.

High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) was introduced in 1969 and since then it has undergone extensive modifications and innovation, which leads to its emergence as the foremost analytical tool for quantitative analysis. HPLC is a type of liquid chromatography that employs a very finely divided stationary phase and liquid mobile phase. To obtain a satisfactory flow rate mobile phase must be pressurized to a few thousands of pounds per square inch.

The rate of distribution of drugs between mobile phase and the stationary phase is achieved by a diffusion process. Faster and effective separation can be achieved if diffusion is minimised. The technique of high performance liquid chromatography is so called because of its improved performance when compared to classical column chromatography. Advances in column technology, high-pressure pumping system and sensitive detectors have transformed liquid column chromatography into high speed, efficient, accurate and highly resolved method of separation.\textsuperscript{21}

The HPLC method was considered as the choice of estimation, since this method is the most powerful of all chromatographic and other separative methods. The HPLC method has enabled analytical chemist to attain great success in solving his analytical problems.
The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise, accurate and the limit of detection is low and also it offers the following advantages:

- Speed (many analyses can be accomplished in 20 minutes or less).
- Greater sensitivity (various detectors can be employed).
- Improved resolution (wide variety of stationary phases).
- Reusable columns (expensive columns but can be used for many analyses).
- Ideal for the substances of low volatility.
- Easy sample recovery, handling and maintenance.
- Instrumentation leads itself to automation and quantitation (less time and less labour).
- Precise and reproducible.
- Calculations are done by integrator.
- Suitable for preparative liquid chromatography on a much large scale.  

**Types of HPLC**

The type of HPLC methods include,

- Normal phase chromatography
- Reversed phase chromatography

**Normal Phase Chromatography**

The concept of normal phase refers to a system where a stationary phase is polar and the mobile phase is non-polar liquid (hexane, benzene, chloroform etc.). In this mode most probably used stationary phase is silica gel. The silica structure is saturated with silanol groups at the end and ‘OH’ groups attached to silicon atoms
are the active binding sites. Best separating compounds include plasticizer, dyes, steroids, amines, alkaloids, alcohols, phenols, aromatic and metal complexes.

**Reversed Phase Chromatography**

A most popular mode for analytical and preparative method for separation of compounds of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode the stationary phase is a non-polar hydrophobic packing with Octyl or octadecyl functional group bonded to the silica gel and the mobile phase is a polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibria (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity.

The polar compound gets eluted first in this mode and non-polar compounds are retained for a longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer time and eluted faster. The different columns used are octadecyl silane (ODS) or C\textsubscript{18}, C\textsubscript{8}, C\textsubscript{4} etc. (In order to increase the polarity of the stationary phase). Hence by varying the organic moiety in the silanization reagent (dimethylchlorosilane derivatives) different stationary phase of polarities can be realized\textsuperscript{38, 39}.

**Importance of Polarity in HPLC**

In both normal phase and reversed phase HPLC, the eluting power or solvent strength of the mobile phase is mainly determined by its polarity. The relative distribution of solute between two phases is determined by the interactions of the solute species with each phase. The relative strengths of these interactions are determined by the polarity of the sample and the mobile and stationary phase.
Reverse Phase Mobile Phases

The power of HPLC in terms of being able to resolve many compounds is mainly due to the diversity of mobile phases or mobile solvents available. The mobile phase in HPLC, however as a great influence on the retention of the solutes and the separation of component mixtures.

The primary constituent of reverse phase-mobile phase is water (distilled or demineralised). Water miscible solvents such as ethanol, dimethylformamide, acetonitrile, tetrahydrofuran, dioxane and methanol are added to adjust the polarity of the mobile phase. They should be of high quality either distilled or demineralised. The most widely used organic modifiers methanol, acetonitrile and tetrahydrofuran. Methanol and acetonitrile have comparable polarities but the latter is an aprotic solvent. This factor may be important if hydrogen bonding plays a significant role in the separation. When inorganic salts and ionic surfactants are used, the mobile phase should be filtered before use since these additives frequently contain a significant amount of water-insoluble contaminants that may damage the column. Degassing is quite important with reverse-phase mobile phases.

Polarity is a term that is as an index of the ability of compounds to interact with one another used in chromatography. It is applied very freely to the solute, stationary and mobile phase. If the polarities of stationary phase and the mobile phase are similar, it is likely that the interactions of a solute with each phase may also be similar, resulting in poor separation. Changing the polarity of the mobile phase usually alters retention of solutes. Successful chromatographic separation
requires a proper balance of intermolecular force among three participants in separation process i.e. Analyte, Mobile phase and Stationary phase.\textsuperscript{40}

\textbf{Methods of Quantitative Analysis in HPLC}

The sample or solute is analyzed quantitatively by HPLC either by peak height or peak area measurements. Peak areas are proportional to the amount of constant rate. Peak heights are proportional to the amount of material only when peak width is constant and are strongly affected by the sample injection techniques. Once the peak height or the peak areas are measured, there are five principle evaluation methods for quantifying the solute.\textsuperscript{21}

\textbf{a) Calibration by Standards}

In this method a calibration curve is constructed for each component is prepared from pure standards, the concentration of solute is read from its curve if the curve is linear.

\begin{align*}
\text{Where,} & \quad X = K \times \text{area} \\
X & = \text{Concentration of solute} \\
K & = \text{Proportionality constant (slope of the curve)}
\end{align*}

In this evaluation method only the area of the peaks of interest is measured. Relative response factors must be considered when converting areas to volume and when the response of a given detector differs for each molecular type of compound.

\textbf{b) Internal Standard Method}

In this technique, a known quantity of the internal standard is chromatographed and area v/s concentration is ascertained. Then a quantity of the
internal standard is added to the raw sample prior to any sample pre-treatment or separation operations.

The internal standard will compensate the changes in sample size or concentration due to instrumental variations, with this method a calibration plot is produced by analysing different calibration solutions with different concentrations of API with the fixed concentration of internal standard added.\textsuperscript{41}

c) Area Normalization

The technique is often used for the sample having identical components. It is used to evaluate the absolute purity of the sample. The procedure is to total up the areas under all peaks and then calculate the percentage of the total area that is contributed by the compound of interest. For this method the entire sample must be eluted, all components must be separated and each peak must be completely resolved.

d) Standard Addition Method

This method is often used in trace analysis, the chromatogram of the unknown is recorded, then a known amount of analyte(s) is added and the chromatogram is repeated using same reagents, instruments and other conditions. Extrapolation of graph against response and the amount added gives the original concentration in an unknown sample.

The important point to be considered in this method i.e. response prior to additional analyte should be high to provide reasonable s/n ratio greater than 10, if not it yields a poor precision.
If an instrumental reading (area/height) ‘Rx’ is obtained, from a sample of unknown ‘x’ and a reading ‘Rt’ is obtained from the sample to which a known concentration ‘a’ of analyte has been added, then ‘x’ can be calculated from,

\[
\frac{x}{x+a} = \frac{Rx}{Rt}
\]

A correction for dilution must be made if the amount of the standard added changes the total sample volume significantly. It is always advisable to check the result by adding at least one other standard.42

e) External Standard Method

It employs a separate injection of a fixed volume of sample and standard solution. The peaks are integrated and concentration is calculated.

\[
\text{Sample concentration} = \frac{\text{Peak area of sample}}{\text{Peak area of standard}} \times \text{Conc. of Standard}
\]

The selection of suitable chromatographic (HPLC) system for a given mixture of solutes cannot be made with certainty and must be confirmed by experiment.

If the chemical nature of the sample components is known, then the phase system can be selected from the literature references. If nothing is known about the chemical nature of the sample, then the sample solubility will give some indication as to which chromatographic method to employ.
The essential parts of high performance liquid chromatographic system are,\textsuperscript{42,43}

1. Solvent reservoir
2. Pump
3. Injection port
4. Column
5. Detector
6. Recorder

1. Solvent Reservoir

Material used for making solvent reservoir is usually stainless steel or glass. Stainless steel reservoir should not be preferred when the solvents contain halide ions, glass should be avoided when the reservoir is exposed to pressure. They should be inert to a variety of aqueous and non-aqueous mobile phases. The capacity of the reservoir should be greater than 500ml. The aqueous and organic solvents are degassed prior to use in order to prevent the formation of gas bubbles in the detector. Degassing is done by the following methods.

- By stirring the mobile phase in a vacuum.
- Purging with helium gas.
- Ultrasonication.

Finally the solvent is filtered through a Millipore filter before introducing into the reservoir.

2. Pumps

The pumps must be fabricated from materials that are inert to all mobile phases. Materials used are stainless steel, glass and Teflon. They should generate a pressure up to 800 psi at a flow rate of up to 3ml/minute and should provide pulse
less solvent flow. The pump should produce reproducible and constant flow rate, HPLC pumps can be classified into two groups according to the manner in which they function,

a) Constant flow rate pump

b) Constant pressure pump

a) Constant flow rate pump

The two principle types of constant flow rate pump are

i) Reciprocating piston pump

ii) Syringe drive pump.

i) Reciprocating piston pump

Reciprocating pump has two cycles one is filling and pumping. During the filling cycle a piston is withdrawn from a syringe type chamber. Two check-valves are connected to this chamber such that during the piston withdrawal, solvent flows from the reservoir to the pump outlet. The volume of solvent discharged from the pump in unit time can be changed by altering the distance that the piston travels (or) the number of cycles. The advantage of this pump is unlimited volume of the solvent reservoir since it is external to the pump.

ii) Syringe drive pump

The syringe drive pump is a single stroke displacement pump in which all of the mobile phase is contained within the pump. A screw feed drive connected to a stepping motor actuates the piston inside the chamber. The voltage applied controls the volume displaced by the pump per unit time. This pump produces a pulse less flow and requires no check valves.44, 45
b) Constant pressure pump

Constant pressure pumps can deliver a steady flow rate if the pump operates against a constant column back pressure, when the viscosity of the mobile phase remains constant. Constant pressure pump is a simple gas displacement pump (or) pneumatic amplifier pump, simple gas displacement pump is a reservoir like a coil of tubing to which pressure is applied from a gas cylinder. The disadvantage of this pump is narrow solvent capacity. The pneumatic amplifier pump is similar with a slight modification of simple gas displacement pump. The gas pressure is applied to a large piston, which is connected to a small diameter piston, which is in link with the mobile phase. When the mobile phase is used up, the piston returns quickly by pneumatic means, thus refilling the chamber. The advantage of this pump is pulseless flow and unlimited solvent capacity.

3. Sample injection system

The injection of sample onto the column experiences some unique problems because of the high pressure involved in HPLC. The volume of sample employed ranges from 0 to 500 µl. The various injection methods are,

a) Syringe Injection

There are two methods for injecting samples by micro syringes, which are designed to withstand pressure up to 1500 psi. The injection is made through a self-sealing elastomeric septum.
b) Stop Flow Injection

This method is simple and convenient, here the flow of the solvent is momentarily stopped and the sample is directly injected onto the head of the column.

c) Loop Injection (or) Sampling Valve

In this method a sample loop of fixed capacity is connected to a high-pressure valve and the sample is filled onto the sample loop through a syringe. This system is the most widely used injection system, which provides precise injection volumes against high back pressures.

4. Columns

HPLC columns are made up of glass or stainless steel, which differs in inside diameter and length depending on its application. Two types of columns are available they are analytical column and preparative column. Internal diameter of standard analytical columns are 4-5mm and 10-30 cm in length. The particle size used ranges from 5-10 micrometers. Preparative columns are 20-50 mm in internal diameter and 20-100 cm in length. The particle size used ranges from 37-50 micrometers.

5. Detector

All detectors used in HPLC may be of selective type and non-selective type in which the former detectors are a part of the components and the later detects almost all the components. The choice of the detector depends on the mobile phase, nature of the analyte and the required sensitivity. The detectors used are Refractive index detector, U.V. absorption detector, Fluorescence detector, electrochemical detector, Mass detector and radioactive detector.
6. Recorder

The deviation from baseline is recorded as signals from the detector. Generally two open recorders are used with instruments having two detectors. The peak position and the curve relative to the starting point gives the information about the particular component, by proper calibration the height or area of peak is a measure of the amount of the component present in the sample.

Best mobile phase, best column, best detection wavelength, efforts in their selection can make a great difference in the development of an HPLC method for routine analysis. By selecting an ideal combination of these factors assures faster delivery of desired results.21

METHOD VALIDATION

1. Specificity

It is a process of measuring the analyte response accurately in presence of all possible sample components, which is referred as sample matrix (synthesis intermediates, excipients, degradation products, impurities). Spectrums of API are obtained by measuring different concentrations, which are overlaid with blank spectrum to find if any interference is present.46, 47

2. Linearity

It is a procedure of obtaining results by calibration curve/standard curve method in which there is a relationship between response and known concentration (usually directly proportional) of the API. Minimum of five concentrations are recommended for linearity.46, 47
3. Range

It is a process of measuring the interval between the upper and lower level of API concentrations.\(^{48}\)

4. Accuracy

Accuracy is determined by adding a known amount of drug to solvent and by recovering that amount using the method developed. This is carried out at three different concentration levels in triplicate and twice in one day, accuracy is reported as \(\%\) accuracy.\(^{49, 50}\)

\[
\% \text{ Accuracy} = \frac{\text{Average concentration}}{\text{Original concentration}} \times 100
\]

5. Precision

It is a method which expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same sample under prescribed conditions.\(^{49}\)

There are four types of precision that can be determined for an analytical method.

- a) Instrument precision or injection repeatability
- b) Repeatability or intra-assay precision
- c) Intermediate precision
- d) Reproducibility

a) **Instrument precision or injection repeatability**

It is determined to test the performance of the instrument used for analytical methodology, by measuring one sample solution repeatedly.
b) Repeatability or intra-assay precision

This method is carried out with at least five determinations of three different concentrations at low, medium and high range of calibration in triplicate and twice in one day. This method is carried out in one laboratory, by one operator, using one piece of instrument/equipment and one set of reagents in one day. Repeatability is reported in % RSD (Relative Standard Deviation).

c) Intermediate precision

This method is carried out by multiple analysts, multiple set of reagents, multiple equipment and multiple days, the objective of this method is to identify the factors within a single laboratory that will contribute to the variability in results. It is carried out at three different concentration levels in triplicates. Intermediate precision is reported in % RSD (Relative Standard Deviation).

d) Reproducibility

This method is carried out in different laboratories when a method is transferred from one laboratory to another where it may come across analysts with different experience and thoroughness, differences in humidity and room temperature, variations in quality and nature of materials, consumables and instrument conditions (HPLC mobile phase composition, column specifications, pH, flow rate of mobile phase) and equipment with different characteristics.51
6. Limit of Detection

Limit of detection (LOD) is the lowest concentration of analyte that can be detected but not quantified under experimental conditions.

In chromatographic methods which exhibit a constant background noise which can be estimated based on signal to noise ratio, which correspond to the concentration at which the response signal to noise ratio is 3.3.

In spectrophotometric method it can be calculate using the relation. \(3.3 \times \text{SD}/S\), Where SD is the standard deviation of response and S is the slope of the calibration curve.\(^{46, 47}\)

7. Limit of Quantitation

Limit of quantitation (LOQ) is defined as, the concentration at and above which the analyte can be performed.

In chromatographic methods which exhibit a constant background noise which can be estimated based on signal to noise ratio, which correspond to the concentration at which the response signal to noise ratio is 10.

In spectrophotometric method it can be calculate using the relation. \(10 \times \text{SD}/S\), Where SD is the standard deviation of response and S is the slope of the calibration curve.\(^{46, 47}\)
8. Robustness

This method is carried out to check the ability of the method to remain unaffected by small changes in method parameters which include temperature, injection volume, solvent system, buffer concentration and pH of solvent or mobile phase. 47, 52

9. Ruggedness

It is defined as the degree of reproducibility of results of the same sample under a variety of conditions which includes different laboratories, different analysts, different environmental and operational conditions. 52, 53

System Suitability

The system suitability test is very important to ensure the performance of analytical system while developing analytical methods. The aim of the system suitability test is to ensure that the whole testing system (including instrument, reagents, columns and analysts) is suitable for the intended method.

System suitability parameters

Capacity factor (K’)

\[ K' = (t_R - t_o) \]

It is the measure of where the peak of interest is located with respect to the void volume i.e. elution time of non retained components.

Relative retention (α)

\[ \alpha = \frac{K'_1}{K'_2} \]

Relative retention is a measure of relative location of two peaks . This is not an essential parameter as long as the resolution Rs is stated.
Resolution ($R_S$)

$$R_S = \frac{(t_{R2} - t_{R1})}{(1/2) (t_{w1} + t_{w2})}$$

Resolution ($R_S$) is a measure of how well two peaks are separated.

Tailing factor ($T$)

$$T = \frac{W_x}{2f}$$

The accuracy of quantitation decreases with increase in peak tailing because of difficulties encountered by the integrator in determining where/when the peak ends.

Theoretical plate number ($N$)

$$N = 16 \left( \frac{t_R}{t_w} \right)^2$$

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

In the current FDA guidelines on validation of chromatographic methods, the following acceptance limits are proposed as initial criteria.\textsuperscript{54}

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacity</td>
<td>Greater than 2</td>
</tr>
<tr>
<td>Injection precision</td>
<td>RSD lesser than 1%, for $n \geq 5$</td>
</tr>
<tr>
<td>Resolution</td>
<td>$R_S$ greater than 2</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>$T$ lesser than 2</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>$N$ more than 2000</td>
</tr>
</tbody>
</table>
Stability

This method is intended specially for biosamples to generate reproducible and reliable results, this method recommends freeze thaw stability for three freeze thaw cycles. Short term temperature stability which is carried out at room temperature for 4-24hrs based on the sample. Long term stability is assessed over a time period greater than the time difference between the date of first sample collection and date of last sample analysis.51

UV Spectrophotometric method development and validation

Spectrophotometry is generally adopted by small scale industries as the cost of the equipment is low and maintenance is minimal. The method of analysis is based on measuring the absorption of a monochromatic light by colourless compounds in the near ultraviolet path of spectrum (200-380nm). The photometric methods of analysis are based on the Bouger-Lambert-Beer’s law, which establishes the absorbance of a solution is directly proportional to the concentration of the analyte. The fundamental principle of operation of the spectrophotometer is covering the UV region consists in that light of definite interval of wavelength passes through a cell with solvent and falls onto the photoelectric cell that transforms the radiant energy into electrical energy measured by a galvanometer.

The important applications are,

- Identification of many types of organic, inorganic molecules and ions.
- Quantitative determination of many biological, organic and inorganic species.
- Quantitative determination of mixtures of analytes.
- Monitoring and identification of chromatographic effluents.
Determination of equilibrium constants.

Determination of stoichiometry and chemical reactions.

Monitoring of environmental and industrial process.

Monitoring of reaction rates.

Typical analysis time ranges from 2 to 30 min for sample 37.

**UV Spectrophotometric Method Development and Validation**

Many molecules absorb ultraviolet (UV), visible (Vis) or near infrared (NIR) radiation. In terms of the electromagnetic spectrum, UV radiation covers the region from 190–350 nm, Visible radiation covers the region 350–800 nm and NIR radiation covers the region 800–2500 nm (and maybe a little higher). Absorption of UV and/or Vis radiation corresponds to the excitation of outer electrons in the molecule. Typically, radiation with a specific intensity is passed through a liquid sample, often in a quartz cuvette. When the radiation emerges on the other side of the cuvette, it is reduced in intensity owing to losses from,

- Reflection of the cuvette windows,
- Scattering and
- Absorption by the sample itself.

Often, a reference solution which has no analyte is also analysed to account for the losses due to reflection and scattering, thereby the intensity attenuation due to absorption alone can be worked out by simple subtraction. Inorganic molecules, this absorption is restricted to certain functional groups (chromophores) that contain electrons of low excitation energy. Aromatic molecules, for example, mostly absorb UV in the 200–300 nm region. An absorption spectrum is usually a plot of
absorbance versus wavelength and is normally continuous and broad with little fine structure. The broad spectrum is due to the fact that the higher energy radiation involved means that vibrational and rotational transitions co-occur as well as electronic transitions; all of these are superimposed on each other resulting in broad bands rather than sharp peaks. In UV–Vis absorption spectrometry, concentration of the species is related to absorbance by the Beer–Lambert Law

$$A_\lambda = \varepsilon_\lambda cl$$

Where $A_\lambda$ - Absorbance at a particular wavelength ($\lambda$),

$\varepsilon_\lambda$ - Extinction coefficient at a particular wavelength ($\lambda$),

c - Concentration and

l - Path length.

During most experiments, $\varepsilon$ and l remain constant, so absorbance is proportional to concentration, a relationship that is exploited for quantitative analysis $^{55}$.

$$A = \varepsilon cl$$

$$\varepsilon = A/cl$$

**Solubility of the given Active Pharmaceutical Ingredient (API)**

Solubilities of pharmacopoeial substances are provided primarily for information, and are increasingly being expressed in descriptive terms. Specific pharmacopoeial statements of solubility as weight of solute in specified volume of solvent at a particular temperature. Solubilities are also expressed as one part of the solute in x parts of solvent, meaning either 1gm solid solute or 1ml liquid solute in x ml solvent.$^{40,56}$
Take the required number of test tubes and dissolve the given API in the given solvent system and shake for some time and identify for the solubility.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Solvent System</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Water</td>
<td>+/-</td>
</tr>
<tr>
<td>2</td>
<td>0.1N HCL</td>
<td>+/-</td>
</tr>
<tr>
<td>3</td>
<td>2% PHOSPHATE BUFFER 6.8</td>
<td>+/-</td>
</tr>
<tr>
<td>4</td>
<td>2% PHOSPHATE BUFFER 7.4</td>
<td>+/-</td>
</tr>
<tr>
<td>5</td>
<td>20% METHANOLIC WATER</td>
<td>+/-</td>
</tr>
<tr>
<td>6</td>
<td>20% METHANOLIC WATER + 0.1 N HCl</td>
<td>+/-</td>
</tr>
<tr>
<td>7</td>
<td>20% ETHANOLIC WATER</td>
<td>+/-</td>
</tr>
<tr>
<td>8</td>
<td>20% ETHANOLIC WATER + 0.1 N HCl</td>
<td>+/-</td>
</tr>
<tr>
<td>9</td>
<td>20% METHANOL+2%PHOSPHATE BUFFER pH 6.8</td>
<td>+/-</td>
</tr>
<tr>
<td>10</td>
<td>20% METHANOL+2%PHOSPHATE BUFFER pH 7.4</td>
<td>+/-</td>
</tr>
<tr>
<td>11</td>
<td>20% ETHANOL+2%PHOSPHATE BUFFER pH 6.8</td>
<td>+/-</td>
</tr>
<tr>
<td>12</td>
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<td>+/-</td>
</tr>
</tbody>
</table>

**Note:** ‘+VE’ indicates solubility of the given API,

‘-VE’ indicates insolubility of the given API.

**Determination of Maximum Absorbance (\(\lambda_{\text{max}}\))**

\(\lambda_{\text{max}}\) can be defined as the wavelength at which maximum absorbance or maximum fraction of the light is absorbed by a solution containing API.

Labindia UV 3092 spectrophotometer with 1 cm matched quartz cells were used for the absorbance measurements connected to a computer and loaded with UV win 5.2.0.1104 software. In the process of determination of \(\lambda_{\text{max}}\), different APIs are dissolved in different solvents based on their solubility. A stock solution of 100 \(\mu g/ml\) is prepared by dissolving 10mg of API in 5ml of solvent and transferred in to 100ml volumetric flask sonicate it for 5mins, final volume is made up to the mark.
with the same solvent. The maximum absorbance is determined in the UV-Visible range 200-800 nm using an appropriate blank.\textsuperscript{57}

**Method validation of UV-Visible Analytical Method**

Method validation can be defined as (ICH) establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

Method validation is a fundamental part of the method development. It is the process of proving that the analytical procedures are suitable for their intended use and they give evidence about the identity, quality, purity and potency of the API.

Method Validation, however, is generally a one-time process performed after the method has been developed to demonstrate that the method is scientifically sound and that it serves the intended analytical purpose.

**Validation Parameters**

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BIBLIOGRAPHY


32. International Organization for Standardization. Accuracy (Trueness and Precision) of Measurement Methods and Results. 1994; 5725-1 to 5725-3.


52. AOAC. Manual on policies and procedures 1993; 6-11.


