Chapter - II

Experimentation
Analytical chemistry is a key component of chemistry education and research. It is also essential to determine the properties of materials, ensuring quality products and services, and used to monitor quality of the product. Analytical chemistry is the Science of making qualitative and quantitative measurements. In practice, qualifying an analyte in a complex sample becomes exercise in problem solving. To be efficient and effective an analytical chemist must know the tools that are available to tackle a wide variety of problems. A qualitative method provides information about the identity of atomic or molecular species or functional group in a sample. A quantitative method provides numerical information as to the relative amount of one or more of the components.

In the past decades, a number of elegant instrumental techniques were reported which are rapid, selective and having a high degree of accuracy. Analytical research studies are carrying out both in basic and advanced levels. There are three large research groups. First group, Chromatography and Separation Research Group focuses on chromatographic separation techniques involving fundamental studies on separation techniques, sample preparation techniques, Gas Chromatography (GC), Liquid Chromatography (LC), Ion Chromatography techniques, Capillary Electrophoresis (CE) and hyphenated techniques with mass spectrometry: GC-MS and LC-MS. They work on various fields of interest including drug and pharmaceutical, food and agricultural analysis, enantiomeric separation, biochemical analysis, medicinal analysis, as well as analysis of natural products, medicinal plants, environmental, petrochemical and polymer. These studies cover theory, development of instrumentation and techniques, validation of analytical methods as well as applications.

The second group is the environmental Analysis Research Group, which aims to develop analytical methods for determination of toxic and trace level pollutants in the last group is Electrochemical Research Group. The research interests include electro analytical detection of dissolved gas and trace metal analysis in water,
electrochemical catalysis, fuel cell technology, flow-based system. Future prospect for the Analytical Chemistry education expect to help each student get a strong foundation in chemical analysis, learn something new about analytical chemistry, and understand modern analytical techniques.

Among these spectrophotometry and chromatography techniques are most important, which is widely used in pharmaceutical industry to determine a wide variety of compounds. High accuracy, precision, sensitivity and the ease of availability of spectrophotometer made this technique indispensable for the modern analytical chemists. Besides, it offers the advantage of having calibration of graphs that are linear over a wide range of concentration of substances may be covered. Analytical chemistry plays an important role in the modern especially pharmaceutical industries, which rely upon both the qualitative and quantitative chemical analysis include UV-VIS, IR, GC and HPLC methods.

The analytically important functional groups of selected drugs do not seem to be fully exploited for designing visible spectrophotometric methods for their determinations. The chemical features of selected drug molecules still offer a lot of scope for the development of new UV and Visible Spectrophotometer and HPLC methods hopefully with better sensitivity, selectivity, precision and accuracy.

In this present study the author has made some attempts in this direction and succeeded in developing some new analytical methods having advantages of one or more of the above desirable features. Among the several HPLC methods reported for the selected drugs estimation of many of them, are in biological fluids and very few in pharmaceutical formulations.

This chapter summarizes the basic introduction of research in analytical chemistry and the basic principles and components of the techniques spectrophotometer and High Performance Liquid Chromatography (HPLC) used for this study and also explains brief introduction of method development, method validation.
2.1 Brief Introduction of Ultraviolet - Visible Absorption Spectroscopy

Ultraviolet and visible spectrometers have been in general use for the last 35 years and over this period it has become the most important analytical instrument in the modern day laboratory. In many applications other techniques could be employed but none rival UV-Visible spectrometry for its simplicity, versatility, speed, accuracy and cost-effectiveness.

In ultra violet (200-380 nm) and visible (380-800 nm) spectroscopy absorption of radiation is the result of excitation of bonding (sigma and pi) and non bonding (n) electrons. The frequencies of the absorption can be influenced by solvents and by delocalization in conjugated systems. Spectrophotometer is an instrument for measuring the intensity of light of various wavelength transmitted by a solution. The intensity of light is determined by electric detectors, which convert radiant energy to electric energy and can eliminate the need for subjective measurements.

Radiation is a form of energy and we are constantly reminded of its presence via our sense of sight and ability to feel radiant heat. It may be considered in terms of a wave motion where the wavelength, $\lambda$, is the distance between two successive peaks. The frequency, $\nu$, is the number of peaks passing a given point per second. These terms are related so that:

$$c = \nu \lambda.$$

Where $c$ is the velocity of light in a vacuum.

The wavelength $\lambda$ of electromagnetic radiation

The full electromagnetic radiation spectrum is continuous and each region merges slowly into the next. For spectroscopy purposes, we choose to characterize light in the ultraviolet and visible regions in terms of wavelength expressed in
nanometers. Other units which may be encountered, but whose use is now discouraged, are the Angstrom (Å) and the millimicron (mµ).

$$1\text{nm} = 1\text{mµ} = 10\text{Å} = 10^{-9}\text{meters}$$

<table>
<thead>
<tr>
<th>Region</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Far ultraviolet</td>
<td>10-200</td>
</tr>
<tr>
<td>Near ultraviolet</td>
<td>200-380</td>
</tr>
<tr>
<td>Visible</td>
<td>380-780</td>
</tr>
<tr>
<td>Near infrared</td>
<td>780-3000</td>
</tr>
<tr>
<td>Middle infrared</td>
<td>3000-30,000</td>
</tr>
<tr>
<td>Far infrared</td>
<td>30,000-300,000</td>
</tr>
<tr>
<td>Microwave</td>
<td>300,000-1,000,000,000</td>
</tr>
</tbody>
</table>

The human eye is only sensitive to a tiny proportion of the total electromagnetic spectrum between approximately 380 and 780 nm and within this area we perceive the colors of the rainbow from violet through to red. If the full electromagnetic spectrum shown in Fig. 2.1 was redrawn on a linear scale and the visible region was represented by the length of one centimeter, then the boundary between radio and microwaves would have to be drawn approximately 25 kilometers away!

![Electromagnetic Spectrum](image)

**Fig-2.1 Electromagnetic Spectrum**
The beam of light consists of a stream of photons, represented by the purple balls in the simulation shown below. When a photon encounters an analyte molecule (the analyte is the molecule being studied), there is a chance the analyte will absorb the photon. This absorption reduces the number of photons in the beam of light, thereby reducing the intensity of the light beam.

**Experimental Procedure:**

The following simulation illustrates the procedures for making spectrophotometer measurements.

First, the intensity of light ($I_0$) passing through a blank is measured. The intensity is the number of photons per second. The blank is a solution that is identical to the sample solution except that the blank does not contain the solute that absorbs light. This measurement is necessary, because the cell itself scatters some of the light.

Second, the intensity of light ($I$) passing through the sample solution is measured. (In practice, instruments measure the power rather than the intensity of the light. The power is the energy per second, which is the product of the intensity (photons per second) and the energy per photon).

Third, the experimental data is used to calculate two quantities:

- the transmittance ($T$) and the absorbance ($A$),
  \[ T = \frac{I}{I_0} \]
  \[ A = - \log_{10} T \]

The transmittance is simply the fraction of light in the original beam that passes through the sample and reaches the detector. The remainder of the light, $1 - T$, is the fraction of the light absorbed by the sample.

In most applications, one wishes to relate the amount of light absorbed to the concentration of the absorbing molecule. It turns out that the absorbance rather than the transmittance is most useful for this purpose. If no light is absorbed, the absorbance is zero (100% transmittance). Each unit in absorbance corresponds with an order of magnitude in the fraction of light transmitted. For $A = 1$, 10% of the light
is transmitted \((T = 0.10)\) and 90\% is absorbed by the sample. For \(A = 2\), 1\% of the light is transmitted and 99\% is absorbed. For \(A = 3\), 0.1\% of the light is transmitted and 99.9\% is absorbed.

Using the simulation below, perform the following steps:

- Measure the intensity of light passing through the blank.
- Measure the intensity of light passing through the sample.
- Calculate the transmittance.
- Calculate the absorbance.

Note: For each measurement, run the simulation long enough to detect at least 1000 photons. There is substantial random error in the intensity, and the more photons that are counted, the lower the relative uncertainty in the results.

<table>
<thead>
<tr>
<th>Color absorbed</th>
<th>Color observed</th>
<th>Absorbed radiation (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Violet</td>
<td>Yellow-green</td>
<td>400-435</td>
</tr>
<tr>
<td>Blue</td>
<td>Yellow</td>
<td>435-490</td>
</tr>
<tr>
<td>Green-blue</td>
<td>Orange</td>
<td>480-490</td>
</tr>
<tr>
<td>Blue-green</td>
<td>Red</td>
<td>490-500</td>
</tr>
<tr>
<td>Green</td>
<td>Purple</td>
<td>500-560</td>
</tr>
<tr>
<td>Yellow-green</td>
<td>Violet</td>
<td>560-580</td>
</tr>
<tr>
<td>Yellow</td>
<td>Blue</td>
<td>580-595</td>
</tr>
<tr>
<td>Orange</td>
<td>Green-blue</td>
<td>595-695</td>
</tr>
<tr>
<td>Red</td>
<td>Blue-green</td>
<td>605-750</td>
</tr>
</tbody>
</table>

A close relationship exists between the color of a substance and its electronic structure. A molecule or ion will exhibit absorption in the visible or ultraviolet region when radiation causes an electronic transition within its structure. Thus, the absorption of light by a sample in the ultraviolet or visible region is accompanied by a change in the electronic state of the molecules in the sample.

The energy supplied by the light will promote electrons from their ground state orbital to higher energy, excited state orbital or anti-bonding orbital.
2.2 Brief Introduction of High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) \(^6\text{-}^{21}\) is a special branch of column chromatography in which the mobile phase is forced through the column at high speed. As a result the analysis time is reduced by 1-2 orders of magnitude relative to classical column chromatography and the use of much smaller particles of the adsorbent or support becomes possible increasing the column efficiency substantially. The main components of the High performance Liquid Chromatography are shown in the schematic diagram.

Operculum …..Injection Port ……..Column ……………..Detector ………..Effluent ………..Thermostat ………Recorder

Columns\(^{22\text{-}23}\)

The heart of the system is the column. The choice of the common packing material and mobile phase depend upon the physical properties of the drug. The column selection, a flow chart in Table 2.1 can assist one in determining which columns to examine. Many different reverse phase columns will provide excellent specificity for any particular separation. It is therefore best to routinely attempt separation with a standard C-8 or C-18 column. (E.g. Zorbax, Rx C-8) and determine if it provides good separations. If this column does not provide good separation or the mobile phase is unsatisfactory, alternate methods or columns should be explored. Reverse phase columns differ by the carbon chain length, degree of end capping and percent carbon loading Diol cyano and amino groups can also be used for reverse phase chromatography.
### Table: 2.1

**Column selection flow chart**

<table>
<thead>
<tr>
<th>Sample</th>
<th>LC mode</th>
<th>Column choice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic or Acidic</td>
<td>Reverse phase- ion pair (Allows neutral and charged compounds to be simultaneously analyzed)</td>
<td>C18, C8, C6, C4, C2, TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1(PH1-13)</td>
</tr>
<tr>
<td>Ionizable</td>
<td>Ion Separation</td>
<td>C18, C8, C6, C4, C2, TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1(PH1-13)</td>
</tr>
<tr>
<td></td>
<td>Ion exchange</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anionic</td>
<td>Strong anion exchange</td>
</tr>
<tr>
<td></td>
<td>Cationic</td>
<td>Strong cation exchange</td>
</tr>
<tr>
<td></td>
<td>Normal phase</td>
<td>Increasing polarity of Bonded phases</td>
</tr>
<tr>
<td>Neutral</td>
<td>Diol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NH₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse phase</td>
<td>Silica</td>
</tr>
<tr>
<td></td>
<td>Alumina</td>
<td>Increasing Polarity of bonding phase</td>
</tr>
<tr>
<td></td>
<td>Silica</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alumina</td>
<td>Increasing Polarity of bonding phase</td>
</tr>
<tr>
<td></td>
<td>C-18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phenyl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TMS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hamilton PRP-1</td>
<td></td>
</tr>
</tbody>
</table>
Mobile phase

Mobile phases used for HPLC typically are mixtures of organic solvent and water or aqueous buffers. Table 2.2 lists the physical properties of organic solvents commonly used for HPLC. Isocratic methods are preferable to gradient methods. Gradient methods will sometime be required when the molecules being separated have vastly different portioning properties. When a gradient elution method is used care must be taken to ensure that all solvents are miscible.

The following points should be considered when choosing a mobile phase:

1. It is essential to establish that the drug is stable in the mobile phase for at least the duration of the analysis.

2. Excessive salt concentrations should be avoided. High salt concentrations can result in preparation, which can damage HPLC equipment.

3. The mobile phase should have a pH 2.5 and pH 7.0 to maximize the life time of the column.

4. Reduce cost and toxicity of the mobile phase by using methanol instead of acetonitrile when possible.

5. Minimize the absorbance of buffer.

6. Since trifluoroacetic acid, acetic acid or formic acid absorb at shorter wavelengths, they may prevent detection of products without chromophores above 220 nm. Carboxylic acids modifier can be frequently replaced by phosphoric acid, which does not absorb above 200 nm.

7. Use volatile mobile phases when possible to facilitate collection of products and LC-MS analysis. Volatile mobile phases include ammonium acetate, ammonium phosphate, and formic acid, acetic acid and trifluoroacetic acid.

8. Some caution is needed as these buffers absorb below 220 nm.

Based on the nature of the stationary and mobile phases and use of different columns, in the present investigation five HPLC methods (M10 to M15) have been reported.
Table: 2.2

**Physical Properties of Common HPLC Solvents**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>M.W (25°C)</th>
<th>B.P</th>
<th>R.I Off nm</th>
<th>UV&lt;sup&gt;a&lt;/sup&gt; Cut</th>
<th>Density g/ml(25&lt;sup&gt;0&lt;/sup&gt;C)</th>
<th>Viscosity C&lt;sub&gt;p&lt;/sub&gt; (25&lt;sup&gt;0&lt;/sup&gt;C)</th>
<th>Dielectric constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>41.02</td>
<td>82</td>
<td>1.342</td>
<td>190</td>
<td>0.787</td>
<td>0.358</td>
<td>38.8</td>
</tr>
<tr>
<td>Dioxane</td>
<td>88.1</td>
<td>101</td>
<td>1.420</td>
<td>215</td>
<td>1.034</td>
<td>1.26</td>
<td>2.21</td>
</tr>
<tr>
<td>Ethanol</td>
<td>46.1</td>
<td>78</td>
<td>1.359</td>
<td>205</td>
<td>0.789</td>
<td>1.19</td>
<td>24.5</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>88.1</td>
<td>77</td>
<td>1.372</td>
<td>256</td>
<td>0.901</td>
<td>0.450</td>
<td>6.02</td>
</tr>
<tr>
<td>Methanol</td>
<td>32.0</td>
<td>65</td>
<td>1.326</td>
<td>205</td>
<td>0.792</td>
<td>0.584</td>
<td>32.7</td>
</tr>
<tr>
<td>CH₂Cl₂</td>
<td>84.9</td>
<td>40</td>
<td>1.424</td>
<td>233</td>
<td>1.326</td>
<td>0.44</td>
<td>8.93</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>60.1</td>
<td>82</td>
<td>1.375</td>
<td>205</td>
<td>0.785</td>
<td>2.39</td>
<td>19.9</td>
</tr>
<tr>
<td>n-Propanol</td>
<td>60.1</td>
<td>97</td>
<td>1.383</td>
<td>205</td>
<td>0.804</td>
<td>2.20</td>
<td>20.3</td>
</tr>
<tr>
<td>THF</td>
<td>72.1</td>
<td>66</td>
<td>1.404</td>
<td>210</td>
<td>0.889</td>
<td>0.51</td>
<td>7.58</td>
</tr>
<tr>
<td>Water</td>
<td>18.0</td>
<td>100</td>
<td>1.333</td>
<td>170</td>
<td>0.998</td>
<td>1.00</td>
<td>7.58</td>
</tr>
</tbody>
</table>

Ionizable compounds in some cases can present some problems when analyzed by reverse phase chromatography, two modifications of the mobile phase can be useful in reverse phase HPLC for the ionizable compounds. One is called ion suppression and other ion pairing chromatography. In both techniques, a buffer is used to ensure that the P<sub>H</sub> of the solution is constant and usually 1.5 P<sub>H</sub> units from a P<sub>Ka</sub> of the drug to ensure that one form predominates. If P<sub>H</sub> is approximately equal to P<sub>Ka</sub> peak broadening can occur. In ion suppression chromatography the P<sub>H</sub> of the aqueous portion of the mobile phase is adjusted to allow the neutral form of the drug to predominate. This ensures that the drug is persistent in only one form and results in improvement of the peak shape and consistency of retention times. In ion pairing chromatography the P<sub>H</sub> of the mobile phase is adjusted so that the drug is completely ionized. If necessary to improve peak shape or lengthen retention time, an alkyl sulphonic acid salt or bulky anion such as trifluoroacetic acid is added to the ion pair to cationic drugs or a quaternary alkyl ammonium salt is added to ion pair to anionic drugs. Ion pairing chromatography also allows the simultaneous analysis of both neutral and charged compounds.
**Temperature**

Room temperature is the first choice. Elevated temperatures are sometimes used to neither reduce column pressure nor enhance selectivity. Typically temperatures in excess of 60°C are not used.

**Retention Time**

Due to a number of samples assayed in the course of pre formulation study it is advisable to have as short a retention time as possible. However, the retention time should be long enough to ensure selectivity. When choosing the optimum mobile phase, consideration should be given to the retention time of degradation products. So that these compounds do not elute in the solvent front and remain in the column.

**Detectors**

The detections of UV light absorbance offer both convenience and sensitivity for molecules. In a chromophore the wavelength of detection for a drug should be based on its UV spectrum in the mobile phase and not in pure solvents, the most selective wavelength for detecting a drug is frequently the longest wavelength, maximum to avoid interference from the solvents, buffers and excipients. Other method of detection can be useful or required in some instances.

1. Solute specific detectors (UV, Visible, fluorescence, electrochemical Infrared radioactivity)
2. Bulk property detector (Reractive index, Viscometric, conductivity)
3. Desolvation detectors (Flame ionization etc.)
4. LC-MS detectors and.
5. Reaction detectors

**Performance Calculations**

Calculating the following values (which can be included in a custom report) used to access overall system performance.

1. Relative retention
2. Theoretical plates
3. Capacity factor
4. Resolution

5. Peak asymmetry

6. Plates per meter

The following information furnishes the parameters used to calculate these systems performance values for the separation of two chromatographic components. (Note: Where the terms W and t appear in the same equation they must be expressed in the same units).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative Retention(Selectivity)</td>
<td>$\alpha = \frac{(t_2-t_a)}{(t_1-t_a)}$</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>$n = 16\left(\frac{t}{W}\right)^2$</td>
</tr>
<tr>
<td>Capacity factor</td>
<td>$K' = \frac{(t_2-t_a)-1}{2}$</td>
</tr>
<tr>
<td>Resolution</td>
<td>$R = \frac{2(t_2-t_1)}{(W_2 + W_1)}$</td>
</tr>
<tr>
<td>Peak asymmetry</td>
<td>$T = \frac{W_{0.05}}{2f}$</td>
</tr>
<tr>
<td>Plates per meter</td>
<td>$N = \frac{n}{L}$</td>
</tr>
<tr>
<td>HETP</td>
<td>$L/n$</td>
</tr>
</tbody>
</table>

$\alpha$: Relative retention

$t_2$: Retention time of the second peak measured. Measured from the point of injection

$t_1$: Retention time of the first peak measured. Measured from the point of injection

$t_a$: t Retained by the Column measured from point of injection

$N$: Theoretical plates

$T$: Retention time of the component

$W$: width of the base of the component peak

$K'$: Capacity factor

Using tangent method
2.3 Method Development and Method Validation

Analytical method development and validation are key elements and also an important regulatory requirement of any drug and pharmaceutical development program. Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. Pharmaceutical products formulated with more than one drug, typically referred to as combination.

In developing a quantitative method for determining an unknown concentration of a given substance by absorption spectrophotometry, the first step will be the selection of analytical wavelength at which absorption measurements are made. The analytical wavelength can be chosen either from literature or experimentally by means of scanning with a spectrophotometer. In order to enhance the sensitivity of the method and signal to noise ratio, the wavelength of maximum absorbance is chosen as analytical wavelength.

Analytical methods including spectrophotometer and chromatographic methods are commonly used for the quantitative and qualitative analysis of raw materials, drug substances, drug products, and compounds in biological samples in
pharmaceutical industry. The components monitored include chiral or achiral drugs, process impurities, residual solvents, excipients such as preservatives, degradation products, extractable and leachable from container and closure or manufacturing process, pesticide in drug product from plant origin, and metabolites.

Slope analysis method and mole ratio methods were followed for finding out the composition of the components in the ion association complexes and charge – transfer complexes, respectively.

Validation of an analytical method which is used during drug development and drug manufacturing is required to demonstrate that the methods are fit for their intended purpose. Additionally, the pharmaceutical industry around the world is subject to extensive regulations due to the nature of its products.

Analytical chemists play important roles in monitoring the drugs in their dosage forms and biological samples. From the viewpoints mentioned above, the title of this special issue was chosen so as to ask chemists to appreciate their great roles in chemistry science. This special issue features 8 research articles. In this special issue, development of chromatographic methods such as high performance liquid chromatography, gas chromatography, micellar liquid chromatography, and their validations is presented.

The ability to provide accurate, reliable, and consistent data is central to the role of the analytical chemist at Eagle Analytical Services. Method development is the process of proving that an analytical method is acceptable for use in our laboratory to measure the concentration of an API in a specific compounded dosage form.

Full method development procedures are complex, extended, and expensive endeavors. These studies usually are required when data from the analysis is going to be submitted to regulatory authorities for certification of a new or amended drug application. The analytical method is tested extensively for specificity, limit of detection, Limit of quantification, linearity, accuracy, precision, range and robustness.

For established pharmaceutical preparations, which are not new drug species, the analytical method process can be much simpler. Modern analytical instrumentation, like high performance liquid chromatography with versatile detectors such as the ultraviolet photodiode array spectrometer (HPLC-PDA), UV-VIS
Spectrophotometer, allow simplified procedures to be employed to verify that an analysis procedure accurately and consistently will deliver a reliable measurement of an active ingredient in a compounded preparation.

**Optimization of Analytical Method**

In each type of basic reaction, the colored species is formed or the final color of the reaction mixture whose absorbance is measured and thus the sensitivity of the method, rate of color formation and stability is affected by the concentration of the reagent in the solution, nature of solvent, temperature, $\text{pH}$ of the medium, order of addition of reactants and intervals between additions. For simple systems having no interaction between variables, the one variable at a time (OVAT) strategy appears to be simple, efficient and effective to establish the optimum conditions. The OVAT approach requires all variables but one to be held constant while a univariate search is carried out on the variable of interest. The details of fixing optimum conditions used in different procedures of present study are furnished in subsequent chapters.

**Method Validation Performance Characteristics:**

**Selectivity**$^{30,31}$

Matrix and interference effects may disturb the determination of an analyte. Some of the excipients, incipient and additives present in pharmaceutical formulations may sometimes interfere in the assay of drug and in such instance appropriate separation procedure is to be adopted initially; the selectivity of the method is separation procedure. The selectivity of the method is ascertained by studying the effect of a wide range of excipients and other additives usually present in the pharmaceutical formulations to be determined under optimum conditions.

Initially, interference studies are carried out by the determination of fixed concentration of the drug several times by the optimum procedure in the presence of a suitable (1-100 fold) molar excess of the foreign compound under investigation and its effect on the absorbance of the solution is noticed. The foreign compound is considered to be interfering at these concentrations if it constantly produces an error of less than 3.0% in the absorbance produced in pure solution.

The method is said to be sensitive, small changes in concentration causes larger changes in response function. The sensitivity of an analytical method is
determined from the slope of the calibration line. The limits of quantitation (LOQ) or working dynamic range of bio analytical method are defined as the highest and lowest concentrations, which can be determined with acceptable accuracy. It is suggested that, this be set at ± 15% for both the upper and lower limit of quantitation respectively. Any sample concentration that falls outside the calibration range cannot be interpolated from the calibration line and extrapolation of the calibration curve is discouraged. If the concentration is over range, the sample should be diluted in drug free matrix and re-assayed. 

**Linearity**

Knowledge of the sensitivity of the color is important and the following terms are commonly employed for expressing sensitivity.

**According to Bougher – Lambert – Beer’s Law**

\[ A = \log \frac{\text{Intensity of incident radiations}}{\text{Intensity of transmitted light}} = \varepsilon \cdot c \cdot t \]

The absorbance (A) is proportional to the concentration (c) of the absorbing species, if absorptivity (\( \varepsilon \)) and thickness of the medium (t) are constant. Where c is in moles per liter, the constant is called molar absorptivity. Beer’s law limits and \( \lambda_{\text{max}} \) values are expressed as \( \mu \text{g.ml}^{-1} \) and \( 1 \text{ mole}^{-1}.\text{cm}^{-1} \), respectively.

Sandell’s sensitivity refers to the number of \( \mu \text{g} \) of the drug to be determined, converted to the colored product, which in a column solution of cross section 1 cm\(^2\) shows an absorbance of 0.001 (expressed as \( \mu \text{g cm}^{-2} \)).

**Limit of Detection**

The limit of detection (LOD) of an analytical method may be defined as the concentration, which gives rise to an instrument signal that is significantly different from the blank. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements the IUPAC approach employs the standard deviation of the intercept (\( S_a \)), which may be related to LOD and the slope of calibration curve, b, by

\[ \text{LOD} = 3S_a / b \]
**Ringbom's Plot** \(^{33-35}\)

The relative concentration error depends inversely upon the product absorbance and transmittance. The relative error increase at the extremes of the transmittance scale. The slope of plot C versus T, i.e. Ringbom plot gives relative error coefficient (i.e. plot of log C vs T) the main limitations Ringbom plot is that it provides no concerning the concentration range of good precision unless it is combined with ΔT versus T relation. The above expression is valid whether Beer’s law is followed or not.

**Precision and Accuracy**

The purpose of carrying out a determination is to obtain a valid estimate of a ‘true’ value. When one considers the criteria according to which an analytical procedure is selected, precision and accuracy are usually the first time to come to mind. Precision and accuracy together determine the error of an individual determination. They are among the most important critical for judging analytical procedures by their results.

**Precision** \(^{36-38}\)

Precision refers to the reproducibility of measurement within a set that is to the scatter of dispersion of a set about its central value. The term ‘set’ is defined referring to a number (n) of independent replicate & measurements of some property. One of the most common statistical terms employed is the standard deviation population of observation.

The precision of analytical method is measure of the random error and is defined as the agreement between replicate measurements of the sample. It is expressed as the percentage coefficient of variation (\(\%\ CV\)) or relative standard deviation (RSD) of the replicate measurements.

\[
%\ CV = \frac{\text{Standard deviation} \times 100}{\text{mean}}
\]

Precision can be considered as having a within assay batch component or replicability which defines the ability to repeat the same methodology with the same analyst, using the same equipment and same reagents in a short interval of time e.g. within a day. This is known as intra-array precision.
The ability to repeat the same methodology under different conditions e.g., change of analyst, reagent or equipment or on subsequent occasions, e.g. across several weeks or months, is covered by the between batch precision and reproducibility, also known as inter-assay precision. The reproducibility of a method is of most interest to the analyst, since this will give better representation of the precision during routine use as it includes the variability from many sources.

For validation of new analytical method for routine use, it is suggested that precision be assessed at four unique concentrations in replicates of six, on four separate conditions i.e. 4x6x4. This approach will allow the data for individual analytes to be analyzed by a one-way analysis of variance, which gives estimates of both the intra-assay and inter assay precision of the method at each concentration. To be acceptable, both measures should be within ± 15% at all concentrations.

Accuracy

Accuracy normally refers to the difference between the mean of the set of results and the true or correct value for the quantity measured. According to IUPAC accuracy relates to the difference between results (or mean) and the true value. For analytical methods, there are two possible ways of determining the accuracy, absolute method and comparative method.

The absolute recovery of a method is measured as the response of a processed spiked matrix standard expressed as a percentage of the response of pure standard, which has not been subjected to sample pre-treatment and indicates whether the method provides a response for the entire amount of analyte that is present in the sample. It is best established by comparing the responses or extracted samples at low, medium and high concentrations in replicates of at least 6 with those of non-extracted standards, which represent 100% recovery.

Absolute recovery + \frac{\text{response of analyte spiked in a matrix (processed)}}{\text{response of analyte of pure standard (Unprocessed)}} \times 100

If an internal standard is used, its recovery should be determined independently at the concentration level used in the method.
The accuracy of the analytical method is a measure of the systematic error or bias and is defined as the agreement between the measured value and the true value. Accuracy is the best reported as percentage bias, which is calculated from the expression.

\[
\% \text{ Bias} = \frac{\text{Measured value} - \text{true value} \times 100}{\text{True value}}
\]

Since the true value is not known for real samples, an approximation is obtained, based on spiking drug-free matrix to a nominal concentration. The accuracy of analytical method is then determined at each concentration by assessing the agreement between the measured and nominal concentrations of the analytes in the spiked drug-free matrix samples. For the validation of a new analytical method for the use, the measured concentrations will be those obtained during the estimation of precision i.e., from the 4*6*4 experiment. All results other than those rejected for analytical reasons, i.e., poor chromatography, should be used in the calculations and accuracy of the method should be within ± 15% at all concentrations.

**Recovery experiments (standard addition method)**

A known amount of the constituent being determined is added to the sample, which is analyzed for the total amount of constituent present. The difference between the analytical results for sample with and without the added constituent gives the recovery of the amount of the added constituent. If the recovery is satisfactory, confidence in the accuracy of the procedure is enhanced.

**2.4 Evaluation of Precision and Accuracy by Comparison of Two Procedures**

To evaluate the accuracy of the method, one often compares the method being investigated of ‘test method’ with an existing method called the ‘reference method’.

**Student t-test**

Student t-test is used to compare the means of two related (paired) samples analyzed by reference and test methods. It gives answer to the correctness of the null hypothesis with a certain confidence such as 95% or 99%. If the number of pairs (n) are small than 30, the condition normality of x is required or at least the normality of the difference (d,). If this is the case the quantity has a student
\[ t = \frac{\overline{d_i}}{s_d / \sqrt{n}} \]

t-distribution with (n-1) degrees of freedom, where \( d_i = X_R \) (Reference method) - \( X_T \) (Test method) and \( s_d \) is the standard deviation.

**F-test**

By the f-test the significance of the difference in variances of reference and test methods can be tested. Let us suppose that one carried out \( n_1 \) replicate measurements by test methods and \( n_2 \) replicate measurements by using reference method. If the null hypothesis is true then the estimates \( S_T^2 \) (variance of the test method) and \( S_R^2 \) (variance of reference method) do not differ very much and their ratio should not differ much from unity. In fact, one uses the ratio of variances

\[ F = \frac{S_T^2}{S_R^2} \]

It is conventional to calculate the F-ratio by dividing the larger variance by the smallest variance in order to obtain a value equal or larger than unity. If the calculated F-values are smaller than F-value from the Table, one can conclude that the procedures are not significantly different in precision at given confidence level.
REFERENCES


17. Beckman Model 330 HPLC Manuel, Beckman Instruments, Fulleron, CA.


