1.1 INTRODUCTION TO ANALYTICAL METHOD DEVELOPMENT [1-8]

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

1.1.1 NEED FOR METHOD DEVELOPMENT:

New analytical methods are needed for the following reasons:

- Methods are not available
- Existing methods are not sufficiently reliable, sensitive, or cost effective.
- New instrumentation or technique has better performance (ease of use, rapid turnaround, automation, higher sensitivity).
- An alternate (orthogonal) method is required for regulatory compliance.

1.1.2 STRATEGY FOR METHOD DEVELOPMENT:

Steps in a common strategy for method development are summarized below:

- Define method and separation goals
- Gather sample and analyte information
- Initial method development—“scouting” runs and getting the primary data
- Method fine-tuning and optimization
- Method validation
1.1.3 LIFE CYCLE OF AN ANALYTICAL METHOD

![Life cycle of analytical method diagram]

**Figure 1.1: Life cycle of analytical method**

1.1.4 VALIDATION OF ANALYTICAL METHODS

Validation of analytical procedures is the process of determining the suitability of a given methodology for providing useful analytical data. Validation is the formal and systematic proof that a method complies with the requirements for testing a product when observing defined procedures.

The various validation parameters to validate method are:

- Accuracy
- Precision (repeatability and reproducibility)
- Linearity and range
- Selectivity/ Specificity
- Robustness/ Ruggedness
- Limit of detection (LOD)
- Limit of quantitation (LOQ)
1.4.1 ACCURACY

The accuracy of an analytical method may be defined as the closeness of the test results obtained by the method to the true value. It is the measure of the exactness of the analytical method developed. Accuracy may often be expressed as percent recovery from the assay of a known amount of analyte added. Accuracy should be established across the specified range of the analytical procedure.

1.4.2 PRECISION

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous samples. This is usually expressed as the standard deviation or the relative standard deviation (coefficient of variation). Precision is a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances. Repeatability involves analysis of replicates by the analyst using the same equipment and method and conducting the precision study over short period of time.

1.4.2.1 Determination of Repeatability:

Repeatability can be defined as the precision of the procedure when repeated by same analyst under the same operating conditions (same reagents, equipments, settings and laboratory) over a short interval of time.

It is normally expected that at least six replicates be carried out and a table showing each individual result provided from which the mean, standard deviation and co-efficient of variation should be calculated for set of n values. The RSD values are important for showing degree of variation expected when the analytical procedure is repeated several times in a standard situation. (RSD below 1% for bulk drugs and RSD below 2% for assay in finished product).

1.4.2.2 Determination of reproducibility:

Reproducibility means the precision of the procedure when it is carried out under different conditions-usually in different laboratories-on separate, identical samples taken from the same homogenous batch of material. Comparisons of
results obtained by different analysts, by the use of different equipments, or by carrying out the analysis at different times can also provide valuable information.

1.4.2.3 Determination of Intermediate Precision

a) Intra-day reproducibility

A variation of results within same day is called intra-day variation. It was determined by repeating calibration curve 3 times on same day.

b) Inter-day reproducibility

Variation of results amongst day is called inter-day variation. It was determined by repeating calibration curve daily for 3 different days.

1.4.3 LINEARITY AND RANGE

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

1.4.4 LIMIT OF DETECTION AND LIMIT OF QUANTITATION

1.4.4.1 Limit of detection (LOD)

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

a) Based on Visual Evaluation

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

b) Based on Signal-to-Noise Approach

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

c) Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as:

$$\text{LOD} = 3.3 \sigma / S$$

Where, $\sigma$ = the standard deviation of the response

$S$ = the slope of the calibration curve

1.4.4.2 Limit of Quantitation (LOQ)

Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

a) Based on Visual Evaluation

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

b) Based on Signal-to-Noise Approach

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

c) Based on the Standard Deviation of the Response and the Slope

The Limit of Quantitation (LOQ) may be expressed as:

$$\text{LOQ} = 10 \sigma / S$$

Where, $\sigma$ = the standard deviation of the response

$S$ = the slope of the calibration curve
1.4.5 SELECTIVITY AND SPECIFICITY

1.4.5.1 Selectivity
The selectivity of an analytical method is its ability to measure the analyte of interest qualitatively.

1.4.5.2 Specificity
The specificity of an analytical method is its ability to measure specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix quantitatively.

1.4.6 ROBUSTNESS AND RUGGEDNESS

1.4.6.1 Robustness:
The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage.

1.4.6.2 Ruggedness:
The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay.

1.1.5 INTRODUCTION TO UV-VISIBLE SPECTROPHOTOMETRY [9-11]
Measurement based on light and other forms of electromagnetic radiation are widely used through analytical chemistry. The interaction of radiation and matter are the subject of science called spectroscopy. Spectroscopic analytical methods are based on measuring the amount of radiation produced or absorbed by molecular or atomic species of interest.
The technique of UV-Visible spectrometry is one of the most frequently employed in pharmaceutical analysis. It involves measurement of the amount of UV (190-380nm) or Visible (380-800nm) radiation absorbed by substance in the solution.
1.1.5.1 PRINCIPLE

The absorption of light by analyte (by raising the electrons to higher level i.e. $\pi$-$\pi^*$ or $n$-$\pi^*$) is due to presence of chromophore in their molecules which are specific portion of molecule that can absorb radiant energy.

This method of analysis is based on Lambert-Beer’s Law

![Diagram of electron transition in UV-Visible spectroscopy]

**Figure 1.2: Electron transition in UV-Visible spectroscopy**

1.1.5.2 THEORY:

In absorption spectroscopy a beam of electromagnetic radiation passes through a sample.

Much of the radiation is transmitted without a loss in intensity. At selected frequencies, however, the radiation’s intensity is attenuated. This process of attenuation is called absorption. Two general requirements must be met if an analyte is to absorb electromagnetic radiation.

The first requirement is that there must be a mechanism by which the radiation’s electric field or magnetic field interacts with the analyte. For ultraviolet and visible radiation, this interaction involves the electronic energy of valence electrons.

The second requirement is that the energy of the electromagnetic radiation must exactly equal the difference in energy, between two of the analytes quantized energy states. Figure 1.1.2 shows a simplified view of the absorption of a photon.
Lambert’s Law is defined as follows:
The intensity of a beam of parallel monochromatic radiation decreases exponentially as it passes through a medium of homogeneous thickness.

Beer’s Law is defined as follows:
The intensity of a beam of parallel monochromatic radiation decreases exponentially with the number of absorbing molecules.
The combination of two laws yields Beer-Lambert law:

\[
A = \varepsilon b c
\]

Where, \(A\) = absorbance of analyte
\(\varepsilon\) = molar absorptivity
\(b\) = path length
\(c\) = concentration of analyte

1.1.5.3 INSTRUMENTATION
The basic components of Instrument are:

1.1.5.3.1 Radiation source:
It emits electromagnetic radiation in UV and Visible region
   a. Deuterium lamp  b. Tungsten lamp  c. Xenon discharge lamp  d. Mercury lamp

1.1.5.3.2 Monochromator:
It spreads the beam of light into its component wavelengths.
   • Diffraction gratings and Slits

1.1.5.3.3 Detectors:
It amplifies the response.
   • Photomultiplier tube, Barrier layer cell and Photo diode array

1.5.3.4 Sample cell:
Made up of quartz or fused silica which is transparent to UV-Visible region.

1.1.5.4 ADVANTAGES
• Wide Applicability, High sensitivity, Moderate to high selectivity, Ease and convenience
1.1.5.5 LIMITATIONS

- Generally, sample concentration must be low (absorbance less than 2). Photosensitive compounds may be difficult to analyse. Light scattering can limit the precision of measurement. Overlapping absorption band in complex mixtures can reduce precision of measurements. Limit of detection can be considerably higher than in fluorescence or chemiluminescence methods.

1.1.5.6 APPLICATIONS

- Identification of compounds by spectrum matching with reference spectra
- Trace analysis of organic, inorganic and biological species
- Detector for chromatography and electrophoresis
- Environmental remote sensing
- Field testing (pH, metals, non-metals and organics)
- Pharmaceutical analysis

1.1.5.7 For the assay of substance in multi-component samples, following methods are routinely being used. They are as follows:

- Simultaneous equation method
- Absorbance ratio method
- Geometric correction method
- Dual wavelength method
- Derivative spectrophotometric method
- Ratio Spectra derivative method
- Difference spectrophotometry
- Orthogonal polynomial method

1.1.5.7.1 ABSORBANCE RATIO METHOD (Q ANALYSIS METHOD)

The absorbance ratio method is a modification of the simultaneous equations procedure. It depends on the property that for a substance which obeys Beer's Law at all wavelengths, the ratio of absorbance’s at any two wavelengths, is a constant value independent of concentration or path length. In the USP this ratio is referred as a Q value. The British Pharmacopoeia also uses a ratio of absorbances at specified wavelengths in certain confirmatory tests of identity.
In the quantitative assay of two components in admixture by the absorbance ratio method, absorbance are measured at two wavelengths, one being the $\lambda_{\text{max}}$ of one of the components ($\lambda_2$) and the other being a wavelength of equal absorptivity of the two components ($\lambda_1$) i.e. isoabsorptive point. Two equations are constructed from the simultaneous equation method.

Figure 1.3: Selection of wavelength for Q-Absorbance Ratio method

$$QM - QY \quad A_1$$

$$C_x = \frac{QM - QY}{A_1} \quad Q_x - QY \quad a_x 1$$

$$QM - QX \quad A_1$$

$$C_Y = \frac{QM - QX}{A_1} \quad Q_Y - Q_X \quad a_x 1$$

Where,

$C_X$ = Concentration of X

$C_Y$ = Concentration of Y

$QM = Q \text{ absorbance ratio of sample at } \lambda_2 \text{ and at } \lambda_1$

$QX = \text{Ratio of absorptivity of X at } \lambda_2 \text{ and } \lambda_1$

$QY = \text{Ratio of absorptivity of Y at } \lambda_2 \text{ and } \lambda_1$

$A_1 = \text{Absorbance of mixture at } \lambda_1$

$A_2 = \text{Absorbance of mixture at } \lambda_2$

$a_x1 = \text{Absorptivity of X at } \lambda_1$
ay1 = Absorptivity of Y at λ1

Equation (1) gives the concentration of X in terms of absorbance ratio, the absorbance of the mixture and absorptivity of the compounds at the iso-absorptive wavelength.

Accurate dilutions of the sample solution and of the standard solutions of X and Y are necessary for the accurate measurement of A1 and ax1 respectively.

1.1.5.7.2 SIMULTANEOUS EQUATION METHOD

If a sample contains two absorbing drugs (X and Y) each of which absorbs at the λ max of the other (as shown in Figure 1.. λ1 and2), it may be possible to determine both drugs by the technique of simultaneous equation (Vierodt’s method) provided that certain criteria apply.

The information required are:

- The absorptivities of X at λ1 and λ2, ax1 and ax2 respectively
- The absorptivities of Y at λ1 and λ2, ay1 and ay2 respectively
- The absorbance of the diluted sample at λ1 and λ2, A1 and A2 respectively.

Let Cx and Cy be the concentration of X and Y respectively in the diluted samples.

Two equations are constructed based upon the fact that at λ1 and λ2, the absorbance of the mixture is the sum of the individual absorbance of X and Y.

At λ1

\[ A_1 = a_{x1}bC_x + a_{y1}bC_y \]  \hspace{1cm} (1)

At λ2

\[ A_2 = a_{x2}bC_x + a_{y2}bC_y \]  \hspace{1cm} (2)

For measurements in 1 cm cells, b = 1.
Rearrange equation (2)

\[ C_y = \frac{(A_2 - a_{x2} C_x)}{ay2} \]

Substituting for \( C_y \) in eq. (1) and rearranging gives

\[ C_x = \frac{(A_2 ay_1 - A_1 ay_2)}{(ax_2 ay_1 - a_{x1} ay_2)} \]

\[ C_y = \frac{(A_1 a_{x2} - A_2 a_{x1})}{(ax_2 ay_1 - a_{x1} ay_2)} \]

\[ \text{Figure 1.4: The overlain spectra simultaneous equation method.} \]

Criteria for obtaining maximum precision have been suggested by Glenn. According to him absorbance ratio place limits on the relative concentrations of the components of the mixture.

\[ \frac{(A_2/A_1)}{(a_{x2}/a_{x1})} \text{ and } \frac{(a_{y2}/ay_1)}{(A2/A_1)} \]

The criteria are that the ratios should lie outside the range 0.1-2.0 for the precise determination of \( Y \) and \( X \) respectively. These criteria are satisfied only when the \( \lambda_{\text{max}} \) of the two components are reasonably dissimilar. An additional criterion is that the two components do not interact chemically, thereby negating the initial assumption that the total absorbance is the sum of the individual absorbance. The additive of the absorbance should always be confirmed in the development of a new application of this technique.
Simultaneous equation method using Matrices and Cramer's Rule can be explained as follows:

Consider a binary mixture of component X and Y for which the absorption spectra of individual components and mixture are shown in Figure 1.4.

-1 is the $\lambda_{\text{max}}$ of component X

-2 is the $\lambda_{\text{max}}$ of component Y

The total absorbance of a solution at a given wavelength is equal to the sum of the absorbance of the individual components at the wavelength. Thus the absorbance of mixture at the wavelength 1 and 2 may be expressed as follows:

At $\lambda_1$

$$A_1 = a_x b C_x + a_y b C_y \quad \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots 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minimum.

Easy measurements on separate peaks, higher values of the analytical signals and no need to work only at zero crossing points (sometimes coexisting compounds have no maximum or minimum at these wavelengths) are advantages for ratio spectra derivative spectrophotometry in comparison with the zero crossing derivative spectrophotometry. Also the presence of lot of maxima and minima in ratio spectra derivative data was another advantage, since these wavelengths give an opportunity for the determination of these compounds in the presence of other active compounds and excipients that possibly interfered with the assay.

1.1.5.7.4 DERIVATIVE ZERO CROSSING SPECTROPHOTOMETRY

For the purpose of spectral analysis in order to relate chemical structure to electronic transitions, and for analytical situations in which mixture contribute interfering absorption, a method of manipulating the spectral data is called derivative spectroscopy.

Derivative spectrophotometry involves the conversions of a normal spectrum to its first, second or higher derivative spectrum. In the context of derivative spectrophotometry, the normal absorption spectrum is referred to as the fundamental, zero order, or $D^0$ spectrum.

1.1.5.7.5 GEOMETRIC CORRECTION METHOD

It is a method of eliminating the background irrelevant absorption that may be present in the biological origin samples. The simplest of them being three point geometric correction methods.

1.1.5.7.6 ORTHOGONAL POLYNOMIAL METHOD

A mathematical correction procedure, involving complex calculation on the basis that absorption can be represented in terms of orthogonal functions.

1.1.5.7.7 DIFFERENCE SPECTROPHOTOMETRY
It is a sensitive method for detecting small change in the chemical environment of a chromophore. The essential feature of this method is that the measured absorbance between the equimolar solution of analyte in different chemical forms exhibits different spectral characteristics.

1.1.5.7.8 DUAL WAVE LENGTH METHOD

This method is applicable to calculate the concentration of component of interest found in a mixture containing it along with some unwanted interfering component. The absorbance difference between two points of the mixture spectra is directly proportional to the concentration of the analyte irrespective of the interferon.

1.1.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1.1.6.1 Definition

HPLC is a physical separation technique conducted in the liquid phase in which a sample is separated into its constituent components (or analyte) by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column). HPLC is the most widely used analytical technique for the quantitative analysis of Pharmaceuticals, Biomolecules, Polymers and other organic compounds.

1.1.6.2 Principle of High Performance Liquid Chromatography

1.1.6.2.1 Normal-Phase Chromatography (NP-HPLC)

Normal-phase HPLC explores the differences in the strength of the polar interactions of the analyte in the mixture with the stationary phase. The stronger the analyte-stationary phase interaction, the longer the analyte retention. As with any liquid chromatography technique, NP-HPLC separation is a competitive process. Analyte molecules compete with the mobile-phase molecules for the adsorption sites on the surface of the stationary phase. The stronger the mobile-phase interactions with the stationary phase, the lower the difference between the stationary-phase interactions and the analyte interactions, and thus the lower the analyte retention. Mobile phases in NP-HPLC are based on nonpolar solvents (such as hexane, heptane, etc.) with the small addition of polar modifier (i.e.,
methanol, ethanol etc).

1.1.6.2.2 Reversed-Phase HPLC (RP-HPLC)

As opposed to normal-phase HPLC, reversed-phase chromatography employs mainly dispersive forces (hydrophobic or Vander Waals interactions). The polarities of mobile and stationary phases are reversed, such that the surface of the stationary phase in RP-HPLC is hydrophobic and mobile phase is polar, where mainly water-based solutions are employed. Reversed-phase HPLC is by far the most popular mode of chromatography. Almost 90% of all analyses of low-molecular-weight samples are carried out using RP-HPLC. One of the main drivers for its enormous popularity is the ability to discriminate very closely related compounds and the ease of variation of retention and selectivity.

1.1.6.3 Instrumentation

Typical HPLC system consists of the following main components:

- Solvent Reservoirs
- Pump
- Injector
- Column
- Detector
- Data Acquisition and Control System
Figure 1.5: Schematic diagram of HPLC Instrument

1.1.6.4 Strategy for Method Development in HPLC

HPLC method development follows a series of steps which are summarized as below:

1. Information on a sample, define separation goals
2. Need for special HPLC procedure, sample pretreatment, etc
3. Choose detector and detector settings
4. Choose LC method; preliminary run; estimate best separation conditions
5. Optimize separation conditions
1.1.6.4.1 Nature of sample

Before proceeding with development of method for a particular sample, it is absolutely essential to have detailed information about sample.

Some important information concerning sample:
- Number of components present.
- Chemical structures (functionality) of compounds.
- Molecular weights of compounds.
- pKa values of compounds.
- UV spectra of compounds.
- Sample solubility.

1.1.6.4.2 Sample pre-treatment

Sample pre-treatment is very important in development of a new method. Most of the samples require dilution before injection. Samples come in various forms:
- Solution ready for injection.
- Solution that requires dilutions, buffering, and addition of an internal standard.
- Solid that must be dissolved or extracted.
- Samples that require sample pretreatment to remove interference and/or to protect the column or equipment from damage.

1.1.6.4.3 Detector and detector settings

Before the first sample is injected during HPLC method development, we must be reasonably sure that the detector selected will sense all sample components of...
interest. Variable-wavelength ultraviolet (UV) detectors normally are the first choice, because of their convenience and applicability for most samples.

1.1.6.4.4 Developing the separation

a) Selecting an HPLC Method and Initial Conditions

An exact recipe for HPLC method development cannot be provided because method development involves considerable trial and error procedures. The first consideration when developing an HPLC method is to determine the solubility of the sample components. Knowing the nature of analyte will allow the most appropriate mode of HPLC to be selected. For the selection of a suitable chromatography method for organic compounds first reversed-phase should be tried, if not successful, normal-phase should be taken into consideration.

1.1.6.5 Getting Started on Method Development

“Best column, best mobile phase, best detection wavelength, efforts in separation can make a world of difference while developing HPLC method for routine analysis. Determining the ideal combination of these factors assures faster delivery of desired results – a validated method of separation. ”

1.1.6.5.1 The Best Mobile Phase

In reverse-phase chromatography, the mobile phase is more polar than the stationary phase. Mobile phase in these systems is usually mixtures of two or more individual solvents with or without additives or organic solvent modifiers. The usual approach is to choose what appears to be the most appropriate column, and then to design a mobile phase that will optimize the retention and selectivity of the system. Separations in these systems are considered to be due to different degrees of hydrophobicity of the solutes. The polarity of organic modifier and its proportion control the rate of elution of the components in the mobile phase. The rate of elution is increased by reducing the polarity.

1.1.6.5.2 The Best Detector

The next consideration should be the choice of detector. There is little use in running a separation if detector used cannot “see” all the components of interest, or conversely, if it “sees” too much. UV-visible detectors are the most popular as
they can detect a broad range of compounds and have a fair degree of selectivity for some analyte. Unfortunately UV-Visible detectors are not universal detectors so it is worthwhile to look at the chemical structure of the analyte to see if it has suitable chromophores, such as aromatic rings, for UV-Visible detection.

**Table 1.1: Different Detector options for HPLC**

<table>
<thead>
<tr>
<th>Detector</th>
<th>Analytes</th>
<th>Solvent Requirements</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-Visible</td>
<td>Any with chromophores</td>
<td>UV-grade non-UV absorbing solvents</td>
<td>Has a degree of selectivity and is useful for many HPLC applications</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Fluorescent compounds</td>
<td>UV-grade non-UV absorbing solvents</td>
<td>Highly selective and sensitive. Often used to analyze derivatized compounds</td>
</tr>
<tr>
<td>Refractive Index (RI)</td>
<td>Compounds with a different RI to the mobile phase</td>
<td>Cannot run mobile phase gradients</td>
<td>Virtually a universal detector but has limited sensitivity</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>Readily oxidized or Reduced compounds especially biological samples</td>
<td>Mobile phase must be conducting</td>
<td>Very selective and sensitive</td>
</tr>
<tr>
<td>Evaporative Light Scattering (ELSD)</td>
<td>Virtually all compounds</td>
<td>Must use volatile Solvents and volatile buffers</td>
<td>A universal detector which is highly sensitive. Not selective</td>
</tr>
<tr>
<td>Mass Spectrometer (MS)</td>
<td>Broad range of compounds</td>
<td>Must use volatile Solvents and volatile buffers</td>
<td>Highly sensitive and is a powerful 2nd dimensional analytical tool. Many modes available. Needs trained operators</td>
</tr>
</tbody>
</table>

1.1.6.5.3 The Best Column Length

Many chromatographers make the mistake of simply using what is available. Often this is a 250 × 4.6mm C18 column. These columns are able to resolve a wide variety of compounds (due to their selectivity and high plate counts) and are common to most laboratories. While many reverse phase separations can be carried out on such column, its high resolving capabilities are often unnecessary, as illustrated in Figure 1.7.

![Figure 1.7: Effect of Column length](image)

1.1.6.5.4 The Best Stationary Phase

Selecting an appropriate stationary phase can also help to improve the efficiency of method development. For example, a C8 phase (reversed phase) can provide a further time saving over a C18, as it does not retain analytes as strongly as the
C18 phase. For normal phase applications, cyano (nitrile) phases are most versatile.

1.1.6.5.5 Retention

Analytes may be too strongly retained (producing long run times). If this occurs, the solvent strength should be increased. In reverse phase analysis this means a higher % of organic solvent in the mobile phase.

1.1.6.5.6 Peak Shape

This is often a problem, especially for basic compounds analyzed by reversed phase HPLC. To minimize any potential problems always use a high purity silica phase such as Wakosil II. These modern phases are very highly deactivated so secondary interactions with the support are minimal. Buffers can be used effectively to give sharp peaks. If peak shape remains a problem, use an organic modifier such as triethylamine, although this should not be necessary with modern phases like Wakosil.

1.1.6.5.7 Buffer selection

In reverse phase HPLC, the retention of analyte is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, therefore, its retention decreases. When separating mixtures containing acid and/or bases by reversed phase HPLC, it is necessary to control the pH of mobile phase using appropriate buffer in order to achieve reproducible results.

When separating acids and bases, a buffered mobile phase is recommended to maintain consistent retention and selectivity. A buffered mobile phase, by definition, resists changes in pH so that the analyte and silica will be consistently ionized, resulting in reproducible chromatography. If the sample is neutral, buffers or additives are generally not required in the mobile phase. Acids or bases usually require the addition of a buffer to the mobile phase.

Buffers play an additional role in the reproducibility of a separation. The buffer salts reduce peak tailing for basic compounds by effectively masking silanols. They also reduce potential ion-exchange interactions with unprotonated silanols (Figure 1.8). To be most effective, a buffer concentration range of 10 - 50 mM is
recommended for most basic compounds.

![Silica Particle](image)

**Figure 1.8: Peak Tailing Interaction**

1.1.6.5.8 Selection of pH

The pH range most often used for reversed-phase HPLC is 1 - 8 and can be divided into low pH (1 - 4) and intermediate pH (4 - 8) ranges. Each range has a number of advantages. Low pH has the advantage of creating an environment in which peak tailing is minimized and method ruggedness is maximized. For this reason, operating at low pH is recommended.

At a mobile phase pH greater than 7, dissolution of silica can severely shorten the lifetime of columns packed with silica-based stationary phases.

The pKa value (acid dissociation [ionization] constant) for a compound is the pH at which equal concentrations of the acidic and basic forms of the molecule are present in aqueous solutions. Analytes may sometimes appear as broad or tailing peaks when the mobile phase pH is at or near, their pKa values. A more rugged mobile phase pH will be at least 1 pH unit different from the analyte pKa. This shifts the equilibrium so that 99% of the sample will be in one form. The result is consistent chromatography.

Dramatic changes in the retention and selectivity (peak spacing) of basic and acidic compounds can occur when the pH of the mobile phase is changed. This is often a result of different interactions between the column and the analytes when the ionization of these compounds changes. It is important to evaluate these changes when a method is developed in order to select the mobile phase pH that provides the most reproducible results.
1.1.6.6 System Suitability Tests

System suitability tests are an integral part of liquid chromatography. These tests include tests for resolution (RS), column efficiency (N) and tailing factor (Tf) or asymmetry factor (AS).

1.1.6.1.1 Resolution (RS)

The resolution or resolution factor R, is specified to ensure that closely eluting compounds, are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug. Resolution between peaks of similar heights of two compounds may be defined by the expression:

Table 1.2: Commonly used buffers for reversed phase HPLC

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pKₐ (25°C)</th>
<th>Maximum Buffer Range</th>
<th>UV cutoff (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA</td>
<td>0.3</td>
<td>-</td>
<td>210</td>
</tr>
<tr>
<td>Phosphate,pK₁H₂PO₄</td>
<td>2.1</td>
<td>1.1-3.1</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Phosphate,pK₂HPO₄⁻</td>
<td>7.2</td>
<td>6.2-8.2</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Phosphate,pK₃PO₄⁻</td>
<td>12.3</td>
<td>11.3-13.3</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Citrate, pK₁</td>
<td>3.1</td>
<td>2.1-4.1</td>
<td>230</td>
</tr>
<tr>
<td>C₃H₂O(COOH)₂(COO)⁻</td>
<td>4.7</td>
<td>3.7-5.7</td>
<td>230</td>
</tr>
<tr>
<td>Citrate, pK₂</td>
<td>6.4</td>
<td>4.4-6.4</td>
<td>230</td>
</tr>
<tr>
<td>C₃H₂O(COOH)₁(COO)²⁻</td>
<td>6.1</td>
<td>5.1-7.1</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Carbonate, pK₁</td>
<td>10.3</td>
<td>9.3-11.3</td>
<td>&gt;200</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>3.8</td>
<td>2.8-4.8</td>
<td>210</td>
</tr>
<tr>
<td>Acetate</td>
<td>4.8</td>
<td>3.8-5.8</td>
<td>210</td>
</tr>
<tr>
<td>Ammonia</td>
<td>9.3</td>
<td>8.3-10.3</td>
<td>200</td>
</tr>
<tr>
<td>Borate</td>
<td>9.2</td>
<td>8.2-10.2</td>
<td>N/A</td>
</tr>
<tr>
<td>TEA</td>
<td>10.8</td>
<td>9.8-11.8</td>
<td>&lt;200</td>
</tr>
</tbody>
</table>
Where,

\[ R = \frac{2(t_{Rb} - t_{Ra})}{W_2 + W_1} \]

\( t_{Rb} \) and \( t_{Ra} \) = Retention times or distance along the baseline between the point of injection and perpendicular dropped from the maxima of two adjacent peaks.

\( W_2 \) and \( W_1 \) = Corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

### 1.1.6.6.2 Tailing Factor (Tf) or Asymmetry Factor (AsF)

Tailing factor, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increase as tailing becomes more pronounced. As peak asymmetry increases, integration and hence precision become less reliable. It was calculated as shown below.

![Asymmetry Factor](image)

**Figure 1.9: Asymmetrical factor calculation**

### 1.1.6.6.3 Column Efficiency

Efficiency is the measure of the degree of peak dispersion in a particular column; as such it is essentially the characteristic of the column. Efficiency is expressed as the number of theoretical plates (N) calculated as shown in Figure 1.10. Where \( t_R \) is the analyte retention time and \( w \) the peak width at the baseline.
1.1.7 INTRODUCTION TO HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) \(^{21-24}\)

High performance thin layer chromatography (HPTLC) is a sophisticated instrumental technique based on the full capabilities of thin layer chromatography. It explains that HPTLC has strong potentials as a surrogate chromatographic model for estimating partitioning properties in support of combinatorial chemistry, environmental fate, and health effect studies.

1.1.7.1 PRINCIPLE

The separation depends on the relative affinity of compounds towards stationary and mobile phase. The compounds under the influence of mobile phase (driven by capillary action) travel over the surface of stationary phase. During this movement the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus separation of components in the mixture is achieved.
1.1.7.2 SALIENT FEATURES:

![Diagram of HPTLC Features]

Figure 1.11: Salient features of HPTLC

1.1.7.3 SCHEMATIC DIAGRAM FOR HPTLC METHOD DEVELOPMENT

![Schematic Diagram for HPTLC Method Development]

Figure 1.12: Schematic Diagram for HPTLC Method Development
1.1.7.4 Steps for Method Development

1.1.7.4.1 Choose Stationary Phase

Choose a scalable TLC plate, preferably that has an identical media as the preparative column. Choose between normal and reverse phase based on sample polarity and solubility.

1.1.7.4.2 Choose a Mobile Phase

Criteria for choosing a preparative solvent

- Solubility
- Affinity
- Resolution

1.1.7.4.3 Apparatus

Prepare a developing chamber as indicated in the picture using a large beaker as the chamber, a half-piece of filter paper inside, and foil or plastic wrap to cover. Pour the eluting solvent into the beaker to a depth of approximately 1 cm. Place the prepared TLC plates in the developing chamber.

After the solvent has risen to near the top of the plate (about 1 cm from the top), remove the plate and mark the solvent front with a pencil. Keep the plates in the hood until the majority of the eluting solvent has evaporated from the plates. Examine the plate under UV light to see the components as dark spots against a bright green-blue background.

1.1.7.4.4 Stationary Phase

As stationary phase, a special finely ground matrix (silica gel, alumina, or similar material) is coated on a glass plate, a metal or a plastic film as a thin layer (~0.25 mm). In addition a binder like gypsum is mixed into the stationary phase to make it stick better to the slide. In many cases, a fluorescent powder is mixed into the stationary phase to simplify the visualization later on (e.g. bright green when you expose it to 254 nm UV light).

The stationary phase - silica gel

Silica gel is a form of silicon dioxide (silica). The silicon atoms are joined via oxygen atoms in a giant covalent structure. However, at the surface of the silica gel, the silicon atoms are attached to -OH groups.
So, at the surface of the silica gel you have Si-O-H bonds instead of Si-O-Si bonds. The diagram shows a small part of the silica surface.

The surface of the silica gel is very polar and, because of the -OH groups, can form hydrogen bonds with suitable compounds around it as well as Van der Waals dispersion forces and dipole-dipole attractions.

1.1.7.4.5 Preparing the Plate

A pencil line is drawn near the bottom of the plate and a small drop of a solution of the mixture is placed on it. Any labeling on the plate to show the original position of the drop must also be in pencil. The start line should be 0.5-1 cm from the bottom of the plate. When the spot of mixture is dry, the plate is stood in a shallow layer of solvent in a covered beaker. It is important that the solvent level is below the line with the spot on it.

1.1.7.4.6 Capillary spotters

Place a melting point capillary in the dark blue part of the bunsen burner flame. Hold it there until it softens and starts to sag. Quickly remove the capillary from the flame and pull on both ends to about 2-3 times its original length. Allow the capillary to cool down, and then break it in the middle.
1.1.7.4.7 Spotting the plate

The thin end of the spotter is placed in the dilute solution; the solution will rise up in the capillary (capillary forces). Touch the plate briefly at the start line. Allow the solvent to evaporate and spot at the same place again. This way you will get a concentrated and small spot.

1.1.7.4.8 Developing a Plate

A TLC plate can be developed in a beaker or closed jar chamber. Place a small amount of solvent (= mobile phase) in the container. The solvent level has to be below the starting line of the TLC, otherwise the spots will dissolve away. The lower edge of the plate is then dipped in a solvent. The solvent (eluent) travels up the matrix by capillarity, moving the components of the samples at various rates because of their different degrees of interaction with the matrix (=stationary phase) and solubility in the developing solvent.

1.1.7.4.9 Measuring R\textsubscript{f} value

These measurements are the distance travelled by the solvent, and the distance travelled by individual spots.

When the solvent front gets close to the top of the plate, the plate is removed from the chamber and the position of the solvent is marked with another line before it has a chance to evaporate.

The R\textsubscript{f} value for each is then worked out using the formula:

\[
R_f = \frac{\text{distance travelled by component}}{\text{distance travelled by solvent}}
\]

1.1.7.4.10 Visualization

There are various techniques to visualize spots of the compounds.

- **Sulfuric acid/heat**: destructive, leaves charred blots behind
- **Ceric stain**: destructive, leaves a dark blue blot behind for polar compounds
- **Iodine**: semi-destructive, iodine absorbs onto the spots, not permanent
- **UV light**: non-destructive, long wavelength (background green, spots dark), short wavelength (plate dark, compounds glow).

### 1.1.7.5 ADVANTAGES

Simultaneous processing of sample and standard - better analytical precision and accuracy less need for Internal Standard
- Several analysts work simultaneously
- Lower analysis time and less cost per analysis
- Low maintenance cost
- Simple sample preparation - handle samples of divergent nature
- No prior treatment for solvents like filtration and degassing
- Low mobile phase consumption per sample
- No interference from previous analysis - fresh stationary and mobile phase for each analysis - no contamination
- Visual detection possible - open system

### 1.1.7.6 LIMITATIONS

- The separation power of the HPTLC is lower than of HPLC and UPLC.
  - Particularly for complex samples like botanical, it is often difficult to achieve sufficient resolution for all components.
  - Volatile and sensitive samples require special care. In order to achieve reproducible results.
1.2 INTRODUCTION TO CVS ACTING DRUG

1.2.1 HYPERTENSION: 25-26

- Hypertension is the most common cardiovascular disease. As many as 50 million people in the United States have systolic and/or diastolic blood pressure above 140/90. Elevated arterial pressure causes pathological changes in the vasculature and hypertrophy of the left ventricle. As a consequence, hypertension is the principal cause of stroke, leads to disease of the coronary arteries with myocardial infarction and sudden cardiac death, and is a major contributor to cardiac failure, renal insufficiency, and dissecting aneurysm of the aorta.

- Hypertension is defined conventionally as blood pressure 140/90; this serves to characterize a group of patients who carry a risk of hypertension-related cardiovascular disease that is high enough to merit medical attention.

- Although many of the clinical trials classify the severity of hypertension by diastolic pressure, progressive elevations of systolic pressure are similarly predictive of adverse cardiovascular events; at every level of diastolic pressure, risks are greater with higher levels of systolic blood pressure. Indeed, in elderly patients, systolic blood pressure predicts outcome better than diastolic blood pressure.

- The pathological basis of the syndrome of malignant hypertension, which is associated with rapidly progressive microvascular occlusive disease in the kidney (with renal failure), brain (hypertensive encephalopathy), retina (hemorrhages, exudates, and disc edema), and other organs. The severe endothelial disruption can lead to microangiopathic hemolytic anemia. Untreated malignant hypertension is rapidly fatal and requires in-hospital management on an emergency basis.

- Left ventricular hypertrophy defined by electrocardiogram, or more accurately by echocardiography, is associated with a substantially worse long-term outcome that includes a higher risk of sudden cardiac death. The risk of cardiovascular disease, disability, and death in hypertensive patients also is increased markedly by concomitant cigarette smoking and by elevated low density lipoprotein; the
coexistence of hypertension with these risk factors increases cardiovascular morbidity and mortality to an extent that is supra additive.

Robust evidence from multiple controlled trials indicates that pharmacological treatment of patients with diastolic pressures of 95 mm Hg or greater will reduce morbidity, disability, and mortality from cardiovascular disease. Effective antihypertensive therapy will almost completely prevent the hemorrhagic strokes, cardiac failure, and renal insufficiency due to hypertension. There is a marked reduction in total strokes.

**Classification of Anti Hypertensive Agents**

1) **Diuretics:**

- Thiazides and related agents (Hydrochlorothiazide, Chlorthalidone)
- Loop agents (Furosemide)
- Potassium-sparing agents (Spironolactone, Triamterine)

2) **Calcium channel blockers:**

   Dihydropyridine: Nifedipine, Amlodipine, Felodipine, Nicardipine.

   Non dihydropyridine: Phenylalkylamine: Verapamil.

   Benzothiazepine: Diltiazem

3) **Beta adrenergic blockers:**

   - Beta-Adrenergic antagonists (Propranolol, Atenolol, Carvedilol, Pindolol, Metoprolol, Labetalol)

4) **Angiotensin converting enzyme (ACE) inhibitors:**

   Types: • Class I: Captopril

   • Class II (prodrug) : e.g., Ramipril, Enalapril, Perindopril

   • Class III ( water soluble) : Lisinopril

5) **Angiotensin Receptor Blockers:**

   Olmesartan, Telmisartan, Saralasin, Losartan
6) Sympatholytics and Alpha Adrenergic Blockers:

Types

1. Alpha 1-receptor blockers: Prazocin, Doxazocin.
2. Centrally acting alpha 2-agonists: Methyldopa, Clonidine.
4. Imidazoline receptor agonists: Rilmenidine, Moxonidine.

7) Direct arterial vasodilators:

Hydralazine, Minoxidil, Nitroprusside, Diazoxide

Mechanism of action of anti hypertensive agent:

1) Diuretics

Mechanisms of action
Initial effects: through reduction of plasma volume and cardiac output.
Long term effect: through decrease in total peripheral vascular resistance.

Advantages:

• Documented reduction in cardiovascular morbidity and mortality.

• Least expensive antihypertensive drugs and best drug for treatment of systolic hypertension and for hypertension in the elderly.

• Can be combined with all other antihypertensive drugs to produce synergetic effect.

2) Calcium channel blockers:

Mechanisms of action

• Decrease in the concentration of free intracellular calcium ions results in decreased contraction and vasodilation.

• Diuretic effect through increase in renal blood flow and glomerular
filtration rate and inhibition of aldosterone secretion.

**Advantages**

- No metabolic disturbances: no change in blood glucose, potassium, uric acid and lipids.
- May improve renal function.
- Maintain optimal physical, mental, and sexual activities.

3) **Beta adrenergic blockers:**

**Mechanisms of Action**

- Initial decrease in cardiac output, followed by reduction in peripheral vascular resistance.
- Other actions include decrease plasma renin activity, resetting of baroreceptors, release of vasodilator prostaglandins, and blockade of prejunctional beta-receptors.

**Advantages**

- Documented reduction in cardiovascular morbidity and mortality.
- Cardioprotection: primary and secondary prevention against coronary artery events (i.e. ischemia, infarction, arrhythmias, death).
- Relatively not expensive.

4) **Angiotensin converting enzyme (ACE) inhibitors:**

**Mechanism of Action**

- Inhibition of circulating and tissue angiotensin- converting enzyme.
- Increased formation of bradykinin and vasodilatory prostaglandins.
- Decreased secretion of aldosterone; help sodium excretion.

**Advantages**

- Reduction of cardiovascular morbidity and mortality in patients with atherosclerotic vascular disease, diabetes, and heart failure.
- Favorable metabolic profile.
- Improvement in glucose tolerance and insulin resistance.
- Renal glomerular protection effect especially in diabetes mellitus.
• Do not adversely affect quality of life.

5) **Angiotensin receptor blockers:**

**Mechanism of action**
They act by blocking type I angiotensin II receptors generally, producing more blockade of the renin - angiotensin - aldosterone axis.

**Advantages**
• Similar metabolic profile to that of ACE-I.
• Renal protection and they do not produce cough.

6) **Sympatholytics And Alpha Adrenergic Blockers:**

**Mechanism of action**
• Alpha1- receptor blockers and imidazoline receptor agonists improve lipid profile and insulin sensitivity.
• Methyldopa: increases renal blood flow. Drug of choice during pregnancy.
• Reserpine: neutral metabolic effects and cheap.

7) **Direct Arterial Vasodilators:**

**Mechanism of action**
• Act as blood vessel dilators by producing relaxation of the vascular smooth muscle, which decrease peripheral vascular resistance and therefore blood pressure.

1.2.2 **HYPERLIPIDEMIA**

Lipid is the scientific term for fats in the blood. At proper levels, lipids perform important functions in your body, but can cause health problems if you have too much. The term hyperlipidemia means high lipid levels or high cholesterol. Hyperlipidemia includes several conditions, but it usually means that you have high cholesterol and high triglyceride levels.

**Causes of hyperlipidemia**
- Most hyperlipidemia is caused by lifestyle habits or treatable medical conditions.
- Lifestyle contributors include obesity, not exercising, and smoking.
Conditions that cause hyperlipidemia include diabetes, kidney disease, pregnancy, and an under active thyroid gland.

The cause may be genetic if you have a normal body weight and other members of your family have hyperlipidemia.

The more risk factors for heart disease, the more aggressively physician will treat hyperlipidemia.

Most blood tests measure levels of LDL (sometimes called "bad") cholesterol, HDL (sometimes called "good") cholesterol, total cholesterol (LDL plus HDL), and triglycerides. To have a low risk of heart disease, your desirable lipid levels are:

- LDL less than 130 mg/dL or < 70 if you have established diagnosis of diabetes
- HDL greater than 40 mg/dL (men) or 50 mg/dL (women);
- Total cholesterol less than 200 mg/dL; and
- Triglycerides less than 200 mg/dL or 150 if you have established heart disease or Diabetes.

Classification of Anti Hyperlipidemic Agents:

1. **HMG CoA Reductase Inhibitors**: Rate-limiting step in the biosynthesis of cholesterol (Rosuvastatin, Atorvastatin, Simvastatin, Pravastatin etc.)

2. **Fibrates**: decreases serum triglycerides and more effective in lowering serum LDL cholesterol. (Ipofibrate, Fenofibrate)

3. **Niacin (vitamin B5)**: Reduce serum cholesterol and TG levels in types II, III, IV, and V hyperlipoproteinemias. TG and VLDL are reduced by 20 to 40% in 1 to 4 days; LDL reduction may be seen in 5-7 days. The decrease in LDL is usually greater if niacin is used with a BAS resin. HDL is increased by 20%.

4. **Bile acid Sequestrants (BAS)**: which prevent body from reabsorbing the cholesterol in bile. Bile is a liquid secreted into small intestine that helps to digest dietary fats.
5. Cholesterol absorption inhibitors:
Mechanism of Anti-Hyperlipidemic Agents\textsuperscript{32-33}:

1. HMG CO-A Reductase Inhibitors:

a. Inhibition of HMG CoA reductase:

- Statins inhibit HMG CoA reductase, leading to a decreased concentration of cholesterol within the cell.
- Low intracellular cholesterol stimulates the synthesis of LDL receptors.
- Increased number of LDL receptors promotes uptake of LDL from blood and low intracellular cholesterol decreases the secretion of VLDL.

b. Increase in LDL receptors:

- Depletion of intracellular cholesterol causes the cell to increase the number of specific cell-surface LDL receptors that can bind and internalize circulating LDLs.
- Thus, the end result is a reduction in plasma cholesterol, both by lowered cholesterol synthesis and by increased catabolism of LDL.

2. Fibrates

- Fibrates stimulate lipoprotein lipase (LPL) activity and the hydrolysis of triglycerides in the plasma.
- They reduce the incorporation of fatty acids into VLDL in the liver, thus inhibiting the synthesis and secretion of VLDL.
- They are almost completely absorbed from the gut, are highly protein bound (more than 90%), are metabolized in the liver and are excreted in the urine as glucuronides.
3. Niacin:

- It inhibits the lipoprotein secretion by the liver thereby reducing hepatic production of VLDL, with consequent reduction in the serum levels of triglycerides, VLDL cholesterol and LDL cholesterol.
- It consistently increases HDL cholesterol.

4. Bile acid Sequestrants (BAS):

- The bile-acid sequestrants are highly positively charged and bind negatively charged bile acids.
- Because of their large size, the resins are not absorbed, and the bound bile acids are excreted in the stool.
- Since over 95% of bile acids are normally reabsorbed, interruption of this process depletes the pool of bile acids, and hepatic bile acid syntheses are increased.
- The increase in hepatic LDL receptors increases LDL clearance and lowers LDL-C levels, but this effect is partially offset by the enhanced cholesterol synthesis caused by up regulation of HMG-CoA reductas.
- Inhibition of reductase activity by a statin substantially increases the effectiveness of the resins.

6. Cholesterol absorption inhibitors:

- Ezetimibe selectively inhibits the intestinal absorption of dietary and biliary cholesterol in the small intestine, leading to a decrease in the delivery of intestinal cholesterol to the liver.
- This causes a reduction of hepatic cholesterol stores and an increase in clearance of cholesterol from the blood.
- Ezetimibe lowers LDL cholesterol by 17% and triacylglycerols by 6%, and it increases HDL cholesterol by 13%.
- Ezetimibe is primarily metabolized in the small intestine and liver via glucuronide conjugation, with subsequent biliary and renal excretion. Both ezetimibe and ezetimibe-glucuronide are slowly eliminated from plasma, with a half-life of approximately 22 hours.
1.2.3 ANTIANGINAL AGENTS\textsuperscript{30-34}: 

An antianginal is any drug used in the treatment of angina pectoris, a symptom of ischaemic heart disease.

**Types of Angina:**

1. **Stable Angina**
   Pain onset with strenuous activity or emotional strain. Alleviated with the removal of stress.

2. **Unstable Angina**
   Pain at unknown conditions due to low supply of Oxygen. also called vaso spasmatic angina.

**Classification of Anti-anginal Agents\textsuperscript{35}**

1) **Nitrates**
   i) **Short Acting:** Glycerol trinitrate (GTN, Nitroglycerine)
      
   ii) **Long Acting:** Isosorbide dinitrate (short acting by sublingual route),
      Isosorbide mononitrate, Erythrityl tetranitrate, etc.

2) **ß-Blockers:** Propranolol, Metoprolol, Atenolol and others.

3) **Calcium Channel Blockers:**
   i) Phenyl Alkylamine: Verapamil

   ii) Benzothiazepine: Diltiazem

   iii) Dihydropyridines: Nifedipine, Felodipine, Amlodipine, Nitrendipine

4) **Potassium Channel Opener:** Nicorandil, Penacidil and Dizoxide

5) **Others:** Dipyridamole, Trimetazidine, Oxyphedrine
Mechanism of Action of Anti-anginal agents:

1) **Nitrates**
   - Decreased cardiac oxygen uptake, secondary to cardiac loads.
   - Coronary flow redistribution through collaterals to ischaemic areas and relief from spasm of coronary arteries.
   - Their action involves the release of nitric oxide and this gas activates soluble guanylate cyclase that elevates the CGMP formation and this CGMP activates protein kinase G and this leads to muscle relaxation.

2) **ß-Blockers**: discussed in hypertensive mechanism of action

3) **Calcium Channel Blockers**: discussed in hypertensive mechanism of action

4) **Potassium Channel Opener**:
   - Open KATP Enhance K+ efflux Membrane Hyperpolarization, decrees Ca$^{2+}$ entry Reduced intracellular calcium Smooth muscle relaxation.
   - Hyperpolarization induced by K ATP CO inhibits production of 1,4,5 IP3 and hence Ca$^{2+}$ release from intracellular stores, Hyperpolarization may also be linked with decreasing sensitivity of contractile elements of vascular smooth muscles.
REFERENCES:


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