Part – [A]

Introduction

High Performance Liquid Chromatography
1. **Importance of drug analysis**

‘Health is wealth’. It is vital fact that a healthy body is desire of every human being. Good health is first condition to enjoy the life and all other things which mankind is having. Nowadays peoples are more concentrating towards health. Even governmental bodies of different countries and World health organization (WHO) are also focusing for health of human being. Health care is prevention, treatment and management of illness and preservation of mental and physical well being. Health care embraces all the goods and services designed to promote health including preventive, curative and palliative in interventions. The Health care industry is considered an industry or profession which includes people’s exercise of skill or judgment or providing of a service related to the prevention or improvement of the health of the individuals or the treatment or care of individuals who are injured, sick, disabled or infirm. The delivery of modern health care depends on an Interdisciplinary Team.

The medical model of health focuses on the eradication of illness through diagnosis and effective treatment. A traditional view is that improvement in health results from advancements in medical science. Advancements in medical science bring varieties of medicines. Medicines are key part of the health care system. The numerous medicines are introducing into the world-market and also, that is increasing every year. These medicines are being either new entities or partial structural modification of the existing one. So, to evaluate quality and efficacy of these medicines is also important factor. Right from the beginning of discovery of any medicine quality and efficacy of the same are checked by quantification means. Quality and efficacy are checked by either observing effect of drug on various animal models or analytical means. The option of animal models is not practically suitable for every batch of medicine as it’s require long time, high cost and more man-power. Later option of analytical way is more suitable, highly precise, safe and selective.

The analytical way deals with quality standards which are assigned for products to have desirable efficacy of the medicines. Sample representing any batch are analyzed for these standards and it is assumed that drug/medicine which is having such standards are having desire effect on use. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stage of production. The decision to release or reject a product is based on one or more type of control action.
Due to rapid growth of pharmaceutical industry during last several years, number of pharmaceutical formulations are enter as a part of health care system and thus, there has been rapid progress in the field of pharmaceutical analysis. Developing analytical method for newly introduced pharmaceutical formulation is a matter of most importance because drug or drug combination may not be official in any pharmacopoeias and thus, no analytical method for quantification is available. To check the quality standards of the medicine various analytical methods are used. Modern analytical techniques are playing key role in assessing chemical quality standards of medicine. Thus analytical techniques are required for fixing standards of medicines and its regular checking. Out of all analytical techniques, the technique which is widely used to check the quality of drug is known as ‘CHROMATOGRAPHY’.

2. History of chromatography and HPLC

In 1903 a Russian botanist Mikhail Tswett produced a colorful separation of plant pigments through calcium carbonate column. Chromatography word came from Greek language chroma = color and graphein = to write i.e. color writing or chromatography\[1, 2\].

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time, pressure liquid chromatography began to be used to decrease flow through time, thus reducing purification times of compounds being isolated by column chromatography. However, flow rates were inconsistent, and the question of whether it was better to have constant flow rate or constant pressure was debated\[3\]. High pressure liquid chromatography was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds.

By the 1980's HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far above the previous techniques. Computers and automation added to the convenience of HPLC. Improvements in type of columns and thus reproducibility were
made as such terms as micro-column, affinity columns, and Fast HPLC began to immerge.

By the 2000 very fast development was undertaken in the area of column material with small particle size technology and other specialized columns. The dimensions of the General Introduction typical HPLC column are 100-300 mm in length with an internal diameter between 3-5 mm. The usual diameter of micro-columns, or capillary columns, ranges from 3 μm to 200 μm\(^4\). In this decade sub 2 micron particle size technology (column material packed with silica particles of < 2μm size) with modified or improved HPLC instrumentation becomes a popular with different instrument brand name like UPLC (Ultra Performance Liquid Chromatography) of Waters and RRLC (Rapid Resolution Liquid Chromatography) of Agilent.

3. Modern High Performance Liquid Chromatography (HPLC)

The highly sophisticated reliable and fast liquid chromatographic (LC) separation techniques are become a requirement in many industries like pharmaceuticals, agrochemicals, dyes, petrochemicals, natural products and others. Early LC used gravity fed open tubular columns with particles 100s of microns in size; the human eye was used for a detector and separations often took hours (days?) to develop.

Isocratic and Gradient LC System Operation

Two basic elution modes are used in HPLC. The first is called isocratic elution. In this mode, the mobile phase, either a pure solvent or a mixture, remains the same throughout the run. A typical system is outlined in Figure 1. A schematic instrumentation of HPLC is given through figure 1-3 as under:
The second type is called gradient elution, wherein, as its name implies, **the mobile phase composition changes during the separation**. This mode is useful for samples that contain compounds that span a wide range of chromatographic polarity. As the separation proceeds, the elution strength of the mobile phase is increased to elute the more strongly retained sample components.
In the simplest case, shown in Figure 2, there are two bottles of solvents and two pumps. The speed of each pump is managed by the gradient controller to deliver more or less of each solvent over the course of the separation. The two streams are combined in the mixer to create the actual mobile phase composition that is delivered to the column over time. At the beginning, the mobile phase contains a higher proportion of the weaker solvent [Solvent A]. Over time, the proportion of the stronger solvent [Solvent B] is increased, according to a predetermined timetable. Note that in Figure 2, the mixer is downstream of the pumps; thus the gradient is created under high pressure. Other HPLC systems are designed to mix multiple streams of solvents under low pressure, ahead of a single pump. A gradient proportioning valve selects from the four solvent bottles, changing the strength of the mobile phase over time [Figure 3].

Today's HPLC requires very special apparatus which includes the following.
1. Extremely precise gradient mixers.
2. HPLC high pressure pumps with very constant flow.
3. Unique high accuracy, low dispersion, HPLC sample valves.
4. Very high efficiency HPLC columns with inert packing materials.
5. High sensitivity low dispersion HPLC detectors.
6. High speed data acquisition systems.
7. Low dispersion connecting tubes for valve to column and column to detector.
**HPLC Gradient mixtures**

HPLC gradient mixers must provide a very precise control of solvent composition to maintain a reproducible gradient profile. This can be complicated in HPLC by the small elution volumes required by many systems. It is much more difficult to produce a constant gradient when mixing small volumes than when mixing large volumes. For low pressure systems this requires great precision in the operation of the miniature mixing General Introduction valves used and low dispersion flows throughout the mixer. For multi-pump high pressure systems it requires a very precise control of the flow rate while making very small changes of the flow rate.

**HPLC Pumps**

Because of the small particles used in modern HPLC, modern LC pumps need to operate reliably and precisely at pressures of 10,000 psi or at least 6,000 psi. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical purposes HPLC pumps should have flow rates that range from 0 to 10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required. It is extremely difficult to provide a very constant flow rate at very low flow rates. If 1% is considered acceptable then for 1ml/min a flow variation of less than 10μl/min is required. This level of constancy is required because most HPLC detectors are flow sensitive and errors in quantization will result from change in flow rate.

**HPLC Sample Valves**

Since sample valves come between the pump and the column it follows that HPLC sample valves must also tolerate pressures up to 10,000 psi. For analytical HPLC, the sample volume should be selectable from sub micro liter to a few micro liters, whereas in preparative HPLC the sample volume may be even greater than 10 ml. To maintain system efficiency the sample valve must be designed to have very low dispersion characteristics, this is true not only for flow dispersion but also for the less obvious problems of dispersion caused by sample adsorption/desorption on valve surfaces and diffusion of sample into and out of the mating surfaces between valve moving parts. It goes without saying that the valves must deliver a very constant sample size but this is usually attained by the use of a constant size sample loop.
HPLC Columns

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. Packing these small particles into the column is a difficult technical problem but even with good packing a great amount of care must be given to the column end fittings and the inlet and outlet connection to keep dispersion to a minimum. The main consideration with HPLC is the much wider variety of solvents and packing materials that can be utilized because of the much lower quantities of both which are required. In particular very expensive optically pure compounds can be used to make Chiral HPLC stationary phases and may even be used as (disposable) HPLC solvents.

HPLC Detectors \([5-10]\)

UV/Vis spectrophotometers, including diode array detectors, are the most commonly employed detectors. Fluorescence spectrophotometers, differential refractometers, electrochemical detectors, mass spectrometers, light scattering detectors, radioactivity detectors or other special detectors may also be used.

Detector consists of a flow-through cell mounted at the end of the column. A beam of UV radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes. Fixed (mercury lamp), variable (deuterium or high-pressure xenon lamp), and multi-wavelength detectors are widely available. Modern variable wavelength detectors can be programmed to change wavelength while an analysis is in progress. Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell, and then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths, spectra of the eluting peaks and also peak purity.
Differential refractometer detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographic compounds as it emerges from the column. Refractive index detectors are used to detect non-UV absorbing compounds.

Fluorometric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups.

Potentiometric, voltametric, or polarographic electrochemical detectors are useful for the quantitation of species that can be oxidized or reduced at a working electrode. These detectors are selective, sensitive, and reliable, but require conducting mobile phases free of dissolved oxygen and reducible metal ions. Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nanogram quantities of easily oxidized compounds, notably phenols and catechols.

In order to give an accurate chromatographic profile the detector sampling (cell) volume must be a small fraction of the solute elution volume. If the detector volume were larger than the elution volume then you would have peaks that appeared with flat tops as the whole peak would be resident in the detector at the same time. This means that as column volumes decrease and system efficiencies increase the volume of the detector cell volume must also decrease. It is odds for the requirement for detector to maintain high sensitivity as this is usually dependant on having a larger cell volume. Again, this requires the very careful design of modern detectors. Many types of detectors can use with HPLC system like UV-Visible or PDA (Photo Diode Array), RI (Refractive Index), Fluorescence, ECD (Electro Chemical Detector), ELSD (Evaporative Light Scattering detector) and many others hyphenated techniques like MS, MS/MS and NMR as well as evaporative IR.

HPLC Data acquisition

In HPLC data acquisition system the higher sampling rate needed for the rapidly eluting narrow peaks of the HPLC chromatogram. Although the theoretical number of samples needed for good quantization are actually quite small, for real systems a hundred samples or more per peak is recommended; thus, for a 4 sec wide peak, a rate of 25 samples per second may be required. The same data analysis and reporting software can be used as in ordinary LC.
Conclusion

HPLC is probably the most universal type of analytical procedure; its application areas include quality control, process control, forensic analysis, environmental monitoring and clinical testing. In addition HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multi-component mixtures. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster and faster analysis times with a constant incorporation of new highly selective column packings.

3.1 Introduction to HPLC Methods of Analysis for Drugs [11-13]

Most of the drugs in single/multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures. Some of the advantages are:

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

There are different modes of separation in HPLC. They are normal phase mode, reversed phase mode, reverse phase ion pair chromatography, affinity chromatography and size exclusion chromatography (gel permeation and gel filtration chromatography).

In the normal phase mode, the stationary phase is polar and the mobile phase is non-polar in nature. In this technique, non-polar compounds travel faster and are eluted first. This is because of the lower affinity between the non-polar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute.
Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence takes longer time to elute.

Reversed phase mode is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is non-polar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and nonpolar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are Octa Decyl Silane (ODS) or C18, C8, C4, etc. (in the order of increasing polarity of the stationary phase).

In ion exchange chromatography, the stationary phase contains ionic groups like $\text{NR}_3^+$ or $\text{SO}_3^{2-}$, which interact with the ionic groups of the sample molecules. This is suitable for the separation of charged molecules only. Changing the pH and salt concentration can modulate the retention.

Ion pair chromatography may be used for the separation of ionic compounds and this method can also substitute for ion exchange chromatography. Strong acidic and basic compounds may be separated by reversed phase mode by forming ion pairs (coulumbic association species formed between two ions of opposite electric charge) with suitable counter ions. This technique is referred to as reversed phase ion pair chromatography or soap chromatography.

Affinity chromatography uses highly specific biochemical interactions for separation. The stationary phase contains specific groups of molecules which can absorb the sample if certain steric and charge related conditions are satisfied. This technique can be used to isolate proteins, enzymes as well as antibodies from complex mixtures.

Size exclusion chromatography separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).
3.2 Method Development and Design of Separation Method

Methods for analyzing drugs in single or multi component dosage forms can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter. An exact recipe for HPLC, however, cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble.

The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient can be started with 5-10 % organic phase in the mobile phase and the organic phase concentration (methanol or acetonitrile) can be increased up to 100 % within 30-45 min. Separation can then be optimized by changing the initial mobile phase composition and the slope of the gradient according to the chromatogram obtained from the preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, namely at what mobile phase composition.

Changing the polarity of mobile phase can alter elution of drug molecules. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, higher is the elution. Ionic samples (acidic or basic) can be separated, if they are present in undissociated form. Dissociation of ionic samples may be suppressed by the proper selection of pH.

The pH of the mobile phase has to be selected in such a way that the compounds are not ionized. If the retention times are too short, the decrease of the organic phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increase of the organic phase concentration is needed.

In UV detection, good analytical results are obtained only when the wavelength is selected carefully. This requires knowledge of the UV spectra of the individual components present in the sample. If analyte standards are available, their UV spectra can be measured prior to HPLC method development.

The molar absorbance at the detection wavelength is also an important parameter. When peaks are not detected in the chromatograms, it is possible that the sample quantity is not enough for the detection. An injection of volume of 20 μL from a solution of 1
mg/mL concentration normally provides good signals for UV active compounds around 220 nm. Even if the compounds exhibit higher \( \lambda_{\text{max}} \), they absorb strongly at lower wavelength.

It is not always necessary to detect compounds at their maximum absorbance. It is, however, advantageous to avoid the detection at the sloppy part of the UV spectrum for precise quantitation. When acceptable peaks are detected on the chromatogram, the investigation of the peak shapes can help further method development.

The addition of peak modifiers to the mobile phase can affect the separation of ionic samples. For example, the retention of the basic compounds can be influenced by the addition of small amounts of triethylamine (a peak modifier) to the mobile phase. Similarly for acidic compounds small amounts of acids such as acetic acid can be used. This can lead to useful changes in selectivity.

When tailing or fronting is observed, it means that the mobile phase is not totally compatible with the solutes. In most case the pH is not properly selected and hence partial dissociation or protonation takes place. When the peak shape does not improve by lower (1-2) or higher (8-9) pH, then ion-pair chromatography can be used. For acidic compounds, cationic ion pair molecules at higher pH and for basic compounds, anionic ion-pair molecules at lower pH can be used. For amphoteric solutes or a mixture of acidic and basic compounds, ion-pair chromatography is the method of choice.

The low solubility of the sample in the mobile phase can also cause bad peak shapes. It is always advisable to use the same solvents for the preparation of sample solution as the mobile phase to avoid precipitation of the compounds in the column or injector.

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that more or less symmetrical peaks on the chromatogram detect all the compounds. By sight change of the mobile phase composition, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time.

The peak resolution can be increased by using a more efficient column (column with higher theoretical plate number, \( N \)) which can be achieved by using a column of smaller particle size, or a longer column. These factors, however, will increase the analysis time. Flow rate does not influence resolution, but it has a strong effect on the analysis time.
Unfortunately, theoretical predictions of mobile phase and stationary phase interactions with a given set of sample components are not always accurate, but they do help to narrow down the choices for method development. The separation scientist must usually perform a series of trial and error experiments with different mobile phase compositions until a satisfactory separation is achieved.

The parameters that are affected by the changes in chromatographic conditions are:
1. Resolution ($R_S$).
2. Capacity factor ($k'$).
3. Selectivity ($\alpha$).
4. Plate number (N).
5. Asymmetry factor (T).

1. **Resolution ($R_S$):** Resolution is the parameter describing the separation power of the complete chromatographic system relative to the particular components of the mixture.

\[
R = \frac{2(tR, 2 - tR, 1)}{Wb, 1 + Wb, 2} = \frac{1.177(tR, 2 - tR, 1)}{W0.5, 1 + W0.5, 2}
\]

If the peak base widths $w_{b,1}$ and $w_{b,2}$ are approximately the same, the resolution $R$ signifies the number of times the peak width $w_b$ can be fitted into the distance between
the peak maxima. At a resolution of R=0.5, two maxima can still be perceived separately. For quantitative analysis, a resolution of up to R=1.5 is desirable; greater values of the resolution lead only to unnecessarily long analysis times.

The resolution R is dependent on the parameters $k_2'$ (capacity factor of the later eluted substance), selectivity $\alpha$ and plate number N of the column:

$$R = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_2'}{1 + k_2'}$$

2. **Capacity factor ($k'$):** The retention time $t_R$ is the qualitative information of a chromatogram. It is constant for a given component provided the chromatographic conditions remain unchanged (column, mobile phase, temperature etc.). For the characterization of substance, it is more convenient to quote the capacity factor $k'$ since, in contrast to the retention times, this is dependent neither on the flow of the eluent nor on the column length:

$$k' = \frac{t_{R'}}{t_0} = \frac{t_R - t_0}{t_0} = \frac{t_R}{t_0} - 1$$

3. **Selectivity ($\alpha$):** The selectivity (or separation factor) is a measure of relative retention of two components in a mixture. Selectivity is the ratio of the capacity factors of both peaks, and the ratio of its adjusted retention times. Selectivity represents the separation power of particular adsorbent to the mixture of these particular components. This parameter is independent of the column efficiency, it only depends on the nature of the components, eluent type, eluent composition and adsorbent surface chemistry. In general, if the selectivity of two components is equal to 1, then there is no way to separate them by improving the column efficiency.

The ideal value of $\alpha$ is 2. It can be calculated by using formula,

$$\alpha = \frac{k_2'}{k_1'} = \frac{t_{R,2} - t_0}{t_{R,1} - t_0} \quad (k_2' > k_1')$$

4. **Plate number (N):** An additional useful quantity to characterize a separation system is the plate number N (number of theoretical plates). A theoretical plate is defined as that zone of separation system within which a thermodynamic equilibrium is established.
between the mean concentration of a component in the stationary phase and its mean concentration in the mobile phase. Efficiency is calculated by using the formula,

\[ N = 16 \left( \frac{t_R}{w_b} \right)^2 \]

Where, \( t_R \) is the retention time.
\( w_b \) is the peak width.

5. **Asymmetry factor (T):** The elution of chromatographic signals as Gaussian peaks is often not achieved in practice. An asymmetric peak shape, known as tailing, is often found. The peak asymmetry is quantified by the asymmetry factor (tailing factor) \( T \) with \( a \) and \( b \) being determined at 10% peak height:

\[ T = \frac{b}{a} \]

For the trouble-free evaluation of the area of a peak, \( T \) must be < 2.5, above this, the end of the peak can be recognized only with difficulty. For a well-packed column, an asymmetry factor of 0.9 to 1.1 should be achievable.

3.3 **Separation Mode of HPLC**
Various modes of HPLC utilized to separate compounds are classified as follows:
1) Adsorption chromatography
2) Normal-phase chromatography
3) Reversed-phase chromatography
4) Ion-pair chromatography
5) Ion-exchange chromatography
6) Size exclusion chromatography
1. **Adsorption chromatography**

Adsorption chromatography uses polar stationary phases with relatively non-polar mobile phases. Separations in adsorption chromatography result to a great extent from the interaction of sample polar functional groups with discrete adsorption sites on the stationary phase. Adsorption chromatography is usually considered appropriate for the separation of nonionic molecules that are soluble in organic solvents.

2. **Normal-phase chromatography**

In HPLC, if stationary phase is more polar than the mobile phase, it is termed as normal-phase liquid chromatography. Polar bonded phases that have a diol, cyano, diethylamino, amino, or diamino functional groups are used as stationary phase in normal-phase chromatography. Due to lower affinity of non-polar compounds to the stationary phases used, non-polar compounds are elute first while polar compounds are retained for longer time. Normal-phase chromatography is widely applied for chiral separations.

3. **Reversed-phase chromatography**

In HPLC, if stationary phase is less polar than the mobile phase, it is termed as reversed-phase liquid chromatography. In this technique, C18, C8, Phenyl, and cyano-propyl functional groups that chemically bonded to micro porous silica particles are used as stationary phase. Retention in reversed phase chromatography occurs by nonspecific hydrophobic interactions of the solute with stationary phase. The ubiquitous application of reversed-phase chromatography arise from the fact that practically all organic molecules have hydrophobic regions in their structures and effectively interact with the stationary phase. It is estimated that over 65% (possibly as high as 90%) of all HPLC separations are executed in the reversed-phase mode. The rationale for this includes the simplicity, versatility, and scope of the reversed-phase method\[^{[14]}\].

4. **Ion-pair chromatography**

Ionic or partially ionic compounds can be chromatographed on reversed phase columns by using ion-pairing reagents. These reagents are typically long chain alkyl anions or cations that, when used in dilute concentrations, can increase the retention of analyte ions. C-5 to C-10 alkyl sulfonates are commonly used for cationic compounds while C-5 to C-8 alkyl ammonium salts are generally used in the cases of anionic solutes.
5. Ion-exchange chromatography

Ion-exchange chromatography is an adaptable technique used primarily for the separation of ionic or easily ionizable species. The stationary phase is characterized by the presence of charged centers having exchangeable counter ions. Both anions and cations can be separated by choosing the suitable ion-exchange medium. Ion-exchange chromatography employs the dynamic interactions between charged solute ions and stationary phases that have oppositely charged groups.

6. Size exclusion chromatography

Size exclusion chromatography separates molecules according to their molecular mass. In Size exclusion chromatography, column is filled with material having precisely controlled pore sizes and the sample is simply screened or filtered according to its solvated molecular size. Largest molecules are eluted first and the smallest molecules last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).

4. Analytical method validation

The developed analytical procedure used to measure the quality of pharmaceutical products. It is necessary to assure that the performance characteristics of the developed analytical procedure meet the requirements for the intended analytical application. The procedure which provides assurance for the same by the means of laboratory studies is defined as method validation.

Method validation is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, strength and quality, for the quantification of the drug substances and drug products. Method validation has received considerable attention in the literature and from industrial committees and regulatory agencies. The U.S. FDA CGMP\textsuperscript{[15]} states for validation for the test methods employed by the firm. The U.S. FDA has also proposed industry guidance for Analytical Procedures and Methods Validation\textsuperscript{[16]}. ISO/IEC 17025 includes a chapter on the validation of methods\textsuperscript{[17]} with list validation parameters. The ICH\textsuperscript{[18]} has developed a consensus text on the validation of analytical procedures. ICH also developed guidance
with detailed methodology\cite{19}. The U.S. EPA prepared guidance for method’s development and validation for the Resource Conservation and Recovery Act (RCRA)\cite{20}.

The AOAC, the EPA and other scientific organizations provide methods that are validated through multi-laboratory studies. The USP has published specific guidelines for method validation for compound evaluation\cite{21}. The WHO published validation guidelines under the title, ‘Validation of analytical procedures used in the examination of pharmaceutical materials’ in the 32nd report of the WHO expert committee on ‘specifications for pharmaceutical preparations’. Representatives of the pharmaceutical and chemical industry have published papers on the validation of analytical methods. Hokanson\cite{22, 23} applied the life cycle approach, developed for computerized systems, to the validation and revalidation of methods. Green\cite{24} gave a practical guide for analytical method validation, with a description of a set of minimum requirements for a method. Wegscheider\cite{25} has published procedures for method validation with a special focus on calibration, recovery experiments, method comparison and investigation of ruggedness. Seno et al.\cite{26} have described how analytical methods are validated in a Japanese QC laboratory. The AOAC\cite{27} has developed a Peer-Verified Methods validation program with detailed guidelines on exactly which parameters should be validated. Winslow and Meyer\cite{28} recommend the definition and application of a master plan for validating analytical methods. J. Breaux and colleagues have published a study on analytical methods development and validation\cite{29}.

### 4.1 Strategy for the Validation of Methods

Method development and validation are an iterative process. The influence of operating parameters on the performance of the method can be assessed at the validation stage which was not done during development/optimization stage of the method. The most significant point raised for validation is that the validity of a method can be demonstrated only through laboratory studies. It is not sufficient to simply review historical results; instead, laboratory studies must be conducted which are intended to validate the specific method, and those studies should be pre-planned and described in a suitable documentation. This documentation should clearly indicate the method’s intended use and principles of operation, as well as the validation parameters to be studied, and a rationale for why this method and these parameters were chosen. It also must include pre-defined acceptance criteria and a description of the analytical procedure.
4.2 Parameters for Method Validation

The parameters for method validation have been defined in different working groups of national and international committees and are described in the literature. An attempt at harmonization was made for pharmaceutical applications through the ICH\cite{18, 19}. The defined validation parameters by the ICH and other regulatory bodies are summarized as under:

a) Specificity study
b) Linearity and range study
c) Limit of detection and Limit of quantitation study
d) Precision study
e) Accuracy study
f) Robustness study
g) Solution stability study
h) System suitability

A brief introduction of above parameters is as below:

a) Specificity study

Specificity of an analytical method is its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers and known (or likely) degradation products that may be expected to be present in the sample matrix. The term specificity is also referring to selectivity when a number of chemical entities that may or may not be distinguished from each other.

Specificity study should also assess interferences that may be caused by the matrix, e.g., urine, blood, soil, water or food. Optimized sample preparation can eliminate most of the matrix components, e.g. placebo. The absence of matrix interferences for a quantitative method should be demonstrated by the analysis of control matrix in specificity. An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and the assay. In order to check the interference of degradation products, analyte is forcibly subject to chemical (acid, alkali and oxidative) and physical (thermal and photolytic) degradation, known as stress application. In each stress application, peak purity of the analyte peak is also evaluated\cite{30}.

b) Linearity and range study

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration (amount) of analyte in samples within a given
range. Linearity may be demonstrated directly on the test substance (by dilution of a
standard stock solution) and/or by using separate weighings of synthetic mixtures of the
test product components, using the proposed procedure.

Linearity is determined by replicate injections of 5 or more concentrations level
within the range of 40–160 %. The response should be directly proportional to the
concentrations of the analytes or proportional by means of a well-defined mathematical
calculation. Linearity is evaluated graphically by plotting a graph of the relative responses
on the y-axis and the corresponding concentrations on the x-axis. A linear regression
equation is applied to the results to evaluate correlation coefficient. In addition, y-
intercept, slope of the regression line and residual sum of squares should also calculate.

The range of an analytical method is the interval between the upper and lower
centations (amounts) of analyte in the sample (including these concentrations) for
which it has been demonstrated that the analytical procedure has a suitable level of
precision, accuracy and linearity. The range is normally expressed in the same units as the
test results (e.g., percentage, parts per million) obtained by the analytical method.

c) Limit of detection (LOD) and Limit of quantitation (LOQ) study

The detection limit of an analytical method is the lowest amount of analyte in a
sample which can be detected but not necessarily quantitated as an exact value. In
chromatography, the detection limit is the injected amount that results in a peak with a
height at least two or three times as high as the baseline noise level. Besides this
signal/noise method, LOD can be measured by another three different methods; (i) visual
inspection (ii) standard deviation of the blank response (iii) standard deviation of the
response based on the slope of the calibration curve.

The quantitation limit of an analytical method is the lowest amount of analyte in a
sample which can be quantitated with suitable precision and accuracy. In
chromatography, the quantitation limit is the minimum injected amount that produces
quantitative measurements in the target matrix with acceptable precision, typically
requiring peak heights 10 to 20 times higher than the baseline noise. Beside this
signal/noise method, LOQ can be measured by another three different methods; (i) visual
inspection (ii) standard deviation of the response (iii) standard deviation of the response
based on the slope of the calibration curve.
d) Precision study

The precision of an analytical method expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the homogeneous sample under the prescribed conditions.

The measurement of precision of an analytical method is performed on replicate standard preparations and replicate sample preparations. The results for the same are usually expressed as the variance, standard deviation or confidence level of a series of measurements. Precision is performed by means of repeatability, reproducibility and intermediate precision (ruggedness).

Repeatability: Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Reproducibility: Reproducibility expresses the precision between laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from same homogeneous lots.

Intermediate Precision: Intermediate precision expresses within-laboratories variations; different days, different analysts, different equipment, etc. The objective of intermediate precision validation is to verify that in the same laboratory the method will provide the same results once the development phase is over. The objective is also extent to verify that the method will provide the same results in different laboratories (ruggedness).

e) Accuracy study

The accuracy of the analytical method is the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value, and the value found. The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. The true value for accuracy assessment can be assessed by analyzing a sample with known concentrations (e.g., a control sample or certified reference material) and comparing the measured value with the true value as supplied with the material. If certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume. After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent (without matrix).
The ICH document on validation methodology recommends accuracy to be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations/three replicates each). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value.

f) Robustness study

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

Robustness tests examine the effect that operational parameters have on the analysis results. For the determination of a method’s robustness, method parameters like pH, flow rate, column temperature, column lot or mobile phase composition, are varied within a realistic range, and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method’s robustness range.

g) Solution stability study

Many solutes readily decompose prior to chromatographic investigations, for example, during the preparation of the sample solutions, extraction, cleanup, phase transfer or storage of prepared vials (in refrigerators or in an automatic sampler). Under these circumstances, method validation should investigate the stability of the analytes and standards in solution form (in analytical preparations). The standard and test preparations are stored up to specified period at specified temperature and its stability is evaluated by comparing solution preparations at different time intervals to that of initial.

h) System suitability study

In addition, prior to the start of laboratory studies to demonstrate method validity, some type of system suitability must be done to demonstrate that the analytical system is performing properly. System suitability should be determined by replicate analysis of the standard or reference solution. System suitability is considered appropriate when the RSD, theoretical plates, tailing factor and resolution parameters calculated on the results obtained at different time intervals, does not exceed more than of specified limit of the corresponding value of the system precision.
4.3 Prior steps of Validation

Prior to start method validation, validation aim should be a well-planned according to scientific soundness and completeness with pre-defined acceptance criteria. Because the type of analysis and the other information of a sample have so much influence on the validation, the objective and scope of the method should always be defined as the first step of any method validation. For an efficient validation process, it is most importance to specify the right validation parameters.

Subsequent to the execution of the validation, results, conclusions and deviations should present in report. Provided the pre-defined acceptance criteria are met, and the deviations (if any) do not affect the scientific interpretation of the data, then the developed analytical method can be considered as valid.

5. Objective of the Work

The specific and main objectives of the work are:

- Development and validation of a stability indicating HPLC assay method for determination of lercanidipine hydrochloride in tablet formulation.
- Development and validation of HPLC assay method for simultaneous determination of atenolol and lercanidipine hydrochloride in combined tablet dosage form in presence of degradation product formed under ICH recommended stress condition.
- Development and validation of UPLC assay method for simultaneous determination of aspirin, clopidogrel bisulphate and atorvastatin calcium in capsule dosage form.
- UPLC assay method development and validation of six drug used for combined hypertension therapy.
6. References

2. From Wikipedia, the free encyclopedia


