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High Performance Liquid Chromatography

K.Malleswari*, D.Rama Brahma Reddy, T.Nandini, S.Ganesh, N.Sneha latha
Corresponding authour: K.Malleswari
Associate professor
Nalanda Institute of Pharmaceutical Sciences, Kantepudi (v),sattenapalli(M),Guntur (dt)

Abstract
Chromatography is defined as a set of techniques which is used for the separation of constituents in a mixture. This technique involves 2 phases stationary and mobile phases. The separation of constituents is based on the difference between partition coefficients of the two phases. The chromatography term is derived from the greek words namely chroma (colour) and graphein (to write). The chromatography is very popular technique and it is mostly used analytically. There are different types of chromatographic techniques namely Paper Chromatography, Gas Chromatography, Liquid Chromatography, Thin Layer Chromatography (TLC), Ion exchange Chromatography and lastly High Performance Liquid Chromatography (HPLC). This review mainly focuses on the HPLC technique its methodology, evaluation studies and testing methods.
Keywords: *HPLC,Chromatography, Mobile phase, Stationary phase, Analyte*

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I. Introduciton[(1)(2)]

High Performance Liquid Chromatography which is also known as High Pressure Liquid Chromatography. It is a popular analytical technique used for the separation, identification and quantification of each constituent of mixture. HPLC is an advanced technique of column liquid chromatography. The solvent usually flows through column with the help of gravity but in HPLC technique the solvent will be forced under high pressures upto 400 atmospheres so that sample can be separated into different constituents with the help of difference in relative affinities. HPLC is able to separate macromolecules and ionic species, labile natural products, polymeric materials and a wide variety of high molecular weight compounds. Sample recovery is easy in HPLC.HPLC offers a greater variety of stationary phases, which allows a greater variety of interactions and more possibilities for separation.Russian botanist Tswett invented chromatography in the year 1967, as a separation technique. HPLC is the fastest growing analytical technique for analysis of drugs.

II. Methodology [(3)(4)]

There are many factors to consider when developing methods. The initially collect the information about the analyte's physiochemical properties (pKa, log P, solubility) and determining which mode of detection would be suitable for analysis. The majority of the analytical development effort goes into validating a stability indicating HPLC–method. The goal of the HPLC-method is to try & separate quantify the main active compound, any reaction impurities, all available synthetic inter-mediates and any degradant.

2.1. Physicochemical properties of the active compound

Physicochemical properties of the active compound play an important role in method development. For method development one has to study the physical properties like solubility, polarity, pKa and pH of the molecule. Polarity is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase. Selection of diluents is based on the solubility of analyte. The analyte must be soluble in the diluents and must not react with any of the diluent components. The diluent should match to the starting eluent composition of the assay to ensure that no peak distortion will occur, especially for early eluting components. The acidity or basicity of a substance is defined most typically by the pH value. The pH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion, pH = - log10 [H3O+]. Selecting a proper pH for ionizable analytes often leads to symmetrical and sharp peaks in HPLC. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits, low relative standard deviations between injections, and reproducible retention times. The pKa is characteristic of a particular compound, and it tells how readily the compound gives up a proton.An acid dissociation constant is a particular example of equilibrium constant. For the specific equilibrium between a monoprotic acid HA and its

conjugate base A−.It turns that the pKa of an acid is the pH at which it is exactly half dissociated. This can be shown by rearranging the expression for Ka:
pH = pKa – log10([AH]/[A-])
At half-neutralization the pH is numerically equal to pKa. Conversely, when pH = pKa, the concentration of HA is equal to the concentration of A−.
The buffer region extends over the approximate range pKa ± 2, though buffering is weak outside the range pKa ± 1. At pKa ± 1,[A−]/[HA] = 10 or 1/10. If the pH is known, the ratio may be calculated. This ratio is independent of the analytical concentration of the acid. When the pKa and analytical concentration of the acid are known, the extent of dissociation and pH of a solution of a monoprotic acid can be easily calculated.

2.2. Column selection

The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Generally, modern reverse phase HPLC columns are made by packing the column housing with spherical silica gel beads which are coated with the hydrophobic stationary phase. The stationary phase is introduced to the matrix by reacted a chlorosilane with the hydroxyl groups present on the silica gel surface. There are several types of matrices for support of the stationary phase, including silica, polymers, and alumina. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One shortcoming of a silica solid support is that it will dissolve above pH 7. In recent years, silica supported columns have been developed for use at high pH.

2.3. Shape and particle size effect

Generally, Smaller particle results in a greater number of theoretical plates, or increased separation efficiency. However, the use of smaller particles also results in increased backpressure during chromatography and the column more easily becomes plugged .

2.4. Common stationary phases

The Common stationary phases are C4 (butyl), C8 (octyl), C18 (octadecyl), nitrile (cyanopropyl), and phenyl (phenyl propyl) columns. In general, longer alkyl chains, higher phase loading, and higher carbon loads provide greater retention of non-polar analytes. Commonly used reverse phase columns and their uses are listed below. Propyl (C3), Butyl (C4), and Pentyl (C5) columns are useful for ion- pairing chromatography. Examples: include Zorbax SB-C3, YMC-Pack C4, and Luna C5. These columns are generally less stable to hydrolysis than columns with longer alkyl chain. Octyl (C8) columns have wide applicability. This phase is less retentive than the C18 phases, but is still quite useful for pharmaceuticals. Example: include (Zorbax SB-C8, Luna C8 and YMC-Pack-MOS). Octadecyl (C18, ODS) columns are the most widely used and tend to be the most retentive for non-polar analytes. Examples include Zorbax SB-C18, YMC- Pack ODS and Luna C18. Xterra RP-C18 and Zorbax Extend-C18 columns have been formulated to tolerate high pH systems (pH >7, normally up to pH 11). Phenyl (Ph) columns offer unique selectivity from the alkyl phases and are generally less retentive than C8 or C18 phases. Phenyl columns are commonly used to resolve aromatic compounds. Examples include Zorbax SB-Phenyl, YMC-Pack Phenyl and Luna Phenyl-Hexyl. Nitrile (CN or cyano) columns are polar and can be used for both reverse and normal phase applications. This phase is often used to increase retention of polar analytes. Examples include Zorbax SB-CN, Luna-CN, and YMC-Pack CN.The type of column chosen for a particular separation depends on the compound and the aim of analysis.

2.4.1. Column temperature

Column temperature control is important for long-term method reproducibility as temperature can affect selectivity. A target temperature in the range of 30–40 °C is normally sufficient for good reproducibility. Use of elevated temperature can be advantageous for several reasons. First, operating at a temperature higher than ambient reduces the viscosity of the mobile phase and thus the overall backpressure on the column. Lower system pressures allow for faster flow rates and thus faster analyses. The temperature may also affect selectivity patterns because analytes will respond dissimilarly to different temperature.

2.5. Mobile phase solvent type[5]

Acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) are commonly used solvents in RP-HPLC having low UV cut-off of 190, 205 and 212nm respectively. These solvents are miscible with water. Mixture of acetonitrile and water is the best initial choice for the mobile phase during method development.

The mobile phase effects resolution, selectivity and efficiency. In reverse phase chromatography, the mobile phase consists of an aqueous buffer and a non-UV active water miscible organic solvent. The effect of

the organic and aqueous phase and the proportions in which they are mixed will affect the analysis of the molecule.

Selection of the mobile-phase and gradient conditions is dependent on the ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixture respectively. Acidic analytes in buffers of sufficiently low pH will remain unchanged, increasing retention. Conversely, at higher pH neutral basic compounds will be more retained, and ionized acidic compounds will elute earlier. Peak splitting may be observed if the pKa of a compound is similar to the pKa of the buffer, and the analyte elutes as both a charged and uncharged species. The pH of a buffer will not greatly affect the retention of non-ionizable sample components.

2.5.1. Isocratic elution

A separation that employs a single solvent or solvent mixture of constant composition.

2.5.2. Gradient elution

Here two or more solvent systems that differ significantly in polarity are employed. After elution is begun; the ratio of the solvents is varied in a programmed way, sometimes continuously and sometimes in a series of steps. Separation efficiency is greatly enhanced by gradient elution.

2.6. Buffer selection

Choice of buffer is typically governed by the desired pH. It is important that the buffer has a pKa close to the desired pH since buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value < 2 units of the desired mobile phase pH.

Table 1: HPLC Buffers, pKa Values and Useful pH range

Buffer	pKa	Useful pH Range
Trifluoroacetic acid (TFA)	<2	1.2-2.5
KH_2PO_4/K_2PO_4	7.2	6.2-8.2
Phosphoric acid	2.1	1.1-3.1
Ammonium hydroxide/ammonia	9.2	8.2-10.2
Potassium formate/ formic acid	3.8	2.8-4.8
Potassium Acetate/ acetic acid	4.8	3.8-5.8
Borate	9.2	8.2-10.2

Generally, a buffer concentration of 10-50 mM is adequate for small molecules. Generally, no more than 50% organic should be used with a buffer. This will depend on the specific buffer as well as its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC. Phosphate buffers can be replaced with sulfate buffers when analyzing organophosphate compounds.
General considerations for buffer selection:

- TFA can degrade with time, is volatile, absorbs at low UV wavelengths.
- Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
- At pH greater than 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns. If possible, organic buffers should be used at pH greater than 7.
- Ammonium bicarbonate buffers usually are prone to pH changes and are usually stable for only 24 to 48 hours. The pH of this mobile phase tends to become more basic due to the release of carbon dioxide.
- Ammonium salts are generally more soluble in organic/water mobile phases.

2.7. Selection of detectors

Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analyses, potential interference, limit of detection required, availability and/or cost of detector.

Table 2: HPLC Detector Choice

Detector	Type of compound can be detected
Fluorescence detector	Fluorescent compounds, usually with fused rings or highly conjugated planer system
Conductivity detector	Charged compounds, such as inorganic ions and organic acid
UV-visible	Compounds with chromophores, such as multiple alternating double bonds

UV-visible detector is versatile, dual wavelength absorbance detector for HPLC. This detector offers the high sensitivity required for routine UV-based applications to low-level impurity identification and quantitative analysis. Fluorescence Detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds.

2.8. Preparation of sample solutions for method development[6]

The components being analyzed should be stable in solution (diluent). During initial method development, preparations of the solutions in amber flasks should be performed until it is determined that the active component is stable at room temperature and does not degrade under normal laboratory conditions. The sample solution should be filtered, the use of a 0.22 or 0.45 µm pore-size filter is generally recommended for removal of particulates. Filtration is a preventive maintenance tool for HPLC analyses.Sample preparation is a critical step of method development that the analyst must investigate. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants/insoluble components without leaching undesirable artifacts into the filtrate. If any additional peaks are observed in the filtered samples, then the diluent must be filtered to determine if a leachable component is coming from the syringe filter housing/filter.

2.9. Optimization method

The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. Stability indicating assay experimental conditions will be achieved through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, sample amounts, Injection volume and diluents solvent type .

III. Validation Method [7][8]

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures. All analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH guidelines.

The main typical analytical performance characterstics which may be tested during methods validation are Accuracy, Precision, Repeatability, Intermediate precision, Reproducibility Linearity, Detection limit, Quantification limit, Specificity, Range, Robustness and Solution stability studies.

3.1 Accuracy

The accuracy of an analytical procedure expresses 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.

3.2 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

3.2.1 Repeatability

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

3.2.2 Intermediate precision

Intermediate precision expresses within laboratories variations: different days, different analysts, different equipment, etc.

3.2.3 Reproducibility

Reproducibility expresses the precision between laboratories.

3.3 Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample.

3.4 Detection limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value.

3.5 Quantification limit

The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantification limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

3.6 Specificity
Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

3.7 Range
The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

3.8 Robustness
The robustness of an analytical procedure 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.

3.9 Solution stability studies
During validation the stability of standards and samples is established under normal conditions, normal storage conditions, and sometimes in the instrument to determine if special storage conditions are necessary, for instance, refrigeration or protection from light.

IV. Testing Methods [9][10]
The HPLC instrumentation involves solvent reservoir, pump, sample injector, column, detector, integrator and display system. In the column the separation occurs. The parts include:

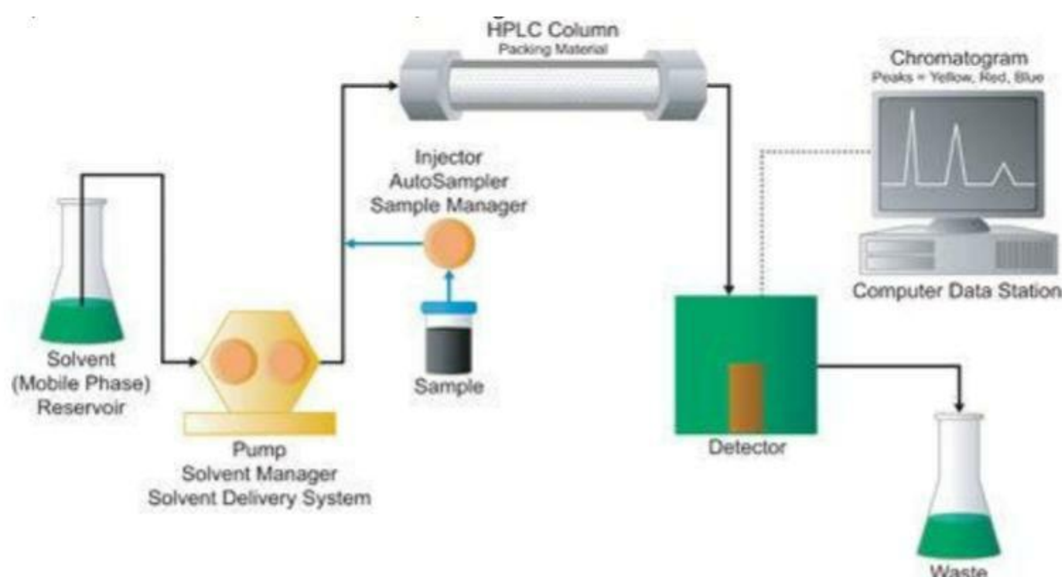

Fig.1 : HPLC INSTRUMENTATION[11]

1. **SolventReservoir:** The contents of mobile phase are present in glass container. In HPLC the mobile phase or solvent is a mixture of polar and non-polar liquid components. Depending on the composition of sample, the polar and non-polar solvents will be varied.
2. **Pump:** The pump suctions the mobile phase from solvent reservoir and forces it to column and then passes to detector. 42000 KPa is the operating pressure of the pump. This operating pressure depends on column dimensions, particle size, flow rate and composition of mobile phase.
3. **SampleInjector:** The injector can be a solitary infusion or a computerized infusion framework. An injector for a HPLC framework should give infusion of the fluid specimen inside the scope of 0.1 mL to 100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).
4. **Columns:** Columns are typically made of cleaned stainless steel, are somewhere around 50 mm and 300 mm long and have an inward distance across of somewhere around 2 and 5 mm. They are generally loaded with a stationary phase with a molecule size of 3 µm to 10 µm. Columns with inner diameters of <2 mm are regularly alluded to as microbore segments. Preferably the temperature of the mobile phase and the column should be kept consistent during investigation[12].
5. **Detector:** The HPLC detector, situated toward the end of the column distinguishes the analytes as they elute from the chromatographic column. Regularly utilized detectors are UV-spectroscopy, fluorescence, massspectrometric and electrochemical identifiers.

V. Conclusion

It can be concluded from the entire review that HPLC is a versatile, reproducible chromatographic technique for the estimation of drug products. The HPLC is mostly used analytical technique. It is having several advantages. With the use of HPLC one can produce extremely pure compounds. It can be used in both laboratory and clinical science. With the use of HPLC the accuracy, precision and specificity can be increased. The only disadvantage of HPLC is high cost.The method was validated as per ICH guidelines in terms of linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ), robustness, and reproducibility. We believe that the HPLC method presented by this work has a lot of merits over the earlier reported methods; it doesn't need internal standard making it more cost effective and simple to apply.

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