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HPLC method development and validation- A general Concept

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ABSTRACT

Chromatography, although primarily a separation technique, is mostly employed in chemical analysis. In which High-performance liquid chromatography (HPLC) is an extremely versatile technique where analytes are separated by passage through a column packed with micrometer-sized particles. Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC. The reasons for this include the simplicity, versatility, and scope of the reversed-phase method as it is able to handle compounds of a diverse polarity and molecular mass.This article involves the strategies and the issues for designing HPLC method development and validation. The method development often follows the well established steps, like selection of buffer, selection mobile phase, selection of column. The method so developed should be as simple as possible, the best strategy being some blend of theoretical and empirical approach.

Keywords: HPLC, Method development, Optimization, Validation.

1. INTRODUCTION

Analytical chemistry is often described as the area of chemistry responsible for characterizing the composition of matter, both qualitatively and quantitatively [1], for analyzing the drug sample in bulk, pharmaceutical formulations and biological fluids, many analytical techniques are used [2]. Chromatography may be regarded as an analytical technique employed for the purification and separation of organic and inorganic substances. It is also very useful for the fractionation of complex mixtures, separation of closely related compound like isomesrs. Chromatography relatively new technique which was 1st developed by M. Tswett, a botanist in 1906 in warsaw.

Basically, this technique is based on differences in the rate at which component of a mixture move through a porous medium (Stationary phase) under the influence of some solvent or gas (mobile phase) at a high pressure [3]. The instrumental method like High Performance Liquid Chromatography (HPLC) was derive from the classical column chromatography and, is widely used tool of analytical chemistry now a days. High Performance Liquid Chromatography is more versatile than gas chromatography since (a) it is not limited to volatile and thermally stable samples, and (b) the choice of mobile and stationary phases is wider.[4]

2. METHOD DEVELOPMENT

Analytical methods development and validation has important roles in the analysis such as discovery, development, and manufacture of pharmaceutical products. The product containing one or more drug called combination product.

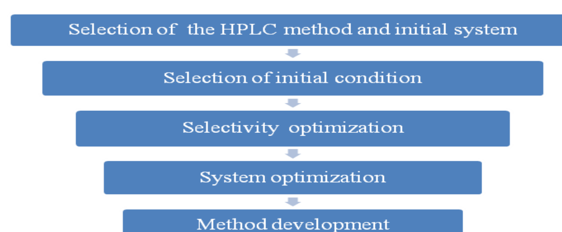


Figure 1: Steps in method development.

These combination products create a different challenge to the analytical chemist

responsible for the development and validation of analytical methods. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products[5].

2.1. Basic criteria for new method development of drug analysis

- If the drug or drug combination is not be added in any pharmacopoeias,
- If a proper analytical procedure is not available due to patent problems
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients or stability problems
- Analytical methods for the quantitation of the drug in biological fluids may not be available,
- Analytical methods for a drug in combination with other drugs may not be available,

The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable [6,7].

3. Chromatographic principles

3.1. Retention

The retention of a drug with a given packing material and eluent can be expressed as a retention time or retention volume. Retention or elution volume is the quantity of the mobile phase required to pull the sample through the column. Retention time is defined as how long a component is retaining the column by the stationary phase relative to the time it resides in the mobile phase. The retention is best described as column capacity ratio (k´), which can be used to evaluate the efficiency of columns. The longer a component is retained by the column, more is the capacity factor. The column capacity ratio of a compound (A) is defined by the following equation:

$$k' = \frac{TA - TO \quad VA - VO}{TO \quad VO}$$

Where, VA is the elution volume of component A and V0 is the elution volume of a non retained compound. At constant flow rate, retention times (TA and T0) can be used instead of retention or elution volumes.

3.2 Resolution

Resolution is the ability of the column to separate `peaks on the chromatograph. Resolution (R) is expressed as the ratio of the distance between two peak maxima to the mean value of the peak width at the base line

R=(TB−TA)2wA + wB

Where, TB is the retention time of component B, TA is the retention time of component A. wA is the peak width of component A and wB is the peak width of component B. If R is equal to or more than 1, then components are completely separated, but if R is less than 1, then components overlap.

3.3. Sensitivity

Sensitivity is a measure of the smallest detectable level of a component in a chromatographic separation and is dependent on the signal-to-noise ratio in a given detector. Sensitivity can be increased by derivatization of the compound of interest, optimization of chromatographic system or miniaturization of the system[8].

3.4. Physicochemical properties of drug

Physicochemical properties of a drug molecule play an important role in method development. For Method development has to consider the physical properties like solubility, polarity, pKa and pH of the drug molecule. Polarity helps an analyst, to decide the solvent and composition of the mobile phase. In a nonpolar covalent bond, the electrons are shared equally between two atoms. A polar covalent bond is one in which one atom has a greater attraction for the electrons than the other atom.

The solubility of molecules can be explained on the basis of the polarity of molecules. Polar, e.g. water, and nonpolar, e.g. benzene, solvents do not mix. In general, like dissolves like i.e., materials with similar polarity are soluble in each other. 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.

pH and pKa plays an important role in HPLC method development. The pH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion.

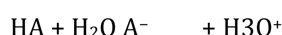
pH = - log10[H3O+]

The acidity or basicity of a substance is defined most typically by the pH value. 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 acidity of an aqueous solution is determined by the concentration of [H3O+] ions. Thus, the pH of a solution indicates the concentration of hydrogen ions in the solution. The concentration of hydrogen ions can be indicated as [H+] or its solvated form in as [H3O+] whose value normally lies between 0 and 14. The lower the pH, the more acidic is the solution. The pH of a solution can be changed simply by adding acid or base to the solution. 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−,

$$HA + H_2O\ A^- \quad + H3O^+$$



The position of equilibrium is measured by the equilibrium constant, K eq.

$$K\ eq = \frac{[H3O+]\ [A-]}{H2O]\ [HA]}$$

Now, in dilute solutions of acid, [H2O] stays roughly constant. Therefore define a new equilibrium constant- the *acidity constant* Ka.

$$Ka = \frac{[H3O+]\ [A-]}{[HA]}$$

This is also in logarithmic form are follows:

$$pKa = -\log 10\ Ka.$$

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:

$$pH = pKa - \log([AH]/[A-])$$

At half-neutralization [A−] / [HA] = 1; since log(1) = 0, the pH at half-neutralization 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

$$pKa \pm 1, [A-]/[HA] = 10 \text{ or } 1/10.$$

If the pH is known, the ratio may be calculated. This ratio is independent of the analytical concentration of the acid[9].

4. HPLC CONDITIONS

4.1. Buffer

Obtaining satisfactory peak shape for your analytes requires the proper choice of a mobile phase buffer. Buffers improve peak shape of basic compounds and can help modify the band spacing (or selectivity) and retention of acidic or basic compounds. We strongly recommend 10 - 20 mM of an ammonium salt of your choice as a starting buffer (i.e. acetate, formate, carbonate, or phosphate salts) for electrolyte solutes.

Table - 1: Merck Index and CRC Handbook of Chemistry and Physics [10]

pKa (25°C)	Compound
0.3	Trifluoroacetic acid2
2.15	Phosphoric acid (pk1)
3.13	Citric acid (pk1)
3.75	Formic acid
4.76	Acetic acid
4.76	Citric acid (pk2)
4.86	Propionic acid
6.35	Carbonic acid (pk1)
6.4	Citric acid (pk3)
7.2	Phosphoric acid (pk2)
8.06	Tris
9.23	Boric acid
9.25	Ammonia
9.78	Glycine (pk2)
10.33	Carbonic acid (pk2)
10.72	Triethylamine
11.27	Pyrrolidine3
12.33	Phosphoric acid (pk3)

Table - 2: Common HPLC Buffers [10]

Buffer	pH range
Phosphate(pk1)	1.1–3.1
Phosphate(pk2)	6.2 – 8.2
Phosphate(pk3)	11.3 – 13.3
Acetate1	3.8 – 5.8
Citrate(pk1)	2.1 – 4.1
Citrate(pk2)	3.7 – 5.7
Citrate(pk3)	4.4 – 6.4
Trifluoroacetic acid (0.1%)	2
Phosphoric acid (0.1%)	2
Formic acid (0.1%)	2.7
Ammonium formate	2.7 – 4.7
Ammonium bicarbonate	6.6 – 8.6
Borate	8.3 -10.3

Further considerations for the proper choice of a buffer system and the manipulation of

buffers as a method development tool are given below in the section method development for ionizable compounds. A buffer maintains the pH when a small amount of acid or base is added.(10) ,some of these additives are mentioned below. A buffer is most effective when used within ±1 pHunit of its pKa,but may provide adequate buffering ±2 pH units from the pKa [10].

The most popular buffers for HPLC with UV detection are phosphate and acetate. Phosphate and acetate are particularly useful buffers because they can be used at wavelengths below 220 nm.

4.2. Buffering Capacity

"Maximum amount of either strong acid or strong base that can be added before a significant change in the pH will occur". Buffering Capacity increases as the molar concentration (molarity) of the buffer salt/acid solution. The closer the buffered pH is to the pKa, the greater the Buffering Capacity, Buffering Capacity is expressed as the molarity of Sodium Hydroxide required to increase pH by 1.0 [9].

4.3. Buffer Selection

Buffers are solutions of a weak acid and its conjugate base, or a weak base and its conjugate acid. They mitigate the influence of hydrogen/hydronium and hydroxide ions and subsequently reduce the pH fluctuations, even upon dilution. The typical pH range for reversed-phase on a silica-based packing is pH 2 to 8. Choice of buffer is typically governed by the desired pH. It is mandatory that the buffer has a pKa near to the desired pH .since buffers control pH best at their pKa. A rule of thumb is to choose a buffer with a pKa value <2 units of the desired mobile phase pH[1].

5. Detectors for HPLC

As with gas chromatography, numerous detectors have been developed for use in monitoring HPLC separations. Majority of HPLC detectors are not unique to the method, but are either stand-alone instruments or modified versions of the same. Spectroscopic Detectors The most popular HPLC detectors are based on spectroscopic measurements, including UV/Vis absorption, and fluorescence. These detectors range from simple designs, in which the analytical wavelength is selected using appropriate filters, to essentially a modified spectrophotometer equipped with a flow cell. When using a UV/Vis detector, the resulting chromatogram is a plot as a function of elution time. Instruments utilizing a diode array spectrophotometer record entire spectra, giving a three-dimensional chromatogram showing absorbance as a function of wavelength and elution time[11].

6. Column selection

Selection of column is the very important criteria. The 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. In general, the nature of stationary phase has the greatest effect on elution, capacity factor, effeciency and selectivity.

Various types of matrices are present for the support of the stationary phase, including the silica, polymers, and alumina. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily derivatized, can be manufacture to consistent size and shape, 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.

The nature, shape and particle size of the silica support effects separation. 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.

In reverse phase chromatography the stationary phase is non-polar and the mobile phase is Polar causing polar peaks to generally elute earlier than non-polar peaks. To create a stationary phase for reverse phase chromatography on silica support, the free silanols are reacted with a chlorosilane with hydrophobic functionality to introduce the non-polar surface. Due to steric constraints, only about 1/3 of the surface silanols are derivatized. The remaining free silanols can interact with analytes, causing peak tailing. Typically, after the derivitization of a column with the desired stationary phase, the column is further reacted with chlorotrimethylsilane to end cap the remaining free silanols and improve the column efficiency. 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[12].

7. Mobile phase

Selecting the correct composition and type of mobile phase is important because it is a variable that governs separation. However, choice is restricted because of the column used, *i.e.*, the type of stationary phase employed. The main distinction is between reversed phase and normal phase chromatography. In normal phase systems, nonpolar solvents such as hexane or iso-octane are used whereas reversed phase requires polar solvents such as water, acetonitrile or methanol. The choice of mobile phase is governed by the physical properties of the solvent. Factors to consider are polarity, miscibility with other solvents, chemical inertness, UV cut off wavelength and toxicity. [13]

Table - 3: Typical solvents for HPLC mobile phases [7]

Solvent	Polarity index	UV cutoff (nm)
Normal phase		
Hexane	0.1	210
Isooctane	0.1	205
Diethyl ether	2.8	218
Dichloromethane	3.1	205
Reverse phase		
Water	10.2	200
Methanol	5.1	210
Acetonitrile	8.8	210
Tetrahydrofuran	4.0	280

8. Practical tips for handling mobile phase

a) Detector Consider the purity of solvents and only use HPLC grade materials:
- Impurities give rise to noisy baselines, *e.g.*, UV detection at low wavelengths;
- De-mineralised water should be employed for mobile phases.

b) Ensure that the mobile phase is free from dust; connect a stainless steel filter element to the end of the tube leading from the reservoir to the pump.

c) Remove dissolved air, because this can cause irregular pumping action and fluctuating signals from the , by performing one or more of the following:
- Degas the mobile phase with helium.
- Place the mobile phase under vacuum.
- Agitate the mobile phase in an ultrasonic bath.

d) When mixing solvents to form mobile phases:
- The analyst must understand the terminology which is used to describe the constituents of the mobile phase - a common expression is for example, 75 /25 v/v methanol / water which indicates a volume measurement, *e.g.*, 75 mLof methanol + 25 mL of water);
- Most systems have a facility to mix the solvents from different reservoir bottles – the analyst only has to set the percentages (*e.g.* 75% methanol, 25%water).
- If a mobile phase of mixed composition is to be prepared manually; the volume of each solvent should be measured separately before they are mixed together.

e) Be aware that volatile components in a mobile phase of mixed composition may evaporate.

This can be minimised by:
- Keeping the solution cool during the degassing procedure;
- Keeping the reservoir bottle stopper at all times.

f) Ensure that the sample to be analyzed is soluble in the mobile phase.

g) When using UV detectors you must consider the UV absorption of the mobile phase. This is indicated by the UV cut-off value. For example, tetrahydrofuran has a UV cut off of 280nm, therefore it cannot be used for analysis of samples for pyridine as the peak maximum for pyridine is ca 260nm.

h) Ensure that the mobile phase does not react with the stationary phase. Buffers and pH modifiers may contain, for example, ammonia which can replace one of the NR groups of an amide on an amino propyl stationary phase.

i) It is also important to monitor the levels of the mobile phases and ensure that they are constantly topped up and therefore the system is never allowed to run dry.

j) Whenever there is a change in the mobile phase, ensure that the labels on the bottles are also changed and label correctly with the following information [16]

11. Method optimization

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, temperature, sample amounts, Injection volume and diluents solvent type.

12. Method validation

Validation is defined by the International Organization for Standardization (ISO) as verification, where the specified requirements are adequate for an intended use", where the term verification is defined as "provision of objective evidence that a given item fulfills specified requirements"

The various validation parameters include linearity, accuracy, precision, ruggedness, robustness, LOD, LOQ and selectivity or specificity.[14]

12.1. 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.

12.2. 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.

12.3. 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.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

12.4. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

12.5. Range

The range of an analytical procedure is the interval between the upper and lower concentration (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.

12.6. 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.

12.7. 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 quantitated as an exact Value.

12.8. Quantitation Limit

The quantitation 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 quantitation 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. [16]

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