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Review Article

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An Overview on HPLC Method Development, Optimization and Validation process for drug analysis

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Abstract Many different strategies of high performance liquid chromatographic method development are being used today. This overview describes a strategy for the systematic development of High performance liquid chromatographic (HPLC) methods. It is an analytical tool which is able to separate, detect and quantify the drug, its various impurities and drug related degradants that can form on synthesis or storage. HPLC involves the understanding of chemistry of drug substance and facilitates the development of the analytical method. Many chromatographic parameters were evaluated in order to optimize the method. Appropriate mobile phase, stationary phase, column, column size, temperature, wavelength and gradient must be found that affords suitable compatibility and stability of drug as well as impurities and degradants. In this paper we have discussed the different physical and chemical parameters that govern the HPLC process and operation and suggested method development for the most optimum conditions based on the analytes.

Keywords HPLC, Chromatography, Drug, Optimization, Validation

Introduction

Chromatography is a procedure that is used for resolving a complex mixture into its individual particular fractions or components. It is a separation technique and the separated unites can be identified by using any analytical technique like UV-visible, Infrared, Mass spectroscopy, NMR etc. For doing quantitative analysis the measurement of the area under the curve in the chromatogram is done.

"Chromato" "graphy" derives its name from two words as chromo means color and graphy means writing. i.e color bands are formed in the procedure which are measured or analyzed. These colour bands are formed due to the separation of individual compounds at different lengths on the column as seen in column chromatography and on paper in paper chromatography.

But in the modern methods like HPLC colour bands cannot be seen and detectors are used.

Principle of chromatography

Chromatography can be simply defined as the process of separation of the individual components of a mixture based on their relative affinities towards mobile phases and stationary phases.

Principle: The samples are subjected to flow by a mobile liquid phase through the stable stationary phase. The sample compounds are separated into individual components based on their relative affinity towards the two phases during their travel.

The sample compound with the greater affinity to the stationary layer will travel slower and for a shorter distance in comparison to compounds with less affinity which travel faster and for a longer distance [1].

Types of Chromatography:

Based on the technique employed in separation of individual components, chromatography is broadly classified as:

1. **Adsorption based**: Here the stationary layer is a solid surface while the mobile phase is liquid. The compounds travel onto the solid surface under the influence of mobile liquid. The separation depends on the extent of physical adsorption of compounds to the solid surface.
2. **Partition based**: In this method, both the stationary and mobile phases are liquids. So the compounds are separated because of affinity based on their partition coefficients into the individual liquid layers. The compound with greater partition coefficient to the mobile liquid has higher affinity to it so travels faster and vice versa.

Based on the type of stationary material used for the separation, it is of two types:

1) **Normal phase:** The stationary material in normal phase is polar in nature and therefore, the compounds with higher polarity elute out last while non polars come out first.
2) **Reverse phase:** The stationary material in reverse phase is non-polar in nature and therefore, the compounds with lower polarity elute out last and vice-versa.

Mostly in HPLC analysis, the type that is used nowadays is reverse phase as many of the biological, phyto-chemical compounds and drugs that are being analysed by using HPLC are polar in nature [1].

What is HPLC?

High pressure liquid chromatography is the full form for HPLC and as given in the name, there is use of high pressure in the *principle* of its operation. Also due to its efficiency in analysis of compounds it is regarded as **High performance liquid chromatography.** Some have even gone to the extent of calling it as **High patience liquid chromatography** based on the long human time requirement and patience needed in its operation.

HPLC is one of the modern chromatography systems which are widely used in the fields of clinical research, biochemical research, industrial quality control etc. Applications of HPLC include detection, analysis, determination, quantification, derivation of molecules from mixtures of biological, plant and medical importance.

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of allowing the solvent to drip through a column under just the force of gravity, it is externally forced through the column under high pressures of up to 400 atm. This makes the chromatographic process a lot faster.

It also allows the use of very small particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing through it. Thus, it allows a much better separation of the components of the mixture.

High performance liquid chromatography is now one of the most powerful tools in analytical chemistry as it has the ability to identify, separate and quantitate the compounds that are present in any sample that can be dissolved in any liquid. Today, trace concentrations of compounds as low as *parts per trillion* [ppt] may easily be identified. HPLC can be, and has been, applied to just about any sample, such as food, pharmaceuticals, forensic samples, nutraceuticals, cosmetics, industrial chemicals and environmental matrices [2-3].

Two variants are in use in HPLC based on the relative polarity of the solvent and the stationary phase.

Normal phase HPLC

This is essentially just the same as you will already have read about in thin layer chromatography or column chromatography. Although it is described as "normal", it isn't the most commonly used form of HPLC.

The column is filled with tiny silica particles, and the solvent is non-polar - hexane, for example. A typical column has an internal diameter of around 4.6 mm, and a length in the range of 150 to 250 mm.

Polar compounds in the mixture that are passed through the column will stick longer to the polar silica than the non-polar compounds. Therefore, the non-polar ones will pass more quickly through the column.

Reversed phase HPLC

In reverse phase, the column size is the same, but silica is modified and made non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent, for example, a mixture of an alcohol such as methanol and water is used [3].

There will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. The attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution won't be as much. Therefore, polar molecules in the mixture will spend most of their time moving with the solvent.

Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van-der Waals forces. They will be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules. They spend less time in solution in the solvent and this will slow them down on their way through the column. This means that now it is the polar molecules that will travel through the column more quickly. Reversed phase HPLC is the most commonly used form of HPLC [2-3].

Principles of method development in HPLC

To understand the principles of method development in hplc one needs to know the basic operation, elution processes and physical and chemical parameters of the technique and the selection of detectors for detecting the analyte. In this review article, we will discuss the above mentioned principles and suggest the most optimum modifications for method development in HPLC.

Operation

The sample to be analyzed is injected in a small volume into the stream of the mobile phase. The motion of analyte through the column is slowed by specific chemical or physical interactions with the stationary phases as it traverses the length of the column. The amount the analyte is slowed depends on the nature of the analyte and on the compositions of the stationary and mobile phases. Time taken by a specific analyte to elute is called retention time; the retention time under particular conditions is considered a reasonably unique identifying characteristic of a given analyte. Smaller particle size column packing (which creates a higher back-pressure) increases the linear velocity giving the components less time to diffuse within the column, which leads to improved resolution in the resulting chromatogram. Commonly used solvents include any miscible combination of water or various organic liquids (most common being methanol and acetonitrile). Water may contain buffers or salts to help in separation of the analyte components or compounds such as trifluoroacetic acid which acts as an ion pairing agent.

A further refinement to HPLC has been to change the mobile phase composition during the analysis. This is known as gradient elution. A general gradient for reversed phase chromatography might start at 5% methanol and progresses gradually to 50% methanol over 25 minutes; the gradient chosen depends on the hydrophobicity of the analyte. The analyte mixtures are separated as a function of the affinity of the analyte for the current mobile phase composition relative to the stationary phase. This process of partioning is similar to that which occurs during a liquid-liquid extraction but this is continuous and not step-wise. For example, when using a low water/ high methanol gradient, the more hydrophobic components will elute from the column due to a relatively hydrophobic mobile phase. The hydrophilic compounds will elute under conditions of relatively low methanol/high water.

The choice of solvents, additives and gradient depend on the nature of the analyte and the stationary phase. Generally a series of tests are performed on the analyte and a number of trial runs may be processed in order to find the optimum HPLC method giving the best separation of peaks.

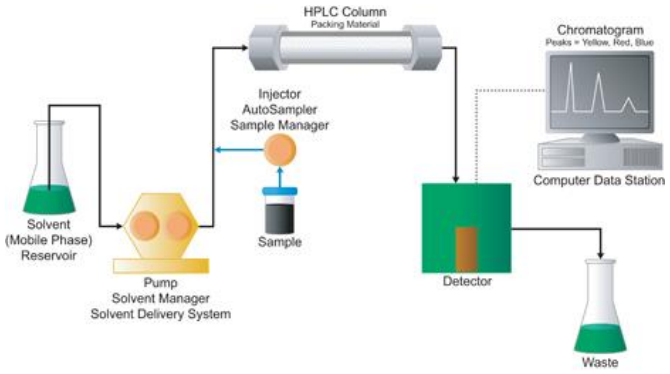


Figure1: *Basic instrumentation of HPLC*

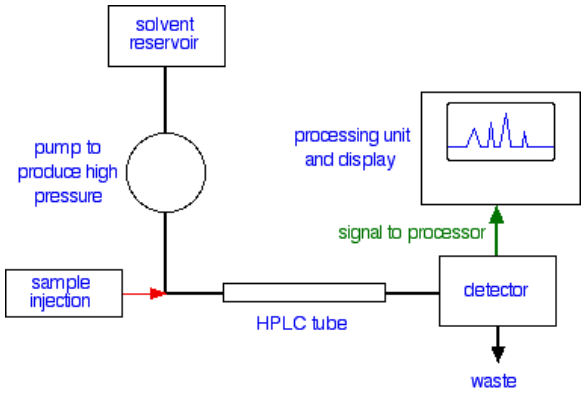


Figure 2: *A flow scheme for HPLC*

Isocratic flow and gradient elution

The separation which has the mobile phase composition remaining constant throughout the procedure is termed as isocratic, meaning constant composition.

The mobile phase composition does not necessarily have to remain constant. When the mobile phase composition is changed during the separation process it is described as a gradient elution. The two components of the mobile phase are typically termed "A" and "B"; component A is the weak solvent which allows solute to elute only slowly and B is the strong solvent which rapidly elutes the solutes from the column. Solvent A is usually water while B is an organic solvent miscible with water, such as methanol, isopropanol, THF or acetonitrile.

In isocratic elution, peak width linearly increases with retention time according to the equation for N, the number of theoretical plates. This has a disadvantage that late-eluting peaks become very flat and broad. The shape and width may keep them from being recognized as peaks.

Gradient elution reduces the retention of the later-eluting components so that they elute quicker, and give narrow and taller peaks for most of the components. This elution also improves the peak shape for tailed peaks because the increasing concentration of the organic eluent pushes the tailing part of a peak forward. The height of the peak increases which makes it looks sharper and this is important in trace analysis. The gradient program may include sudden step increases in percentage of organic component according to the desire for optimum separation in minimum possible time. In isocratic elution, the solutes elute in the same order as the selectivity does not change even if the column dimensions (length and inner diameter) are changed whereas in gradient elution, the elution order my change as the dimensions or flow rate change.

The role of the organic component of the mobile phase is to reduce the high order of water structure and thus reduce the retarding strength of the aqueous component.

Parameters

Internal Diameter

The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded into a column. Larger columns are usually seen in industrial applications such as the purification of a drug product for later use. Low – ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity. Larger – ID columns (over 10 mm) are used to purify usable amounts of material because of their large loading capacity. Analytical scale columns (4.6 mm) have been the most common type of columns though smaller columns are rapidly growing in popularity. Analytical scale columns are used in traditional quantitative analysis of samples and often use a UV – Vis absorbance detector.

Narrow-bore columns (1-2 mm) are used for applications when more sensitivity is desired either with fluorescence detection, UV – Vis detectors or with other detection methods like liquid chromatography – mass spectrometry. Capillary columns having a size under 0.3 mm are used most exclusively with alternative detection means such as mass spectrometry. These columns are usually made from fused silica capillaries, rather than the stainless steel tubing that are employed by larger columns [4].

Particle size

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles. These silica particles come in many sizes with 5 μm beads being the most commonly used. The smaller particles usually provide more surface area and better separations but the pressure required for the optimum linear velocity increases by the inverse of the particle diameter squared. This implies that changing to particles that are half as big while keeping the size of the column the same will definitely double the performance but also increase the required pressure by a factor of four. Larger particles are used in preparative HPLC where column diameters are in range of 5 cm to >30 cm and for non-HPLC applications such as solid – phase extraction [5-6].

Pore size

Many stationary phases are porous to provide greater surface area for the solvent. A small pore will provide greater surface area while a larger pore size has better kinetics, especially large analytes are used. Example, a protein which is only slightly smaller than a pore might enter the pore but does not easily leave once inside [7-8].

Pump Pressure

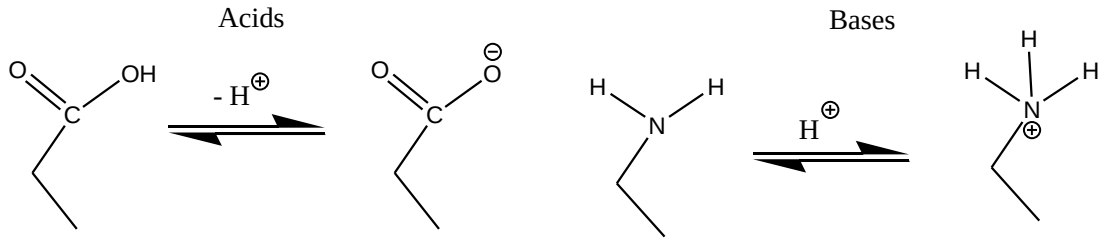
Pumps vary in capacity of pressure but their performance is measured on their ability to yield a consistent and reproducible flow rate. Pressure may reach as high as 40 MPa (6000 lbf/in^2 or about 400 atm). Modern HPLC systems have been improved to work at much higher pressure and therefore are able to use much smaller particle sizes in the columns (<2 μm). These "Ultra High Performance Liquid Chromatography" systems or RSLC/ UHPLCs can work upto 100 MPa (15000 lbf/in^2 or 1000 atm) [9].

Solvent Selectivity

The elution strength of a given solvent is determined by its hydrophobicity but the selectivity of a solvent is determined by its polar characteristics. Heptane and hexane have the same elution strength but different selectivity. For example, Methanol is a strong proton donor and a strong proton acceptor in hydrogen bonding. Acetonitrile has a dipole moment but is only a very weak proton acceptor in hydrogen bonding. Tetrahyrofuran accepts a proton in hydrogen bonding but cannot donate a proton [10].

Effect of pH on analyte ionization

The primary mechanism of retention in RP chromatography is hydrophobic interaction. Ionizing compounds will cause them to behave as more polar species, and reduce their hydrophobic interaction with the stationary phase, leading to decreased retention.



- More hydrophobic, More strongly retained
- Less hydrophobic, Less strongly retained

The ionization state of a molecule will be determined by the pH of the mobile phase and therefore pH of mobile pnase will dictate the retention behavior of analytes with ionizable functional groups.

Effect of temperature

Temperature conditions in HPLC method development present a challenge because it can have unpredictable effects on selectivity. The use of elevated temperatures will:

1. **Reduce mobile phase viscosity** and back-pressure. This can allow you to operate at higher flow rates or to use longer columns/smaller particle sizes.
2. **Reduce elution time**.
3. Improve method **reproducibility** (as opposed to operating at room temperature).

However, it is impossible to determine if the use of elevated temperatures will help or hinder a specific separation because for complex separations, improvements in one portion of the chromatogram are almost always accompanied by disimprovement in another part of the same chromatogram [12].

Retention time

The time taken for a particular compound to travel through the column to the detector is known as its retention time. Retention time is measured from the time at which the sample is injected into the system to the point at which the display shows a maximum peak height for that compound.

Different compounds have different retention times. The retention time for a particular compound will vary depending on:

- the pressure used (because that affects the flow rate of the solvent)
- the nature of the stationary phase (material and particle size)
- the exact composition of the solvent
- the temperature of the column

If you are using retention times as a way of identifying compounds conditions have to be carefully controlled.

Buffer Selection

Choice of buffer is governed by the pH that is desired. The typical pH range for reversed phase on silica based packing is pH 2 to 8. 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.

General contemplations during buffer selection:

- Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
- Some salt buffers are hygroscopic and this may lead to changes in the chromatography like increased tailing of basic compounds and possibly selectivity differences.
- Ammonium salts are generally more soluble in organic/water mobile phases·
- Trifluoroacetic acid can degrade with time. It is volatile and absorbs at low UV wavelengths.
- Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier at all. The growth accumulates on column inlets and can damage chromatographic performance.
- 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 - 48 hrs. The pH of this mobile phase tends to become more basic due to the release of carbon dioxide.
- After buffers are prepared, they should be filtered through a 0.2-µm filter.
- Mobile phases should be degassed.

Buffer Concentration

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. Sulfonate buffers can replace phosphonate buffers when analyzing organophosphate compounds [13].

Selection of detectors

HPLC detectors are important **accessories of the HPLC** instrument.

This part of HPLC helps in detection and identification of compounds in the sample injected.

The detectors are designed to have certain properties like

- they should be inert (non-reactive) to the samples injected and the mobile phases passing through.
- they should be preferably non-destructive to the sample.
- should be able to produce quick and quantitative response.
- reliable, uniform and reproducible detection and analytic data.
- compatible with all types of compounds under testing.
- should have good sensitivity (ablility to detect compounds at very low concentration in the ranges below µg, ng, etc.) as the sample quantity may be lower in many cases.

All types of *HPLC detectors* fulfill most of the above properties [13].

HPLC Detector types

Based on the principle used in detection the detectors available are UV detectors, Fluorescent detectors, Electrochemical detectors and Photo diode-array detectors (PDA) and Refractive index detectors.

UV detectors

The sample detection depends on absorption of UV ray energy by the analyte. The detector comprises of accessories in order as UV source, grating (for light defraction), sample passing through a tubing exposed to rays, photo cell, charge conductor etc.

When the UV rays emitted by lamp pass through gratings, rays split into different wavelengths. One specific wavelength rays are passed through sample. Some amount of light is absorbed by sample and the unabsorbed rays which fall on photo cell.

These rays on collision on photo cells produce electrons whose current is recorded. This is indicative of nature and quantity of sample. This UV wavelength range of absorption is specific for sample.

These are the HPLC detectors used in general, unless there is requirement for analysis of special compounds. They are capable to detect very wide range of compounds. The sensitivity ranges till microgram quantity of estimation.

PDA detectors

These are detectors which follow principle similar to UV detectors but the range of detection extends from UV, visible and to some extent to IR region. Thus, the advantages are higher sensitivity and it measures the entire absorption range i.e it gives the scan of the entire spectrum.

Fluorescence detector

In this detector the fluorescence rays emitted by sample after absorbing incident light is measured as a function of quality and quantity of the sample.

The equipment comprises of accessories in order as light source, sample passing through a tubing exposed to rays, grating (for light defraction), photo cell, charge conductor etc.

Xenon arc lamp is used to produce light for excitation of sample molecules. These light rays excite the sample molecules. These excited molecules emit florescence, which pass through gratings. These gratings pass the florescence at specific wavelength to photo cell which is recorded. The detector is suitable for compounds which can produce florescence.

Some compounds are chemically altered to produce fluorescence by chemical derivatization to estimate by this detector. These detectors have high precision and sensitivity (with less noise in data). Compounds are measurable till nanogram quantities.

Electrochemical detectors

This detector is especially suitable to estimate oxidisable & reducible compounds. The principle is that when compound is either oxidized or reduced, the chemical reaction produces electron flow. This flow is measured as current which is the function of type and quantity of compound.

The system has electrodes like working electrode where oxidation or reduction takes place and reference electrode which acts to subtract conductivity of mobile phase to that of sample. This electrode is suitable for compounds which can't be assayed by UV detector especially due to their similarities in light absorption properties ex: monoamines. This detector has super sensitivity which ranges till picograms measurement. So, very minute quantities of compounds that are present in the sample can be measured.

This electrochemical detector produces severe noise or fluctuations in peaks. So it is difficult to work with when compared to other detectors. As such all types of HPLC detectors are used based on the requirement of labs.

Refractive Index detectors

These are detectors which measure the change of refractive index of the eluant from the column with respect to pure mobile phase. They have several disadvantages like lack of high sensitivity, non suitability for gradient elution, and also require strict temperature control $\pm 0.001^{o}C$ to operate at their highest sensitivity.

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.

Validation of method

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 procedure [14-15]. 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.

Components of method validation

The following are typical analytical performance characteristics which may be tested during methods validation:

- Accuracy
- Precision
- Repeatability
- Intermediate precision
- Linearity
- Detection limit
- Quantitation limit
- Specificity
- Range
- Robustness
- System suitability determination
- Forced degradation studies
- Solution stability studies

Accuracy is the nearness of a measured value to the true or accepted value. Accuracy indicates the deviation between the mean value found and the true value. lt is determined by applying the method to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. It may often be expressed as the recovery by the assay of known, added amounts of analyte [16].

The **precision** of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample. Precision is a measure of the reproducibility of the whole analytical method [17]. It consists of two components: repeatability and intermediate precision.

Intermediate precision is the variation within a laboratory such as different days, with different instruments, and by different analysts [18-19] The precision is then expressed as the relative standard deviation.

Accuracy and precision are not the same. A method can have good precision and yet not be accurate.

Repeatability is the variation experienced by a single analyst on a single instrument. It does not distinguish between variation from the instrument or system alone and from the sample preparation process. During validation, repeatability is performed by analyzing multiple replicates of an assay composite sample by using the analytical method. The recovery value is calculated.

Linearity is the ability of analytical procedure to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample. If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to concentration of analyte in samples within a given range. Linearity is usually expressed as the confidence limit around the slope of the regression line.

Detection Limit

The detection limit (DL) or limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. In analytical procedures that exhibit baseline noise, the LOD can be based on a signal-to-noise (S/N) ratio (3:1), which is usually expressed as the concentration of analyte in the sample.(book) The signal-to-noise ratio is determined by: s = H/h Where H = height of the peak corresponding to the component. h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

Quantitation Limit

The limit of Quantitation (LOQ) or Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. For analytical procedures such as HPLC that exhibit baseline noise, the LOQ is generally estimated from a determination of S/N ratio (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviation as well.

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products, and excipients. Specificity measures only the desired component without interference from other species that might be present; separation is not necessarily required.

Range is defined as the interval between the upper and lower concentrations of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity.

Robustness is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g. pH, mobile phase composition, temperature and instrumental settings) and provides an indication of its reliability during normal usage.

Determination of robustness is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability and/or the analysis of samples.

System Suitability Determination is the evaluation of the components of an analytical system to show that the performance of a system meets the standards required by a method [19]. These parameters can be calculated experimentally to provide a quantitative system suitability test report: number of theoretical plates (efficiency), capacity factor, separation (relative retention), resolution, tailing factor, relative standard deviation (precision). These are measured on a peak or peaks of known retention time and peak width [20].

Forced Degradation Studies

Forced degradation or stress studies are considered to deliberately degrade the sample. These studies have been used to evaluate an analytical method's ability to measure an active ingredient and its degradation products, without interference, by generating potential degradation products. During validation of the method, drug substance have been exposed to acid, base, heat, light and oxidizing agent to produce approximately 10% to 30% degradation of active substance. The studies may also provide information about the degradation pathways and degradation products that could form during storage. These studies may also help in the formulation development, manufacturing, and packaging to improve drug product stability. Reasons for carrying out forced degradation studies include: development and validation of stability-indicating methodology, determination of degradation pathways of drug substances and drug products, discernment of degradation products in formulations that are related to drug substances versus those that are related to non–drug substances in the formulation (e.g., excipients). [18,19]

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 [21-22].

Conclusion

This review describes the general technique of HPLC method development and validation of optimized method. A general and simple approach for the method development for the separation of compounds was discussed. Knowledge of the pH, pKa and solubility of the primary compound is of utmost importance prior to the HPLC method development. Having knowledge of pH can help to discern the ionizable nature of the other impurities (i.e., degradation products, synthetic by-products, metabolites, etc) in the mixture. The selection of buffer and mobile phase composition (organic and pH) plays a dramatic role on the separation selectivity. Final optimization can be performed by changing the gradient slope, temperature and flow rate as well as the type and concentration of

mobile-phase modifiers. Optimized method is validated with various parameters (e.g. specificity, precision, accuracy, detection limit, linearity, etc.) as per ICH guidelines.

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