IJNRD.ORG ISSN : 2456-4184
INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG
An International Open Access, Peer-reviewed, Refereed Journal

A BRIEF REVIEW ON METHOD DEVELOPEMENT AND VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

[1]K.A.Suryawanshi, [2]V.K.Suryawanshi, [3]S.R.Nikam, [4]D.M.Suryawanshi, [5]P.B.Mairal
[1]Student, [2]Student, [3]Student, [4]Student, [5]Assistant Professor
Department Of Pharmaceutical Analysis
Dr.Babasaheb Ambedkar Technological University, Lonere, Raigad, India

ABSTRACT

Due to its very effective separations and often high detection sensitivity, HPLC is the most widely used separation method in recent pharmaceutical and biomedical analysis. Many medications in multiple component dosage forms can be examined using the HPLC method due to its many benefits, including speed, specificity, accuracy, precision, and ease of automation.

The development and validation of HPLC procedures are crucial to novel discoveries, the production of pharmaceutical medications, and numerous other human and animal investigations. To compare a defining characteristic of the drug substance or drug product to predetermined acceptance criteria for that characteristic, an analytical technique is designed. This review provides details on the multiple stages that are involved in developing and validating an HPLC technique.

The validation of an HPLC technique includes testing for system suitability as well as accuracy, precision, specificity, linearity, range, the limit of detection, the limit of quantification, robustness, and other performance parameters.

KEYWORDS: High-Pressure Liquid Chromatography (HPLC), Method development, Validation.

INTRODUCTION

High-Performance Liquid Chromatography is also called as High-Pressure Liquid Chromatograph. It is a well-liked analytical technique used to separate, recognize, and quantify each component of a mixture. A more sophisticated kind of column liquid chromatography is HPLC.[1-2] When it was first developed as an analytical procedure in the early 20th century, liquid chromatography was used to separate coloured substances. This is the origin of the term "chromatography," where "Chroma" refers to colour and "graph" to writing. Mikhail S. Tweet, a Russian botanist, employed a crude kind of chromatographic separation to separate combinations of plant colours into their individual pure elements [3]

The components to be separated are divided between two immiscible phases when using High-performance Liquid Chromatography (HPLC) for separations. These phases are divided into two categories: the stationary phase and the mobile phase [4, 5] Elution, frontal, and displacement modes are the three different ways that chromatography is used. The sample parts are introduced to the chromatographic system at the start of the elution process by injection or placement. Consider a system that is made up of a column. When this occurs, the components elute from the column in accordance with their distributions between the stationary and mobile phases [6], with the concentration distribution often being symmetrical and Gaussian. When a tiny sample size is employed, symmetrical peaks arise.[7] In this scenario, efficiency is at its best, hence when moving quickly, the size of the liquid chromatography sample is relatively small.[8] The various interactions that might take place during the separation are known as the "modes of separation." The Six criteria describe the interactions' mechanisms:

- Bonded phase

- Size exclusion
- Adsorption, Partition
- Ion Exchange
- Affinity

BASIC PRINCIPLE OF HPLC

The distribution of the analyse (sample) between a mobile phase (eluent) and a stationary phase is the basis of the HPLC separation principle (packing material of the column).

The molecules travel through the stationary phase somewhat slowly depending on the chemical nature of the analyse. The duration of a sample's "on-column" time is determined by the specific intermolecular interactions between the sample's molecules and the packing material.

As a result, distinct parts of a sample elute over time at varying rates. Thus, the sample ingredients are successfully separated.

After leaving the column, the analyses are recognised by a detecting device (such a UV detector). A data management system (computer software) converts and records the signals, which are subsequently shown in a chromatogram.

The mobile phase is then subjected to further detector units, a fraction collecting unit, or the waste after passing the detection unit.

A solvent reservoir, a pump, an injection valve, a column, a detector unit, and a data processing unit are the typical components of an HPLC system.

The pump circulates the solvent (eluent) throughout the system at high pressure and steady speed.

A continuous and pulseless flow from the pump is essential to minimize the drift and noise of the detector signal. The injection valve delivers the analyse (sample) to the eluent.[9]

figure 1 hplc

HPLC INSTRUMENTATION

The pump, injector, column, detector, integrator, and display system make up the HPLC instrumentation. The separation takes place in the column. The components are:

Solvent reservoir: Mobile phase's contents are enclosed in a glass container. In HPLC, polar and non-polar liquid components are combined to form the mobile phase, or solvent. The selection of polar and non-polar solvents will vary depending on the sample's nature.

Pump: The pump propels the mobile phase into the column from the solvent reservoir before passing it on to the detector. The pump's operating pressure is 42000 KPa. This operating pressure is influenced by the mobile phase's composition, flow rate, and column dimensions.

Sample Injector: The injector might be a computerized infusion system or a single injection. An injector for an HPLC framework should provide high-reproducibility, high-pressure infusion of the fluid specimen in the volume range of 0.1 mL to 100 milk (Up to 4000 psi).

Columns: Columns are normally formed of polished stainless steel, range in length from 50 to 300 mm, and have an inside diameter between 2 and 5 mm. They typically contain a stationary phase with molecules that range in size from 3 to 10 m. Microbore segments, or columns with inner diameters of less than 2 mm, are frequently mentioned. Ideally, the mobile phase and column temperatures should be maintained.

Detector: As the analyses eluting from the chromatographic column, the HPLC detector, which is located toward the end of the column, identifies them. Electrochemical identification, fluorescence, mass spectrometric, and UV-spectroscopy detectors are frequently used.

Sampling Techniques or Integrator: Signals from the detector may be recorded on graph recorders or electronic integrators, which vary in their ability to analyse, store, and reprocess chromatographic data as well as in their multidimensional quality. The PC coordinates the indicator's response to each component and inserts it into an easily readable chromatograph[10-17]

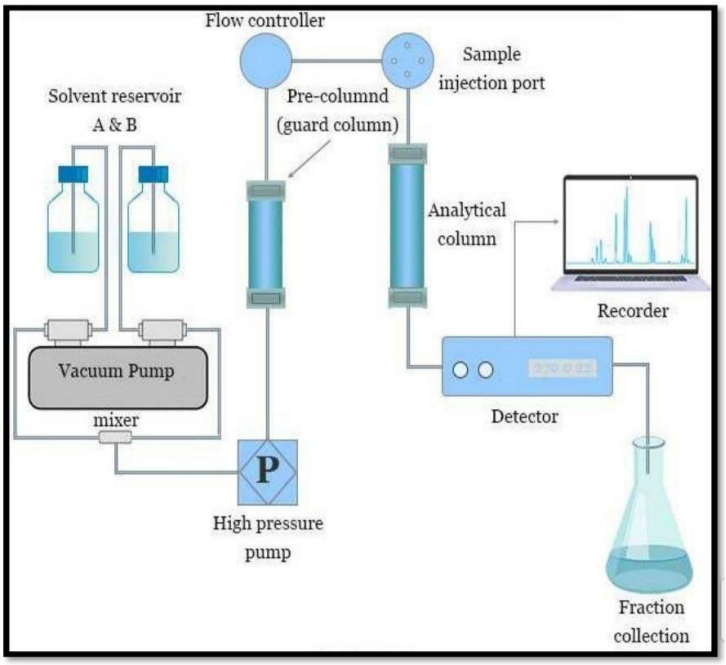

figure 2 instrumentation of hplc

CLASSES OF HPLC

The following categories of HPLC exist according to the stationary phase or substrate use: [18-27]

Normal phase HPLC: Polarity is employed as the basis of separation. Hexane, chloroform, and diethyl ether are employed as the non-polar stationary phase while silica serves as the primary polar stationary phase. In a column, the polar samples are kept.[22]

Reverse phase HPLC: is the opposite of HPLC in the normal phase. The stationary phase is hydrophobic or non-polar while the mobile phase is polar. The non-polar nature will be held more the more of it there is.

Size-exclusion HPLC: Carefully regulated substrate molecules will be incorporated into the column. The separation of constituents will take place based on the variation in molecular sizes.

Ion-exchange HPLC: The stationary phase has an ionized surface that is opposite the charge of the sample. Aqueous buffer is employed as the mobile phase and will regulate the pH and ionic strength.[20]

METHOD DEVELOPMENT

The development and validation of analytical methods are important steps in the development and production of drugs. These techniques are employed to assure the identification, purity, potency, and effectiveness of pharmacological products. When developing methods, there are numerous things that must be considered. The first is to collect data on the physiochemical characteristics of the analyse (pKa, log P, solubility), and then decide which mode of detection would be appropriate for analysis in the case of UV detection. The validation of a stability indicating HPLC method takes up most of the analytical development work. The fundamental objective of the HPLC method is to attempt and quantify the separation of the primary active substance, any reactive impurities, all readily available synthetic intermediaries, and any degradants.[28-30]

The Various steps in the method development process include:

- Physicochemical nature Of the Drug
- HPLC conditions setup
- sample preparation
- Method optimization
- Developed method validation.

DRUG PHYSICOCHEMICAL CHARACTERISTICS:

When developing a method, drug physicochemical characteristics are crucial. One must research the physical characteristics of the drug molecule, such as its solubility, polarity, pKa, and pH, in order to build a procedure. A compound's physical characteristic of polarity. An analyst can use it to choose the mobile phase's solvent and chemical makeup. The electrons in a non-polar covalent bond are distributed equally among the two atoms. One atom has a stronger attraction to the electrons than the other atom in a polar covalent connection. Solvents that are non-polar, like benzene, and polar, like water, do not mix. Like generally dissolves like, which means that substances with comparable polarities can be dissolved in one another. The choice of diluents depends on how soluble the analyse is. The diluents' components must not react with the analyse, which must be soluble in them. To prevent peak distortion, particularly for early eluting components, the diluent should match the starting eluent composition of the assay. In the development of HPLC methods, pH and pKa are crucial factors. The pH value is calculated as the negative of the hydrogen ion concentration's logarithm to base 10, $[H3O^+]$. The pH value is often used to determine whether a substance is acidic or basic. In HPLC, choosing the right pH for ionisable analyses frequently produces symmetrical and acute peaks. For quantitative analysis to achieve low detection limits, low relative standard deviations between injections, and consistent retention times, sharp, symmetrical peaks are required. The quantity of $[H3O^+]$ ions in an aqueous solution determine its acidity. As a result, a solution's pH reflects the number of hydrogen ions present in the solution. The hydrogen ion concentration can be represented by the symbol $[H^+]$ or by its solvated version, $[H3O^+]$, whose value typically ranges from 0 to 14. The solution is more acidic the lower the pH value.

The simple addition of an acid or base will alter the pH of a solution. A property of a certain chemical, the pKa indicates how easily the component releases a proton. One type of equilibrium constant is the acid dissociation constant. When a monocratic acid, HA, and its conjugate base are specifically in equilibrium, A^-,

$HA + H2O \longrightarrow A^- + H3O^+$

The equilibrium constant, Key, determines the state of equilibrium.

$$Key = \frac{[A^-][H3O^+]}{[H2O][HA]}$$

Now, [H2O] essentially remains constant in diluted acid solutions.

As a result, establish the acidity constant Ki as a new equilibrium constant.

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

The following are also in logarithmic form:

$pKa = -\log 10\ Ki.$

It turns out that an acid's pKa is the pH at which it is the same. Dissociated in half. This can be demonstrated by the rearrangement of phrasing for Ki: $pH = pKa - \log([AH]/[A^-])$.

Given that log (1) = 0, the pH at half-neutralization is precisely equal to pKa.

At this point, [A-] / [HA] = 1.

The concentration of HA is equal to the concentration of A^- when pH = pKa, on the other hand.

Although buffering is weak outside the range pKa 1, the buffer region roughly covers the range pKa ± 2. $[A^-]/[HA]$ = 10 or 1/10 when pKa 1. It is possible to calculate the ratio if the pH is known. This ratio is unaffected by the acid's analytical concentration. It is simple to determine the degree of dissociation and pH of a monocratic acid solution when the pKa and analytical concentration of the acid are known.[31-35]

COLUMN SELECTION

The column is the heart of an HPLC device. During technique development, changing a column will have the biggest impact on the resolution of analyses. In general, hydrophobic stationary phase-coated spherical silica gel beads are packed into the column housing to create modern reverse-phase HPLC columns. The hydroxyl groups on the silica gel surface react with chlorosilane to form the stationary phase, which is then added to the matrix. In general, the stationary phase's characteristics have the biggest impact on elution, selectivity, capacity factor, and efficiency. Alumina, polymers, and silica are a few examples of matrices for the support of the stationary phase. The most typical matrix for HPLC columns is silica. The sphere sizes of silica matrices are uniform, they can be easily derivative, and they do not typically compress under pressure. In low-pH environments and with most organic

solvents, silica is chemically stable. A silica solid support has the drawback of dissolving above pH 7. Silica-supported columns have been created recently for application at high pH levels. The silica support's composition, shape, and particle size affect separation. A smaller particle produces more theoretical plates or a higher level of separation efficiency. Smaller particles are used in chromatography, but they also cause higher backpressure and make the column more prone to plugging. Polar peaks often elute earlier than non-polar peaks in reverse-phase chromatography because the stationary phase is nonpolar and the mobile phase is Polar. The free sialons are treated with hydrophobic chlorosilane to introduce the non-polar surface and produce a stationary phase for reverse-phase chromatography on a silica support. Only roughly one-third of the surface sialons are derivative because of steric restrictions. Peak tailing may result from interactions between the remaining free sialons and analyses. Typically, a column is subsequently treated with chlorotrimethylsilane to end cap the residual free sialons and increase column efficiency after being derivative with the appropriate stationary phase. The C4 (butyl), C8 (octyl), C18 (octadecyl), nitrile (cyanopropyl), and phenyl (phenyl propyl) columns are typical stationary phases. In general, increased phase loading, higher carbon loads, and longer alkyl chains all result in enhanced retention of non-polar analyses.

The following is a list of common reverse phase columns and their applications. For ion-pairing chromatography, propyl (C3), butyl (C4), and pentyl (C5) columns are helpful. Zorbax SB-C3, YMC-Pack C4, and Luna C5 are a few examples. Compared to columns with longer alkyl chains, these columns are typically less resistant to hydrolysis. Wide applications can be made with octyl (C8) columns. Despite having a lower retention than the C18 phases, this phase is nonetheless very helpful for medications. Example: comprise (Zorbax SB-C8, Luna C8 and YMC-PackMOS). The most popular and often used columns for non-polar analyses are octadecyl (C18, ODS) columns. Zorbax SB-C18, YMC- Pack ODS, and Luna C18 are a few examples. The Xterra RP-C18 and Zorbax Extend-C18 columns were designed to withstand high pH environments (pH > 7, typically up to pH 11).

The selectivity and resolution of polar analyses can be impacted by pH variation, particularly for ionisable substances. Phenyl (Ph) columns are often less retentive than C8 or C18 phases and offer special selectivity from the alkyl phases. To resolve aromatic compounds, phenyl columns are frequently employed. Examples are Luna Phenyl-Hexyl, YMC-Pack Phenyl, and Zorbax SBPhenyl. Polar nitrile (CN or cyano) columns can be utilised for applications in both reverse and normal phases. To improve polar analytes' retention, this phase is frequently utilised. Examples include YMC-Pack CN, Luna-CN, and Zorbax SB-CN. The type of column selected for a certain separation is determined by the compound and the analysis's purpose.[42-45]

COLUMN TEMPERATURE

Temperature can alter selectivity; column temperature control is crucial for long-term technique repeatability. For satisfactory reproducibility, a target temperature in the range of 30 to 40 °C is typically sufficient. There are various benefits to using an increased temperature. First, running at a temperature greater than ambient lowers the mobile phase's viscosity and, consequently, the column's overall backpressure. Faster flow rates and quicker analyses are made possible by lower system pressures. Because analyses react differently to different temperatures, the temperature may also impact selectivity patterns. Finally, using a column oven removes variability brought on by regular variations in the air temperature surrounding the column.

Although the temperature is a factor that can influence selectivity, its impact is minimal. Additionally, for neutral substances, the k' often drops with temperature, although less noticeably for partially ionised analyses. When there is a noticeable difference in size and shape, there is some influence.

Overall, because it has a considerably more noticeable effect, using solvent strength to regulate selectivity is preferable to using temperature. Both ionic and neutral samples have reportedly shown considerable variations in temperature changes, with an increase of 1°C decreasing the k' by 1 to 2%. It is advised that the column be thermo-stated to regulate the temperature to prevent potential temperature variations during method development and validation.

MOBILE PHASE

Solvent Type

Selectivity will be impacted by the kind of solvent (methanol, acetonitrile, or tetrahydrofuran). Depending on the analyses' solubility and the buffer being employed, methanol or acetonitrile may be preferred. The least polar of these three solvents, tetrahydrofuran, frequently causes significant changes in selectivity and is incompatible with the low-wavelength detection needed for most medicinal chemicals. The mobile phase has an impact on efficiency, selectivity, and resolution. The mobile phase in reverse-phase chromatography comprises of a non-UV active water-miscible organic solvent and an aqueous buffer. The impact of the organic and aqueous phases, as well as the ratios in which they are combined, will have an impact on how the drug molecule is analysed. The choice of the gradient and mobile-phase conditions depends on the mixture's hydrophobicity and the analyses' monogenic nature are each considered. The aqueous buffer has several uses. The mobile phase protonates unbound sialons on the column at low pH, which minimizes peak tailing. Basic analyses are protonated at low enough pH levels; as a result, they will elute

more quickly but with better peak form. Acidic analyses will not change in buffers with a low enough pH, boosting retention. In contrast, neutral basic compounds will be held more and ionized acidic compounds would elute earlier at higher pH levels. If a compound's pKa is close to that of the buffer and the analyse elutes as both a charged and an uncharged species, peak splitting may be seen.

The retention of sample components that are not ionisable is not much impacted by a buffer's pH. Usually, an aqueous buffer solution between 10 and 50 mM is utilized. The most widely used aqueous phase is phosphate buffer or H3PO4 in water. Mono-, di-, or tribasic phosphate salts can be used to quickly change the pH of a phosphate buffer. However, when using phosphate salts, the solution needs to be filtered using 0.22-m filter paper to get rid of any insoluble particles. It is also possible to use additional acids and bases that are not UV-active to alter peak shape and retention.

MOBILE PHASE PH

There is no separation when the sample is eluted using a mobile phase that is 100% organic because the sample is eluted in the void space. This is due to the sample not being kept; however, retention is shown when the strength of the mobile phase solvent is reduced to allow for solute molecule competition between the bonded phase and the mobile phase. It may be necessary to try another organic solvent with a different polarity or even a mixture of two organics to achieve separation when the separation is complex, that is, when there are numerous components to be separated, and when the solvent strength is reduced but there is still no resolution between two close peaks. Additionally, by replacing C18/C8 with cyano or phenyl, mobile phase optimization can be strengthened when combined with bonded phase optimization. For most of the regular product release or stability runs, a target band spacing of a solute (K') should be in the range of 4 to 9 and a run time of approximately 15 minutes or 20 minutes at most.

SEPARATION TECHNIQUES

Isocratic separations:

When the eluent composition is constant, the equilibrium conditions in the column and the actual velocity of compounds moving through the column are also constant, as are the interactions between the analyse and the eluent and the analyse stationary phase throughout the entire run. Although the separation power (the number of compounds that could be resolved) is not very high, this makes isocratic separations more predictable. The peak capacity is limited, and the width of the resulting peak increases with the retention time of the component on the column.

Gradient separation:

Gradient separation considerably boosts a system's capacity for separation, mostly due to the sharp rise in apparent efficiency (reduced peak width). Gradient separation dramatically boosts the separation power of a system, primarily due to the sharp increase in apparent efficiency (decrease of the peak width). The lowering of the peak width is caused by the circumstance where the tail of a chromatographic zone is constantly influenced by a stronger eluent composition. Peak width varies in proportion to the rate of change in eluent composition (gradient slope). This results in the general elution problem, where no single set of circumstances can reliably remove all components from a column in a timely manner while still achieving component resolution. Gradient implementation is required as a result. Utilizing steep or shallow gradients enables achieving variations in chromatographic selectivity. This can be explained by the fact that each analyse in the combination has a different slope of the retention versus organic composition. Prior to the next sample injection and the beginning of the next gradient run, the column must be given time to equilibrate at the starting mobile-phase conditions when a gradient method is being employed. The number of active components that need to be resolved or separated determines whether to use an isocratic or gradient method. An initial gradient run is conducted, and the ratio between the total gradient duration and the difference in gradient time between the first and last components is calculated to determine if a gradient would be necessary or whether the isocratic mode would be sufficient. When the determined ratio is less than 0.25, isocratic is suitable; when the ratio is greater than 0.25, a gradient would be advantageous.[42,46,47]

PREPARATION OF SAMPLES FOR METHOD DEVELOPMENT

The drug substance being examined ought to be soluble (diluent). The preparation of the solutions in amber flasks should be carried out during the earliest stages of method development until it is established that the active component is stable at room temperature and does not deteriorate under typical laboratory conditions. Filtering the sample solution is necessary; for particle removal, a 0.22 or 0.45 m pore-size filter is typically advised. For HPLC analysis, filtering is a preventive maintenance tool. The analyst must look at the crucial stage of technological development known as sample preparation. Syringe filters' efficiency is largely dependent on their capacity to filter out impurities and insoluble substances without introducing unwanted artifacts (i.e., extractables) into the filtrate. To

determine whether a leachable component is coming from the syringe filter housing/filter the diluents must be filtered if any additional peaks are seen in the filtered samples.

METHOD ADVANCEMENT

After achieving the necessary separations and sensitivity, the experimental conditions should be optimized. Through systematic/planned analysis of parameters such as pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample quantities, injection volume, and diluents solvent type, stable indicative assay experimental conditions will be attained.

METHOD VALIDATION

The process by which it is determined, by laboratory tests, that the performance characteristics of the procedure match the needs for its intended usage is known as validation of an analytical procedure. The methodical and planned collection is the first step in the technique's validation process for analytical procedures. validation data to support analytical processes submitted by the applicant. Validation is required for all analytical techniques intended for use with clinical material. According to ICH standards, the validation of analytical procedures is carried out.[49-54]

VALIDATION STANDARDS

Typical analytical performance traits that could be evaluated during technique validation include the following:

- Accuracy
- Precision
- Repeatability
- Intermediate precision
- Linearity
- Detection limit
- Quantitative limit
- Specificity
- Range
- Robustness
- System Suitability Determination
- Studies on forced degradation
- Stability studies

ACCURACY

A measured value's accuracy refers to how closely it matches the true or accepted value. The difference between the mean value obtained and the true value is what is meant by accuracy.

Applying the technique to samples that have known dosages of analyse added yields the result. To make sure there is no interference, these should be compared to both standard and blank solutions. The accuracy is then computed as a percentage of the analyse recovered by the assay using the test findings. The recovery by the test of known added amounts of analyse is a common way to represent it.

PRECISION

The level of consistency between individual test findings produced from multiple sampling of a homogeneous sample is known as the precision of an analytical method. A measure of precision is the reproducibility of the entire analytical process. Repeatability and intermediate precision constitute their two elements.

REPEATABILITY:

The fluctuation that a single analyst encounters on a single instrument is known as repeatability. It makes no distinction between changes resulting from the equipment or system or from the method used to prepare the sample. Repeatability is assessed during validation by employing the analytical procedure to examine numerous replicates of an assay composite sample. A recovery value calculation is made. The fluctuation inside a laboratory, such as different days, with different tools, and by different analysts, is known as intermediate precision. The relative standard deviation is then used to express the precision.

$$\text{RSD} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

The diagram below shows that accuracy and precision are not the same things. Even with good precision, a procedure may not be accurate.

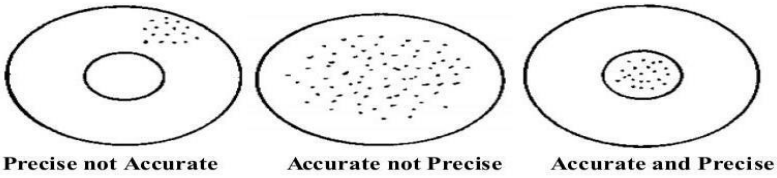

figure 3 accuracy and precision

LINEARITY

A method's capacity to produce a response that is directly proportional to the concentration (quantity) of analyse in the sample is known as linearity. If the procedure is linear, the test findings are directly proportional to the analyse concentration in samples falling within a specified range, or they can be transformed mathematically in a specific way. The confidence limit surrounding the slope of the regression line is typically used to express linearity.

DETECTION LIMIT

The lowest concentration of an analyse in a sample that can be detected but not necessarily quantitated as an accurate value is known as the detection limit (DL) or limit of detection (LOD) of a specific method. The LOD can be predicated on a signal-to-noise (S/N) ratio (3:1), which is typically reported as the concentration of analyse in the sample, in analytical techniques that exhibit baseline noise.

The signal-to-noise ratio is calculated using the formula: s = H/h, where H is the height of the component-specific peak. h is the absolute value of the biggest noise fluctuation from the chromatogram of a blank solution's baseline.

QUANTITATIVE LIMIT

The smallest amount of analyse in a sample that can be quantitatively identified with adequate precision and accuracy is known as the limit of quantitation (LOQ) or quantitation limit of a specific analytical process. The LOQ is typically estimated from a determination of S/N ratio (10:1) for analytical processes like HPLC that exhibit baseline noise, and is typically confirmed by injecting standards that yield this S/N ratio and have an acceptable percent relative standard deviation as well.

SPECIFICITY

The capacity to clearly evaluate the analyse in the presence of elements that might be anticipated to be present, such as contaminants, degradation products, and excipients, is known as specificity.

Specificity does not necessarily require separation because it simply measures the desired component without interfering with any other species that may be present.

The range is the distance between the analyse concentrations in the sample that fall between the upper and lower bounds for which it has been shown that the analytical technique has a sufficient degree of precision, accuracy, and linearity.

ROBUSTNESS

It is described as the measure of an analytical method's resistance to being influenced by the minute but intentional changes in method parameters (such as pH, mobile phase composition, temperature, and instrument settings) and offers a clue as to how reliable the method will be under typical conditions. Determining robustness is a systematic process that involves changing a parameter and evaluating the impact on the methodology through system suitability monitoring and/or sample analysis.

SYSTEM SUITABILITY PARAMETERS

The evaluation of an analytical system's parts to demonstrate that its performance satisfies method performance standards is known as system suitability determination. To generate a quantitative system suitability test report, the following parameters can be calculated experimentally: the number of theoretical plates (efficiency), capacity factor, separation (relative retention), resolution, tailing factor, and relative standard deviation (precision). These are assessed on a peak or peaks with known peak width and retention period. The limits of the chromatographic system are determined using system suitability criteria. The following System Suitability (SST) parameters have been investigated:

RESOLUTION (RS)

Resolution refers to the measurements used to describe how well the entire chromatographic system can separate various mixture components from one another.

The ratio of the separation between the two peak maxima is used to define the resolution Rest two adjoining peaks. It is calculated by dividing the average peak width of two solutes by the difference in their retention periods. The optimal RS value for baseline separation is 1.5. With the use of the formula, it is calculated (Figure 1).

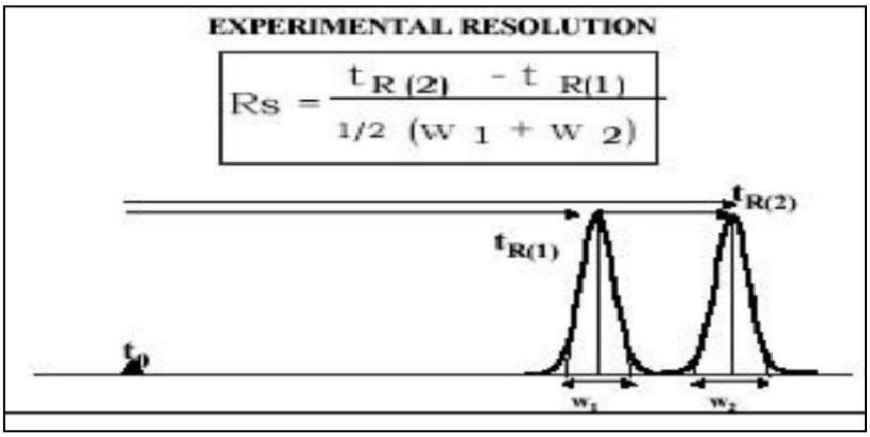

figure 4: resolution between two peaks

Where W_1 and W2 are the peak widths of components 1 and 2, and $t_{R(1)}$ and $t_{R(2)}$ are the retention times of components 1 and 2, respectively.

CAPACITY FACTOR (K')

The number of solute molecules in the stationary phase divided by the number of the same molecules in the mobile phase is known as the capacity factor, or k'. The capacity factor gauges how effectively a column or TLC plate retains the sample molecule during an isocratic separation. The K' ranges from 2 to 10. The formula used to calculate the capacity factor,

$$K' = \frac{V_1 - V_0}{V_0}$$

V_1 = retention volume at the peak's (Solute) apex and V_0 = System void volume

COLUMN EFFICIENCY (N)

It is a measurement of a peak's band widening. Greater the number of theoretical plates, the smaller the band spread, and the better column and system performance. For a good system, columns with N between 5,000 and 100000 Plates/meter are optimal. Utilizing the following formula, efficiency is determined: (Figure 2)

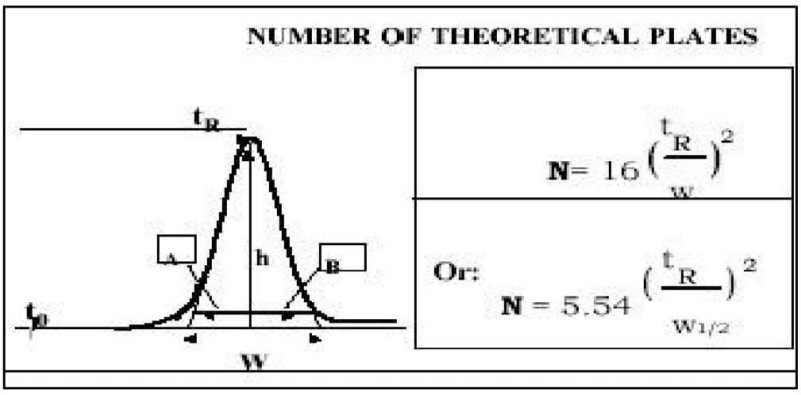

figure 5: number of theoretical plate

where W is the peak width and t_R is the retention period.

PEAK ASYMMETRY FACTOR (AS) AND TAILING FACTOR

Peak asymmetry factor (As) can be used to assess the effectiveness of a column. The peak asymmetry is calculated by dividing the corresponding front half width by 10% of the complete peak height

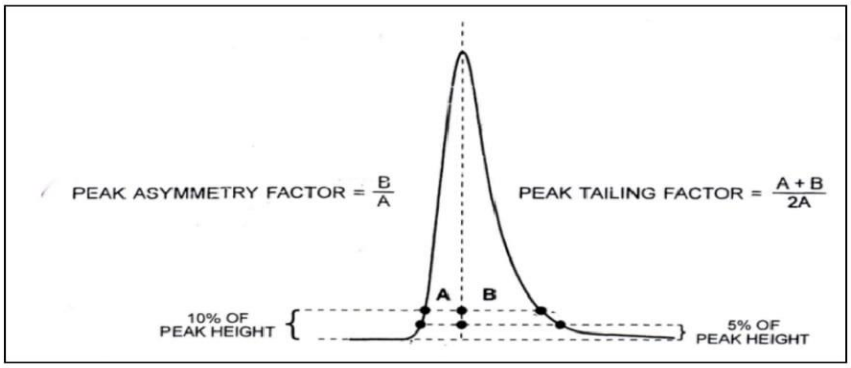

Figure 6: Peak asymmetry and tailing factor

Symmetry Factor = B/A

A: Front half-width; B: Peak half width

Peaks from good columns have values between 0.95 and 1% (perfectly symmetric peaks have an as the value of 1.0%).

PEAK PURITY

Analysis of the main peak's peak purity (or peak homogeneity), to determine whether impurities are present beneath the main peak. It is a crucial component of method validation.

STUDIES OF FORCED DEGRADATION

It is decided to purposely deteriorate the sample by forced degradation or stress research. By producing probable degradation products, these investigations are used to assess an analytical method's capacity to measure an active ingredient and its degradation products without interference. Drug compounds are subjected to acid, base, heat, light, and oxidizing agents during the technique validation, resulting in a 10% to 30% degradation of the active ingredient. Studies can also reveal the degradation pathways and any degradation products that might develop during storage. In order to improve a drug product, these studies may also be useful in formulation development, manufacturing, and packaging. Development and validation of stability-indicating methodologies, identification of the degradation pathways of drug substances and drug products, and differentiation of degradation products in formulations related to drug substances from those related to non-drug substances are some of the reasons why forced degradation studies are carried out. (e.g. Excipients).

Benefits of Analytical Methods Validation

- It increases confidence to some extent, not just for both the user and the developer.
- Even though the validation process may have seemed expensive and Despite being time-consuming, it turns out to be cheap by reducing tedious repeats makes things better. Ultimately, time management.
- The method validation reduces the impact of changes in analytical conditions and returns on investment are more than the process.

STABILITY STUDIES

The stability of standards and samples is checked during validation. Established under typical circumstances, with typical storage situations, and occasionally in the tool to ascertain if, for example, particular storage conditions are required. refrigeration or light shielding.[55-61]

APPLICATION OF HPLC

Chemical Separations are based on the concept that given a specific column and mobile phase; various compounds migrate at different rates. The extent or degree of separation is mostly influenced by the stationary phase and mobile phase selection.

Purification is the process of separating or removing the desired product from a mixture of impurities or chemicals. Each substance displayed a distinctive peak under specific chromatographic circumstances. For the pure desired compound to be collected or extracted without causing any other undesirable compounds, the migration of the compounds and contaminants through the column needs to differ sufficiently from one another.

Identification In general, HPLC is used for the compound assay. The assay's settings should allow a clear peak of the known sample on the chromatograph. At the detection levels where the test will be run, the identifying peak should have a respectable retention period and be clearly distinguished from unrelated peaks.

Other applications for HPLC include: Additional uses for HPLC include

Pharmaceutical Applications [62-65]

- Research on the dissolving of pharmaceutical dosage forms in tablets.
- Determining the medicinal items' shelf lives
- Identification of dosage forms' active components
- Control of pharmaceutical quality

Environment Application [66-69]

- Identifying diphenhydramine in sediment samples and detecting phenolic chemicals in drinking water
- Pollutant bio-monitoring

Forensic [70-72]

- Measuring the drug's effect in biological samples.
- Finding anabolic steroids in urine, perspiration, serum, and hair
- Investigative study of textile dyes.
- Blood tests to check for cocaine and its metabolites.[73-76]
- Measuring the ions in human urine Antibiotics in blood plasma are examined.
- Blood plasma estimation of bilirubin and biliverdin in the event of hepatic diseases.
- Finding endogenous neuropeptides in the brain's extracellular fluids.

Flavour and Food [76]

- Ensuring the quality of drinking water and soft drinks.
- Fruit juices' sugar analyses.
- Vegetable polycyclic compound analysis.
- A trace examination of high explosives used by the military in crops.

CONCLUSION

The overall methodology for developing an improved HPLC method and validating it is described in this article. It addressed how to establish a method for separating pharmaceutical substances in general. Before developing the HPLC process, information on the pKa, pH, and solubility of the primary compound is essential. Understanding pH can be useful in determining the ignitability of various contaminants in a combination, such as synthetic by-products, metabolites, degradation products, etc. The choice of buffer and mobile phase (organic and pH) composition has a significant impact on separation selectivity. Changing the temperature, gradient slope, flow rate, type, and concentration of mobile-phase changes will all result in the final optimization. According to ICH standards, the effective method is evaluated using several parameters, including accuracy, precision, specificity, linearity, and detection limit.

ACKNOWLEDGMENT

I wish to express my sincere thanks and gratitude to my esteemed Mentor **"Puja Mairal"** Who has contributed so much for the successful completion of my review Article by his thoughtful reviews and valuable guidance.

REFERENCE

1. Rogalski E. Modern high-performance liquid chromatography and HPLC 2016 International Symposium. JChromatogr Sep Tech.2016;7:e135.
2. Mulubwa M, et al. Development and validation of high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for determination of tenofovir in small volumes of human plasma. J Chromatogr Sep Tech. 2015;6:300.
3. Joseph C. Touchstone (2006), History of Chromatography, Journal of liquid chromatography, volume 8 (16), 1647-1665.https://doi.org/10.1080/10826079308021679. 4. Miller JM. Chromatography concepts and contrasts. 2nd ed. New Jersey. 2005:35-182
4. Dincer Z, Basan H, Goger NG. Quantitative determination of ambroxol in tablets by derivative UV spectrophotometric method and HPLC. J Pharm Biomed Anal. 2003;31(5):867-72.

5. Meiling Qi, Peng Wang, Ruihua Cong, Jianjun Yang. Simultaneous determination of roxithromycin and ambroxol hydrochloride in a new tablet formulation by liquid chromatography. J Pharm Biomed Anal.2004;35(5):1287-91.
6. Morita MR, Berton D, Boldin R. Determination of levocetirizine in human plasma by liquid chromatography-electrospray tandem mass spectrometry: application to a bioequivalence study. J Chromatogra B.2008;862(1-2):132-9
7. Rafferty JL, Siepmann JI Schure MR. Molecular-level comparison of alkylsilane and polarembedded reversed-phase liquid chromatography systems. Anal Chem. 2008;80(16):6214-221. https://www.knauer.net/Application/application_notes/VSP0019_HPLC%20Basics%20%20principles%20and%20parameters_final%20-web-.pdf
8. Reinhardt TA, et al. A Microassay for 1,25-dihydroxyvitamin D not requiring highperformance liquid chromatography: application to clinical studies. JCEM. 1983;58.
9. Parker JMR, et al. New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and x-ray-derived accessible sites.Biochemistry 1986;25:5425-5432.
10. Shephard GS, et al. Quantitative determination of fumonisins b1and b2 by highperformance liquid chromatography with fluorescence detection. J Liquid Chromatogr. 2006:13.
11. Hamscher G, et al. Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Anal Chem.2002;74:1509-1518.
12. Mesbah M, et al. Precise measurement of the g+c content of deoxyribonucleic acid by highperformance liquid chromatography. Int J Syst Evol Microbiol. 1989;39:159-167.
13. Tamaoka J and Komagata K. Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microb let. 1984.
14. Svec F and Frechet MJJ. Continuous rods of the macroporous polymer as high-performance liquid chromatography separation media. Anal Chem. 1992;64:820-822.
15. Shintani H. Validation Study in membrane chromatography adsorber and phenyl hydrophobic membrane chromatography adsorber for virus clearance and removal of many other components. Pharm Anal Acta.2013; S2:005
16. Badgujar DC, et al. Pathogenicity of mutations discovered in BRCA1 BRCT domains is characterized by destabilizing the hydrophobic interactions. J Cancer SciTher. 2012;4:386-393.
17. Ukuku DO, et al. Effect of thermal and radio frequency electric fields treatments on Escherichia coli bacteria in apple juice. J MicrobBiochem Technol. 2012;4:76-81.
18. Qiao G, et al. Modified a colony-forming unit microbial adherence to hydrocarbons assay and evaluated cell surface hydrophobicity and biofilm production of vibrio scophthalmi. J Bacteriol Parasitol. 2012;3:130
19. Pandarinath P, et al. A Python-based hydrophilicity plot to assess a protein's exposed and buried regions. J Proteomics Bioinform. 2011;4:145-146.
20. Lu M, et al. Hydrophobic fractionation enhances novel protein detection by mass spectrometry in triple-negative breast cancer. J Proteomics Bioinform. 2010;3:029-038.
21. Morgante PG, et al. Establishment of simple and efficient methods for plant material harvesting and storage to allow DNA extraction from a Myrtaceae species with medicinal Potential. Int J Genomic Med. 2013;1:109.
22. Patelia EM and Rakesh Jayesh PT. Estimation of balsalazide by HPTLC-Densitometry method in pharmaceutical formulation. J Chromatograph SeparatTechniq. 2013;4:189.
23. Shah DA, et al. Simultaneous estimation of pregabalin and methyl cobalamin in pharmaceutical formulation by HPTLC-densitometry method. J Chromat Separation Techniq. 2013;4:169.
24. Mehta FA, et al. Simultaneous estimation of ambroxol hydrochloride and doxofylline in pharmaceutical formulation by HPTLC-densitometric method. J Chromat Separation Techniq. 2013;4:168.
25. Boadu RF, et al. In vitro activity and evaluation of the quality of some selected penicillins on the Ghanaian market using developed HPLC methods. Med chem. 2015;5:1-14.
26. United States Pharmacopoeia and National Formulary, (24th) Asian Edition, The United States Pharmacopoeia Convention Inc. U.S.A. 2126.
27. Sethi PD, HPLC: Quantitative Analysis of Pharmaceutical Formulation New Delhi, CBS Publishers, and Distributors, 1996; 113-202.
28. Sankar SR, Textbook of Pharmaceutical Analysis. 5th Edition 2006. Rx publications, Tirunelveli. 2006; 13-1,2.
29. Jeffery GH, Bassett J, Mendham J, Denny RC, Vogel's Textbook of Quantitative Chemical Analysis, fifth edition, Longman scientific & technical.

30. Kaushal C, Srivastava B, A Process of Method Development: A Chromatographic Approach. J Chem Pharm Res, 2010; 2(2): 519-545.
31. Buffers and pH Buffers: available from: www.xtremepapers.com. Accessed April 05, 2013.
32. Understanding pH Buffers: which one to use, and at what concentration: available from: www.laserchrom.co.uk. Accessed April 05, 2013.
33. Technical Tips: Selecting Buffers pH in Reversed-phase HPLC: available from: download.5117.com/data/file/30.pdf. Accessed April 05, 2013.
34. Reversed-phase HPLC Buffers: High-Quality Buffers (solutions, solids, or concentrates): Available from: ccc.chem.pitt.edu/wipf/web/HPLC_RP_buffers.pdf. Accessed April 05, 2013.
35. Effective HPLC method development. Available from http://www.cvg.ca/images/HPLCMethodDevelopment.pdf. Accessed April 05, 2013.
36. Changhe Wen, Designing HPLC Methods for Stability Indication and Forced Degradation Samples For API, Collected from American Pharmaceutical Review at http://www. americanpharmaceuticalreview.com. Accessed April 05, 2013.
37. Chandra M., Buffers: A guide for the preparation and use of buffers in the biological system: available from: www.calbiochem.com. Accessed April 05, 2013.
38. Lindholm J, Development and Validation of HPLC Method for Analytical and Preparative Purpose, Acta Universities Upsaliensis Uppsala, 2004; 13-14.
39. Snyder LR, Kirkland JJ, Glajch JL. Practical HPLC method development. 2nd edition. New York. John wiley.1997; 233-291.http://dx.doi.org/10.1002/9781118592014
40. Columns. From http://www.waters.com/watersdivision/pdf/Ic3AC.pdf. Accessed April 05, 2013.
41. Wagaw S, Tedrow J, Grieme T, Bavda L, Wang W, Viswanath S, et al HPLC Guide; Departments R450, R452, R45R.
42. Weston A, Brown PR, HPLC and CE Principles and Practise, Academic Press, California, 1997.
43. Donald D. Hong and Mumtaz Shah, Development, and validation of HPLC Stabilityindicating Assays, In Sens T. Carstensen, C.T.Rhodes, editors Drug Stability-Principle & Practice. 3rd Edition. New York:Marcel Dekker Inc. 2008; p. 332.
44. John W. Dolan, "Stability-Indicating Assays", LC Troubleshooting 2005, 275.
45. Dean JA, Analytical Chemistry Handbook, Mc Graw-Hill, New York,1995.
46. Mayer ML. HPLC for Pharmaceutical scientists. Am. Lab. 1997; 29, 34-37.
47. Merrill JC. HPLC for Pharmaceutical scientist Am. Lab. 1987; 74-81.
48. Bliesner D.M., Validating Chromatographic Methods, john wliey & sons, Inc. 2006; 88-92
49. A Guide to Validation in HPLC Based on the Work of G.M. Hearn Perkin Elmer. R.A. van Iterson Drenthe College Emmen Holland for www.standardbase.com. . Accessed April 05, 2013.
50. Mayer ML, Am. Lab. 1997; 29, 34-37.
51. Monika Bakshi and Saranjit Singh. Development of validated stability-indicating assay methods a critical review. J. Pharm. Biomed. Anal.2002;28(6):1011-1040.http://dx.doi.org/10.1016/S0731-7085(02)00047-X
52. FDA Guidance for Industry: Analytical procedures and methods validation (Draft guidance) August 2000
53. Ngwa G, Forced Degradation Studies. Forced degradation as an Integral part of HPLC Stability Indicating Method Development Drug Delivery Technology.2010; 10(5).
54. ICH, Q2A, Text on Validation of Analytical Procedures, International Conference on Harmonization, October 1994, Geneva.
55. ICH, Q2B, Validation of Analytical Procedures, Methodology, International Conference on Harmonization, November.1996; Geneva.
56. ICH, Stability testing of new drug substances and products (QIAR) international conference on harmonization IFPMA, 2000; Geneva.
57. Reynolds DW, Facchine KL, Mullaney JF, Alsante KM, Hatajik TD, Mott MG. Available Guidance and Best Practices for Conducting Forced Degradation Studies. Pharmaceutical Technology, 2002; 48-56.
58. Kats M, Forced degradation studies: regulatory considerations and implementation. BioPharm Int, 2005; 18:7.
59. Alsante KM, Ando A, Brown R, Ensing J, Hatajika TD, Kong Tsuda Y, The role of degradant profiling in active pharmaceutical ingredients and drug products. Adv Drug Deliv Rev. 2007; 59: 29–37 http://dx.doi.org/10.1016/j.addr.2006.10.006.
60. Bergh J. J., Breytenbach, J. C.Stability-indicating High-performance Liquid- chromatographic Analysis of Trimethoprim in Pharmaceuticals. J.Chromatogr. 1987; 387: 528531.
61. Stubbs C., Kanfer, I. Stability-indicating High-performance Liquidchromato- graphic Assay of Erythromycin Estolate in Pharmaceutical Dosage Forms. Int. J.Pharm. 1990; 3(2): 113-119.

62. MacNeil L., Rice J. J., Muhammad N. Lauback R. G. Stability-indicating Liquidchromatographic Determination of Cefapirin, Desacetylcefapirin and Cefapirin Lactone in Sodium Cefapirin Bulk and Injectable Formulations. J. Chromatogr. 1986; 361: 285-290.
63. Bounine J. P., Tardif B., Beltran P.Mazzo D. J. High-performance Liquid- chromatographic Stability-indicating Determination of Zopiclone in Tablets. J. Chromatogr. 1994; 677(1): 87-93.
64. Lauback R. G., Rice J. J., Bleiberg B.,Muhammad N., Hanna, S. A. 1984.Specific Highperformance Liquidchromato- graphic Determination of Ampicillin in Bulks, Injectables, Capsules, and Oral Suspensions by Reversed-phase Ion-pair Chromatography. J. Liq. Chromatogr.1984; 7(6): 1243-1265.
65. Wiklund A E., Dag B., Brita S.Toxicity evaluation by using intact sediments and sediment extracts.Marine Pollution Bulletin (2005);50(6): 660-667.
66. Kwok Y. C., Hsieh D. P. H., Wong P.K. Toxicity identification evaluation(TIE) of pore water of contaminated marine sediments collected from Hong Kong waters. Marine Pollution Bulletin. 2005; 51(8-12): 1085-1091.
67. Hongxia Yu., Jing C., Cui Y., Shang H., Ding Z., Jin H. Application of toxicity identification evaluation procedures on wastewaters and sludge from a municipal sewage treatment works with industrial inputs. Ecotoxicology and Environmental Safety. 2004; 57(3): 426-430.
68. Ayrton ton J. Assay of ceftazidime in biological fluids using high-pressure liquid chromatography. J. Antimicrob.Chemother. 1981; 8: 227-231.
69. Bowden R.E., Madsen P.O. High-pressure liquid chromatographic assay of sulbactam in plasma, urine, and tissue. Antimicrob. Agents Chemother.1986; 30: 31-233.
70. Haginaka J., Yasuda H., Uno T., Nakagawa T. Alkaline degradation, and determination by high-performance liquid chromatography. Chem. Pharm. Bull.1984; 32: 2752-2758
71. Fredj G., Paillet Aussel M. F., Brouard A., Barreteau H., Divine C., Michaud M. Determination of sulbactam in biological fluids by high-performance liquid chromatography. J. Chromatogr.
72. 1986; 383: 218-222.
73. Rodenas V., Garcia M.S., SanchezPedreno C., Albero M.I. Flow injection spectrophotometric determination of frusemide or sulphathiazole in pharmaceuticals. J.Pharm. Biomed. Anal. 1997;1687-1693.
74. Shah A.J., Adlard M.W., Stride J.D. A sensitive assay for clavulanic acid and sulbactam in biological fluids by high-performance liquid chromatography and pre-column derivatization. J.Pharm. Biomed. Anal. 1990; 5: 437- 443.
75. Abidi S.L. High-performance liquid chromatography of phosphatidic acids and related polar lipids. J.Chromatogr.1991; 587: 193-203.
76. Christie W.W., Gill S., Nordbäck J.,Itabashi Y., Sanda S., Slabas A.R. New procedures for rapid screening of leaf lipid components from Arabidopsis.Phytochemical Anal. 1998; 9: 53-57.