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# A PERSPECTIVE REVIEW ON METHOD DEVELOPMENT AND VALIDATION BY HPLC

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## ABSTRACT

Due to the rapid advancements in the medicinal chemistry as well as analytical chemistry, the number of novel drug molecules entering the market is sky rocketing but these drugs at times, due to the toxicities and ADR's take way too long to enter the pharmacopoeias, therefore there is a need of highly sophisticated method development and validation techniques. The objective of the present review work is aimed at exploring the diversity of the method development and validation techniques available. We are trying to present an in depth study of method development and validation primarily by HPLC method. The technique that we are discussing here is RP-HPLC. This technique offers flexibility in the use of a variety of columns, detectors, pumps etc. as per the drug requirement.

**Keywords:** HPLC**,** Method development, Validation, Chromatography.

## INTRODUCTION

In present days, the analytical chemistry has been practiced largely in laboratories in many diverse ways in both theoretical as well as practical manner. Due to such advancements in the analytical chemistry the introduction of number new drugs in the market is increasing every year. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This is because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors [1]. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. Therefore, it is a necessity to develop, improve and validate the newer analytical methods for such drugs so that these analytical methods can be studied collaboratively & applied practically for the analysis of different new compounds [2].

Development of a new method of analysis is a very tedious work but the performing a complete method development for the first time should have several advantages i.e.

- A well developed method never requires any modification or redevelopment for all over its life.
- A well developed method also indicates a brief knowledge/information about the various working parameters like percent organic modifier, pH, temperature, column reproducibility, reproducibility of method etc. [3]. Inspite of variations in individual approach, the method development often follows the well-established steps. In most cases the desired separation can be achieved with only few experiments, while in other cases considerable amount of experimentation may be required. However, the method so developed should be as simple as possible, the best strategy being theoretical & empirical approach [4] .
- Before proceeding with development of method for a particular sample, it is absolutely essential to have detailed information about the sample & separation goal should be clearly defined, but the complete composition of the sample may not always known since, it may contain impurities, degradation/ decomposition products. Thus while developing separation of component of similar structure; supplementing informations are obtained from the literature [5].

**Aim of Separation**

Before doing analysis of any sample our aim and objective of separation must be clear, following points must be kept in mind:

- Methods for quantitative analysis of components of interest, detection of impurities, characterization of unknown sample components or isolation of pure substance.
- Whether all the impurities or degradation products to be individually separated or total sum of these products is to be estimated.
- For quantitative analysis, level of precision/accuracy required.
- Type of different sample matrices for while the method is expected to be applied.
- Number of samples to be analyzed at a given time, when one is required to analyze large number of sample [6].

**Different methods used for the analysis of drugs**

The various methods of analysis can be grouped into two categories i.e. chemical methods and instrumental methods.

**Chemical methods**

In these methods, volume and mass are used as means of detection. These methods are further divided into different methods i.e. titrimetric methods (acid-base, oxidation-reduction, non-aqueous, complexometric, precipitation titration), gravimetric and thermogravimetric methods, volumetric methods.

**Instrumental methods**

These methods are dependent on the measurement of physical properties of the compounds

**Based on measurement of signals**

Different methods based on measurement of signals are tabulated (Table I) below:

**TABLE I: METHODS BASED ON MEASUREMENT OF SIGNALS**

| S. NO. | TYPE OF SIGNAL | TYPE OF INSTRUMENTAL METHOD |
|---|---|---|
| 1. | Emission of radiation | X-ray emission spectrometry, fluorescence spectrometry |
| 2. | Absorption of radiation | UV/Visible and I.R. spectrophotometry<br>NMR spectroscopy<br>ESR spectroscopy<br>Atomic absorption spectrometry |
| 3. | Mass to charge ratio | Mass spectrophotometry |
| 4. | Refraction of radiation | Refractometry |
| 5. | Scattering of radiation | Nephlometry |
| 6. | Rotation of radiation | Polarimetry |
| 7. | Electrical potential | Potentiometry |
| 8. | Electrical current | Amperometry, polarography |
| 9. | Electrical resistance | Conductometry |
| 10. | Thermal properties | Differential thermal analysis<br>Differential scanning calorimetry<br>Thermogravimetry |

**Based on separation techniques**

Some of the chromatographic techniques are differentiated on the basis of different separation techniques used for ex. adsorption chromatography, partition chromatography, ion exchange HPLC, ion pair chromatography, size exclusion chromatography, thin layer chromatography, high performance thin layer chromatography, high performance capillary electrophoresis and gas chromatography. Among these methods, spectrophotometric and chromatographic methods are most widely used.

**Liquid Chromatography**

Liquid chromatography has these days, became one of the most versatile techniques available to analyst, because of its simplicity & capacity for high resolution separation. It refers to those methods, in which the separation takes places with in a packed column and mobile phase is forced through these packed columns under high pressure, i.e. 1000 to 3000 psi that is why it is known as high pressure liquid chromatography [7] .

Different modes of separation of a mixture are available in liquid chromatography depending upon the nature of stationary phase. Modes available are as adsorption chromatography, ion-exchange chromatography, size-exclusion chromatography, affinity chromatography [8] .

**Partition chromatography**

It is an example of adsorption chromatography, mixture of solutes are separated according to the relative tendencies of their components to partitioned between a mobile phase and stationary phase consisting of a layer of liquid coated or bonded onto the surface of a solid support. The liquid is present as an extremely thin layer so that equilibrium between the phases may be attained rapidly by minimizing the diffusion of the solute into the stationary phase [9] . The surface of the solid support is frequently treated in order to eliminate adsorptive effects. Bonded phase chromatography has been developed so as to have stable stationary phase, here liquid phase permanently is bonded chemically to the surface of the solid support. Silica gel with its high surface population of hydroxide groups provides an excellent medium onto which various substances can be bounded using appropriately substituted silylating agents, for example

octadecyldimethylsilylchloride reacts with silica gel to form a stable, non polar stationary phase called ODS (Octadecylsilyl) [10] .

Partition chromatography may be conducted in either or the two ways i.e. normal phase chromatography and reversed phase chromatography.

**Normal phase chromatography**

In this stationary phase is a polar substance, such as polyethylene glycol or the untreated silica surface itself, and the mobile phase is non polar, e.g. n-hexane. Under these circumstances polar compounds are retained preferentially and non polar substances elute more quickly [11].

**Reverse phase chromatography**

Here stationary phase is non polar, e.g. ODS and mobile phase in polar usually a mixture of water and methanol and/acetonitrile. Non-polar compounds are retained more strongly by this system, while polar substance elute first. Reserved phase separations are most frequently used methods in HPLC [12] . Now days, several techniques are used to alter the mobile phase to permit successful chromatography of ionic compounds using non popular stationary phase. These methods are called as ion suppression chromatography, ion-pairing chromatography and 'Soap' chromatography. This technique is used for substance such as weak acidic (pka < 2) and weak basic (pka < 8), which are only ionized partially at the neutral pH values characteristic of the usual mobile phase. In order to enhance the retention of the substance in a reversed phase system, the pH of the mobile phase is adjusted to a value low enough to suppress the ionization of the substance [13].

**Instrumentation**

To achieve successful analysis HPLC system must have at least following components.

**Solvent reservoirs**

These are glass or stainless steel containers capable of holding up to 1L mobile phase, which may be consists of pure organic solvents or aqueous solutions of salts or buffers. The substances used to prepare these mixtures should be of highest purity available as to avoid any damage to column. The mobile phase is filtered to remove

particulate matters and degassed by vacuum, sonication or purging with helium to eliminate out gassing in the pump or detector [14].

**Pumps**

These are used to generate pressure upto 5000 psi to force the mobile phase through the column. There are two types of pumps i.e. mechanical pumps and pneumatic pump / constant pressure [15] .

**Mechanical pump** - The most frequently used mechanical pump is reciprocating piston type. There are check valves to control the flow of solvent into and out of the liquid end and prevent back flow.

**Pneumatic Pumps** - These may be either gas-displacement type or pneumatic amplifier type. They have advantages as these produces pulse less operation [16].

**Injector device**

The solute mixture is introduced into the chromatography by means of a suitable injector device. Septum injector is available, in which the sample solution is injected through a self scaling rubber or Teflon disk a micro liter syringe. Rotary valve- and-loop injector is also used. This consists of a stainless steel and Teflon block, which has been drilled to provide two alternate paths for solvent flow each selectable by a rotating valve [17].

**Precolumn**

This is optional and may be used to protect the main column by trapping particulate matter and retain substances, which would be irreversibly adsorbed on the analytical column. That is why it is also known as a guard column. This is packed with a stationary phase identical to that in the main column, except its particle size may be larger, so that it will not restrict the flow [18] .

**Analytical column**

Actual separation takes place in this. It is made up stainless steel, usually 5 to 25cm in length, with internal diameter 2 to 9.6 mm. It is packed with stationary phase. The material used for packing are of two types: superficially porous or pelliculor and totally porous. Silica gel is the material most frequently employed for micro particulate column packing. Alkyl group with carbon chain lengths of 1, 2, 8 or 18 (ODS) carbon, cyano, phenyl groups and on exchange one attached to it [19] .

**Gradient controller**

Some time gradient analysis is necessary to achieve a particular separation. This is an electronic device, which synchronized the operations of the two pumps to provide a mobile phase mixture of the desire concentration.

**Solvent –conditioning column**

This is used only under special circumstances. Most of HPLC column packing materials are prepared from silica gel, which will dissolve slowly in solvents whose pH values are below 5 or above 7. This will lead to loss of resolution. Therefore to minimize this occurrence and protect the expensive silica based packing materials, a small column (5 to 10 cm) packed with HPLC grade silica gel is inserted into the liquid stream after the pump and before the injector. The material in this column dissolved preferentially, saturating the mobile phase and protect the analytical column [20] .

**Detector**

There are a number of sensitive and specific detection systems based on the spectrometric, refractrometric or electrochemical properties of the solutes are available. The most frequently used instrument is an ultraviolet visible spectrometer, which has been fitted with a flow cell of very small volume (8µl). There may be of fixed wavelength or variable wavelength type detector, more elaborate models also available, which can scan the entire UV spectrum repeatedly during elution of peak to determine if more than one substance is co eluting. Fluorescence spectrometer is much more sensitive but less broadly applicable. Differential refractometer, which measures refractive index, electrochemical which is based on electrochemical measurement is few examples of some other detectors.

**Method development using HPLC**

It is an effort to select best chromatographic conditions like the best column, the best mobile phase, the detection wavelength etc. to be used for routine analysis of any drug. For the method development by HPLC method some information about the sample is very essential i.e. number of components present in the sample, pka values of different components, uv-vis Spectra of each analyte, solubility in different solvents, concentration range of each component, nature of sample etc. Prior to method development there must be some technical informations i.e. chromatography method selection according to the

sample properties, the sample is analyzed with HPLC in condition where all compounds elute in a reasonable time, the HPLC method is optimized with regard to analysis time, resolution selectivity and sensitivity [21].

**Information about Sample**

While developing new method we must have some essential information about the sample such as number of components present in sample, UV-VIS spectra of each analyte, solubility in different solvents, concentration range of each component, nature of sample etc [22]. Development of a new analytical method also requires some technical information about the sample to be analysed, chromatographic method to be used, conditions using in method development etc. Some of the technical informations which are essential for a method development are given below

- The selection of the chromatographic method should be based on the properties of the sample to be analysed.
- The sample is analyzed with HPLC in condition where all compounds elute in reasonable time.
- The HPLC method is optimized with regards to analysis time, resolution, selectivity & sensitivity.

**Commonly used chromatographic methods**

The most commonly used chromatographic methods are normal phase chromatography, reverse phase chromatography, reverse phase ion-pair chromatography and ion-exchange chromatography. There are some strategies for method development using HPLC i.e. selection of suitable chromatographic method for organic compounds, first reversed phase should be tried, if not successful, normal phase should be tried, then reverse phase, ion-pair chromatography should be tried, ion-exchange chromatography at the end [23].

**Reverse phase chromatography**

It is a trend to try RP–HPLC at the first. For this a quick method is developed to separate a sample initially using a column of dimensions 15cm x 4.6mm, packed with particles of 5 µm diameter of C-18 or C-8 with a sample gradient for example, 10:9(v/v) acetonitrile: 50mM phosphate buffer (pH-3) to 9:10(v/v) acetonitrile: 50mM phosphate buffer (pH=3) in 20 minute at a flow rate of 1.0 ml/min. From this gradient separation,

appropriate isocratic condition can be developed. In reversed phase chromatography the selection of mobile phase is very important for the analysis of the drug. For RP-HPLC eluent of choice are as follows, we can use acetonitrile frequently as it is suitable for the entire UV range, methanol and isopropanol are not suitable below wavelength of 210nm, acetic acid is suitable above a wavelength of 240 nm, both $K_2HPO_4$ and $KH_2PO_4$ can be use in entire UV range, freshly distilled THF is suitable for HPLC above a wave length of 240nm, TEA is suitable above 240 nm, ammonium acetate can be used above 215 nm, EDTA can be used in entire UV range, sodium phosphate is suitable above 210 nm [24].

**Choice of buffer**

When separating acid or bases, buffered mobile phase is required to maintain consistency in retency and selectivity. Buffered salts reduce peak tailing for basic compounds by effectively masking silanol groups and also reduce potential ion-exchange interactions with a protonated silanol groups. As potassium is a stronger counter ion than sodium, it provides improved results compared to sodium ($Na^+$) [25]. Potassium phosphate is used for preparation of buffers of various pH. If band tailing is observed for basic amphoteric compounds few drops of diluted triethylamine or ammonium acetate is added, for acidic or amphoteric compounds few drops of diluted triethylamine or ammonium acetate is tried. The aqueous eluent used in method development are Water for neutral compounds, 100 mM $H_3PO_4$ buffer of pH 2.3 for weak to medium acidic compounds, 100 mM $H_3PO_4$ buffer of pH 4.0 for weak to medium acidic compounds, 50 mM $H_3PO_4$ buffer of pH 7.5 for week to medium basic or acidic compound in ionized form. Unknown sample should be analyzed first with water, then with an acidic and a neutral buffer. In most of the cases the mobile phase is binary or ternary system it is advisable to filter each solvent before mixing. Regardless of the type of filter used, it is recommended to rinse the filter first discarding first few ml of the solvent, so that filter is free from potential soluble contaminants [26].

**Mixing different components of mobile phase**

Before mixing different components of mobile phase, some basic information about the mixing, compatibility, selectivity is very essential some of them are given below

**Preparation /mixing**

The chromatographers are usually not aware of the difference in partial pressure of individual solvent at ratio they are to be combined. It is important and known that adding 50ml of water to 50ml of alcohol dose not produce 100ml of solution.

**Solvent compatibility**

Solvent system must be miscible with the previously used mobile phase, if not intermediate solvent may be used, the one that is miscible with previous mobile phase and new mobile phase.

**Solvent selectivity**

It is an ability of a solvent to resolve two or more peak on a given stationary phase. For reverse phase separation water, acetonitrile, tetrahydrofuran (THF) and methanol or isopropyl alcohol are most commonly used as binary mixtures 100% acetonitrile, 100% water or 100% methanol is rarely gives optimal separation [27] .

**Solvent degassing**

Degassing of solvent used in mobile phase in very important it has following advantages i.e. stability of the baseline and enhanced sensitivity reproducible retention times for eluting peaks, reproducible injection volume stable pump operation. Different methods that are used for the solvent degassing are, parging (on line degassing), heat (off line degassing), vacuum (off line degassing method), sonication (off line degassing method) [28] .

**Solvent delivery system (pump) selection**

For the delivery of the solvent a suitable pump is very essential. Different types of pumps are used in the HPLC for the delivery of the solvent for ex. syringe pump, reciprocating pump and pneumatic pump. The reciprocating pump is further classified into three type's i.e. single piston reciprocating pump, dual piston reciprocating pump, reciprocating diaphragm pump. The pneumatic pump is also classified into direct pump and amplifier pump.

For the delivery of the solvent the pump must fulfill certain desired parameters i.e. the pressure (psi) must be 3000-5000, flow range (ml / min) should be 0.5-2ml, accuracy must be greater than 5%, the reproducibility shown by the pump must be greater than 1%, pump should have the solvent storage capacity upto 200-500 ml, optional

capacity for routine analysis but suitable for method development. More than 90% of HPLC system use reciprocating pump for the pulse free delivery of the mobile phase [29].

**HPLC column selection**

We can call column as the heart of HPLC separation process. Stable, high performance column is essential requirement for rugged and reproducible method. Type and configuration of the column used is depends upon the separation required. Prior to method development and subsequent routine use certain basic information about column specifications and its performance must be known for example, plate number (N) for given value of k, peak asymmetry factor (As), selectivity value for two different solutes, retention (k), reproducibility, column stability [30].

**Plate number (N)**

It reflects column quality of separation and its ability to produce sharp, narrow peak and achieving good resolution of peaks.

Plate number = 3500 x L (cm) / dp (µm)

L = Length of the column in cm, dp = diameter of particle (µm) [31].

**Peak symmetry factor**

Ideally if should be unit for a symmetric peak

T = W 0.05 / 2f

Where, W 0.05 is peak width at 5% high from base line, f is the distance measured at 5% height from base, peak maxima to the leading edge of peak [32].

**Resolution**

Measures the quality of separation of adjacent peaks/bands. It is calculated from width and retention time of two adjacent peaks/bands.

$Rs = 2(t_2-t_1) / W_1+W_2$

Where $t_1$ and $t_2$ are the retention times of the first and second adjacent bands where as $W_1$ and $W_2$ are their base line bandwidths [33].

**Capacity factor (k')**

It is measure of the position of a sample peak in the chromatogram, being specific for a given compound, a parameter which specifies the extent of delay of substance to be separated. K' depends at stationary phase, mobile phase, temperature and quality of column packing. For good chromatographic performance with isocratic separation, k'

value should be in the range of 1-10. If k' is less than 1.0, the bands are inadequately separated from excessively unretained maternal if k' > 10 separation takes too long and bands broadened, if k' > 30, one can't expect satisfactory isocratic separation using present column and mobile phase, gradient elution should be tried [34].

**Theoretical plate number (n)**

It is calculated using following formula

(i) $N = 16 (t / W) 2$

N = Number of theoretical plates, t = Retention time of the components, W = Width at the base of the peak

(ii) $N = 5.54 (t / W_{1/2}) 2$

$W_{1/2}$ = band width at half-height

Particle size and column dimensions are optimized after obtaining a separation and establishing that the analysis is robust. The column must have certain characterstics i.e.

**Column length**

For better resolution always choose longer columns where as we can get shorter analysis times, less solvent consumption, lower back pressure by the use of shorter column [35].

**Column internal diameter**

Diameters of a column also plays a very important role in the analysis, so choose wider diameter columns for greater sample loading and choose narrow columns for more sensitivity and reduced mobile phase consumption [36].

**Particle shape**

Choose spherical particle for lower back pressure, column stability and greater efficiency. For getting high surface area and high capacity irregular particles must be used.

**Particle size**

For complex mixtures the particle size should be small (3-4 µm) but for sample with structurally different compounds large particles are required (5 -10 µm) [37].

**Surface area**

Surface area for a column is very essential for the elution of the solvent so selection of column on the basis of surface area is very important. Before choosing a column some informations must be taken in consideration i.e. choose high surface area for more capacity, greater resolution and longer retention, choose low surface area packing for quick equilibration time [38] .

**Carbon load**

Choose high carbon load for greater capacities and resolution Choose low carbon load for fast analysis time [39].

**Flow rate Selection**

Column can be operated at any flow rate that is consistent with the backpressure limitations. Flow rates should be optimized to provide the highest efficiency of separation of sample. Flow rate with different internal diameter (ID) of columns with different packing particle size are tabulated (Table II) below [40].

**TABLE II: DEPENDENCE OF FLOW RATE ON PACKING PARTICLE SIZE AND INTERNAL DIAMETER**

| Packing particle size (µm) | Internal diameter of column (µm) | Flow rate (ml/min) |
|---|---|---|
| 3 | 4.6 | 0.5 |
| 5 | 1.0 | 0.1 |
| 5 | 2.0 | 0.2 |
| 5 | 3.2 | 0.5 |
| 5 | 4.6 | 1.0 |
| 5 | 10.0 | 5.0 |
| 10 | 4.6 | 2.0 |
| 10 | 21.2 | 21.0 |

**Detector Selection**

Detectors are eyes of the liquid chromatography system and measure the compounds after their separation on the column. Selected detector should be capable of

responding to change in concentrations of all the components in the sample with adequate sensitivity even to measure trace substances. Basically there are two types of detectors are present i.e. bulk property detector and solute property detector. Bulk property detectors function on some bulk property of the eluent, such as refractive index and solute property that is specific to the solute only. The detectors must have certain characteristics i.e. high sensitivity, higher linear dynamic range, application to most of the solutes, does not contribute to band broadening, non-destructive, faster response. In the HPLC method development the selection of suitable wavelength is very essential because absorption maximum of complex formulations containing several ingredients with variable concentrations spread over longer UV range. So, it is desired to explore the possibility of selecting a suitable wavelength at which entire chromatogram can be scanned without sacrificing vital information about different components of the sample. This single wavelength is usually referred as most suitable wavelength. This problem is not encountered with variable wavelength or PAD detector. On the basis of applications different detectors are tabulated (Table III) below [41].

**Further Optimization**

Shorter analysis time

- Change to an isocratic method, the suitable eluent composition can be estimated from the gradient run.
- Shorter column, if enough resolution was obtained.

Better resolution

- Longer column
- Use of sorbent with smaller particles (3-4 µm) [42].

Better selectivity and sensitivity

- Other stationary phases e.g. phenyl, CN etc.
- pH control with ion-forming compounds
- Use of methanol or THF instead of acetonitrile.
- Detection at the absorption maximum of the substance
- All factors which leads to narrower and higher peaks as gradient elution, smaller particle, microbore columns.

**TABLE III: DETECTORS AND THEIR APPLICATIONS**

| S. No. | Detector | Analytes | Solvent requirement |
|---|---|---|---|
| 1. | UV-VS | Any with chromophores | UV-grade non-UV absorbing solvents |
| 2. | Fluorescence | Fluorescent compound | UV-grade non-UV absorbing solvents |
| 3. | Refractive index (RI) | Compounds with a different RI to that of mobile phase | Cannot run mobile phase gradients |
| 4. | Conductivity | Charged/polar compounds | Mobile phase must be conducting |
| 5. | Mass spectrometer (MS) | Broad range of compounds | Must use volatile solvent and volatile buffers |

**Quantitative methods**

Quantitation involves comparison of standard and sample (their area or height) based on two requirements i.e. reproducible chromatogram linear response of the detector for analytes of interest.

**Calculation methods**

**1) Normalized peak area**

After integrating all significant peaks in a chromatogram total peak may be calculated. Area (%) of any individual peak is called as normalized peak area.

% Peak area = (Area of peak) x 100 / Total area of peaks

**2) External standardization**

A pure reference standard material corresponding to the substance to be determined is dissolved in a solvent at a known concentration. Exactly measured quantity of this solution i.e. 1,2,3,4 etc µ/ml is injected successively. The areas of each of the peaks produced are plotted verses the mass of solute injected and a calibration curve is produced. Next, the known solution is injected, the area of determined and concentration found by interpolation [43].

**3) Internal standardization**

This procedure requires two standards; the analytical standard, being a pure sample of compound to be analyzed and the other as internal standard. This is normally a substance, which elutes at a position near the substance being analyzed and well resolved (Rs >1.25). A series of solution is prepared containing varying amounts of the analytical standard and constant amount of the internal standard. These are chromatographed and calibration curved is determined by plotting the ratio of the areas of the two peaks verses their concentration. The unknown is dissolved in a suitable solvent, the same amount of internal standard added and this mixture is chromatographed. The ratio of area is calculated and by interpolation on the calibration curve the amount is determined [44].

**Method Validation**

Analytical test method validation is completed to ensure that analytical methodology is accurate, specific, reproducible and robust over the specified range that an analyte will be analyzed. Method validation provides assurance reliability during normal use and some time referred to as "The process of providing documented evidences that what has intended to do" [45].

**Types of analytical procedure to be validated**

There are different types of analytical methods which can be validated i.e. identification tests, quantitative test for impurities contents, limit tests for control of impurities and quantitative tests of the active moiety in sample of drug substance and drug products or other selected components in the drug products. The typical validation characteristics, which should be considered, are accuracy, precision (repeatability, intermediate, precision reproducibility), specificity, detection limit, quantitation limit, robustness, linearity and range (Table IV) [46].

**TABLE IV: DATA ELEMENTS REQUIRED FOR ASSAY VALIDATION**

| Analytical performance parameters | Identification | Testing of impurities | | Assay dissolution contents/potency |
|---|---|---|---|---|
| | | Quantitative Test | Limit Test | |
| Accuracy | - | + | - | + |
| Precision | | | | |
| Repeatability | - | + | - | + |
| Intermediate Precision | - | + | - | + |
| Specificity | + | + | + | + |
| Detection Limit | - | - | + | - |
| Quantitation limit | - | + | - | + |
| Linearity | - | + | - | + |
| Range | - | + | - | + |

- signifies that this characteristics is not normally evaluated

+ signifies that this characteristics is normally evaluated

**Specificity**

It is the ability to assess unequivocally the analyte in the presence of components, which may expect to be present8. Typically these may include impurities, degradents, matrix etc. This definition has following implications [47].

**Identification**

To ensure identity of analyte i.e., ability to discriminate between compounds of closely related structures, which are likely to be present [48] .

**Assay and impurity tests**

- Assay (contents or potency) – To provides an exact result allows an accurate statement on the content and potency of the analyte in the sample.
- Purity tests- To ensures that all analytical procedure performed allows an accurate statement of the content of impurities of an analyte i.e. related substance test, heavy metals, residual solvents, etc.

**Accuracy**

The accuracy of the analytical procedure express 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, this is sometimes termed as trueness. Accuracy should be established across the specific range of the analytical procedure (49).

**Assay for drug substance**

Several methods of determining accuracy are available

- Application of analytical procedure to an analyte of known purity.
- Comparison of the results of the proposed analytical procedure with those of a second well characterized procedure.
- Accuracy may be inferred once precision, linearity, and specificity have been established.

**Assay for drug product**

Several methods of determining accuracy are available

- Application of the analytical procedure to synthetic mixture of the drug products components to which known quantities of the drug substance to be analyzed have been added.
- Accuracy may be inferred once precision, linearity and specificity have been established.

**Impurities (Quantitation)**

Accuracy should be assessed on samples spiked with known amounts of impurities.

**Recommended data**

Accuracy should be assessed on samples using a minimum of nine determinants over a minimum of three concentration levels covering the specified range. Accuracy should be reported as percentage recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

**Precision**

Validation of tests for assay and quantitative determination of impurities includes an investigation of precision [50].

**Repeatability**

It should be assessed using

- A minimum of nine determinants covering the specified range.

- A minimum of six determinants at 100% of the test concentration [51].

**Intermediate Precision**

Here effects of random events on precision of analytical procedure are established. Typical variation to be studied includes days, analyst, equipments, etc [52].

**Reproducibility**

It is assessed by means of an inter laboratory trails. Reproducibility should be considered in case of standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias [53].

**Limit of Detection**

It is the lowest amount of an analyte in a sample, which can be detected but not necessarily quantitated as exact value. Several approaches for determination of detection limit are possible, depending on whether the procedure is a non instrumental or instrumental.

**Based on visual evaluation**

It is generally used for non instrumental method but may be used for instrumental method.

Based on signal to noise

This approach is only applied to analytical methods, which exhibit base line noise.

LOD= 3S / N

Where, S is signal and N is noise value.

Based on standard deviation and slope of response

LOD = 3.3 x SD / S

Where, SD = Standard Deviation and S = Slope of Calibration Curve [54].

**Limit of Quantitation**

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. Several approaches for the Quantitation limit are possible depending upon whether the procedure is a non instrumental or instrumental [55].

**Based on visual evaluation**

Visual evaluation is generally used for non instrumental methods but may also be used with instrumental methods [56].

**Based on signal to noise**

This approach is only applied to analytical methods, which exhibit base line noise.

LOD = 10 S / N

Where, S is signal and N is noise value [57].

**Based on standard deviation and slope of response**

LOD = 10 x SD / S

Where, SD = Standard deviation and S = Slope of calibration Curve [58].

**Linearity and Range**

The linearity of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration of analyte in the sample. For establishment of linearity, five concentrations are recommended. The correlation coefficient, y-intercepts, slope of regression line etc. are calculated. Range is derived from linearity studies and depends upon the intended application of the procedure [59].

**Robustness**

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

**Most encountered variations**

There are some variations which can be encountered in the method validation. Some of the variations are exemplified below as

- Stability of analytical solutions
- Variation in extraction time
- Variation of pH in mobile phase
- Variation in mobile phase composition
- Different columns (different lots and /or suppliers)
- Temperature variation
- Flow rate variation

**System suitability**

It is an integral part of many analytical procedures. These tests are based on the concept that the equipment, electronics, analytical operation and sample to be analyzed constitute an integral system that can be evaluated as such. System suitability test

parameters to be established for a particular procedure depending on the type of procedure being validated [60] .

## CONCLUSION

HPLC is a highly versatile and efficient technique for the analysis of a wide variety of drugs because it offers superior qualitative and quantitative results, reproducibility, high detection sensitivity, and unsurpassed reliability. Therefore, by using this technique we can develop and validate a new method for the analysis of any drug.

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