ABSTRACT
A review suggests systematic way for analytical method development for the separation of related substances or impurities from API and after development how we can perform a validation of that developed method as according to regulatory guidelines. Need of analytical method development in nowadays increases continuously due to number of alternative drug molecules are developing for customer satisfaction. Analytical technique as high performance liquid chromatography (HPLC) generally used to identify, separate, detect and quantities main active ingredient or API and its related substances. Method development is essential in case of discovery of drug molecule, drug development phase and in analysis of API and its related substances. Various prodrugs, Intermediates and organic impurities during product development phase do not have any pharmacopoeial method so we can develop the analytical method by own to set the specifications. The article focuses on HPLC method development, optimization of developed method and validation of developed analytical method as per ICH guideline Q2 (R1).

KEYWORDS: HPLC, method development, related substances, validation, stability study.

INTRODUCTION
Analytical chemistry is a science relates to physical chemistry, it is a branch of pure chemistry. The aim of analytical chemistry is substantially to develop and apply advanced methodology and instrumentation with the target of providing information on the
characteristics and configuration of matter. Analytical chemistry also grants the determination of a compound’s structure, either moderately or entirely, in samples of differing complexity. Finally, part of the aspect of analytical chemistry is to furnish an interpretation of the results obtained.[1] “The separation of a chemical entity into its imminent or eventual parts; the identification of its components or of the related substances it may contain” this definition suggest in very wide terms the application of analytical chemistry. Whenever an analytical chemist deals with highly unfamiliar sample, first specification usually start with finding out that which kind of components are present in it. This general issue may sometimes be challengeable to decide that what kind of related substances or impurities are present in a given sample or as it may be not confirms that certain stated impurities are present. Solution of such kind of problems related to uncertainty solve by the application of qualitative analysis. First the person involve in testing assure the characteristics of the components of a given sample, the analyst is then basically determine that how much of each component or specified components, is present in sample matrix. This kind of determinations can discover by the application of quantitative analysis which involve various methods to quantify the amount of components present in sample according to the nature of that particular compound as most of the drug can easily quantify by HPLC method and organic solvents can determine by the use of gas chromatography. To supply the needed information a number of other techniques are available.[2] Analytical methods for separation and resolution of closely related substance involve common separation methods like chromatography, distillation, extraction, ion exchange, fractional crystallization, and selective precipitation.[3] Healthcare system involves single API medication and their combinations which are familiarize into the pharmaceutical marketing and increasing every year due to the customer satisfaction, such drugs may be a new molecule or partial structural transformation of the existing one. Getting rational, predictable and accurate data is the main role of any analytical measurement for quality assurance purpose. Validated analytical methods play an extensive role in accomplishes this objective. Validation of methods give assurance that specifications limits can be used to set benchmark for accuracy, reproducibility and flexibility of results obtain from analytical method for testing, which serve as an integral concern of any appropriate analytical practice. Most of the regulatory bodies as USFDA, ICH focuses on analytical method development as well as validation of that analytical method so ICH give their guideline for that purpose as ICH Q2(R1). After the development of new drug molecule or new drug formulation, it takes time to enroll those drugs into pharmacopoeias but these drug or drug formulations comes into the market, this arise due to the possible doubt in the
continuation and extensive use of these drugs by users, sometime drugs fails in clinical trial phase fourth which involve marketing survey and drug may show toxicity or any adverse effects in broad spectrum of patients (results removal of those drugs from the market), patient resistance also can affect the removal of drug from market or sometime in a market there is a introduction of more suitable drugs by the competitors. So there is a need of analytical method development because under these conditions standard analytical procedures for testing of these new drug molecules or drug formulation may not be available in official methods as suggest by the pharmacopoeias. Here we have to develop an analytical method for qualitative and quantitative analysis for drug molecule as single API and in formulation. As analytical method development is important for analysis of such drug which does not have analytical method for testing in pharmacopoeia, validation is also an important parameter after developing that method as it is documented evidence which assure the predetermined specifications. Pharmaceutical drug formulation which manufactured by the combination of more than one active pharmaceutical ingredient designed by joining the therapeutic effects of two or more drugs in one product needs analytical method development to check the amount of release of each drug form combination. Such kinds of drug products can make challenges to the analytical chemist who involve in development of analytical method for determination of such kind of drug combinations and also validate them. These developed method after proper validation is used by quality control (QC) department to assure the identity, potency and purity of new drug formulation. Quantitative and Qualitative analysis of related substance or impurities is an important feature in process development phase to increase the quality and safety of drug product which is finalized to export.

Related substances
These are the impurities (may be organic or inorganic) present in active pharmaceutical ingredients (API). They are undesirable or unwanted chemical entities which remain in main component known as API. It may be developing during formulation process, during stability testing method development or especially by the storage of drug product for long term. Availability of such kind of unwanted components known as related impurities even in trace quantity may affects the pharmaceutical product’s efficacy and safety. There are number of analytical techniques available for determination of related substance present in the matrix of main component as HPLC is one of them. There is a great demand to develop an analytical method for related substance of new arising drug which not introduce into the pharmacopoeia for quality assessment purpose.\(^4\)
Chromatographic separation

There are numerous separation techniques available in pharmaceutical analysis and one of them is chromatography. Separation technique through which each drug component from drug matrix can easily separate by their migration rates. The chromatographic technique involves movement of analyte through solvents known as mobile phase and get separated over a solid stationary phase in case of high performance liquid chromatography. Analytes have different migration rate or we called say affinity towards mobile phase and stationary phase thus they gets separated according to their retardation factor. Component which have greater affinity with mobile phase elute first from the column thus which have greater affinity with stationary phase require more time to elute. According to Stationary phase used chromatographic separation classify into different categories as Thin-layer chromatography in which a thin layer of silica on a glass slide used as a stationary phase for separation, Paper chromatography which involves a stationary phase of paper so the separation take place between the liquid bounded within paper and mobile phase so it’s called liquid-liquid chromatography. High performance liquid chromatography (HPLC) technique is a highly advanced technique and also has a complex instrumentation as compare to paper and thin layer chromatography. HPLC is applicable for separation of organic components as most of the pharmaceutical drugs are organic compounds so this is widely used in pharmaceutical industries. Technique involves separation of each component from sample mixture by the column which acts as a heart of HPLC system. These columns are made up of stainless steel packed with silica attached by hydrocarbon chain as C₈ or C₁₈. Resolution or separation of two analyte from a mixture can enhance by the use of smaller particle size of packing material in columns but when we decrease the particle size of column packing material, requirement of solvent delivery pressure increased randomly so there is a need of such system which allows high pressure limits. Further minimization in column material particle size can show higher solvent flow rates, decreasing analysis time period without losing resolution. So there is a newly developed technique known as Ultra High Pressure Liquid Chromatography (UPLC) which allows higher pressure limits than HPLC. Columns use in UPLC generally has lower particle size than HPLC columns which show higher resolution in less time hence; this new technique is more suitable than HPLC.[5]

ANALYTICAL METHOD DEVELOPMENT BY HPLC

One of the most popular separation techniques because most of the pharmaceutical drug molecules can separate into its components by the use of HPLC so it can also called as a
classic method for separation. Mikhail Tswett in 1903 originally developed the chromatographic technique. Chromatographic method can use for qualitative as well as quantitative analysis which involve determination of amount of analyte present in sample matrix. Qualitative analysis involves identification of analyte in sample matrix on other hand quantitative analysis provides all information related to the potency or purity by providing the exact amount of each analyte in sample mixture. HPLC technique is completely based on variability in migration rates so this variability allows each analyte to get separated over solid stationary phase which is packed into the columns. Mobile phase also play an important role in HPLC as analyte which have greater affinity toward mobile phase eluted first so the choice of both as column and mobile phase keep in consideration to develop an analytical method for separation. There are two kind of HPLC method is there according to stationary phase used, known as normal phase-HPLC and reversed phase-HPLC. Both techniques have difference in their stationary phase and mobile phase characteristics. T-swett, during his plant pigments separation experiment, used a polar stationary phase as unmodified silica filled in a glass column and non-polar mobile phase (hexane) for the separation this is known as normal phase chromatography on the other hand reversed phase-HPLC is based on opposite nature of normal phase so in this a non- polar stationary phase as modified silica with hydrocarbon chain and a polar mobile phase like water, methanol and acetonitrile are used. Reversed phase chromatography widely used for most of the pharmaceutical drug compounds. Due to the huge advancement in column configuration reversed phase chromatography can also used for sample mixture that possesses some degree of hydrophobic characteristic, such as proteins nucleic acids and peptides, can also be separated by reversed phase chromatography. A variety of methods are available for analyzing pharmaceutical compounds; however, high-pressure liquid chromatography is one of them but it is a most classic and suitable technique for separation of most of the drug substance. In Pharmaceutical industries HPLC play an important role in research and development and quality control department for qualitative as well as quantitative estimation of drug substance.\[6\]

COMPONENT INVOLVE IN HPLC SYSTEM
HPLC system in their overall instrumentation have following components as:

- Solvent reservoir
- Pump module
- Injector
- Column compartment and column
Detector
Data integration system

Solvent Reservoir
Mobile phase storage glass container used in HPLC known as solvent reservoir. There are four channels or mobile phase lines as A, B, C and D are connected to system for mobile phase supply. These mobile phase channels are dipped into mobile phase which store in the solvent reservoir bottles made up of glass.

Pump Module
As we know in HPLC mobile phase play an important role for the separation so to force up the mobile phase into the stationary phase pumps are used. In most of the HPLC system pumps can operate about 5000psi pressure but now ultimate HPLC system launched by dionex can operate 8000psi pressure which is the latest advancement in HPLC pump modules.

Sample Injector
To inject a sample into HPLC system there is a sample injector. In most of the HPLC system Auto samplers are used which controlled by software programming but in some models of HPLC we have manual samplers too in which we inject the sample by the help of injection or syringe. In pharmaceutical industries auto sampler are used so for this purpose different sample solutions are filled in a different vials and load into sampler plate then set the sequence programming by the software. According to sequence set by the user sample get injected automatically.

Columns
Columns are the heart for HPLC so there is a great impact of column packing configuration on separation of drug components. In HPLC columns are made up of stainless steel. Columns are available in different sizes as between 50-300 mm in length having an internal diameter between 2-5mm. The packing materials used in columns are generally having particle size between 3-10 µm. In HPLC, column compartment play an essential role to maintain the temperature of mobile phase and column as temperature affects the resolution of two peaks so it should maintain during overall process of separation.
Detector
In HPLC, detectors are used to generate electric signals in form of absorbance with respect to time (retention time of components). A separate compartment is situated at the bottom of HPLC system for detector. To observe an accurate and error less signals, detectors should sensitive to analyte with lower detector noise. UV-visible, photo diode array, fluorescence, mass spectroscopic are the most common HPLC detectors but UV-visible detector is the most common detector used for analytical methods of most of the pharmaceutical drug components.

Data integration system
Computer operating systems to integrate and store the data obtain in form of signals, used in HPLC system. Various software are used to integrate the data as per the requirement like ‘Empower’ is the most common software used to integrate the data which is developed by the popular HPLC brand known as ‘WATERS’. On the other hand ‘Chromeleon’ is software used to integrate data in HPLC system made by the brand ‘DIONEX’.[7]

FACTOR AFFECTING CHROMATOGRAPHIC PURITY
Signals obtain by the detectors in HPLC are highly affected by some parameters which are the result of chromatographic separation. As we know resolution of two peaks (signals) depends on the column configuration and mobile phase composition. Therefore there are some factors which relate to column configuration as well as characteristic of mobile phase which highly affects the chromatographic separation.[8]

A. Void volume
The concept of column void volume ($V_0$) is depending on flow rate and void time. Void volume is defined as the volume of the empty column minus the volume occupied by the solid packing materials. It is the liquid holdup volume of column that each analyte must elute from. Void volume is equal to the void time multiplied by the flow rate ($F$). It can be described by the formula:

$$V_0 = t_o F$$

B. Capacity Factor
While retention time is used for peak identification, it is dependent on the flow rate, the column dimension and other parameters. A more fundamental term that measures the degree of retention of the analyte is the capacity factor calculated by normalizing the net retention
time minus the void time by the void time. The capacity factor measures how many times the analyte is retained relative to an unretained component.\[^9\]

**Capacity factor = \( t_R - t_0 / t_o \)**

### C. Selectivity (\(\alpha\))

Separation between two components is only possible if they have different migration rates through the column. Selectivity can determine by the capacity factors of two peaks as it is the ratio of capacity factor of two peaks obtain in a chromatogram. For peak resolution it is the important factor and should obtain within limits as it is always greater than one for better peak separation. It is dependent on characteristics of stationary phase (i.e. C18, C8, phenyl, cyano etc) and the mobile phase composition. The selectivity effects of the mobile phase can be skillfully exploited by experienced chromatographers to enhance separation of key analytes in the sample.\[^10\]

### D. Theoretical Plate or Plate Height (HETP or \(H\))

The concept of plate is derived from industrial distillation process using a distillation column consisting of individual plates where the condensing liquid is in equilibrium with the rising vapor. Thus, a longer distillation column would have more “plates” or equilibration steps. As per the plate theory of chromatography, separation occurs in columns in form of imaginary plates and high number of theoretical plates shows greater efficiency in separation. This we can suggest in form of equation as Column efficiency \(\alpha\) Number of theoretical plates.

Number of theoretical plates can be calculated by the formula as given in United States Pharmacopoeia:

\[ N = 16[R_t/W_b] \]

Where, \(R_t\) = Retention time of peak  
\(W_b\) = Peak Width  
\(N\) = Number of theoretical plates.\[^10\]

### WHY WE NEED METHOD DEVELOPMENT PROCESS

- There is a chance that drug substance as API doesn’t available in pharmacopoeia. For example Lisdexamphetamine, is a prodrug of dextroamphetamine which is used in attention deficit hyperactivity disorder, does not available in USP. Not even this drug; there are so many drugs which do not have analytical method for testing in
pharmacopoeia. So here we have to develop an appropriate non-official method for those drugs.

- Sometime there are so many non-official methods are available for such drugs but due to the patent regulation it can’t be share with others so various pharmaceutical industries who involve in analytical method development process, develop their own methods for analysis.
- As we know drugs are also available in form of combination formulations so sometime analytical methods may not be available for the drugs in the form of combination. On the other hand, Drug formulation can’t be prepared without excipients and these sometime also affects the testing of the main ingredient (API) so here we need to develop a specific analytical method for that drug.
- During the formulation development phase it is a challenge for researcher to know the actual amount of that drug present in plasma after metabolism so for that purpose there is need of development of such analytical methods which can be used for biological fluids also.
- If the already developed methods are not reliable and cost of analysis is also high then we can develop a cost effective as well as reliable method for analysis but it should accurate, precise and reproducible also.

GENERAL STEPS IN METHOD DEVELOPMENT

- Sample information
- Selection of detector
- Selection of mobile phase
- Selection of column
- Method optimization
- Method validation
Sample Information
Nature of sample as physicochemical characteristics of a drug molecule shows an important aspect in method development process. It should necessary to know the physicochemical characteristics of sample for which we develop a method. As we know HPLC separation highly depends on the polarity of the mobile phase, stationary phase as well as sample which we want to separate. So the first thing which we have to know is that what is the nature of sample as it is hydrophobic or hydrophilic. After that we can easily select the solvents or mobile phase for the sample, as well as the choice of stationary phase can also be made. Partition coefficient value for sample should also know that’s how we determine the distribution of drug within stationary and mobile phase. pH of sample in all aspects should also determine for separation purpose because pH can also affects the retention time of analyte. In general, before selection of column and mobile phase we know the all chemistry of the molecule which we want to separate. The first step during method development is that to select diluents in which a drug dissolve completely so it can be easily determine if know the solubility of drug substance in each solvent as it may be organic solvent as acetonitrile, methanol or non-organic solvent as water. Diluents which select to dissolve the drug should not have any interference with the drug substance as well.

Selection of detector
Chromatographic signals are obtain by the help of detector situated in HPLC system. Signals are the results of analyte separation within stationary phase and mobile phase so it is necessary to choose a suitable detector for analysis. We always find the detector which is sensitive to analyte. Physicochemical properties of analytes, limit of detection, availability and detector cost are the criteria for selection of suitable detector. Uv-Visible detector is adjustable, dual wavelength absorbance detector for HPLC. High sensitivity is the characteristic of these Uv-visible detectors which is important for routine analysis. Uv – based detector have application to identification of low level impurity and quantitative analysis. Photodiode array detector are the another one which used to detect signals with low noise. Peak purity is the function of specificity parameter which can be detect by these PDA detectors. These detectors give a way to find drug substance in multi wavelength as caffeine can be detect at different wavelength maxima as at 205nm as well as at 273nm. Some analyte do not show absorbance at Uv-visible range so it can be detect by refractive index detector which propose high sensitivity, stability and reproducibility in signals. Selection of detector
totally based on the nature of component so before selection of detector we have to know about the chemical nature of the analyte.

**Selection of Mobile Phase**
Choosing the appropriate composition and type of mobile phase is necessary because it is one of the variables that affect separation. First we decide during method development that which kind of separation we going to develop as normal phase or by reversed phase which is depend on the nature of analyte for which the method is develop. The main difference between reversed phase and normal phase chromatography is that in reversed phase requires polar solvents such as water, acetonitrile or methanol whereas normal phase systems, non-polar solvents such as hexane or iso-octane are used. Mobile phase selection is also based on the physicochemical properties of the analyte. For proper selection of mobile phase factors to deal with are miscibility with other solvents, polarity, chemical inertness, toxicity, UV cut off wavelength.\[^{12}\] Mobile phase greatly affects the retention time of the component. In some cases when we use composition of two mobile phase as one of them is buffer and another one is solvent like acetonitrile so their composition can also affect the retention time of components as when we change the mobile phase composition there is a change in retention time. For this purpose we set a gradient program for proper separation. This gradient programming can also be use for enhance the chromatographic separation of two overlapping peaks. Most of the pharmaceutical drugs are soluble in polar solvents so we first start with polar solvent water and organic solvents as acetonitrile and methanol. At different composition we mix them and check the retention time for analyte. Mobile phase compositions which show lesser retention time for analyte is selected for overall method for separation. We can also use composition of 3-4 solvent if require for greater selectivity. It is show by various practices that mixture of THF, methanol, water and acetonitrile show separation for most of the components in reversed phase chromatography in short duration.\[^{14}\]

**Selection of column**
Column is work as a heart for HPLC. As when we develop an analytical method, our first priority is to know the characteristic of analyte then for column selection we should also know the classification of columns and their configuration so we can easily select an appropriate column for separation. Columns are classified by the USP which is known as L nomenclature for column. As it is subdivided into categories like L1 is a category of octadecilsilane (C\(_{18}\)) as bonded phase. According to their bonded phase there are other classes.
for columns in USP nomenclature of columns. A small variation in column configuration can highly affects the chromatographic separation so for this purpose there is no specific guideline therefore we use hit and trial method for selecting a column. Literature on that drug like their chemistry and previous related method for that drug for identification can helps to find a column for separation. Generally, the properties of stationary phase have the tremendous effect on selectivity, capacity factor, efficiency and elution. Different kinds of matrices are used to support stationary phase as silica, alumina and polymers. Silica is the one supporting material for columns which used from many years and it has a great absorbing power. It can easily derivatized, stable over a high range of high pressure and easily available in spherical size. Inertness of silica over a wide range of chemical also makes this choice of selection, as at low pH system it doesn’t change their configuration. As silica has a number of good properties as a supporting media but it also show a one weak aspect as it gets dissolve above neutral pH.[13] For this purpose polystyrene matrices packing are utilize which get stable over a high pH range, as these kind of polystyrene supporting material have no silanol groups their selectivity is also different from silica based material. Selectivity of column is totally based on the molecular structure and interactions of molecules within stationary phase and mobile phase which can’t be judge theoretically so we have to focuses on different experimental trials for selection of column for method development.[14]

Method optimization
After developing a method we should optimize it for every factor as mobile phase ratio, gradient programming and pH of buffer, column temperature, flow rate and diluents which affects the chromatographic separation so the developed method will show reproducible results. Sensitivity of analytical method can determine during method optimization, for this purpose we used stability indicating assay methods for drugs.[15]

Method Validation
After developing an analytical method we should validate that particular method as per the protocol. It is defined as the method of collecting the documented evidence which reveals that method performs according to the intended motive and it follow all predetermined specification. Quality of data is assured by the use of the four major components as: analytical instrument qualification, analytical method validation, system suitability tests and quality control checks. Validation is not only a requirement of researcher to validate their method but it is a regulatory parameter as International Council for Harmonization (ICH)
regulate the method development and validation so they give a proper guideline for method validation known as ICH (Q2 R1). Different parameters are comes under this guideline to validate a method as mention here:

1. Specificity or selectivity
2. Linearity & Range
3. Precision
4. Accuracy (Recovery)
5. Solution stability
6. Detection Limit or limit of detection (LOD)
7. Quantification Limit or limit of quantification (LOQ)
8. Robustness

1) Specificity
The main objective of specificity is to demonstrate the ability of analytical method to measure accurately and specifically the analyte in the presence of other components as it may be an organic impurity or related substances which can be a part of sample matrix. Specificity determines by injecting an individual solution of each component as main component and their related substance then after a composite solution of all impurities and main component also prepare and inject. Peak resolution and peak purity analysis should check after obtaining a chromatogram. Peak should be well resolved in a composite solution’s chromatogram and purity angle should be less than purity threshold which is obtained by software programming after integration of data. For different drug there are different criteria involve to check the specificity as we can also check relative retention time, retention time and resolution for peaks obtain. Sometime a sensitivity solution is also made to check the relative standard deviation in area at lower concentration limits.[16]

2) Linearity and Range
A linear relationship should be evaluated across the range of analytical procedure. Linearity is performed to demonstrate that the analytical method is capable for obtaining the test results within a selected specified range of concentration. As we know results obtain in chromatogram are in form of absorbance so these results should directly proportional to the concentration range as selected for analysis. For chromatographic purity methods linearity can be obtain within a range of LOQ% to 120% of specified limits for each impurity and main component individually. Three replicates of LOQ, 50, 80, 100 and 120% are injected.
and plot a graph between these levels of concentration and area obtain. Regression line equation is used to determine the correlation coefficient, slope of the regression line and y-intercept. Detect ability of linear range which should obey Beer-Lambert law is dependent on the detector which used in process and on the compound nature. The relation who obeys Beer-lambert law is acceptable as according to this law absorbance is directly proportional to the concentration of analyte. A perfect relation between concentration and absorbance is shown by the value of correlation coefficient which should near 1 as not less than 0.999 for assay methods and not less 0.99 for chromatographic purity methods. The range of an analytical procedure is the upper and lower interval levels of concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.[1]

3) Precision
Precision of an analytical method has an objective to demonstrate that the analytical method for testing is capable to show closeness in data values between a series of measurements which are obtained from multiple sampling of the homogenous sample. As it is determine by three ways:

- **System Precision:** Six replicates (same preparation) inject from homogenous sample and check the percentage relative standard deviation in areas of peak obtains by individual analyte to check the method precision.
- **Method Precision:** Six different preparation of test are inject and check the percentage relative standard deviation in areas of peak obtains by individual analyte to check the system precision.
- **Intermediate Precision:** Analysis of a homogenous sample by different analyst on different days in different instrument and finally check the percentage relative standard in areas of peak obtain by individual analyte to check the intermediate precision.[13]

4) Accuracy
Objective of accuracy determination is to demonstrate that the analytical method is capable to yield data values close to true values. Accuracy of an analytical method determines by the recovery studies. For methods of chromatographic purity or related substance accuracy can be determine by these recovery studies as we have to spike individual impurity in test solution within a range of LOQ, 100 and 120% individually and at these three levels we calculate the recovery amount from test solution. Accuracy is then calculated from the test
results as the percentage of the analyte recovered by the test solution in form of percentage recovery.[16]

5) Solution stability
Solution stability is a function of shelf life or storage period or date of expiration. Stability of solution should unchangeable by environmental conditions as humidity, temperature with respect to time. Analytical method for related substance or chromatographic purity also involve solution stability parameter in validation process so to check the solution stability we have to inject a standard solution at different time period, start with 0 hours (freshly prepared) then up to 48 hours then evaluate the system suitability data in which we check the retention time, resolution, peak symmetry, relative retention time, peak tailing.[16]

6) Limit of detection (LOD)
Lowest amount of analyte which can be detected by the chromatographic separation but it is not necessary that this amount can quantify as an exact value. LOD can determine for drug substance and its related impurity. A blank solution is injected and peak to peak noise ratio we have to calculate from blank chromatograms. Then calculate the concentration at which the signal to noise ratio is about 3:1(the data from standard solution and reference solution is considered for this calculation). After selecting the LOD level we have to prepare six different preparations at LOD level and then check the signal to noise ratio by each which should under limit. This approach is known as signal to noise ratio approach to determine LOD for analyte.[16] There are some another methods available to detect LOD as per the ICH guideline like visual evaluation, standard deviation method. By this standard deviation method we can calculate LOD using a formula after plotting a calibration curve as:

\[ \text{LOD} = 3.3 \frac{\delta}{S} \]

Where, \( \delta \) = Standard deviation of intercept obtain by calibration curve.
\( S \) = Linearity plot slope.[17]

7) Limit of Quantification (LOQ)
It is lowest amount of analyte which can be quantified with suitable precision. The LOQ is determined for drug substance and its related substances or impurity individually. A blank solution is injected and peak to peak noise ratio we have to calculate from blank chromatograms. Then calculate the concentration at which the signal to noise ratio is about 10:1(the data from standard solution and reference solution is considered for this calculation).
After selecting the LOQ level we have to prepare six different preparations at LOQ levels and then check the signal to noise ratio by each which should under limits. This approach is known as signal to noise ratio approach to determine LOQ for analyte. Calculated the concentration at which the signal to noise ratio is about 10:1 (the data from LOD can considered for this calculation). This signal to noise approach can limited to those process which exhibit baseline noise. A typical signal-to-noise ratio is 10:1. LOQ can then be used for linearity study for selecting a range between LOQ to 120%.\[16\] ICH advises the following four methods for determination of LOQ. The acceptable approaches are:

- Visual evaluation.
- Signal-to-noise ratio.
- Standard deviation by calibration plot, which involve the calculation of LOQ by the formula as: \[
    \text{LOQ} = 10 \frac{\delta}{S}
\]

Where, \( \delta \) = Standard deviation
\( S \) = Slope obtain from calibration curves.\[17\]

8) Robustness
The main objective to check the robustness of analytical method is to demonstrate that the analytical method is capable to yield reproducible results under small but deliberate variations in method parameters during normal usage such as column temperature, Buffer pH and flow rate etc. The robustness study is performed for the following parameters:

- **Effect of flow rate:** The flow rate of mobile phase change by \(+0.1\) mL/min and the effect of same on system suitability parameters are study.

- **Effect of column temperature:** The column temperature change by \(\pm5.0^\circ\text{C}\) and the effect of same on system suitability are studied.

- **Effect of pH:** Mobile phase as buffer pH change by \(\pm0.2\) and effect of same on system suitability are studied.\[16\]

**NEED OF METHOD VALIDATION**
It is actually to apply well-define and completely validated analytical methods to yield certain results in the laboratories while analyzing the registration batch and accelerated stability testing samples. It is also important to affirm that each analytical technique has its own properties, which will change from analyte to analyte. In this case, particular validation criteria may require to be developed for each analyte. Additionally, the fitness of the
technique may also be affect by the ultimate aim of the study. Sometime the analysis of sample perform in different sites by different users so it should produce accurate and precise results in every aspects so here we should validate the analytical method according to ICH guidelines and to accommodate proper validation information for various sites and different specification and to fixed inter and intra laboratory accuracy.[18]

CONCLUSION
Analytical method development drastically utilizes by the pharmaceutical industry in research and development department for the drug substance which do not involve in pharmacopoeias or sometime analytical methods are not suggested on a particular instrument of choice. Also a number of alternative drugs are introduced day by day for competition and customer satisfaction so all of them do not have analytical method for testing which require the utilization of method development skills. Regulatory bodies like ICH therefore focus on method development and validation so introduce a guideline for validation as ICH (Q2 R1). There is no proper guideline to develop an analytical method for drug substance assay and chromatographic purity but by the use of literature we can apply a hit and trial method for development of analytical method. Selection of chromatographic conditions, mobile phase and column basically depends on chemical nature of the components which we want to separate out by HPLC so we should have a large data on chemical nature of the analyte. On the other hand we should have a column classification data so the choice of column will be easy for researcher. At every stage of development of method we should also optimize the conditions which select for separation by making small change in the conditions. Method Validation is also a key point to judge the reproducibility of developed method. For method development researchers can use the guideline given by ICH, all the parameters for all factors involve in method validation should under limits for successful method development.

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