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A Review on Analytical Method Development and Validation by High Performance Liquid Chromatography Technique

Siddhata Deshmukh [1]*, Ganesh Chavan [1], Suvarna Vanjari [2], Rajendra Patil [2]

[1]*Department of Pharmaceutical Quality Assurance, J.S.P.M's Rajarshi Shahu College of Pharmacy and Research, Survey No.80, Tathawade, Pune 411033.*

[2] *Department of Pharmaceutical Chemistry, J.S.P.M's Rajarshi Shahu College of Pharmacy and Research, Survey No.80, Tathawade, Pune 411033.*

Abstract

In pharmaceutical industry, the products which are produce of quality, reliable and cost effective. Method development is essential for discovery and evaluation of drugs in the pharmaceutical formulation. It is an analytical tool used to detect, separate and quantify the drug, impurities and drug related degradants that can form on synthesis or storage. It involves the chemistry of drug substance and facilitates the development of analytical method. To optimize the method; a number of chromatographic parameters are evaluated such as mobile phase, wavelength, column and column temperature While performing different steps in method development the problem analysis is performed. The article describes different steps in method development by QbD approach as per ICH guideline Q8, Q9 and Q10.Validation parameters are explained in terms of accuracy, precision, specificity, limit of detection (LOD) , limit of quantitation (LOQ), robustness and system suitability testing. All validation parameters are used in the analysis.

Keywords: Chromatographic technique, method development, QbD approach, validation.

INTRODUCTION

The most powerful tool in analytical chemistry is High performance Liquid Chromatography. High-performance liquid chromatography (HPLC) is a type of column chromatography used for analysis to identify and separate the active compounds. The HPLC column holds the stationary phase, mobile phase moves by pump through the column and detector gives the retention time of the drug molecule. On the basis of rates of migration through column from sample to stationary and mobile phase. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent used. The sample compound with the greater affinity to the stationary layer will travel slower and for a shorter distance in comparison to compounds with less affinity which travel faster and for a longer distance. In small volume to the stream of mobile phase, the sample compound is introduced and it is dense by interaction with stationary phase the amount of retardation depends on the nature of the analyte and composition of both stationary and mobile phase. The time at which a specific analyte elutes from the column called the retention time. Common solvents used include any miscible combinations of water or organic liquids (the most common are methanol and acetonitrile.) Separation has been done to vary the mobile phase composition during the analysis; this is known as gradient elution. Based on the nature of the stationary phase and analyte the solvents to be selected.[1-4]

Types of HPLC

Following types of HPLC generally used in analysis-

Normal phase chromatography

Also known Normal phase HPLC (NP-HPLC), this method separates analytes based on polarity. This type of chromatography uses a polar stationary phase and a non-polar mobile phase. The polar analyte interacted with and is retained by the polar stationary phase. The increase of adsorption strength with increasing analyte polarity and the interaction between the polar analyte and the polar stationary phase increases the elution time.

Reversed phase chromatography

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and polar mobile phase. RPC operates on the principle of hydrophobic interactions, in which result from non-polar analyte, and the non-polar stationary phase.

Size exclusion chromatography

Size exclusion chromatography (SEC),or gel filtration chromatography separates particles on the basis of size. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids.For the molecular weight determination of polysaccharides, this technique is used.

Ion exchange chromatography

Retention is based on the attraction between solute ions and charged sites bound to the stationary phase in this chromatography.Ions of the same charge are removed. This form of chromatography is widely used in purifying water, Ligand exchange chromatography, Ion-exchange chromatography of proteins, High-pH anion-exchange chromatography of carbohydrates and oligosaccharides, etc.[5-7]

HPLC Method Development

There is no availability of an official methods it is required. Alternate method for existing (Non Pharmacopoeial) products is to reduce the cost and time for better precision and ruggedness. Separation quantify the active drug, impurities, any degradants is the main goal of HPLC method.

The criteria for the development of novel methods of drug analysis are:

a) If there is no official drug or drug combination obtained in the pharmacopoeias.

b) When there is no courteous analytical process for the existing drug in the literature due to patent regulations.
c) If there is no analytical method for the drug due to the interference caused by the formulation excipients.
d) Analytical methods for the quantitation of the analyte in biological fluids are found to be unavailable.
e) The analytical procedures may need costly reagents and solvents.[13]
Steps involved in method development of HPLC is as follows:
1) Understand the Physicochemical properties of drug molecule.
2) Selection of chromatographic conditions.
3) Developing the approach of analysis.
4) Sample preparations
5) Method optimization
6)Method validation

1) Understand the Physicochemical properties of drug molecule

For Method development one has to study the physical properties like solubility, polarity, pKa and pH of the drug molecule. The solubility of molecules can be explained on the basis of the polarity of molecules. Polar, e.g. water, and nonpolar, e.g. benzene, solvents do not mix. Based on the solubility of analyte, the mobile phase is selected. Selecting a proper pH leads to symmetrical and sharp peaks in HPLC. To achieve low detection limits, , and reproducible retention times the sharp and symmetrical peaks are necessary. The pH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion.[15]

$pH = - \log10[H_3O^+]$.

2) Selection of Chromatographic conditions

Buffer Selection:
By the desired pH, buffer is selected. pH 2 to 8 is range for reversed phase chromatography.
General considerations during buffer selection:

- Phosphate is more soluble in methanol/water.
- Some salt buffers are hygroscopic. This may lead to changes in the chromatography (increased tailing of basic compounds, and possibly selectivity differences).
- TFA can degrade with time, is volatile, absorbs at low UV wavelengths.
- Microbial growth can damage chromatographic performance.
- At pH < 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns.
- Ammonium bicarbonate buffers are usually stable for only 24 to 48 hours.
- The buffers should be filtered through a 0.2-µm filter.
- Mobile phases should be degassed.

Buffer Concentration:
A buffer concentration of 10-50 mM is adequate for small molecules, no more than 50% organic should be used with a buffer. It is based on the specific buffer and its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC.[8]
Selection of the column
Stationary phase/column selection is most important step in method development. Without the availability of a stable, high performance column, the development of rugged and reproducible method is difficult. A C8 or C18 column suitable for all samples and is strongly recommended.[9]
Selection of the mobile phase:
The mobile phase consists of an aqueous buffer and a non-UV active water miscible organic solvent in reverse phase chromatography. Acetonitrile (ACN) ,methanol (MeOH) and tetrahydrofuran (THF) are commonly used solvents in RP-HPLC. Based on the ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixture, the mobile phase and gradient conditions are selected. The aqueous buffer serves several purposes. At low pH, the mobile phase protonates free silanols on the column and reduces a peak tailing. Conversely, at higher pH neutral basic compounds will be more retained, and ionized acidic compounds will elute earlier.If the pKa of a compound is similar to the pKa of the buffer then peak splitting may be observed, and the analyte elutes as both a charged and uncharged species.Mixture of acetonitrile and water is the best initial choice for the mobile phase during method development.[10]
Increasing Polarity Order: Acetonitrile > Methanol > Ethanol > Pyridine > 2-propanol > Acetone > Ethyl acetate > Diethyl ether > t-Butyl ether > Chloroform > Toluene > Benzene > n-hexane > Cyclohexane
Selection of detector:
Selection of detector depends on the chemical nature of analyses, potential interference, limit of detection required, availability and/or cost of detector. UVvisible detector is dual wavelength absorbance detector for HPLC. Photodiode Array (PDA) Detector used for Waters analytical HPLC, preparative HPLC.Multi-wavelength Fluorescence Detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds.[11]Table(a)

3) Developing the approach for analysis

The analytical method on RP-HPLC the first step performed is the selections of various chromatographic parameters like selection of mobile phase, selection of column, selection of selection of pH of mobile phase, flow rate of mobile phase in developing method. All of these parameters are selected on the basis of trials and followed by considering the system suitability parameters. Detection wavelength is usually isobestic point in the case of simultaneous estimation of 2 components. After this the linearity of the drug is studied in order to know the range of concentrations up to which the drug follows the linear pattern. Analysis of the laboratory mixture is also carried out in order to know practicability of developed method for simultaneous estimation. After that analysis of marketed formulation is carried out by diluting the marketed formulation up to concentration range of linearity.[8]

4) Sample preparation

Sample preparation is a critical step.The purpose of sample preparations to create a processed sample that leads to better analytical results compared with the initial sample.

5)Method Optimization

To get desired separations and sensitivity, experimental conditions should be optimized. The planned or systematic examination on parameters such as mobile phase. pH, flow rate, injection volume, sample amounts will be used for stability indicating assay.[14]

6) Method Validation

The analytical method validation is helpful for new method to give reproducible results in same or different laboratories Method validation can be used to check or inspect the reliability, quality of analytical results. Use of equipment that is within specification, working correctly and adequately calibrated is fundamental to the method validation process. Analytical methods need to be validated or revalidated.[16-18]

Components of method validation

1. Linearity
2. Accuracy
3. Precision
4. Limit of detection
5. Limit of quantitation
6. Specificity
7. Range
8. Robustness

Linearity- Its ability to obtained results, which are directly proportional to the concentration of analyte in the sample.

Linearity is usually expressed as the confidence limit around the slope of the regression line. For the establishment of linearity, minimum of five concentrations is recommended by ICH guideline.[18]

$R^2>0.995$

Range- It is the interlude or intermission between the higher and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of accuracy, precision and linearity.

Precision- It is defined as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision may be considered at three levels such as repeatability, intermediate precision and reproducibility.[19]

1) Repeatability

It is the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

2) Intermediate precision

It is performed within-laboratories variations such as different analysts, different equipments, different days, etc.

3) Reproducibility

It is performed between laboratories for collaborative studies.

Accuracy- It is the nearness of obtained value to the true or accepted value. It differentiates between the mean value found and the accepted value.These should be checked by compairing with standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. It may often be expressed as the recovery by the assay of known, added amounts of analyte.[8]

Limit of detection- The detection limit of procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. In analytical procedures that exhibit baseline noise, the LOD can be based on a signal-to-noise (S/N) ratio (3:1), which is usually expressed as the concentration of analyte in the sample.

The signal-to-noise ratio is determined by: s = H/h Where H = height of the peak corresponding to the component. h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.[21]

$$LOD = \frac{3.3\sigma}{s}$$

Where σ-Standard deviation
s-Average of slope

Limit of quantitation- The limit of Quantitation is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. For analytical procedures such as HPLC that exhibit baseline noise, the LOQ is generally estimated from a determination of S/N ratio (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviation as well.[21]

$$LOQ = \frac{10\sigma}{s}$$

Where σ-Standard deviation
s-Average of slope

Robustness- It is defined as the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters such as pH, mobile phase composition, temperature and instrumental settings and provides an indication of its reliability during normal usage.[22]

System suitability- System suitability testing performed by the pharmaceutical industries to decide whether a chromatographic system is used in a routine manner in pharmaceutical laboratories. In the system suitability tests, parameters are as follows:

1. Number of theoretical plates or Efficiency (N).
2. Capacity factor (K).
3. Separation or Relative retention (α).
4. Resolution (Rs).
5. Tailing factor (T).
6. Relative Standard Deviation (RSD)

Number of theoretical plates or Efficiency(N): Efficiency is defined as the measurement of the degree of peak dispersion and it should have the column characteristic in specific column. The efficiency is explained in terms of

number of theoretical plates. The formula of calculation of N is illustrated bellow in the following.

$$N=16 \left(\frac{t_R}{W}\right)$$

N = Efficiency / Number of theoretical plates.
t_R = Retention time of analyte.
h = Height of the peak.
W = Gaussian e peak width .

Tailing factor- Under ideal conditions the chromatographic peak have a Gaussian shape. The regulatory organizations like USP and EP have mainly recommended this system suitability parameter. In most cases the asymmetry factor are roughly same and rarely accurate. Values should between 1.0-1.5 shown in Table(b) . The peak asymmetry is computed by utilizing the following formula.
As = B/A
Where: As = peak asymmetry factor.
B = distance from the peak midpoint to the trailing edge.
A = distance from the leading edge of peak to the midpoint.
The acceptance criteria (limits) of system suitability parameters are shown in the following table(c)

Problem Analysis in Method Development-
Successful chromatographer is not the one who can only develop analytical methods/procedures, but the one who can properly maintain the instrument and has good experience of trouble-shooting. While performing different steps in HPLC method such as mobile phase preparation, sample preparation, introduction of sample, column equilibrium, the concept of Preventive Maintenance (PM) should be routine.[2]Table(d)

Quality by Design approach in Analytical Method Development:
The concept of quality by design (QbD) has been implemented in the pharmaceutical industry through several initiatives such as the FDA's cGMP for the 21st Century as well as with the regulatory documents ICH Q8, Q9 and Q10 and the FDA guidance on Process Validation. The basic concept of QbD is "The Quality cannot be tested into the product, but it should be built into it." Quality is "Degree to which a set of inherent characteristics fulfils requirements" (ICH Q9) .Quality by Design (QbD) has become an significant concept for the pharmaceutical industry that is further defined in the International Conference on Harmonisation (ICH) guidance on pharmaceutical development as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and quality risk management." The foundation of Quality by Design is ICH Guidelines. The outcome of using QbD concepts is a well-understood product and process that consistently delivers its intended performance. During development of analytical methods, same QbD principles can been applied to the development of analytical methods. For the purpose of QbD for HPLC methods, robustness and ruggedness should be verified early in the method development stage to ensure method performance over the lifetime of the product.[23-25](Fig.1)
Method development by QbD approach
Step 1: Defining method intent:
Pharmaceutical QbD is a systematic practical approach that begins with predefined objectives and lay emphasis on product and process understanding and control so the goals of HPLC method development have to be clearly defined.The eventual goal of the analytical method is to separate and quantify the main compound.
Step 2: Performing experimental design:
A systematic experimental design is needed with obtaining in-depth method understanding and performing optimization. It forms a chromatographic database that will help out with method understanding, optimization, and selection. Design of experiments (DOE) has been often executed to optimize analytical methods because of its application, like a decrease in total number of attempts that need be carried out bringing about less reagent utilization and extensively lower laboratories task.
Step 3: Evaluation of experimental results and selection of final method conditions*:*
The conditions for the method need to be evaluated using the threetiered approach. At first the conditions should be evaluated for peaks symmetry, peaks fronting and peaks tailing.The best suited experimental conditions shall be optimized using design expert software.
Step 4: Performing risk assessment with robustness and ruggedness evaluation*:*
Once the final method is selected against method attributes, it is highly likely that the selected method is reliable and will remain operational over the lifetime of product. The fourth step of method development is mainly for the method verification and finalization and the evaluation of method robustness and ruggedness to be carried out. Structured methodologies for risk assessment such as Ishikawa diagram can be implemented.[25-27](Fig.2)
Methods of risk assessment are mentioned in ICH guideline Q9 as follows:
Failure Mode Effects Analysis (FMEA);
Failure Mode, Effects and Criticality Analysis (FMECA);
Fault Tree Analysis (FTA);
Hazard Analysis and Critical Control Points (HACCP);
Preliminary hazard Analysis (PHA);

ICH Guideline and QbD:
The underlying principles of QbD i.e. science- and risk-based product development, risk assessment, lifecycle approach and method design are explained in the quality guidelines of international conference on harmonization i.e. ICH Q8 Pharmaceutical Development, ICHQ9 Quality Risk Management and ICH Q10 Pharmaceutical Quality System.[28,29]

Table (a): Detectors used in HPLC	
Detector	Type of compound can be detected
UV-Visible & Photodiode array	Compounds with chromophore,such as aromatic rings or multiple alternating double bonds.
Fluorescence detector	Fluorescent compunds,usually with fused rings or highly conjugated planer system.
Conductivity detector	Charged compounds,such as inorganic ions and organic acid.
Electrochemical detector	For easily oxidized compounds like quinones or amines.
Refractive Index detector & Evaporative light scattering detector	Compounds that do not show characteristics usable by the other detectors,eg. Polymers,saccharides

Table(b):Acceptance Criteria for Tailing factor	
Tailing Factor	Character
1.0-1.05	Excellent
1.2	Acceptable
2	Unacceptable
4	Very poor

Table (c):Acceptance criteria (limits) of system suitability parameters		
Sr.No.	Parameter Name	Acceptance Criteria
1	Number of theoretical plates or Efficiency(N)	>2000
2	Capacity factor(K)	<1
3	Separation or Relative retention(α)	>1
4	Resolution(Rs)	>1.5
5	Tailing factor or Asymmetry(T)	<2
6	Relative Standard Deviation(RSD)	<2

Table (d): Troubleshooting in HPLC		
Problem	Reason	Resolution
Tailing peaks.	Wrong pH (some peaks are tailing while others are symmetrical).	The pH of the mobile phase should be 1.5 units or more above or below the pKa value of the analyte to have all molecules either in the charged or in the neutral state.
	Void volumes (all peaks are tailing).	Check connections, replace guard column, replace column.
	Stationary phase degradation.	Loss of ligands when the column is exposed to extreme pH or when the column is very old can lead to peak fronting. Replace the column.
Broad but symmetrical peaks.	Column over loading/sample volume too large.	Reduce the amount of sample injected or use a column with a larger ID.
	Late eluting sample components from the previous injection.	Use double injections-late eluters only appear in the second run.Extend run time, use strong eluting wash step, use gradient.
Ghost peaks.	Carry-over from contaminated injector.	Clean system/injector until obtaining a clean blank.
Shifting retention times.	Change in temperature.	Use column oven or operate in a temperature controlled laboratory.
	Mobile phase not mixed properly.	Make sure the mobile phase is well mixed (isocratic) or the solvent mixer (proportioning valve or pump heads a and B) is working correctly (gradient).
	Solvent evaporation.	Make sure solvent bottles are capped.
Low sensitivity	. Broad peaks.	Optimize running conditions (flow rate,temperature) use column with smaller particles,reduce extra column volumes. Use stronger eluent, use gradient elution

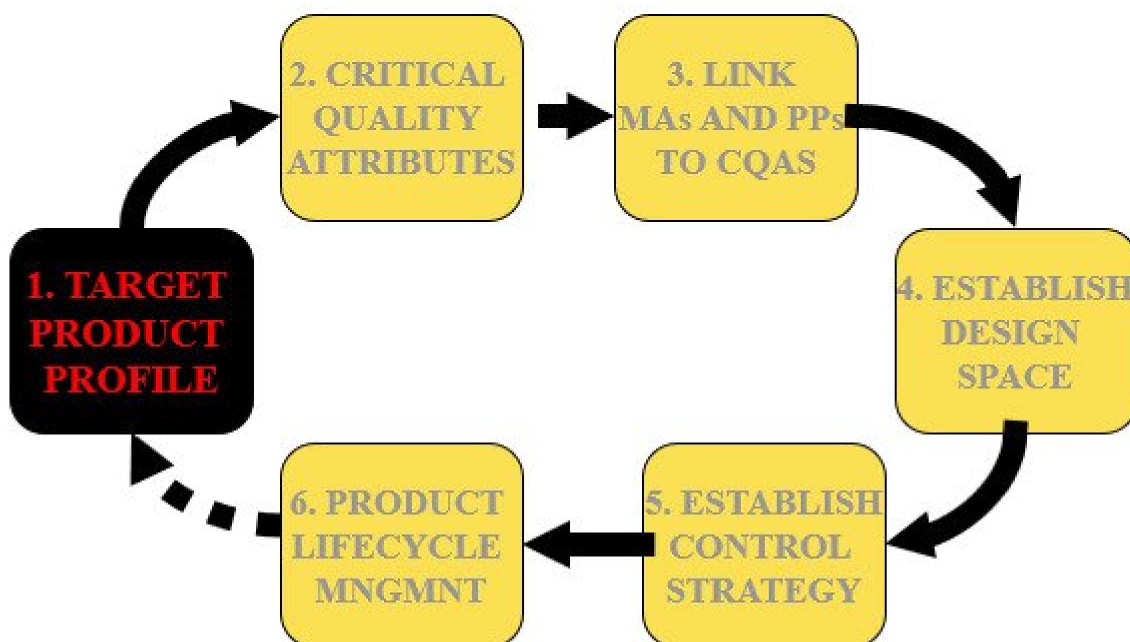

Figure 1 : Overview of QbD

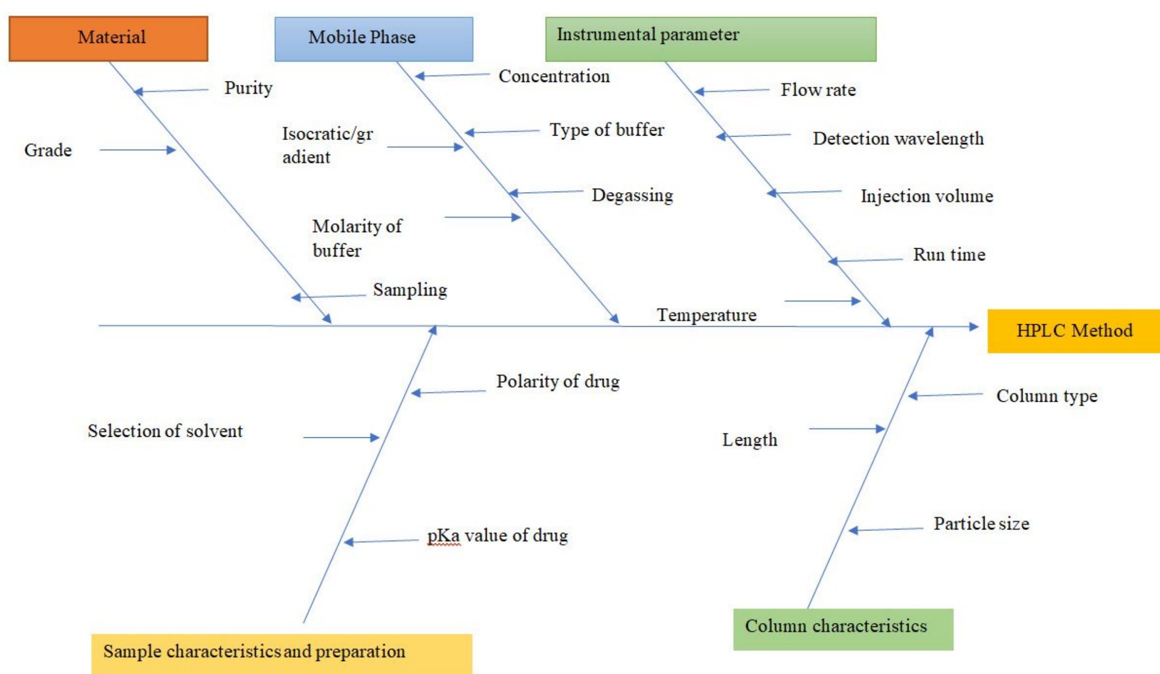

Figure 2: Ishikawa diagram

CONCLUSION

HPLC method development and validation of optimized method is described in this review article. The study of the pKa, pH and solubility of the compound is important in the HPLC method development. Selection of buffer and mobile phase composition plays a important role in the separation. The information about method validation parameters are well explained. n as per ICH guidelines. Optimized method is validated with various parameters (e.g. accuracy, precision, linearity robustness, detection limit, quantitation limit). It is an important procedure in the pharmaceutical industry and it is utilized to ensure that quality is built in to the processes supporting drug development and manufacture. By changing the temperature, gradient slope, and flow rate, the type and concentration of mobile-phase. In chromatographic technique is the most common analytical tool in pharmaceutical quality control, analytical method development, formulation and development protocols for dosage form so implementation of QbD provides regulatory flexibility and requires high degree of robustness, product quality, and analytical method understanding. Method transfer in QbD is useful for

analytical methods and more efficient, and continuous improvements for future methods.

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