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Review Article

PARTICLE SIZE ROLE, IMPORTANCE AND STRATEGY OF HPLC ANALYSIS-AN UPDATE

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ABSTRACT

In HPLC analysis particle size of column play a very big role in new method development and also for modification of exiting method. Method development and validation is an act of proving that any procedure, process, equipment, material, activity or system performs as expected under given set of conditions and also give the required accuracy, precision, sensitivity, ruggedness, etc. When extended to an analytical procedure, depending upon the application, it means that a method works reproducibly, when carried out by same or different persons, in same or different laboratories, using different reagents, different equipments, etc. In this review article we discussed about the strategy and importance of validation of analytical Methods.

Keywords: Particle size; Validation; Analysis; Accuracy; Precision.

INTRODUCTION

In pharmaceutical industry the role of analytical chemistry is very important in quality control It is one of the important branch of chemistry which is both theoretical and practical science, practical analytical chemistry in a large number of laboratories in many diverse ways. The analytical procedure or method direct to performing the analysis. Analytical method development and validation is essential for any substance either it is herbal product, new process and reaction, new molecules, active ingredients, residues, impurity profiling and component of interest in different matrices. An analytical methodology consists of the techniques, method, procedure and protocol. This methodology includes the required data for a given analytical problem, required sensitivity, required accuracy, required range of analysis and required precision to the analyst. In pharmaceutical industry the quality control play a role as checking point where HPLC is main tools to authenticate the bulk product as well as finished product. The data produced is required for assuring quality, achieving acceptance of products by the international agencies, mandatory requirement purposes for accreditation as per ISO 17025 guidelines, mandatory requirement for registration of any pharmaceutical product. The main aim is to demonstrate that the procedure is suitable for its intended purpose. The World Health Organization (WHO) has a guideline under the title, 'Validation of analytical procedures used in the examination of pharmaceutical materials'. It appeared in the 32nd report of the WHO expert committee on 'specifications for pharmaceutical preparations' which was published in 1992.

The International Conference on Harmonization (ICH), which has been on the forefront of developing the harmonized tripartite guidelines for adoption in the US, Japan and EC, has issued two guidelines under the titles-'Text on validation of Analytical procedures (Q2A) and validation of Analytical procedure Methodology (Q2B)'.

SEPARATION PROCESS IN HPLC:

The current status in pharmaceutical industry HPLC is one the authentic and reliable technique that has been used in quality control and research laboratories worldwide over the past four decades. It is a well-known and commonly used classical separation technique in laboratories. In Chromatography discovery M. S. Tswet defined the fractional adsorption process, with the explanation that molecules of different analytes have different affinity (interactions) with the adsorbent surface, and analytes with weaker interactions are less retained. In modern high-performance liquid chromatography the separation of the analytes is still based on the differences in the analyte affinity for the stationary phase surface, and the original definition of the separation process given at its inception almost 100 years ago still holds true. Liquid chromatography has come a long way with regard to the practical development of HPLC instrumentation and the theoretical understanding of different mechanisms involved in the analyte retention as well as the development of adsorbents with different geometries and surface chemistry.

Prof. Horvath was introduced the term HPLC in 1970. He builds an instrument that allowed development of a continuous flow of the liquid through the column. This was the origin of high-performance liquid chromatography (HPLC).

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Principles of HPLC:

The principle of HPLC can be defined as the type of sorbent use in separation like adsorption, partition, exclusion, and ion exchange.

Limitation of HPLC: lack of efficiency compared with other techniques. Low diffusion coefficients in the liquid phase involved in slow diffusion of analytes in the stationary phase results in long analysis time. Therefore, the evaluation of the HPLC technique has led to the reduction in the size of the packing materials. As the size of the packing decreased, the efficiency, resolution, and the ability to work with short analysis time increased, resulting in use of different dimension of column.

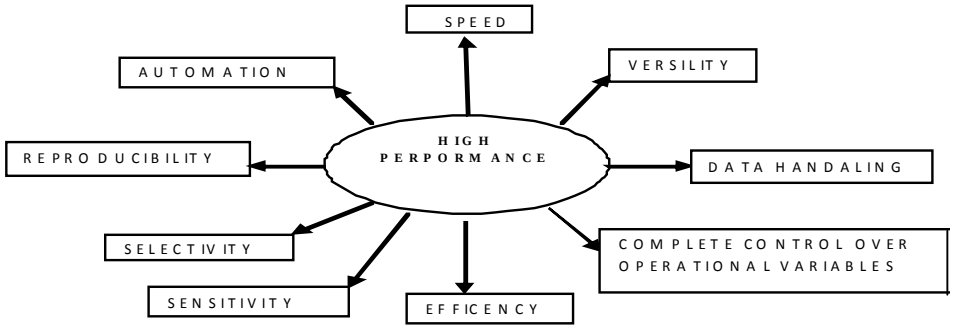
HPLC System:

Typical HPLC system consists of the following main components:

Solvent Reservoirs: Adequate amount of HPLC solvents stored for continuous operation of the system. In modern HPLC could be equipped with an online degassing system and special filters to isolate the solvent from the influence of the environment.

Pump: This provides the constant and continuous flow of the mobile phase through the system. Most modern pumps allow controlled mixing of different solvents from different reservoirs.

Injector: This allows an introduction (injection) of the analyte



mixture into the stream of the mobile phase before it enters the column, most modern injectors are auto-samplers, which allow programmed injections of different volumes of samples that are withdrawn from the vials in the Auto-sampler tray.

Column: This is the heart of HPLC system; Column is a place where separation of constituents takes place. Here the mobile phase is in contact with the stationary phase, forming an interface with enormous surface. Most of the chromatography development in recent years went toward the design of many different ways to enhance this interfacial contact.

Detector: This is a device for continuous registration of specific physical (sometimes chemical) properties of the column effluent. The most common detector used in pharmaceutical analysis is UV (ultraviolet), which allows monitoring and continuous registration of the UV absorbance at a selected wavelength or over a span of wavelengths (diode array detection). Appearance of the analyte in the detector flow cell causes the change of the absorbance. If the analyte absorbs greater than the background (mobile phase), a positive signal is obtained.

Data Acquisition and Control System: Computer-based system that controls all parameters of HPLC instrument (eluent composition (mixing of different solvents); temperature, injection sequence, etc.) and acquires data from the detector and monitors system performance (continuous monitoring of the mobile-phase composition, temperature, backpressure, etc.).

Modern HPLC Column

The separation of analyte mixtures in modern HPLC is performed in the device called the "column." Current HPLC columns in most cases are a stainless steel tube packed with very small (1–5μm) particles of rigid porous material. Packing material is retained inside the column with special end-fittings equipped with porous frits allowing for liquid line connection (to deliver mobile phase to the column). Stainless steel or titanium frits have a pore size on the level of 0.2–0.5μm, which allows for the mobile phase to pass through while small particles of packing material are retained inside the column. The column is the "heart" of the chromatographic system; and it is the only device where actual separation of the analyte mixture takes place.

Role of small particles:

Scaling factors for different size columns (Column Diameters). In practically it is found on the basis of scaling equation (I.D. 1) ^2 / (I.D. 2) ^2.

A relatively large savings in solvent can be gained by moving from a 4.6 mm to a 3.0 mm column. The increases in sensitivity are theoretical. The actual gain will depend on the extra column band spreading of the peak caused by tubing volume and flow cell geometry.

The performance of an HPLC column is found a relation with the particle diameter or particle size. Smaller particle size gives result of higher peak efficiencies. Analyte resolution is dependent upon fundamental relationship of resolution

Type	Column Diameter (mm)	Relative Flow Rate	Relative Sensitivity	Relative Sample Load	Relative Solvent Savings (%)
LC	4.6	1.32	0.7	1.32	-30
LC	4	1	1	1	0
LC	3.9	0.95	1.1	0.95	5
LC	3	0.56	1.8	0.56	44
LC	2.1	0.28	3.6	0.28	72
LC	1	0.06	16	0.06	94
GPC	7.8	1	1	1	0
GPC	4.6	0.25	4	0.25	75

equation.

$$R - 1/4\ (\sqrt{N})\ x\ (k'/k'+1)\ x\ (\alpha\text{-}1)$$

Efficiency Retention Selectivity

This resolution equation comprises three terms: selectivity, retention capacity, and efficiency. Each of these terms is affected by the specific components of an analytical method. A column's particle size, in particular, affects the efficiency term of the resolution equation.

An analyst can keep the same resolution and decrease the length of the column by the same factor as the particle size, shortening the analysis time. It is also beneficial that efficiency is inversely proportional to the square of the peak width—higher efficiencies produce narrower peak widths. Narrow peak widths enhance resolution by lengthening the baseline between two adjacent peaks. (An important note—this does not imply that by simply lowering the particle size we can separate all test mixes. Stationary phase selectivity is still the driving force behind resolution.) Smaller particle size columns improve resolution Efficiency, as measured by the number of theoretical plates (N), is inversely proportional to particle size (dp) and directly proportional to column length (L).

$$N \propto \frac{1}{dp} \qquad N \propto \frac{L}{dp}$$

Pore Size vs Particle Size:

Pore size and particle size both properties are not related. It is observed that most of the reversed –phase HPLC columns are based on a stationary phase bonded to silica particles. The particle sizes generally used of diameters are 5-, 3.5- and 3-µm. Particles of <3-µm are becoming increasingly popular because of their increased column efficiency and relative independence of column efficiency on flow rate. Regarding pore size in HPLC it is vary widely from product to product. There are two general pore size observe that is 6-15 nm (60-150 Å), and 8-12-nm range. Sample molecule molecular weight less than <≈1000 Da separation takes place by the above pore size range but molecules like protein pores have ≥ 30 nm (300 Å).

Observations: Column length, Relative analysis time, Relative Back pressure and Relative solvent saving

It is found in a traditional experiment in HPLC a relation between four parameter – Column length, Relative analysis time, Relative back pressure and Relative solvent saving. As the column length decrease the analysis time, back pressure and solvent saving percentage all are decreased. From this result a new version of HPLC started that is UPLC.

Column Length (cm)	Relative Analysis (time)	Relative Back pressure	Relative Solvent Savings (%)
30	2	2	-100
25	1.67	1.67	-83
15	1	1	0
10	0.67	0.67	33
5	0.33	0.33	67
3	0.2	0.2	80
2	0.13	0.13	87

TYPES OF HPLC

HPLC are broadly classified in four main types-

(A) Normal Phase Chromatography (NPC)

(B) Reverse Phase Chromatography (RPC)

(C) Ion Exchange Chromatography (IEX-C)

(D) Size Exclusion Chromatography (SEC).

HPLC classification is based on the dominant type of molecular interaction employed between the solute and stationary phase.

S. No.	Types of Force	Types of HPLC
1	Polar forces	Normal-phase HPLC
2	Dispersive forces	Reversed-phase HPLC
3	Ionic forces	Ion-exchange HPLC
4	Size-exclusion HPLC is based on the absence of any specific analyte interactions with the stationary phase.	

High performance liquid chromatography (HPLC) is an important tool for the analysis of pharmaceutical drugs, for drug monitoring and for quality assurance. This tool is very authentic and reliable in the field of pharmacy. Here analyst performs qualitative and quantitative analysis both. The method differentiate complex mixtures, for example, herbal medicine plant extracts, to be separated into individual compounds, which can be identified and quantified by suitable detectors and data handling systems. Separation and detection occurs at ambient temperature or slightly above. Therefore, the method is ideally suited for compounds of limited thermal stability. State-of-the-art HPLC equipment can automate HPLC separations, using automatic samplers, injectors, microprocessor-controlled analytical conditions and Chem. Stations for data evaluation.

Basic needs for automation are:

• Very well precision of the liquid chromatography system,

• Authentic data evaluation with report printouts,

• The facility to store chromatograms and results,

• The possibility to detect leaks and other errors for safety reasons, and

• Implemented OQ/PV tools in the HPLC system.

Automation makes very well précised result by minimize the human errors along with this it also enhance the throughput in pharmaceutical laboratories and companies. An observation found that the mobile phase in NP HPLC are based on non-polar solvents (such as hexane, heptane, etc.) with the small addition of polar modifier (i.e., methanol, ethanol).Variation of the polar modifier concentration in the mobile phase allows for the control of the analyte retention in the column. For this the typical polar additives are alcohols (methanol, ethanol, or isopropanol) added to the mobile phase in relatively small amounts. In NP-HPLC the dominant force are polar in nature and these force makes more strong by even adding only 1 % v/v variation of the polar modifier in the mobile phase and by this the results are significant shift in the analyte retention. For the analysis of NP-HPLC the packing materials traditionally used are usually porous oxides such as silica (SiO2) or alumina (Al2O3). Surface of these stationary phases is covered with the dense population of OH groups, which makes these surfaces highly polar. Analyte retention on these surfaces is very sensitive to the variations of the mobile-phase composition. Chemically modified stationary phases can also be used in normal-phase HPLC. Silica modified with trimethoxy glycidoxypropyl silanes (common name: diol-phase) is typical packing material with decreased surface polarity. Surface density of OH groups on diol phase is on the level of 3–4μmol/ml, while on bare silica silanols surface density is on the level of 8μmol/ml. The use of diol-type stationary- phase and low-polarity eluent modifiers [esters (ethyl acetate) instead of alcohols] allow for increase in separation ruggedness and reproducibility, compared to bare silica.Selection of using normal-phase HPLC as the chromatographic method of choice is usually related to the sample solubility in specific mobile phases. Since NP uses mainly nonpolar solvents, it is the method of choice for highly hydrophobic compounds (which may show very stronger interaction in reversed-phase HPLC), which are insoluble in polar or aqueous solvents.

METHOD DEVELOPMENT STRATEGY

In HPLC method development considering the systematic approach is need to develop the method for this scientist first define the goal and then according to that perform the task. For this work can divided in three steps which is useful to perform.

(A) Literature: perform thorough literature search for similar separations through scientific journals, application databases on the internet.

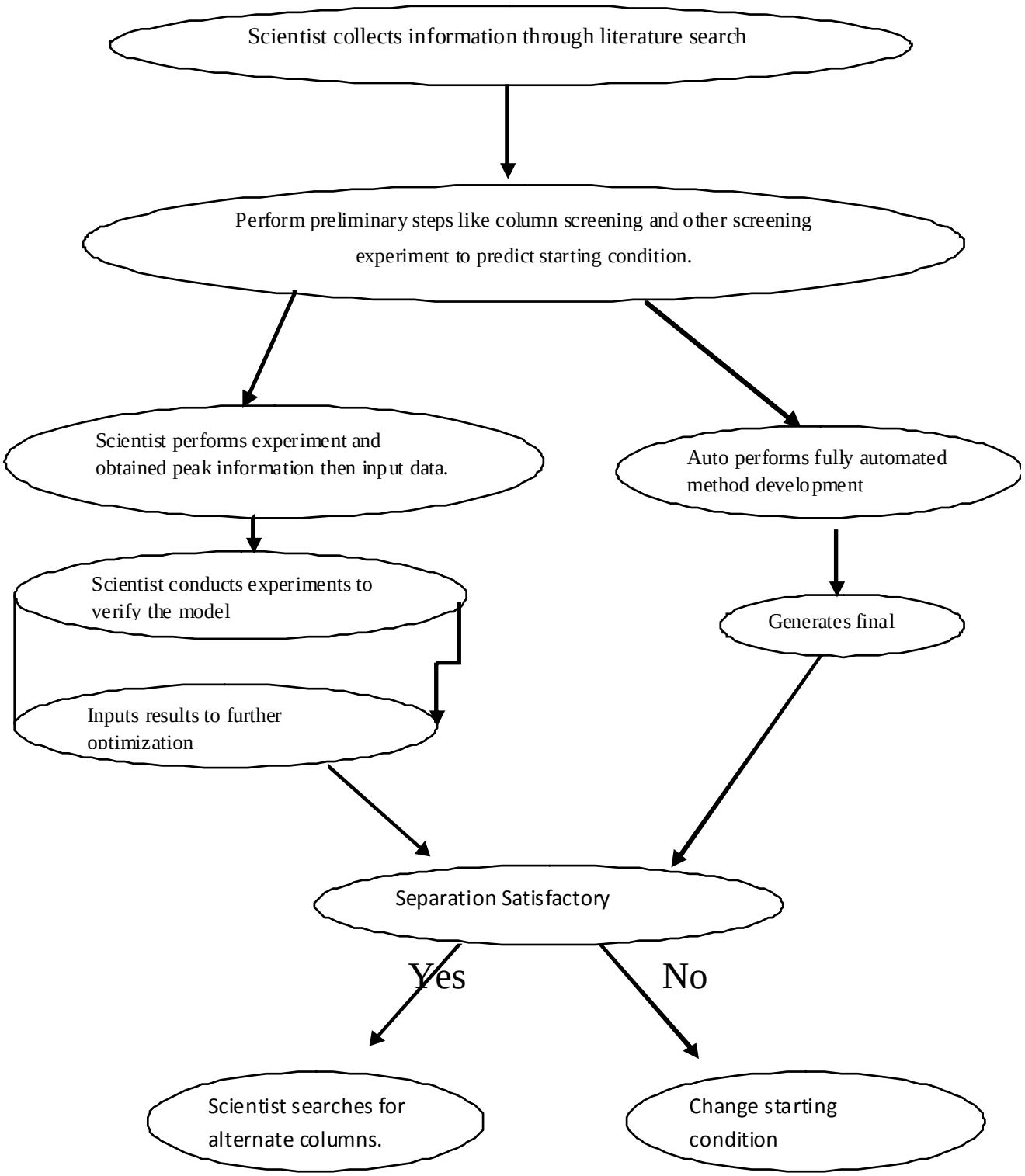
(B) Determination of Chemical nature of the analytes: Find out the physicochemical characteristics of the analytes such as UV absorbance, solution solubility, stability, and pKa, functional groups, acid/base properties.

(C) Use modern technologies such as computer-assisted method development tool and automated column switching system to carry out mobile phase and stationary phase screening to obtain preliminary/ promising chromatographic conditions, then examine the data and finalize the HPLC method, identify equivalent columns, and validate the final method.

Method Development:

If in literature there is no any perfect method described new method are produced for the analysis of novel drugs. To purpose the analyze the existing either pharmacopoeial or non-pharmacopoeial products are reduce the cost with better result in respect to accuracy, precision and ruggedness. These methods are optimized and validated through trial runs.

Strategy of method development can be describe in flow diagram



Alternate methods are proposed and put into practice to replace the existing procedure in the comparative laboratory data with all available merits and demerits.

The following points are taken in consideration for the development of method for drug analysis.

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

b) When there is no decorous analytical process for the existing drug in the literature due to patent regulations.

c) When there are no analytical methods for the formulation of 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 existing analytical procedures may need costly reagents and solvents. It may also involve burdensome extraction and separation procedures.

In HPLC analysis method development define and follow the following steps to achieve the goal.

a) Describe the sample,
b) Establish goals,
c) Consider sample preparation
d) Choose detector
e) Solubility of Analytes
f) Choice of Column

a) Describe the sample: Number of compounds, Chemical structures/functional groups, Molecular weights,pKa values for ionizable species,UV spectra,Sample matrix,Solubility, Concentration level (analytes).

b) Establish Goals: Are all components in the sample known? (Raw material source may have changed in generic product), Are there different sample matrices or packaging to be investigated.(Packaging and matrix extractables may vary),Number of samples,(How many tests will need to be performed on each sample),What LC equipment is available in the lab.

c) Sample Preparation: what is the Nature of the Sample? Solution ready for injection (filtration?) Solution that needs dilution, pH adjustment or addition of additives/preservatives Solid substances that are soluble in the mobile phase Solution containing interfering substances or "column killers" that must be removed before injection. (Accelerated Oxidation, addition of acid or base) Analytes in an insoluble matrix (extraction).

d) Choice of Detector: Selection of detector in HPLC depends on the nature of the analyte. Universal versus selective detection, UV-detector the PDA detector is very well suited for method development RI-detector Universal, no gradients, less sensitive Fluorescence Very sensitive and selective Others Electrochemical/conductivity Evaporative/laser light scattering Mass Spectrometry (MS),Nuclear Magnetic Resonance (NMR),Choice of Chromatographic Mode, Reversed phase is preferred, exceptions are High molecular weight compounds Size exclusion or ion exchange Non-porous reversed-phase (NPRP) occasionally used —Optical isomers (enantiomers) Chiral columns —Other isomers (stereo or positional isomers) Normal-phase chromatography is often best —Special compound groups Carbohydrates (e.g. NH2 column with MeCN/water) Proteins/peptides (RP with TFA/MeCN gradient, IEX, SEC) Amino acids (IEX with post column derivatization, Pico•Tag®, AccQ•Tag™) Inorganic ions (ion chromatography).

e) Solubility of Analytes: Check the solubility of the analyte and final the column for method development, the approach of selection of stationary phase is like. First check the solubility in water and buffer if it positive decide column reversed phase if not then check in organic system and column may be normal phase.

f) Choice of Column: For selection of column take important parameter in consideration like material surface properties, particle size, elutent strength of mobile phase which improve the quality chromatogram.

Method Validation:

The following parameters are performed for the perfect method validation in analytical method development. Components of method validation the following are typical analytical performance characteristics which may be tested during methods validation.

a) Accuracy
b) Precision
c) Repeatability
d) Intermediate precision
e) Linearity
f) Limt of detection (LOD)

g) Limt of quantitation (LOQ)

h) Specificity

i) Range

j) Robustness

k) System suitability determination

l) Forced degradation studies

m) Solution stability studies

Accuracy is defined as the nearness of a measured value to the true or accepted value. Practically accuracy indicates the deviation between the mean value found and the true value. It is determined by applying the method to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. It may often be expressed as the recovery by the assay of known, added amounts of analyte. The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample. Precision is a measure of the reproducibility of the whole analytical method. It consists of two components: repeatability and intermediate precision. Intermediate precision is the variation within a laboratory such as different days, with different instruments, and by different analysts. The precision is then expressed as the relative standard deviation. Accuracy and precision are not the same. A method can have good precision and yet not be accurate. Repeatability is the variation experienced by a single analyst on a single instrument. It does not distinguish between variation from the instrument or system alone and from the sample preparation process. During validation, repeatability is performed by analyzing multiple replicates of an assay composite sample by using the analytical method. The recovery value is calculated. Linearity is the ability of analytical procedure to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample. If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to concentration of analyte in samples within a given range. Linearity is usually expressed as the confidence limit around the slope of the regression line.

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