Review Article

Analytical Method Validation Parameters: An Updated Review

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Received: 08-02-2020; **Revised:** 22-03-2020; **Accepted:** 27-03-2020.

ABSTRACT

Analytical method development aids to understand the critical process parameters and to minimize their influence on accuracy and precision. A validated systematic approach ensures that it provides consistent, reliable, and accurate data. The parameters depicted here are according to ICH guidelines and include accuracy, precision, specificity and limit of detection, the limit of quantitation, linearity, range and robustness. Method validation ensures that the selective method will give reproducible, reliable, and consistent results adequate for the intended purpose. It is, therefore, necessary to define precisely both the conditions in which the procedure is to be used and the purpose for which it is intended. Method validation is, therefore, a fundamental component of the measures that a laboratory should establish to be able to create reliable analytical data.

Keywords: Validation, precision, specificity, accuracy, ICH guidelines.

INTRODUCTION

Analytical method validation is the process of demonstrating that analytical procedures are suitable for their intended use. More specifically, analytical method validation is a matter of establishing documented evidence that the specified method will consistently provide accurate test results that evaluate a product against its defined specification and quality attributes. The method should be validatable, transferable, robust, reliable, accurate and precise for day-to-day activities in the Quality Control environment. The method should not enter the validation phase unless it is fully developed. Validation experiments must be properly documented and performed on qualified and calibrated instrumentation and equipment.[1-8]

There are different types of formulation compositions available:

Dose proportional formulation composition.

Pseudo dose proportional formulation composition.

Look alike formulation composition.

Dose proportional formulation composition:

In these types of formulations, composition of active and inactive ingredients proportionally increases as the strength increases. In this case, method validation can be performed on any of the strengths.

Pseudo Dose proportional formulation composition:

In this type of formulations, composition of the active ingredient proportionally increases as the strength increases but the average weight of dosage form remains constant.

The weight of constituents of the matrix is modified so as to keep constant average weight.

Look like formulation composition:

In these types of formulations, composition of the active ingredient proportionally increases as the strength increases but the average weight of dosage form remains constant by a minor change in weight of one of the excipient.

Look alike formulation concept is applicable only for the Drug Product having less content of active ingredient. These current validation characteristics describe the validation parameters stated by the International Conference on Harmonization [ICH] guidelines Q2 (R1)[9-11].

Different Types of Validation characteristics:

- Precision.
- Accuracy.
- Specificity.
- Linearity.
- Range.
- Detection Limit.
- Quantitation Limit.
- Ruggedness.
- Robustness.

System Suitability

System suitability is defined by ICH as "the checking of a system, before or during the analysis of unknowns, to ensure system performance." System suitability criteria may include such factors as plate count, tailing, retention, and/or resolution. System suitability criteria should also

include a determination of reproducibility (%RSD) when a system suitability "sample" (a mixture of main components and expected by-products/interferences) is run.

System suitability testing is an integral part of analytical procedures.

- If the % RSD specification is below 2.0 % five replicates are used.
- If the % RSD specification above 2.0 %, six replicates are used.

The parameters used in the system suitability tests (SST) report are as follows:

- The number of theoretical plates or Efficiency (N).
- Capacity factor (K).
- Separation or Relative retention (α).
- Resolution (Rs).
- Tailing factor (T).
- Relative Standard Deviation (RSD).

Number of theoretical plates/Efficiency (N)

In a specified column, efficiency is defined as the measurement of the degree of peak dispersion and it should have the column characteristics. The efficiency is conveyed in terms of the number of theoretical plates'. The formula of calculation of N is illustrated bellow in the following Figure 1. (Half height method).

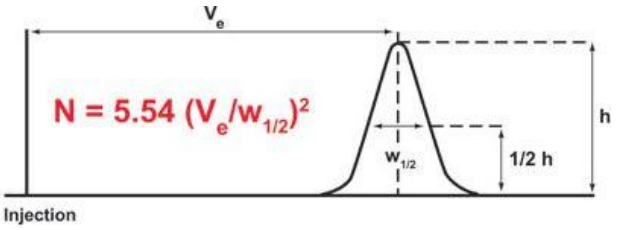

Figure 1: Half height method relating to determination of N.

N = Efficiency / Number of theoretical plates.
V_e = Retention time of analyte.
h = Height of the peak.
$w_{1/2}$ = Gaussian function of the peak width at the half-height.

4-Sigma/tangential method (USP method)

With the help of signa/tangential method N is calculated which is shown in the following figure 2 duly noting the formula for calculation of N.

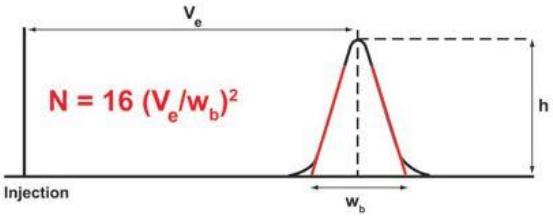

Figure 2: Sigma/tangential method relating to the determination of N.

N = Number of theoretical plates.
V_e = elution volume, retention time or retention distance (mL, sec, or cm).
h = peak height.
w_b = width of the peak at the base line (mL, sec, or cm).

The plate number depends on column length. The theoretical plate number is the measure of column efficiency. As stated by plate theory, the analyte will be in instant equilibrium with the stationary phase and the column has to be divided into the number of hypothetical plates and each plate consists of a fixed height and analyte spends finite time in the plate. Height equivalent to a theoretical plate (HETP) is given by the following formula:

HETP = L/N, Where, (1)

L = length of column.

N = plate number.

Capacity ratio or Capacity factor (k′)

$$k' = \frac{t_R - t_M}{t_M} \quad (2)$$

The above said capacity factor sometimes is called as a retention factor which has no dimension and independent from the flow rate of mobile phase as well as column dimensions which is the measure of the extent of retention relating to an analyte relative to an un-retained peak. Where t_R implies the retention time of the sample peak and retention time of an un-retained peak is t_M.

k' = 0 means no compound is left in the column. Generally the value of k' is > 2.

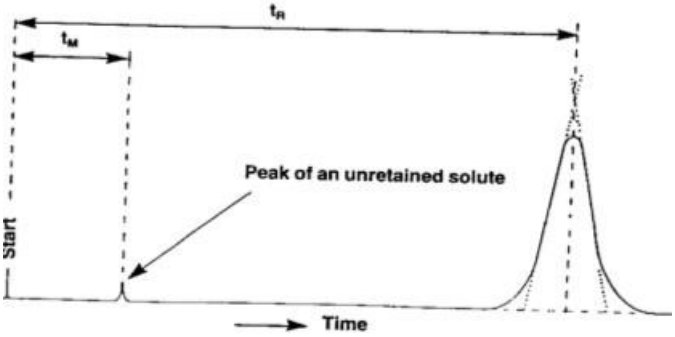

Figure 3: Determination of capacity factor/ capacity ratio.

Relative retention or separation factor (α)

$$\alpha = t_2 - t_a / t_1 - t_a \quad (3)$$

α = Relative retention.

t_2 = Retention time calculated from point of injection.

t_a = Unretained peak time (Retention time (t_R) of an inert component not retained by the column).

t_1 = the retention time from the point of injection of reference peak defined. (Suppose no reference peak is found, the value would be zero).

Resolution (Rs)

Resolution is the capability of the column to separate 2 drugs in 2 individual peaks or chromatographic zones and it is improved by enhancing column length, reduction of particle size and rising temperature, altering the eluent or stationary phase. It can be told in terms of the ratio of separation of the apex of two peaks by the tangential width average of the peaks. By using the following formula, the resolution is calculated.

$$R_s = (t_{R_2} - t_{R_1}) / 0.5 (t_{w_1} + t_{w_2})$$

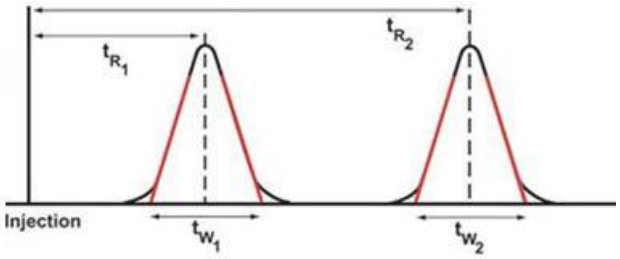

Figure 4: Determination of resolution between two peaks.

t_{R1} and t_{R2} are the retention times for the two peaks of components.

t_{w1} and t_{w2} = At the baseline lies between tangents drawn to the sides of the peaks. (Tangents are drawn at 0.6 times the peak height). If the peaks are correctly symmetric, provided the valley between the two peaks should touch the baseline Rs is 1.5. Generally good value of resolution is Rs ≥2 should be adequate and preferred normally.

Resolution factor (R)

Resolution is a function of capacity factor, the function of selectivity and a function of efficiency (or) number of theoretical plates (N). In order to separate any two peaks, you must have the right capacity factor ideally between 2 and 10, but appropriate selectivity is required i.e., ideally 1.2 and enough efficiency i.e., a number of theoretical plates (more than 2000 theoretical plates). The resolution should be ≥ 1.5. 1.5 defines baseline resolution.

$$R = \frac{k'}{1+k'} \times \frac{\alpha-1}{\alpha} \times \sqrt{\frac{N}{4}} \qquad \text{- (5)}$$

Tailing factor or Asymmetry factor

Chromatographic peak assumed to have a Gaussian shape under ideal conditions. However in practical conditions, there is always a deviation from the normal distribution which indicates non-uniform migration and non-uniform distribution process. Hence the regulatory organizations like USP and EP have recommended this as one of the system suitability parameters. The asymmetry factor and tailing factor are roughly the same and rarely accurate and equal in most cases. Values should normally between 1.0-1.5 and values greater than 2 are unacceptable. The peak asymmetry is computed by utilizing the following formula.

$$A_s = B/A \qquad (6)$$

Where:

A_s = peak asymmetry factor.

B = distance from the point at peak midpoint to the trailing edge. (measured at 10 % of peak height).

A = distance from the leading edge of peak to the midpoint. (measured at 10 % of peak height).

Ideally, peaks should be Gaussian in shape or totally symmetrical. Determination of tailing and asymmetric factors is shown in Figure 5.

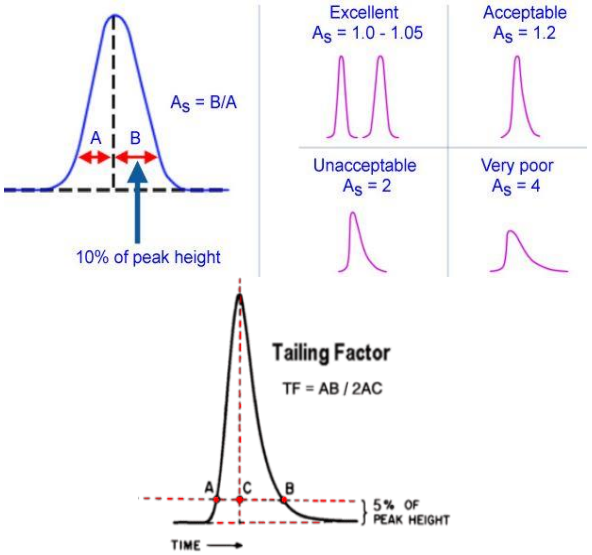

Figure 5: Determination of tailing and asymmetric factor.

Acceptance criteria (limits) of system suitability parameters are shown in the following Table 1.

Table 1: Acceptance criteria for system suitability parameters.

S.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

Specificity

One of the significant features of HPLC is its ability to generate signals free from interference. Specificity refers to the strength of the analytical method to differentiate and quantify the analyte in complex mixtures. An investigation of specificity is to be conducted during the determination of impurities and validation of identification tests.

An ICH guideline defines specificity as the ability to assess unequivocally the analyte in the presence of other compounds that may be likely to be present. Typically these might be impurities, degradants, matrix, etc.

The definition has the following implications:

- **Identification test:** Identification tests should be able to differentiate compounds of closely related structure which are expected to be present i.e., to assure the identity of an analyte.
- **Purity test:** To ensure that the analytical procedure performed allows an accurate statement of the content of the impurity of an analyte i.e. related substances, residual solvents content, heavy metals, etc.
- **Assay:** To arrive at an accurate result, this permits a correct report on the potency or content of analyte in a sample.

Precision

Definition: The closeness of agreement between a series of measurements multiple samplings of the same homogeneous sample under prescribed condition. The precision of test method is usually expressed as the standard deviation or relative standard deviation of a series of measurements.

Precision may be considered at three levels: Repeatability, Intermediate Precision and reproducibility.

Method precision (Repeatability):

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

Intermediate Precision:

It expresses with in laboratory variations; different days, different analysts, different equipment, etc.

Reproducibility:

Precision between laboratories (mostly performed during analytical method transfer).

Relative standard deviation:

This serves as a daily evaluation of the repeatability of the system. Often, the relative standard deviation calculated as % RSD for five or six replicate injections of a reference standard or working standard is measured at the beginning of each set of analyses. Standard deviation is calculated using the formula

$$s = \sqrt{\frac{\sum(x - \overline{x})^2}{N - 1}}$$

$$\overline{x} = \frac{\sum x}{N}$$

Where

s = standard deviation

x = each value in the sample

$\overline{x}$ = mean of the values

N = the no. of values (sample size)

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

Accuracy

Definition: It is the closeness of agreement between the actual value and measured value.

Accuracy is calculated as the percentage of recovery by the assay of the known added amount of the analyte in the sample or the difference between the mean and accepted true value together with confidence intervals.

The ICH guidance recommended to take a minimum of 3 concentration levels covering the specified range and 3 replicates of each concentration are analyzed (totally 3 * 3 = 9 determination)

Specificity

Definition: The ability to assess unequivocally the analyte in the presence of components that may be expected to present, such as impurities, degradation products and matrix components, etc.

Methodology:

Specificity shall be demonstrated by performing Placebo / blank interference and forced degradation studies.

1. Blank interference:

Prepare blank solutions as per the test method and analyze them as per the test method.

2. Placebo interference (In case of Drug products):

Prepare the placebo solution equivalent to the test concentration (Subtract the weight of active ingredient) and analyze it as per the test method.

3. Force Degradation studies[12]:

Degrade the sample forcefully under the various stress conditions like Light, heat, humidity, acid/base/water hydrolysis, and oxidation and ensure the degradation and for peak purity.

Note: Based on the physio-chemical properties and literature stress conditions can be decided.

Linearity and Range

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

Range

The range of analytical procedure is the interval between the upper and lower concentrations of analyte in the analytical procedure has a suitable level of precision, accuracy, and linearity.

Methodology:

At least 6 replicates per concentrations to be studied. Plot a graph of concentration (on the x-axis) Vs mean response (on Y-axis). Calculate the regression equation, Y – intercept and correlation coefficient. Linearity shall be established across the range.

If linearity is not meeting the acceptance criteria, establish the range of concentration in which it is linear.

Correlation Co-efficient

A measure of the strength of linear association between two variables. The correlation will always between -1.0 and +1.0. If the correlation is positive, we have a positive relationship. If it is negative, the relationship is negative.

$$r = \frac{n\sum(xy) - \sum x \sum y}{\sqrt{\left[n\sum(x^2) - (\sum x)^2\right]\left[n\sum(y^2) - (\sum y)^2\right]}}$$

Where,

N = Number of values or elements
X = First Score
Y = Second Score
Σ*xy* = Sum of the product of first and Second Scores
Σ*x* = Sum of First Scores
Σ*y* = Sum of Second Scores
Σx^2 = Sum of square First Scores
Σy^2 = Sum of square Second Scores

Detection Limit

Definition: It is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions.

Methodology:

Following are different approaches:

Visual Evaluation Method:

The visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Based on Signal to Noise Ratio Method:

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Based on the standard Deviation of the Response and the Slope:

The detection limit (DL) may be expressed as:

The formula for calculating LOD is

$$LOD = 3.3\ \delta/S \qquad (7)$$

Where δ = standard deviation of intercepts of calibration curves.

S = the slope of the linearity plot.

The slope shall be estimated from the calibration curve of the analyte.

Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Quantitation Limit

Definition: It is lowest amount of analyte in a sample, which can be quantitatively determined with acceptable accuracy and precision.

Methodology:

Following are different approaches:

Visual evaluation method:

The visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on signal to noise ratio method:

This approach can only be applied to analytical procedures that exhibit baseline noise.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Based on the standard Deviation of the Response and the Slope:

The formula for calculating LOQ is

$$LOQ = 10 \frac{\delta}{S} \quad (8)$$

Where δ = standard deviation of response.

S = Mean of slopes of the calibration curves.

Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Ruggedness

Definition: Ruggedness is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of test conditions such as different laboratories, analysis, instruments, reagent lots, elapsed assay times, temperature, days, etc. It can be expressed as a lack influence of the operation and environmental variable on the test results of the analytical method.

Robustness

Definition: It is a measure of the method's ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure.

Examples of typical variations are:

• Stability of analytical solutions

• Extraction time

In the case of liquid chromatography, examples of typical variations are

• Influence of variations of ph in a mobile phase

• Influence of variations in mobile phase composition

• Different columns (different lots and/or suppliers)

• Temperature

• Flow rate.

The following are the typical method parameters need to change deliberately and verify during method validation:

Flow rate: (+/- 0.2ml/minutes).

Mobile phase composition: (+/- 10% of organic phase).

Column oven temperature: (+/- 5°C).

PH of buffer in mobile phase: (+/- 0.2 units).

Filter suitability: (At least two filters).

Methodology:

a) Mobile phase variation: Prepare the mobile phases by changing organic phase to +/-10 % of the mobile phase composition.

b) Flow rate: Change the flow rate by +/- 0.2 ml/minutes of the target flow rate mentioned in test method.

c) Temperature of the Column*:* Change the temperature of the column by +/- 5.0°C of the target temperature mentioned in Test method.

d) PH of the buffer of mobile phase: Prepare the mobile phases by changing the pH of the buffer by +/- 0.2 units of the pH mentioned in the test method.

e) Filter Suitability: Prepare the test solution as per the test method and filter through two different types of filters. Analyse the sample as per the test method and compare the results against the unfiltered / centrifuged sample.

CONCLUSION

HPLC is probably the most important analytical technique used in pharmaceutical analysis[13,14]. A skilled operator is required to perform HPLC analysis. Method validation is an significant requirement for any package of information submitted to international regulatory agencies in support of novel product marketing or clinical trial applications. Analytical methods should be validated, including methods published in the relevant pharmacopoeia or other recognized standard references. The suitability of all test methods used should always be verified under the real conditions of use and should be well documented. Methods should be validated to include consideration of characteristics included in the International Conference on Harmonization (ICH) guidelines[14] addressing the validation of analytical methods. Generally to evaluate and interpret bioequivalence, bioavailability, toxicokinetic study and pharmacokinetic data bioanalytical method validation[15,16] plays an important role. In this, infact the quantitative determination of drug and its metabolites in the biological fluid can be performed.

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Source of Support: Nil, **Conflict of Interest:** None.