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Chapter 7

Validation of Analytical Methods

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Additional information is available at the end of the chapter

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Abstract

Method validation is a key element in the establishment of reference methods and within the assessment of a laboratory's competence in generating dependable analytical records. Validation has been placed within the context of the procedure, generating chemical data. Analytical method validation, thinking about the maximum relevant processes for checking the best parameters of analytical methods, using numerous relevant overall performance indicators inclusive of selectivity, specificity, accuracy, precision, linearity, range, limit of detection (LOD), limit of quantification (LOQ), ruggedness, and robustness are severely discussed in an effort to prevent their misguided utilization and ensure scientific correctness and consistency among publications.

Keywords: method validation, accuracy, precision, linearity, LOD, LOQ

1. Introduction

Analytical method validation is an essential requirement to perform the chemical evaluation [1–3]. Method validation is a procedure of performing numerous assessments designed to verify that an analytical test system is suitable for its intended reason and is capable of providing beneficial and legitimate analytical data [4–8]. A validation examine includes testing multiple attributes of a method to determine that it may provide useful and valid facts whilst used robotically [9–11]. To accurately investigate method parameters, the validation test ought to consist of normal test conditions, which includes product excipients [11–14]. Therefore, a method validation examine is product-specific.

2. Procedure

2.1. Parameters to be checked for method validation

- Selectivity/Specificity
- Precision

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- Accuracy
- Linearity
- Range
- Stability
- Limit of Detection (LOD) and Limit of Quantitation (LOQ)

2.1.1. Selectivity/specificity

Selectivity of an analytical method is its ability to measure accurately an analyte in the presence of interferences that may be expected to be present in the sample matrix.

Selectivity is checked by examining chromatographic blanks (from a sample that is known to contain no analyte) in the expected time window of the analyte peak. And the raw data for selectivity will be recorded in the raw data in approved formats.

2.1.2. Precision

Precision of a method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings.

Precision is measured by injecting a series of standards or analyzing series of samples from multiple samplings from a homogeneous lot. From the measured standard deviation (SD) and Mean values, precision as relative standard deviation (% rsd) is calculated.

$$\%\text{rsd or CV} = \frac{\text{SD}}{\text{Mean}} \times 100 \tag{1}$$

The raw data for precision will be recorded in the approved format and the acceptance criteria for precision will be given in the respective study plan or amendment to the study plan.

OR

Precision can be also calculated by using Horwitz equation:

The acceptable percent of relative standard deviation results for precision may be based on the Horwitz equation, an exponential relationship between the among-laboratory relative standard deviation (RSD_R) and Concentration (C): [15]

$$\%\text{RSD}_\text{R} = 2^{(1-0.5\log\text{C})} \tag{2}$$

For estimation of repeatability (RSDr), is modified to:

$$\%\text{RSD}_\text{r} = \%\text{RSD}_\text{R} \times 0.67 \tag{3}$$

The Horwitz curve has been empirically derived and has been proven to be more or less independent of analyte, matrix and method of evaluation over the concentration range C = 1 (100%) to C = 10^{-9} by the evaluation of vast numbers of method precision studies. The

modified Horwitz values for repeatability CV given under may be used for guidance. If measured repeatability is outside those values, suggested explanation must be submitted for consideration. The details were presented in **Table 1**.

2.1.3. Accuracy

The accuracy of an analytical method is the degree of agreement of test results generated by the method to the true value.

Accuracy is measured by spiking the sample matrix of interest with a known concentration of analyte standard and analyzing the sample using the "method being validated." The procedure and calculation for Accuracy (as% recovery) will be varied from matrix to matrix and it will be given in respective study plan or amendment to the study plan.

2.1.4. Linearity

The linearity of an analytical method is its capability to elicit check consequences which might be at once, or with the aid of well described mathematical adjustments, proportional to the concentration of analytes in within a given range.

Linearity is determined by injecting a series of standards of stock solution/diluted stock solution using the solvent/mobile phase, at a minimum of five different concentrations in the range of 50–150% of the expected working range. The linearity graph will be plotted manually/using Microsoft Excel or software of the computer (Concentration vs. Peak Area Response) and which will be attached to respective study files.

2.1.5. Range

The range of an analytical method is the interval between the upper and lower levels that have been demonstrated to be determined with precision, accuracy and linearity using the set method. This range will be the concentration range in which the Linearity test is done.

Percent of analyte	**Proposed acceptable % RSD_r (Horwitz value × 0.67)**
100.00	1.340
50.00	1.490
20.00	1.710
10.00	1.900
5.00	2.100
2.00	2.410
1.00	2.680
0.25	3.300

Note: The unmodified Horwitz equation is used as a criterion of acceptability for methods collaboratively tested by CIPAC.

Table 1. Details of Horwitz values.

2.1.6. Stability

Many analytes readily decompose prior to chromatography investigations, for example during the preparation of the sample solutions, during extraction, clean-up, phase transfer, and during storage of prepared vials. Under these circumstances, method development should investigate the stability of the analyte. Accuracy test takes care of stability. It is required to mention in the method how long a sample after extraction can be stored before final analysis, based on the duration taken for accuracy test.

2.1.7. Limit of detection and limit of quantitation

The term LOD is defined as the lowest concentration at which the instrument is able to detect but not quantify and the noise to signal ratio for LOD should be 1:3. The term LOQ is defined as the lowest concentration at which the instrument is able to detect and quantify. The noise to signal ratio for LOQ should be 1:10.

Determination of Limit of Detection (LOD) and Limit of Quantitation (LOQ) from Detector Linearity experiments (applicable to only instrument sensitivity).

LOD and LOQ values are calculated manually by taking Noise to signal ratio of a lowest/known concentration of linearity samples and it will be expressed in μg/ml or ppm. To calculate in %, values of LOD and LOQ will be multiplied by 100/lowest or known concentration of test item (mg/L) taken for analysis of that particular a.i. or impurity analysis.

Calculations of LOD and LOQ values for instrument sensitivity:

$$\mathrm{LOD\,(mg/L)} = 3 \times \frac{\mathrm{Noise}}{\mathrm{Signal}} \times \text{Lowest concentration of the linearity samples}$$

$$\mathrm{LOQ\,(mg/L)} = 10 \times \frac{\mathrm{Noise}}{\mathrm{Signal}} \times \text{Lowest concentration of the linearity samples}$$

Calculations of LOD and LOQ values for method:

$$\mathrm{LOD\,(\%)} = \frac{\mathrm{LOD\,(mg/L)}}{\text{Test item conc.used} \quad \text{for quantification}} \times 100$$

$$\mathrm{LOQ\,(\%)} = \frac{\mathrm{LOD\,(mg/L)}}{\text{Test item conc.used} \quad \text{for quantification}} \times 100$$

OR

2.1.8. Mathematical derivations

2.1.8.1. Determination of limit of detection (LOD) and limit of quantitation (LOQ)

Prepare a series of standard solutions (minimum five concentrations covering working concentrations used for routine analysis) and analyze each solution minimum twice and record the instruments response.

- Using the concentrations and corresponding instrument response, LOD and LOQ can be calculated as follows:

Let the linear regression equation be $Y = a + bX$.

Where, X and Y are the variables (data of two parameters). Generally, X is called the independent variable and Y, the dependent variable.

Take concentration on X-axis and instrument response on Y-axis.

"a" and "b" are the regression constants. Further, "a" is known as the intercept and "b," the slope of the line.

Let (X_1, Y_1), (X_2, Y_2), $(X_3, Y_3)\ldots(X_n, Y_n)$ be the set of values required to be fit in the linear equation.

a. Method of arriving at "a" and "b" $\overline{y}$

i. Tabulate as given below:

X_1	Y_1
X_2	Y_2
.	.
.	.
.	.
X_n	Y_n
Mean, $= \overline{X} = \Sigma X/n$	$\overline{Y} = \Sigma X/n$

ii. Calculate the following parameters:

$$\Sigma xx = \Sigma\left(X - \overline{X}\right)^2 = \Sigma X^2 - (\Sigma X)^2/n$$
$$\Sigma yy = \Sigma\left(Y - \overline{Y}\right)^2 = \Sigma Y^2 - (\Sigma Y)^2/n$$
$$\Sigma xy = \Sigma XY - (\Sigma X)(\Sigma Y)/n$$

iii. Calculate the slope "b," and intercept "a" as given below:

$$b = \frac{\sum xy}{\sum xx}$$

$$a = \overline{Y} - b\overline{X}$$

b. Method of calculation r (correlation coefficient)

$$r = \frac{\sum xy}{\sqrt{\sum xx . \sum yy}}$$

c. Method of calculation standard deviation for "a" and "b"

The standard deviation of the individual deviations of measured values in Y, above and below the linear line (fitted line) is:

$$Sy.x = \sqrt{\frac{\left(\sum yy - \left\{(\sum xy)^2 / \sum xx\right\}\right)}{n-2}}$$

From this, the standard deviation for "a" and "b" are calculated.

Standard deviation

for "a," represented = $Sy.x\sqrt{\frac{\sum X^2}{n\sum xx}}$

as S_a

Standard deviation.

For "b," represented = $Sy.x\sqrt{\frac{1}{n\sum xx}}$

as S_b

2.1.8.2. Application of a, b, and S_a to obtain limit of detection and limit of quantitation

When S_a is obtained for a linear calibration line, then it provides a clear information on the standard deviation of the "Blank" (or Control) response from the instruments.

The LOD and LOQ can be worked out, as given below:

$$\text{LOD} = \frac{|a| + 3S_a}{b}$$

$$\text{LOQ} = \frac{|a| + 10S_a}{b}$$

Note:

- The above calculations can be programmed in a computer but before every use, the computer program must be validated using the example given in section
- The above procedure can also be used for obtaining LOD and LOQ of the method from recovery test results by taking fortified concentration on X-axis and obtained concentrations on Y-axis.

3. Example

In this example, the linear regression equation is employed to find out the extent of linear response of an Detector to a reference analytical standard in the concentration range of about 0.2–3.0 ppm.

Each of these working standards is injected thrice (1 μl per injection), and the peak area counts corresponding to the active ingredient peak are given below.

From the peak areas corresponding to each concentration level, the mean, standard deviation (SD) and coefficient of variation (%CV) are also calculated. The details were presented in **Table 2**.

Fitting the data of concentration of standard solution and mean detector response (peak area counts) in a linear equation

Let the equation be $Y = a + bX$.

Where, Y = Mean peak area counts and X = Concentration of standard solution, μg/ml.

The calculations were presented in **Table 3**.

Conc. of standard solution (μg/ml)	Peak area			Mean	SD (n − 1)	%CV
	1	2	3			
0.1956	32,827	33,299	32,731	32,952	304	0.923
0.4890	87,783	88,480	87,446	87,903	527	0.600
0.9780	176,037	174,675	177,203	175,972	1265	0.719
1.467	246,212	250,786	246,849	247,949	2477	0.999
1.956	319,143	319,615	315,316	318,025	2358	0.741
2.934	415,059	410,773	418,407	414,746	3827	0.923

%CV = SD × 100/Mean: The coefficient of variation (CV) shows that the Injection variation is less than 1%.

Table 2. Calculation details of mean, SD, and %CV.

Sl. no.	Y	X
1.	32952	0.1956
2.	87903	0.4890
3.	175972	0.9780
4.	247949	1.4670
5.	318025	1.9560
6.	414746	2.9340

Table 3. Calculation details of additional parameters.

$$\sum Y = 1277547 \qquad \sum X = 8.0196 \qquad \sum XY = 2424193.441$$
$$\overline{Y} = 212924.5 \qquad \overline{X} = 1.3366 \qquad n = 6$$
$$\sum Y^2 = 3.7441177 \times 10^{11} \qquad \sum X^2 = 15.820245$$

Using the above parameters, calculate the following

$$\begin{aligned}\sum xx &= \sum X^2 - (\sum X)^2/n \\ &= 15.820245 - (8.0196)^2/6 \\ &= 5.101248\end{aligned}$$

$$\begin{aligned}\sum yy &= \sum Y^2 - (\sum Y)^2/n \\ &= 3.7441176 \times 10^{11} - (1277547)^2/6 \\ &= 1.0239070 \times 10^{11}\end{aligned}$$

$$\begin{aligned}\sum xy &= \sum XY - (\sum X)(\sum Y)/n \\ &= 2424193.441 - (1277547)(8.0196)/6 \\ &= 716624.12\end{aligned}$$

Calculation of a, b, and r

$$\begin{aligned}b &= \frac{\sum xy}{\sum xx} \\ &= \frac{716624.12}{5.101248} \\ &= 140480.16\end{aligned}$$

$$\begin{aligned}b &= \frac{\sum xy}{\sum xx} \\ &= \frac{716624.12}{5.101248} \\ &= 140480.16\end{aligned}$$

$$\begin{aligned}a &= \overline{Y} - b\overline{X} \\ &= 212924.5 - 140480.16 \times 1.3366 \\ &= 25158.718\end{aligned}$$

$$r = \frac{\sum xy}{\sqrt{\sum xx \cdot \sum yy}}$$

$$r = \frac{716624.12}{\sqrt{1.0239070X10^{11}X5.101248}} = 0.99157$$

Note: Sometimes r^2 is also used to express the goodness of fit.

Calculation of standard deviation for a and b:

$$\begin{aligned} Sy.x &= \sqrt{\frac{\sum yy - \left\{(\sum xy)^2 / \sum xx\right\}}{n-2}} \\ &= \sqrt{\frac{(1.0239070X10^{11}) - \left\{(716624.12)^2/(5.101248)\right\}}{6-2}} \\ &= 20731.806 \end{aligned}$$

The standard deviation for a is calculated as:

$$\begin{aligned} S_a &= Sy.x\sqrt{\frac{\sum X^2}{n\sum xx}} \\ &= 20731.806\sqrt{\frac{15.820245}{6\times 5.101248}} \\ &= 14905 \end{aligned}$$

The standard deviation for b is calculated as

$$\begin{aligned} S_b &= Sy.x\sqrt{\frac{1}{n\cdot\sum xx}} \\ &= 20731.806\sqrt{\frac{1}{6\times 5.101248}} \end{aligned}$$

Note: Assay procedures vary from highly exacting analytical determinations to subjective evaluations of attributes. Therefore different test methods require different validation schemes.

Category I

Analytical methods for quantitation of major excipients and/or active ingredients, and preservatives in finished goods.

Category II

Analytical methods for determination of impurities or degradation compounds in finished goods. These methods include quantitative assays and limit tests, titrimetric and bacterial endotoxin tests.

Category III

Analytical methods for determination of performance characteristics, e.g., sterility testing, dissolution and drug release for pharmaceutical products.

Data Elements Required for Assay Validation.

Details of required validation parameters of assay presented in **Table 4**.

Analytical parameters	Assay category 1	Assay category 2 quantitative	Limit Test	Assay category III
Assay accuracy	Yes	Yes	*	*
Precision	Yes	Yes	No	Yes
Specificity	Yes	Yes	Yes	*
Limit of detection	Yes	Yes	Yes	*
Limit of quantitation	Yes	Yes	No	*
Linearity	Yes	Yes	No	*
Range	Yes	Yes	*	*
Robustness	*	*	*	*

*May be required depending on the specific test.

Table 4. Validation parameters of assay [16].

4. Conclusions

Analytical validation data playing a fundamental role in pharmaceutical industry, pesticide industry for releasing the economic batch and long term stability information consequently, the records must be produced to suited regulatory authority requirements.

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