# Validation of an Analytical Method

Refer to: ICH **Guideline Q2(R1), Validation of Analytical Procedures: Teaxt andMethodology**.

### Introduction

All major laboratories eg. in the industry operates with clearly defined procedures for how an analytical method should be validated. The validation procedures are build around the same parameters but can often be very comprehensive if the analytical methods is to be used over longer time-frame by different persons and possibly in many different laboratories. Such a validation therefore often encompasses intermediate precision (different operators, apparatus, and reagents) and reproducibility (studies performed in different laboratories).

This document is based on an analytical method developed for a specific - shorter - timeframe eg. a masters thesis work or a research project. The purpose of the validation in this case is to document, that all samples analysed using this method fulfils the specified quality parameters regarding precision and accuracy. Such a validation shall encompass determination of:

- linear range
- precision
- limits of detection and quantitation
- accuracy
- selectivity

### Determination of linear range (at lest five concentration levels).

The calibration curve must contain (two times) the limit of quantitation and the maximum expected concentration:

| Standard solution | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Named | $C_1$ | $C_2$ | $C_3$ | $C_4$ | $C_5$ |
| Concentration | 2*LOQ | (C1+C3)/2 | (C1+C5)/2 | (C3+C5)/2 | Max. Koncentration*1,25 |
| Replicates | 6 | 2 | 6 | 2 | 6 |

The rationale behind this strategy can be found in the section: “Calibration Curves”.

A plot containg all measured data points is created and the following calculated:

- Slope and intercept (linear model)
- Coefficient of correlation, r, with sign
- Standard deviation for slope, intercept, and regression
- Standard deviation for calculated x-values
- Confidence intervals for calculated x-values

All measurements on sample material are performed in duplo with full sample preparation.

**A SPREADSHEET HAS BEEN CREATED FOR CALCULATING THESE PARAMETERS AND THE CONTENT OF SAMPLES**

For biological sample material or other complex sample matrices test for interference from the sample matrix should be performed. The sample is spiked at 5 concentration levels, the standard-addition curves is plotted and the above mentioned calculations are repeated. This procedure should be repeated for six different samples of the matrix.

The calibration curve should always be measured in the matrix in question.

*Spectroscopic methods:*

If the slope of the standard-addition curve is significantly different form the slope of the calibration curve (t-test), the samples should be quantitated using the standard-addition curve.

If there is no significant difference of the slopes, the quantitation is performed using the calibration curve. A new calibration curve is measured every day using only 2 replicates for each concentration.

*Chromatography:*

If the slope of the standard-addition curve is significantly different from the calibration curve, a response factor is used to correct for the difference. The response-factor should not be less than 0.8. or more than 1.25.

On a daily basis it is checked, that at least one standard yields the same response as during validation.

**Precision:**

Repeatability is calculated on 3 levels as the standard deviation of 6 replicates on the calibration curve – typically the same day (intra-day precision)..

Inter-day variation is calculated by determination of the same samples at 2 concentration levels in the analysis series on different days (at least 3 different days). Two replicates are measured for each sample resulting in a total of (at least) 6 measurements on each sample at each concentration level. These data are used for calculating day-to-day variation.

(The rationale for using 6 replicates is that by using this number of replicates a significantly uncertainty is obtained than if using fewer replicates. At the same time, using a larger number of replicates does not result in a significantly smaller uncertainty, se figure 1)

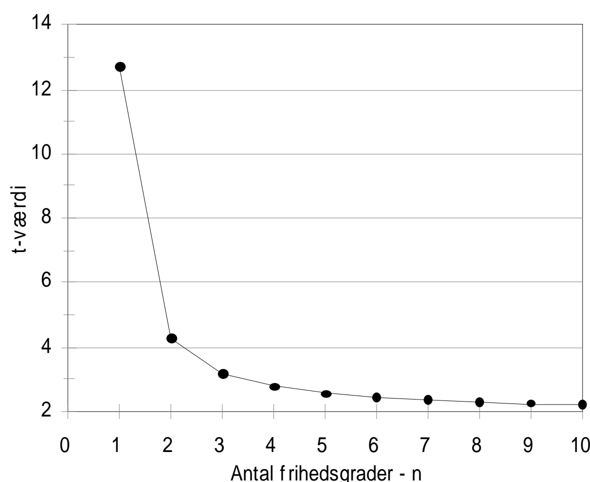


**Figure 1. t-values as function of number of degrees of freedom. 6 replicates results in 5 degrees of freedom.**

The relative standard deviation in % - calculated according to the equation below - is often used as a measure of precision

$$RSD\% = \frac{100}{\bar{y}}\sqrt{\frac{\sum\left(y_i - \bar{y}\right)^2}{n-1}}$$

**Limits of determination,, LOD and LOQ:**

Limits of determination and quantitation are determined using one of the three following methods. Methods one and two are preferred to method three.

The Limit of Detection (LOD) for an analytical method is the smallest amount (lowest concentration) of an analyte, that can be detected in a sample. Or put in another way: the amount (concentration) of the analyte, that causes a signal (Y), that can be distinguished from noise (signal from blank[1]).

Defined according to IUPAC: $Y = Y_B + 3s_B$

Where Y is the signal, $Y_B$ is the mean value of the signal from the blank sample and $s_B$ is the standard deviation of the signal from the blank sample.

In this definition a value of the signal to noise ratio (S/N) of 3 is used.

When calculating the Limit of Quantitation (LOQ) the signal to noise ratio is set to 10:

$$Y = Y_B + 10s_B$$

LOQ is the smallest amount/lowest concentration that can be determined with a given precision. If the purpose of the analysis is to analyse a pharmaceutical preparation, the precision must be high, as the quantitative limits for content are narrow. If a biological matrix is to be analysed, a relative standard deviation on LOQ of 15-20% is acceptable.

An estimate of $s_B$ can be obtained in several ways:

*Spectroscopic methods:*

1. A number of replicates of the blank sample is measured (n=20). The mean value $Y_B$ and the standard deviation $s_B$ are calculated. The concentration (LOD) is calculated from the value of the signal Y using the slope (a) of the regression-line (calibration curve):

$$LOD = (Y_B + 3s_B)/a$$

If no signal is obtained from the blank sample, a low concentration (close to the expected detection limit) of the analyte can be used instead

If the value of the signal from the blank sample is zero, the equation is simplified to:

$$LOD = 3s_B/a$$

and the Limit of Quantitation is likewise calculated as:

$$LOD = (Y_B + 10s_B)/a \qquad \text{or} \qquad LOQ = 10s_B/a \; (Y_B = 0)$$

*All methods:*

2. The Limit of Detection is calculated from the regression line (calibration curve):

The standard deviation of the regression line(curve) is used as an estimate of $s_B$, and the slope is used to calculate the concentration (LOD) from the signal.

*Chromatographic methods:*

3. The Limit of Detection is calculated after a visual estimation of the noise:

a). According to Ph.Eur.: ”peak-to-peak noise” (=N) is measured on the baseline covering an area corresponding to 20 times the width of the peak at half height (=20·FWHH, see figure 2):

[1] A sample containing matrix, but no analyte is designated blank. The designation blind is not used in this context.

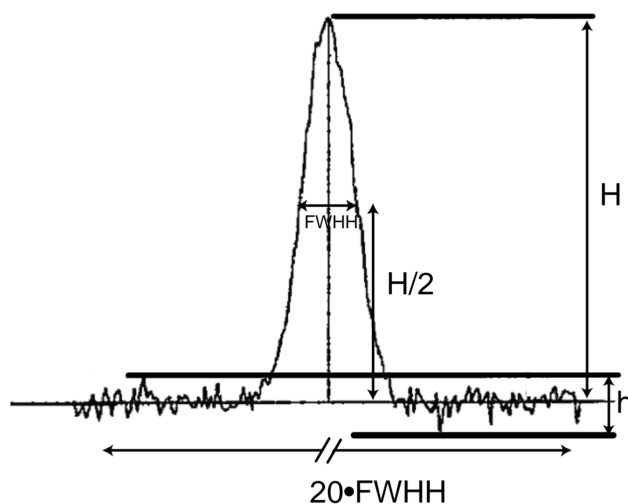


**Figure 2. Measuring Signal (S) and Noise (N) in a chromatogram according to Ph.Eur.**

The Signal to Noise ratio is calculated according to the following equation:

$$S/N = \frac{2H}{h}$$

b). The baseline is plotted with large vertical expansion, two parallel lines delimiting the noise are drawn. The distance between these two lines is designated the "peak-to-peak noise", $N_{p-p}$. The standard deviation is estimated as af fifth of this:

$$s_B = N_{p-p}/5$$

The Limit of Detection is then calculated as for method 1.

Note that method 3a and 3b does not give the same result, but as these methods are only estimates, and the S/N-ratio is not a physical constant – depending on several parameters in the chromatographic system – this is to be expected.

**Important**

After calculation of the Limit of Detection, it should be tested if the result is realistic. This is done by analysing a sample with the calculated concentration and evaluate, if the signal can be distinguished from noise.

Analogously, the Limit of Quantitation should be tested by analysing a concentration of the analyte close to the calculated LOD in order to verify that the relative standard deviation does not exceed the specified value.

**Accuracy**

Accuracy can be tested in several ways:

1. Analysis of a reference material with a known, certified content of the analyte. The reference material should resemble the actual samples as closely as possible so the concentrations of the analyte and matrix in the actual sample and the reference sample are comparable.

2. Comparison with an independent and validated method of analysis. The less the two methods resemble each other, the better.

If sample preparation is a part of the analytical method, Recovery must be determined (Recovery). The sample is spiked with analyte standard at 3 concentration levels with at least 3 determinations at each level (preferably 5 to 6). The result is presented as % recovery.

In many cases, this is the only possibility for determining the accuracy of the method. The assumption is then, that this test combined with traceable standards, the use of qualified apparatus, and the other parts of the validation corrobates the estimate of accuracy.

### Selectivity / (Specificity)

This is the most difficult to examine. If the method of analysis encompasses a separation – as does HPLC – it should be examined whether the signal from the analyte is caused by the analyte only. If (all) possible interferences are known (eg. impurities from synthesis, degradation products or metabolites) and these are available, it should be demonstrated that these does not interfere (coelutes) with the signal from the analyte.

A way of examining this is to utilise a diode array detector (DAD) to explore whether the analyte peak consists of one and only one UV-VIS spectrum during all of the elution of the peak and that this spectrum is identical to that of the reference compound. As an alternative, LC-MS or LC-NMR can be used to examine the mass spectra resp. the proton spectra during the elution of the peak.

Selectivity and specificity are distinguished such that the selectivity relates to the ability of the analytical method to determine one or several analytes in a complex matrix without interference from other compounds present. On the other hand, a specific method can only determine the analyte. Specific methods are rare and specificity is almost impossible to prove.

### Robustness

The robustness of an analytical method describes how reliable the method works over an extended period, using different batches of reagents, and many different operators. Immediate robustness can be tested by systematically varying analytical parameters such as temperature, amount of reagents etc. Therefore robustness is not discussed further here.

### Referencer.

- ICH guideline on validation of Analytical Procedures Q2(R1): - Text and Methodology. (step 4).

- IUPAC Compendium of Analytical Nomenclature. The Orange Book - 3rd Ed. J Inczedy, T. Lengyel, and A.M. Ure. Blackwell Science, 1998. ISBN 0-632-05127-2