Method Validation and Verification Protocols for Test Methods

What is it ?

- **Method validation & verification** provides **objective evidence that a test method is fit for purpose,**

  i.e. that the particular requirements for a specific intended use are fulfilled.

- *The term 'method' includes kits, individual reagents, instruments, platforms and software.*

- ***Method Validation*** *:* in-house and modified standard methods
- ***Method Verification*** *:* standard methods

When it is required ?

- ***Method Validation*** *:* in-house and modified standard methods
- ***Method Verification*** *:* standard methods

Method	Requirement
Fully validated standard methods	Verification
Standard methods – modifications	Validation
Standard methods – outside their intended scope	Validation
Laboratory developed and non-standard methods	Validation

Why it is necessary ?

- *A test method must be shown to be fit for purpose by validation and verification for the customers to gain confidence in the test results*

Verification

- Standard validated methods - AOAC, ASTM, ISO, etc
- Peer accepted methods published in scientific literature
- Commercial test kits

*Laboratory needs to verify that **analysts** using their **equipment** in their **laboratory environment** obtain the **same outcomes** as defined in the validation data*

Verification

- **Method performance demonstrated by**
  - blanks or un-inoculated media - to assess contamination;
  - laboratory control samples - to assess accuracy;
  - duplicates - to assess precision
  - calibration check standards - for quantitative analyses
  - monitoring quality control samples, and
  - participation in a PT testing program

Some examples

Method	Requirement
using the same type of chromatographic column from a different manufacturer	Verification
a slight change in a non-critical incubation temperature	Verification
use of a different non-selective growth medium,	Verification
differences in details of sample dilutions as a consequence of expected counts	Verification

Some examples

Method	Requirement
use of a different extraction solvent; use of HPLC instead of GLC	Validation
differences in the formulation of the selective/differential medium (e.g. addition of an alternative antibiotic)	Validation
different antibiotic concentration to the base medium	Validation
a change to a critical incubation temperature or time (e.g. 3 days rather than 5 days incubation)	Validation
different confirmation procedure (e.g. use of an alternative suite of biochemical tests other than those specified)	Validation

Key parameters for verification

Tests	Parameters
For quantitative results	measurement of bias and measurement of precision - minimum requirements
For trace analyses	**limit of detection (LOD)** and **limit of quantification (LOQ)**
For qualitative methods	correlation studies with validated methods or comparisons with known outcomes
For diagnostic methods	sensitivity and selectivity (specificity)

Validation

- Non-standard and in-house-developed methods
- Scope and validation criteria to be defined and documented

Tools to demonstrate the method performance

- *Blanks*
- *Certified Reference Material (CRMs)*
- *Fortified materials*
- *Replication*
- *Statistical analysis*

Types of Validation

- Comparative Validation
  - To demonstrate equivalent performance between two methods (validated and revised analytical method)

- Primary Validation
  - an exploratory process to establish operational limits and performance characteristics for alternative or new method

Validation

Two steps

1. to specify what you intend to identify or measure
2. to determine selected performance parameters

Validation Parameters

1. Linearity range
2. Measuring interval
3. Matrix effects
4. Selectivity
5. Sensitivity
6. Accuracy .
7. Precision
8. Repeatability
9. Reproducibility
10. Trueness
11. Limit of detection (LOD) and limit of quantitation (LOQ)
12. Ruggedness
13. Measurement Uncertainty.

Analytical Performance Characteristics Procedure

- *Before validation, design, maintain, calibrate and validate the* ***analytical system (protocol, conc. range and specified material)***
- ***Train*** *all the personnel who perform the validation testing*
- *Get* ***approval*** *of method validation protocol from CA before execution.*

1. Specificity

Test procedure: Investigate by injecting of the extracted sample to demonstrate the absence of interference with the elution of analyte

Documentation : Print chromatograms.

Acceptance criteria : The excipient compounds must not interfere with the analysis of the targeted analyte.

2. Linearity

- **Test procedure :**
- Prepare standard solutions at six concentrations, typically 25, 50, 75, 100, 150, and 200% of target conc.
- Analyze three individually prepared replicates at each concentration.
- Use same method of standard preparation and number of injections as in the protocol

- **Documentation**:
- Record results on a datasheet.
- Calculate the mean, standard deviation, and RSD for each conc.
- Plot concentration (x-axis) versus mean response (y-axis) for each conc.
- Calculate the simple regression or weighted regression equation & correlation coefficient and record.

2. Linearity

- **Acceptance criteria** :
- The correlation coefficient for six conc. levels will be ≥ 0.999 for the range of 80 to 120% of the target conc.
- The y-intercept must ≤ 2% of the target conc. response.
- A plot of response factor vs conc. must show all values within 2.5% of the target level response factor.
- The coefficient for active ingredients should be ≥ 0.997, for impurities 0.98 and for biologics 0.95

3. Range

- **Test procedure** :
- Use the data obtained during linearity and accuracy studies to assess the range of the method.
- We can use the precision data for this assessment, if precision of the three replicate samples is analyzed at each level in the accuracy studies.
- **Documentation** : Record the range on the datasheet.

- **Acceptance criteria**

  Acceptable range (- defined as the conc. interval over which linearity and accuracy are obtained)

  It yields a precision of **≤ 3% RSD**.

4. Accuracy

- **Test procedure**
- Prepare spiked samples at three conc. over the range of 50 to 150% of the target conc.
- Analyze three individually prepared replicates at each conc..
- When it is impossible or difficult to prepare known sample, use a low concentration of a known standard.
- **Documentation** :
- For each sample, report the theoretical value, assay value, and percent recovery.
- Calculate the mean, standard deviation, RSD, and percent recovery for all samples.
- Record results on the datasheet.

4. Accuracy

- **Acceptance criteria**
- The mean recovery will be within 90 to 110% of the theoretical value for non-regulated products.
- For the U.S. pharmaceutical industry, 100 ± 2% is typical for an assay of an active ingredient in a drug product over the range of 80 to 120% of the target concentration.
- Lower percent recoveries may be acceptable based on the needs of the methods.
- Health Canada states that the required accuracy is a bias of ≦ 2% for dosage forms and ≤ 1% for drug substance.

5. Precision - Repeatability

- **Test procedure:**
- Prepare one sample solution containing the target level of analyte
- Make ten replicates from this sample solution
- **Documentation:**
- Record retention time, peak area, & peak height on datasheet.
- Calculate the mean, standard deviation, and RSD.
- **Acceptance criteria:**
- FDA states - typical RSD should be 1% for drug substances and drug products, ± 2% for bulk drugs and finished products.
- HC states - RSD should be 1% for drug substances and 2% for drug products. For minor components, it should be ± 5% but may reach 10% at the LOQ.

6. Intermediate Precision

- **Test procedure:**
- Demonstrate Intermediate precision (within-laboratory variation) by two analysts, using two HPLC systems on different days and evaluate the relative percent purity data across the two HPLC systems at three conc. levels (50%, 100%, 150%) covering range of 80 to 120%.
- **Documentation**:
- Record the relative % purity (% area) of each conc. on the datasheet.
- Calculate the mean, standard deviation, and RSD for operators and instruments.
- **Acceptance criteria:**
- The results obtained by two operators using two instruments on different days should have a statistical RSD ≤ 2%.

7. Limit of Detection

- **Test procedure**
- Determine the lowest concentration of the standard solution by sequentially diluting the sample.
- Make six replicates from this sample solution.
- **Documentation**
- Print the chromatogram and record the lowest detectable concentration and RSD on the datasheet.
- **Acceptance criteria**
- The International Conference on Harmonization (ICH) references a signal-to-noise ratio of 3:1.2
- Health Canada recommends a signal-to-noise ratio of 3:1.
- Some analysts calculate the standard deviation of signal (or response) of a number of blank samples and then multiply this number by 2 to estimate the signal at LOD

8. Limit of Quantitation

- **Test procedure**
- Determine the lowest concentration at which an analyte in the sample matrix can be measured with the accuracy & precision.
- This value may be the lowest concentration in standard curve.
- Make six replicates from this solution.

- **Documentation**
- Print the chromatogram and record the lowest quantified concentration and RSD on the datasheet.
- Provide data that demonstrates the accuracy and precision required in the acceptance criteria.

8. Limit of Quantitation

- **Acceptance criteria:**
- The limit of quantitation for chromatographic methods is described as the conc. that gives a signal-to-noise ratio of 10:1.2
- Quantitation limit is the best estimate of a low conc. that gives an RSD of approx. 10% for a minimum of six replicate determinations.

9. System Suitability

- **Test procedure**
- Perform system suitability tests on both HPLC systems to determine the accuracy and precision of the system by injecting six injections of a solution containing analyte at 100% of test conc..
- Determine plate count, tailing factors, resolution, & reproducibility (% RSD of retention time, peak area, & height)

- **Documentation:**
- Print the chromatogram and record the data on the datasheet

9. System Suitability

- **Acceptance criteria:**
- Retention factor (k): the peak of interest be well resolved from other peaks and the void volume; generally k should be ≥2.0.
- Resolution (Rs): Rs should be ≥2 between the peak of interest and the closest eluted peak (impurity, excipient, and degradation product).
- Reproducibility: RSD for peak area, height, and retention time will be 1% for six injections.
- Tailing factor (T): T should be 2.
- Theoretical plates (N): ≥2000

10. Robustness

- Measures the capacity of an analytical method to remain unaffected by small but deliberate variations in method parameters.
- Provides some indication of the reliability of an analytical method during normal usage.
- Parameters investigated - % organic content in the mobile phase or gradient ramp, pH of the mobile phase, buffer concentration, temperature, and injection volume.
- Evaluate these parameters - one factor at a time or simultaneously as part of a factorial experiment.

10. Robustness

- Compare the chromatography obtained for a sample containing representative impurities, when using modified parameter(s), to the chromatography obtained using the target parameters.
- Determine the effects of the following changes in chromatographic conditions :
  - methanol content in mobile phase adjusted by ± 2%,
  - mobile phase pH adjusted by ± 0.1 pH units,
  - Column temperature adjusted by ± 5˚C.

- If these changes are within the limits that produce acceptable chromatography, incorporate in the method procedure.

11. Measurement Uncertainty

- Calculation of measurement uncertainty by mathematical model according to law of propagation of uncertainty

$$u\,[y\,(x1.\ x2\ldots..)] = \sqrt{\sum_{i=1,n} c_i^2\, u(x_i)^2}$$

Where

u [y (x1. x2…..)] is a function of several independent variables x1, x2, …

c_i is a sensitivity coefficient evaluated as ci = δy/ δx, the partial differential of y with respect to x_i

u(xi) and u(y) are **standard uncertainties** i.e measurement uncertainties expressed as SD

So, u [y (x1. x2…..)] is referred as a **combined standard uncertainty**

Estimation of Uncertainty

Uncertainty calculation for Chloramphenicol analysis

- Type A and Type B errors are the sources to calculate uncertainty.
- **Type A** – Due to sample (Repeatability Measurement) ($\mathbf{U_{Rep}}$)
- **Type B** – a). Due to Equipments **($\mathbf{U_{Equip}}$)**

  b). Due to Purity of Chemicals and CRM **($\mathbf{U_{Pur}}$)**

  c). Due to Glassware **($\mathbf{U_{g}}$)**
- **Coverage factor k = 2** at 95 % confidence level.

Type A Error

Repeatability Readings Xi	Average X	Std.	$U_{STD} = Sd/\sqrt{n}$
0.28000	0.2912	0.02100	0.00860
0.27300			
0.30000			
0.26700			
0.32000			
0.30700			

Type B

i. Uncertainty due to Equipments

Equipment	Uncertainty	k	U. Equip= U/k
Weighing Balance	0.09	2	0.045
Refrigerated Centrifuge	0.06	2	0.03
Vortex Mixer	0.06	2	0.03

ii. Uncertainty due to Chemicals and CRM (Upur)

Chemical	Purity %	U. Chem %	% Conv = U	k	Std Uncertainty = U/k
Chloramphenicol (CRM)	99.7	0.3	0.003	2	0.0015
Acetonitrile	99.9	0.1	0.001	1.732	0.0006
Carbon Tetrachloride	99	1	0.01	1.732	0.0058
Ethyl Acetate	99.7	0.3	0.003	1.732	0.0017

iii. Due to Standard Uncertainty Glassware (Ug)

Glassware	Capacity	Std Uncertainity
Volumentric Flask (U_{Vol})	10 ml	0.00200
Measuring Cylinder	25 ml	0.00200
Micro Pipette (U_{Pip})	1000 µl	0.11000
Micro Pipette (U_{Pip})	100 µl	0.01000
Micro Pipette (U_{Pip})	20 µl	0.09500

Calculation of Combined Standard Uncertainity

Uncertainity Sources	Value X	Standard Uncertainity $U_{(X)}$	Relative Uncertainity $U_R = U_{(X)} / X$
U_{Rep}	0.2912	0.0086	0.029536
U_{Bal}	2.0050	0.04500	0.022444
U.RF	6000.00	0.03000	0.000005
U. VM	2500.00	0.03000	0.000012
$U_{pur\ (CAP)}$	99.70	0.0015	0.000015
Upur (ACN)	99.90	0.0006	0.000006
Upur (Ethyl Acetate)	99.70	0.0017	0.000017
Upur (Carbon Tetrachloride)	99.00	0.0058	0.000059
Volumentric Flask (U_{Vol})	10.00	0.0020	0.000200
Measuring Cylinder	25.00	0.00200	0.000080
Micro Pipette (U_{Pip})	1000.00	0.00200	0.000002
Micro Pipette (U_{Pip})	100.00	0.11000	0.001100
Micro Pipette (U_{Pip})	20.00	0.01000	0.000500
Combined Standard Uncertainity		$\sqrt{U_R^2} =$	**0.01080707**

Therefore, Chloramphenicol residues in shrimp (ppb) = 0.2912 ± 0.011