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Calibration and Validation of HPLC, GC and UV-VIS Spectroscopy

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Abstract:

There are as many definitions of calibration as there are methods. According to ISA's The Automation, Systems, and Instrumentation Dictionary, the word calibration is defined as "a test during which known values of measured are applied to the transducer and corresponding output readings are recorded under specified conditions." The definition includes the capability to adjust the instrument to zero and to set the desired span. An interpretation of the definition would say that a calibration is a comparison of measuring equipment against a standard instrument of higher accuracy to detect correlates e, adjusts, rectify and document the accuracy of the instrument being compared. Typically, calibration of an instrument is checked at several points throughout the calibration range of the instrument. The calibration range is defined as "the region between the limits within which a quantity is measured, received or transmitted, expressed by stating the lower and upper range values." The limits are defined by the zero and span values. The zero value is the lower end of the range. Span is defined as the algebraic difference between the upper and lower range values. The calibration range may differ from the instrument range, which refers to the capability of the instrument.

KEYWORDS: HPLC, GC, UV-VIS, CALIBRATION AND VALIDATION

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1. Definition of Validation: USP:

"Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications."

1.1. Validation Protocol:

A written plan stating how validation will be conducted and defining acceptance criteria. For example, the protocol for a manufacturing process identifies processing equipment, critical process parameters/operating ranges, product characteristics, sampling, test data to be collected, number of validation runs, and acceptable test results.

1.2. Equipment validation:

Demonstrate that equipment used in validation studies is suitable for use and is comparable to equipment used for routine analysis Calibrated (as applicable) Qualifications should have been performed Installation Qualification Operation Qualification Performance Qualification Routine maintenance performed- per working order

2. Qualification:

Action of proving and documenting that equipment or ancillary systems are properly installed, work correctly, and actually lead to the expected results. Qualification is part of validation but the individual qualification steps alone do not constitute process validation.

2.1. Installation Qualification (I.Q):

The purpose of I.Q is to check the\installation site/environment, confirms equipment specifications and verifies the condition of installed equipment.

I.Q protocol shall include the following:

- Confirmation of the specifications of the analytical equipment.
- Confirmation and maintenance of documents (Instruction manuals, qualification protocol and certificates).
- Confirmation of installation site and conditions
- Confirmation of delivered equipment.
- Confirmations of Software and Firmware i.e., verify that the equipment is consistent with actual versions displayed when power is turned on.

In I.Q, connect each unit (Electrical system, Flow line system) and confirm that the connections are correct. Any problems identified in I.Q must be investigated and appropriate actions must be taken. All such actions must be documented and approved by higher authority.

2.2. Operational Qualification (O.Q):

O.Q includes procedures and documentation of O.Q of analytical instrument. When all procedures are executed and all items pass the inspection, it is verified that the system operates to satisfy the intended purpose.

O.Q protocol shall include the following:

- Operation check on each unit: Due to modular nature of the system, the operation of each unit is checked properly.
- Operation check on overall system: Confirm that the system controller and work station control each unit during analysis and that the analysis results meet the prescribed criteria.
- Software and Firmware check: Here Firmware checking is conducted based on version display and Software certificate of Compliance. The Software and Firmware must be properly managed and change procedures must be properly clarified.

Any problems identified in O.Q must be investigated and appropriate actions must be taken. All such actions must be documented and approved by higher authority. Prior to implementing O.Q, check the system configuration, determine the items to be evaluated and record them in O.Q record and have they approved.

2.3. Performance Qualification (PQ):

The objective is to ensure that the instrument is performing within specified limits. Hence documented verification that the equipment and ancillary systems, as connected together, can perform effectively and reproducibly based on the approved process method and specifications.

The PQ represents the final qualification of equipment or system. This incorporates a range of testing to simulate your production process options and provide assurance that systems and operating documentation are capable of subsequent process validation activities. It is used to establish and or confirm;

1. Definition of performance criteria and test procedures.
2. Selection of critical parameters, with predefined specifications.
3. Determination of the test intervals, e.g.,
   (a) - Everyday.
   (b) - Every time the system is used.
   (c) - Before, between and after a series of runs.
4. Define corrective actions on what to do if the system does not meet the established criteria.

3. Definition of Calibration: ICH

The demonstration that a particular instrument or device produces results within specified limits by comparison with those produced by a reference or traceable standard over an appropriate range of measurements.

3.1. Calibration of HPLC: Various Calibration parameters are:

1) Flow rate accuracy
2) Injector accuracy
3) System Precision
4) Wavelength accuracy
5) Detector linearity
6) Injector linearity
7) Gradient Performance Check
8) Column oven temperature accuracy

3.1.1. Flow Rate Accuracy:

1. Prime all the solvent lines with Milli Q water.
2. Set the flow rate to 0.500 ml/m.
3. Wait for about 15 m to stabilize the system and ensure that the pressure is stable.
4. Insert the outlet tubing into a 10 ml volumetric flask and start the stop watch simultaneously.
5. Stop the stopwatch when the lower meniscus reaches the 10 ml mark on the flask.
6. Record the elapsed time.
7. Similarly check the flow for 1.0 ml/m and 2.0 ml/m.

Acceptance criteria: The time taken to collect the water should be with in ± 2.0% of the actual value.

Table 1: I.Q and O.Q and P.Q Report

System Name:
System ID No.:
Installation site:
Performer_____Signature _____Date:_____
Reviewer_____Signature_____ Date:_____
Manager_____Signature_____Date:_____

Table 2: Flow Rate Accuracy

Set Flow to collect	Actual time required Up to the mark	Acceptance criteria (in m)
0.5 ml/m	20.0	19.6 – 20.4
1.0 ml/m	10.0	9.8 – 10.2
2.0 ml/m	5.0	4.9 – 5.1

3.1.2. Injector Accuracy:

1. Connect the pump and detector inlet with union.
2. Prepare mobile phase consisting of a mixture of water and Methanol (70:30 v/v)
3. Set a flow rate of 0.5 ml/m and a run time of 1 m..
4. Set the column temperature at 25±2°C.
5. Fill a standard HPLC vial to 2/3rd with Milli-Q water. Seal the vial properly with a cap.
6. Weigh the vial and record the weight as W_1 grams.
7. Place the vial in the chromatographic system and perform 6 injections of 50µl volume from this vial.
8. Weigh the vial again and note the weigh after the injections as W_2 grams. Calculate the mean volume injected per injection as follows:

Mean injected volume (µl) = $(W_1 – W_2) \times 100/6$

Acceptance criteria: The mean injected volume should be 50.0±1.0 µl

3.1.3. System Precision:

Standard Preparation: Accurately weigh and transfer about 60mg of Caffeine into a100ml volumetric flask. Dissolve and dilute to the volume with mobile phase. Transfer 10ml of this solution into a 100ml volumetric flask and dilute to the volume with mobile phase.

Procedure: Inject blank, followed by standard preparation in 6 replicates. Note down the areas and retention times. Now calculate the %RSD of retention time and peak areas for 6 replicates injections.

Acceptance criteria: The %RSD of retention time & peak area should be <1.0%.

3.1.4. Wavelength Accuracy:

Procedure: Create and instrument method with a wavelength in nm and inject blank, followed by Standard preparation and note down the height or absorbance.

Acceptance criteria: The maximum absorbance should be ±2nm.

Table 3: Chromatographic Conditions for System Precision

Column	C18, 150mm×4.6 mm, 5µm
Flow rate	1.0 ml/m
Detection	UV at 272 nm
Injection volume	20 µl
Run time	15 m
Column oven temperature	25°C ± 2°C
Mobile phase	Water: Methanol (70:30v/v)

3.1.4.1. PDA Detector Accuracy:

Select 3D mode and set the wavelength range as 200-400nm.Inject 20 µl of standard preparation once into the chromatographic system. Extract and record the chromatograms at wavelengths of 202 to 208nm with an interval of 1nm and at 269 to 275 nm with an interval of 1nm.Note down the height or absorbance.

Acceptance criteria: The maximum absorbance should be at 205±2nm and 272±2nm.

3.1.5. Detector Linearity:

Standard Preparation: Accurately weigh and transfer about 60mg of Caffeine into a100ml volumetric flask. Dissolve and dilute to the volume with mobile phase.

- **Detector linearity solution 1(0.06mg/ml):** Transfer 10ml of Standard Preparation into a
- 100ml volumetric flask and dilute to the volume with mobile phase.

Procedure: Inject blank, followed by Detector linearity solutions and record the peak responses of Caffeine standard plot between the concentration Vs the peak responses.

100ml volumetric flask and dilute to the volume with mobile phase

- **Detector linearity solution 2(0.048 mg/ml):** Transfer 8ml of Standard Preparation into a 100ml volumetric flask and dilute to the volume with mobile phase.
- **Detector linearity solution 3(0.03mg/ml):** Transfer 5ml of Standard Preparation into a 100ml volumetric flask and dilute to the volume with mobile phase.
- **Detector linearity solution 4(0.24mg/ml):** Transfer 4ml of Standard Preparation into a 100ml volumetric flask and dilute to the volume with mobile phase.
- **Detector linearity solution 5(0.012mg/ml):** Transfer 2ml of Standard Preparation into a

Acceptance criteria: The plot should be linear and regression coefficient (R2) should not be less than 0.99.

3.1.6. Injector Linearity:

Standard Preparation: Accurately weigh and transfer about 60mg of Caffeine into a 100ml volumetric flask. Dissolve and dilute to the volume

with mobile phase. Transfer 1Standard Preparation into a 100ml volumetric flask and dilute to the volume with mobile phase.

Procedure: Inject 5 µl of the mobile phase as blank injection. Inject 5 µl, 10 µl, 20 µl, 50 µl and 80 µl of the Standard Preparation and record the peak 0ml of areas. Plot a curve for the volume injected Vs peak area.

Acceptance criteria: The plot should be linear and regression coefficient (R2) should not be less than 0.99.

3.1.7. Gradient performance check:

Add 5ml of acetone to 1000ml of methanol filter and degas. Connect the pump and detector inlet with union. Set the detector wave length at 254 nm. Place Channels A and C in methanol and channel B and D in 0.5% acetone in methanol. Set binary gradient with a total flow rate of 2.0ml/m.Set gradient program as shown below for channels A, B and C, D individually.

Table 4. Gradient Performance Check

Time (m) (ethanol)	A or C	B or D (0.5% Acetone in Methanol)
Initial	100.0	0
4.00	100.0	0
4.01	90.0	10.0
8.00	90.0	10.0
8.01	100.0	0
12.00	100.0	0
12.01	75.0	25.0
16.00	75.0	25.0
16.01	100.0	0
20.00	100.0	0

Purge all the channels at a flow rate of 2ml/m for about 5 m. Set the flow rate at 2.0ml/m and wait until the base line is stable. Set the gradient profile for A and B and run the gradient profile by injecting "0.0"volume of methanol. Record the height of the peaks. Consider the height of the peak resulting from B at 100% concentration as 100 and calculate the percentage height of other peaks. Perform the gradient performance check similarly for channels C and D. Consider the height of the peak resulting from D at 100% concentration as 100 and calculate the percentage height of other peaks.

Acceptance criteria: The resulting oven temperature from the thermometer display should be within ±2°C of the set temperature.

Calculations: Height (%) of B/D =Height of B/D peak×100/Height of full scale peak

Acceptance criteria: The calculated percentage composition (Height (%) should be with in±1.0% of the set composition.

3.1.8. Column Oven Temperature Accuracy:

It is evaluated with a calibrated digital thermometer at 30°Cand 60°C .Place the thermometer probe in the column oven and set the column oven temperature at 30°C.Wait till the temperature stabilizes. Record the temperature displayed on the thermometer. Similarly performs the column oven temperature accuracy test at 60°C.

NOTE: a) for oven Temperature Accuracy, Chromatographic conditions and mobile phase refer to system precision test.

3.2. Calibration of Gas Chromatography:

Various Calibration parameters are:

1) Flow rate accuracy 2) Column oven temperature accuracy 3) System precision 4) System precision for head space auto sampler 5) Detector linearity 6) Detector noise and drift test

Table 10: Chromatographic Conditions

Column	15m ×0.53mm,3.0µ,DB-1
Detector	Flame ionization detector
Injector temperature	150°C
Detector temperature	200°C
Flow mode	Pressure
Septum purge flow	3 ml/m
Oven program	60°C
Split ratio	1:10
Hydrogen flow	40 ml/m
Air flow	400 ml/m
Run time	15 m

3.2.1. Flow rate accuracy:

1. Connect the digital flow meter to the detector outlet port.
2. Set the carrier gas (Helium) flow and wait till it reaches the set flow.
3. Note the observed flow in replicate.
4. Repeat the procedure for other carrier gases such as Hydrogen and Air.
5. Record the result in GC calibration protocol.

Acceptance criteria: The flow rate of carrier gas should be ±10% of set flow.

Table 5: Flow Rate Accuracy:

S.No .	Carrier gas	Acceptance criteria in ml/m
1.	Helium	125
2.	Hydrogen	40
3.	Air	400

3.2. 2. Column Oven Temperature Accuracy:

1. Connect the column to the detector port.
2. Place the thermometer probe in the column oven and set the column oven temperature at 40°C.Wait till the temperature stabilizes.
3. Note the observed temperature as read by the probe in triplicate over a period of 10m.
4. Repeat the procedure for 100°C, 150°C and 190°C.

Acceptance criteria: The resulting oven temperature from the thermometer display should be within ±2°C of the set temperature

3.2.3. System Precision:

Preparation of Standard solution:

Transfer 20 ml of Methanol, Ethanol and Acetone into 100ml volumetric flask and make up with Ethyl acetate

Procedure: Inject blank, followed by Standard preparation in 6 replicates. Note down the areas and Retention times.

Table 6: Chromatographic Conditions for System Precision

Column	30m × 0.32mm, 1.8µ, DB-624
Detector	Flame ionization detector
Injector temperature	80°C
Detector temperature	250°C
Flow mode	Pressure
Carrier Gas flow rate Helium	25 kpa
Oven program	50°C(hold 5 m) raise to 10°0C
Split ratio	1:10
Injection volume	0.2 µl
Hydrogen flow	40 ml/m
Air flow	400 ml/m

Acceptance criteria: The %RSD of retention time should be not more than 1.0%& peak area should be not more than 5.0%.

3.2. 4. System precision for head space auto sampler:

Preparation of standard solution: Prepare a standard mixture solution by taking Methylene dichloride (0.6g), Chloroform (0.06g), Trichloroethane (0.08g), 1, 4-Dioxane (0.38g) in 50ml volumetric flask containing about 40ml of Dimethyl formamide. Finally makeup to volume with DMF (Solution-A).

Procedure: Take 0.5 ml of standard solution-A in 6 different vials and seal with septum, then magnetic caps and crimp. Place these vials on head space sampler; prepare a blank vial also. Load the vials in head space sampler tray. Blank vials followed by the standard vials.

Acceptance criteria: The %RSD of retention time should be NMT 1.0%& peak area should be NMT 15.0%.

3.2.5. Detector linearity:

Preparation of standard solutions:

- **Detector linearity solution A:** Transfer 10ml of each Methanol, Ethanol and Acetone into a 10ml volumetric flask and dilute to the volume with Ethyl acetate.
- **Detector linearity solution B:** Transfer 15ml of each Methanol, Ethanol and Acetone into a 100ml volumetric flask and dilute to the volume with Ethyl acetate.

- **Detector linearity solution C:** Transfer 20ml of each Methanol, Ethanol and Acetone into a 100ml volumetric flask and dilute to the volume with Ethyl acetate.
- **Detector linearity solution D:** Transfer 25ml of each Methanol, Ethanol and Acetone into a 100ml volumetric flask and dilute to the volume with Ethyl acetate.

Table 7: Chromatographic Conditions for Head Space Auto Sampler

Column	30m × 0.32mm, 1.8µ, DB-624
Detector	Flame ionization detector
Injector temperature	220°C
Detector temperature	260°C
Flow mode	Pressure
Carrier Gas flow rate Helium	25 kpa
Oven program	40°C(hold 5 m) raise to 200°C(hold 5 m)
Split ratio	1:10
Injection volume	0.2 µl
Hydrogen flow	40 ml/m
Air flow	400 ml/m

Table 8: Head Space Conditions

Vial equilibrium	22 m
Vial pressure	0.5 m
Loop fills	0.5 m
Loop equilibrium	0.05 m
Inject	1.00 m
GC cycle time	38 m
Oven temperature	80°C
Loop temperature	100°C
Vial pressure	10.8 psi

- **Detector linearity solution E:** Transfer 30ml of each Methanol, Ethanol and Acetone into a 100ml volumetric flask and dilute to the volume with Ethyl acetate.
- Procedure: Inject blank, followed by Detector linearity solutions and record the peak responses .Draw a standard plot between the concentrations Vs the peak responses.

Acceptance criteria: The plot should be a linear and regression coefficient (R2) should not be less than 0.99.

Table 9: Chromatographic Conditions for Detector Linearity

Column	30m ×0.32mm, 1.8µ, DB-624
Detector	Flame ionization detector
Injector temperature	180°C
Detector temperature	250°C
Flow mode	Pressure
Carrier Gas flow rate Helium	25 kpa
Oven program	50°C(hold 5 m) raise to 100°C
Split ratio	1:10
Injection volume	0.2 µl
Hydrogen flow	40 ml/m
Air flow	400 ml/m

3.2.6. Detector Noise and Drift Test:

After GC is ready run the system up to 15 m through single run. After completion of run calculate noise and drift through software.

Acceptance criteria:

Noise NMT: 100 µV

Drift NMT: 2500 µV/hr

3.3. Calibration of UV-Visible pectrophotometer:

3.3.1. Spectral calibration: visible spectral region

- Ensure-the socket of the power cord of the instrument is inserted properly-cuvettes are clean
- Switch ON the instrument. Allow 15m to warm up.
- Keep dummy cuvette in position of sample holder.

- Set the λ to 485 nm and press %T button.
- Press 0%T in appropriate direction to adjust 0.00 reading on read out.
- Now remove dummy cuvette from sample holder. Close the lid.
- By adjusting coarse and fine control set a reading of around 80.0 on read out
- Now set the value of wavelengths in increments of 0.1 nm up to λ of 487 nm and read the value of %T at each increment of λ
- Draw a curve %T Vs λ.
- If the peak value of %T is occurring at a λ 486.1 ± 0.5 nm, the spectral calibration of the instrument in the visible spectral region is proper.
- This can be confirmed by repeating the above steps with a maximum value of %T of around 30.0 on the read out and λ setting from 655 to 657 nm.
- If the maximum %T is obtained at a λ 656.2 ± 0.5 nm, the spectral calibration of the instrument in the visible spectral region is confirmed to be proper.

3.3. 2. Spectral calibration: U.V spectral region:

- Keep blank (distilled water) filled cuvette and sample (benzene vapor) filled cuvette.
- Set the λ to 253 nm and press absorbance button.
- Adjust blank to 0.000 on the read out by using coarse and fine adjustment
- Now place the sample into optical path, value of Absorbance of sample at the λ set appears on the read out.
- Again set the values of wavelengths increments of 0.1nm up to a λ of 255nm. Measure the A at each increment. If maximum A is obtained at λ 253.9 ± 0.5nm the "spectral calibration" of the instrument in U.V region is confirmed to be proper

3.3.3. Photometric Calibration:

Absorbance: Visible region

- Place dummy cuvette in sample holder and set %T to "zero". Now remove dummy cuvette, by using fine & coarse control set a reading exactly 40.0 on the read out.
- Press Absorbance push button. If the maximum absorbance obtained at λ of 485nm is 0.398 ± 0.002, the photometric calibration of instrument is confirmed to be proper.
- To confirm, repeat above steps, and set 10.00 on read out
- Press Absorbance botton.If the λ at 485nm is 1.000±0.002 then it is confirmed the photometric performance in the visible region is proper.

Absorbance: U.V region

- Place blank0.1N H_2SO_4 cuvette and 60ppm $K_2Cr_2O_7$ as sample
- Set λ exactly to 257 nm, if the value of Absorbance of sample at the set λ is0.864±0.005, the instrument is measuring Absorbance properly.

%Transmittance: As the value of %T is delivered from Absorbance itself, if the instrument is measuring Absorbance properly it is deemed that it measures %T properly.

Concentration:

- Place blank 0.1N H_2SO_4 cuvette and 60 ppm $K_2Cr_2O_7$ (0.06006g/l of $0.1NH_2SO_4$) as standard and 20 ppm (0.02002 g/l of $0.1NH_2SO_4$) as sample.
- Press "Concentration" push button and adjust Concentration control to 600 for standard on read out.
- Now place sample holder into optical path, if the value o f Concentration appearing on the read out for sample is 200±5, the instrument is measuring "concentration" properly.

3.3.4. Calibration of Wavelength:

a) Holmium filter: For routine calibrations, holmium filter is satisfactory. Record the absorption spectrum from 500 to 230 nm using slowest scan speed and narrowest slit setting. Identify 3 fused absorption bands centered on 452.2nm and single band at around 360.9nm.Instruments with accurately calibrated λ scales will show λ max at 453.2, 418.4, 360.9, 287.5, 279.4 and 241.5nm.

b**) Holmium per chlorate solution:** Prepare a solution of Holmium (III) per chlorate by dissolving 0.5g of holmium oxide in 2.4 ml perchloric acid (72% AR grade) by warming
gently and diluting to 10ml with water.Record the absorption spectrum from 500-230nm.The wavelengths of principal bands (Absorbance- 0.4) should be 485.8, 450.8,
416.3, 361.5, 287.1, 278.7, 241.1 nm.

c) **Discharge lamps: A** low pressure discharge lamp is suitable. Record the transmission spectrum from 600 to 240 nm of Mercury lamp place near the entrance to monochromatic, using minimum slit setting and slowest scan speed. The principal emission lines of Mercury are at 579.0, 576.9, 546.1, 435.8, 404.5, 364.9 and 253.7 nm.

d) Prepare standard solution by dissolve 100 mg of Potassium dichromate in 0.05N Potassium hydroxide solution in 100ml volumetric flask. Make up to volume with the same. From the standard solution take 20ml and make up to 500ml with

0.05N Potassium hydroxide solution. Now scan the wave length from 340 to 400nm using blank 0.05N Potassium hydroxide solution. The maximum wave length is observed at 370 nm.

3.3.5. Limit of stray light:

Weigh accurately 1.2g of dried Potassium chloride in 100 ml volumetric flask and makeup to mark with Double distilled water. Measure the absorbance at 200 nm.

Acceptance criteria: Tolerance limit NLT 2.0

Resolution:

Prepare 0.02%v/v solution of Toluene and make up with Hexane. Scan the wavelength from 250 to 280nm.Maximum absorbance is 269 nm and Minimum absorbance is 266nm

Acceptance criteria: Ratio limit NLT 1.5

3.3.6. Photometric linearity:

- Weigh accurately 100mg of Potassium chromate in 100ml volumetric flask and dissolve in 0.05N Potassium hydroxide solution. Make up with the same solvent.
- From the above solution take 20ml and make up to 500ml with 0.05N Potassium hydroxide solution.
- Now prepare dilution of 4,8,16,24,32 μg/ml
- Measure the absorbance at 370nm using blank.
- **Acceptance criteria:** The plot should be linear and regression coefficient (R2) should NLT 0.99

Table 11: Schedule for Calibration/Inspection of Some Major Instruments

INSTRUMENT	INTERVAL (MONTHS)
HPLC	3 months ±7 days
Gas Chromatography	3 months ±7days
UV-Visible spectrophotometer	Monthly once ±3days
IR spectrophotometer	Monthly once ±3days
NMR spectrophotometer	6 ±15days
Flour meter	3±7 days
Polari meter	Monthly once ±3days
PH meter	daily
Analytical balance	daily

The calibration of the entire analytical instrument or its components (which ever is Appropriate) should be performed after any major maintenance.

4. Conclusion: During all phases of clinical development, including the use of small scale facilities or Laboratories to manufacture batches of APIs for use in clinical trials, procedures should be in place to ensure that equipment is calibrated, clean and suitable for its intended use. Procedures for the use of facilities should ensure that materials are handled in a manner that minimizes the risk of contamination and cross-contamination. So validation and calibration is very important for analytical instruments.

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