HPLC CALIBRATION PARAMETERS

CALIBRATION: Calibration is the testing of Instruments with the primary standards for their reproducibility by confirming that the Instrument is within specification range and capable of working precisely and accurately.
For HPLC, Physical and chemical parameters are analysed for Calibration.

Physical Parameters include: -

- Flow rate Accuracy
- Temperature Accuracy
- Gradient proportional Valve Accuracy (GPV)
- Injector Accuracy

Chemical Parameters include: -

- Drift and Noise
- Injector Precision
- Injector Linearity
- Detector Linearity and Detector Sensitivity
- Wavelength Accuracy
- Carryover Test

The Instrument under calibration should meet the Acceptance criteria for each parameter and thereby the Instrument can be used for regular analysis for mentioned grace period.

Physical Parameters: -

- **Flow rate Accuracy: -**

  Put the solvent pumps into the HPLC grade water.
  Set the flow rate 1.0ml/min.
  Flow each solvent pump individually with 1.0ml/min flow rate (Solvent pump-A, Solvent pump-B, Solvent pump-C and Solvent pump-D)
  Perform multiple (two or three) times and average the value.
  The solvent is collected in the volumetric glassware for the set timer using stop-watch.
  Repeat with 2.0ml/min and 3.0ml/min Flow rate.
  Calculate the flow rate with the formulae: -

$$\text{Flow rate} = \frac{\text{Volume collected in ml}}{\text{Time in minutes}}$$

Acceptance criteria for the Flow rate accuracy is ±0.5% of the set flow.

- **Temperature Accuracy: -**

  Temperature is checked for Column compartment and Sample compartment (Auto sampler).
  Digital Thermometer is used for checking the set temperature and compared with the displayed temperature of the Instrument.

  For Column compartment: -
  Set the column compartment temperature for 60°C.
  Wait for column compartment to achieve the set temperature.
  Record the observed temperature using a calibrated temperature probe with digital thermometer.
  Repeat the procedure with different set temperatures, 50°C, 30°C, 20°C and 10°C.

  For sample compartment (Auto sampler): -
  Set the sample compartment temperature for 40°C.
  Wait for sample compartment to achieve the set temperature.
  Record the observed temperature using a calibrated temperature probe with digital thermometer.
  Repeat the procedure with different set temperatures, 30°C, 15°C, 10°C and 5°C.

  Acceptance criteria for the Temperature accuracy is ±0.2°C with the set temperature.

- **Gradient Proportion valve Accuracy (GPV): -**
  Column is replaced with Union to the column compartment.

Following Chromatographic conditions shall be used for Gradient proportion valve Accuracy

Column	Union
Mobile phase	Solent A & B in 0.0056 mg/ml of Propyl paraben Solvent C & D in Methanol
Flow rate	2.0ml/min
Injection volume	0µl
Detection	257mn
Run time	According to the Programme
Pump Mode	Gradient
Programme Curve	11

Gradient programme				
Time (minutes)	Mobile pump-A % Flow	Mobile pump-B % Flow	Mobile pump-C % Flow	Mobile pump-D % Flow
0	50	50	0	0
2	0	0	50	50
6	50	50	0	0
10	45	45	10	0
12	50	50	0	0
14	45	45	0	10
16	50	50	0	0
20	50	50	0	0

Note: - ***Above mentioned Gradient programme is mentioned based on literature available. You could use any available programme with you for the calculation of GPV Accuracy***

Acetonitrile: Methanol Proportions are taken as Mobile phase for some Programmes based on availability

Calculation of GPV: -

Calculate the GPV by ratio of heights of peaks obtained by inclusion of A, B, C and A, B, D with Full scale peak

$$GPV = \frac{\text{Height at A, B, C (or) Height at A, B, D} * 100}{\text{Height at Full scale}}$$

OR

Calculate the GPV by ratio of areas of peaks obtained by inclusion of A, B, C and A, B, D with Full scale peak

$$GPV = \frac{\text{Area at A, B, C (or) Area at A, B, D} * 100*2}{\text{Height at Full scale}}$$

Acceptance Criteria: -

The value of the ratio shall be 10.0±0.5%

- **Injector Accuracy: -**

  Fill the HPLC vial with Water and close with cap.
  Weigh the vial and record the weight, W1
  Place the vial in the HPLC into the sample compartment.
  Inject the required volume, say 20µl for mentioned times, say 10times.
  After completion, weigh the vial and record the weight, W2.
  Average µl per injection is calculated by using the formulae: -

$$\text{Average volume (µl)} = \frac{[(W1-W2)] * 1000}{\text{No. of Injections} * \text{Density of water at 25°C}}$$

Acceptance criteria for Injector accuracy is ±0.4µl with the set Injection volume.

Drift and Noise: -

Following Chromatographic conditions shall be used for Drift & Noise test

Column	C8 or C18 column with dimensions (250*4.6) mm, 5µm or (150*4.6) mm, 5µm
Mobile phase	50:50%v/v Methanol: Water
Flow rate	1.0ml/min
Injection volume	0µl
Detection	273mn
Run time	60minutes
Pump Mode	Isocratic

Noise and Drift parameter is tested for Detector's stability.

Acceptance Criteria: -

Noise should have less than or equal to 60µAU for UV Detector and less than or equal to 80 µAU for PDA Detector.
Drift should be less than 10milli AU/Hr

Chemical Parameters: -

Note: - Caffeine is used as a primary standard for calibration of HPLC for Chemical parameters.

Injector Precision: -

Primary standard is prepared at certain concentration (Say 100µg/ml) is injected with mentioned amount of injection volume (5µl and 100µl) for repeated number of injections (six) for calculating the difference in area of the standard with repeatability of Injections for the given chromatographic conditions.

Following Chromatographic conditions shall be used for Injector Precision

Column	C8 or C18 column with dimensions (250*4.6) mm, 5µm or (150*4.6) mm, 5µm
Mobile phase	50:50%v/v Methanol: Water
Flow rate	1.0ml/min
Injection volume	5µl & 100µl
Detection	273mn
Run time	Two times the Retention of Caffeine
Pump Mode	Isocratic
No. of Injections	Six repeated injections each

Acceptance Criteria: -

% RSD for Area of Caffeine should be NMT 1.0% for 5µl & 100µl

Injector Linearity: -

Primary standard is prepared at certain concentration (Say 100µg/ml).

Inject caffeine solution with Injection volume as 5 µl, 10 µl, 20 µl, 50 µl and 100 µl from the same vial.

Calculate the R square value by plotting Injection volume (x-axis) versus Area obtained for the injection (y-axis).

Following Chromatographic conditions shall be used for Injector Linearity

Column	C8 or C18 column with dimensions (250*4.6) mm, 5µm or (150*4.6) mm, 5µm
Mobile phase	50:50%v/v Methanol: Water
Flow rate	1.0ml/min
Injection volume	5 µl, 10 µl, 20 µl, 50 µl and 100 µl
Detection	273mn
Run time	Two times the Retention of Caffeine
Pump Mode	Isocratic
No. of Injections	Each 01 of mentioned volume from the same vial

Acceptance Criteria: -

Correlation co-efficient of Injection volume versus Area obtained should NLT 0.999

Detector Linearity & Detector sensitivity: -

Linear concentrations of Primary standard are prepared as 5µg,10µg, 25 µg, 100 µg, 125µg and 250µg per ml.

Inject the Linearity level solutions and record the area for the respective concentrations.

Calculate the R square value by plotting Concentration (x-axis) versus Area obtained for the injection (y-axis).

Following Chromatographic conditions shall be used for Detector Linearity

Column	C8 or C18 column with dimensions (250*4.6) mm, 5µm or (150*4.6) mm, 5µm
Mobile phase	50:50%v/v Methanol: Water
Flow rate	1.0ml/min
Injection volume	5 µl
Detection	273mn
Run time	Two times the Retention of Caffeine
Pump Mode	Isocratic
No. of Injections	Each 01

Acceptance Criteria: -

Correlation co-efficient of Concentration versus Area obtained should NLT 0.999

Detector sensitivity Calculation: -

Following formulae used for Calculating the Detector sensitivity

$$\text{Detector sensitivity} = \frac{\text{Area obtained}}{\text{Concentration}}$$

Acceptance Criteria: -

%RSD for the given concentrations of the Detector sensitivity should NLT 4.75

Wavelength Accuracy: -

Primary standard is prepared at certain concentration (Say 100µg/ml).

Inject the preparation at mentioned wavelengths and record the Area obtained for the respective wavelength.

Following Chromatographic conditions shall be used for Wavelength Accuracy

Column	C8 or C18 column with dimensions (250*4.6) mm, 5µm or (150*4.6) mm, 5µm
Mobile phase	50:50%v/v Methanol: Water
Flow rate	1.0ml/min
Injection volume	5 µl
Detection	200-400 (select 3D channel for PDA detector) and 200-210, 240-250 & 270-280 for UV detector, create number of programmes accordingly.
Run time	Two times the Retention of Caffeine
Pump Mode	Isocratic

No. of Injections	Each 01

Acceptance Criteria: -

For PDA detector

Wavelength maxima found should be between 273±2 nm.
Wavelength maxima found should be between 205±2 nm.
Wavelength minima found should be between 245±2 nm.

For UV detector

270-280nm: - Wavelength maxima found should be between 273±2 nm.
200-210nm: - Wavelength maxima found should be between 205±2 nm.
240-250nm: - Wavelength minima found should be between 245±2 nm.

Carryover Test: -

Primary standard is prepared at certain concentration (Say 500µg/ml).
Inject the sequence as Standard (Caffeine) and Carryover Blank.
Record the Areas obtained in the respective injections at Retention time of Caffeine.

Following Chromatographic conditions shall be used for Carryover Test

Column	C8 or C18 column with dimensions (250*4.6) mm, 5µm or (150*4.6) mm, 5µm
Mobile phase	50:50%v/v Methanol: Water
Flow rate	1.0ml/min
Injection volume	5 µl
Detection	273nm
Run time	Two times the Retention of Caffeine
Pump Mode	Isocratic
No. of Injections	Each 01

Calculate the carryover of the caffeine by the below mentioned equation.

$$\%\text{Carryover} = \frac{\text{Area obtained in carryover blank}*100}{\text{Area obtained in Standard (Caffeine)}}$$

Acceptance Criteria: -

% Carryover should NMT 0.01%

Calibration frequency: -

Calibration of HPLC is performed on six-month basis or after any major failure or after maintenance.

On completion of calibration, the HPLC calibration report shall be filled as per the given format.