Guidance Document

Calibration of HPLC

Document ID	Version	Issue Date
IPC/GD/05	1.0	16th September 2021

Indian Pharmacopoeia Commission

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Introduction

Guidance is provided on general procedure applicable for calibration of High Performance Liquid Chromatography (HPLC) taking Agilent Infinity Series (Quaternary Channel) system as an example. Parameters to be considered for calibration of HPLC are:

1. Calibration of Pump

Calibration of pump shall be done on the basis of the following parameters:

a) Flow rate accuracy
b) Flow rate consistency
c) Compositional accuracy (gradient profile)
d) Delay volume of the system

2. Calibration of Autosampler

Calibration of autosampler shall be done on the basis of the following parameters:

a) Injection volume accuracy
b) Injection volume precision
c) Injection volume linearity
d) Autosampler temperature accuracy

3. Calibration of Column Oven

4. Calibration of Detector

Calibration of detector shall be done on the basis of the following parameters:

a) Detector linearity
b) Wavelength accuracy

General Maintenance

Remove the column from the system and replace with dead volume connector or union. Flush the system using all channels at a flow rate of 2 ml/min with hot (50-70°) HPLC grade water for about half an hour. Following composition can be used: Channel A, 25%; B, 25%; C, 25%; and D, 25%.

1. Calibration of Pump

Calibration of pump should be done on the basis of the following parameters:

a) Flow Rate Accuracy

- Remove the column and put all the channels inlets in reservoirs of HPLC grade water. Set the flow rate at 1.0 ml/min. using the following composition: Channel A, 25%; B, 25%; C, 25%; and D, 25%.
- Collect the HPLC grade water from column inlet into a dry 10.0 ml of calibrated volumetric flask and note down the time taken to fill the volumetric flask till the mark using a calibrated stopwatch. Perform the exercise in duplicate.
- Calculate the flow rate as follows:
  Flow rate = Volume in ml /Time in minutes.
- Set the flow rate at 2.0 ml/min. and 3.0 ml/min. and perform the same exercise in duplicate.
- **Acceptance Criteria:** The flow rate should be within ±2.0% of the set value.

b) Flow Rate Consistency

- Accurately weigh about 100 mg of Caffeine IPRS into a 100.0 ml volumetric flask. Dissolve in about 10 ml of methanol and make up the volume with mobile phase.

Further dilute accordingly with mobile phase to get solution having concentration of 10 ppm.

- **Chromatographic conditions:**

Column	: Octadecylsilane (C-18) or Octylsilane (C-8) (250 mm x 4.6 mm x 5 µm)
Mobile phase	: Methanol : Water (50 : 50)
Flow rate	: 1 ml/min.
Injection volume	: 20 µL
Detection	: UV at 272 nm
Run time	: 10 minutes
Retention time of caffeine	: About 5 minutes

- Inject 10 ppm caffeine solution six times and calculate %RSD of the retention time of caffeine as obtained from the chromatograms.
- **Acceptance criteria:** The %RSD of retention time of caffeine should not be more than 1.0%.

c) Compositional Accuracy (Gradient Profile)

- Remove the column from the system and replace with dead volume connector.
- Prepare a 0.25% v/v solution of acetone in water. Flush the channels at a flow rate of 1.0 ml/min. using the composition given below:

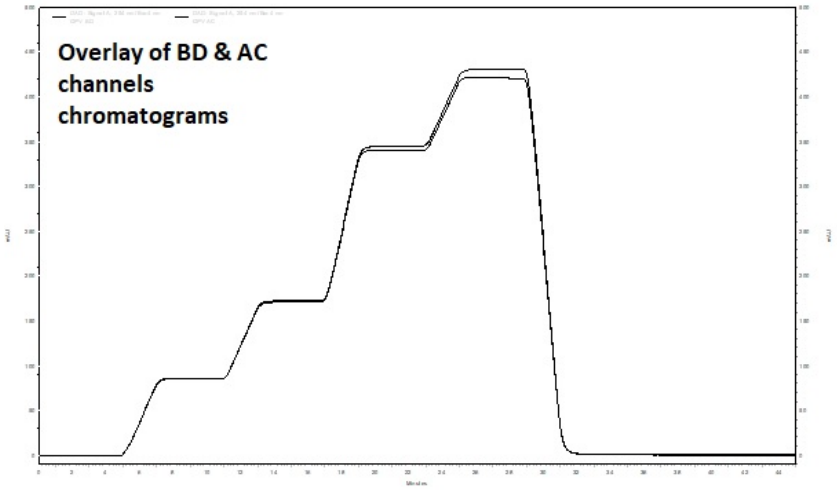
Time (min)	HPLC grade water (Channel A, B) (%)	0.25% v/v Acetone in water (Channel C, D) (%)
0	25 + 25	25 + 25
10	25 + 25	25 + 25
12	50 + 50	0 + 0
20	50 + 50	0 + 0

- Check the compositional accuracy of the HPLC system with the conditions given below:

Flow rate	: 1 ml/min.
Detection	: UV at 254 nm
Run time	: 30 minutes
Injection delay	: 15 minutes

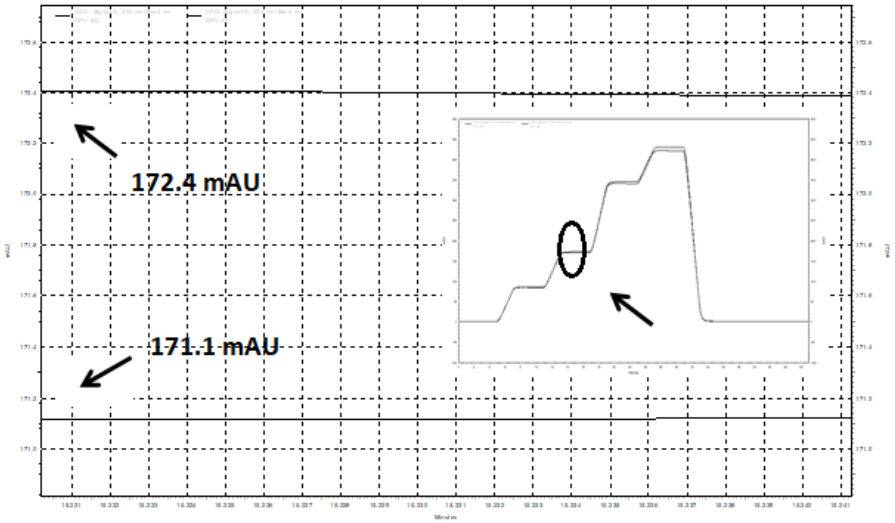
Time (min.)	HPLC grade water (Channel A) (%)	0.25% v/v Acetone in water (Channel C) (%)
0	100	0
4	100	0
6	80	20
10	80	20
12	60	40
16	60	40
18	20	80
22	20	80
24	0	100
28	0	100
30	100	0

- Run the gradient using channel combination A and C and repeat the same gradient using channel combination B and D.
- Inject 0 µL or minimum volume of HPLC grade water and record the gradient profile.
- Print the overlay plot of gradient profile of A/C and B/D. Chromatogram is attached for reference.



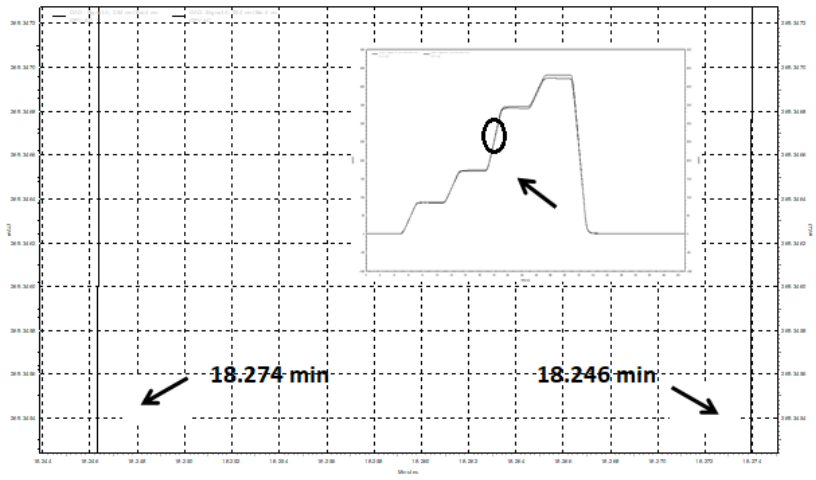
- **Acceptance Criteria:** The gradient profile of A/C and B/D should overlay with each other with difference in absorbance should not more than 0.01 AU and the difference in time should not more than 20 seconds. Chromatogram is attached for reference.

Difference in Absorbance (Limit NMT 0.01 AU)



Difference in Absorbance	172.4-171.1	mAU
	1.3	mAU
	1.3/1000	
	0.0013	AU

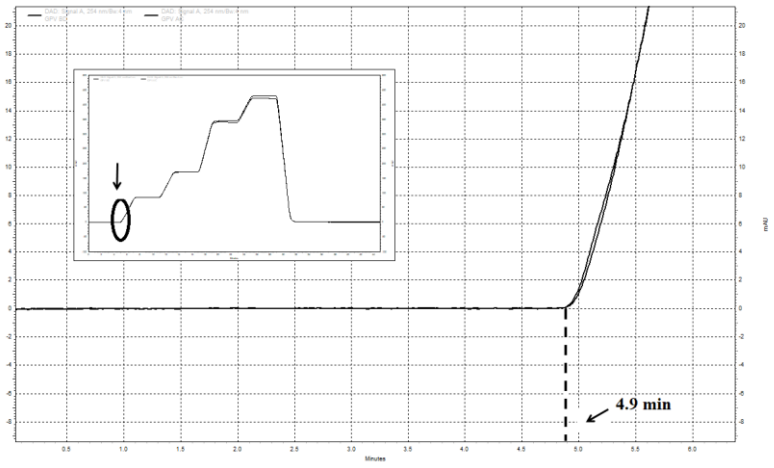
Difference in time (Limit NMT 20 seconds)



Difference in Time	18.274-18.246	min
	0.028	min
	0.028 x 60	seconds
	1.662	seconds

d) Delay Volume of the System

- Review the gradient profile performed under - Compositional accuracy (Gradient profile). Note the time in minutes taken for the actual first change in absorbance (Lift of the baseline).
- The delay volume of the system can be calculated in terms of ml by subtracting 4 minutes from the actual time in minutes taken for change in absorbance.
- **Acceptance criteria:** The delay volume of the system should be not more than 1.0 ml. Chromatogram is attached for reference.



Delay time	4.9-4.0	min
	0.9	min
Delay volume	Delay time (min) X Flow rate (ml/min)	
	0.9 X 1.0	
	0.9 ml	
Limit	NMT	1.0 ml

2. Calibration of Autosampler

Calibration of autosampler shall be done on the basis of the following parameters:

a) Injection Volume Accuracy

- Purge the instrument with HPLC grade water.
- Fill the HPLC vial with HPLC grade water and close with a cap. Weigh this vial and record weight in gram (W1). Water is used in auto injection volume accuracy test because its density is 0.9982 g/ml at 20°C and 0.9970 g/ml at 25°C. This introduces less than 0.3% error when volume is assumed equal to weight.
- Program HPLC system with a flow rate of 1.0 ml/min. with water and run time of 1 minute.
- Inject 20 µL from HPLC Vial and repeat it for 10 times from the same HPLC vial.
- After completion of 10 injections, remove the vial and weigh again (W2).
- Calculate the average volume (in µL) injected per injection using formula:

  Average Volume = [(W1-W2)/10] x1000= µl/injection

- **Acceptance Criteria:** Average volume of injection (µl/injection) should be 20 µl±0.4 µl.

b) Injection Volume Precision

- Flush the HPLC system with HPLC grade water for about half an hour.
- **Chromatographic conditions:**

Column	: Octadecylsilane (C-18) or Octylsilane (C-8) (250 mm x 4.6 mm x 5 µm)
Mobile phase	: Methanol : Water (50 : 50)
Flow rate	: 1 ml/min.
Injection volume	: 20 µL
Detection	: UV at 272 nm
Run time	: 10 minutes
Retention time of caffeine	: About 5 minutes

- Accurately weigh about 100 mg of caffeine in a 100 ml volumetric flask. Dissolve in 10 ml of methanol and make up the volume with mobile phase. Further dilute accordingly with mobile phase to get solution having concentration of 10 ppm.
- Inject this solution six times and calculate %RSD for the area obtained in the chromatograms.
- **Acceptance criteria:** %RSD for the area should not be more than 1.0%.

c) Injection volume linearity

- Inject 10 ppm caffeine solution with duplicate injection of 5 µl, 10 µl, 20 µl, 50 µl and 100 µl.
- From the data obtained, plot a graph of mean area count of duplicate injections on y-axis versus injection volume on the x-axis and calculate the value of R-square.
- **Acceptance criteria:** R-square should be not less than 0.9990.

d) Autosampler Temperature Accuracy

- Set the sample compartment temperature at 40º.
- After about 10 minutes record the observed temperature using a calibrated probe with digital thermometer.

- Repeat the same at 30°, 15°, 10° and 5°.
- **Acceptance Criteria:** The observed temperature should be within ±2ºC of the set temperature.

3. Calibration of Column Oven

- Set the column oven temperature at 60º.
- After about 10 minutes, record the temperature using a calibrated probe with a digital thermometer.
- Repeat the same at 50°, 30°, 20° and 10°.
- **Acceptance Criteria:** The observed temperature should be within ±2ºC of the set temperature.

4. Calibration of Detector

Set up the HPLC system using chromatographic conditions mention below:

Column	: Octadecylsilane (C-18) or Octylsilane (C-8) (250 mm x 4.6 mm x 5 µm)
Mobile phase	: Methanol : Water (50 : 50)
Flow rate	: 1 ml/min.
Injection volume	: 10 µL
Detection	: UV at 272 nm
Run time	: 10 minutes
Retention time of caffeine	: About 5 minutes

a) Detector Linearity

- Accurately weigh about 100.0 mg of caffeine in a 100 ml volumetric flask. Dissolve in 10 ml of methanol and make up the volume with mobile phase.
- Further dilute accordingly with mobile phase to get solution having concentration of about 0.001, 0.01 and 0.10 mg/ml (1.0, 10.0 and 100.0 µg/ml).
- Inject in duplicate 10 µl of 0.001, 0.01 and 0.10 mg/ml solutions of caffeine prepared above.
- From the data obtained plot a graph of mean area counts of duplicate injection on y-axis versus concentration (µg/ml) on x-axis and calculate the value of R-square.
- **Acceptance Criteria:** R-square should not be less than 0.9990.

b) Wavelength Accuracy

For Photo Diode Array Detectors (PDA)

- Set detector wavelength at 200 nm to 400 nm.
- Inject 20 µl of 0.01 mg/ml solution of caffeine.
- Record spectrum and report maxima and minima.
- **Acceptance Criteria**
  - 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 Variable Wavelength Detectors

- Create 32 acquisition program with same parameters except changing wavelength 269 to 278, 239 to 249 and 200 to 210.
- Inject 20 µL of 0.01 mg per mL solution of Caffeine.
- Record spectrum and report maxima and minima.

- **Acceptance Criteria**
  - 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.

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.

	INDIAN PHARMACOPOEIA LABORATORY
	HPLC CALIBRATION REPORT

Instrument No. :
Instrument Make :
Calibration Frequency :

SOP No. :
Calibrated on :
Next Due on :

PUMP				
S. No.	**Parameter**	**Limit**	**Observed Value**	**Complies/ Does Not Comply**
1	FLOW RATE ACCURACY	±2.0 % of set value		
	1.0 mL/minute	0.98 – 1.02 mL/minute		
	2.0 mL/minute	1.96 – 2.04 mL/minute		
	3.0 mL/minute	2.94 – 3.06 mL/minute		
2	FLOW RATE CONSISTENCY	%RSD of retention time of Caffeine NMT 1.0%		
3	COMPOSITIONAL ACCURACY	Difference in Absorbance NMT 0.01 AU		
		Difference in Time NMT 20 sec.		
4	DELAY VOLUME	NMT 1.0 mL		
AUTOSAMPLER				
1	INJECTION VOLUME ACCURACY	Average volume for 10 injections 20 ± 0.4 µL		
2	INJECTION VOLUME PRECISION	%RSD NMT 1.0 %		
3	INJECTION VOLUME LINEARITY	R-SQUARE NLT 0.9990		
4	AUTO SAMPLER TEMPERATURE ACCURACY	5 ºC ± 2ºC		
		10 ºC ± 2ºC		
		15ºC ± 2ºC		
		30 ºC ± 2ºC		
		40ºC ± 2ºC		
COLUMN OVEN				
1	TEMPERATURE ACCURACY	10 ºC± 2ºC		
		20 ºC± 2ºC		
		30 ºC± 2ºC		
		50 ºC± 2ºC		
		60ºC± 2ºC		
DETECTOR				
1	LINEARITY	R-Square NLT 0.9990		
2	WAVELENGTH ACCURACY	Maxima 273 ± 2 nm		
		Maxima 205 ± 2 nm		
		Minima 245 ± 2 nm		

Performed By _____ Verified By _____