HPLC Method Description for In-process Control of Intermediates

Project	**Pyronaridine_INV-054926**
Compound	**PNDa01, PNDa02, PNDa06, PNDa04 and PND**
Purpose	**Method Description**
Category	**Methods**
Substance Type	**Intermediate**
Report ID	**INV_054926_HPLC_M1 Version 1.0**

Authors

Cai Alain

Approvers

By: DocuSigned by: Alain Cai 9DCF20AFFA87456... Title: AD Manager Date: 1/21/2024

By: DocuSigned by: Qi 55362BFE31B84BD... Title: Head of Technical Development Date: 1/21/2024

Distribution

Zhou Irvin, Pyronaridine (INV-054926) project team, Approvers

Table of contents

1. Objective 3

2. Summary and conclusion 3

3. Experimental 6

3.1. HPLC 6
3.2. Equipment and reagents 7
3.3. Solutions 7
3.3.1. Diluent 7
3.3.2. Mobile phase preparation 7
3.3.3. Solution preparations 7
3.4. Proposed injection sequence and system suitability test 9
3.5. Calculation 10

4. Figures 11

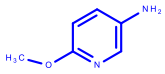
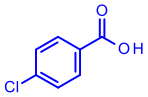
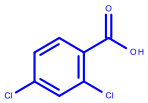
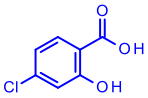
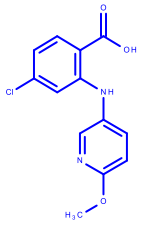
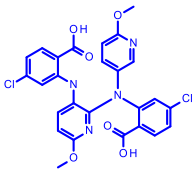
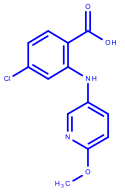
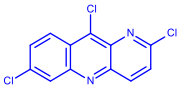
1. Objective

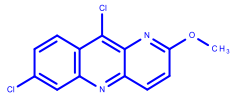
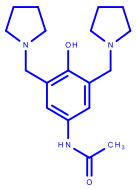
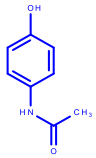
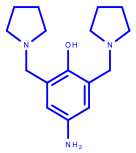
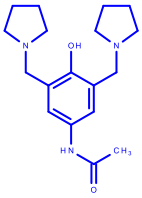
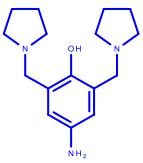
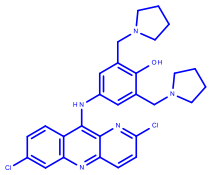
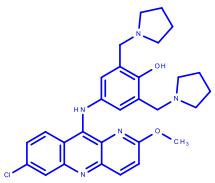
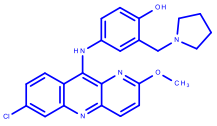
This method 'INV_054926_HPLC_M1' for intermediates of Pyronaridine (INV-054926) project is developed by HPLC. The parameters of the reversed phase HPLC method suitable for in-process control of intermediate steps shall be described in this document.

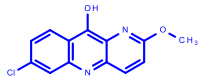
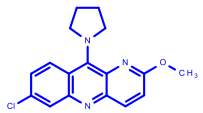
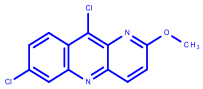
2. Summary and conclusion

The method is applied for the HPLC testing of all intermediates (in-process control). This method is based on reversed phase liquid chromatography with UV detection and gradient elution using a Waters Atlantis T3, 3µm, 150 x 4.6mm HPLC column.

Table 1 Structure, Retention time and RRT of all intermediates and their related substances

Compound	Structure	Retention time (RT)	RRT
PNDa01 step			
6-methoxy-3-aminepyridine (SM 2)		ca. 6.6 min	ca. 0.34
PNDa01 impurity 2		ca. 17.4 min	ca. 0.91
2,4-dichlorobenzoic (SM 1)		ca. 18.0 min	ca. 0.94
PNDa01 impurity 1		ca. 18.2 min	ca. 0.95
PNDa01		ca. 19.2 min	1.00
PNDa01 impurity 3		ca. 20.5 min	ca. 1.07
PNDa02 step			
PNDa01		ca. 19.2 min	ca. 0.85
BIA		ca. 21.9 min	ca. 0.97

Compound	Structure	Retention time (RT)	RRT
PNDa02		ca. 22.5 min	1.00
PNDa06-HCl step			
PNDa06		ca. 7.7 min	1.00
Acetaminophen (SM5)		ca. 8.1 min	ca. 1.05
PNDa04-HCl step			
PNDa04		ca. 6.4 min	1.00
PNDa06		ca. 7.7 min	ca. 1.20
PND step			
PNDa04		ca. 6.4 min	ca. 0.54
DIA		ca. 11.4 min	ca. 0.97
PND		ca. 11.8 min	1.00
DIN		ca. 13.4 min	ca. 1.14

Compound	**Structure**	**Retention time (RT)**	**RRT**
TGF-001 impurity 1		ca. 16.2 min	1.37
TGF-001 impurity 2		ca. 17.1 min	1.45
PNDa02		ca. 22.5 min	1.91

Example chromatograms and extracted HPLC-PDA spectra are given in Section 4.

3. Experimental

Equivalent equipment or grade of materials can be used.

3.1. HPLC

- HPLC System: Quaternary pump module (e.g.: Waters Alliance 2695)
  PDA detector (e.g.: Waters Alliance 2998)
  Auto sampler (e.g.: Waters Alliance 2695)
  Column oven (e.g.: Waters Alliance 2695)
- Empower-control and integration software or equivalent
- Column: Waters Atlantis T3, 3µm, 150 x 4.6mm
- Flow rate: 1.0 mL/min
- Elution: Gradient mode
- Run time: 30.0 min
- Detection:

PNDa01 step	254 nm
PNDa02 step	254 nm
PNDa06-HCl step	254 nm
PNDa04-HCl step	278 nm
PND step	278 nm

- Injection: 10 µL
- Column temp.: 35°C ± 5°C
- Auto sampler temp.: Room temperature
- Mobile phase (see section 3.3.2):
  - **A:** 0.1% TFA in Water
  - **B:** 0.1% TFA in Acetonitrile
- Diluent:

PNDa01 step	1M Na_2CO_3 in water/Acetonitrile=90/10 v/v
PNDa02 step	2-MeTHF
PNDa06-HCl step	Acetonitrile/ water = 20/80 v/v
PNDa04-HCl step	Acetonitrile/ water = 5/95 v/v
PND step	Acetonitrile/ water = 50/50 v/v

- Needle wash: Water/ Acetonitrile (50:50 v/v)
- Equilibration time: 5 min
- Gradient:

Table 2 Gradient Table

Time (min)	% A	% B
0.0	100.0	0
3.0	100.0	0
13.0	65.0	35.0
20.0	1.0	99.0
25.0	1.0	99.0
25.1	100.0	0

Time (min)	% A	% B
30.0	100.0	0

3.2. Equipment and reagents

- Balance: e.g.: Mettler Toledo XP56
- Acetonitrile: HPLC grade, e.g.: Merck LiChrosolv
- Water: HPLC grade, e.g.: from Milipore ultra-pure water system
- TFA: HPLC grade, e.g.: Sigmer-Aldrich
- 2-MeTHF: HPLC grade, e.g.: Innochem
- DMSO: HPLC grade, e.g.: Innochem
- Glassware: 10, 50, 100 mL volumetric flasks, 1L graduated cylinders
- Pipette: e.g.: 1.0, 5.0 mL Pipette

3.3. Solutions

3.3.1. Diluent

Different volumes can be prepared as soon as the solvent ratio is the same.

Diluent 1: 1M Na_2CO_3 in water/Acetonitrile=90/10 v/v for PNDa01

Diluent 2: 2-MeTHF for PNDa02

Diluent 3: Acetonitrile/ water = 20/80 v/v for PNDa06-HCl

Diluent 4: Acetonitrile/ water = 5/95 v/v for PNDa04-HCl

Diluent 5: Acetonitrile/ water = 50/50 v/v for PND

3.3.2. Mobile phase preparation

Preparation is described for a volume of 1 liter. Different volumes can be prepared as soon as the solvent ratio is the same.

Mobile phase A (0.1% TFA in Water):

In a suitable container, add 1000 mL of water and 1 mL of TFA. Mix well.

Mobile phase B (0.1% TFA in Acetonitrile):

In a suitable container, add 1000 mL of acetonitrile and 1 mL of TFA. Mix well.

3.3.3. Solution preparations

Other volumes and weigh-ins might be used as long as the final concentration remains the same. Min. weight of used balance must be considered during sample preparation.

3.3.3.1. Standard solutions

- PNDa01 step

IPC Standard Solution (10% SM1, 10% SM2 in 100% PNDa01):

Weigh approx. 2 mg of SM1, 2 mg of SM2 and 20 mg of PNDa01 reference standards into a 100-mL volumetric flask. Dissolve and dilute to volume with diluent 1. Mix well (SM1/ SM2: 0.02 mg/mL, PNDa01: 0.2 mg/mL).

- PNDa02 step

IPC Standard Solution (10% PNDa01 in 100% PNDa02):

Weigh approx. 0.8 mg of PNDa01 and 8 mg of PNDa02 reference standards into a 100-mL volumetric flask. Dissolve and dilute to volume with diluent 2. Mix well (PNDa01: 0.008 mg/mL, PNDa02: 0.08 mg/mL).

- PNDa06-HCl step

IPC Standard Solution (10% SM5 in 100% PNDa06):

Weigh approx. 3 mg of SM5 and 39 mg of PNDa06-HCl reference standards into a 100-mL volumetric flask. Dissolve and dilute to volume with diluent 3. Mix well (SM5: 0.03 mg/mL, PNDa06 free base: 0.3 mg/mL).

- PNDa04-HCl step

IPC Standard Solution (10% PNDa06 in 100% PNDa04):

Weigh approx. 6.5 mg of PNDa06-HCl and 83 mg of PNDa04-HCl reference standards into a 100-mL volumetric flask. Dissolve and dilute to volume with diluent 4. Mix well (PNDa06 free base: 0.05 mg/mL, PNDa04 free base: 0.5 mg/mL).

- PND step

IPC Standard Solution (10% PNDa02, 10% PNDa04 in 100% PND):

Weigh approx. 1.8 mg of PNDa02, 3 mg of PNDa04-HCl and 18 mg of PND reference standards into a 100-mL volumetric flask. Dissolve and dilute to volume with diluent 5. Mix well (PNDa04 free base/ PNDa02: 0.018 mg/mL, PND: 0.18 mg/mL).

3.3.3.2. Sample solutions

The IPC sample is submitted as a solution of each intermediate. Dilute the sample to a suitable concentration based on the estimated reaction solution concentration.

- PNDa01 step

For example: If the reaction solution is 360 mg/mL of PNDa01, transfer 0.5 mL of sample into a 50-mL volumetric flask and dilute to volume with diluent 1 (3.6 mg/mL), and then transfer 3 mL of that solution into a 50-mL volumetric flask and diluent to volume with diluent 1 (0.216 mg/mL).

- PNDa02 step

For example: If the reaction solution is 110 mg/mL of PNDa02, transfer 0.5 mL of sample into a 50-mL volumetric flask and dilute to volume with diluent 2 (1.1 mg/mL), and then transfer 0.7 mL of that solution into a 10-mL volumetric flask and diluent to volume with diluent 2 (0.077 mg/mL).

- PNDa06-HCl step

For example: If the reaction solution is 410 mg/mL of PNDa06 free base, transfer 0.5 mL of sample into a 50-mL volumetric flask and dilute to volume with diluent 3 (4.1 mg/mL), and then transfer 0.8 mL of that solution into a 10-mL volumetric flask and diluent to volume with diluent 3 (0.328 mg/mL of free base).

- PNDa04-HCl step

For example: If the reaction solution is 100 mg/mL of PNDa04 free base, transfer 0.5 mL of sample into a 50-mL volumetric flask and dilute to volume with diluent 4 (1 mg/mL), and then transfer 5 mL of that solution into a 10-mL volumetric flask and diluent to volume with diluent 4 (0.5 mg/mL of free base).

- PND step

For example: If the reaction solution is 230 mg/mL of PND, transfer 0.5 mL of sample into a 50-mL volumetric flask and dilute to volume with diluent 5 (2.3 mg/mL), and then transfer 0.8 mL of that solution into a 10-mL volumetric flask and diluent to volume with diluent 5 (0.184 mg/mL).

3.4. Proposed injection sequence and system suitability test

Table 3 Proposed injection sequence and SST criteria

Sample name	No. of injections	SST acceptance criteria
Blank (sample diluent)	1 + N [1]	No interference between the blank peaks and the components of interest
IPC standard Solution	1	For identity
Sample solution	1	N/A

[1] Additional blanks may be run until an acceptable baseline is obtained.

3.5. Calculation

Reaction monitoring by formula (%area):

- ***PNDa01 step***

$$\%\ SM2\ (remaining\ by\ conversion) = \frac{A_{SM2}}{A_{SM2}+A_{PNDa01}} * 100\%$$

Where:

A_{SM2} = peak area of SM2 in sample solution (diluted reaction solution)

A_{PNDa01} = peak area of PNDa01 in sample solution (diluted reaction solution)

- ***PNDa02 step***

$$\%\ PNDa01\ (remaining\ by\ conversion) = \frac{A_{PNDa01}}{A_{PNDa01}+A_{PNDa02}} * 100\%$$

Where:

A_{PNDa01} = peak area of PNDa01 in sample solution (diluted reaction solution)

A_{PNDa02} = peak area of PNDa02 in sample solution (diluted reaction solution)

- ***PNDa06-HCl step***

$$\%\ SM5\ (remaining\ by\ conversion) = \frac{A_{SM5}}{A_{SM5}+A_{PNDa06}} * 100\%$$

Where:

A_{SM5} = peak area of SM5 in sample solution (diluted reaction solution)

A_{PNDa06} = peak area of PNDa06 in sample solution (diluted reaction solution)

- ***PNDa04-HCl step***

$$\%\ PNDa06\ (remaining\ by\ conversion) = \frac{A_{PNDa06}}{A_{PNDa06}+A_{PNDa04}} * 100\%$$

Where:

A_{PNDa06} = peak area of PNDa06 in sample solution (diluted reaction solution)

A_{PNDa04} = peak area of PNDa04 in sample solution (diluted reaction solution)

- ***PND step***

$$\%\ PNDa02\ (remaining\ by\ conversion) = \frac{A_{PNDa02}}{A_{PNDa02}+A_{PND}} * 100\%$$

Where:

A_{PNDa02} = peak area of PNDa02 in sample solution (diluted reaction solution)

A_{PND} = peak area of PND in sample solution (diluted reaction solution)

4. Figures

Figure 1 Example HPLC chromatogram of PNDa01 IPC solution, method INV_054926_HPLC_M1, 254nm

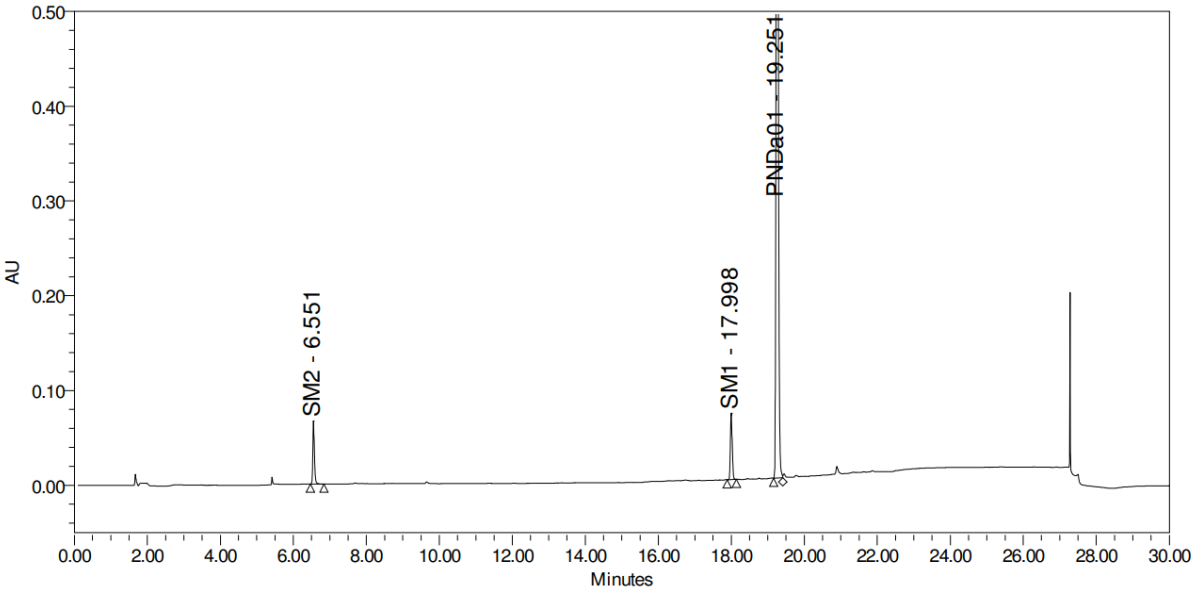


Figure 2 Example HPLC chromatogram of PNDa02 IPC solution, method INV_054926_HPLC_M1, 254nm

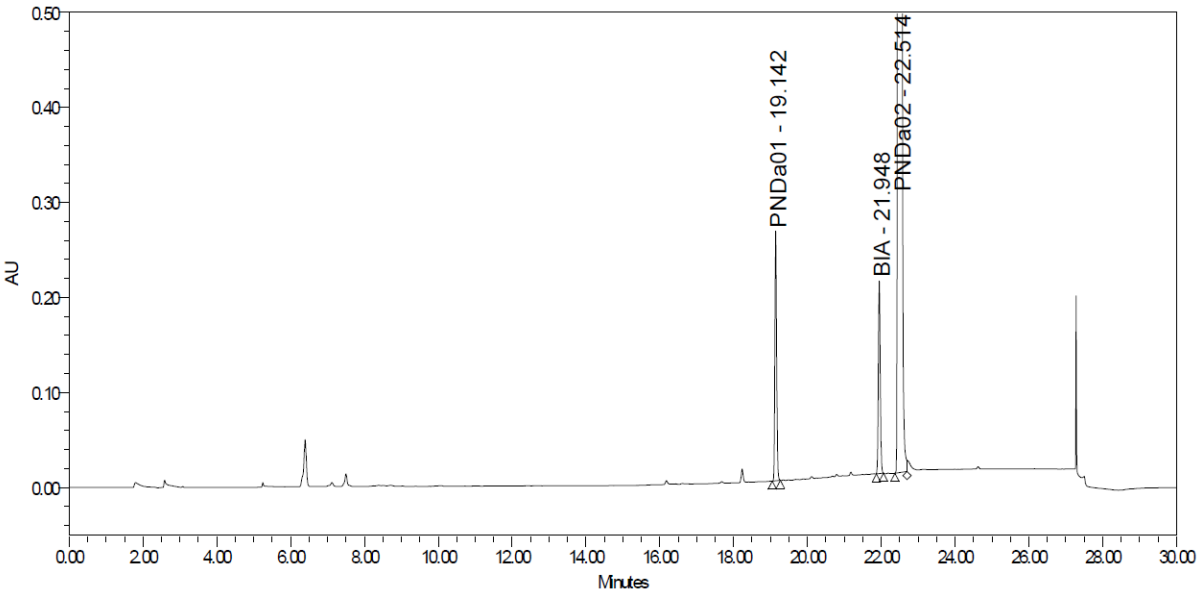


Figure 3 Example HPLC chromatogram of PNDa06-HCl IPC solution method INV_054926_HPLC_M1, 254nm

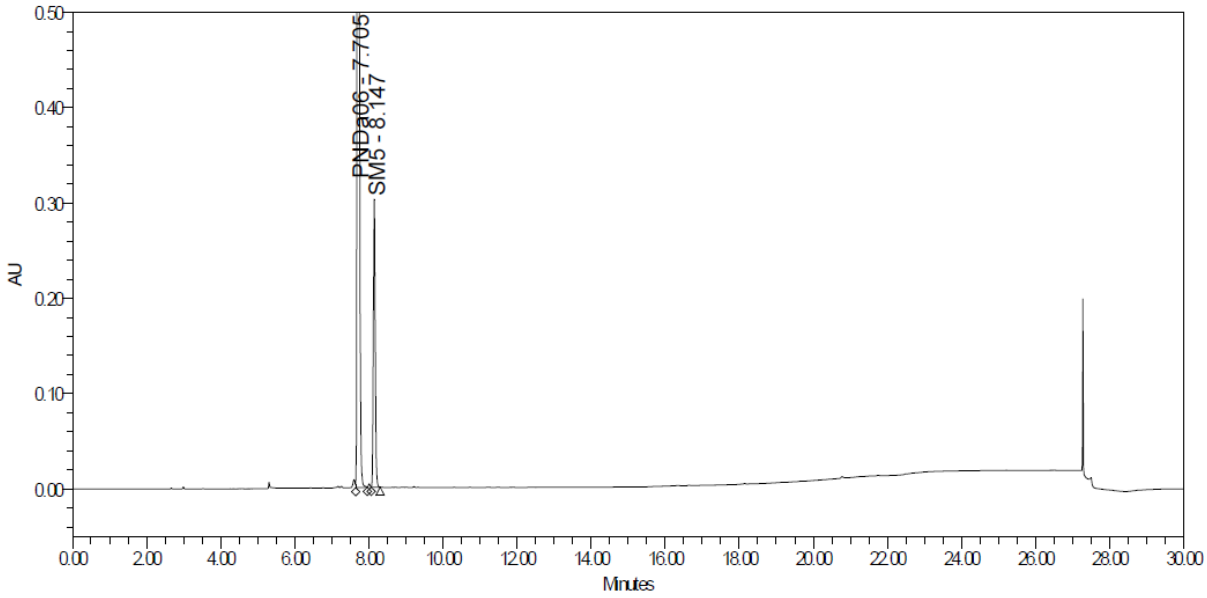


Figure 4 Example HPLC chromatogram of PNDa04-HCl IPC solution, method INV_054926_HPLC_M1, 278nm

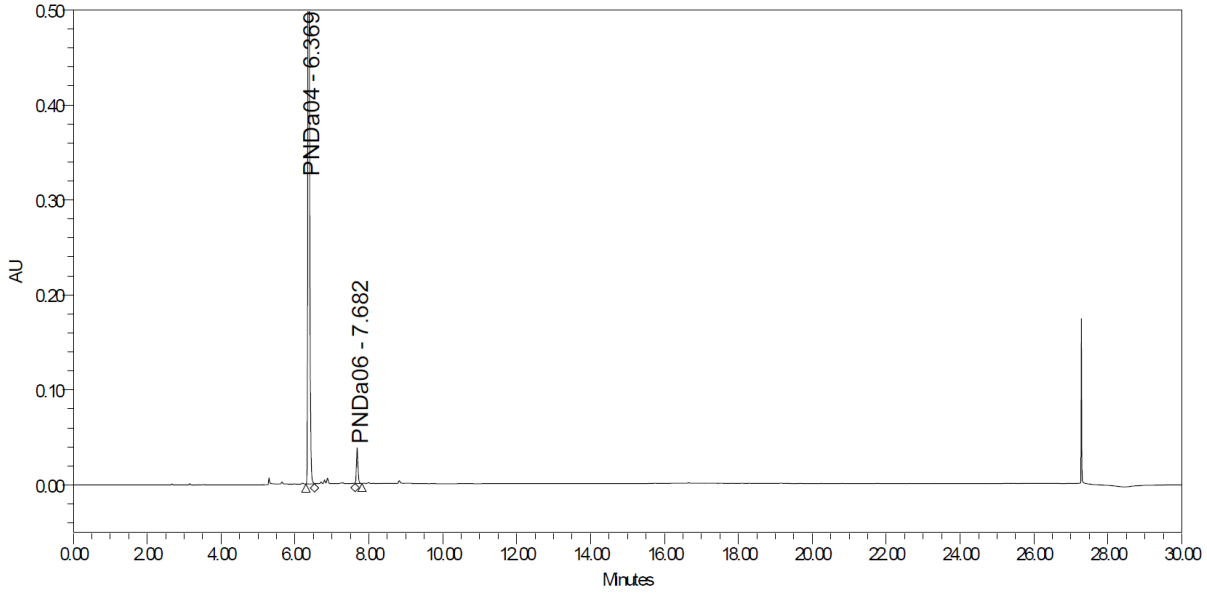


Figure 5 Example HPLC chromatogram of PND IPC solution, method INV_054926_HPLC_M1, 278nm