HPLC Method Description for Identity, Assay and Related Substances of PNDa02

Project	**Pyronaridine_INV-054926**
Compound	**PNDa02**
Purpose	**Method Description**
Category	**Methods**
Substance Type	**Intermediate**
Report ID	**INV_054926_HPLC_M3 Version 1.0**

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Distribution

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1. Objective

This method 'INV_054926_HPLC_M3' for intermediate PNDa02 of Pyronaridine (INV-054926) project is developed by HPLC. The parameters of the reversed phase HPLC method suitable for identity, assay, and related substances of PNDa02 shall be described in this document.

Related reports:

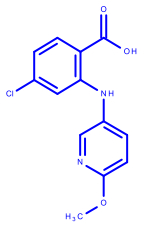
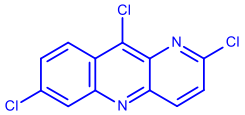
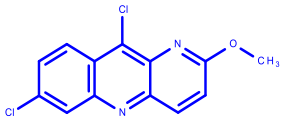
INV_054926_HPLC_M1: HPLC Method Description for In-process Control of Intermediates

INV_054926_HPLC_V3: HPLC Method Limited Validation for PNDa02 (non-GMP)

2. Summary and conclusion

The method is applied for the HPLC testing of PNDa02 (identity, assay and related substances). 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 PNDa02 and its related substances

Compound	Structure	Retention time (RT)	RRT
PNDa01		ca. 3.3 min	ca. 0.32
BIA		ca. 8.5 min	ca. 0.84
PNDa02		ca. 10.2 min	**1.00**

Example chromatograms and extracted HPLC-PDA spectrum of PNDa02 are given in Section 4.

Specificity, LOQ, Linearity of PNDa02, Accuracy, Repeatability was performed and reported in report 'INV_054926_HPLC_V2'.

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: 25.0 min
- Detection: 248 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: Dimethyltetrahydrofuran (2-MeTHF)
- Needle wash: Water/ Acetonitrile (50:50 v/v)
- Equilibration time: 5 min
- Gradient:

Table 2 Gradient Table

Time (min)	% A	% B
0.0	30.0	70.0
5.0	30.0	70.0
17.0	1.0	99.0
20.0	1.0	99.0
20.2	30.0	70.0
25.0	30.0	70.0

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: AR grade, e.g.: Innochem
- Glassware: 10, 50, 100-mL volumetric flasks, 1L graduated cylinders
- Pipette: e.g.: 1.0 mL Pipette

3.3. Solutions

3.3.1. Diluent

Dimethyltetrahydrofuran (2-Me THF).

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

Standard Solution 1 & 2 (conc.: 0.1 mg/mL):

Accurately weigh approx. 10 mg of PNDa02 reference standard into a 100-mL volumetric flask.

Dissolve and dilute to volume with diluent. Mix well.

Prepare in duplicate if needed.

Standard Solution 3 (0.05%, corresponding to 0.00005 mg/mL):

Transfer 0.5 mL of Standard Solution 1 into a 50 mL volumetric flask. Fill up to volume with diluent and mix well.

Transfer 0.5 mL of above solution into a 10 mL volumetric flask. Fill up to volume with diluent and mix.

3.3.3.2. Sample solutions

Accurately weigh approx. 10 mg of PNDa02 sample into a 100-mL volumetric flask. Dissolve and dilute to volume with diluent. Mix well.

Number of sample preparations depends on the samples under analysis.

3.4. Proposed injection sequence and system suitability test

Table 3 Proposed injection sequence and SST criteria

Sample name	No. of injections [1]	SST acceptance criteria
Blank (diluent)	1 + N [1]	No interference between the blank peaks and the components of interest
Standard Solution 3 (0.05%)	1	S/N ≥ 10
Standard Solution 2	6	%RSD (main peak area) ≤ 2 %
Standard Solution 1	1	Recovery: 98% - 102% (6 injection Std 2 to be used as reference)
Sample solution prep.1	1	N/A
Sample solution prep.2	1	for Identification purpose just 1 sample preparation is required
Standard Solution 2 [2]	1	Recovery: 98% - 102% (6 injection Std 2 to be used as reference)

[1] Additional blanks may be run until an acceptable baseline is obtained. - [2] For multiple sample analysis, 1 injection of standard solution 2 is recommend every 6 sample preparation injections.

3.5. Calculation and Reporting

Calculations should be performed individually for each sample weighing. Only then should the calculation of the average result be performed.

3.5.1. Identification by HPLC

The main peak retention time of standard injections should not differ by more than 5% from the main peak retention time of the sample injections.

3.5.2. Purity by HPLC in %area

Calculate the purity using the following formula, for each sample preparation:
100 - %(area) Total impurities

Calculate the average of the 2 individual preparations by: (P1+P2) /2

Where:
Pi = PNDa02 purity % (area)

3.5.3. Related substances by HPLC: Total impurities in %area

Sum of the % area of all impurities (Report only the peaks for which the % area is not less than 0.05%).

3.5.4. Related substances by HPLC: Individual impurities in %area (by RRT)

Report all individual impurities ≥ 0.05% (area) by their RRT.
Analyses with two sample weighings: (A1+A2)/2
Where:
Ai = impurity peak % (area)

In case of the specified impurities, report:
- For impurity content below LOQ concentration, report "Less than 0.05 %(area)";
- If impurity is not detected, report "Not detected".

3.5.5. Assay by HPLC in %w/w

$$\text{Assay}_{\text{PNDa02}} \quad (\%\text{w/w}) \quad = \frac{\text{Area}_{\text{sam}} \times \text{W}_{\text{std}} \times \text{V}_{\text{sam}}}{\text{Area}_{\text{std}} \times \text{W}_{\text{sam}} \times \text{V}_{\text{std}}} \times \text{P}_{\text{std}}$$

Where:
Area $_{sam}$= PNDa02 peak area obtained in the sample chromatogram
Area $_{std}$= Average PNDa02 peak area obtained for the 6 standard injections (std 2)
W $_{std}$ = Standard weight (Std 2)
W $_{Sam}$ = Sample weight (mg)
V $_{sam}$ = Volume of the flask used in the sample preparation
V $_{std}$ = Volume of the flask used in the standard preparation (Std 2)
P $_{std}$ = Potency determined for the standard in used. Use value from CoA for PNDa02

4. Figures

Figure 1 Example HPLC chromatogram of blank, method INV_054926_HPLC_M3, 248nm

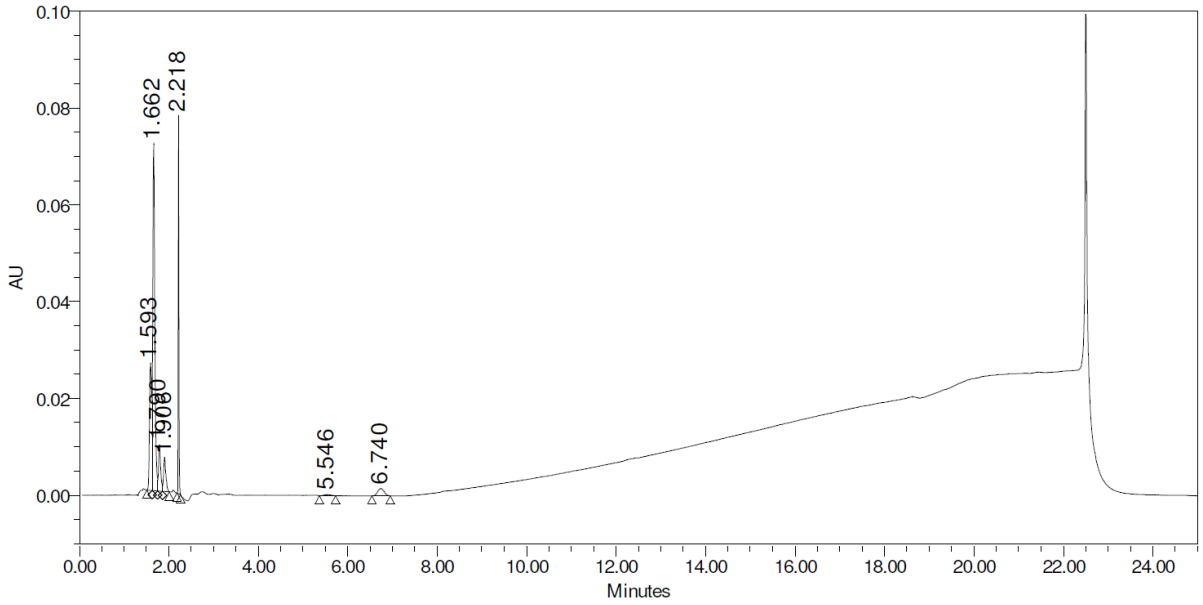


Figure 2 Example HPLC chromatogram of PNDa02 batch DQW447-REF, method INV_054926_HPLC_M3, 248nm

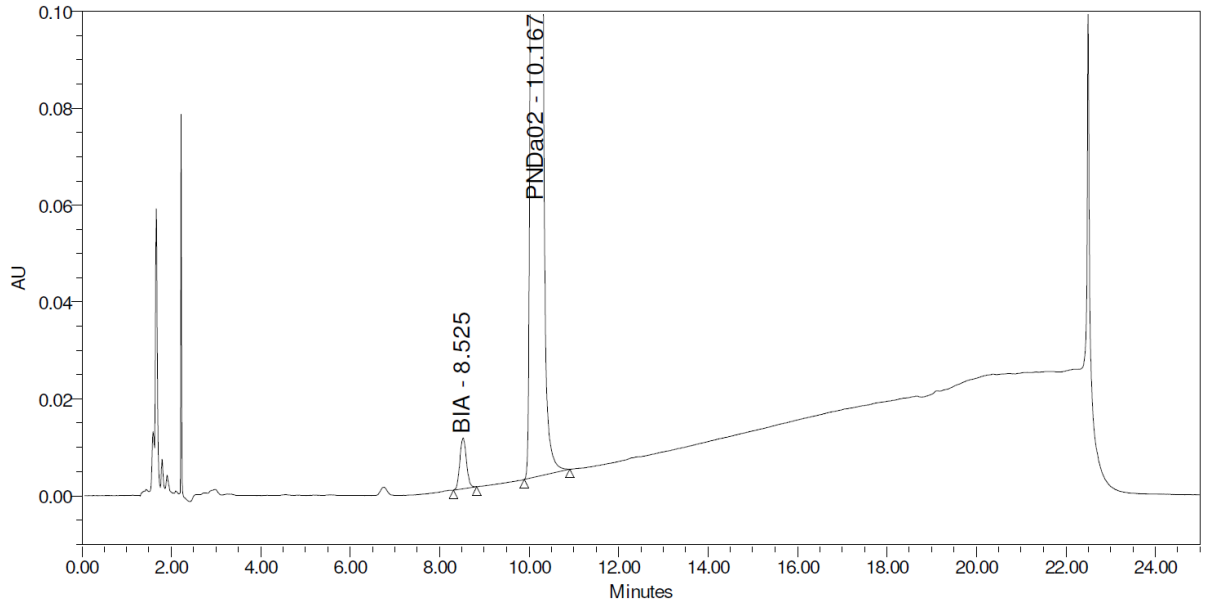


Figure 3 Extracted HPLC-PDA Spectrum of PNDa02, method INV_054926_HPLC_M3

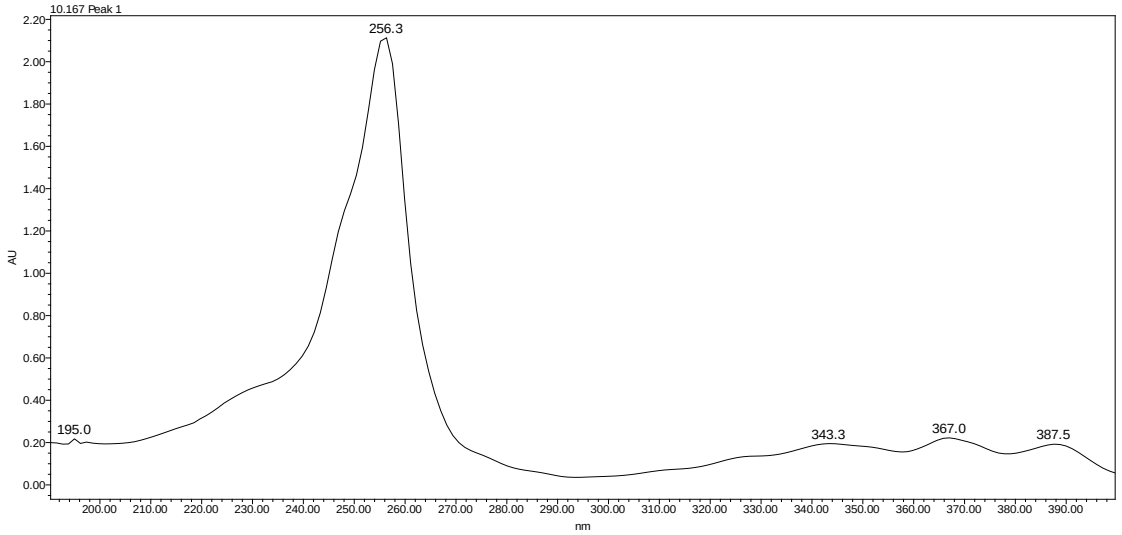


Figure 4 Extracted HPLC-PDA Spectrum of impurity BIA, method INV_054926_HPLC_M3

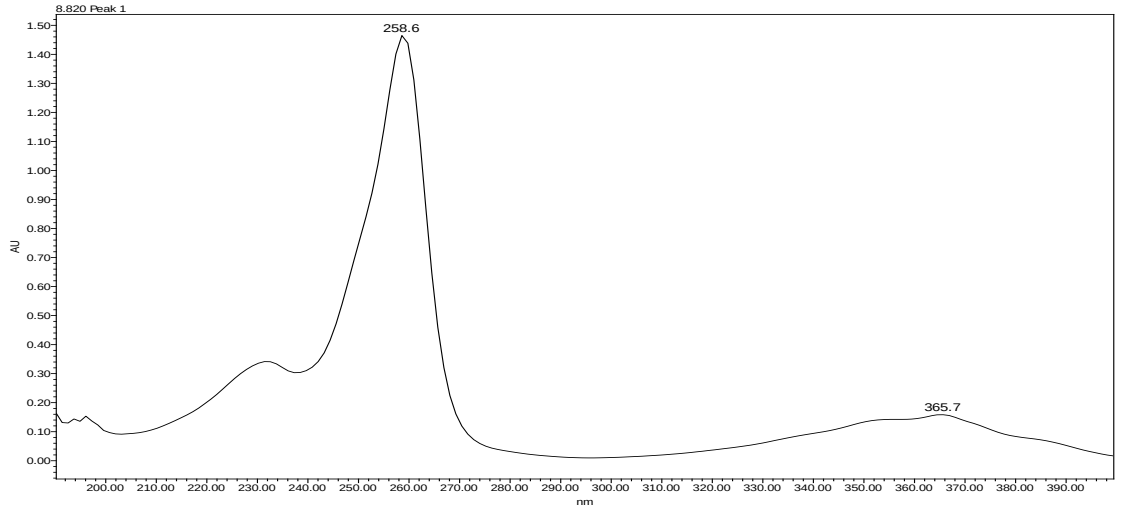


Figure 5 Extracted HPLC-PDA Spectrum of PNDa01, method INV_054926_HPLC_M3