

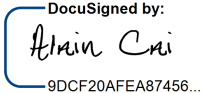
HPLC Method Limited Verification for PNDa02 (non-GMP)

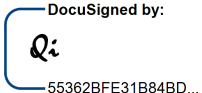
Project	Pyronaridine_INV-054926
Compound	PNDa02
Purpose	Method Verification
Category	Methods
Substance Type	Intermediate
Report ID	INV_054926_HPLC_V3 Version 1.0

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Distribution

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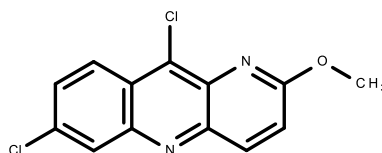
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1. Objective

PNDa02 is analyzed by the newly developed HPLC method 'INV_054926_HPLC_M3' using a reversed phase C18 column, e.g.: Waters Atlantis T3 column. In this document, the limited verification of the new HPLC method shall be performed at non-GMP level.

Related reports:

INV_054926_HPLC_M3: HPLC Method Description for Identity, Assay and Related Substances of PNDa02



PNDa02

2. Summary and conclusion

The method verification has been performed at non-GMP level. The following analytical parameters have been assessed:

- Specificity/ selectivity
- LOQ
- Linearity of PNDa02
- Repeatability
- Accuracy

The obtained results are summarized in Table 1.

Table 1 non-GMP verification of the new developed HPLC method for PNDa02

Parameter	Acceptance criteria	Results	Conformity	Remark
Specificity/ selectivity	No interference between the blank peaks and the components of interest in blanks. All impurity peaks should be separated with PNDa02 peak	No interference peaks in blanks. All impurity peaks are separated with PNDa02 peak.	Pass	See section 3.1
LOQ	S/N (0.05% PNDa02) \geq 10	S/N (0.05% PNDa02) = 12.5	Pass	See section 3.2
Linearity of PNDa02	$R^2 \geq 0.995$, y intercept \leq 5% of the nominal concentration, Report slope, show figure.	$R^2 = 0.9994$ y intercept = 0.46% Slope = 122011696	Pass	See section 3.3
Repeatability	%RSD (100% PNDa02, n=6) \leq 2%	%RSD (100% PNDa02) = 0.19%	Pass	See section 3.4
Accuracy	Recovery: 98% - 102% (calculated as determined concentration vs. nominal concentration)	Recovery = 98.5%	Pass	See section 3.5

3. Results

3.1. Specificity/ selectivity

Specificity/ selectivity was performed using PNDa02 batch DQW-447-REF.

There is no interference between the blank peaks and the components of interest. All impurity peaks are separated with PNDa02 peak.

Table 2 Results for specificity/ selectivity of PNDa02

PNDa02			Batch: DQW-447-REF			
Name	RT, min	RRT	Area	% Area	Peak Purity Angle	Peak Purity Threshold
BIA	8.757	0.84	101140	0.84%	0.48	0.79
PNDa02	10.428	1.00	11925526	99.16%	0.91	1.03

Figure 1 Example HPLC chromatogram of blank of PNDa02, 248 nm

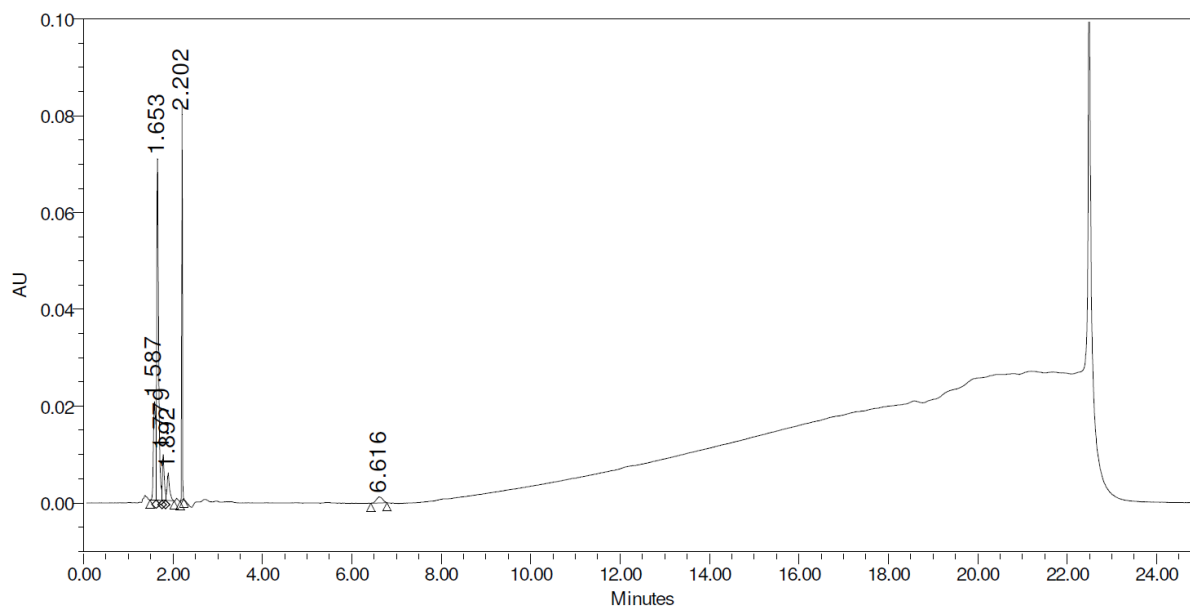
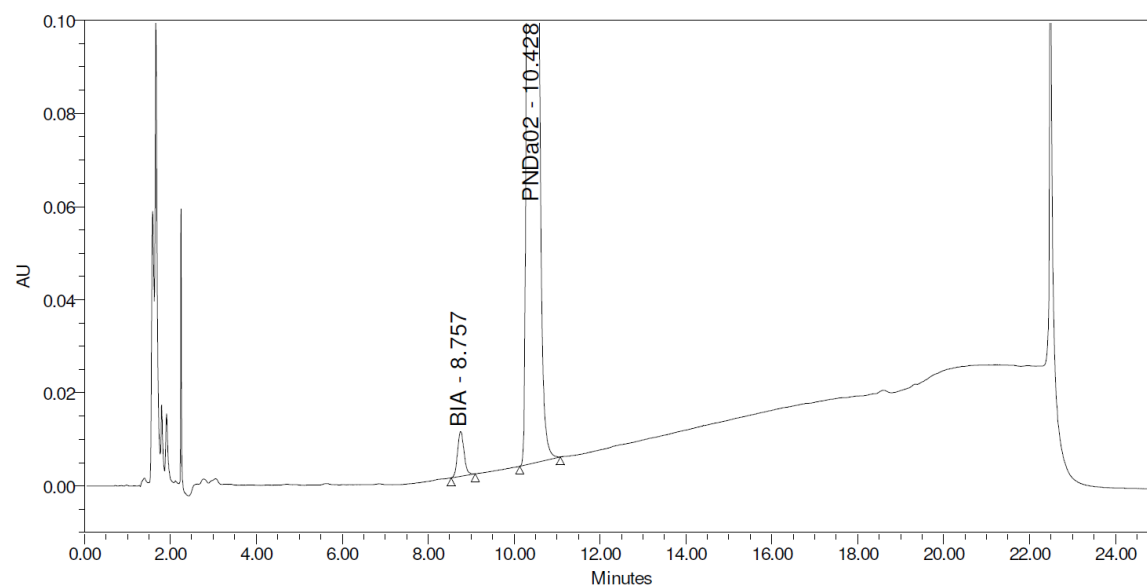


Figure 2 Example HPLC chromatogram of PNDa02 batch DQW-447-REF, 248 nm

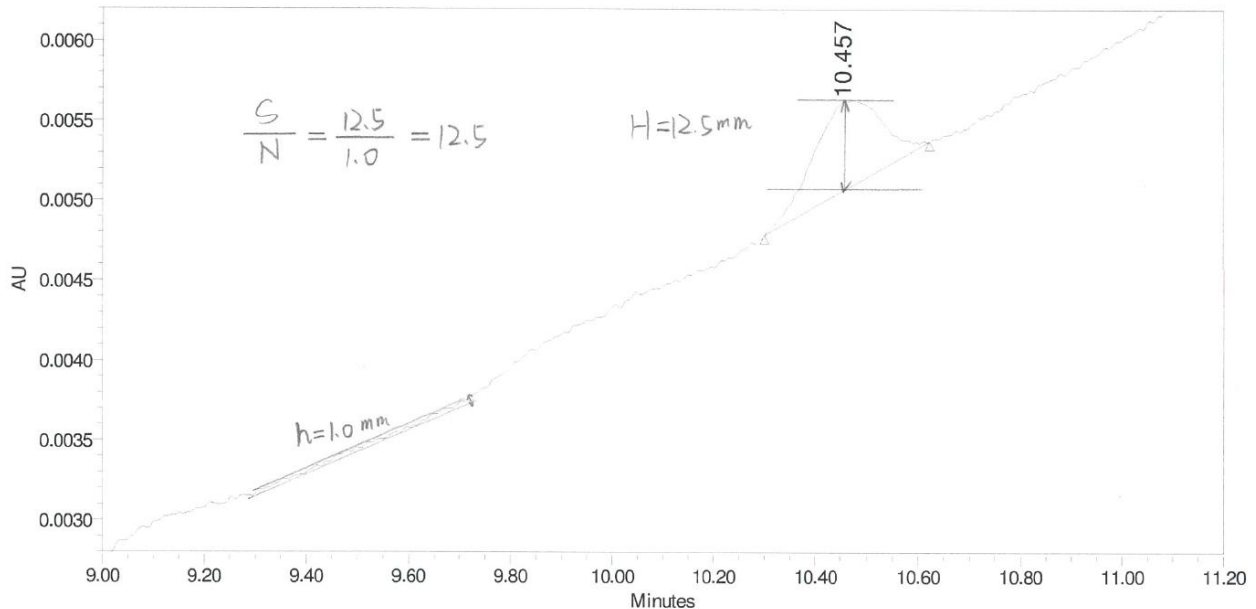


3.2. LOQ

LOQ were performed using PNDa02 batch DQW-447-REF.

The signal to noise of PNDa02 peak is calculated to be 12.5 in LOQ solution (0.05%). The result meets the acceptance criteria ($S/N \geq 10$).

Figure 3 Example Chromatogram of signal to noise calculation for 0.05% PNDa02 solution (LOQ).



3.3. Linearity of PNDa02

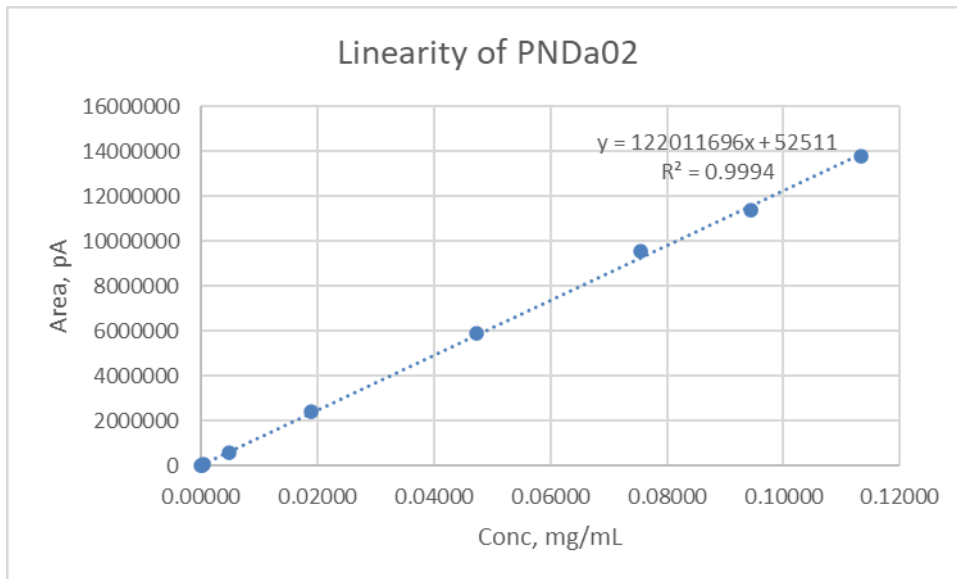
Linearity was performed using PNDa02 batch DQW-447-REF.

The correlation coefficient (R^2) was found to be 0.9994 for the range from LOQ to 120%. The y intercept was calculated to be 0.46% and the slope was found to be 122011696.

All results meet the acceptance criteria.

Table 3 Concentration and area counts of linearity for PNDa02

% of the conc.	Conc. (mg/mL)	Area counts [AU*min]
0.05%	0.00005	5049
0.1%	0.00009	12611
0.5%	0.00047	58722
5%	0.00472	604531
20%	0.01889	2404844
50%	0.04722	5933636
80%	0.07554	9556034
100%	0.09443	11413151
120%	0.11332	13764781

Figure 4 Linearity of PNDa02 from LOQ to 120%

3.4. Repeatability

Repeatability was performed using PNDa02 batch DQW-447-REF.

The results showed that the %RSD of peak area counts is 0.2% for PNDa02. The result meets the acceptance criteria.

Table 4 Results for repeatability for PNDa02

100% PNDa02	Area of PNDa02 [AU*min]
Inj.1	11392109
Inj.2	11390690
Inj.3	11401851
Inj.4	11414397
Inj.5	11350097
Inj.6	11382826
AVG	11388662
STDV	21787.85
RSD%	0.19%
Acceptance criteria	≤ 2%

3.5. Accuracy

Recovery was performed using PNDa02 batch DQW-447-REF.

Recovery of PNDa02 is calculated to be 98.5% as determined concentration vs. nominal concentration. The result meets the acceptance criteria (98-102%).

Table 5 Calculations for accuracy of PNDa02

PNDa02	Weigh in [mg]	Area counts [AU*min]	Recovery
100% prep.1 6 injections (average)	4.97	11388662	-
100% prep.2	4.98	11239027	98.5%
Acceptance criteria	-	-	98-102%

4. Experimental

4.1. Equipment

- HPLC System: Quaternary pump module (Waters Alliance 2695)
PDA detector (Waters Alliance 2998)
Auto sampler (Waters Alliance 2695)
Column oven (Waters Alliance 2695)
- Empower-control and integration software or equivalent
- Column: Waters Atlantis T3, 3 μ m, 150 x 4.6mm
- Flow rate: 1.0 mL/min
- Elution: Gradient mode
- Run time: 25.0 min
- Detection: 248 nm
- Injection: 10 μ L
- Column temp.: 35°C \pm 5°C
- Auto sampler temp.: Room temperature
- Mobile phase:
 - **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 5 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

4.2. Equipment and reagents

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

4.3. Solutions

4.3.1. Diluent

Dimethyltetrahydrofuran (2-Me THF).

4.3.2. 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.

4.3.3. Standard Solutions

➤ **Specificity/ selectivity and Repeatability**

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

➤ **LOQ and Linearity**

Stock Standard Solution (conc.: 0.2 mg/mL, 200%):

Accurately weigh approx. 20 mg of PNDa02 reference standard into a 100-mL volumetric flask. Dissolve and dilute to volume with diluent. Mix well.

120% standard Solution:

Transfer 6 mL stock standard solution into a 10 mL volumetric flask. Fill up to volume with sample diluent and mix well.

100% standard Solution (conc.: 0.1 mg/mL):

Transfer 5 mL stock standard solution into a 10 mL volumetric flask. Fill up to volume with sample diluent and mix well.

80% standard Solution:

Transfer 4 mL stock standard solution into a 10 mL volumetric flask. Fill up to volume with sample diluent and mix well.

50% standard Solution:

Transfer 2.5 mL stock standard solution into a 10 mL volumetric flask. Fill up to volume with sample diluent and mix well.

20% standard Solution:

Transfer 1 mL stock standard solution into a 10 mL volumetric flask. Fill up to volume with sample diluent and mix well.

5% standard Solution:

Transfer 2.5 mL 20% standard solution into a 10 mL volumetric flask. Fill up to volume with sample diluent and mix well.

0.5% standard Solution:

Transfer 1 mL 5% standard solution into a 10 mL volumetric flask. Fill up to volume with sample diluent and mix well.

0.1% standard Solution:

Transfer 1 mL 5% standard solution into a 50 mL volumetric flask. Fill up to volume with sample diluent and mix well.

0.05% standard Solution (LOQ solution):

Transfer 0.5 mL 5% standard solution into a 50 mL volumetric flask. Fill up to volume with sample diluent and mix well.

➤ **Accuracy**

Accurately weigh approx. 5 mg of PNDa02 reference standard into a 50-mL volumetric flask. Dissolve and dilute to volume with diluent. Mix well. Prepare in duplicate.