

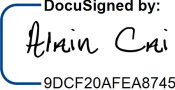
# HPLC Method Limited Verification for PNDa01 and PNDa06-HCl (non-GMP)


<b>Project</b>	<b>Pyronaridine_INV-054926</b>
<b>Compound</b>	<b>PNDa01 and PNDa06-HCl</b>
<b>Purpose</b>	<b>Method Verification</b>
<b>Category</b>	<b>Methods</b>
<b>Substance Type</b>	<b>Intermediate</b>
<b>Report ID</b>	<b>INV_054926_HPLC_V1 Version 1.0</b>

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## Distribution

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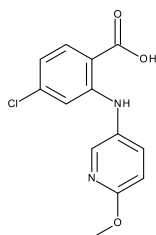
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## 1. Objective

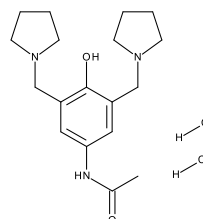
PNDa01 and PNDa06-HCl are analyzed by the newly developed HPLC method 'INV\_054926\_HPLC\_M1' using a reversed phase C18 column, e.g.: Waters Atlantis T3 column. In this document, the limited verification of the new HPLC method shall be performed at non-GMP level.

### Related reports:

INV\_054926\_HPLC\_M1: HPLC Method Description Identity, Assay and Related Substances of PNDa01 and PNDa06-HCl



**PNDa01**



**PNDa06-HCl**

## 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 PNDa01 and PNDa06-HCl
- Repeatability
- Accuracy

The obtained results are summarized in Table 1.

**Table 1** non-GMP verification of the new developed HPLC method for PNDa01 and PNDa06-HCl

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 from the main peak	No interference peaks in blanks.  All impurity peaks are separated from the main peak.	Pass	See section 3.1
LOQ	S/N (0.05% PNDa01) $\geq 10$ S/N (0.05% PNDa06-HCl) $\geq 10$	S/N (0.05% PNDa01) = 15.0 S/N (0.05% PNDa06-HCl) = 19.8	Pass	See section 3.2
Linearity of PNDa01 and PNDa06-HCl	$R^2 \geq 0.995$ , y intercept $\leq 5\%$ of the nominal concentration, Report slope, show figure.	For PNDa01: $R^2 = 1.0000$ y intercept = 0.19% Slope = 20081470  For PNDa06-HCl: $R^2 = 0.9971$ y intercept = 0.76% Slope = 3163913	Pass	See section 3.3
Repeatability	%RSD (100% PNDa01, n=6) $\leq 2\%$  %RSD (100% PNDa06-HCl, n=6) $\leq 2\%$	For PNDa01: %RSD (100% PNDa01) = 0.06%  For PNDa06-HCl: %RSD (100% PNDa06-HCl) = 0.75%	Pass	See section 3.4
Accuracy	Recovery: 98% - 102% (calculated as determined concentration vs. nominal concentration)	For PNDa01: Recovery = 99.9%  For PNDa06-HCl: Recovery = 99.6%	Pass	See section 3.5

### 3. Results

#### 3.1. Specificity/ selectivity

Specificity/ selectivity was performed using PNDa01 batch PHTHARRYS-434-REF and PNDa06-HCI batch PHTRACKD-450-REF respectively.

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

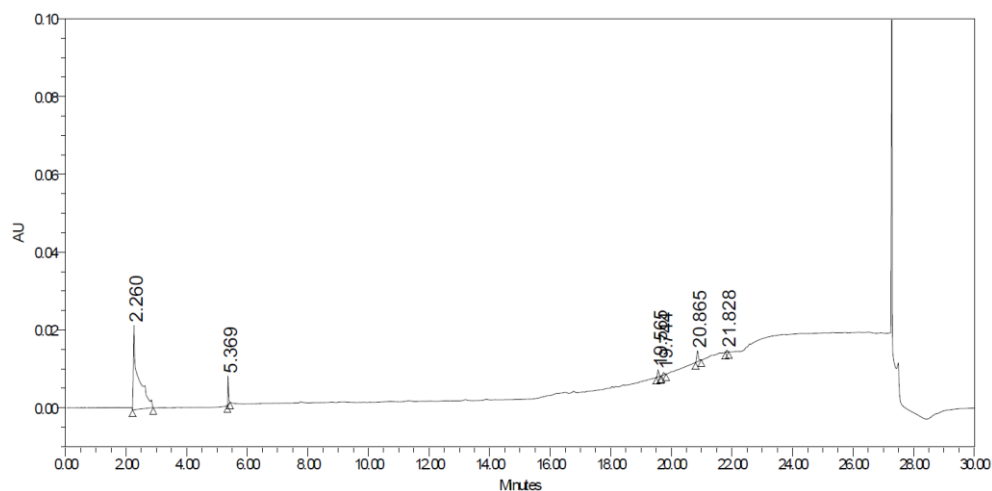
**Table 2** Results for specificity/ selectivity of PNDa01

PNDa01			Batch: PHTHARRYS-434-REF			
Name	RT, min	RRT	Area	% Area	Peak purity Angle	Peak purity Threshold
PNDa01	19.246	1.00	3967622	99.67%	1.71	3.60

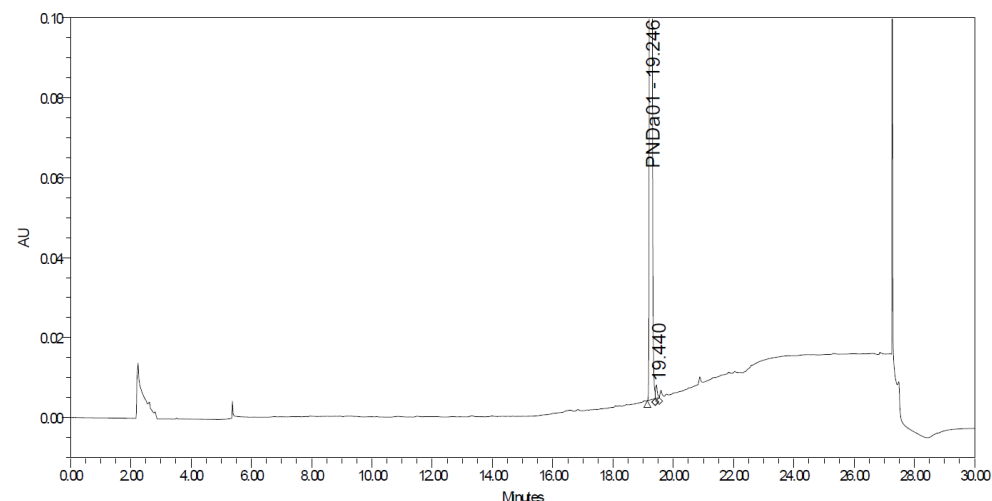
**Table 3** Results for specificity/ selectivity of PNDa06-HCI

PNDa06-HCI			Batch: PHTRACKD-450-REF			
Name	RT, min	RRT	Area	% Area	Peak purity Angle	Peak purity Threshold
PNDa06-HCI	7.657	1.00	2307693	100.00	3.98	5.36

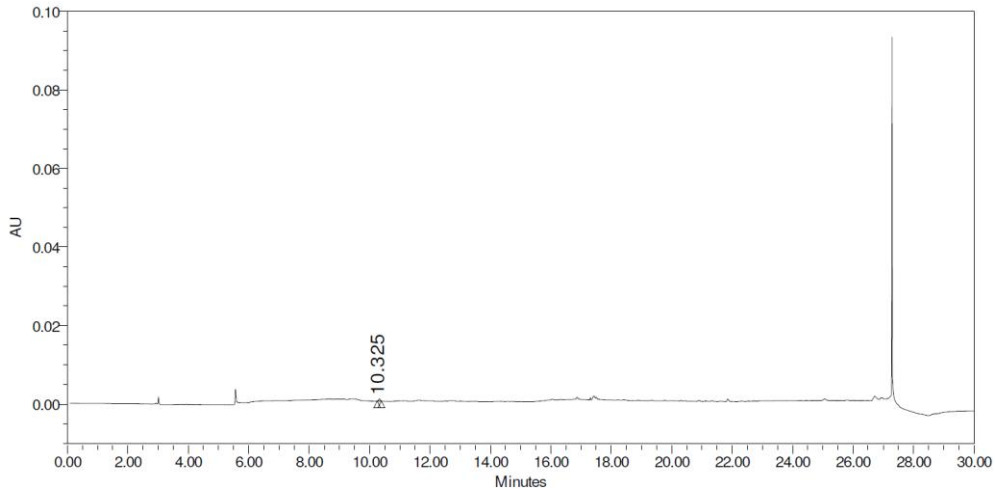
**Figure 1** Example HPLC chromatogram of blank of PNDa01, 254 nm



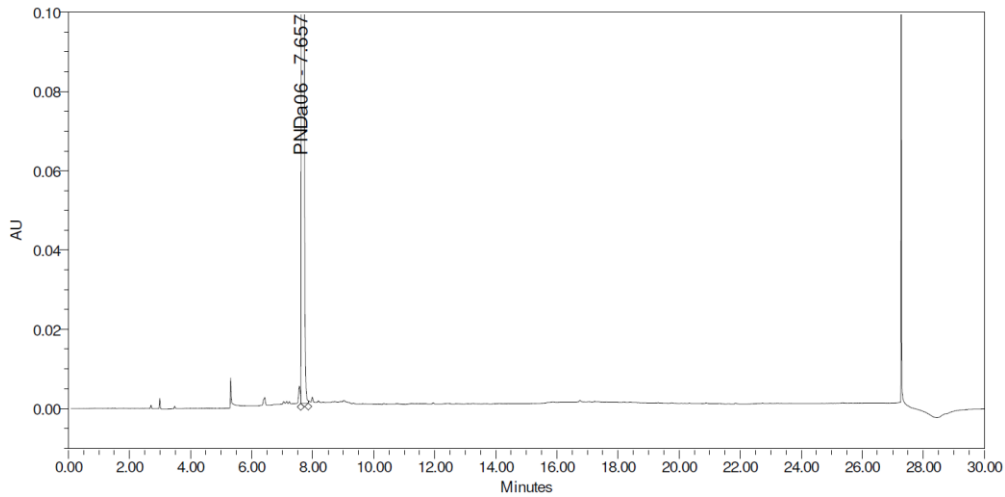
**Figure 2** Example HPLC chromatogram of PNDa01 batch PHTHARRYS-434-REF, 254 nm



**Figure 3** Example HPLC chromatogram of blank of PNDa06-HCl, 278 nm



**Figure 4** Example HPLC chromatogram of PNDa06 batch PHTRACKD-450-REF, 278 nm



### 3.2. LOQ

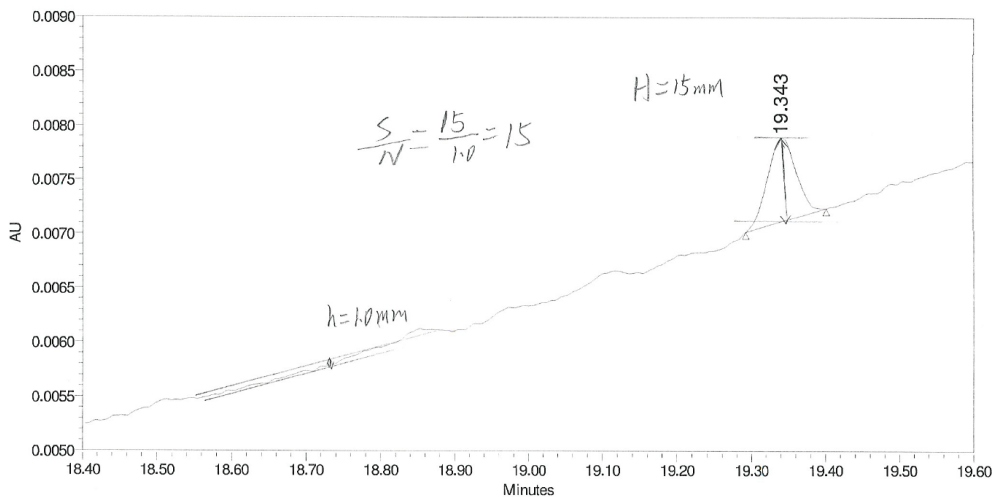
LOQ were performed using PNDa01 batch PHTHARRYS-434-REF and PNDa06-HCl batch PHTRACKD-450-REF respectively.

The signal to noise of PNDa01 peak is calculated to be 15.0 in LOQ solution (0.05%).

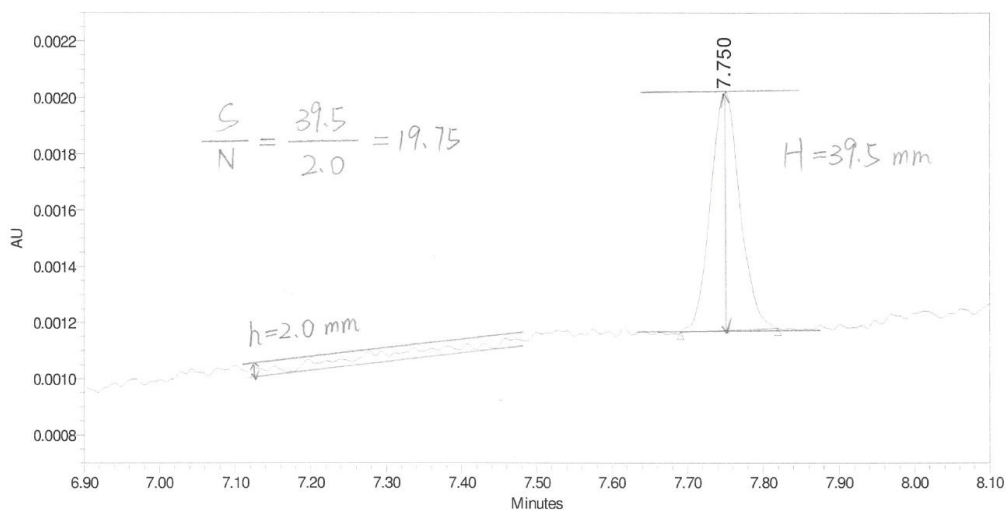
The signal to noise of PNDa06-HCl peak is calculated to be 19.8 in LOQ solution (0.05%).

All results meet the acceptance criteria.

**Figure 5** Example Chromatogram of signal to noise calculation for 0.05% PNDa01 solution (LOQ).



**Figure 6** Example chromatogram of signal to noise calculation for 0.05% PNDa06-HCl solution (LOQ).



### 3.3. Linearity of PNDa01 and PNDa06-HCl

Linearity was performed using PNDa01 batch PHTHARRYS-434-REF and PNDa06-HCl batch PHTRACKD-450-REF respectively.

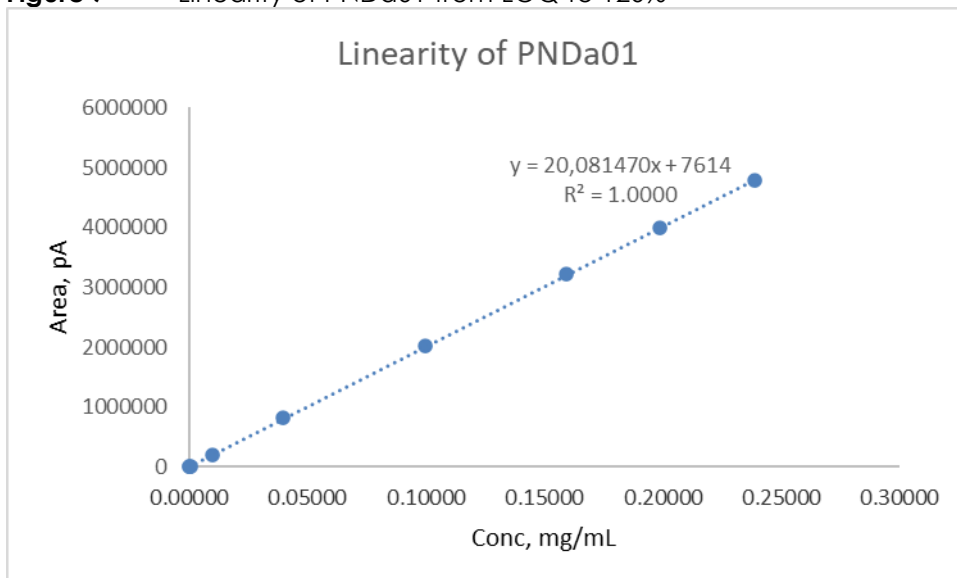
**PNDa01:** The correlation coefficient ( $R^2$ ) was found to be 1.0000 for the range from LOQ to 120%. The y intercept was calculated to be 0.19% and the slope was found to be 20081470. All results meet the acceptance criteria.

**PNDa06-HCl:** The correlation coefficient ( $R^2$ ) was found to be 0.9971 for the range from LOQ to 120%. The y intercept was calculated to be 0.76% and the slope was found to be 3163913. All results meet the acceptance criteria.

**Table 4** Concentration and area counts of linearity for PNDa01

% of the conc.	Conc. (mg/mL)	Area counts [AU*min]
0.05%	0.00010	2055
0.1%	0.00020	4122
0.5%	0.00099	20320
5%	0.00995	206326
20%	0.03980	817144
50%	0.09950	2022640
80%	0.15919	3222911
100%	0.19899	3995711
120%	0.23879	4788530

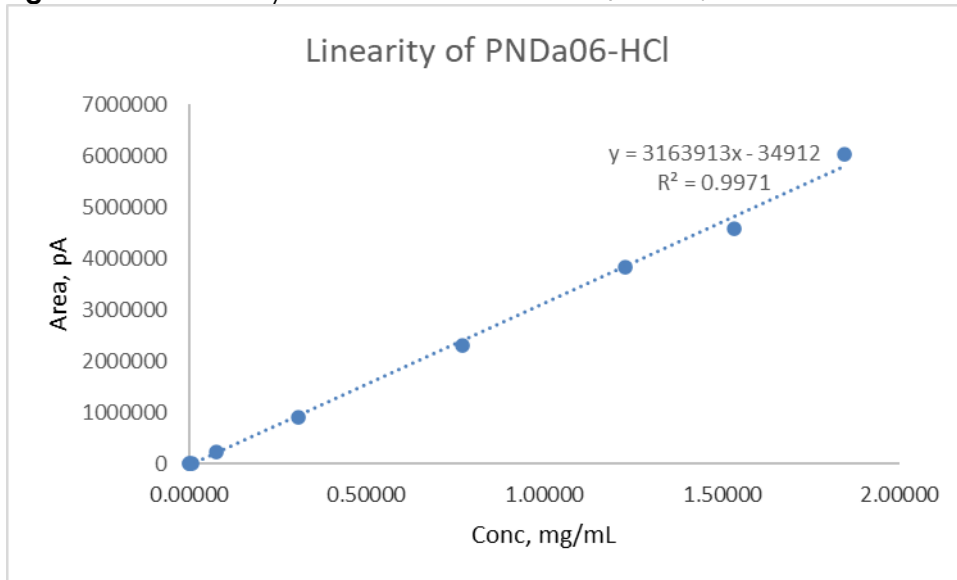
**Figure 7** Linearity of PNDa01 from LOQ to 120%





**Table 5** Concentration and area counts of linearity for PNDa06-HCl

% of the conc.	Conc. (mg/mL)	Area counts [AU*min]
0.05%	0.00077	2296
0.1%	0.00154	3731
0.5%	0.00768	22931
5%	0.07677	229907
20%	0.30708	903214
50%	0.76771	2307693
80%	1.22833	3831950
100%	1.53542	4593838
120%	1.84250	6039020

**Figure 8** Linearity of PNDa06-HCl from LOQ to 120%

### 3.4. Repeatability

Repeatability was performed using PNDa01 batch PHTHARRYS-434-REF and PNDa06-HCl batch PHTRACKD-450-REF respectively.

The results showed that the %RSD of peak area counts is 0.1% for PNDa01, 0.8% for PNDa06-HCl. All results meet the acceptance criteria.

**Table 6** Results for repeatability for PNDa01

100% PNDa01	Area of PNDa01 [AU*min]
Inj.1	3992814
Inj.2	3987793
Inj.3	3988702
Inj.4	3988332
Inj.5	3988214
Inj.6	3985439
AVG	3988549
STDV	2392.34546
RSD%	<b>0.06%</b>
Acceptance criteria	≤ 2%

**Table 7** Results for repeatability for PNDa06-HCl

100% PNDa01	Area of PNDa06 [AU*min]
Inj.1	4559168
Inj.2	4610646
Inj.3	4646378
Inj.4	4655067
Inj.5	4633829
Inj.6	4632353
AVG	4622906.833
STDV	34660.24432
RSD%	<b>0.75%</b>
Acceptance criteria	≤ 2%

### 3.5. Accuracy

Recovery was performed using PNDa01 batch PHTHARRYS-434-REF and PNDa06-HCl batch PHTRACKD-450-REF respectively.

Recovery of PNDa01 is calculated to be 99.9% as determined concentration vs. nominal concentration. The result meets the acceptance criteria (98-102%).

**Table 8** Calculations for accuracy of PNDa01

PNDa01	Weigh in [mg]	Area counts [AU*min]	Recovery
100% prep.1 6 injections (average)	10.04	3988549	-
100% prep.2	9.96	3952393	<b>99.9%</b>
Acceptance criteria	-	-	98-102%

Recovery of PNDa06-HCl is calculated to be 99.6% as determined concentration vs. nominal concentration. The result meets the acceptance criteria (98-102%).

**Table 9** Calculations for accuracy of PNDa06-HCl

PNDa06-HCl	Weigh in [mg]	Area counts [AU*min]	Recovery
100% prep.1 6 injections (average)	79.98	4622906.8	-
100% prep.2	79.96	4603882	<b>99.6%</b>
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 $\mu$ m, 150 x 4.6mm
- Flow rate: 1.0 mL/min
- Elution: Gradient mode
- Run time: 30.0 min
- Detection: 254 nm for PNDa01  
278 nm for PNDa06-HCl
- Injection: 10  $\mu$ 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: DMSO/Acetonitrile (50: 50 v/v) for PNDa01  
Water/Acetonitrile (80: 20 v/v) for PNDa06-HCl
- Needle wash: Water/ Acetonitrile (50:50 v/v)
- Equilibration time: 7.0 min
- Gradient:

**Table 10** Gradient Table

Time (min)	% A	% B
0.0	100.0	0.0
1.0	100.0	0.0
13.0	65.0	35.0
20.0	1.0	99.0
25.0	1.0	99.0
25.1	100.0	0.0
30.0	100.0	0.0

### 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
- DMSO: HPLC grade, Sigmer-Aldrich
- Glassware: 10, 20, 25, 50-mL volumetric flasks, 1L graduated cylinders
- Pipette: 1.0 mL Pipette

### 4.3. Solutions

#### 4.3.1. Diluent

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

**PNDa01:** DMSO/Acetonitrile (50:50 v/v).

**PNDa06-HCl:** Water/Acetonitrile (80: 20 v/v).

#### 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

**PNDa01:**

➤ **Specificity/ selectivity and Repeatability**

Accurately weigh approx. 10 mg of PNDa01 reference standard into a 50-mL volumetric flask. Dissolve and dilute to volume with diluent. Mix well.

➤ **LOQ and Linearity**

Stock Standard Solution (conc.: 0.4 mg/mL, 200%):

Accurately weigh approx. 10 mg of PNDa01 reference standard into a 25-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.: 0.2 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. 10 mg of PNDa01 reference standard into a 50-mL volumetric flask. Dissolve and dilute to volume with diluent. Mix well. Prepare in duplicate.

**PNDa06-HCl:**

➤ **Specificity/ selectivity and Repeatability**

Accurately weigh approx. 80 mg of PNDa06-HCl reference standard into a 20-mL volumetric flask. Dissolve and dilute to volume with diluent and mix well. Transfer 5 mL of above solution into a 10 mL volumetric flask. Fill up to volume with diluent and mix well.

➤ **LOQ and Linearity**

Stock Standard Solution (conc.: 4.0 mg/mL, 200%):

Accurately weigh approx. 80 mg of PNDa06-HCl reference standard into a 20-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.: 2.0 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. 80 mg of PNDa06-HCl reference standard into a 20-mL volumetric flask. Dissolve and dilute to volume with diluent and mix well. Transfer 5 mL of above solution into a 10 mL volumetric flask. Fill up to volume with diluent and mix well. Prepare in duplicate.