

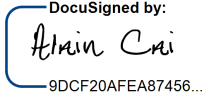
# HPLC Method Description for Identity, Assay and Related Substances of PNDa01 and PNDa06-HCl

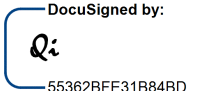
<b>Project</b>	<b>Pyronaridine_INV-054926</b>
<b>Compound</b>	<b>PNDa01 and PNDa06-HCl</b>
<b>Purpose</b>	<b>Method Description</b>
<b>Category</b>	<b>Methods</b>
<b>Substance Type</b>	<b>Intermediate</b>
<b>Report ID</b>	<b>INV_054926_HPLC_M1R Version 1.0</b>

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## Distribution

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

## Table of contents

1.	Objective	3
2.	Summary and conclusion	3
3.	Experimental	5
3.1.	HPLC	5
3.2.	Equipment and reagents	5
3.3.	Solutions	6
3.3.1.	Diluent	6
3.3.2.	Mobile phase preparation	6
3.3.3.	Solution preparations	6
3.4.	Proposed injection sequence and system suitability test	7
3.5.	Calculation and Reporting	7
3.5.1.	Identification by HPLC	7
3.5.2.	Purity by HPLC in %area	7
3.5.3.	Related substances by HPLC: Total impurities in %area	8
3.5.4.	Related substances by HPLC: Individual impurities in %area (by RRT)	8
3.5.5.	Assay by HPLC in %w/w	8
4.	Figures	9

## 1. Objective

This method 'INV\_054926\_HPLC\_M1R' for intermediates PNDa01 and PNDa06-HCl 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 PNDa01 and PNDa06-HCl shall be described in this document.

### Related reports:

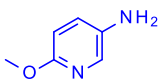
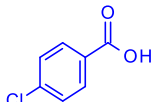
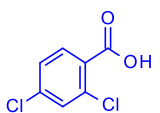
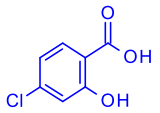
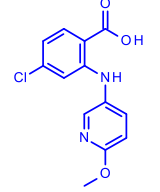
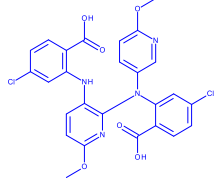
INV\_054926\_HPLC\_M1: HPLC Method Description for In-process Control of Intermediates

INV\_054926\_HPLC\_V1: HPLC Method Limited Validation for PNDa01 and PNDa06-HCl (non-GMP)

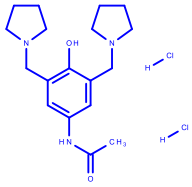
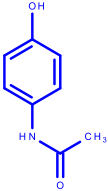
## 2. Summary and conclusion

The method is applied for the HPLC testing of PNDa01 and PNDa06-HCl (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, 3 $\mu$ m, 150 x 4.6mm HPLC column.

**Table 1** Structure, Retention time and RRT of PNDa01 and its related substances

Compound	Structure	Retention time (RT)	RRT
6-methoxy-3-aminepyridine (SM2)		ca. 6.8 min	ca. 0.35
PNDa01 impurity 2		ca. 17.4 min	ca. 0.90
2,4-Dichlorobenzoic acid (SM1)		ca. 18.0 min	ca. 0.94
PNDa01 impurity 1		ca. 18.2 min	ca. 0.95
<b>PNDa01</b>		ca. 19.2 min	<b>1.00</b>
PNDa01 impurity 3		ca. 20.5 min	ca. 1.07

**Table 2** Structure, Retention time and RRT of PNDa06-HCl and its related substances

Compound	Structure	Retention time (RT)	RRT
PNDa06-HCl		ca. 8.1 min	1.00
Acetaminophen (SM5)		ca. 8.5 min	ca. 1.04

Example chromatograms and extracted HPLC-PDA spectra of PNDa01 and PNDa06-HCl are given in Section 4.

Specificity, LOQ, Linearity, Accuracy, Repeatability was performed and reported in report 'INV\_054926\_HPLC\_V1'.

### 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, 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 (see section 3.3.2):
  - **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 3** 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

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

### 3.3. Solutions

#### 3.3.1. 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).

#### 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:**

Standard Solution 1 & 2 (conc.: 0.2 mg/ml):

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 if needed.

Standard Solution 3 (0.05%, corresponding to 0.0001 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.

**PNDa06-HCl:**

Standard Solution 1 & 2 (conc.: 2.0 mg/ml):

Accurately weigh approx. 20 mg of PNDa06-HCl reference standard into a 10-mL volumetric flask. Dissolve and dilute to volume with diluent. Mix well.

Prepare in duplicate if needed.

Standard Solution 3 (0.05%, corresponding to 0.001 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

Number of sample preparations depends on the samples under analysis.

#### PNDa01:

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

#### PNDa06-HCl:

Accurately weigh approx. 20 mg of PNDa06-HCl sample into a 10-mL volumetric flask. Dissolve and dilute to volume with diluent. Mix well.

## 3.4. Proposed injection sequence and system suitability test

**Table 4** Proposed injection sequence and SST criteria

Sample name	No. of injections <sup>[1]</sup>	SST acceptance criteria
Blank (diluent)	1 + N <sup>[1]</sup>	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 <sup>[2]</sup>	1	Recovery: 98% - 102% (6 injection Std 2 to be used as reference)

<sup>[1]</sup> Additional blanks may be run until an acceptable baseline is obtained. - <sup>[2]</sup> For multiple sample analysis, 1 injection of standard solution 2 is recommended 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:  
 $100 - \%(area) \text{ Total impurities}$

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

Where:

$P_i = \text{PNDa01 or PNDa06-HCl purity \% (area)}$

### 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  $\geq 0.05\%$  (area) by their RRT.

Analyses with two sample weighings:  $(A1+A2)/2$

Where:

$A_i$  = 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

#### PNDa01:

$$\text{Assay}_{\text{PNDa01}} (\%w/w) = \frac{\text{Area}_{\text{sam}} \times W_{\text{std}} \times V_{\text{sam}}}{\text{Area}_{\text{std}} \times W_{\text{sam}} \times V_{\text{std}}} \times P_{\text{std}}$$

Where:

$\text{Area}_{\text{sam}}$  = PNDa01 peak area obtained in the sample chromatogram

$\text{Area}_{\text{std}}$  = Average PNDa01 peak area obtained for the 6 standard injections (std 2)

$W_{\text{std}}$  = Standard weight (Std 2)

$W_{\text{sam}}$  = Sample weight (mg)

$V_{\text{sam}}$  = Volume of the flask used in the sample preparation

$V_{\text{std}}$  = Volume of the flask used in the standard preparation (Std 2)

$P_{\text{std}}$  = Potency determined for the standard in used. Use value from CoA for PNDa01

#### PNDa06-HCl:

$$\text{Assay}_{\text{free base}} (\%w/w) = \frac{\text{Area}_{\text{sam}} \times W_{\text{std}} \times V_{\text{sam}}}{\text{Area}_{\text{std}} \times W_{\text{sam}} \times V_{\text{std}}} \times P_{\text{std}}$$

Where:

$\text{Area}_{\text{sam}}$  = PNDa06 peak area obtained in the sample chromatogram

$\text{Area}_{\text{std}}$  = Average PNDa06 peak area obtained for the 6 standard injections (std 2)

$W_{\text{std}}$  = Standard weight (Std 2)

$W_{\text{sam}}$  = Sample weight (mg)

$V_{\text{sam}}$  = Volume of the flask used in the sample preparation

$V_{\text{std}}$  = Volume of the flask used in the standard preparation (Std 2)

$P_{\text{std}}$  = Potency determined for the standard in used. Use value (free base) from CoA for PNDa06-HCl

$$\text{Assay}_{\text{salt}} (\%w/w) = \text{Assay}_{\text{free base}} \times \frac{MW_{\text{salt}}}{MW_{\text{free base}}}$$

Where:

$\text{Assay}_{\text{free base}}$  = Potency determined for the free base of PNDa06 sample

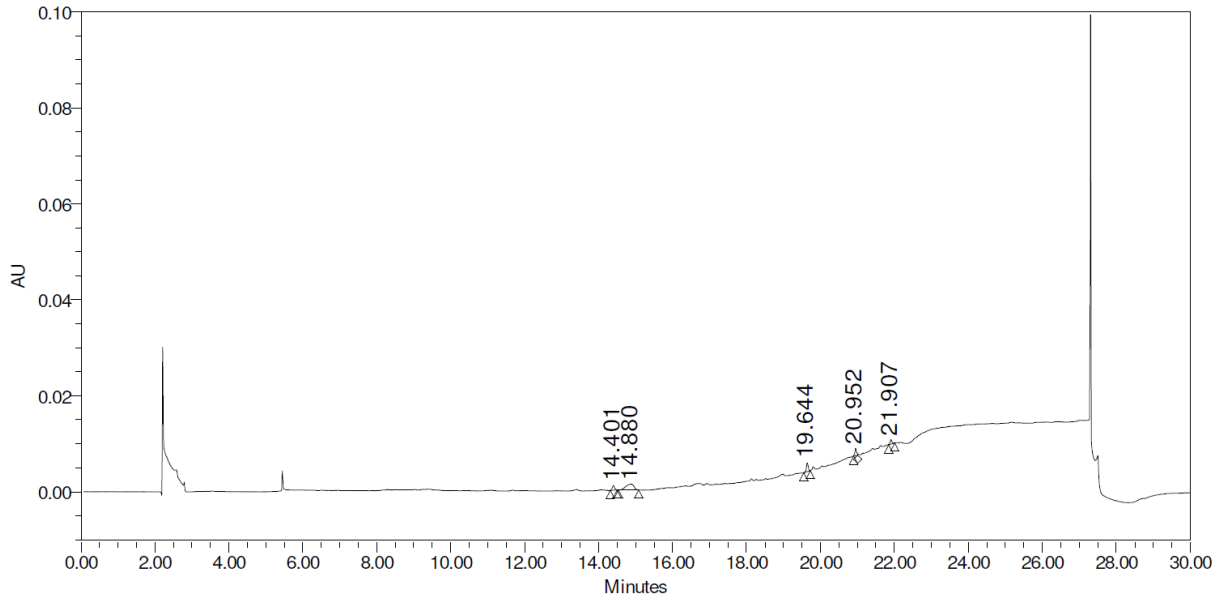
$MW_{\text{salt}}$  = Molecular weight of PNDa06-HCl salt (390.35 g/mol)

$MW_{\text{free base}}$  = Molecular weight of PNDa06 free base (317.43 g/mol)

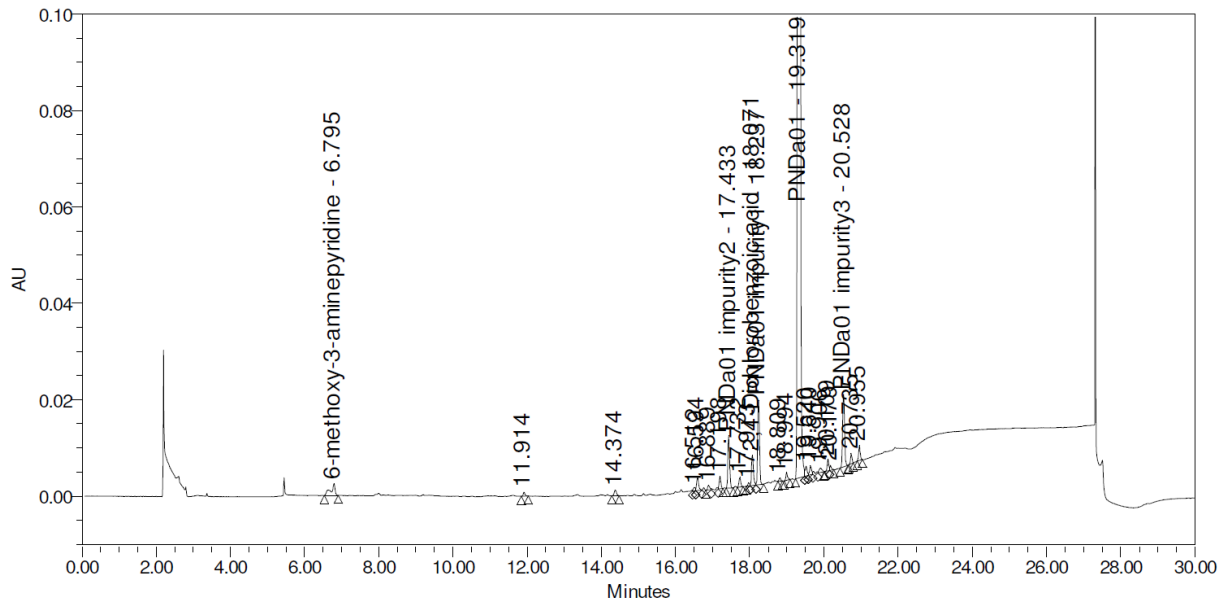


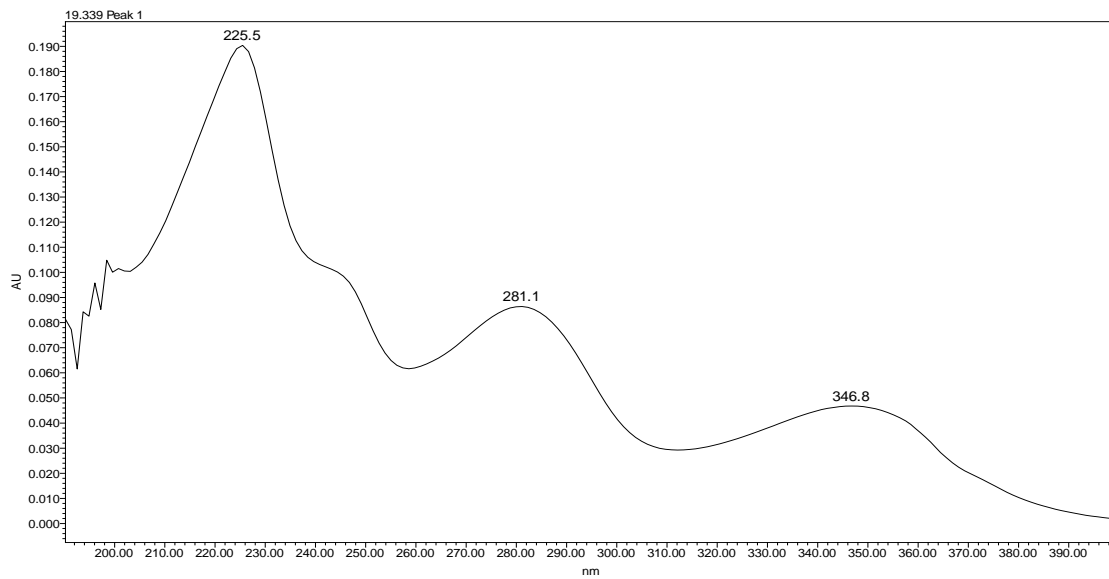
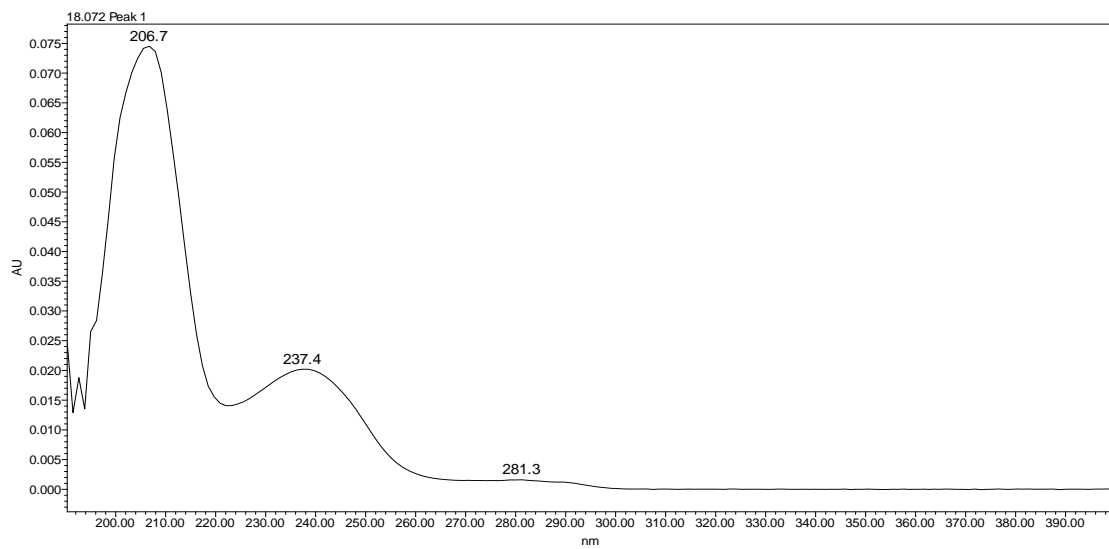
#### 4. Figures

**Figure 1** Example HPLC chromatogram of blank for PNDa01, method INV\_054926\_HPLC\_M1, 254nm

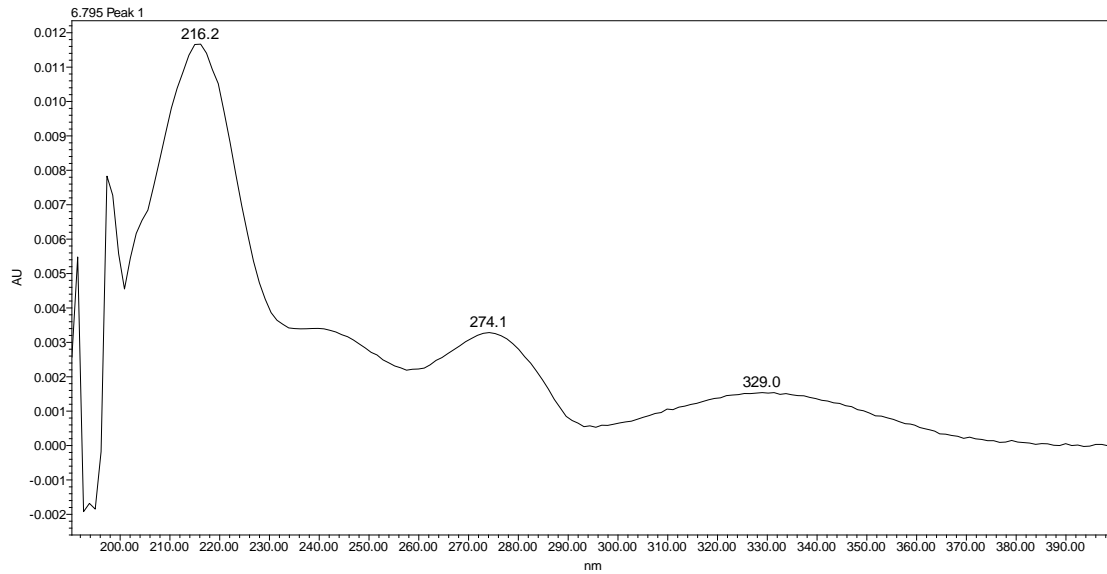


**Figure 2** Example HPLC chromatogram of PNDa01 batch PHTANWARL-528-1, method INV\_054926\_HPLC\_M1, 254nm

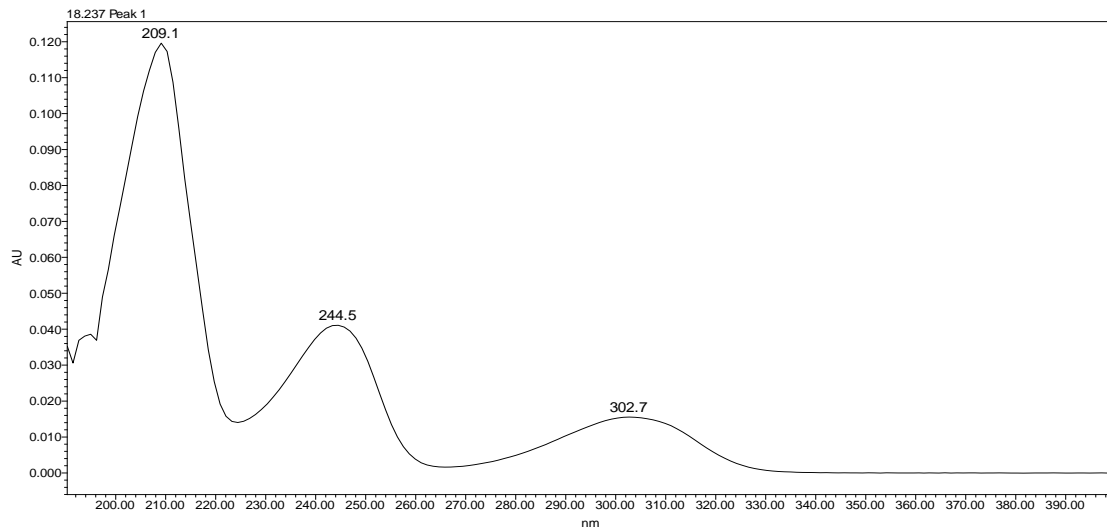


**Figure 3** Extracted HPLC-PDA Spectrum of PNDa01, method INV\_054926\_HPLC\_M1**Figure 4** Extracted HPLC-PDA Spectrum of 2,4-Dichlorobenzoic acid (SM1), method INV\_054926\_HPLC\_M1

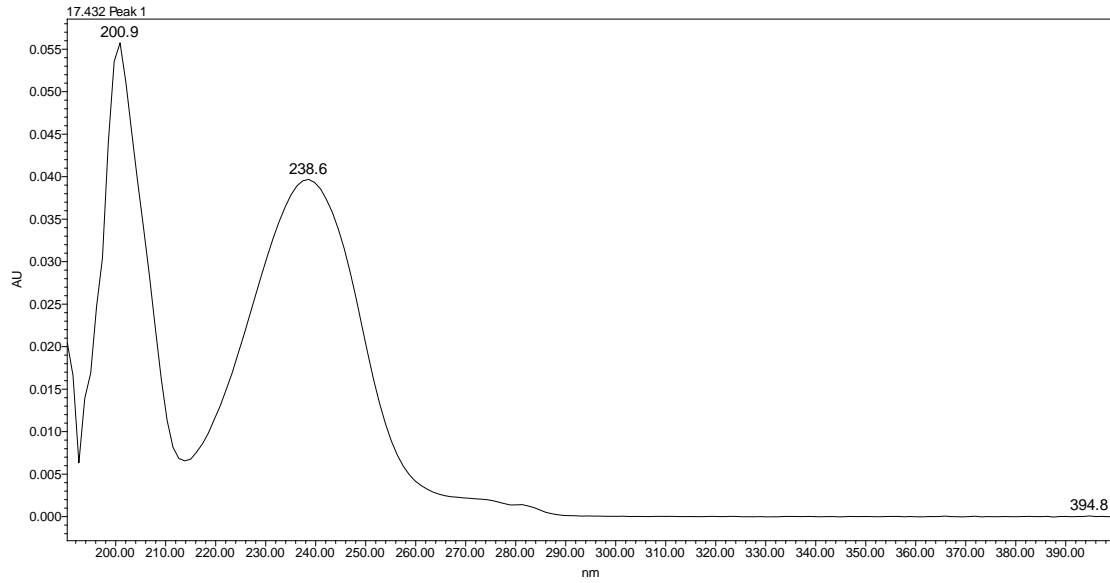
**Figure 5** Extracted HPLC-PDA Spectrum of 6-methoxy-3-aminepyridine (SM2), method INV\_054926\_HPLC\_M1



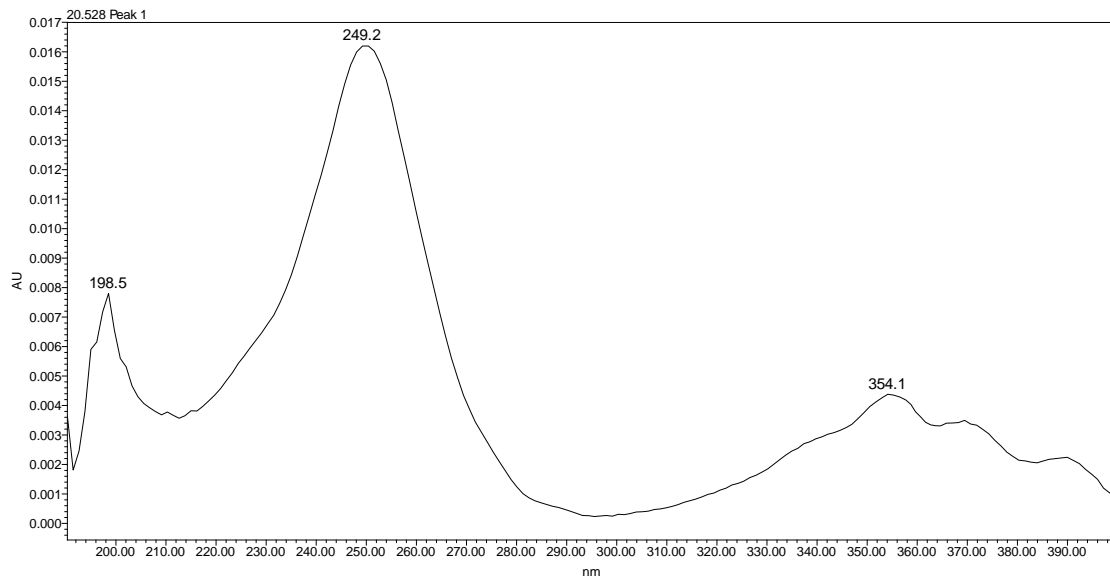
**Figure 6** Extracted HPLC-PDA Spectrum of PNDa01 impurity 1, method INV\_054926\_HPLC\_M1



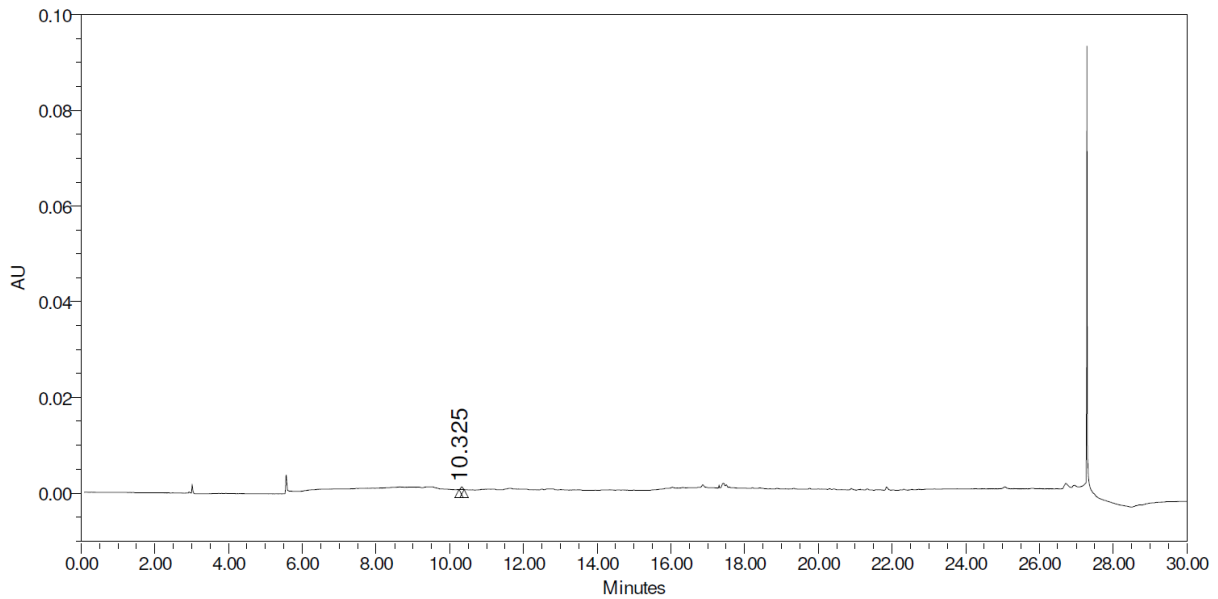
**Figure 7** Extracted HPLC-PDA Spectrum of PNDa01 impurity 2, method INV\_054926\_HPLC\_M1



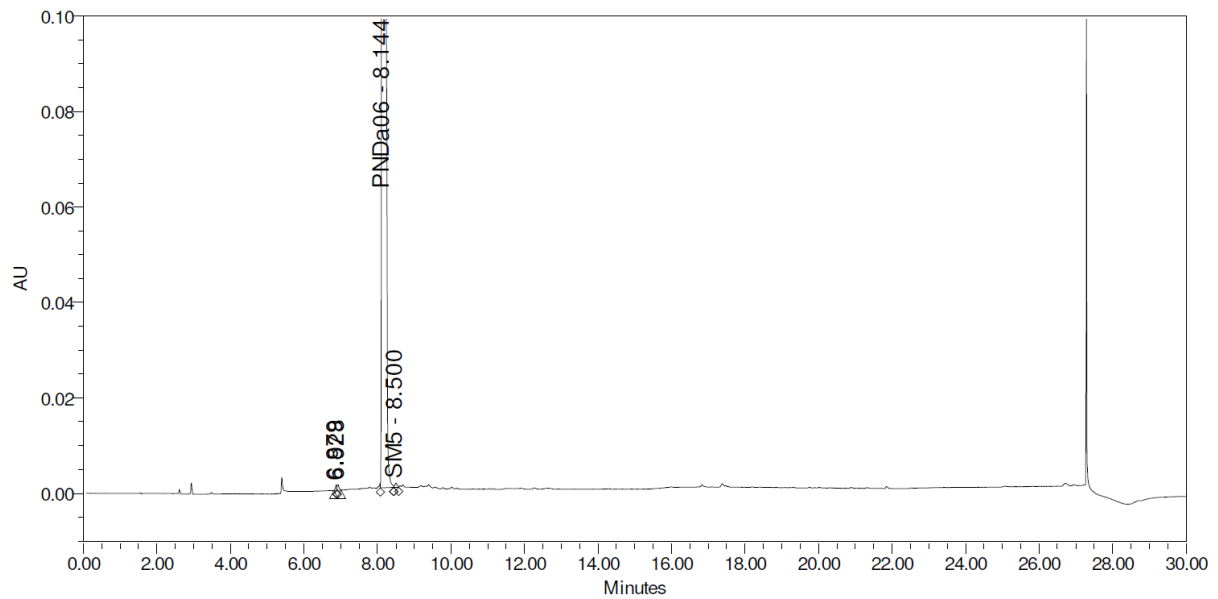
**Figure 8** Extracted HPLC-PDA Spectrum of PNDa01 impurity 3, method INV\_054926\_HPLC\_M1



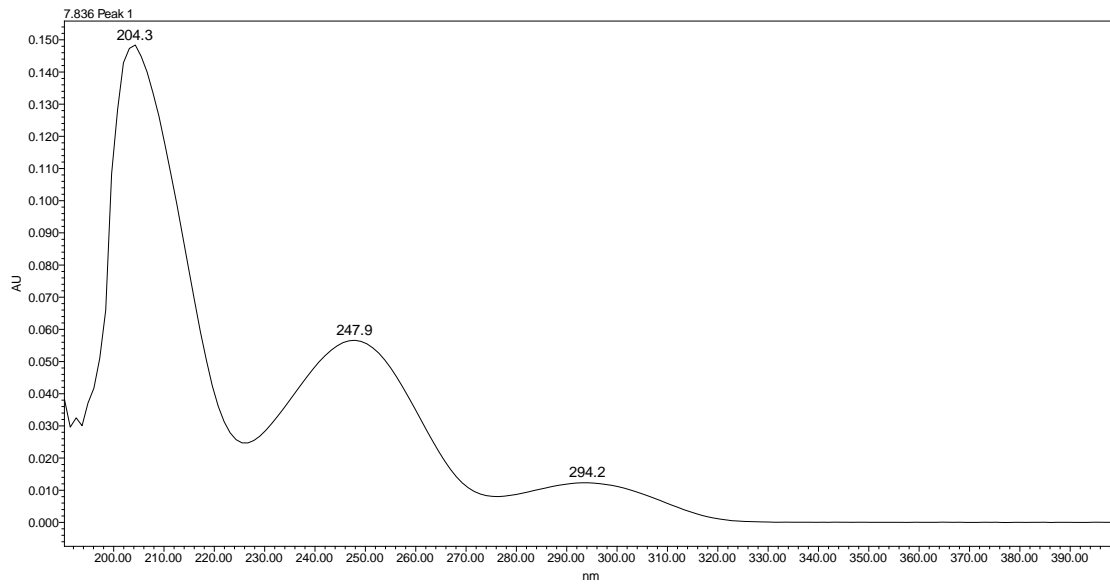
**Figure 9** Example HPLC chromatogram of blank for PNDa06-HCl, method INV\_054926\_HPLC\_M1, 278nm



**Figure 10** Example HPLC chromatogram of PNDa06-HCl batch PHTANWARL-528-1, method INV\_054926\_HPLC\_M1, 278nm



Name	Retention Time, min	RRT	Area	% Area
	6.879	0.84	3443	0.07
	6.928	0.85	3577	0.07
PNDa06	8.144	1.00	5067326	99.78
Acetaminophen (SM5)	8.500	1.04	4279	0.08

**Figure 11** Extracted HPLC-PDA Spectrum of PNDa06-HCl, method INV\_054926\_HPLC\_M1**Figure 12** Extracted HPLC-PDA Spectrum of Acetaminophen (SM5), method INV\_054926\_HPLC\_M1