HPLC Method Description for Identity, Assay and Related Substances of TGF-001

Project	**Pyronaridine_INV-054926**
Compound	**TGF-001**
Purpose	**Method Description**
Category	**Methods**
Substance Type	**Drug substance**
Report ID	**INV_054926_HPLC_M4 Version 1.0**

Authors

Cai Alain

Approvers

By: DocuSigned by: Alain Cai 9DCF20AFEA87456... 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

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1. Objective

This method 'INV_054926_HPLC_M4' for API TGF-001 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 TGF-001 shall be described in this document.

Related reports:

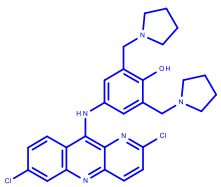
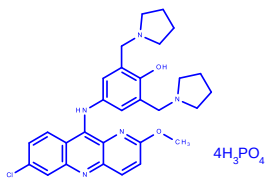
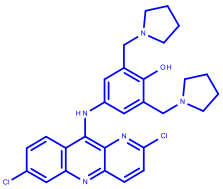
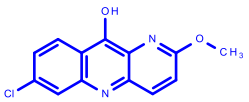
INV_054926_HPLC_M1: HPLC Method Description for In-process Control of Intermediates

INV_054926_HPLC_V4: HPLC Method Limited Validation for TGF-001 (non-GMP)

2. Summary and conclusion

The method is applied for the HPLC testing of TGF-001 (identity, assay and related substances). This method is based on reversed phase liquid chromatography with UV detection and gradient elution using a Waters X-Bridge C18, 150*4.6mm, 3.5µm HPLC column.

Table 1 Structure, Retention time and RRT of TGF-001 and its related substances

Compound	Structure	Retention time (RT)	RRT	RRF [1]
DIA		ca. 10.4 min	ca. 0.86	1.00
TGF-001		ca. 12.1 min	**1.00**	-
DIN		ca. 25.3 min	ca. 2.09	1.00
TGF-001 impurity 1		ca. 29.9 min	ca. 2.47	1.00 [2]

[1] RRF value was tested in report 'INV_054926_HPLC_V4'. - [2] Use 1.00, in case there is no RRF determined.

Example chromatograms and extracted HPLC-PDA spectrum of TGF-001 are given in Section 4.

Autosampler stability, Specificity, LOD, LOQ, Linearity, Accuracy and Repeatability were performed and reported in report 'INV_054926_HPLC_V4'.

3. Experimental

Equivalent equipment or grade of materials can be used.

3.1. HPLC

- HPLC System: e.g.: Agilent 1260 Infinity II system
  Open Lab CDS-control and integration software or equivalent
- Column: Waters X-Bridge C18, 150*4.6mm, 3.5µm PN: 186003034
- Flow rate: 1.0 mL/min
- Elution: Gradient mode
- Run time: 40.0 min
- Detection: 278 nm
- Injection: 10 µL
- Column temp.: 35°C ± 2°C
- Auto sampler temp.: Room temperature
- Mobile phase (see section 3.3.2):
  - **A:** 0.1% H_3PO_4 in Water
  - **B:** Acetonitrile
- Diluent: 0.1% H_3PO_4 in Water/Acetonitrile (80/20 v/v)
- Needle wash: Water/ Acetonitrile (50:50 v/v)
- Equilibration time: 10.0 min
- Gradient:

Table 2 Gradient Table

Time (min)	% A	% B
0.0	98	2
3.0	98	2
5.0	92	8
25.0	85	15
32.0	15	85
37.0	15	85
37.1	98	2
40.0	98	2

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
- Phosphoric acid: 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.

0.1% H_3PO_4 in Water/Acetonitrile (80/20 v/v):

Combine 800 mL of water and 200 mL of acetonitrile into a suitable container, and then add 1 mL of Phosphoric acid. Mix well.

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% H_3PO_4 in Water):

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

Mobile phase B (Acetonitrile):

Acetonitrile.

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.
All TGF-001 Materials must be weighted out in a hunidity-controlled environment (≤ 10%).

3.3.3.1. Standard solutions

Standard Solution 1 & 2 (conc.: 0.3 mg/ml):

Accurately weigh approx. 15 mg of TGF-001 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 0.00005 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. 15 mg of TGF-001 sample into a 50-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 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) Total impurities

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

Where:
Pi = TGF-001 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

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

Where:

Area $_{sam}$= TGF-001 peak area obtained in the sample chromatogram
Area $_{std}$= Average TGF-001 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 (free base) from CoA for TGF-001

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

Where:

Assay $_{free\ base}$ = Potency determined for the free base of TGF-001 sample
MW $_{salt}$ = Molecular weight of TGF-001 salt (910.04 g/mol)
MW $_{free\ base}$ = Molecular weight of TGF-001 free base (518.05 g/mol)

3.5.6. Related substances by HPLC: Total impurities in %w/w

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

3.5.7. Related substances by HPLC: Individual impurities in %w/w

$$Content_{impurity} \ (\%w/w) = \frac{Area_{imp.\ sam.} \times W_{std} \times V_{sam.}}{Area_{TGF-001\ std} \times W_{sam.} \times V_{std}} \times RRFimp. \times P_{TGF-001\ std\ (free\ base)}$$

Where:

Area $_{imp.\ sam.}$ = Impurity peak area obtained in the sample chromatogram
Area $_{TGF\text{-}001\ std}$ = Average TGF-001 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)
RRF $_{imp.}$ = Relative response factor determined for the impurity.
(For values see Table 1. Use 1.00, in case there is no RRF determined)
P $_{TGF\text{-}001\ std\ (free\ base)}$ = Potency determined for the standard in used. Use value from the CoA for TGF-001 (free base).

- For impurity content below LOQ concentration, report "Less than 0.05 %(w/w)" (ICH reportable threshold);
- If impurity is not detected, report "Not detected'.

4. Figures

Figure 1 Example HPLC chromatogram of blank, method INV_054926_HPLC_M4, 278nm

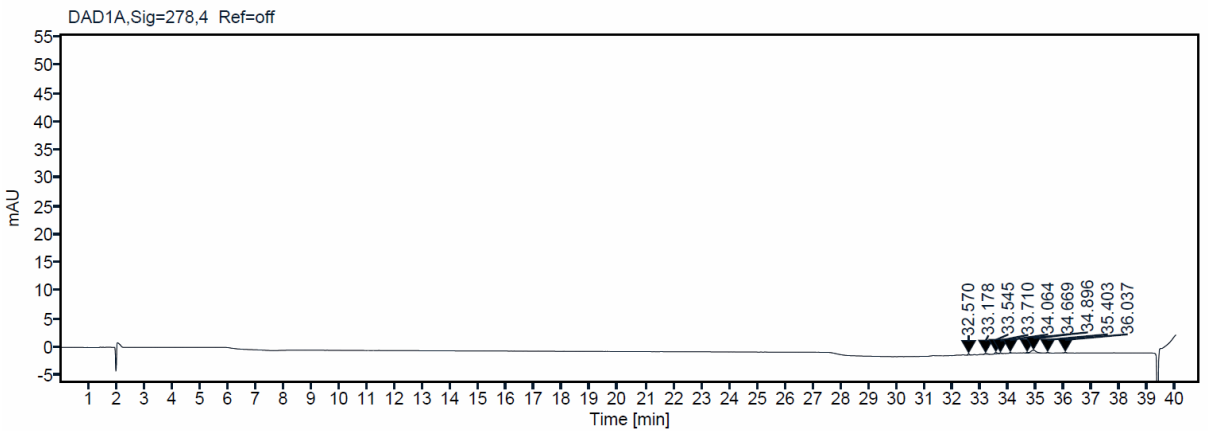


Figure 2 Example HPLC chromatogram of TGF-001 batch 100144-200502-REF, method INV_054926_HPLC_M4, 278nm

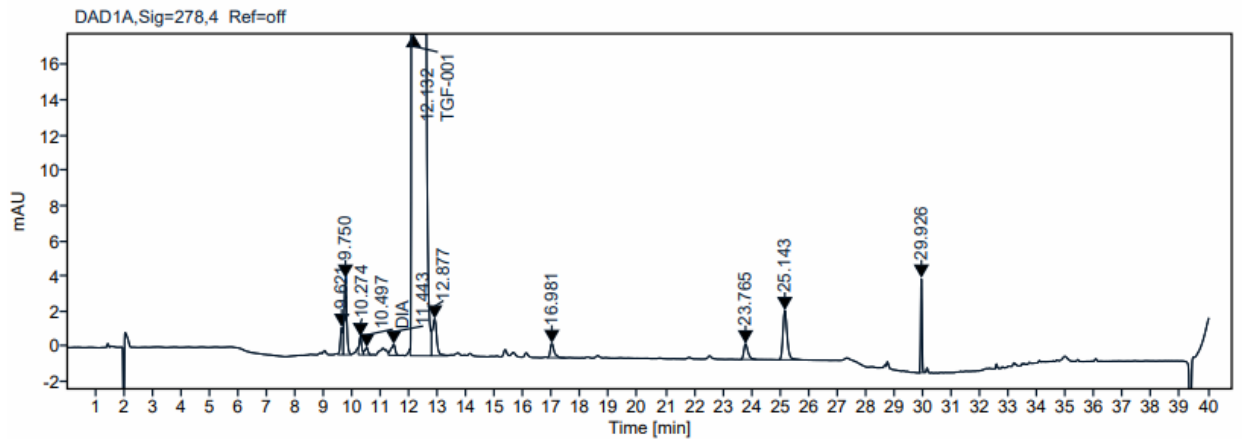


Figure 3 Example HPLC chromatogram of TGF-001 batch 100144-200502-REF spiked with impurity DIA batch PHTRACKD-394-REF, 278 nm

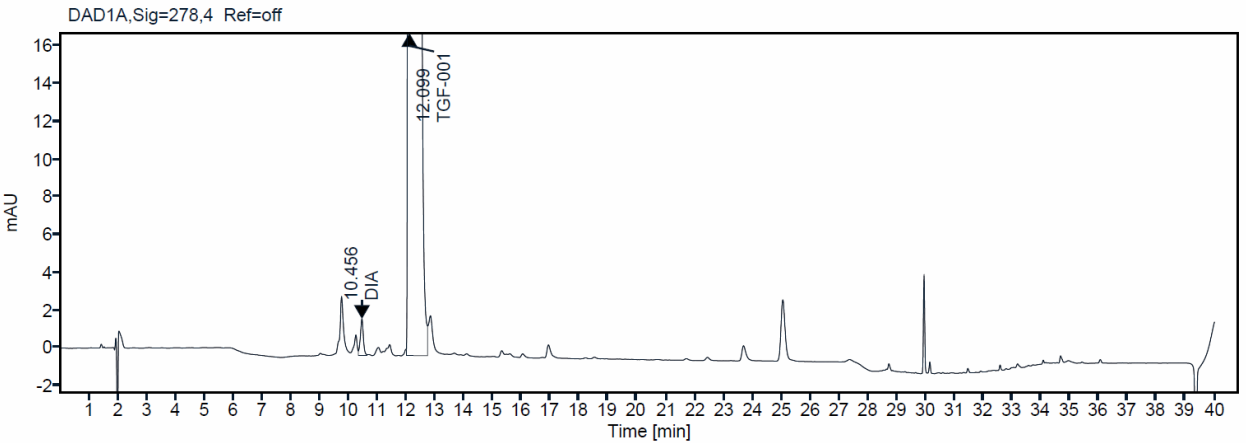


Figure 4 Example HPLC chromatogram of TGF-001 batch 100144-200502-REF spiked with impurity DIN batch PHTRACKD-389-REF, 278 nm

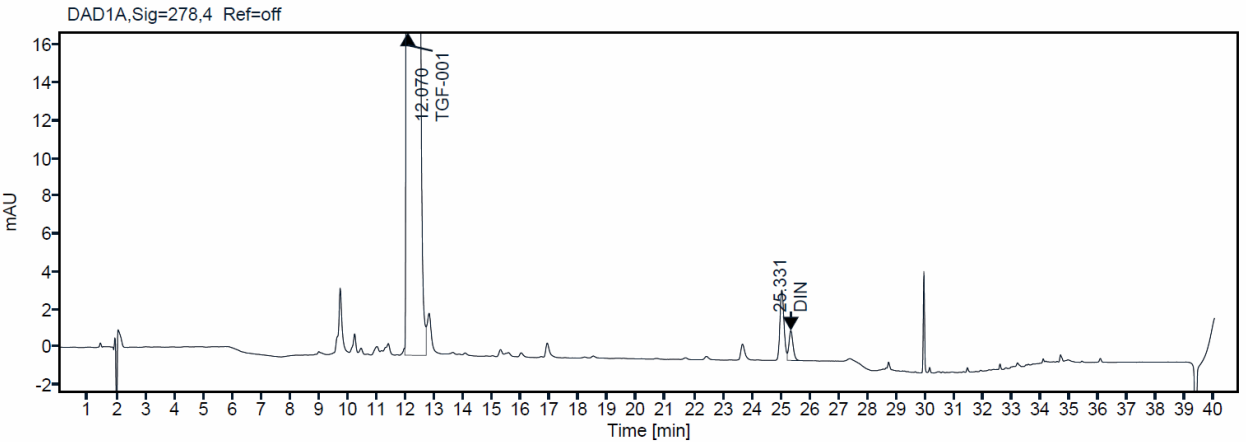


Figure 5 Extracted HPLC-PDA Spectrum of TGF-001, method INV_054926_HPLC_M4

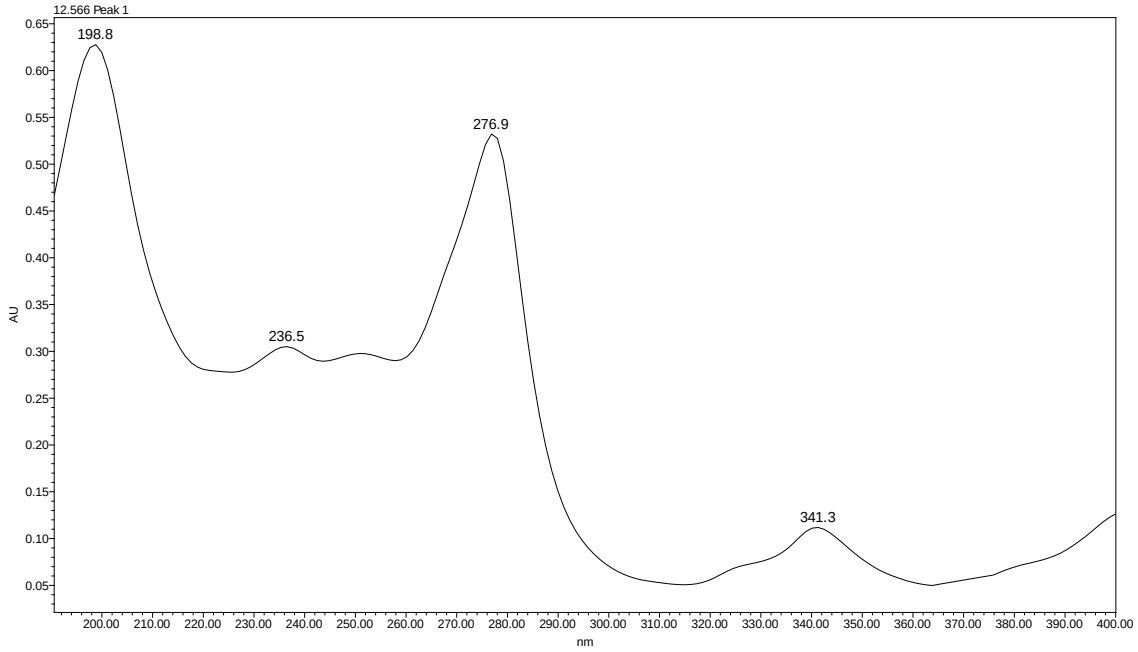


Figure 6 Extracted HPLC-PDA Spectrum of impurity DIA, method INV_054926_HPLC_M4

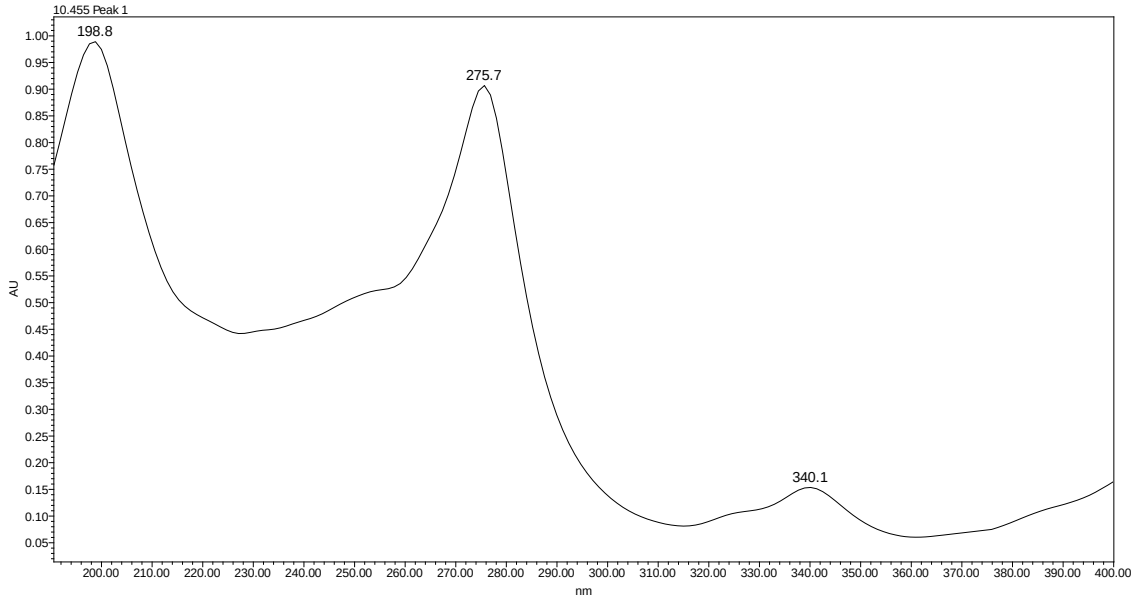


Figure 7 Extracted HPLC-PDA Spectrum of impurity DIN, method INV_054926_HPLC_M4