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| **Notebook Reference** | ARD-0618/64-131 |
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# Introduction

This report summarizes the execution of method validation protocol PRO-02816 (v1.0), which pertains to the early phase method validation of the *Assay*, *Related Substances* and *Identification* analytical procedures for TYRA-300 Sprinkle Capsules (1 mg, 5 mg, and 10 mg).

Appropriate studies were performed in order to demonstrate that the proposed method is suitable for intended use. The corresponding protocol describes the methodology for the validation of the analytical procedure and defines the criteria to assess the results.

The composition of the TYRA-300 Sprinkle Capsules is summarized in **Table 1-1**. The three strengths are dose proportional.

Table 1-1. Ingredient Composition for TYRA-300 Sprinkle Capsules

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Ingredients** | **mg/unit** | | | **%w/w** | | |
| **1 mg** | **5 mg** | **10 mg** | **1mg** | **5mg** | **10mg** |
| TYRA-300-B01 salt | 1.282 | 6.41 | 12.82 | 6.41 | | |
| Lactose Monohydrate, NF (Fast Flo 316) – Part I | 1.784 | 8.92 | 17.84 | 8.92 | | |
| Lactose Monohydrate, NF (Fast Flo 316) – Part II | 3.568 | 17.84 | 35.68 | 17.84 | | |
| Lactose Monohydrate, NF (Fast Flo 316) – Part III | 3.568 | 17.84 | 35.68 | 17.84 | | |
| Microcrystalline Cellulose, NF (Avicel PH 102) | 9.00 | 45.00 | 90.00 | 45.00 | | |
| Croscarmellose Sodium NF (Ac-Di-Sol) | 0.4 | 2.00 | 4.00 | 2.00 | | |
| Colloidal Silicon Dioxide, NF (Cab-O-Sil) | 0.10 | 0.50 | 1.0 | 0.50 | | |
| Sodium Stearyl Fumarate, NF | 0.3 | 1.5 | 3.0 | 1.50 | | |
| **Core Mini-Tablets Total** | 20 | 100 | 200 | **100.00** | | |
| Opadry AMB II white 88A180040 | 2 | 10 | 20 | 10.00 | | |
| Purified Water | NA | N/A | NA | n/a | | |
| **Talc Blending** |  |  |  |  | | |
| Talc, USP | 0.04 | 0.20 | 0.40 | 0.20 | | |
| **Capsule Fill Weight** | 22 | 110 | 220 | **110.00** | | |

The specified process impurities of TYRA-300 are listed in **Table 1-2**. These process impurities are controlled in the drug substance and therefore will not be monitored in the final drug product. It is worth noting that impurity R-191-2 may be present in the enantiomeric forms as two peaks which will be referred to as R-191-2a and R-191-2b.

**Table 1-2: Potential Impurities of TYRA-300**

| Chemical Name | Synonym | Impurity Type | Specified Impurity |
| --- | --- | --- | --- |
| Unknown | IMP\_RRT 0.81 | Process Impurity | Yes |
| Unknown | IMP\_RRT 0.90 | Process Impurity | Yes |
| Unknown | IMP\_RRT 0.97 | Process Impurity | Yes |
| 5-((R)-1-(3,5-Dichloropyridin-4-yl)ethoxy-3-((3-((methansulfonyl)amino)methyl)-(3-(benzensulfonyloxy)methyl)azetidine-3-yl)pyridine-3-yl-1*H*-indazole | EtOH Adduct | Process Impurity | Yes |
| 5-((R)-1-(3,5-Dichloropyridin-4-yl)ethoxy-3-((3-((methansulfonyl)amino)methyl)-(3-ethoxymethyl) azetidine-3-yl)pyridine-3-yl-1*H*-indazole | BSA Adduct | Process Impurity | Yes |
| 5-((R)-1-(3,5-Dichloropyridin-4-yl)ethoxy-3-(6-(6-(methylsulfonyl)-2,6-diazaspiro[3.3]heptan-2-yl)pyridine-3-yl-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazole | R-191-2  (R-191-2a,  R-191-2b) | Process Impurity | Yes |

The method validation was performed in accordance with Frontida’s Standard Operating Procedure for Validation of Analytical Methods, SOP-01377 (SOP MPC QC/RD-017) (current version), which is based on the ICH guidelines Q2(R1). The following characteristics/parameters were evaluated:

* System Suitability
* Specificity (Interference and Identification)
* Linearity and Range
* Accuracy by Spiked Recovery
* Precision
* Quantitation Limit
* Filtration
* Solution Stability for the standard solution, sample solution, and mobile phases

# Analytical Procedure

The following section describes the final procedure performed for method validation and has been updated to include changes or deviations from those described in the corresponding section in the protocol.

## Chromatographic Parameters

Table 2-1. HPLC Parameters

|  |  |  |  |
| --- | --- | --- | --- |
| **Column** | Waters Cortecs, C18: 2.1 x 100 mm, 1.6 µm  PN: 186007095 | | |
| **Mobile Phase A** | 0.1% TFA in water | | |
| **Mobile Phase B** | 0.1% TFA in acetonitrile | | |
| **Needle Wash** | 90% methanol/ 10% water | | |
| **Purge/Seal Wash** | 20% methanol/ 80% water | | |
| **Needle Wash** | Extended | | |
| **Gradient Program** | **Time (min)** | **Mobile Phase A** | **Mobile Phase B** |
| 0 | 95 | 5 |
| 2.0 | 95 | 5 |
| 18.0 | 57 | 43 |
| 24.0 | 53 | 47 |
| 27.0 | 40 | 60 |
| 31.0 | 25 | 75 |
| 31.5 | 5 | 95 |
| 33.5 | 5 | 95 |
| 35.0 | 95 | 5 |
| 40.0 | 95 | 5 |
| **Detection** | 262 nm | | |
| **Detector Sampling Rate** | 10 pts/sec | | |
| **Flow Rate** | 0.4 mL/min | | |
| **Column Temperature** | 40°C ± 3°C | | |
| **Sample Compartment Temperature** | 5°C ± 4°C | | |
| **Injection Volume** | 2 μL | | |
| **Run Time** | 40 minutes | | |

## Reagents and Materials

Purified Water, Millipore

Acetonitrile, HPLC Grade

Methanol, HPLC Grade

Trifluoroacetic Acid (TFA), HPLC Grade

TYRA-300-B01, Reference Standard (RS)

TYRA-300 Sprinkle Capsules, 1 mg, 5 mg, 10 mg

Pall Acrodisc, 0.2-µm PTFE 25 mm syringe filter

## Mobile phase A (0.1% TFA in Water)

Combine 1.0 mL of trifluoroacetic acid with 1000 mL of purified water in a suitable container. Mix well and degas.

## Mobile phase B (0.1% TFA in Acetonitrile)

Combine 1.0 mL of trifluoroacetic acid with 1000 mL of acetonitrile in a suitable container. Mix well and degas.

## Diluent Preparation

Prepare a mixture of methanol and purified water at a ratio of 90:10. Mix well.

## Standard Solution Preparation

**NOTE – Protect standard solutions from light.**

Prepare a check standard solution in a similar manner.

### Stock Standard Solution Preparation

Accurately weigh and quantitatively transfer about 65 mg of TYRA-300-B01 RS into a 100-mL volumetric flask. Add *diluent* to about 2/3 of flask volume and briefly sonicate (about 5 minutes) to dissolve the standard. Equilibrate to room temperature then dilute to volume with diluent, mix well and label as the Stock standard solution.

The concentration of TYRA-300 free base is about 0.5 mg/mL.

### Working Standard Solution Preparation

Pipette 10.0 mL of stock standard into a 50-mL volumetric flask. Dilute to volume with *diluent*, mix well and label as the working standard solution.

The concentration of TYRA-300 free base is about 0.1 mg/mL.

## Sensitivity Standard Solution Preparation

Note—The sensitivity solution is required only for the Related Substances test method.

### Intermediate Sensitivity Solution

Pipette 1.0 mL of *stock standard solution* into a 100-mL volumetric flask. Dilute to volume with *diluent* and mix well. Label as sensitivity intermediate solution.

The concentration of TYRA-300 free base is about 5 µg/mL.

### Working Sensitivity Solution

Pipette 5.0 mL of Intermediate Sensitivity solution into a 100-mL volumetric flask. Dilute to volume with *diluent* and mix well. Label as sensitivity solution.

The concentration of TYRA-300 free base is about 0.25 µg/mL.

## Placebo Solution Preparation

Weigh not less than (NLT) 420 mg placebo mixture into a 20-mL volumetric flask. Fill with *diluent* to about 2/3 of flask volume and sonicate 30 minutes and shake 30 minutes. Equilibrate to room temperature then fill flask to volume with *diluent* and mix well. Centrifuge portion of sample at 12000 rpm for 10 minutes and transfer the supernatant to an HPLC vial for analysis.

Alternatively, filter a portion of sample through a Pall Acrodisc, 0.2-µm PTFE 25 mm syringe filter, after discarding the first 2 mL.

## Assay and Related Substances Sample Solution Preparation

**NOTE – Protect sample solutions from light.**

Accurately weigh Ten (10) capsules, then carefully open and transfer the contents into the volumetric flask indicated in **Table 2-2**. Weigh the empty capsules and calculate the sample weight.

**Table 2-2: Stock Sample Preparation**

|  |  |  |  |
| --- | --- | --- | --- |
| **Strength  (mg)** | **Number of capsules** | **Volumetric flask  (mL)** | **Concentration  (mg/mL)** |
| 1 mg | 10 | 20 | 0.5 |
| 5 mg | 10 | 100 | 0.5 |
| 10 mg | 10 | 200 | 0.5 |

Add *water* to 10% of flask volume and briefly sonicate to disperse coating (about 2 minutes). Fill with *methanol* to about 2/3 of flask volume and sonicate 30 minutes and shake 30 minutes. Equilibrate to room temperature then fill flask to volume with *methanol* and mix well.

Related Substances Sample Preparation:

Centrifuge a portion of the stock sample at 12000 rpm for 10 minutes and transfer the supernatant to an HPLC vial for analysis.

Assay Sample Preparation:

Pipette 5.0 mL of stock sample into a 25-mL volumetric flask. Fill flask to volume with *diluent* and mix well.

Centrifuge portion of stock sample at 12000 rpm for 10 minutes and transfer the supernatant to an HPLC vial for analysis.

Alternatively, filter a portion of sample through a Pall Acrodisc, 0.2-µm PTFE 25 mm syringe filter, after discarding the first 2 mL.

## Procedure

Separately inject equal volumes (2 µL) of the diluent, sensitivity standard solution, working standard and check standard, placebo, sample solutions, and bracketing standards. Record the chromatograms and measure the peak area responses of the TYRA-300 peak and related impurities.

**Note**: Equilibrate the column at the initial conditions until a stable baseline is achieved.

**Example of Injection Sequence**

|  |  |
| --- | --- |
| Solutions | Number of Injections |
| Diluent | ≥1 |
| Placebo Solution\* | 1 |
| Sensitivity Solution\* | 1 |
| Standard Solution | 5 |
| Check Standard Solution† | 1 |
| Bracketing Standard | 1 |
| Sample Solution | ≤6 |
| Bracketing Standard | 1 |

\* Required for Related Substance only  
† Required for Assay only

## System Suitability Requirements

* The blank prior to the identity injection does not contain a peak at the retention time of TYRA-300 peak with an area count of above 0.2% of the first injection of standard.
* The tailing factor of TYRA-300 in the first injection of standard should be not more than (NMT) 2.0.
* The relative standard deviation (RSD) of the TYRA-300 area responses for the five (5) consecutive injections of the standard solution is NMT 2.0%.
* The percent deviation between mean system suitability standards and each bracketing standard injection must be NMT 3.0%.

Additional requirements applicable to only the Assay analysis:

* The percent recovery of TYRA-300 in the check standard solution is within 98.0% - 102.0%.

Additional requirements applicable to only the Related Substances analysis:

* The signal-to-noise (S/N) ratio of TYRA-300 peak in the sensitivity solution should be NLT 10.

## Chromatogram Integration

Integrate the chromatogram of each standard and sample, exclude peaks present in system blank and/or placebo as well as process impurities noted in **Table 2-10**.

## Peak Identification

The Relative Retention Times (RRT) of known impurities are provided in the table below:

**Table 2-10: TYRA-300 Process Impurities**

|  |  |
| --- | --- |
| **Compound Name** | **Approximate RRT** |
| Impurity @ 0.81 | 0.81 |
| Impurity @ 0.90 | 0.90 |
| Impurity @ 0.97 | 0.97 |
| EtOH Adduct | 1.09 |
| BSA Adduct | 1.19 |
| R-191-2a | 1.24 |
| R-191-2b | 1.26 |

## Calculations

Calculate the % Label Claim as follows:

For Assay:

Where,

|  |  |  |
| --- | --- | --- |
| Ru | : | The area response of TYRA-300 peak in the sample solution |
| Rs | : | The area response of TYRA-300 peak in the standard solution |
| W | : | Weight of TYRA-300 in the standard solution, in mg |
| P | : | Purity of standard expressed as % Purity/100% |
| CF | : | Free base conversion factor, 0.7796 |
| DF | : | Dilution Factor used for sample preparation, in mL |
| LC | : | Label claim, in mg |
| N | : | Number of capsules |

**Note** – CF may already be included in calculation for Purity. If so, then CF should be omitted from above calculation.

**Note – Report % Impurity of specified process impurities as “n/a”. The total area of all integrated peaks should not include peaks below reporting limit (0.05%), diluent peaks, or system peaks. For total impurities exclude any specified process impurities and include only impurities ≥ 0.05%.**

For Related Substances:

Where,

|  |  |  |
| --- | --- | --- |
| Aimp | : | Area of Individual Impurity |
| Atotal | : | Total area of all integrated peaks |

# Instruments and Equipment

* Waters Acquity UPLC system
* UPLC Instrument: ARDUPLC09, Cal Due 03/24
* Column: Cortecs C-18, 100x2.1 mm, 1.6 µm, S/N 01623311118584

# Reagents and Materials

Reagents:

* Purified water, Millipore, In-House, ARD-WA-01, Cal Due 02/24
* Methanol, HPLC Grade, Supelco, Lot #62167, Exp Date: 11/26, RT (room temperature)
* Acetonitrile, HPLC Grade, Supelco, Lot #62167, Exp Date: 11/26, RT
* Trifluoroacetic acid, HPLC Grade, Lot#62273319, Exp Date: 10/26, RT

Materials:

* TYRA-300-B01 Reference Standard, Cambrex, Lot# 006BJF062, Exp Date: 10/24, purity = 76.09%
* TYRA-300 10mg Sprinkle Capsule, Lot #NB1806:73
* 0.2-µm PTFE 25 mm syringe filter, Pall, P/N 4521

# System Suitability/System Precision

The system suitability was successfully demonstrated for HPLC analysis. Results are summarized below in **Table 5-1**:

**Table 5-1: Representative System Suitability Results**

|  |  |
| --- | --- |
| **Criteria** | **Result** |
| **Diluent Interference** | None detected (ND) |
| **Tailing Factor** | 1.7 |
| **Standard % RSD (n=5)** | 0.1% |
| **Bracketing Standard % Deviation** | 0.1%-1.0% |
| **Check Std % Recovery (for Assay)** | 99.7% |
| **Sensitivity S/N (for Related Substance)** | 33 |
| Acceptance Criteria:   * The blank prior to the identity injection does not contain a peak at the retention time of TYRA-300 peak with an area count of above 0.2% of the first injection of standard. * The tailing factor of TYRA-300 in the first injection of standard should be NMT 2.0. * The RSD of the TYRA-300 area responses for the five (5) consecutive injections of the standard solution is NMT 2.0%. * The percent deviation between mean system suitability standards and each bracketing standard injection must be NMT 3.0%.   Additional requirements applicable to only the Assay analysis:   * The percent recovery of TYRA-300 in the check standard solution is within 98.0% - 102.0%.   Additional requirements applicable to only the Related Substances analysis:   * The signal-to-noise ratio of TYRA-300 peak in the sensitivity solution should be NLT 10. | |

Reference: ARD-0618/96

# SPECIFICITY STUDY (INTERFERENCE AND IDENTIFICATION)

Specificity studies were performed in order to determine whether there are any significantly interfering peaks arising from the diluent or placebo that may affect the quantitation of the intended analytes.

## Diluent Interference Solution Preparation

Used diluent as the diluent interference solution.

## Placebo Solution Preparation

Prepared a placebo solution as directed in **Section 2.8**.

## TYRA-300 Sample Solution Preparation

Prepared a sample solution as directed in **Section 2.9**.

## Identification by Retention Time (RT)

The successful establishment of system suitability was considered fulfillment of Identification by RT test method.

## Results and Discussion

All acceptance criteria were met. Results for specificity are summarized in **Table 6-1**:

**Table 6-1: Specificity Results**

|  |  |
| --- | --- |
| **Solution** | **Result** |
| Diluent | Not Detected |
| Placebo | Not Detected |
| Acceptance Criteria:   * The diluent and placebo solutions do not show any significantly interfering peaks near the retention time of TYRA-300 (NMT 0.2%). | |

Reference: ARD-0618/96-97

**Figure 1** is a representative chromatogram of the diluent solution.

**Figure 2** is a representative chromatogram of the placebo solution.

**Figure 5** is a representative chromatogram of the working standard solution.

# Linearity

For Assay, linearity was assessed from TYRA-300 concentrations of 0.05 mg/mL to 0.15 mg/mL, which corresponds to 50% to 150%, respectively, of the nominal TYRA-300 concentration in the Assay sample and standard solutions.

For Related Substances, linearity was evaluated from TYRA-300 concentrations of 0.25 µg/mL to 7.5 µg/mL, which corresponds to 0.05% to 1.5% of the impurity level with respect to the nominal sample concentration. Linearity was evaluated from TYRA-300 concentrations of 0.25 mg/mL to 0.625 mg/mL, which corresponds to 50% to 125% of TYRA-300 concentration with respect to the nominal Related Substances sample concentration.

## Stock Assay Linearity Solution Preparation

Used the *Stock Standard* solution (**Section 2.6.1**).

The concentration of TYRA-300 free base was about 0.5 mg/mL.

## Working Assay Linearity Solution Preparation

Prepared the working linearity solutions for the L1 to L5 levels as directed in **Table 7‑1**. Diluted each to volume with the diluent and mixed well.

Table 7-1. Preparation of working TYRA-300 linearity solutions

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Assay Linearity Level | Nominal Conc.  (%) | Volume of Stock TYRA-300 Solution  (mL) | Flask  Volume  (mL) | Approx. Conc. of TYRA-300  (mg/mL) |
| L1 | 50 | 2.5 | 25 | 0.05 |
| L2 | 80 | 4.0 | 25 | 0.08 |
| L3 | 100 | 10.0 | 50 | 0.10 |
| L4 | 120 | 6.0 | 25 | 0.12 |
| L5 | 150 | 7.5 | 25 | 0.15 |

## Related Substance Linearity Solution Preparation

Accurately weighed and quantitatively transferred about 130 mg of TYRA-300-B01 RS into a 100-mL volumetric flask. Added diluent to about 2/3 of flask volume and briefly sonicated (about 5 minutes) to dissolve the standard. Equilibrated to room temperature then diluted to volume with diluent, mixed well and labeled as the Stock standard solution.

The concentration of TYRA-300 free base was about 1 mg/mL.

## Intermediate Impurity Linearity Solution Preparation

Transferred 2.5 mL of the *Stock Related Substance* solution into a 100-mL volumetric flask. Diluted to volume with the diluent and mixed well.

The concentration of TYRA-300 free base was about 0.025 mg/mL.

## Working Related Substance Linearity Solution Preparation

Prepared the working linearity solutions for the L1 to L5 levels as directed according to **Table 7-2**. Diluted each to volume with the diluent and mixed well.

**Table 7-2. Preparation of working Related Substance linearity solutions**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Related Substance Linearity Level | Nominal Conc.  (%) | Volume of Related Substance Linearity Solution  (mL) | Flask  Volume  (mL) | Approx. Conc. of TYRA-300/Impurities  (mg/mL) |
| L1 | 40 | 10.0 | 50 | 0.2 |
| L2 | 60 | 6.0 | 20 | 0.3 |
| L3 | 80 | 10.0 | 25 | 0.4 |
| L4 | 100 | 10.0 | 20 | 0.5 |
| L5 | 125 | 12.5 | 20 | 0.625 |

## Working Impurity Linearity Solution Preparation

Prepared the working linearity solutions for the L1 to L5 levels as directed according to **Table 7-3**. Diluted each to volume with the diluent and mix well.

**Table 7-3. Preparation of working Impurity linearity solutions**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Impurity Linearity Level | Nominal Conc.  (%) | Volume of Intermediate Impurity Linearity Solution (mL) | Flask  Volume  (mL) | Approx. Conc. of TYRA-300/Impurities  (µg/mL) |
| L1 | 0.05 | 1.0 | 100 | 0.25 |
| L2 | 0.2 | 5.0 | 100 | 1.25 |
| L3 | 0.5 | 5.0 | 50 | 2.5 |
| L4 | 1.0 | 5.0 | 25 | 5.0 |
| L5 | 1.5 | 7.5 | 25 | 7.5 |

## Results and Discussion

All acceptance criteria were met. Linearity data and plots are given in **Table 7-4**, **Table 7-5**, **Table 7-6**, **Figure 10**, **Figure 11**,and **Figure 12** for Assay, Related Substances, and Impurity levels, respectively.

**Table 7-4**: **Linearity Results for Assay**

|  |  |  |  |
| --- | --- | --- | --- |
| Sample | Level (%) | Conc. TYRA-300  Free Base (µg/mL) | Peak Area |
| L1 | 50 | 49 | 477486 |
| L2 | 80 | 79 | 777920 |
| L3 | 100 | 98 | 970063 |
| L4 | 120 | 118 | 1161510 |
| L5 | 150 | 147 | 1454202 |
| Y-Intercept | | | 6033 |
| % Intercept (Relative to L3) | | | 0.6% |
| Correlation Coefficient (r) | | | 1.000 |
| Acceptance Criteria:   * Meet the linearity range of a minimum of five consecutive levels. * The correlation coefficient, r, is NLT 0.999. * The Y-intercept relative to nominal 100% level is NMT 2%. | | | |

Reference: ARD-0618/118-119

**Table 7-5**: **Linearity Results for Related Substances**

|  |  |  |  |
| --- | --- | --- | --- |
| Sample | Level (%) | Conc. TYRA-300  Free Base (µg/mL) | Peak Area |
| L1 | 40 | 197 | 1925205 |
| L2 | 60 | 296 | 2883678 |
| L3 | 80 | 394 | 3834931 |
| L4 | 100 | 493 | 4860538 |
| L5 | 125 | 616 | 5958453 |
| Slope | | | 9691 |
| Y-Intercept | | | 22217 |
| % Intercept (Relative to L4) | | | 0.5% |
| Correlation Coefficient (r) | | | 0.999 |
| Acceptance Criteria:   * Meet the linearity range of a minimum of five consecutive levels. * The correlation coefficient, r, is NLT 0.999 * The Y-intercept relative to nominal 100% level is NMT 2%. | | | |

Reference: ARD-0618/116-117

**Table 7-6**: **Linearity Results for Impurity Level**

|  |  |  |  |
| --- | --- | --- | --- |
| Sample | Level (%) | Conc. TYRA-300  Free Base (µg/mL) | Peak Area |
| L1 | 0.05 | 0.25 | 2384 |
| L2 | 0.2 | 1.23 | 11072 |
| L3 | 0.5 | 2.47 | 22140 |
| L4 | 1.0 | 4.93 | 44509 |
| L5 | 1.5 | 7.40 | 66302 |
| Slope | | | 8962 |
| Relative Slope (relative to related substances level) | | | 92% |
| Correlation Coefficient (r) | | | 1.00 |
| Acceptance Criteria:   * Meet the linearity range of a minimum of five consecutive levels. * The correlation coefficient, r, is NLT 0.99. * The relative slope of TYRA-300 (impurity level) to TYRA-300 (related substances level) is within 90% to 110%. | | | |

Reference: ARD-0618/114-115

# Accuracy by Spiked recovery

An accuracy study was performed to demonstrate that the method can achieve acceptable recoveries.

The accuracy study for *Assay* and *Impurities* was performed by spiking known amounts of TYRA-300 onto a corresponding amount of composite placebo powder.

For *Assay*, the accuracy was evaluated from TYRA-300 concentrations of 0.05 mg/mL to 0.15 mg/mL, which corresponds to 50% to 150% of the nominal sample solution concentration.

For *Related Substances*, the accuracy study was evaluated from concentrations of 0.25 µg/mL to 7.5 µg/mL, which corresponds to impurity levels of 0.05% to 1.5% with respect to the nominal sample solution concentration.

## Accuracy for Assay

### Assay Recovery Sample Solution Preparation

Accurately weighed and quantitatively transferred portions of TYRA-300 and composite placebo powder into volumetric flasks as shown in **Table 8-1**. Filled with *diluent* to about 2/3 of flask volume and sonicated 30 minutes and shook 30 minutes. Equilibrated to room temperature, then filled flask to volume with *diluent* and mixed well.

Diluted 5.0 mL of each stock recovery solution to 25-mL with the diluent and mixed well.

Filtered a portion of sample through a 0.2-µm PTFE 25 mm syringe filter, after discarding the first 2 mL.

Prepared each level in triplicate.

Table 8-1. Preparation of the stock recovery assay sample solutions

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Recovery Level | Nominal Conc.  (%) | Weight of TYRA-300-B01  (mg) | Weight of Placebo Powder  (mg) | Flask Volume  (mL) | Approx. TYRA-300 Conc.  (mg/mL) |
| R1 | 50 | 32 | 1050 | 100 | 0.25 |
| R2 | 100 | 32 | 525 | 50 | 0.5 |
| R3 | 150 | 48 | 525 | 50 | 0.75 |

## Accuracy for Impurities

### Stock Spiking Solution Preparation

Used the Working standard solution (**Section 2.6.2**) as the Stock Spiking solution.

The concentration of TYRA-300 was about 0.1 mg/mL.

### Working Spiking Solution Preparation

Diluted 12.5 mL of the Stock Spiking Solution (**Section 8.2.1**) to 50-mL with diluent and mixed well.

The concentration of TYRA-300 was about 0.025 mg/mL.

### Recovery Sample Solution Preparation

Accurately weighed and quantitatively transferred an amount of the composite placebo powder into separate volumetric flasks as outlined in **Table 8-2**. Filled with *diluent* to about 2/3 of flask volume and sonicated 30 minutes and shook 30 minutes. Equilibrated to room temperature then filled flask to volume with *diluent* and mixed well. Filled flask to volume with methanol and mixed well.

Filtered a portion of sample through a 0.2-µm PTFE 25 mm syringe filter, after discarding the first 2 mL.

Prepared samples for each recovery level in triplicate.

Table 8-2. Preparation of the recovery impurities sample solutions

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Recovery Level | Impurity Level  (%) | Number of Replicates | Weight of Placebo Powder  (mg) | Volume of Stock Spiking Solution (mL) | Volume of Working Spiking Solution (mL) | Flask Volume (mL) | Approx. Conc. of TYRA-300 (µg/mL) |
| R1 | 0.05 | 3 | 1050 | - | 1.0 | 100 | 0.25 |
| R2 | 0.5 | 3 | 210 | - | 2.0 | 20 | 2.5 |
| R3 | 1.0 | 6 | 210 | 1.0 | - | 20 | 5.0 |
| R4 | 1.5 | 3 | 210 | 1.5 | - | 20 | 7.5 |

### Control Sample Preparation

Accurately weighed and quantitatively transferred 210 mg of the composite placebo powder into 20-mL volumetric flask. Filled with *diluent* to about 2/3 of flask volume and sonicated 30 minutes and shook 30 minutes. Equilibrated to room temperature then filled flask to volume with *diluent* and mixed well.

Filtered a portion of sample through a Pall Acrodisc 0.2-µm PTFE 25 mm syringe filter, after discarding the first 2 mL.

Prepared one (1) control sample solution.

## Results and Discussion

All criteria were met. Accuracy results are given in **Table 8-3** and **Table 8-4** for Assay and Impurities, respectively.

**Table 8-3: Accuracy Results for Assay**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Level** | **Sample** | **Peak Area** | **% Recovery** | **Average % Recovery** | **% RSD** |
| R1 | 1 | 477310 | 99.17 | 99 | 1 |
| 2 | 468949 | 97.77 |
| 3 | 470671 | 98.79 |
| R2 | 1 | 958117 | 98.91 | 99 | 0.4 |
| 2 | 955215 | 99.57 |
| 3 | 974805 | 98.81 |
| R3 | 1 | 1429493 | 99.46 | 99 | 0.4 |
| 2 | 1432726 | 99.06 |
| 3 | 1450254 | 99.94 |
| Acceptance Criteria   * The mean precent recovery of triplicate preparations is within 95%-105%. * The % RSD of the triplicate preparations is NMT 3%. | | | | | |

Reference: ARD-0618/98

**Table 8-4: Accuracy Results for Impurities**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Level** | **Sample** | **Peak Area** | **% Recovery** | **Average % Recovery** | **% RSD** |
| R1 | 1 | 2593 | 106.50 | 105 | 2 |
| 2 | 2508 | 103.00 |
| 3 | 2591 | 106.42 |
| R2 | 1 | 23778 | 97.65 | 98 | 0.3 |
| 2 | 23794 | 97.72 |
| 3 | 23676 | 97.23 |
| R3 | 1 | 48110 | 98.68 | 98 | 1 |
| 2 | 47521 | 97.48 |
| 3 | 48104 | 98.67 |
| 4 | 47585 | 97.61 |
| 5 | 47686 | 97.82 |
| 6 | 47303 | 97.03 |
| R4 | 1 | 69668 | 95.24 | 97 | 1 |
| 2 | 71205 | 97.34 |
| 3 | 71093 | 97.19 |
| Acceptance Criteria   * The mean precent recovery of the R1 level is within 50%-150%. * The mean percent recovery of the R2, R3, and R4 levels is within 80%-120%. * The % RSD of the triplicate preparations for the R1 level is NMT 20% and R2-R4 levels is NMT 15%. | | | | | |

Reference: ARD-0618/101

# Precision Study

## Precision

### Precision – Assay

Prepared six (6) sample solutions as directed in **Section 2.9** using TYRA-300, 5 mg capsules.

### Precision – Impurities

Prepared six (6) sample solutions spiked at the 100% level (R3) as directed in **Section 8.2.3**.

## Results and Discussion

All criteria were met. Precision results are given in **Table 9-1** and **Table 9-2** for Assay and Related Substances, respectively.

**Table 9-1: Precision Results for Assay**

|  |  |
| --- | --- |
| **Sample** | **% LC** |
| 1 | 101.56 |
| 2 | 101.99 |
| 3 | 101.37 |
| 4 | 101.68 |
| 5 | 101.60 |
| 6 | 101.53 |
| **Mean** | **101.6** |
| **% RSD** | **0.2** |
| **Acceptance Criteria:**   * The % RSD (n=6) is NMT 3%. | |

Notebook: ARD-0618/104

**Table 9-2: Precision Results for Related Substances**

|  |  |
| --- | --- |
| **Sample** | **% Recovery** |
| 1 | 96.68 |
| 2 | 97.48 |
| 3 | 98.67 |
| 4 | 97.61 |
| 5 | 97.82 |
| 6 | 97.03 |
| **Mean** | **97.9** |
| **% RSD** | **0.7** |
| **Acceptance Criteria:**   * The % RSD (n=6) is NMT 15%. | |

Notebook: ARD-0618/101-103

# Quantitation limit

The Quantitation Limit (QL) was evaluated at a concentration corresponding to an impurity level of 0.05%. The quantitation limit was represented by the impurity R1 and L1 levels in the Accuracy (**Section 8.2.3**) and Linearity studies (**Section 7.6**).

## Results and Discussion

All criteria were met. The results for the quantitation limit are given in **Table 10-1**:

**Table 10-1: Quantitation Limit Results**

|  |  |  |
| --- | --- | --- |
| **Impurity Level** | **Sample** | **Signal-to-Noise** |
| R1 | 1 | 31 |
| 2 | 30 |
| 3 | 30 |
| **Acceptance Criteria:**   * The impurity R1 level meets the criteria of the Accuracy study (**Table 8-4**) and the Impurity L1 level meets the criteria of the Linearity study (**Table 7-6**). * The signal-to-noise ratio of the TYRA-300 peak from the impurity R1 level solutions is ≥ 10. | | |

Reference: ARD-0618/131

# Filter Study

A filter study was performed to evaluate the suitability of the filters used for the sample solution preparation.

## Filter Study on Diluent

Separately filtered portions of the diluent through a Pall Acrodisc 0.2-µm PTFE membrane filter and collected the first 2 mL of filtrate.

## Filter Study on Sample Solution

Filtered Sample:

In a separate manner, filtered a portion of the assay sample prepared as per **Section 2.9** (Note—A sample solution prepared for **Section 9.1.1** may be used) through a Pall Acrodisc 0.2-µm PTFE filter and collected each portion as shown in **Table 11-1.**

**Table 11-1. Collection of filtrate aliquots for filter study**

|  |  |  |
| --- | --- | --- |
| **Aliquot** | **Filtration Fraction (mL)** | **Volume Collected (mL)** |
| 1 | 0-2 | 2 |
| 2 | 2-4 | 2 |
| 3 | 4-6 | 2 |

Centrifuged Sample:

Additionally, centrifuged a portion of the same sample at 12000 rpm for 10 minutes.

## Results and Discussion

All criteria were met. Results from the filter study are summarized in **Table 11-2**. Discarding the first 2 mL before collecting filtrate aliquots is adequate.

**Table 11-2: Filter Study Results**

|  |  |  |
| --- | --- | --- |
| **Sample** | **Peak Area** | **% Relative Recovery** |
| Centrifuge | 1006713 | — |
| Filter 0-2 mL | 999181 | **99** |
| Filter 2-4 mL | 1006060 | **100** |
| Filter 4-6 mL | 1004122 | **100** |
| **Acceptance Criteria:**   * The relative recovery of TYRA-300 from the filtrate aliquots of the sample solution (calculated against the centrifuged sample solution) is within 98%-102%. | | |

Reference: ARD-0618/105

# Stability Study

The stability of the standard and sample solutions was evaluated at normal laboratory environmental conditions (NLEC) and refrigerated conditions (2°C-8°C) to determine whether they are stable for use within the set time frame at the storage condition.

The stability of the standard solution was determined by periodically evaluating the recovery of TYRA-300 in the solution against freshly prepared standard solutions.

The stability of the sample solution was determined by periodically quantitating the percent of TYRA-300 and impurity levels in the solution against freshly prepared standard solutions.

The stability of the mobile phase was evaluated concomitantly with that of the standard and sample solutions.

## Results and Discussion

For Standard Solution:

Stability results are summarized in **Table 12-1**. The acceptance criteria were not met for solutions stored at NLEC at the evaluated intervals. The acceptance criteria were met for the solutions stored at refrigerated conditions for all evaluated conditions.

**Table 12-2: Standard Solution Stability Results for Assay**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Storage** | **Day** | **% Recovery** | **% Relative Recovery** | **Conclusion** |
| — | Initial | 99.8 | — | — |
| NLEC | 4 | 95.8 | 96.0 | Fail |
| 5 | 94.4 | 94.6 | Fail |
| Refrigerated | 4 | 98.3 | 98.5 | Pass |
| 5 | 99.8 | 100.1 | Pass |
| **Acceptance Criteria:**   * The standard solution is considered stable if the relative recovery of the solution that is tested for stability at the evaluated time interval is within 98.0-102.0% of the original results (t0). | | | | |

Reference: ARD-0619/33&37

For Sample Solution:

Stability results are summarized in **Table 12-2** for Assayand **Table 12-3** and **Table 12-4** for Impurities at NLEC and refrigerated conditions, respectively. The acceptance criteria were not met for solutions stored at NLEC at the evaluated intervals, but they were met for refrigerated conditions for at least 3 days for both Assay and Impurities.

**Table 12-2: Sample Solution Stability Results for Assay**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sample** | **Day** | **Peak Area** | **% Recovery** | **% Relative Recovery** | **Conclusion** |
| — | Initial | 1007517 | 101.37 | — | — |
| NLEC | 2 | 949085 | 95.49 | 94.2 | Fail |
| 3 | 908342 | 91.39 | 90.2 | Fail |
| Refrigerated | 2 | 1011629 | 101.78 | 100.4 | Pass |
| 3 | 1018711 | 102.50 | 101.1 | Pass |
| **Acceptance Criteria:**   * The sample solutions are considered stable if the relative recovery obtained at the evaluated time interval is within 98-102% of the original results (t0). | | | | | |

Reference: ARD-0618/106

**Table 12-3: Sample Solution Stability Results for Related Substances**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Storage** | **Day** | **% Impurity** | | | | | | | | |
| **RRT**  **0.47** | **RRT**  **0.50** | **RRT**  **0.72** | **RRT**  **0.74** | **RRT**  **0.77** | **RRT**  **0.95** | **RRT**  **0.96** | **RRT**  **1.08** | **Total Impurities** |
| — | Initial | ND | ND | ND | ND | ND | 0.11 | ND | 0.06 | 0.17 |
| NLEC | 2 | 0.06\* | 0.11\* | 0.10\* | 0.12\* | 0.28\* | 0.10 | <QL (0.04) | 0.06 | 0.83\* |
| 3 | 0.12\* | 0.15\* | 0.20\* | 0.12\* | 0.48\* | 0.10 | 0.05 | 0.06 | 1.27\* |
| Fridge | 2 | ND | ND | ND | ND | ND | 0.10 | ND | 0.06 | 0.16 |
| 3 | ND | ND | ND | ND | ND | 0.10 | ND | 0.06 | 0.16 |
| ND: Not Detected  \*: Did not meet acceptance criteria  **Acceptance Criteria:**   * The sample solutions are considered stable if there are no significant changes in the levels of impurities (within ±10.0%) when compared to the original results (t0) as defined: Impurities <0.2%: ±0.05% absolute; Impurities ≥0.2%: ±10% relative. | | | | | | | | | | |

Reference: ARD-0618/127-130

For Mobile Phase:

Stability results are summarized in **Table 12-4**. Criteria were met for NLEC conditions for at least 3 days.

**Table 12-4: Mobile Phase Stability Results**

|  |  |  |  |
| --- | --- | --- | --- |
| **Day** | **Retention Time TYRA-300** | **Acceptable Range (min)** | **Conclusion** |
| Initial | 15.4492 | 13.9042-16.9941 | — |
| 1 | 15.4689 | Pass |
| 2 | 15.4367 | Pass |
| 3 | 15.4136 | Pass |
| **Acceptance Criteria:**   * The retention time of the bracketing standard is within 10% of that obtained from the initial run (t0). | | | |

Reference: ARD-0618/113

# Method Range

For Assay, the method range is 0.05 mg/mL to 0.15 mg/mL for TYRA-300 (free base) based on successfully demonstrated linearity and accuracy/precision studies. This range corresponds to 50% to 150% of the nominal sample solution concentration (0.1 mg/mL).

For Impurities, the method range is 0.25 µg/mL to 7.5 µg/mL for TYRA-300 (free base) based on successfully demonstrated linearity and accuracy/precision studies. This range corresponds to 0.05% to 1.5% of the nominal sample solution concentration (0.5 mg/mL).

# Conclusions

The method validation protocol PRO-02816 (v1.0) for TYRA-300 Sprinkle Capsules was successfully executed. The findings from the studies are provided below:

* **Specificity (Interference)**: Specificity (Interference) of the method was demonstrated. There were no peaks in the diluent or placebo solutions at the retention time of the TYRA-300 peak.
* **Linearity**:
  + For Assay, linearity of TYRA-300 (free base) was demonstrated for the concentration range from 0.05 mg/mL to 0.15 mg/mL. This range corresponds to 50% to 150% of the nominal sample solution concentration (0.1 mg/mL).
  + For Related Substance, linearity of TYRA-300 (free base) was demonstrated for the concentration range from 0.2 mg/mL to 0.625 mg/mL. This range corresponds to 40% to 125% of the nominal sample solution concentration (0.5 mg/mL)
  + For Impurities, linearity of TYRA-300 (free base) was demonstrated for the concentration range from 0.25 µg/mL to 7.5 µg/mL. This range corresponds to 0.05% to 1.5% of the nominal sample solution concentration (0.5 mg/mL).
* **Accuracy**:
  + For Assay, accuracy of this method was demonstrated for the concentration range from 0.05 mg/mL to 0.15 mg/mL for TYRA-300 (free base). This range corresponds to 50% to 150% of the nominal sample solution concentration (0.1 mg/mL).
  + For Impurities, accuracy of this method was demonstrated for the concentration range from 0.25 µg/mL to 7.5 µg/mL for TYRA-300 (free base). This range corresponds to 0.05% to 1.5% of the nominal sample solution concentration (0.5 mg/mL).
* **Standard Solution Stability**: The standard solution was found to be stable for at least 5 days stored at refrigerated conditions (2-8°C).
* **Sample Solution Stability**:
  + For Assay, the sample solution was found to be stable for at least 3 days stored at refrigerated conditions (2-8°C).
  + For Impurities, the sample solution was found to be stable for at least 3 days stored at refrigerated conditions (2-8°C).
* **Mobile Phase Stability**: The mobile phase is stable for at least 3 days stored at ambient conditions.

# Figures

**Figure 1: Representative Chromatogram of the Diluent**

A graph of a person with a number of miles

Description automatically generated with medium confidence

**Figure 2: Representative Chromatogram of the Placebo**

A graph with numbers and a line

Description automatically generated

**Figure 3: Representative Chromatogram of the Sensitivity Solution**

A graph with numbers and a line

Description automatically generated

**Figure 4: Expanded Chromatogram of the Sensitivity Solution**

A graph of a person's reaction

Description automatically generated with medium confidence

**Figure 5: Representative Chromatogram of the Working Standard Solution**

A graph with numbers and symbols

Description automatically generated

**Figure 6: Expanded Chromatogram of the Working Standard Solution**

A graph of a patient

Description automatically generated

**Figure 7: Representative Chromatogram of the Assay Sample Solution**

A graph of numbers and lines

Description automatically generated

**Figure 8: Representative Chromatogram of the Related Substances Sample Solution**

A graph showing numbers and a number of times

Description automatically generated with medium confidence

**Figure 9: Expanded Chromatogram of the Related Substances Sample Solution**

A graph of a number of numbers and a line

Description automatically generated with medium confidence

**Figure 10: Area vs Concentration for Assay Level**

**Figure 11: Area vs Concentration for Related Substances Level**

**Figure 12: Area vs Concentration for Impurity Level**

# Changes/Deviations

## Changes to and Deviations from Protocol

|  |  |
| --- | --- |
| **Protocol Section** | **Changes/Deviation** |
| **Section 1, Table 1-1** | Corrected listed Croscarmellose Sodium NF (Ac-Di-Sol) mg/unit for 10 mg. |
| **Section 2.6 & 2.9** | Added notes to protect solutions from light These statements were added based on findings observed during standard solution stability testing during the execution of Dissolution Method Validation protocol PRO-02815**.** |
| **Section 10.1** | Changed quantitation limit acceptance criteria from “specified impurities” to “TYRA-300” in Table 10-1. |
| **Section 12.1** | Changed mobile phase stability criteria from “mean of retention times of the standards in the system suitability” to “retention time of the bracketing standard” in Table 12-5. |