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| **Customer Approvals** |  |

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# Introduction

This protocol pertains to the early phase method validation of the *Dissolution by HPLC* analytical procedure for the TYRA-300 sprinkle capsules. The composition of the TYRA-300 sprinkle capsules is given below.

Table 1-1: TYRA-300 Sprinkle Capsule Formulation

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Ingredients** | **mg/unit** | | | **%w/w** | | |
| **1 mg** | **5 mg** | **10 mg** | **1 mg** | **5 mg** | **10 mg** |
| 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 | 10.00 | 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** | | |

Appropriate validation studies will be performed by the Adare/Frontida BioPharm ARD department in order to demonstrate that the method is suitable for the intended use. The following analytical parameters and procedures will be performed:

• System Suitability  
• Specificity (Interference)  
• Linearity and Range  
• Accuracy  
• Precision   
• Filter Study  
• Solution Stability (Standard, Sample, Mobile Phase)

If during the validation, any changes or deviations are deemed necessary, appropriate additional validation may be performed. The analytical report and method will reflect any changes.

# Analytical Procedure

## Chromatographic Parameters

**Table 2-1: HPLC Parameters**

|  |  |
| --- | --- |
| **Column** | Zorbax Eclipse XDB C-18, 150x4.6 mm, 3.5 µm,  P/N 963967-902 |
| **Mobile Phase** | Water: Acetonitrile (60:40) with 0.1% TFA |
| **Needle Wash** | 50:50 Acetonitrile: Purified Water |
| **Needle Wash Setting** | Normal |
| **Detection** | 325 nm |
| **Flow Rate** | 1.0 mL/min |
| **Injection Volume** | 20 μL |
| **Column Temperature** | 40°C ± 3°C |
| **Run Time** | 6 minutes |

## Dissolution Conditions

**Table 2-2: Dissolution Conditions**

|  |  |
| --- | --- |
| **Medium** | 0.05% CTAB in 0.01 N HCl |
| **Volume** | 900 mL |
| **Apparatus** | USP Type II (Paddles) |
| **Sinkers** | Japanese |
| **Speed** | 75 RPM (raise to 250 rpm after 60 min pull) |
| **Time** | For Profile: 15, 30, 45, 60 and 75 min |
| **Temperature** | 37°C ± 0.5°C |

**Table 2-3: Autosampler Parameters**

|  |  |
| --- | --- |
| **Flush Volume** | 5.0 mL |
| **Offset Volume** | 4.0 mL |
| **Sampling Rate** | 10.0 mL/min |
| **Collection Rate** | 10.0 mL/min |
| **Other Rates** | 10.0 mL/min |
| **Flush Times** | 2 |

## Reagents and Materials

* Purified or D. I. Water
* Acetonitrile, HPLC grade or equivalent
* Methanol, HPLC grade or equivalent
* Trifluoroacetic Acid, HPLC grade or equivalent
* Concentrated HCl, Reagent Grade or better
* Cetyltrimethylammonium Bromide (CTAB), Reagent Grade or equivalent
* TYRA-300-B01 Reference Standard
* Porous UHMW polyethylene filter, 10 µm, QLA P/N FIL010-01
* 0.45 µm wwPTFE filter, Pall P/N 4932

## Mobile Phase Preparation (Water: ACN: TFA, 60:40:0.1)

For 1 L, combine 600 mL water, 400 mL acetonitrile, and 1 mL TFA. Mix well and degas.

## 0.01 N Hydrochloric Acid Solution preparation

Add 0.83 mL of concentrated Hydrochloric Acid per 1 L of water. Mix well and degas.

## Diluent/Dissolution Medium Preparation (0.05% CTAB in 0.01N HCl)

Add 0.5 g of CTAB per 1L of 0.01N HCl. Stir, heat, and/or sonicate until CTAB is in solution.

## Standard Solution Preparation

Prepare a Check Standard Solution using a similar procedure.

### Stock Standard Solution Preparation

Accurately weigh and quantitatively transfer about 65 mg of TYRA-300-B01 standard into a 100-mL volumetric flask. Add methanol: water (90:10) to about 2/3 of flask volume and briefly sonicate (about 5 minutes) to dissolve the standard. Dilute to volume with methanol: water (90:10), 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 1.0 mL from the stock standard into a 100-mL volumetric flask. Dilute to volume with dissolution medium, mix well and label as the working standard solution.

The concentration of TYRA-300 free base is about 0.005 mg/mL.

## Sample Solution Preparation

* Transfer 900 mL of the medium to six dissolution vessels, assemble the apparatus and equilibrate the dissolution medium to 37.0 ± 0.5 °C.
* Weigh 6 capsules individually and record the weights, corresponding to each respective vessel for information only. Secure each capsule into a sinker.
* When the media equilibrates to 37.0 ± 0.5 °C, drop sinker (with capsule) into vessel and start the method.
* At the specified time point(s), manually withdraw an aliquot (5 mL) with a syringe from midway between the surface of the dissolution medium and the top of the rotating paddle and not less than 1-cm away from the wall of the dissolution vessel.
* For manual pull: Filter samples using a 0.45-µm wwPTFE filters, discarding the first 3 mL. Retain the remaining filtrate for analysis.
* For autosampler: Autosampler probes should be equipped with 10-µm full flow filters. For Distek 4300 Automatic Sampler Flush Volume and Offset Volume settings should be properly adjusted to ensure transfer lines and filters are properly flushed before samples are delivered into HPLC vials.

## Injection Procedure

The following injection procedure is recommended. However, the injection sequence can be adjusted accordingly to the actual test situation.

**Table 2-4: The typical injection sequence is shown below**

|  |  |
| --- | --- |
| **Sample Type** | **No. of Injections** |
| Diluent (dissolution medium) | 2 |
| Working standard solution | 6 |
| Check standard solution | 1 |
| Bracketing standard (Working standard solution) | 1 |
| Sample Solution | 1 (bracket up to 12 sample injections with bracketing standard) \* |
| Bracketing standard (Working standard solution) | 1 |

**\***Make one injection of working standard solution after every 12 injections of sample solution and at the end of the run

## System Suitability

* The dissolution media injections should have no peaks that elute at RRT of the TYRA-300 peak or which significantly interfere with the TYRA-300 peak (NMT 2% relative to the first injection of working standard).
* The relative standard deviation (%RSD) for the last six (6) consecutive injections of the working standard solution is NMT 2% for the TYRA-300 peak.
* The percent recovery of the check standard solution is within 97% - 103%.
* The retention time for the TYRA-300 peak of the bracketing standards is within 20% of the average for the last six (6) consecutive injections of the working standard solution.

## Calculation

|  |  |  |
| --- | --- | --- |
| Where: | | |
| Cstd | : | Concentration of standard (mg/mL in free base) |
| Wstd | : | Weight of TYRA-300-B01∙2HCl in standard solution (mg) |
| VF | : | Volumetric flask of standard solution (mL) |
| Purity | : | Purity of reference standard as %/100 |
| CF | : | Free base conversion factor, 0.7796 |
| Achk | : | Area of TYRA-300 peak for *check standard solution* |
| Astd | : | Average area of TYRA-300 peak for *standard solution* (includes PCS) |
| Cstd | : | Standard concentration for *standard solution* (mg/mL in free base) |
| Cchk | : | Concentration in the *check standard solution* (mg/mL in free base) |
| **Note** – CF may already be included in calculation for Purity. If so, then CF should be omitted from above calculation. | | |

Calculation the % dissolved as follows:

For Single Time Point and 1st Time Point of Profile:

% Dissolved of TYRA-300 for dissolution release profile (no dissolution medium replacement)

• Calculate sample concentration at each time point

• Calculate the dissolution release profile

|  |  |  |
| --- | --- | --- |
| Where: | | |
| Asmp | : | Peak area in the sample solution |
| Astd | : | Average peak area of TYRA-300 in all standard and PCS injections |
| Cstd | : | Concentration of standard (mg/mL in free base) |
| Ci | : | Concentration of sample concentration at i time point (mg/mL in free base) |
| V | : | Initial volume of dissolution medium (mL) |
| LC | : | Label claim capsule strength (mg) |
| Vr | : | Volume of dissolution medium removed for each measurement, in mL |
| n | : | Number of the measurements (1, 2, 3......n) |

# System Suitability

The system suitability of the test method will be performed and demonstrated as part of establishing system suitability for the subsequent validation studies. The successful establishment of the system suitability requirements (as described in **Section 2.10**) will be considered fulfillment of this study.

# Specificity study

## Dissolution Medium Interference Solution Preparation

Use the dissolution medium.

## Placebo Interference Solution Preparation

Accurately weigh and quantitatively transfer about 210 mg of TYRA-300 placebo into a vessel containing 900 mL of dissolution medium. Add one empty capsule into the vessel. Perform the dissolution according to **Section 2.2** and prepare the sample solution in **Section 2.8**.

## Validity Criteria

* Meet the system suitability requirements as per **Section 2.10**.

## Acceptance Criteria

* The dissolution medium interference and placebo interference injections should have no peaks that elute at RRT of the TYRA-300 peak or which significantly interfere (NMT 2% relative to first injection of working standard).

# Linearity

The linearity will be studied from about 10% to about 300% of the TYRA-300 concentration of the working standard solution, which corresponds to about 0.5 µg/mL to about 15.0 µg/mL.

## Working Linearity Solution Preparation

Prepare the working linearity solutions as directed in **Table 5-1**. Dilute each to volume with the dissolution medium and mix well.

**Table 5-1: Preparation of Working Linearity Solutions**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample** | **Level (%)** | **Volume of Stock Std**  **(mL)** | **Flask (mL)** | **Approx. Conc. TYRA-300**  **(µg/mL)** |
| L1 | 10 | 10.0 mL of L3 | 100 | 0.5 |
| L2 | 50 | 1.0 | 200 | 2.5 |
| **L3** | **100** | **1.0** | **100** | **5.0** |
| L4 | 150 | 1.5 | 100 | 7.5 |
| L5 | 300 | 3.0 | 100 | 15.0 |

## Procedure

* Establish system suitability per **Section 2.10**.
* Inject each linearity level once.
* Construct a plot of the peak area responses vs. concentration.
* Perform a linear regression analysis and determine the correlation coefficient (r), slope, and y-intercept.

## Validity Criteria

* Meet the system suitability requirements as per **Section 2.10**.

## 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%.

# accuracy by spiked recovery

Accuracy by spiked recovery will be performed to demonstrate that the method can achieve acceptable recoveries.

The accuracy will be performed by spiking TYRA-300 drug substance into an amount of composite placebo corresponding to the 10 mg dosage strength. The accuracy will be evaluated from about 4.5% to 135% of the nominal concentration of TYRA-300 in the sample solution of the 10 mg capsule, which corresponds to about 0.5 µg/mL to 15 µg/mL. (Note—This range corresponds to a nominal sample concentration of 45% to 1350% for the 1 mg capsule and 9% to 270% for 5 mg capsule.)

## Spiking Solution Preparation

Accurately weigh and quantitatively transfer about 32.5 mg of TYRA-300-B01 material into a 100-mL volumetric flask. Add methanol: water (90:10) to about 2/3 of flask volume and briefly sonicate (about 5 minutes) to dissolve the standard. Dilute to volume with methanol: water (90:10), mix well and label as the spiking solution.

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

## Recovery Sample Solution Preparation

Accurately weigh and quantitatively transfer the amounts of placebo and volumes of the spiking solution into flasks as described in **Table 6-1**. Add about 2/3 volume of dissolution medium along with one empty capsule. Place the flasks into a water bath at 37°C and shake for 30 minutes. Equilibrate the solutions to room temperature, dilute to volume with dissolution medium and mix well. Filter an aliquot of the solution through a 0.45-µm wwPTFE filter, discarding the first 2 mL.

Prepare each recovery level in triplicate.

**Table 6-1: Preparation of Recovery Sample Solutions**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Level** | **Nominal Concentration (%) Relative to the 10 mg Capsule** | **Volume of Spike Solution (mL)** | **Placebo (mg)** | **Flask (mL)** | **Approx. Conc. TYRA-300 (µg/mL)** |
| R1 | 4.5 | 0.5 | 52 | 250 | 0.5 |
| R2 | 9 | 1.0 | 52 | 250 | 1.0 |
| R3 | 45 | 5.0 | 52 | 250 | 5.0 |
| R4 | 135 | 15.0 | 52 | 250 | 15.0 |

## Procedure

* Meet system suitability requirements as per **Section 2.10**.
* Inject each solution once.
* Calculate the percent recovery for TYRA-300 as follows:

## Validity Criteria

* Meet the system suitability requirements in **Section 2.10**.

## Acceptance Criteria

* The mean result at each level is within 90-110%.
* The percent RSD of the triplicate preparations at each level is NMT 5%.

# Precision Study

For Precision, a six-capsule dissolution profile on TYRA-300 Capsules 1 mg and 10 mg as per **Section 2**. The API-to-placebo ratio is dose proportional; the 1 mg and 10 mg are considered the worst-case scenarios.

## Procedure

* Perform a six-capsule dissolution profile on TYRA-300 Capsules 1 mg and 10 mg as per Section 2.
* Establish system suitability per **Section 2.10**.
* Inject each solution once.
* Determine the percent dissolved at 30 min.

## Validity Criteria

* Meet the system suitability requirements in **Section 2.10**.

## Acceptance Criteria

The acceptance criteria below will be evaluated only at the time points when the mean of % Released is NLT 85%.

* The %RSD (n=6) is NMT 5%.

# Filter Study

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

## Filter Study on Dissolution Medium

Separately filter portions of the dissolution medium previously heated to about 37°C through a 0.45-µm wwPTFE filter and 10-µm filter and collect the first 2 mL of filtrate for each.

## Filter Study Sample Preparation

Filtered Sample:

For Manual Collection: Collect filtrate aliquots as per **Table 8-1** from a one‑capsule dissolution of the 1 mg and 10 mg capsules as per **Section 2**. (Note—The 75 min timepoint solution prepared for Precision (1 mg and 10 mg) (prepared as per **Section 7.1**) may be used.)

For Autosampler Collection: Collect aliquot from a one-capsule dissolution of the 1 mg and 10 mg capsules as per **Section 2**. (Note—The 75 min timepoint solution prepared for Precision (1 mg and 10 mg) (prepared as per **Section 7.1**) may be used.)

Centrifuge Sample:

Additionally, centrifuge a portion of the same sample solution obtained for the filtered sample at 12000 rpm for 10 minutes.

Table 8-1: Filter Study by Manual Collection

|  |  |
| --- | --- |
| **Solution** | **Fraction of Filtrate Solution (mL)** |
| 0.45 µm wwPTFE filter, Pall P/N 4932 | 0-1 discarded |
| 1-3 |
| 3-5 |

## Procedure

* Establish system suitability per **Section 2.10**.
* Inject each solution once.
* Determine whether any peaks are attributed to the filter.
* Determine the relative recovery of TYRA-300 obtained from each filtrate aliquot of the sample solution and centrifuged sample solution.

Calculate the % relative recovery from the filter as follows:

Where,

A filtered : Peak area of TYRA-300 in filtered sample solution

A centrifuged : Peak area of TYRA-300 in supernatant of centrifuged sample solution

## Validity Criteria

* Meet system suitability requirements as per **Section 2.10**

## Acceptance Criteria

* The percent relative recovery in the filter sample solution against the centrifuged solution is 97%-103%.
* Determine volume of filtrate to be discarded before collecting samples that meet acceptance criteria for each level.

# Solution Stability

The standard and sample solutions will be evaluated at normal laboratory environmental condition (NLEC) to determine the appropriate time frame for use. Their stabilities will be determined by periodically evaluating the solutions for change in TYRA-300 against freshly prepared solutions.

Additionally, stability of the mobile phase will be concurrently evaluated.

## Sample Solution Preparation

Perform a one-capsule dissolution for each of the strengths (1 mg and 10 mg).

(Note—Sample solution stability may be determined from a sample solution prepared for the precision study.)

Record the time at which the preparation of the solution was completed.

## Procedure

* At each evaluation, establish system suitability per **Section 2.10**.
* Prepare a working standard solution as per **Section 2.7**. Record the time at which the preparation of the solution was completed.
* Store a portion of the standard and sample solutions at normal laboratory environmental conditions.
* Periodically evaluate the standard and sample solutions against a freshly prepared standard solution.
* Inject each solution once.
* Determine the percent relative recoveries of the working standard and sample solutions at each time interval.
* Determine the percent RSD of the retention times of the TYRA-300 peak obtained from the injections of the working standard solution to establish system suitability.

## Validity Criteria

* Meet system suitability requirements as per **Section 2.10**.

## Acceptance Criteria

* The standard solution is considered stable if the percent relative recovery at each time interval is within 97.0%-103.0%.
* The sample solution is considered stable if the percent relative recovery at each time interval is within 97%-103%.
* The mobile phase is considered stable if the mean of retention times of the standards in the system suitability is within 10% of that obtained from the initial run (t0).