





|  |  |
| --- | --- |
| **CUSTOMER APPROVAL** |  |

Table of contents

[1 Introduction 4](#_Toc150168414)

[2 Analytical Procedure 5](#_Toc150168415)

[2.1 Chromatographic Parameters 5](#_Toc150168416)

[2.2 Reagents and Materials 5](#_Toc150168417)

[2.3 Mobile phase A (0.1% TFA in Water) 6](#_Toc150168418)

[2.4 Mobile phase B (0.1% TFA in 70% Acetonitrile) 6](#_Toc150168419)

[2.5 Diluent Preparation 6](#_Toc150168420)

[2.6 Standard Solution Preparation 6](#_Toc150168421)

[2.6.1 Stock Standard Solution Preparation 6](#_Toc150168422)

[2.6.2 Working Standard Solution Preparation 6](#_Toc150168423)

[2.7 Blend Assay Sample Solution Preparation 6](#_Toc150168424)

[2.8 Blend Uniformity Sample Solution Preparation 7](#_Toc150168425)

[2.9 Content Uniformity Solution Preparation 7](#_Toc150168426)

[2.10 Procedure 8](#_Toc150168427)

[2.11 System Suitability Requirements 8](#_Toc150168428)

[2.12 Calculations 9](#_Toc150168429)

[3 SPECIFICITY STUDY (INTERFERENCE AND IDENTIFICATION) 10](#_Toc150168430)

[3.1 Diluent Interference Solution Preparation 10](#_Toc150168431)

[3.2 Placebo Solution Preparation 10](#_Toc150168432)

[3.3 Procedure 10](#_Toc150168433)

[3.4 Validity Criteria 10](#_Toc150168434)

[3.5 Acceptance Criteria 10](#_Toc150168435)

[4 Linearity 10](#_Toc150168436)

[4.1 Stock TYRA-300 Linearity Solution Preparation 10](#_Toc150168437)

[4.2 Working TYRA-300 Linearity Solutions Preparation 11](#_Toc150168438)

[4.3 Procedure 11](#_Toc150168439)

[4.4 Validity Criteria 11](#_Toc150168440)

[4.5 Acceptance Criteria 11](#_Toc150168441)

[5 Accuracy by Spiked recovery 11](#_Toc150168442)

[5.1 Working Spiking Solution Preparation 12](#_Toc150168443)

[5.2 Recovery Sample Solution Preparation 12](#_Toc150168444)

[5.3 Control Sample Preparation 12](#_Toc150168445)

[5.4 Procedure 13](#_Toc150168446)

[5.5 Validity Criteria 13](#_Toc150168447)

[5.6 Acceptance Criteria 13](#_Toc150168448)

[6 Precision 13](#_Toc150168449)

[6.1 Precision 13](#_Toc150168450)

[6.2 Procedure 13](#_Toc150168451)

[6.3 Validity Criteria 13](#_Toc150168452)

[6.4 Acceptance Criteria 13](#_Toc150168453)

[7 Filter Study 13](#_Toc150168454)

[7.1 Filter Study on Diluent 14](#_Toc150168455)

[7.2 Filter Study on Sample Solution 14](#_Toc150168456)

[7.3 Procedure 14](#_Toc150168457)

[7.4 Validity Criteria 14](#_Toc150168458)

[7.5 Acceptance Criteria 14](#_Toc150168459)

[8 Stability Study 15](#_Toc150168460)

[8.1 Procedure 15](#_Toc150168461)

[8.2 Validity Criteria 15](#_Toc150168462)

[8.3 Acceptance Criteria 16](#_Toc150168463)

# Introduction

This protocol pertains to the early phase method validation of the *Blend/Bulk Assay*, *Blend Uniformity* and *Uniformity of Dosage Units by Content Uniformity* analytical procedures for TYRA-300 sprinkle capsules (1 mg, 5 mg, and 10 mg).

Appropriate studies will be performed in order to demonstrate that the proposed method is suitable for intended use. This 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 tablets is summarized in **Table 1-1**. The three strengths are dose proportional.

Table 1-1. Ingredient Composition for TYRA-300 Tablets

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **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 | 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** | | |

The method validation will be 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 will be evaluated:

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

If during the validation, any changes or deviations are deemed necessary, additional 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** | 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 Time** | 15 seconds pre/30 seconds post | | |
| **Gradient Program** | **Time (min)** | **Mobile Phase A** | **Mobile Phase B** |
| 0 | 80 | 20 |
| 1.0 | 80 | 20 |
| 2.0 | 50 | 50 |
| 4.0 | 50 | 50 |
| 4.1 | 5 | 95 |
| 5.5 | 5 | 95 |
| 5.6 | 80 | 20 |
| 7.0 | 80 | 20 |
| **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** | 3 μL | | |
| **Run Time** | 7 minutes | | |

## Reagents and Materials

Purified Water, Millipore, In-house

Acetonitrile, HPLC Grade

Methanol, HPLC Grade

Trifluoroacetic Acid (TFA), HPLC Grade

Tyra-300-B01, Reference Standard (RS)

Pall Acrodisc 0.2-µm PTFE 25mm 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 70% 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

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. 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 solution into a 50-mL volumetric flask. Dilute to volume with diluent and mix well.

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

## Blend Assay Sample Solution Preparation

Prepare the sample by weighing an equivalent of 10 capsules into the appropriate flask in order to achieve a free base concentration of TYRA-300 between 0.05 mg/mL and 0.15 mg/mL. Add diluent to about 2/3 of the flask volume to dissolve TYRA-300. Sonicate for 10 minutes. After equilibration to room temperature, dilute to volume with diluent and mix well. Filter a portion of sample through a 0.2-µm PTFE 25 mm syringe filter, after discarding first 2 mL.

## Blend Uniformity Sample Solution Preparation

Wipe the outsides of the sample bottles. Place the bottles upright and tap gently to dislodge any powder adhering to the liner of the caps. Gently remove the cap and weigh the bottle and sample (do not weigh the cap). Quantitatively transfer the entire bottle contents into a suitable volumetric flask as outlined in Table 2-1, rinsing the bottle a few times with the diluent to effect complete transfer.

**Table 2-1: Sample Preparation for Blend Uniformity**

|  |  |  |  |
| --- | --- | --- | --- |
| **Dosage Strength** | **Run Weight** | **Sample Dosage (x = run weight)** | **Volumetric Flask**  **(mL)** |
| 1 mg | 22 mg | 1x | 10 |
| 2x | 20 |
| 3x | 20 |
| 5 mg | 110 mg | 1x | 50 |
| 2x | 100 |
| 3x | 100 |
| 10 mg | 220 mg | 1x | 100 |
| 2x | 200 |
| 3x | 250 |

Add diluent to about 2/3 of the flask volume to dissolve TYRA-300. Sonicate for 10 minutes. After equilibration to room temperature, dilute to volume with diluent and mix well. Filter a portion of sample through a 0.2-µm PTFE 25mm syringe filter, after discarding first 2 mL.

Allow the sample bottles to dry and reweigh. Use this weight as the tare weight for the calculation. Determine the sample weight by subtracting the bottle tare weight from the weight of the bottle and samples as obtained above.

## Content Uniformity Solution Preparation

Weigh ten capsules individually and record the weight (for information only). Open ten capsules and transfer contents to individual volumetric flasks according to the table below and record the empty shell weight (for information only).

Table 2-2. Content Uniformity Sample Preparation

|  |  |  |
| --- | --- | --- |
| **Dosage Strength** | **Volumetric Flask  (mL)** | **TYRA-300 Concentration (mg/mL)** |
| 1 mg | 10 | 0.1 mg/mL |
| 5 mg | 50 | 0.1 mg/mL |
| 10 mg | 100 | 0.1 mg/mL |

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 15 minutes and shake 15 minutes. After equilibration to room temperature, dilute flask to volume with methanol 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 0.2-µm PTFE 25 mm syringe filter, after discarding first 2 mL.

## Procedure

Separately inject equal volumes (3 µL) of the diluent, working standard and check standard, and sample solution. Record the chromatograms and measure the peak area responses of the TYRA-300 peak.

**Example of Injection Sequence**

|  |  |
| --- | --- |
| Solutions | Number of Injections |
| Diluent | ≥1 |
| Working Standard Solution | 5 |
| Check Standard Solution | 1 |
| Procedural Control Standard (PCS) | 1 |
| Sample Solution | ≤12 |
| Procedural Control Standard (PCS) | 1 |

## System Suitability Requirements

* The diluent blank injection should have no peaks which significantly interfere (NMT 0.5% of first injection of working standard) with the quantitation of TYRA-300.
* The RSD of the TYRA-300 peak area responses for the five (5) consecutive injections of working standard solution is NMT 2.0%.
* The percent recovery of TYRA-300 in the check standard solution is within 98.0% - 102.0%.
* The percent deviation between average of 5 consecutive working standard and each bracketing standard injection must be NMT 2.0%.

## Calculations

Calculate the % Label Claim as follows:

For Blend Uniformity:

For Content Uniformity:

Where,

|  |  |  |
| --- | --- | --- |
| Ru | : | The area response of TYRA-300 in the sample solution |
| Rs | : | The area response of TYRA-300 in the standard solution |
| Ws | : | Weight of the TYRA-300 standard, in mg |
| P | : | Purity of standard expressed as % Purity/100% |
| C | : | Free base conversion factor, 0.7796 |
| VF | : | Volumetric flask used for sample solution, in mL |
| WSpl | : | Weight of the TYRA-300 sample, in mg |
| RW | : | Run Weight, in mg |

For Content Uniformity, calculate the Acceptance Value as follows:

Acceptance Value = |M - X̄| + ks

Where,

|  |  |  |
| --- | --- | --- |
| X̄ | : | Mean of individual contents |
| k | : | 2.4 (for sample size of 10 units) or 2.0 (for sample size of 30 units) |
| s | : | Standard deviation for individual contents |
| M | : | Case, |
|  |  | If 98.5% ≤ X̄ ≤ 101.5%, then M = X̄ |
|  |  | If X̄ ≤ 98.5%, then M = 98.5% |
|  |  | If X̄ ≥ 101.5%, then M = 101.5% |

# SPECIFICITY STUDY (INTERFERENCE AND IDENTIFICATION)

Specificity studies will be performed in order to determine peak identities as well as evaluate 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

Use diluent as the diluent interference solution.

## Placebo Solution Preparation

Accurately weigh 210 mg placebo powder and quantitatively transfer into a 100‑mL volumetric flask. Add 2 mL of water and sonicate 5 minutes. Fill with methanol to about 2/3 of flask volume and sonicate 30 minutes and shake 30 minutes. After equilibration to room temperature, dilute flask to volume with methanol 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 NLT first 2 mL.

## Procedure

* Establish system suitability per **Section 2.11**.
* Inject each solution once.

## Validity Criteria

* Meet the system suitability requirements in **Section 2.11**.

## Acceptance Criteria

* The diluent and placebo solutions do not show any significantly interfering peaks near the retention time of TYRA-300 (NMT 0.5%).

# Linearity

Linearity of TYRA-300 will be evaluated from a concentration 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 standard and sample solutions.

## Stock TYRA-300 Linearity Solution Preparation

Accurately weigh and quantitatively transfer about 65 mg of TYRA-300-B01 standard 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. After equilibration to room temperature, dilute to volume with diluent and mix well.

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

## Working TYRA-300 Linearity Solutions Preparation

Prepare the working linearity solutions for the L1 to L5 levels as directed in **Table 4‑1**. Dilute each to volume with the diluent and mix well.

Table 4-1. Preparation of working TYRA-300 linearity solutions

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Assay Linearity Level | Nominal Conc.  (%) | Volume of Stock TYRA-300 Linearity 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 |

## Procedure

* Establish system suitability per **Section 2.11**.
* Inject each linearity level solution in triplicate. (Note—The linearity solutions may be injected consecutively, bracketed by procedural control standards.)
* 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 system suitability as per **Section 2.11**.

## 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 the 100% nominal level is NMT 2%.

# Accuracy by Spiked recovery

An accuracy study will be performed in order to demonstrate that the method can achieve acceptable recoveries.

The accuracy study will be performed by adding known amounts of TYRA-300 onto a corresponding amount of placebo powder. The accuracy will be evaluated from a TYRA-300 concentration of 0.05 mg/mL to 0.15 mg/mL, which corresponds to 50% to 150% of the nominal sample solution concentration.

## Working Spiking Solution Preparation

Use *Stock Standard* solution (**Section 2.6.1**).

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

## Recovery Sample Solution Preparation

Accurately weigh and quantitatively transfer portions of TYRA-300 placebo powder into volumetric flasks as shown in **Table 5-2**. Add volumes of the *Working Spiking* solution as shown in **Table 5-2**. Fill with *diluent* to about 2/3 of flask volume and sonicate 15 minutes and shake 15 minutes. After equilibration to room temperature, dilute flask to volume with diluent and mix well. Alternatively, filter a portion of sample through a 0.2µm PTFE 25mm syringe filter, after discarding NLT first 2-3 mL.

Prepare each level in triplicate.

**Table 5-2. Preparation of the recovery sample solutions**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Recovery Level | Nominal Conc.  (%) | Volume of Working Spike Solution (mL) | Weight of Placebo Powder  (mg) | Volumetric Flask  (mL) | Approx. TYRA-300 Conc.  (mg/mL) |
| R1 | 50 | 5.0 | 105 | 50 | 0.05 |
| R2 | 100 | 10.0 | 105 | 50 | 0.10 |
| R3 | 150 | 15.0 | 105 | 50 | 0.15 |

## Control Sample Preparation

Accurately weigh 105 mg placebo powder and quantitatively transfer into a 50‑mL volumetric flask. Add 5 mL of water and sonicate 5 minutes. Fill with methanol to about 2/3 of flask volume and sonicate 30 minutes and shake 30 minutes. After equilibration to room temperature, dilute flask to volume with methanol 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 first 2 mL.

## Procedure

* Establish system suitability per **Section 2.11**.
* Inject each solution once.
* Calculate the percent recovery for TYRA-300 as follows:

## Validity Criteria

* Meet the system suitability requirements in **Section 2.11**.
* The percent RSD of the triplicate preparations is NMT 3%.

## Acceptance Criteria

* The mean percent recovery is within 95%-105%.

# Precision

## Precision

Prepare ten (10) sample solutions as directed in **Section 2.9** using TYRA-300 sprinkle capsules, 5 mg strength.

## Procedure

* Establish system suitability per **Section 2.11**.
* Inject each solution once.
* Determine the percent label claim and calculate the Acceptance Value (AV).

## Validity Criteria

* Meet the system suitability requirements in **Section 2.11**.

## Acceptance Criteria

* The acceptance value (AV) is NMT 15.0.

# Filter Study

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

## Filter Study on Diluent

Separately filter portions of the diluent through a Pall Acrodisc 0.2-µm PTFE filter and collect the first 2 mL of filtrate for each.

## Filter Study on Sample Solution

Filtered Sample:

Filter a portion of the content uniformity sample solution prepared as per **Section 2.9** (Note**—**A sample solution prepared for **Section 6.1** may be used) through a Pall Acrodisc 0.2-µm PTFE membrane or Titan 0.45-µm PTFE membrane filter, and collect each aliquot portion as shown in **Table 7-1**.

Centrifuged Sample:

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

Note—Centrifuge as necessary to obtain a clear supernatant.

Table 7-1. 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 |

## Procedure

* Establish system suitability per **section 2.11**.
* 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.

## Validity Criteria

* Meet the system suitability requirements in **Section 2.11**.

## Acceptance Criteria

* No interference more than 2% (relative to first injection of working standard) is observed in the filtered diluent solution.
* The relative recovery of TYRA-300 from the filtrate aliquots of the sample solution (calculated against the centrifuged sample solution) is within 98.0%-102.0%.

# Stability Study

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

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

The stability of the sample solution will be determined by periodically quantitating the percent of TYRA-300 in the solution against freshly prepared standard solutions.

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

## Procedure

Establish system suitability per Section 2.11.

Prepare a standard solution as per Sections 2.6. Record the time at which the preparation of the solution was completed.

Prepare the sample solution as per Section 2.9. Record the time at which the preparation of the solution was completed. (Note—Sample solution stability may be determined from a sample solution prepared for the precision study)

Store a portion of the standard and sample solutions in the refrigerator and at normal laboratory environmental conditions in the volumetric flasks.

Periodically evaluate the standard and sample solutions against a freshly prepared standard solution.

Inject each solution once.

Determine the percent relative recoveries of the standard and sample solutions at each time interval.

Evaluate retention times of the TYRA-300 peak obtained from the injections of the working standard solution.

## Validity Criteria

Meet the system suitability requirements in **Section 2.11.**

## 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).
* 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).

For Mobile Phases:

* 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).