METHOD VALIDATION PROTOCOL

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

ASSAY, RELATED SUBSTANCES, AND IDENTIFICATION METHOD BY HPLC

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1 INTRODUCTION

This protocol 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 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 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	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 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 their 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	IMP_RRT 1.09	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	IMP_RRT 1.19	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 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
- Quantitation Limit
- Filtration
- 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.

2 ANALYTICAL PROCEDURE

2.1 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		

2.2 Reagents and Materials

- Purified Water, Millipore
- Acetonitrile, HPLC Grade
- Methanol, HPLC Grade
- Trifluoroacetic Acid (TFA), HPLC Grade
- TYRA-300 standard of known purity
- 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

2.3 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.

2.4 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.

2.5 Diluent Preparation

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

2.6 Standard Solution Preparation

Prepare a check standard solution in a similar manner.

2.6.1 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.

2.6.2 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.

2.7 Sensitivity Standard Solution Preparation

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

2.7.1 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.

2.7.2 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.

2.8 Placebo Solution Preparation

Weigh 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 NLT the first 2 mL.

2.9 Assay and Related Substances Sample Solution Preparation

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 NLT the first 2 mL.

2.10 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

2.11 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 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.

2.12 Chromatogram Integration

Integrate the chromatogram of each standard and sample, exclude peaks present in system blank and/or placebo.

2.13 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

2.14 Calculations

Calculate the % Label Claim as follows:

For Assay:

$$\%\ \text{Label Claim} = \frac{R_u}{R_s} \times \frac{W \times P \times CF}{100\ \text{mL}} \times \frac{10\ \text{mL}}{50\ \text{mL}} \times \frac{DF}{LC \times N} \times 100\%$$

Where,

R_u : The area response of TYRA-300 peak in the sample solution

R_s : 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 specifiedprocess impurities and include only impurities ≥ 0.05%.

For Related Substances:

$$\% \text{ Impurity} = \frac{A_{Imp}}{A_{total}} x100\%$$

$$\% \text{ Total Impurity} = \sum (\% \text{ Individual Impurities})$$

Where,

A_{imp} : Area of Individual Impurity

A_{total} : Total area of all integrated peaks

3 SYSTEM SUITABILITY/SYSTEM PRECISION

The System Suitability/System Precision will be performed and demonstrated as part of establishing system suitability for each of the validation studies. The successful establishment of the suitability requirements (as described in **Section 2.11**) will be considered fulfillment of this study.

4 SPECIFICITY STUDY (INTERFERENCE AND IDENTIFICATION)

Specificity studies will be 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.

4.1 Diluent Interference Solution Preparation

Use diluent as the diluent interference solution.

4.2 Placebo Solution Preparation

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

4.3 TYRA-300 Sample Solution Preparation

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

4.4 Identification by Retention Time (RT)

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

4.5 Procedure

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

4.6 Validity Criteria

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

4.7 Acceptance Criteria

- The diluent and placebo solutions do not show any significantly interfering peaks near the retention time of TYRA-300 (NMT 0.2%).

5 LINEARITY

For Assay, linearity will be assessed from a TYRA-300 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 Assay sample and standard solutions.

For Related Substances, linearity will be evaluated from a TYRA-300 concentration 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 will be evaluated from a TYRA-300 concentration 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.

5.1 Stock Assay Linearity Solution Preparation

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

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

5.2 Working Assay Linearity Solution Preparation

Prepare the working linearity solutions for the L1 to L5 levels as directed in **Table 5-1**. Dilute each to volume with the diluent and mix well.

Table 5-1. 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

5.3 Related Substance Linearity Solution Preparation

Accurately weigh and quantitatively transfer about 130 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 1 mg/mL.

5.4 Intermediate Impurity Linearity Solution Preparation

Transfer 2.5 mL of the *Stock Related Substance* solution into a 100-mL volumetric flask. Dilute to volume with the diluent and mix well.

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

5.5 Working Related Substance Linearity Solution Preparation

Prepare the working linearity solutions for the L1 to L5 levels as directed according to **Table 5-2**. Dilute each to volume with the diluent and mix well.

Table 5-2. 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	50	10.0	50	0.2
L2	70	6.0	20	0.3
L3	90	10.0	25	0.4
L4	100	10.0	20	0.5
L5	125	12.5	20	0.625

5.6 Working Impurity Linearity Solution Preparation

Prepare the working linearity solutions for the L1 to L5 levels as directed according to **Table 5-3**. Dilute each to volume with the diluent and mix well.

Table 5-3. 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

5.7 Procedure

- Establish system suitability per **Section 2.11**.
- Inject each linearity level solution once. (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.

5.8 Validity Criteria

- Meet system suitability as per **Section 2.11**.

5.9 Acceptance Criteria

Assay:

- 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%.

Related Substances:

- The relative slope of TYRA-300 (impurity level) to TYRA-300 (related substances level) is within 90% to 110%.
- Meet the linearity range of a minimum of five consecutive levels. For the impurities level linearity, the correlation coefficient, r, is NLT 0.99.

6 ACCURACY BY SPIKED RECOVERY

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

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

For *Assay*, 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.

For *Related Substances*, the accuracy study will be 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.

6.1 Accuracy for Assay

6.1.1 Assay Recovery Sample Solution Preparation

Accurately weigh and quantitatively transfer portions of TYRA-300 and composite placebo powder into volumetric flasks as shown in **Table 6-1**. 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.

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

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

Prepare each level in triplicate.

Table 6-1. 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

6.2 Accuracy for Impurities

6.2.1 Stock Spiking Solution Preparation

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

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

6.2.2 Working Spiking Solution Preparation

Dilute 12.5 mL of the Stock Spiking Solution (**Section 6.2.1**) to 50-mL with diluent and mix well.

The concentration of TYRA-300 and impurities is 0.025 mg/mL.

6.2.3 Recovery Sample Solution Preparation

Accurately weigh and quantitatively transfer an amount of the composite placebo powder into separate volumetric flasks as outlined in **Table 6-2**. 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. Fill 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 NLT the first 2-3 mL.

Prepare samples for each recovery level in triplicate.

Table 6-2. 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

6.2.4 Control Sample Preparations

Accurately weigh and quantitatively transfer 210 mg of the composite placebo powder into 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 NLT the first 2-3 mL.

Prepare one (1) control sample solution.

6.3 Procedure

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

$$\text{Recovery (\%)} = \frac{\text{Actual Amount Recover–d - Amount in Control}}{\text{Theoretical Amount Spiked}} \times 100\%$$

6.4 Validity Criteria

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

6.5 Acceptance Criteria

Assay:

- The mean percent recovery of triplicate preparations is within 95%-105%.
- The percent RSD of the triplicate preparations is NMT 3%.

Impurities:

- The mean percent 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 level is NMT 15%.

7 PRECISION STUDY

7.1 Precision

7.1.1 Precision – Assay

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

7.1.2 Precision – Impurities

Prepare six (6) sample solutions spiked at the 100% level (R3) as directed in **Section 6.2.3**.

7.2 Procedure

- Establish system suitability per **Section 2.11**.
- Inject each solution once.
- Determine the percent label claim or percent impurities.

7.3 Validity Criteria

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

7.4 Acceptance Criteria

For Assay:

- The RSD of the results from the precision study (n=6) is NMT 3%.

For Related Substances:

- The RSD of the results from the precision study (n=6) is NMT 15%.

8 QUANTITATION LIMIT

The Quantitation Limit (QL) will be evaluated at a concentration corresponding to an impurity level of 0.05%. The quantitation limit is represented by the impurity R1 and L1 levels in the Accuracy (**Section 6.2.3**) and Linearity studies (**Section 5.6**).

8.1 Procedure

From the injections of the impurity R1 level solutions (**Section 6.2.3**), determine the signal-to-noise (s/n) ratio.

8.2 Acceptance Criteria

- The impurity R1 level meets the criteria of the Accuracy study and the impurity L1 level meets the criteria of the Linearity study.
- The signal-to-noise ratio of the specified impurities from the impurity R1 level solutions is ≥ 10.

9 FILTER STUDY

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

9.1 Filter Study on Diluent

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

9.2 Filter Study on Sample Solutin

Filtered Sample:

In a separate manner, filter a portion of the assay sample prepared as per **Section 2.9** (Note—A sample solution prepared for **Section 7.1.1** may be used) through a Pall Acrodisc 0.2-µm PTFE filter and collect each aliquot portion as shown in **Table 9-1.**

Table 9-1. 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, centrifuge a portion of the same sample at 12000 rpm for 10 minutes.

Note—Centrifuge as necessary to obtain a clear supernatant.

9.3 Procedure

- Establish system suitability per **Section 2.11**.
- Inject each solution in 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.

9.4 Validity Criteria

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

9.5 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%.

10 STABILITY STUDY

The stability of the standard and sample solutions will be evaluated at normal laboratory environmental and refrigerated conditions 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 and impurity levels 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.

10.1 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. (Note—The standard solution is the same as for the Blend Assay, Blend Uniformity, Content Uniformity methods. The standard solution stability findings may be taken from PRO-02817.)
- 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 the retention times of the TYRA-300 peak obtained from the injections of the standard solution.

10.2 Validity Criteria

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

10.3 Acceptance Criteria

- The standard solutions are considered stable if the relative recovery of the solution that is tested for stability is within 98.0%-102.0% of the original mean results.

For Assay:

- The sample solutions are considered stable if the relative recovery obtained at the evaluated time interval is within 98-102% of the original results (t_0).

For Related Substances:

- 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 (t_0) as defined: Impurities <0.2%: ±0.05% absolute; Impurities ≥0.2%: ±10% relative.

For Mobile Phases:

- The mean of retention times of the standards in the system suitability is within 10% of that obtained from the initial run (t0).

TYRA-300 Sprinkle Capsules_Assay_Related Substances_ID Method by HPLC

Final Audit Report 2023-11-10

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