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# Introduction

CX-4945 Sodium Salt (Formula: C19H11ClN3O2Na; molecular weight: 371.75 g/mol) is chemically known sodium 5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylate. The structural formula of CX-4945 is represented below:



This protocol pertains to the verification of the Dissolution analytical procedure for CX-4945 capsules (200 mg) by Frontida BioPharm Analytical Research and Development (ARD) department.

The method qualification of analytical procedure has been successfully performed by Alcami, the findings from which are summarized in corresponding method qualification report provided by Senhwa Biosciences, Inc. (Report#: RPT 71219.00). The target dissolution limit is shown in Table 1-1.

**Table 1-1.** Target Limit for CX-4945 Capsules (200 mg)

|  |  |
| --- | --- |
| **Test** | **Target Limit** |
| Dissolution | NLT 80% (Q) of the labeled amount of CX-4945 is dissolved in 45 minutes |

The qualification of the method included and demonstrated the following method parameters/characteristics:

* System Suitability
* Specificity (Interference)
* Linearity
* Method Repeatability
* Filter Study
* Stability of Solutions

Appropriate verification studies will be performed by the Frontida BioPharm ARD department in order to verify the suitability of the method and demonstrate the capability of the laboratory to perform the analysis.

This protocol describes the methodology for the verification of the analytical procedure and defines the criteria to assess the results. The wavelength number was modified using 360 nm in this protocol instead of 265 nm that was used in the Alcami method. The following studies will be performed, which are considered to be sufficient to verify the modification of wavelength, suitability of test method and demonstrate the capability of Frontida BioPharm ARD department to perform this analysis:

* System Suitability
* Specificity (Interference)
* Linearity and Range
* Accuracy
* Precision (Repeatability)
* Filtration Study
* Stability of Solutions

The studies will be performed in accordance with Frontida BioPharm’s Standard Operating Procedure (SOP) for Validation of Analytical Methods, SOP MPC QC/RD-017 (current version).

If during the execution of the verification studies, any changes or deviations are required, additional appropriate studies may be performed, if deemed necessary. The corresponding report and method will reflect any deviations and changes.

In the event an acceptance criterion is not met, a laboratory investigation will be performed in accordance with Frontida BioPharm’s SOPs and the outcome will be reported in the verification/investigation report.

# Analytical Procedure

## Chromatographic Parameters

**Table 2-1.** HPLC Parameters

|  |  |
| --- | --- |
| **HPLC System** | Waters Acquity HClass |
| **Column** | Agilent InfinityLab Poroshell 120 EC-C18 50 x 4.6 mm, 2.7 μm  Part Number: 699975-902 |
| **Mobile Phase A** | 0.1% TFA in Purified Water |
| **Mobile Phase B** | 0.05% TFA in Acetonitrile |
| **Needle Wash** | 50:50 Acetonitrile: Purified Water |
| **Gradient Program** | |  |  |  | | --- | --- | --- | | Time (min) | A (%) | B (%) | | 0 | 90 | 10 | | 6.0 | 10 | 90 | | 6.1 | 90 | 10 | | 9.0 | 90 | 10 | |
| **Detection** | 360 nm |
| **Flow Rate** | 1.0 mL/min |
| **Column Temperature** | 40°C ± 5°C |
| **Autosampler Temperature** | 5°C ± 3°C |
| **Injection Volume** | 2 μL |
| **Sampling Rate** | 10 points/sec |
| **Run Time** | 9 minutes |

## Dissolution Conditions

Table 2-2. Dissolution Conditions

|  |  |
| --- | --- |
| **Medium** | Purified Water |
| **Volume** | 900 mL |
| **Apparatus** | USP Type II (Paddles) |
| **Speed** | 50 RPM |
| **Time** | For single pull: 45 minutes  For profile: 5, 15, 30, 45, 60 minutes (infinity: 60 min at 250 rpm) |
| **Temperature** | 37.0°C ± 0.5°C |
| **Pull Volume** | 10 mL |
| **Filter** | 0.45 µm PVDF filter |

## Reagents and Materials

* Purified Water, Millipore
* Acetonitrile (ACN), HPLC Grade
* Trifluoroacetic Acid (TFA), HPLC Grade
* CX-4945 (free acid) Standard, client provided
* CX-4945 Capsules composite placebo
* Whatman 0.45 μm PVDF membrane filter

## Dissolution Medium Preparation

Transfer 6000 mL of purified water into a suitable flask. Mix well and degas.

## Mobile Phase A Preparation (0.1% TFA in water)

Transfer 1.0 mL of TFA into a suitable flask containing 1000 mL of purified water. Mix well.

## Mobile Phase B Preparation (0.05% TFA in Acetonitrile)

Transfer 0.5 mL of TFA into a suitable flask containing 1000 mL of acetonitrile. Mix well.

## Diluent Preparation

Transfer 50 mL of TFA into a suitable flask containing 950 mL of acetonitrile. Mix well.

## Standard Solution Preparation

*Standard Usage Note: Prior to use, standard must be ground with a mortar and pestle and then equilibrated to ambient laboratory conditions for at least one hour, but not more than 2 hours.*

*Determine the water content of the ground, equilibrated standard on the day of use as per current USP <921> Method Ia (performed as per SOP MPC RD 065, SOP MPC RD 066; SOP MPC QC 197, SOP MPC QC 198) as follows:*

*Diluent: Methanol Dry*

*Titrant: Composite 2*

*Sample Amount: About 100 mg (or adjusted as needed to obtain an amount of water between 2 mg to 250 mg)*

*Perform the water determination in duplicate. The absolute difference between the two results should be NMT 1.0%. Report the mean of two determinations.*

Accurately weigh the equivalent of approximately 22 mg of CX-4945 free acid standard by quantitatively transferring into a 100-mL volumetric flask an amount (in mg) of standard adjusted for its purity as follows:

, where *P* is the purity of reference standard expressed as % Purity/100%. Add about ¾ volume of diluent and mix to dissolve. Sonicate to dissolve if necessary. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well.

The concentration of CX-4945 free acid is 0.22 mg/mL.

Prepare a check standard solution in a similar manner.

## Sample Solution Preparation

At each dissolution analysis time point, withdraw a 10 mL portion of the solution at a zone midway between the surface of the dissolution medium and the top of the rotating paddle and not less than 1 cm from the vessel wall. Filter the solution through a Whatman PVDF 0.45-µm filter, discarding the first 3 mL of the filtrate.

Note—Dispense sample solutions directly into HPLC vials for analysis. The results obtained at all other profile time points besides at endpoint (45 minutes) are only for reporting purposes and will not appear in the finished product Certificate of Analysis.

## Procedure

Separately inject equal volumes (2 µL) of the dissolution media, diluent, standard, and sample solutions. Record the chromatograms and measure the peak area responses of the CX-4945 peak.

A maximum of six injections of sample may be performed between bracketing standard injections

## System Suitability Requirements

* The diluent and dissolution media injections should have no peaks that elutes at RRT 0.98 – 1.02 of the CX-4945 peak, which significantly interfere (NMT 1% relative to the average peak area of the CX-4945 peak from the five replicate injections of working standard) with the quantitation of CX-4945
* The % RSD of the CX-4945 retention time from the five (5) consecutive injections of working standard solution is NMT 2.0%.
* The % RSD of the CX-4945 peak area responses from the five (5) consecutive injections of working standard solution is NMT 2.0%.
* The mean USP Tailing Factor (Tf) for the CX-4945 peak from the five (5) consecutive injections of working standard solution is NMT 2.0.
* Standard check agreement should be between 98.0 – 102.0%.

## Calculations

Calculate the % dissolved as follows:

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

For All Other Profile Time Points:

Where,

Rspl : The area response of CX-4945 in the sample solution

Rs : The area response of CX-4945 in the standard solution

Ws : Weight of the CX-4945 free acid standard, in mg

P : Purity of the CX-4945 free acid standard expressed as % Purity/100%

LC : Nominal Label Claim of CX-4945 Capsules, in mg

V : Withdraw Volume, in mL

n : Sampling time point number

# System Suitability

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

# Specificity (Interference)

## Dissolution medium Interference Solution Preparation

Use the *Dissolution medium* as the dissolution medium interference solution

## Diluent Interference Solution Preparation

Use the *Diluent* as the diluent interference solution.

## 2X Placebo Interference Solution Preparation

Accurately weigh and quantitatively transfer about 252 mg of CX-4945 capsule composite placebo into a 900 mL volumetric flask. Add about ¾ volume of dissolution medium, preheated to about 37ºC, and swirl to avoid clumping. Stir for 45 minutes and remove the stirrer bar, then dilute to volume with dissolution medium and mix well. Filter an aliquot of the solution through a 0.45‑μm Whatman PVDF membrane filter, discarding the first 3 mL to waste.

## 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 dissolution medium interference, diluent interference, and placebo interference should have no peaks which significantly interfere (NMT 1% from injections of dissolution medium and diluent and NMT 2% from injections of placebo, relative to the average peak area of the CX-4945 peak from the five replicate injections of working standard) with the quantitation of CX-4945

# Linearity Study

The linearity study will be evaluated from about 5% to 120% of the CX-4945 concentration of the working standard solution, which corresponds to about 11 µg/mL to 264 µg/mL.

## Stock Linearity Solution

Accurately weigh the equivalent of approximately 55 mg of CX-4945 free acid standard by quantitatively transferring into a 50-mL volumetric flask an amount (in mg) of standard adjusted for its purity as follows:

, where *P* is the purity of reference standard expressed as % Purity/ 100%. Add about ¾ volume of diluent and mix to dissolve. Sonicate to dissolve if necessary. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well.

The concentration of CX-4945 free acid is 1.1 mg/mL.

## Working Linearity Solution Preparations

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

Table 5-1. Preparation of working linearity solutions

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Linearity  Level | Nominal Conc.  (%) | Volume of Stock Linearity Solution  (mL) | Volume of Working Linearity L5 Solution (mL) | Flask Volume (mL) | Approx. Conc.  CX-4945  (µg/mL) |
| L1 | 5 | — | 5.0 | 100 | 11 |
| L2 | 25 | — | 12.5 | 50 | 55 |
| L3 | 50 | 5.0 | — | 50 | 110 |
| L4 | 75 | 7.5 | — | 50 | 165 |
| L5 | 100 | 10.0 | — | 50 | 220 |
| L6 | 120 | 12.0 | — | 50 | 264 |

## Procedure

* Establish system suitability per **Section 2.11**.
* Inject each linearity level solution once.
* For each linearity injection, calculate the response factor and relative response factor relative to the response factor of the 100% level as follows:

Linearity Relative Response Factor to 100% Level

* 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.
* Calculate the percent y-intercept relative to 100% level as follows:

Linearity Relative Response Factor to 100% Level

## Validity Criteria

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

## Acceptance Criteria

* Meet the linearity range of a minimum of five consecutive levels.
* The relative response factors (RRF) at each level is within 97.0% to 103.0%.
* The correlation coefficient, R, is NLT 0.995.
* The percent y‑intercept is NMT 2%.

# Accuracy By Spiked Recovery

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

The accuracy study will be performed by spiking CX-4945 drug substance solution into an amount of composite placebo corresponding to the 200 mg dosage strength. The accuracy will be evaluated from about 5% to 120% of the CX-4945 concentration of the working standard solution, which corresponds to about 11 µg/mL to 330 µg/mL of the CX-4945. In order to exclude effects of potency differences using the drug substance in the sodium salt form, a control solution (Spike Solution II), prepared directly using the CX-4945 sodium salt drug substance, will be used to verify the recovery.

## Spike Solution I Preparation

Accurately weigh and quantitatively transfer about 270 mg of the CX-4945 (sodium salt) drug substance equivalent to about 220 mg of CX-4945 (free acid) into a 50-mL volumetric flask. Add about ¾ volume of dissolution medium and mix to dissolve. Sonicate to dissolve if necessary. Allow solution to cool to room temperature, then dilute to volume with dissolution medium and mix well.

The concentration of CX-4945 free acid is 4.4 mg/mL.

Note—Determine the water content of the CX-4945 sodium salt drug substance as per the analytical procedure in method verification protocol PRO MV 0129.

## Spike Solution II Preparation

Dilute 10.0 mL of the spike I solution to 200 mL with dissolution medium. Mix well.

The concentration of CX-4945 free acid is 0.22 mg/mL.

## Working Recovery Solution Preparation

For each recovery level, prepare samples in triplicate.

Accurately weigh and quantitatively transfer about 31.1 mg of composite placebo and one (1) empty capsule into volumetric flasks. Add volumes of the *spike solution* as described in **Tables 6-1**.Add about ¾ volume of dissolution medium, place the flask into a water bath at 37°C and shake for 45 minutes, swirling the flask occasionally, until the excipients blend and capsule shell are completely dispersed. Allow the solution to cool to room temperature, dilute to volume with dissolution medium and mix well. Filter an aliquot of the solution through a 0.45‑μm Whatman PVDF membrane filter, discarding the first 3 mL to waste.

Table 6-1. Preparation of recovery sample solutions.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Recovery Level | Nominal Conc.  (%) | Volume of Spike Solution I  (mL) | Volume of Spike Solution II (mL) | Flask Volume (mL) | Approx. Conc.  CX-4945  (µg/mL) |
| R1 | 5 | — | 10.0 | 200 | 11 |
| R2 | 50 | 5.0 | — | 200 | 110 |
| R3 | 100 | 10.0 | — | 200 | 220 |
| R4 | 150 | 15.0 | — | 200 | 330 |

## Procedure

* Establish system suitability per **Section 2.11**.
* Inject each recovery level solution once.
* Calculate the percent recovery for CX-4945 as follows:

## Validity Criteria

* Meet the system suitability requirements in **Section 2.11**.
* The percent RSD of results for levels R1 is NMT 6.0%.
* The percent RSD of results for levels R2 to R4 is NMT 3.0%.

## Acceptance Criteria

* The mean percent recovery of the R1 level is within 90%-110%.
* The mean percent recovery of the R2 to R4 levels is within 95%-105%.

# Precision

## Precision

Perform a six-capsule dissolution profile for the CX-4945 capsules (200 mg) as per the analytical test method (**Section 2**).

## Procedure

* Establish system suitability per **Section 2.11**.
* Inject each sample solution once.
* Determine the percent dissolved.

## Validity Criteria

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

## Acceptance Criteria

* The % RSD for the dissolution results at 45 minutes is NMT 6% for mean results < 85% dissolved, and NMT 5% for mean results ≥ 85% dissolved.

# Filter study

A filter study will be performed to evaluate the suitability of the filters used (Whatman 0.45‑µm PVDF membrane filter) for the sample solution preparation.

## Filter Study on Dissolution Medium

Filter a portion of the dissolution medium previously heated to about 37°C through a Whatman 0.45‑µm PVDF filter and collect the first 2 mL of filtrate.

## Filter Study on Sample Solution

Filtered Sample:

Filter a portion of the sample solution (prepared as per **Section 2.9**) through a Whatman 0.45‑µm PVDF filter, and collect each aliquot portion as shown in **Table 8-1.**

Note—Sample solutions prepared for the precision study may be used. Evaluate the filter using the infinity timepoint.

**Table 8-1. Collection of filtrate aliquots for filter study**

|  |  |  |
| --- | --- | --- |
| **Aliquot** | **Filtration Fraction (mL)** | **Volume Collected (mL)** |
| 1 | 0-3 | 2 |
| 2 | 3-6 | 2 |
| 3 | 6-9 | 2 |

Centrifuged Sample:

Centrifuge an aliquot of the sample solutions evaluated for the filter study at 10000 rpm (11400 RCF) for 10 minutes.

Noe—Centrifuge as necessary to obtain a clear supernatant.

## Procedure

* Establish system suitability per **Section 2.11**.
* Inject each test sample solution in once.
* Determine whether any peaks are attributed to the filter.
* Determine the relative recovery of CX-4945 obtained from each filtrate aliquot of the standard solution to the unfiltered standard solution.
* Determine the relative recovery of CX-4945 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

* The relative recovery of CX-4945 in each filtrate aliquot of the sample solution to the centrifuged sample solution is within 97 – 103%.

# Stability Study

The standard and sample solutions will be evaluated at normal laboratory environmental condition to determine the appropriate time frame for use. Their stabilities will be determined by periodically evaluating the solutions for change in CX-4945 against freshly prepared or qualified standard solutions. A dissolution sample from one vessel was analyzed at the 60 minute time point on the day the sample was pulled.

Standard solution stability was considered from the time of preparation to the time of injection of the aged solution. Sample solution stability was considered from the time of initial injection to the time of injection of the aged solution.

## Procedure

* Prepare a working standard solution as per **Sections 2.8**.
* At each evaluation, establish system suitability as per **Section 2.11**.
* Evaluate the stabilities of the working standard solution and sample solution at normal laboratory environmental condition.
* At each evaluation, inject each solution once.
* Determine the percent recovery of the standard and sample solution (tested for stability). Calculate the percent relative recovery at tested interval results to those initially obtained.

## Validity Criteria

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

## Acceptance Criteria

* The standard solutions are considered stable if the relative recovery result at each time interval is within the range of 98.0 – 102.0%.
* The sample solutions are considered stable if the relative recovery result at each time interval is within the range of 98 – 102%.