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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 *Assay (Content Uniformity* and *Blend Uniformity)*, *Related Substances* and *Identification by Retention Time* analytical procedure for CX-4945 (sodium salt) drug substance (Manufacturer: Carbogen Amcis AG) and CX-4945 capsules (200 mg) by Frontida BioPharm Analytical Research and Development (ARD) department.

Note that CX-4945 capsules contain the CX-4945 as a sodium salt. The label claim is calculated based on the free acid.

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 71442.00. The qualification of the method included and demonstrated the following method parameters/characteristics:

* System Suitability
* Specificity (Interference)
* Forced Degradation
* Linearity
* Accuracy by Recovery
* Method Repeatability
* Method Sensitivity (Reporting Limit)
* 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 to perform the analysis.

This protocol describes the methodology for the verification of the analytical procedure and defines the criteria to assess the results. In order to verify the suitability of the test method and demonstrate the capability of Frontida BioPharm ARD department to perform this analysis, the following studies will be performed:

* System Suitability
* Specificity (Interference and Identification)
* Forced Degradation[[1]](#footnote-1) (Oxidation by Peroxide and Metal Oxidation)
* Quantitation Limit
* Accuracy by Spiked Recovery
* Precision (Repeatability)
* Filtration Study
* Stability of Solutions

Note—Modifications to the standard and sample solution preparation for the drug substance and drug product are proposed. There is no impact of the proposed change as the final solution concentration is maintained.

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

|  |  |
| --- | --- |
| **Column** | Phenomenex Gemini C18, 110 Å 100 mm x 4.6 mm, 3 µm  Part number: 00D-4439-E0 |
| **Mobile Phase A** | 0.1% TFA in Purified Water |
| **Mobile Phase B** | 0.05% TFA in Acetonitrile |
| **Needle Wash** | 50:50 Acetonitrile: Purified Water |
| **Needle Wash Setting** | Extended |
| **Gradient Program** | |  |  |  | | --- | --- | --- | | Time (min) | A (%) | B (%) | | 0 | 90 | 10 | | 2.0 | 50 | 50 | | 6.0 | 35 | 65 | | 8.0 | 90 | 10 | | 12.0 | 90 | 10 | |
| **Detection** | 227 nm |
| **Flow Rate** | 1.2 mL/min |
| **Column Temperature** | 30°C ± 5°C |
| **Injection Volume** | 10 μL |
| **Sampling Rate** | 10 points/sec |
| **Run Time** | 12 minutes |

## Reagents and Materials

* Purified Water, Millipore
* Acetonitrile, HPLC Grade
* Trifluoroacetic Acid (TFA), HPLC Grade
* CX-4945 (free acid) Standard, client provided
* CX-4945 Capsules composite placebo
* CX-4945 Capsules, 200 mg
* Millipore 0.45‑μm PVDF membrane filter

## 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.*

### Stock Standard Solution Preparation

Accurately weigh the equivalent of approximately 50 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 if necessary to dissolve. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well.

The concentration of CX-4945 free acid is 1.0 mg/mL.

Prepare a check standard solution in a similar manner.

### Working Standard Solution Preparation

Dilute 5.0 mL of the stock standard solution to 50 mL with the Diluent. Mix well.

The concentration of CX-4945 free acid is 0.1 mg/mL.

Prepare a check standard solution in a similar manner.

## Intermediate Sensitivity Solution

Dilute 2.0 mL of the working standard solution to 100 mL with the Diluent. Mix well.

The concentration of CX-4945 free acid is 2.0 µg/mL (2.0% w/w%).

## Sensitivity Solution

Dilute 2.5 mL of the intermediate sensitivity solution to 100 mL with the Diluent. Mix well.

The concentration of CX-4945 free acid is 0.05 µg /mL (0.05% w/w%).

## Drug Substance Assay/RS Sample Solution Preparation

### Stock Sample Solution Preparation:

Accurately weigh and quantitatively transfer corrected amount of sample equivalent to 50 mg of CX-4945 in the free acid form (approximately 60 mg of CX-4945 as sodium salt) into a 50‑mL volumetric flask. Add about ¾ volume of diluent and mix to dissolve. Sonicate until completely dissolved. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well.

### Working Sample Solution Preparation:

Dilute 5.0 mL of the stock sample solution to 50 mL with the diluent. Mix well.

The concentration of CX-4945 free acid is about 0.1 mg/mL.

## Drug Product Assay/RS Sample Solution Preparation

### Stock Sample Solution Preparation:

Accurately weigh 10 capsules and transferred the capsule contents into a single container and mix well. Weigh the empty capsule shells to determine an average capsule content.

Accurately weigh and transfer the equivalent of 2 capsules into a 200-mL volumetric flask. Add about ¾ volume of diluent and swirl to avoid clumping. Sonicate for 15 minutes with occasional swirling. Mechanically shake for 15 minutes. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well. Filter an aliquot of the solution through a Millipore 0.45‑μm PVDF membrane filter, discarding the first 3 mL to waste.

### Working Sample Solution Preparation:

Dilute 5.0 mL of the stock sample solution to 100 mL with the diluent. Mix well.

The concentration of CX-4945 free acid is about 0.1 mg/mL.

## Drug Product CU Sample Solution Preparation

### Stock Sample Solution Preparation:

Accurately weigh 1 capsule and transfer entire contents into a 100-mL volumetric flask. Rinse capsule shells with diluent into the volumetric flask. Add about ¾ volume of diluent and swirl to avoid clumping. Sonicate for 15 minutes with occasional swirling. Mechanically shake for 15 minutes. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well. Filter an aliquot of the solution through a Millipore 0.45‑μm PVDF membrane filter, discarding the first 3 mL to waste.

### Working Sample Solution Preparation:

Dilute 5.0 mL of the stock sample solution to 100 mL with the diluent. Mix well.

The concentration of CX-4945 free acid is about 0.1 mg/mL.

## Drug Product BU Sample Solution Preparation

### Stock Sample Solution Preparation:

Determine appropriate size of volumetric flask needed to prepare a sample solution in the range of 1.0 – 3.0 mg/mL CX-4945 free acid.

Transfer entire contents into an appropriate volumetric flask. Rinse bottle with diluent to effect complete transfer. Add about ¾ volume of diluent and swirl to avoid clumping. Sonicate for 15 minutes with occasional swirling. Mechanically shake for 15 minutes. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well. Filter an aliquot of the solution through a Millipore 0.45‑μm PVDF membrane filter, discarding the first 3 mL to waste.

Allow the bottles to dry and then record weight.

### Working Sample Solution Preparation:

Dilute 5.0 mL of the stock sample solution to 100 mL with the diluent. Mix well.

## Procedure

Separately inject equal volumes (10 µL) of the diluent, sensitivity, standard (n=5), and sample solutions) – refer to example injection sequence below. Record the chromatograms and measure the peak area responses of the CX-4945 peak.

**Example Injection Sequence**:

|  |  |
| --- | --- |
| **Solution** | **Number of Injections** |
| Diluent | ≥1 |
| Sensitivity | 1 |
| Working Standard | 5 |
| Working Check Standard | 2 |
| Working Standard as Procedural Control Standard (PCS) | 1 |
| Working Sample Solution (Assay, RS, CU, BU, ID) | 1 |
| Working Standard as Procedural Control Standard (PCS) | 1 |

## System Suitability Requirements

* The diluent injection should have no peaks which significantly interfere (NMT 0.2% 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 S/N of CX-4945 peak from the injection of sensitivity solution ≥ 10.
* The mean Tailing Factor (Tf) for the CX-4945 peak 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 1.5%.
* The % RSD of the CX-4945 *retention time* from the five (5) consecutive injections of working standard solution is NMT 2.0%.
* Standard check agreement should be between 98.5 – 101.5%.

Note—The S/N requirement does not apply when only testing Assay, BU, or CU.

## Calculations

Calculate as follows:

DRUG SUBSTANCE ASSAY (% w/w, free acid, as is):

DRUG SUBSTANCE ASSAY (% w/w, free acid, on anhydrous, solvent free basis):

Note— Residual solvents of CX-4945 sodium salt drug substance as per the material Certificate of Analysis

DRUG PRODUCT ASSAY (%LC):

CONTENT UNIFORMITY (%LC):

Calculate the content uniformity acceptance value (AV) as per cUSP <905>.

Acceptance Value =

Where:

|  |  |
| --- | --- |
| : | Mean of individual contents |
| k: | 2.4 (for sample size of 10 units) or k = 2.0 (for sample size of 30 units) |
| s: | Standard deviation of individual contents |
| 1M: | Case,  If 98.5% 101.5%, then M =  If < 98.5% then M = 98.5%  If > 101.5% then M = 101.5% |

BLEND UNIFORMITY (%LC):

RELATED SUBSTANCES (% area):

RETENTION TIME DIFFERENCE (% difference):

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 CX-4945 free acid standard, in mg

Wspl : Weight of CX-4945 Sample powder, in mg

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

: Volume of Stock Sample solution, in mL

ACC : Average capsule content in mg, salt form/capsule

MWC :

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

Rimp : The area response of individual impurity peak in the sample solution

Rtotal : Sum of all peak area responses of all peaks in the sample solution greater than or equal to 0.05%, excluding peaks observed in the diluent or solvent front

RTstd : Retention Time average from bracketing standard.

RTspl : Retention Time from Sample

# 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 2.14**) will be considered fulfillment of this study.

# Specificity (Interference)

## Diluent Interference Solution Preparation

Use the *Diluent* as the diluent interference solution.

## Placebo Interference Solution Preparation

### Stock Placebo Interference Solution Preparation

Accurately weigh and quantitatively transfer about 140 mg of CX-4945 capsule composite placebo into a 100‑mL volumetric flask. Add about ¾ volume of diluent and swirl to avoid clumping. Sonicate for 15 minutes with occasional swirling. Mechanically shake for 15 minutes. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well. Filter an aliquot of the solution through a 0.45‑μm Millipore PVDF membrane filter, discarding the first 3 mL to waste.

### Placebo Interference Solution Preparation

Dilute 5.0 mL of the stock placebo interference solution to 100 mL with the diluent. Mix well.

## Sample Interference Solution Preparation

### Drug Substance Interference Solution Preparation

Prepare sample solution as described in **Section 2.9.**

Note—A sample solution prepared for **Section 8.1** may be used.

### Drug Product Interference Solution Preparation

Prepare sample solution as described in **Section 2.10.**

Note—A sample solution prepared for **Section 8.2** may be used.

## Procedure

* Establish system suitability per **Section 2.14**.
* Inject each solution once.

## Validity Criteria

* Meet the system suitability requirements in **Section 2.14**.

## Acceptance Criteria

* The diluent interference and placebo interference should have no peaks which significantly interfere (NMT 0.2% 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.
* From injections of sample interference solutions, resolution between CX-4945 and the closest eluting peak ≥ 0.05% is NLT 1.5.

# Forced Degradation

Forced Degradation (FD) studies will be performed on the composite placebo and drug product. The placebo and drug product will be exposed to peroxide (~5% H2O2) and metal oxidative (50 mM FeCl3) conditions.

## Control Sample Solution Preparation

### Control Placebo Preparation

Accurately weigh and quantitatively transfer about 20 mg of composite placebo powder into a 250-mL volumetric flask. Add 12.5 mL of purified water and gently swirl. Fill with diluent to ¾ of flask volume and swirl to avoid clumping. Sonicate for 15 minutes with occasional swirling. Mechanically shake for 15 minutes. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well. Centrifuge an aliquot of the control placebo solution at 10000 rpm (11400 RCF) for 10 minutes.

Note—Centrifuge as necessary to obtain a clear supernatant.

### Control Sample Preparation

Accurately weigh and quantitatively transfer about 43 mg of capsule content powder into a 250-mL volumetric flask. Add 12.5 mL of purified water and gently swirl. Fill with diluent to ¾ of flask volume and swirl to avoid clumping. Sonicate for 15 minutes with occasional swirling. Mechanically shake for 15 minutes. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well. Centrifuge an aliquot of the control sample solution at 10000 rpm (11400 RCF) for 10 minutes.

Note—Centrifuge as necessary to obtain a clear supernatant.

## Oxidation by Peroxide (5% Hydrogen Peroxide)

### Peroxide Oxidation Blank Preparation

Dilute 5.0 mL of ~5% hydrogen peroxide solution to 100 mL with diluent. Mix well.

Note—Prepare ~5% hydrogen peroxide solution by diluting 16.5 mL of concentrated hydrogen peroxide to 100 mL with purified water.

### Peroxide Oxidation Placebo Preparation

Accurately weigh and quantitatively transfer about 20 mg of composite placebo powder into a 250-mL volumetric flask. Add 12.5 mL of 5% hydrogen peroxide solution and gently swirl. Allow to stand for at least 24 hours at ambient condition. Fill with diluent to ¾ of flask volume and swirl to avoid clumping. Sonicate for 15 minutes with occasional swirling. Mechanically shake for 15 minutes. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well. Centrifuge an aliquot of the peroxide oxidation placebo solution at 10000 rpm (11400 RCF) for 10 minutes.

Note—Centrifuge as necessary to obtain a clear supernatant.

### Peroxide Oxidation Sample Preparation

Accurately weigh and quantitatively transfer about 43 mg of capsule content powder into a 250-mL volumetric flask. Add 12.5 mL of 5% hydrogen peroxide solution and gently swirl. Allow to stand for at least 24 hours at ambient condition. Fill with diluent to ¾ of flask volume and swirl to avoid clumping. Sonicate for 15 minutes with occasional swirling. Mechanically shake for 15 minutes. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well. Centrifuge an aliquot of the peroxide oxidation sample solution at 10000 rpm (11400 RCF) for 10 minutes.

Note—Centrifuge as necessary to obtain a clear supernatant.

## Metal Oxidation (50 mM Ferric Chloride)

### Metal Oxidation Blank Preparation

Dilute 5.0 mL of ~50 mM Ferric Chloride (FeCl3) solution to 100 mL with diluent. Mix well.

Note—Prepare ~50 mM Ferric Chloride solution by dissolving about 0.8 g of Ferric Chloride (FeCl3) to 100 mL with purified water.

### Metal Oxidation Placebo Preparation

Accurately weigh and quantitatively transfer about 20 mg of composite placebo powder into a 250-mL volumetric flask. Add 12.5 mL of 50 mM Ferric Chloride solution and gently swirl. Allow to stand for at least 5 days at ambient laboratory conditions. Fill with diluent to ¾ of flask volume and swirl to avoid clumping. Sonicate for 15 minutes with occasional swirling. Mechanically shake for 15 minutes. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well. Centrifuge an aliquot of the metal oxidation placebo solution at 10000 rpm (11400 RCF) for 10 minutes.

Note—Centrifuge as necessary to obtain a clear supernatant.

### Metal Oxidation Sample Preparation

Accurately weigh and quantitatively transfer about 43 mg of capsule sample powder into a 250-mL volumetric flask. Add 12.5 mL of 50 mM Ferric Chloride solution and gently swirl. Allow to stand for at least 5 days at ambient laboratory conditions. Fill with diluent to ¾ of flask volume and swirl to avoid clumping. Sonicate for 15 minutes with occasional swirling. Mechanically shake for 15 minutes. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well. Centrifuge an aliquot of the metal oxidation sample solution at 10000 rpm (11400 RCF) for 10 minutes.

Note—Centrifuge as necessary to obtain a clear supernatant.

## Procedure

* Establish system suitability per **Section 2.14**.
* Inject each solution once.
* Collect and report the chromatographic data with a PDA detector from 200 nm – 400 nm. Assess the spectral peak purity of the CX-4945 peak.
* Determine the percent recovery (calculated against the control) and the percent degradation.
* Report UV spectra of active and degradation products obtained.

## Validity Criteria

* Meet system suitability requirements per **Section 2.14**.

## Acceptance Criteria

* Degradation should be between 5% to 25%.
* The resolution between the active and the closest-eluting peak (if present at a level of ≥ 0.05%) is NLT 1.5.
* The resolution between any known impurity and the closest-eluting peak (if present at a level of ≥ 0.05%) is NLT 1.2.
* Degradation peaks ≥ 0.05% must be resolved from each other to the extent that all impurity peaks can be accurately quantified.
* Peak purity analysis of active peak from treated solutions indicate that the peak elutes as a spectrally homogenous peak (purity threshold > purity angle).

# Quantitation Limit

The Quantitation Limit (QL) will be evaluated at a concentration corresponding to an impurity level of 0.05%. The QL is represented by the sensitivity solution (**Section 2.8**). The peak signal-to-noise ratio (S/N) will be assessed in order to ensure that adequate sensitivity can be achieved at this level.

## Procedure

* Establish system suitability per **Section 2.14**.
* Inject sensitivity solution six times and determine the S/N.

## Validity Criteria

* Meet the system suitability requirements in **Section 2.14**.

## Acceptance Criteria

* The S/N is NLT 10 in each injection.
* The % RSD of peak area responses is NMT 15% for the active.

# Accuracy by spiked Recovery

The accuracy of the method will be assessed for the quantitation of the CX-4945 in the drug product.

The accuracy study will be performed by spiking known amounts of CX-4945 drug substance onto a corresponding amount of CX-4945 capsule composite placebo.

For Assay, the accuracy will be evaluated from CX-4945 concentrations corresponding to 50% to 150% of the nominal sample concentration of 0.1 mg/mL.

For Impurities, the accuracy will be evaluated from concentrations corresponding to an impurity level of 0.1% to 0.3% of the nominal sample concentration of 0.1 mg/mL.

## Accuracy for Assay

### Recovery Sample Preparations

Accurately weigh about 140 mg of CX-4945 capsule composite placebo and CX-4945 sodium salt drug substance as directed in **Table 7-1** into 100-mL volumetric flasks. Add about ¾ volume of diluent and swirl to avoid clumping. Sonicate for 15 minutes with occasional swirling. Mechanically shake for 15 minutes. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well. Filter an aliquot of the solution through a Millipore 0.45‑μm PVDF membrane filter, discarding the first 3 mL to waste.

Dilute 5.0 mL of the filtrate to 100 mL with diluent and mix well.

**Table 7-1.** Preparation of Recovery sample solutions for Assay

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Recovery Level** | **Nominal Concentration (%)** | **Weight of CX-4545 sodium salt (mg)** | **Weight of Placebo  (mg)** | **Flask Volume (mL)** | **Dilution** | **Approximate Concentration of CX‑4545  (mg/mL)** |
| R1 | 50% | 125 | 140 | 100 | 5.0 mL to 100 mL | 0.05 |
| R2 | 100% | 250 | 140 | 100 | 0.1 |
| R3 | 150% | 375 | 140 | 100 | 0.15 |

Prepare each level in triplicate.

## Control/Reference Solution Preparation

Prepare a control/reference solution using CX-4945 sodium salt drug substance as per **Section 2.9**.

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.

## Accuracy for Impurities

### Spiking Solution Preparation

Accurately weigh and quantitatively transfer about 123 mg of CX-4945 sodium salt drug substance (equivalent to approximately 100 mg of CX-4945 as free acid) into a 100‑mL volumetric flask. Add about ¾ volume of diluent and mix to dissolve. Sonicate until completely dissolved. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well.

Dilute 5.0 mL of the above solution to 100 mL with diluent and mix well.

### Recovery Sample Preparations

Accurately weigh about 140 mg of CX-4945 capsule composite placebo into 100-mL volumetric flasks. Transfer volumes of recovery spiking solution as directed in **Table 7-2**. Add about ¾ volume of diluent and swirl to avoid clumping. Sonicate for 15 minutes with occasional swirling. Mechanically shake for 15 minutes. Allow solution to cool to room temperature, then dilute to volume with diluent and mix well. Filter an aliquot of the solution through a Millipore 0.45‑μm PVDF membrane filter, discarding the first 3 mL to waste.

Dilute 5.0 mL of the filtrate to 100 mL with diluent and mix well.

**Table 7-2.** Preparation of Recovery sample solutions for Impurities

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Recovery Level** | **Impurity Level  (%)** | **Volume of Spiking Solution (mL)** | **Weight of Placebo  (mg)** | **Flask Volume (mL)** | **Dilution** | **Approximate Concentration of CX‑4945  (µg/mL)** |
| R1 (QL) | 0.1 | 4.0 | 140 | 100 | 5.0 mL to 100 mL | 0.10 |
| R2 | 0.15 | 6.0 | 140 | 100 | 0.15 |
| R3 | 0.3 | 12.0 | 140 | 100 | 0.30 |

Prepare each level in triplicate.

## Control/Reference Solution Preparation

Dilute the 6.0 mL of the spiking solution (**Section 7.3.1**) to 100 mL with the diluent and mix well.

Dilute the 5.0 mL of the above solution to 100 mL with the diluent and mix well.

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.

## Procedure

* Establish system suitability per **Section 2.14**.
* Inject each sample solution once.
* Calculate the % recovery against control/reference solution.
* Calculate the % RSD of results between same level.

## Validity Criteria

* Meet the system suitability requirements in **Section 2.14**.

## Acceptance Criteria

For Assay:

* The % RSD of the triplicate preparations within the same level is NMT 3.0%.
* The mean % recovery within the same level is between 98.0 – 102.0%.

For Impurities:

* The % RSD of the triplicate preparations within the same level is NMT 11.0%.
* The mean % recovery within the same level is between 80 – 120%.

# Precision

## Drug Substance: Assay

Prepare six (6) sample solutions using the CX-4945 drug substance as described in **Section 2.9**.

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.

## Drug Product: Assay

Prepare six (6) sample solutions using CX-4945 drug product capsules as described in **Section 2.10**.

## Drug Product: Content Uniformity

Prepare ten (10) sample solutions as described in **Section 2.11**.

## Drug Product: Related Substances

Prepare six (6) sample solutions at the R2 level as described in **Section 7.3.2**.

## Procedure

* Establish system suitability per **Section 2.14**.
* Inject each sample solution once.
* For drug substance Assay samples, calculate the % Assay.
* For drug product Assay and Content Uniformity samples, calculate the % LC.
* For drug product Related Substances samples, calculate the % Impurity and absolute difference.

## Validity Criteria

* Meet the system suitability requirements in **Section 2.14**.

## Acceptance Criteria

For Drug Substance Assay:

* The % RSD of the results from the Precision study (n=6) is NMT 3.0%.
* The average retention time of CX-4945 in the sample solution corresponds to that of the standard solution is within ± 2.0%

For Drug Product Assay:

* The % RSD of the results from the Precision study (n=6) is NMT 3.0%.
* The retention time of CX-4945 in each sample solution corresponds to that of the standard solution is within ± 2.0%

For Drug Product Content Uniformity:

* The AV as calculated according to USP <905> is NMT 15.0.

For Drug Product Related Substances:

* The % RSD of the impurity results ≥ 0.6% from the Precision study (n=6) is NMT 15.0%.
* The absolute difference between the individual and mean results for each impurity ≥ 0.05% and < 0.6 % must meet the criteria in **Table 8-1**.

**Table 8-1**. Absolute Difference Acceptance Criteria for Related substance

|  |  |
| --- | --- |
| % Related Substance | Absolute Difference |
| ≥ 0.05 and ≤ 0.30 | NMT 0.10 |
| > 0.30 and < 0.6 | NMT 0.20 |

# Filter study

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

## Filter Study on Diluent

Filter a portion of the diluent through a Millipore 0.45‑µm PVDF filter and collect the first 2 mL of filtrate.

## Filter Study on Assay Sample Solution

Filtered Sample:

Filter a portion of the assay sample solution prepared as per **Section 2.9** (Note**—**A sample solution prepared for **Section 8.1** may be used) through a Millipore 0.45‑µm PVDF 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 | 0-3 | 3 |
| 2 | 3-6 | 3 |
| 3 | 6-9 | 3 |

Centrifuged Sample:

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

Note—Centrifuge as necessary to obtain a clear supernatant.

## Filter Study on Related Substance Sample Solution

Filtered Sample:

Filter a portion of the related substance sample solution (prepared as per **Section 8.4**) through a Millipore 0.45‑µm PVDF filter, and collect each aliquot portion as shown in **Table 9-1**.

Centrifuged Sample:

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

Note—Centrifuge as necessary to obtain a clear supernatant.

## Procedure

* Establish system suitability per **Section 2.14**.
* Inject each test sample solution once.
* Determine whether any peaks are attributed to the filter.
* 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 2.14**.

## Acceptance Criteria

* For assay sample filter study, the relative recovery of CX-4945 in each filtrate aliquot of the sample solution to the centrifuged sample solution is within 98.0 – 102.0%.
* For related substances ≥ 0.05% and < 0.4%, the absolute difference of the filtered sample result from the centrifuged sample result is NMT 0.10%.
* For related substances ≥ 0.4%, the percent recovery of CX-4945 in each filtrate aliquot of the sample solution to the centrifuged sample solution is within 85.0 – 115.0%.

# Stability Study

The standard and sample solutions (drug substance and drug product) 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.

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 2.6**.
* At each evaluation, establish system suitability as per **Section 2.14**.
* Prepare a sample solution as per **Sections 2.9**, and **2.10**. (Note—Sample solution stability may be determined from a sample solution prepared for the precision study)
* Evaluate the stabilities of the working standard solution and sample solutions at normal laboratory environmental condition.
* At each evaluation, inject each solution once.
* Determine the percent assay of the standard and sample solutions (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 2.14**.

## Acceptance Criteria

* The standard solutions are considered stable if the relative recovery result at each time interval is within the range of 98.5 – 101.5%.
* The sample solutions are considered stable if the relative recovery result at each time interval is within the range of 98.5 – 101.5% for drug substance and 98.0 – 102.0% for drug product.
* For each related substance ≥ 0.05% and < 0.4%, the absolute difference of the aged sample result from the initial sample result is NMT 0.10%.
* For each related substance ≥ 0.4%, the relative % impurity in the aged sample solution to the initial sample solution is within 85.0 – 115.0%.

# Identification by Retention Time (RT)

Verification of the Identification by Retention Time will be performed and demonstrated as part of establishing system suitability (**Section 2.14**) and execution of the Precision study for Assay (**Section** **8.1** and **8.2**). The successful establishment and completion of these studies will be considered fulfillment of Identification by RT.

1. Based on Alcami’s method validation report (Report#: RPT 71442.00), degradation of the drug product was produced only in oxidation by peroxide conditions. Hence, of the forced degradation conditions performed by Alcami, only the oxidation by peroxide condition of the drug product will be performed herein as part of method verification. In addition, forced degradation of the drug product by metal oxidation, a condition not previously evaluated, will be performed. [↑](#footnote-ref-1)