METHOD VALIDATION PROTOCOL

CX-4945 (Silmitasertib) Tablets, 500 mg

ASSAY, RELATED SUBSTANCES, CONTENT UNIFORMITY, BLEND UNIFORMITY AND IDENTIFICATION BY RETENTION TIME METHOD BY HPLC

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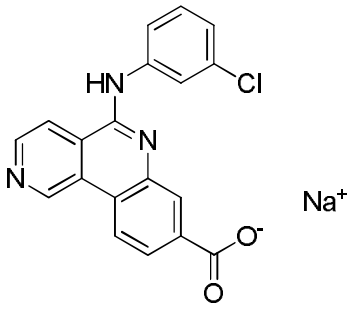
1 INTRODUCTION

CX-4945 Sodium Salt (Formula: $C_{19}H_{11}ClN_3O_2Na$; 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 validation of the *Assay (Content Uniformity* and *Blend Uniformity)*, *Related Substances* and *Identification by Retention Time* analytical procedure for CX-4945 Tablets (500 mg) by Frontida BioPharm Analytical Research and Development (ARD) department.

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

Proposed formulation of CX-4945 Tablets, 500 mg is summarized in **Table 1-1**.

Table 1-1. Proposed formulation of CX-4945 Tablets, 500 mg

Ingredients	%w/w	mg/unit
Intra Granular		
CX-4945 (sodium salt) (a)	71.33	535.00
Mannogem EZ Spray Dried Mannitol	15.67	117.50
Hydroxy Propyl Cellulose (Klucel EF)	1.00	7.50
Croscarmellose Sodium, NF (Ac-Di-Sol)	5.00	37.50
Sodium Lauryl Sulfate(in solution)	1.00	7.50
Sodium Lauryl Sulfate	4.00	30.00
Purified Water	N/A	N/A
Granulation Total	**98.00**	**735.00**
Extra Granular		
Croscarmellose Sodium, NF (Ac-Di-Sol)	1.50	11.25
Magnesium Stearate, NF [Vegetable Source]	0.50	3.75
Fill Weight	**100.0**	**750.00**

Appropriate validation studies will be performed by the Frontida BioPharm ARD department in order to demonstrate that the 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 following studies will be performed:

- System Suitability
- Specificity (Interference and Identification)
- Forced Degradation (Oxidation by Peroxide)
- Linearity
- Quantitation Limit
- Accuracy by Spiked Recovery
- Precision
- Intermediate Precision
- Filtration Study
- Solution stability

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.

2 ANALYTICAL PROCEDURE

2.1 Chromatographic Parameters

Table 2-1. HPLC Parameters

Column	Agilent Zorbax SB-C18 150 mm x 4.6 mm, 3 µm Part number: 863953-902
Mobile Phase A	0.1% TFA in Purified Water and Acetonitrile (90:10)
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 (%)** 0.00 / 100 / 0 0.25 / 100 / 0 3.50 / 55.5 / 44.5 6.50 / 40 / 60 8.50 / 100 / 0 12.5 / 100 / 0
Detection	227 nm
Flow Rate	1.2 mL/min
Column Temperature	30°C ± 3°C
Injection Volume	5 µL
Sampling Rate	10 points/sec
Run Time	12.5 minutes

2.2 Reagents and Materials

- Purified Water, Millipore
- Acetonitrile, HPLC Grade
- Trifluoroacetic Acid (TFA), HPLC Grade
- CX-4945 (free acid) Standard, client provided
- CX-4945 Tablets composite placebo
- CX-4945 Tablets, 500 mg
- Impurity C-028349
- Impurity C-028350
- Millipore 0.45-µm PVDF membrane filter

2.3 Mobile Phase A Preparation (0.1% TFA in water and Acetonitrile, 90:10)

Transfer 1.0 mL of TFA into a suitable flask containing 900 mL of purified water and 100 mL of acetonitrile. Mix well.

2.4 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.

2.5 Diluent Preparation

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

2.6 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.

2.6.1 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:

$\frac{50\ \text{mg}}{P}$, 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.

2.6.2 Working Standard Solution Preparation

Dilute 10.0 mL of the stock standard solution to 50 mL with the Diluent. Mix well.

The concentration of CX-4945 free acid is 0.2 mg/mL.

Prepare a check standard solution in a similar manner.

2.7 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 4.0 µg/mL (2.0% w/w%).

2.8 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 0.1 µg/mL (0.05% w/w%).

2.9 Placebo Solution Preparation

2.9.1 Stock Placebo Solution Preparation

Accurately weigh and quantitatively transfer about 250 mg of CX-4945 tablet composite placebo into a 250-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.

2.9.2 Working Placebo Solution Preparation

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

2.10 Assay and Related Substances Sample Solution Preparation

2.10.1 Stock Sample Solution Preparation

Determine the average tablet weight (ATW) of NLT 10 tablets.

$$\text{ATW} = \frac{Weight\ of\ NLT\ 10\ Tablets}{Quantity\ of\ NLT\ 10\ \ Tablets\ Weighed}$$

Grind tablets into a fine, uniform powder using a mortar and pestle.

Accurately weigh an amount of ground powder equivalent of 500 mg of CX-4945 as follows:

Equivalent to (500 mg) = $\frac{500\ mg\ x\ ATW}{Label\ claim}$

Transfer weight into a 250-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.

2.10.2 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 0.2 mg/mL.

2.11 Content Uniformity Sample Solution Preparation

2.11.1 Stock Sample Solution Preparation:

Accurately weigh 1 tablet and transfer into a 250-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.

2.11.2 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 0.2 mg/mL.

2.12 Blend Uniformity Sample Solution Preparation

2.12.1 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.

2.12.2 Working Sample Solution Preparation:

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

2.13 Procedure

Separately inject equal volumes (5 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
Placebo Solution	1
Working Standard	5
Working Check Standard	1
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

2.14 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 (T_f) 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 2.0%.
- 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 98.0 – 102.0%.

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

2.15 Calculations

Calculate as follows:

DRUG PRODUCT ASSAY (%LC):

$$\%LC = \frac{R_{spl}}{R_s} \times \frac{W_s\ (mg) \times P}{50\ (mL)} \times \frac{10.0\ (mL)}{50\ (mL)} \times \frac{250\ (mL) \times ATW}{W_{spl}\ (mg)} \times \frac{50\ (mL)}{5.0\ (mL)} \times \frac{100\%}{LC}$$

CONTENT UNIFORMITY (%LC):

$$\%LC = \frac{R_{spl}}{R_s} \times \frac{W_s\ (mg) \times P}{50\ (mL)} \times \frac{10.0\ (mL)}{50\ (mL)} \times \frac{250\ (mL)}{1\ tablet} \times \frac{50\ (mL)}{5.0\ (mL)} \times \frac{100\%}{LC}$$

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

$$\text{Acceptance Value} = |M - \bar{X}| + ks$$

Where:

$\bar{X}$: 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
[1]M: Case,
If $98.5\% \leq \bar{X} \leq 101.5\%$, then $M = \bar{X}$
If $\bar{X} < 98.5\%$ then $M = 98.5\%$
If $\bar{X} > 101.5\%$ then $M = 101.5\%$

BLEND UNIFORMITY (%LC):

$$\%LC = \frac{R_{spl}}{R_s} \times \frac{W_s\ (mg) \times P}{50\ (mL)} \times \frac{10.0\ (mL)}{50\ (mL)} \times \frac{V_{spl}\ (mL)}{W_{spl}(mg)} \times \frac{50\ (mL)}{5.0\ (mL)} \times \frac{750\ mg}{LC} \times 100\%$$

RELATED SUBSTANCES (% area):

$$\%\ Impurity = \frac{R_{imp}}{R_{total}} \times 100\%$$

RETENTION TIME DIFFERENCE (% difference):

$$\% \text{ Difference} = \frac{RT_{std} - RT_{spl}}{RT_{std}} \times 100\%$$

Where,

R_{spl} : The area response of CX-4945 in the sample solution

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

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

W_{spl} : Weight of CX-4945 Sample, in mg

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

V_{spl} : Volume of Stock Sample solution, in mL

ATW : Average Tablet Weight in mg

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

R_{imp} : The area response of individual impurity peak in the sample solution

R_{total} : 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

RT_{std} : Retention Time average from bracketing standard, in min

RT_{spl} : Retention Time from Sample, in min

Note: the molecular weights of CX-4945 are as follow:

CX-4945 in free acid form: 349.77 g/mol

CX-4945 sodium salt form: 371.75 g/mol

3 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.

4 SPECIFICITY (INTERFERENCE)

4.1 Diluent Interference Solution Preparation

Use the *Diluent* as the diluent interference solution.

4.2 Placebo Interference Solution Preparation

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

4.3 Specificity (Impurity) Identification (ID) Solution Preparation

4.3.1 Stock Impurity ID Solutions Preparation:

Accurately weigh and quantitatively transfer about 15 mg each of Impurity C-028349 and Impurity C-028350 into separate 100-mL volumetric flasks. To each flask, add diluent to fill about half the flask volume. Sonicate to dissolve. Allow to cool to room temperature. Dilute to volume with diluent and mix well.

4.3.2 Intermediate Impurity ID Solution Preparation:

Transfer 5.0 mL of each Stock Impurity ID solutions into separate 250-mL volumetric flasks. Dilute to volume with diluent and mix well.

4.3.3 Working Impurity ID Solution Preparation:

Transfer 5.0 mL of each Intermediate Impurity ID solutions into separate 50-mL volumetric flasks. Dilute to volume with diluent and mix well.

The concentration of each impurity is about 0.3 µg/mL.

4.4 Procedure

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

4.5 Validity Criteria

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

4.6 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.

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 the following conditions: elevated temperature, ambient, UV photolysis, acid and base hydrolysis, peroxide (~5% H_2O_2).

5.1 Control Sample Solution Preparation

5.1.1 Control Placebo Preparation

Accurately weigh and quantitatively transfer about 40 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 sample solution at 10000 rpm (11400 RCF) for 10 minutes.

Note—Centrifuge as necessary to obtain a clear supernatant.

5.1.2 Control Sample Preparation

Accurately weigh and quantitatively transfer about 86 mg of tablet 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.

5.2 Elevated Temperature Condition

Accurately weigh and quantitatively transfer separate portions of the composite placebo powder (about 40 mg) and tablet powder (about 86 mg) into separate 250-mL volumetric flasks. Place the samples in an oven at 105°C for a minimum of 3 hours.

Following the elapse of the minimum time, take note of any physical changes that occurred. Prepare sample solutions similarly as directed in **Section 5.1** except using the sample specimen exposed to elevated temperatures.

5.3 Short Wavelength UV Condition

Transfer separate portions of composite placebo powder and tablet powder into suitable containers. Place samples under short wavelength UV light for at least 7 days.

Following the elapse of the minimum time, take note of any physical changes that occurred. Prepare sample solutions similarly as directed in **Section 5.1** except using the sample specimen exposed to the UV light.

5.4 Ambient Condition

Transfer separate portions of composite placebo powder and tablet powder into suitable containers. Place samples under ambient conditions for at least 7 days.

Following the elapse of the minimum time, take note of any physical changes that occurred. Prepare sample solutions similarly as directed in **Section 5.1** except using the sample specimen exposed to ambient conditions.

5.5 Acid Hydrolysis Condition

5.5.1 Acid Blank Solution Preparation

Transfer equal volumes, 5.0 mL of 1 N hydrochloric acid and 1 N sodium hydroxide into a 100-mL volumetric flask. Dilute to volume with diluent and mix well.

5.5.2 Acid Hydrolysis Placebo Solution Preparation

Accurately weigh and quantitatively transfer about 40 mg of composite placebo powder into a 250-mL volumetric flask. Add 12.5 mL of 1 N Hydrochloric Acid and swirl. Allow to stand for 24 hours at ambient conditions. Neutralize with 12.5 mL of 1 N Sodium Hydroxide solution. 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.

5.5.3 Acid Hydrolysis Sample Solution Preparation

Accurately weigh and quantitatively transfer about 86 mg of tablet powder into a 250-mL volumetric flask. Add 12.5 mL of 1N Hydrochloric Acid and swirl. Allow to stand for 24 hours at ambient conditions. Neutralize with 12.5 mL of 1 N Sodium Hydroxide solution. 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.

5.6 Base hydrolysis Condition

5.6.1 Base Blank Solution Preparation

Refer to **Section 5.5.1**.

5.6.2 Base Hydrolysis Placebo Solution Preparation

Accurately weigh and quantitatively transfer about 40 mg of composite placebo powder into a 250-mL volumetric flask. Add 12.5 mL of 1N Sodium Hydroxide solution and swirl. Allow to stand for 24 hours at ambient conditions. Neutralize with 12.5 mL of 1 N Hydrochloric Acid solution. 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.

5.6.3 Base Hydrolysis Sample Solution Preparation

Accurately weigh and quantitatively transfer about 86 mg of tablet powder into a 250-mL volumetric flask. Add 12.5 mL of 1 N Sodium Hydroxide and swirl. Allow to stand for 24 hours at ambient conditions. Neutralize with 12.5 mL of 1 N Hydrochloric Acid solution. 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.

5.7 Oxidation by Peroxide (5% Hydrogen Peroxide)

5.7.1 ~5% Hydrogen Peroxide Preparation

Dilute 16.5 mL of concentrated hydrogen peroxide to 100 mL with purified water.

5.7.2 Peroxide Oxidation Blank Preparation

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

5.7.3 Peroxide Oxidation Placebo Solution Preparation

Accurately weigh and quantitatively transfer about 40 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 control sample solution at 10000 rpm (11400 RCF) for 10 minutes.

Note—Centrifuge as necessary to obtain a clear supernatant.

5.7.4 Peroxide Oxidation Sample Solution Preparation

Accurately weigh and quantitatively transfer about 86 mg of tablet 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 control sample solution at 10000 rpm (11400 RCF) for 10 minutes.

Note—Centrifuge as necessary to obtain a clear supernatant.

5.8 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.

5.9 Validity Criteria

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

5.10 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).

6 LINEARITY STUDY

The linearity study will be performed on CX-4945 at appropriate range for Assay and Impurities.

For assay, the linearity will be assessed over the intended range of method of 50% to 150% of the nominal active concentration of 0.2 mg/mL (0.1 mg/mL to 0.3 mg/mL).

For related substances, the linearity will be assessed from the QL level (0.05%) to 0.3% of the nominal active concentration of 0.2 mg/mL (0.1 µg/mL to 0.6 µg/mL).

6.1 Assay Linearity Stock Solution Preparation

Use the Stock Standard Solution prepared as directed in **Section 2.6.1**.

6.2 Impurity Linearity Stock Solution Preparation

Use the intermediate sensitivity solution prepared as directed in **Section 2.7**.

6.3 Assay Linearity Working Solutions Preparation

Prepare the linearity solutions for the L1 to L5 levels as directed in **Table 6-1**. Dilute each to volume with diluent and mix well.

Table 6-1. Assay Linearity Solutions Preparation

Assay Linearity Level	Nominal Conc. (%)	Volume of Assay Linearity Stock Solution (mL)	Flask Volume (mL)	Approx. Conc. of CX-4945 (mg/mL)
L1	50	5.0	50	0.1
L2	75	7.5	50	0.15
L3	100	10.0	50	0.2
L4	125	12.5	50	0.25
L5	150	15.0	50	0.3

6.4 Impurity Linearity Working Solution

Prepare the working linearity solutions for the QL to L5 levels as directed in **Table 6-2**. Dilute each to volume with diluent and mix well.

Table 6-2. Impurity Linearity Solutions Preparation

Impurity Linearity Level	Nominal Conc. (%)	Volume of Impurity Linearity Stock Solution (mL)	Flask Volume (mL)	Approx. Conc. of CX-4945 (µg/mL)
L1	QL	2.5	100	0.1
L2	0.1	5.0	100	0.2
L3	0.15	7.5	100	0.3
L4	0.2	10.0	100	0.4
L5	0.3	15.0	100	0.6

6.5 Procedure

- Establish system suitability as per **Section 2.14**.
- Inject each linearity level solution once.
- For each linearity injection, calculate the response factor and relative response factor relative to the mean response factor of the 100% level as follows:

$$\text{Linearity Response Factor (RF)} = \frac{\text{Peak Area Response}}{\text{Concentration}}$$

$$\text{Linearity Relative Response Factor to 100\% level} = \frac{\text{Response Factor}}{\text{Response Factor of the 100\% Level}}$$

- 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.

- From the slopes, determine the relative slope at impurity level to assay level as follows:

$$\text{Relative Slope} = \frac{\text{Slope of obtained at Impurity level}}{\text{Slope of obtained at Assay level}}$$

6.6 Validity Criteria

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

6.7 Acceptance Criteria

Assay:

- Meet the linearity range of a minimum of five consecutive levels.
- The correlation coefficient, r, is NLT 0.999.
- The linearity relative response factors (RRF) at each level is within 98.0% to 102.0%.

Impurities:

- Meet the linearity range of a minimum of five consecutive levels.
- The linearity relative response factors (RRF) at each level is within 80% to 120%.
- The relative slope of impurity level to assay level is within 90% to 110%.
- For the impurities level linearity, the correlation coefficient, r, is NLT 0.995.

7 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.

7.1 Procedure

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

7.2 Validity Criteria

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

7.3 Acceptance Criteria

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

8 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 tablet composite placebo.

For Assay, the accuracy will be evaluated from CX-4945 concentrations corresponding to 50% to 150% of the nominal sample concentration of 0.2 mg/mL.

For Impurities, the accuracy will be evaluated from concentrations corresponding to an impurity level of 0.05% to 0.3% of the nominal sample concentration of 0.2 mg/mL.

8.1 Accuracy for Assay

8.1.1 Recovery Sample Preparations

Accurately weigh about 100 mg of composite placebo and CX-4945 sodium salt drug substance as directed in **Table 8-1** 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 50 mL with diluent and mix well.

Prepare each level in triplicate.

Table 8-1. Preparation of Recovery sample solutions for Assay

Recovery Level	Nominal Concentration (%)	Weight of CX-4945 sodium salt (mg)	Weight of Placebo (mg)	Flask Volume (mL)	Dilution	Approximate Concentration of CX-4945 * (mg/mL)
R1	50%	125	100	100	5.0 mL to 50 mL	0.1
R2	100%	250	100	100		0.2
R3	150%	375	100	100		0.3

*Approximate concentration based on CX-4945 sodium salt containing ~15% water.

8.2 Control/Reference Solution Preparation

8.2.1 Stock Sample Solution Preparation:

Accurately weigh and quantitatively transfer corrected amount of sample equivalent to 200 mg of CX-4945 in the free acid form (approximately 250 mg of CX-4945 as sodium salt) 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.

8.2.2 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 0.2 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.

8.3 Accuracy for Related Substances

8.3.1 Spiking Solution Preparation

Accurately weigh and quantitatively transfer about 125 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.

The concentration of CX-4945 free acid is 0.05 mg/mL.

8.3.2 Recovery Sample Preparations

Accurately weigh about 100 mg of CX-4945 tablet composite placebo into 100-mL volumetric flasks. Transfer volumes of recovery spiking solution as directed in **Table 8-2**. 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 50 mL with diluent and mix well.

Table 8-2. 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)	0.05	2.0	100	100	5.0 mL to 50 mL	0.1
R2	0.1	4.0	100	100		0.2
R3	0.15	6.0	100	100		0.3
R4	0.3	12.0	100	100		0.6

Prepare each level in triplicate.

8.4 Control/Reference Solution Preparation

Dilute 6.0 mL of the spiking solution (**Section 8.3.1**) to 100 mL with the diluent and mix well.

Dilute the 5.0 mL of the above solution to 50 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.

8.5 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.

8.6 Validity Criteria

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

8.7 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%.

9 PRECISION AND INTERMEDIATE PRECISION

9.1 Precision

9.1.1 Precision – Assay

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

9.1.2 Precision – Content Uniformity

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

9.1.3 Precision – Related Substances

Prepare six (6) sample solutions at the R3 level as described in **Section 8.3.2**.

9.2 Intermediate Precision

9.2.1 Intermediate Precision – Assay

Similarly, as directed for the precision study, have a different analyst than the one who performed the precision study prepare and analyze an additional six (6) sample solutions as directed in **Section 2.10** using a different HPLC system and column and chemical lots (if possible).

9.2.2 Intermediate Precision – Content Uniformity

Similarly, as directed for the precision study, have a different analyst than the one who performed the precision study prepare and analyze an additional ten (10) sample solutions as directed in **Section 2.11** using a different HPLC system and column and chemical lots (if possible).

9.3 Procedure

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

9.4 Validity Criteria

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

9.5 Acceptance Criteria

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 Precision studies (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 9-1**.

Table 9-1. 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

10 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.

10.1 Filter Study on Diluent

Filter a portion of the diluent through a Millipore 0.45-µm PVDF filter and collect the first 3 mL of filtrate.

Dilute 2.5 mL of the filtrate to 25 mL with the diluent.

10.2 Filter Study on Assay Sample Solution

Filtered Sample:

Filter a portion of the assay sample solution prepared as per **Section 2.10.1** (Note—A sample solution prepared for **Section 9.1.1** may be used) through a Millipore 0.45-µm PVDF filter, and collect each aliquot portion as shown in **Table 10-1**.

Table 10-1. 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

Dilute 2.5 mL of the filtrate to 25 mL with the diluent.

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.

Dilute 2.5 mL of the filtrate to 25 mL with the diluent.

10.3 Filter Study on Related Substance Sample Solution

Filtered Sample:

Filter a portion of the related substance sample solution (prepared as per **Section 9.1.3**) through a Millipore 0.45-µm PVDF filter, and collect each aliquot portion as shown in **Table 10-1**.

Dilute 2.5 mL of the filtrate to 25 mL with the diluent.

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.

Dilute 2.5 mL of the filtrate to 25 mL with the diluent.

10.4 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.

10.5 Validity Criteria

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

10.6 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, the percent recovery of CX-4945 in each filtrate aliquot of the sample solution to the centrifuged sample solution is within 90.0% – 110.0%.

11 STABILITY STUDY

The sample solution will be evaluated at normal laboratory environmental condition to determine the appropriate time frame for use. Its stabilities will be determined by periodically evaluating the solutions for change in CX-4945 against freshly prepared or qualified standard solutions.

Sample solution stability is considered from the time of initial injection to the time of injection of the aged solution.

11.1 Procedure

- At each evaluation, establish system suitability as per **Section 2.14**.
- Prepare a working standard solution as per **Sections 2.6.2**. In order to reduce effects of moisture changes of the standard, the aliquots of the standard may be quantitatively weighed into appropriate flasks and tightly sealed and prepared (dissolved in and diluted with diluent) at the time of use.
- Prepare a sample solution as per **Sections 2.10**. (**Note**—Sample solution stability may be determined from a sample solution prepared for the precision or intermediate 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.

11.2 Validity Criteria

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

11.3 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 98.0% – 102.0%.

- For each related substance if present in the sample solution ≥ 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 if present in the sample solution ≥ 0.4%, the relative % impurity in the aged sample solution to the initial sample solution is within 85.0% – 115.0%.

12 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 9.1.1**). The successful establishment and completion of these studies will be considered fulfillment of Identification by RT.

13 DOCUMENT HISTORY

Protocol Revision	Section	Change/Deviation
2	2	Several issues were encountered during execution of protocol revision 1 (e.g. distorted peak shapes obtained from different column batches, difficulty establishing system suitability, system/method precision, and failure to meet accuracy and linearity criteria). These issues were determined to be best addressed by switching to a different column (Agilent Zorbax SB C18 previously Phenomenex Gemini C18) and modifying the chromatographic conditions to optimize system/method precision. Change in test method as follows – standard and sample solution concentrations, chromatographic parameters/conditions (column, mobile phase proportion, gradient program, and injection volume).
	4, 5, 6, 8	Revise solution as necessary to correspond to change in concentration of standard and sample solutions.
	5	Added other stress conditions to the Forced Degradation Study such as Elevated Temperature, Short Wavelength UV, Ambient, Acid Hydrolysis and Base Hydrolysis conditions.
1	N/A	Initial issuance of protocol

Signature Manifest

Document Number: PRO MV 0176 **Revision:** 2

Title: CX-4945 (Silmitasertib) tablets, 500 mg: Assay, Related Substances, Content Uniformity, Blend Uniformity and Identification by Retention Time Method by HPLC

All dates and times are in Eastern Time.

CX-4945 (Silmitasertib) tablets, 500 mg: Assay, Related Substances, Co...

Step 3 Customer Approval

Name/Signature	Title	Date	Meaning/Reason
Chen-Fu Liu (CHENFULIU1)	Director of R& D	07 Dec 2021, 02:26:50 AM	Complete & Quit
Marjorie Cordero (MCORDERO)	Analytical Chemist - CMS	07 Dec 2021, 09:02:05 AM	Complete

P Step 4 Author Approval

Name/Signature	Title	Date	Meaning/Reason
Marjorie Cordero (MCORDERO)	Analytical Chemist - CMS	07 Dec 2021, 09:33:06 AM	Approved

P Step 4a Management Approval

Name/Signature	Title	Date	Meaning/Reason
Timothy Kim (TKIM)	Manager, ARD	07 Dec 2021, 09:13:34 AM	Approved
Shiying Tian (STIAN)	Director, AR&D	07 Dec 2021, 09:43:32 AM	Approved

P Step 4b QA Approval

Name/Signature	Title	Date	Meaning/Reason
Kirit Patel (KPATEL)	Director - QA	07 Dec 2021, 10:12:02 AM	Approved

Step 5 Set Effective Date

Name/Signature	Title	Date	Meaning/Reason
Marjorie Cordero (MCORDERO)	Analytical Chemist - CMS	07 Dec 2021, 10:40:29 AM	Approved