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Formulation for a Novel Inhaled Peptide Therapeutic for Idiopathic Pulmonary Fibrosis

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Abstract

A caveolin-1 scaffolding domain, CSP7, is a newly developed peptide for the treatment of idiopathic pulmonary fibrosis. To develop a CSP7 formulation for further use we have obtained, characterized and compared a number of lyophilized formulations of CSP7 trifluoroacetate with DPBS and in combination with excipients (mannitol and lactose at molar ratios 1:5, 70 and 140). CSP7 trifluoroacetate was stable (>95%) in solution at 5 and 25°C for up to 48 hours and tolerated at least 5 freeze/thaw cycles. Lyophilized cakes of CSP7 trifluoroacetate with excipients were stable (>96%) for up to 4 weeks at room temperature (RT), and retained more than 98% of the CSP7 trifluoroacetate in the solution at 8 hours after reconstitution at RT. The lyophilized CSP7 formulations were stable for up to 10 months at 5°C protected from moisture. Exposure of the lyophilized cakes of CSP7 to 75% relative humidity (RH) resulted in an increase in the absorbed moisture, promoted crystallization of the excipients and induced reversible formation of CSP7 aggregates. Increased molar ratio of mannitol slightly affected formation of the aggregates. In contrast, lactose significantly decreased (up to 20-times) aggregate formation with apparent saturation at the molar ratio of 1:70. The possible mechanisms of stabilization of CSP7 trifluoroacetate in solid state by lactose include physical state of the bulking agent and the interactions between lactose and CSP7 trifluoroacetate (e.g., formation of a Schiff base with the N-terminal amino group of CSP7). Finally, CSP7 trifluoroacetate exhibited excellent stability during nebulization of formulations containing mannitol or lactose.

Keywords

CSP7 trifluoroacetate; idiopathic pulmonary fibrosis (IPF); lyophilization; nebulization; bulking agent; mannitol; lactose; aggregation; crystallization; stabilizing effect

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Novelty statement. To our knowledge, no study has prepared an inhaled formulation of a novel peptide, CSP7, for the treatment of Idiopathic Pulmonary Fibrosis (IPF).

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1. Introduction

Idiopathic Pulmonary Fibrosis (IPF) is a serious, chronic and steadily progressive interstitial lung disease. By definition, IPF is a build-up of scar tissue in the lung parenchyma from unknown origins (1). This condition often occurs in elderly male smokers or ex-smokers and results in restrictive lung dysfunction (2–4). In the general population, prevalence estimates for IPF have varied from 14 to 43 cases per 100,000 people per year and it is known to be increasing (1). Median survival is about three years after the condition is diagnosed (5) and mortality usually results from respiratory failure (6). While not well-appreciated, survival from IPF is actually lower than many malignancies (4). Current treatments for IPF include nintedanib (7), pirfenidone (8), oxygen therapy (1), and in advanced cases, lung transplantation (9). Unfortunately, none of these interventions are curative and lung transplantation is invasive, expensive and carries its own co-morbidities.

Recently identified pharmacotherapy can slow the progression of disease indicating that better treatment is possible. A caveolin-1 scaffolding domain 7-mer peptide (CSP7), FTTFTVT, is being developed by Lung Therapeutics Inc. for treatment of patients with IPF. CSP7 can inhibit tumor suppressor protein (p53), and alveolar epithelial cell (AEC) apoptosis. In AEC, when expression of p53 increases, AEC apoptosis is augmented. Apoptotic AECs are replaced by activated fibroblasts or fibrotic lung fibroblasts (fLFs) which proliferate, resist apoptosis and deposit matrix proteins (10). CSP7 has an effect on both pathways to stop or reverse development of PF. These combined effects of CSP7 result in the prevention of PF development and resolution of existing PF in mice after bleomycin (BLM)-induced lung injury (11–15). A related form of CSP7; CSP20 has been effective in reversing pulmonary fibrosis in mice when delivered by nasal insufflation as well as by intraperitoneal administration (11). Therefore, CSP7 has emerged as a new and promising compound for airway delivery via nebulization to treat IPF. If this treatment is successful in future clinical trial testing, it may benefit thousands of patients suffering from IPF in the US and worldwide. Thus, a stable CSP7 formulation prepared by lyophilization and a reconstituted solution delivered to the lung using a nebulizer was investigated for preclinical assessment and for subsequent clinical trial utilization.

Lyophilization is used to enhance the stability of peptides since the molecular mobility and reactivity of the peptides in the solid state are reduced (16). However, it is known that residual or absorbed moisture can induce physical and chemical instability of peptide and protein drugs in the lyophilized state, such as aggregation and degradation (17). Aggregation often leads to unfavorable outcomes (e.g., reduction of biological activity, solubility or enhancement of immunogenicity) (18,19). Residual moisture content of the lyophilized products may depend on the bulking agent(s) used in formulations (20–22). A number of publications document that the physical states of the bulking agents have a strong impact on stabilization of the lyophilized products (23–27). Mannitol and lactose are the most commonly and widely used bulking agents in lyophilized protein/peptide formulations (27–29). Typically mannitol, a non-reducing sugar, provides a robust cake structure, which undergoes micro-collapse; however, its crystallization behavior leads to an unexpected protective effect (24,25,29,30). On the other hand, lactose, a disaccharide reducing sugar, usually remains in an amorphous state during the freeze-drying process (31,32).

Biopharmaceuticals for treatment of pulmonary disease can be directly delivered to the lungs to minimize their systemic exposure and to increase their efficiency and safety (33,34). Nebulization is used for airway delivery of protein and peptide drugs. However, during nebulization, thermolabile proteins can be partially denatured leading to loss of activity, formation of aggregates, which may lead to an unwanted outcomes like increased immunogenicity (35). These effects result from stress on proteins in the droplets and bulk reservoir liquid. Hence, the evaluation of the effects of nebulization processes on activity and stability is critical to the development of novel protein formulations. Currently used nebulizers are categorized by their mechanism of nebulization such as jet, ultrasonic and vibrating mesh (or membrane) nebulizers (35). The jet and ultrasonic nebulizers cause the greater problem of air- liquid interface than the vibrating mesh nebulizer due to the number of recirculation on the baffle prior to emission of the devices (34). To address/minimize potential degradation and inactivation of biologics, vibrating mesh nebulizers have been recommended to aerosolize protein and peptide formulations (36,37). These devices gently generate the aerosol plume (38,39) with low shear force (40). Thus, vibrating mesh nebulizers are considered suitable/optimal method devices for pulmonary protein/peptide delivery.

The present study was focused on evaluation of stability of CSP7 and finding a formulation for further *in vivo* clinical studies. The physical and chemical stability of CSP7 in both liquid and lyophilized states, and the effect of nebulization on stability of CSP7 solutions were evaluated. Stability assessments include characterization of moisture-induced aggregates using various techniques such as high performance liquid chromatography (HPLC), and liquid chromatography/mass spectroscopy (LC/MS), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS). In addition, effects of moisture-induced crystallization of bulking agents are also reported.

2. Materials and Methods

2.1. Materials

The CSP7 trifluoroacetate (purity > 95%), was kindly provided by Lung Therapeutics, Inc. (Austin, TX). Lactose monohydrate was purchased from Foremost Farms (Baraboo, WI). Dulbecco's phosphate buffered saline (DPBS) without calcium or magnesium was purchased from Lonza (Walkersville, MD). Mannitol, ammonium hydroxide solution (28% w/w in water) ultrapure water, HPLC grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals utilized in this study were of ACS grade.

2.2. Chromatographic analysis

Samples were chemically analyzed with a Dionex 3000 HPLC system (Thermo Fisher Scientific, Fair Lawn, NJ) with a wavelength of 220 nm. CSP7 was eluted by Phenomenex® Luna 5µm C18(2) 100 Å, 150 mm x 4.6 mm (Phenomenex®, Torrance, CA) column at a flow rate of 1.0 mL/min. Two mobile phases were A (0.1% trifluoroacetic acid in water) and B (0.09% trifluoroacetic acid in a mixture of 20:80, water and acetonitrile). The HPLC gradient consisted of 25–35% mobile phase B in 20 min and was run at ambient temperature. 20 µL of each sample was injected onto the column and chromatograms were

acquired at the detection wavelength of 220 nm. Standard curve of the CSP7 trifluoroacetate (concentrations of 0.1–1 mg/mL) were prepared. Duplicate determinations were made for each sample. LC/MS was used for to confirm identity.

2.3. LC/MS and MALDI-TOF MS

The LC/MS system consisted of Agilent model 1260 Infinity system (Agilent Technologies, CA, USA) coupled with an Agilent Technologies 6530 Accurate-Mass Q-TOF mass spectrometer (Agilent Technologies, CA, USA). Positive mode electrospray ionization was used. The chromatographic condition was performed at ambient temperature using an Eclipse Plus C18, 5 μ m, 50 $\times$ 2.1 mm HPLC column (Agilent Technologies, CA, USA), coupled with a Zorbax C18 guard column, 5 μ m (Agilent Technologies, CA, USA). The mobile phases consisted of mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile) under gradient elution started from 0% of mobile phase A until 10 min and then immediately switched to 95% mobile phase A from 10.01–14 min. The flow rate of 0.7 mL/min and the injection volume of 1 μ L were set.

The clear sample solutions were mixed in with matrix alpha-cyano-4-hydroxycinnamic acid (CHCA) and analyzed on a Voyager-DE MALDI mass spectrometer (Applied Biosystems, UK)

2.4. Preformulation studies

2.4.1. Aggregation and chemical stability of the peptide solution—Aggregation and chemical stability of the peptide solution was studied prior to developing the lyophilized formulation. The solutions of CSP7 trifluoroacetate dissolved in DPBS with minimal amount of ammonium hydroxide solution (28% w/w in water) (concentration 0.5 mg/mL CSP7 trifluoroacetate) were prepared to test the effects of agitation, freeze-thaw cycles and storage temperature as follows:

2.4.1.1. Effect of agitation: The solutions of CSP7 trifluoroacetate were shaken on an orbital shaker at 200 RPM at 5°C and 25°C. The samples were collected at 0.5, 1, 3, 6, 12 and 24 hours HPLC as described in section 2.2.

2.4.1.2. Effect of multiple freeze-thaw cycles: Freeze-thaw cycles were studied at –20°C and –80°C. The peptide solutions were frozen at each temperature for 1 hour and then thawed at room temperature for 1 hour. The freeze-thaw cycle was repeated for 5 cycles and the samples were collected after each cycle and analyzed by HPLC as described in section 2.2.

2.4.1.3. Effect of storage temperature: CSP7 trifluoroacetate solutions were kept at –80°C, –20°C, 5°C and room temperature (about 25°C). Samples were collected after 24 and 48 hours and analyzed by HPLC as described in section 2.2.

2.5. Formulation preparation

Solutions of CSP7 trifluoroacetate were prepared by dissolving the peptide (concentration of 0.5 mg/mL CSP7 trifluoroacetate) and bulking agents (mannitol or lactose) in the following

CSP7 trifluoroacetate:excipient molar ratios of 1:5, 1:70 and 1:140 in DPBS with pH adjusted to 8.0 by ammonium hydroxide solution (28% w/w). Resulting solutions were filtered through a 0.2 µm PES filter (Celltreat Scientific Products, Shirley, MA). Aliquots of 2 mL of the solution were filled into 5 mL borosilicate glass vials. Rubber stoppers were placed on top of the vials prior to loading on the lyophilizer. Samples were lyophilized using a VirTis BenchTop lyophilizer (Gardiner, NY). Lyophilized cycle parameters are as presented in Table 1. At the completion of the cycle, nitrogen was introduced into the chamber. Vials were sealed with rubber stoppers and aluminum closure, and kept in a desiccator with desiccant at 5°C until tested.

2.6. Chemical stability of peptide

2.6.1. Chemical stability of peptide in reconstituted solution—The lyophilized formulation of CSP7 trifluoroacetate-mannitol (1:140) cakes was reconstituted to 0.5 mg/mL CSP7 trifluoroacetate and kept at 5°C and 25°C. The samples were collected in duplicate at 0.5, 1, 2, 3, 6, and 8 hours. The aggregation was observed and chemical stability of the peptide was then determined by HPLC as described in section 2.2.

2.6.2. Chemical Stability of peptide in lyophilized state—Vials of lyophilized CSP7 trifluoroacetate-mannitol (1:140) cakes were stored at 5°C and 25°C and sampled in duplicate after 1, 2, and 4 weeks. The lyophilized cakes were reconstituted (0.5 mg/mL CSP7 trifluoroacetate) and the solutions were then investigated for aggregation and chemical stability of the peptide by visual observation, i.e. presence or absence of precipitation of peptide and HPLC/LCMS, respectively. Residual moisture content, osmolality, reconstitution time and pH of the samples were also monitored at each time point.

2.7. Karl Fischer Titration

Measurement of moisture content was demonstrated by Aquatest 2000 moisture analyzer (Photovolt Instruments, Minneapolis, MN); and by Karl Fischer titration using an its, Minneapolis, MN). Anhydrous methanol (1 mL) was added to the pre-weighed sealed sample vial by syringe through through the rubber stopper and mixed using vortex mixer. Sample aliquots were withdrawn and transferred to the Karl Fischer vessel. The vial with remaining suspension was opened, dried in an oven, cooled in the desiccator and re-weighed. The weight of sample tested was the different weight between the pre-weighed sealed sample vial and the weight of vial after drying. Moisture content of 1 mL anhydrous methanol was also determined and subtracted from the moisture content of samples. Percentage of moisture content was calculated and compared between freshly prepared lyophilized cakes and the samples after exposure to 75% RH. Moisture analysis was performed in triplicate for each of the samples. ANOVA test was used to evaluate the results.

2.8. Osmolality measurements

Osmolality of the reconstituted solutions of CSP7 trifluoroacetate-mannitol and lactose (1:140 and 1:70) were performed on a 5004 Micro-osmometer™ High Sensitivity Osmometer (Precision Systems Inc. Natick, MA) with 50 µL sample volume. The equipment was calibrated using osmometry standard solutions of 100 and 500 mOsm/kg H₂O (Precision Systems Inc. Natick, MA) before measurement. The osmolality of the reconstituted CSP7

trifluoroacetate in sterile water for injection (SWI), DBPS and solutions of mannitol (or lact measured in triplicate.

2.9. Measurement of reconstitution time

Reconstitution time of the lyophilized cake into the sample vial. The vial was gently swirled. Two mL of SWI was used as control sample. Clarity of the reconstituted solution was compared with the control every 5 seconds and the reconstitution time was recorded when visual clarity was observed. Samples were tested in duplicate.

2.10. Moisture-induced aggregation and quantitation of CSP 7 aggregates

Lyophilized formulations of CSP7 trifluoroacetate with both sugars (molar ratios of 1:5, 1:70 and 1:140) were initially prepared. The sample vials were then opened and placed in a desiccator containing an aqueous saturated sodium chloride solution at constant relative humidity (75% relative humidity (RH)). The desiccator was stored at room temperature for 12 hours. After incubation, two vials of exposed sample powders were resealed before testing moisture content by Karl Fisher titration. The other four vials were reconstituted with SWI to a concentration of 0.5 mg/mL CSP7 trifluoroacetate, then shaken on an orbital shaker (Lab-Line Instruments, Inc., Melrose Park, IL) at 100 rpm in a cold room (5°C). After 2 hours of shaking, two vials of the aggregated samples were centrifuged at 12,000 rpm for 15 min in the microcentrifuge (model Microfuge 18, Beckman Coulter, Brea, CA). The clear supernatant and aggregate samples were collected. The separated aggregates were re-dissolved in 2 mL of DPBS with ammonium hydroxide solution (28% w/w in water) to render clear solutions (pH 8). Controls were composed of reconstituted solutions of each freshly prepared sample. All clear sample solutions were analyzed by HPLC and LC/MS.

2.11. Powder X-ray diffractometry (PXRD)

Solid structure of the powders was investigated using a Benchtop X-ray diffraction instrument, model Miniflex 600 (Rigaku, Woodlands, TX) with primary monochromated radiation (CuK radiation source, $\lambda = 1.54056 \text{ \AA}$). The instrument was operated at an accelerating voltage of 40 kV and 15 mA. All samples were subjected to the same program (scanned over a 2-theta range of 5° to 40° at a step size of 0.04°/sec and a dwell time of 2 sec), scan speed 1°/min.

2.12. Differential scanning calorimetry (DSC)

Thermal properties of formulations were conducted using a modulated differential scanning calorimetry (mDSC) (model Q20, TA Instruments, New Castle, DE) equipped with a refrigerated cooling system. Each sample of approximately 3 mg was loaded into a standard aluminum pan and press-sealed with an aluminum lid (PerkinElmer, Waltham, MA). Another crimped empty pan was used as a reference. Experiments were performed at a heating ramp rate of 10 °C/min in the range of 30–250 °C with a modulation temperature amplitude of 1 °C/min under dynamic flow of nitrogen purge the sample cell at a flow rate of 40 mL/min. Data was analyzed using TA Universal Analysis 2000 software (TA Instruments, New Castle, DE).

2.13. Determination of water sorption isotherm

Isotherms of peptide-bound water were determined for lyophilized formulations using dynamic vapor sorption (Surface Measurement Systems Ltd., London, UK). A quartz pan was loaded with approximately 10 mg of the lyophilized powder. Water absorbed on the samples prior to assay was removed by leaving the samples at below 0.5% RH until mass changed by less than 0.002% in dm/dt for 120 min. Each formulation was run for a full sorption/desorption cycle from 0% to 90% RH in steps of 10% RH at 25 °C. The instrument was run in step dm/dt mode reach equilibrium, as determined by a dm/dt less than 0.0075% within an interval of 5 min. Percentage of change in mass was calculated and plotted as sorption isotherm. The following equation was used to calculate percent mass sorption (desorption).

$$\text{mass\%sorption(desorption)} = \left(\frac{\text{increase(decrease)in mass form water sorption(desorption)}}{\text{mass of dry powder at initial equilibration(0\%RH)}} \right) \times 100$$

2.14. Long-term stability study (mannitol- and lactose-basedformulations)

To avoid exposure to humidity, the lyophilized CSP7 trifluoroacetate with mannitol and lactose cakes (1:5, 1:70 and 1:140) in the sealed vials were wrapped with parafilm and aluminum foil and kept in a desiccator in the fridge. After 10 months, the cakes were reconstituted and analyzed by HPLC as describe in section 2.2.

2.15. Nebulization of peptide

The lyophilized CSP7 trifluoroacetate: sugar (mannitol and lactose) in the molar ratio of 1:140 and 1:70, respectively were freshly reconstituted with SWI (concentration of 0.5 mg/mL CSP7 trifluoroacetate). The solutions (2 mL) were atomized using two types of vibrating mesh nebulizers, which are Aeroneb® Pro (Aerogen, Mountain View, CA) and EZ Breathe® Atomizer (Model EZ-100, Nephron Pharmaceuticals Corporation, Orlando, FL). Nebulization was performed until the reservoir was empty. Each atomized sample was collected by condensation in the polypropylene tube (35) and analyzed by HPLC as described in section 2.2.

3. Results and Discussion

3.1 Preformulation studies

A common challenge of formulating biopharmaceutical products is their instability. Peptide drugs may exhibit a wide range of aggregation phenomena along with chemical instability (41,42). At this relatively early stage of product development, the chemical stability of the CSP7 trifluoroacetate were evaluated at different conditions (Figure 1). Figure 1A shows the effect of agitation on recovery of CSP7 trifluoroacetate after shaking at 5°C and 25°C for 24 hours. The level of the peptide decreased about 4% after 24 hours of shaking at both temperatures. The solutions were still clear and colorless. Peptide aggregation was not detected.

The assessment of repeated freeze-thaw cycles on quality of the CSP7 trifluoroacetate solution was also conducted. Figure 1B shows that about 100% of the CSP7 trifluoroacetate remained after 5 freeze-thaw cycles. No aggregation was found in any samples following 5 freeze-thaw cycles. These results indicate that freeze-thaw cycling did not affect the stability of CSP7 solution. In addition, Figure 1C shows that the storage temperature (from -80 to 25°C) only slightly affected the stability of CSP7 trifluoroacetate up to 48 hours. The extent of CSP7 trifluoroacetate decreased about 3% after storage at 25°C for 48 hours but it was more stable at lower temperatures and shorter storage periods. At -80°C and -20°C , the remaining CSP7 trifluoroacetate was about 100% after storage for 48 hours. There was no aggregation or precipitation detected in any sample after reconstitution in 2 mL of SWI. These results clearly indicate that CSP7 trifluoroacetate being lyophilized, dissolved at neutral pH or frozen is stable for a short time at wide range of temperatures. Next, the effect of excipients (mannitol and lactose) on long-term storage stability of CSP7 trifluoroacetate was studied.

3.2 Appearance and residual moisture content of lyophilized cakes

The lyophilized CSP7 trifluoroacetate –mannitol (1:70 and 1:140) and CSP7-lactose (1:70) formulations produced smooth and homogeneous cakes. The cakes maintained their structure when tapped and did not exhibit collapse or melt-back after storage at 5°C . The lyophilized products of neat CSP7 trifluoroacetate without bulking agent and the 1:5 molar ratio of both excipient-based formulations showed a loss of pore structure and broke into pieces while the CSP7 trifluoroacetate-lactose (1:140) cake had minimal shrinkage. Behavior of the lyophilized cake significantly affects the stability of the formulations. Proper cake formation results in proper pore formation, which is associated with water escape and moisture content in the product (43). Compared to the cake, which remains in the microstructure, collapsed mass may contain a higher level of residual moisture since the specific surface area of the shrunken structure is decreased (44).

As shown in Figure 5, the residual moisture content from the freshly prepared cakes was in the range of 0.49–4.02% w/w. The results with standard deviation (SD) of each formulation including the neat CSP7 trifluoroacetate, the mannitol- and lactose based in the ratio of 1:5, 1:70 and 1:140 were 0.64 ± 0.22 , 0.66 ± 0.21 , 0.49 ± 0.26 , 0.53 ± 0.15 , 0.75 ± 0.25 , 0.61 ± 0.35 , 4.02 ± 0.45 , respectively. Overall, residual moisture content of the mannitol- and lactose-based formulations were similar except for the CSP7 trifluoroacetate-lactose (1:140), which contained the highest moisture content (3.02% w/w) approximately 8-fold greater than that of the mannitol-based cake of the same molar ratio (0.53% w/w). The difference in residual moisture content resulted from poor structure and improper pore formation of the CSP7 trifluoroacetate-lactose (1:140) cakes. Generally, lyophilized peptides are more stable at lower water content ($< 3\%$) since high levels of residual moisture frequently lead to higher molecular mobility and chemical instability in the solid state of the protein and peptide (28,45,46). These results demonstrate that lyophilization of CSP7 alone or with mannitol or lactose (1:5 or 1:70) result in effective removing of moisture from the formulations.

3.3 Chemical stability of peptide in reconstituted solution

Figure 2A shows the stability of CSP7 trifluoroacetate in reconstituted solutions. Potency, which refers to the amount of peptide in the sample, remained at about 100% after 8 hours at 5°C and 25°C. It is important to obtain isotonic solution after reconstitution of the lyophilized formulations. The non-isotonic solutions can cause the release of histamine, which resulted from changing of the osmotic load around mast cells (47). The release of histamine is responsible for bronchoconstriction (48). Osmolality of the reconstituted solutions of lyophilized CSP7 trifluoroacetate containing mannitol or lactose was 378 and 328 mOsm/kg (slightly hypertonic). The osmolality of DBPS and solutions of mannitol and lactose in water were 294, 98, and 47 mOsm/kg, respectively. The osmolality of DPBS and sugar solutions were thus very close to the osmolality of the reconstituted CSP7 trifluoroacetate-bulking agent solutions. Although the CSP7 trifluoroacetate reconstituted solutions were slightly hypertonic, using vibrating mesh nebulizers for drug delivery can markedly improve the treatment time and dose delivery (49).

3.4 Chemical stability, Osmolality and reconstitution time of peptide in lyophilized state with mannitol

A plot of the percentage remaining CSP7 trifluoroacetate (potency) versus time is presented in Figure 2B. The results show that CSP7 trifluoroacetate was quite stable in the lyophilized state for 4 weeks at 5°C (98.6%) and 25°C (96.8%). The reconstitution times were between 50–55 seconds and the pH of the samples was about 8. Stability results indicated that the CSP7 trifluoroacetate in lyophilized cakes containing mannitol (1.5% w/v; 1:140) can resist chemical degradation in the solid state for up to 1 month. In addition, there was no significant change in osmolality (range between 374–378 mOsm/kg) throughout the study period. Residual moisture content of the samples increased from 0.5% at initial to 1.21% and 0.92% after storage at 5°C and 25°C, respectively, however, aggregation of the peptide was not detected when reconstituted after 1-month storage. Thus, storage of mannitol based formulations without exposure to moisture results in both excellent chemical stability of CSP7 and stable osmolality of the solution upon reconstitution.

The curves with low slope in Figures 1A, 1B and 2 show the good stability of the samples since the potency stays close to 100% independently of the study time. Under agitation (Figure 1A) CSP7 trifluoroacetate is almost equally stable in the solution at 25°C and at 5°C. Whereas, without agitation CSP7 trifluoroacetate is more stable than with agitation (comparing the graphs from Figure 1A and Figure 2A). On the other hand, freeze-thaw cycles, especially at –80°C, do not affect stability of the CSP7 trifluoroacetate. Figure 1B shows the potency of CSP7 trifluoroacetate at –80°C is safer than that of at –20°C.

The results from Figures 2A and 2B can be concluded that inactivation of the peptide in solid state (Figure 2B) tends to be at least an order of magnitude slower than that in the solution (Figure 2A).

3.5 Long term stability of the lyophilized CSP7 trifluoroacetate-bulking agent cakes

To test long term stability of CSP7 trifluoroacetate cakes in the absence of moisture a set of cakes of different formulations was kept at 5°C in a desiccator. Thus, the cakes were

protected from humidity during this stability study to avoid an increase of moisture content. After 10 months at 5°C cakes were reconstituted and analyzed for CSP7 trifluoroacetate using HPLC, as described under Materials and Methods. The results of the analysis (Figure 3) demonstrate high stability of the lyophilized cakes of CSP7 trifluoroacetate with mannitol (1:70 and 1:140) or lactose (1:5; 1:70 and 1:140) stored without moisture. The slightly aggregates were observed in CSP7 trifluoroacetate-mannitol (1:5) cakes (not shown) after reconstitution. Thus, this formulation was not analyzed. The other solutions (Figure 3) were clear and colorless. Figure 3 shows that the percent potency of the samples was between 92.7% (± 1.0) and 97.4% (± 1.9). Regarding this study, without exposure to humidity, the CSP7 trifluoroacetate-bulking agent formulations (except CSP7 trifluoroacetate-mannitol 1:5) were sufficiently stable at 5°C for up to 10 months.

These results demonstrate that mannitol and lactose protect more than 92% of CSP7 trifluoroacetate up to 10 months at 5°C without exposure to moisture and any of formulations shown in Figure 3, if stored properly, could be used for further *in vivo* studies of CSP7 trifluoroacetate. On the other hand, an increase in moisture content in the cake during storage was associated with the lower Tg of the protein formulations. (32,50). The decreased Tg led to an increase in molecular mobility, crystallization of the bulking agent and protein instability (32). Thus, water vapor most likely transferred from around the rubber stoppers could affect the stability of the peptide. Indeed, at 8 weeks, the moisture content of samples stored at 5°C and 25°C without desiccator increased further to 1.99% ± 0.83 and 1.36% ± 0.47 , respectively.

Moreover, both samples exhibited turbidity upon reconstitution due to formation of insoluble aggregates, similar to those observed after 10 months at 5°C in formulation with mannitol (1:5). As the aggregates were observed in the cakes containing higher moisture content, the moisture- induced aggregation became concerned. Water sorption can increase molecular mobility which leads to a change of physical structure (22). Therefore, the effect of moisture on structure of the cake and aggregate formation of CSP7 trifluoroacetate was further studied.

3.5.1 Determination of water sorption isotherm—To evaluate ability of different formulations of CSP7 trifluoroacetate to absorb moisture, water absorption/desorption isotherms were determined as described under Materials and Methods. Figures 4A and 4B display the water sorption/desorption isotherms of the lyophilized CSP7 trifluoroacetate formulations containing mannitol and lactose, respectively. In general, amorphous solids absorb more water than their crystalline counterpart (50). The crystalline material adsorbs water molecules only at the surface, while amorphous organic solids have glassy state that allows water to diffuse through them as well as onto the surface. This theory coincides with our water sorption/desorption isotherms. The almost unchanged weight of bulk materials indicated that the bulk mannitol and lactose monohydrate were non-hygroscopic and in their crystalline state, which adsorbed minimal water vapor onto only their surface. On the other hand, the significant weight changes of the lyophilized formulations determined that they were hygroscopic solids. Figure 4A shows the sorption/desorption cycles of the lyophilized neat CSP7 trifluoroacetate and CSP7 trifluoroacetate with mannitol or lactose (1:5 molar ratio) formulations. The isotherm patterns showed that the lyophilized neat CSP7

trifluoroacetate rapidly absorbed considerable quantities of moisture at high %RH (70–90%RH) and that the lowest level of bulking agents (1:5) slightly affected the moisture sorption of CSP7 trifluoroacetate. Lyophilized CSP7-excipient formulations at all three molar ratios showed that the higher CSP7 trifluoroacetate-excipient molar ratio, the less water was absorbed (Figures 4B and 4C). We hypothesize that the higher the level of bulking agent, the more interactions can occur between peptide and bulking agents occur. At 1:70 and 1:140, the water sorption of both mannitol- and lactose-based formulations was lower than the neat CSP7 trifluoroacetate. Moreover, at the same molar ratio, the lactose-based formulation adsorbed much less water than the mannitol ones. This can be attributed to the greater chemical interaction between CSP7 trifluoroacetate and lactose than the mannitol. Once the hydrogen bonds between CSP7 there are less functional groups available for water to interact with the peptide and the less water capable of being absorbed. Sorption isotherms of CSP7 trifluoroacetate-lactose formulations showed that the lactose-based formulations began adsorbing moisture at about 40% RH. This is attributed to the amorphous state of the formulations. The CSP7 trifluoroacetate-lactose 1:5 formulation showed the highest percentage mass change. Rates of moisture sorption of the 1:70 and 1:140 were slower as a function of % RH, which may result from recrystallization of the lactose at higher % RH and the more interaction between CSP7 trifluoroacetate and lactose.

3.6 Effect of bulking agent on moisture-induced lyophilized CSP7 trifluoroacetate aggregation

Data shown in Figure 4 demonstrate that exposure of CSP7 trifluoroacetate cakes to moisture during storage results in an increase in the moisture content and, potentially, this can affect stability of CSP7 trifluoroacetate and induce crystallization of the bulking agent. Based on the results shown in Figure 4, the 12-hour exposure to 75% RH at 25°C was selected as the condition for further evaluation of the effect of moisture on structure of the cake and CSP7 trifluoroacetate stability. To investigate the effect of the bulking agents, lactose and mannitol, on aggregation of lyophilized CSP7 trifluoroacetate formulations, three drug-excipient molar ratios (1:5, 1:70 and 1:140) were prepared and exposed to 75% RH at 25°C. Mannitol and lactose were selected as bulking agents since they likely exhibit different physical states (crystalline and amorphous) after lyophilization. Exposure of the cakes to 75% RH at 25 °C for 12 hours resulted in a significant (up to an order of magnitude) increase in the moisture content (Figure 5).

3.6.1 Moisture content after exposure to high humidity environment—Karl Fischer titration (Figure 5) measured the moisture content of lyophilized CSP7 trifluoroacetate-bulking agent formulation before (previously discussed in section 3.2) and after exposure to 75% RH at room temperature for 12 hours. The results showed that lyophilized neat CSP7 trifluoroacetate absorbed considerably moisture after exposure to 75% RH. The moisture levels of mannitol- and lactose-based formulations decreased when the amount of bulking agents increased. At the same molar ratio, the lactose-based formulations tended to absorb more moisture than the mannitol-based ones. This was attributed to the addition of amorphous lactose. The amorphous sugars usually absorb water into their bulk structure (51). At the molar ratio of 1:5, mannitol and lactose-based formulations show higher moisture content than the lyophilized neat CSP7 trifluoroacetate,

however, there was no statistically significant difference between the formulations with bulking agents and neat CSP7 trifluoroacetate ($p > 0.05$). The formulations containing less mannitol exhibited greater moisture content. This can be explained since the 1:5 mannitol-based formulation contained amorphous mannitol, which absorbed more moisture than either the 1:70 and 1:140 formulations which contained a higher degree of recrystallized mannitol.

Such a significant ($p < 0.05$) increase in the moisture content (up to 36-fold for CSP7 trifluoroacetate-Mannitol 1:70 formulation) upon exposure to 75% RH (Figure 5) can reflect formation of crystals in the cakes. It is known that moisture induced crystallization of bulking agents, such as mannitol, or inorganic salts, affects chemical stability of proteins and peptides as well as promote their aggregation (24,27,30,52,53). To test this hypothesis lyophilized cakes exposed to 75% RH were analyzed using PXRD and DSC. Lyophilized cakes of the same formulations, which have not been exposed to 75% RH, were used as controls.

3.6.2 Powder X-ray diffraction (PXRD) analysis—Figure 6 shows the X-ray diffractograms of the lyophilized CSP7 trifluoroacetate- stabilizing agent formulations, bulk materials, and lyophilized neat peptide without bulking agent. The sharp peaks at $2\theta = 32^\circ$ and 26° present in all lyophilized samples are phosphate crystal peaks from the DPBS, which is used as solvent buffer in the formulations (54).

The XRD results of neat CSP7 trifluoroacetate and the mannitol containing formulations are shown in Figure 6A. Bulk mannitol as purchased was in β crystalline form and is shown as spectrum “a” while the crystalline mannitol a polymorph is shown as spectrum “b”. The XRD 9.7, 14.5, 19.7, and 24.8 deg, 2θ have been reported (55). The lyophilized neat CSP7 trifluoroacetate in DPBS before and after exposure to 75% RH were amorphous (spectra “c” and “d”). The lyophilized CSP7 trifluoroacetate-mannitol (1:5) formulation was partially transformed to its amorphous state therefore, very low intensity peaks of δ mannitol were observed (spectrum “e”) (56). The XRD patterns of the 1:70 and 1:140 molar ratio of the mannitol-based formulations (spectrum “g” and “i”) indicate the mixtures of polymorphs of crystalline mannitol. The peaks of δ , α and β mannitol are present. Intensity of the crystalline mannitol peaks (at 9.7, 14.5, 19.7, 20.5 and 24.6 deg; Figure 6A) increased as the amount of mannitol in the formulation was increased. Based on the XRD data acquired in this study, β mannitol was predominantly transformed to mixtures of δ (and/or α) polymorphs after lyophilization. In addition, the peak at 9.7, and 18 deg, 2θ maybe the peaks of mannitol hemihydrate, which is the crystalline fraction within the freeze-dried products, and this hydrate is metastable and prone to lose water at room temperature easily (55,57). As a result, water can interact with the peptide and induce aggregation. After exposure to 75% RH, the major peaks of δ , α (and/or hemihydrate form) mannitol were smaller or no longer so apparent while the intensity of the β polymorph peaks increased (spectrum “f”, “h” and “j”). The results indicate that unstable polymorphic forms of crystalline mannitol are converted to the most stable β form when moisture in the cakes is increased. Formation of crystalline mannitol coincided with an increase in the moisture in the formulation (Figure 5) and with aggregation of CSP7 trifluoroacetate upon reconstitution of the lyophilized cakes.

Figure 6B shows the XRD results of CSP7 trifluoroacetate-lactose formulations. The absence of crystalline lactose patterns (peaks 12.5, 20 and 21.5, Figure 6B) indicated that amorphous lactose was fully formed at all CSP7 trifluoroacetate-lactose molar ratios following lyophilization (spectra “d”, “f” and “h”). Amorphous lactose in the 1:5 CSP7 trifluoroacetate- lactose formulation remained unchanged after exposure to 75% RH (spectrum “e”); whereas the crystalline lactose developed in the 1:70 and 1:140 formulations after exposure to 75% RH (spectrum “g” and “i”). Thus, similar to mannitol, lactose recrystallization was also induced by water absorbed during 12h exposure to 75% RH. Thus, an increase in the moisture in the cake (Figure 5) induces crystallization of bulking agents and inorganic buffer salts, which could significantly affect stability and solubility of the CSP7 trifluoroacetate.

3.6.3 Differential scanning calorimeter (DSC) analysis—DSC is used to detect crystallization of various organic materials including excipients (58). In this study, we focus on the endothermic peaks indicating such events as phase transitions, conformational changes or thermal decomposition of crystal hydrates. Figures 7A-D show the effect of exposure to 75% RH on DSC thermograms of the CSP7 trifluoroacetate- mannitol and lactose formulations. The melting peak of peptide was not present indicating the amorphous state. As mentioned earlier, CSP7 trifluoroacetate formulations were prepared in DPBS buffer, which consists of sodium chloride (NaCl), potassium chloride (KCl), dibasic sodium hydrogen phosphate dihydrate (Na₂HPO₄ 2H₂O) and monobasic potassium phosphate (KH₂PO₄). There are various studies reporting the effects of buffer salts on crystallinity of the bulking agents, such as delayed lactose crystallization, polymorphic behavior of mannitol, or inhibition of mannitol crystallization (59–62).

DSC data of the CSP7 trifluoroacetate-mannitol formulations before exposure to 75% RH showed that the low extent of crystalline mannitol in the CSP7 trifluoroacetate-mannitol formulation at the lowest molar ratio (1:5) was present as a very small endothermic peak at 135°C. This melting endotherm indicated the endothermic transition of mannitol polymorphism (59). A similar peak was observed such that the melting point of δ mannitol was detected at 139°C or 136°C, when mannitol was prepared in mixtures with inorganic salts 10% w/w of NaCl and KCl, respectively. These melting temperatures are different from the melting point of pure δ mannitol reported as 155°C (59). Even though the δ polymorph is difficult to produce, it frequently occurs in freeze-dried mate (59).

DSC thermograms of the higher CSP7 trifluoroacetate-mannitol molar ratios of 1:70 and 1:140 showed melting endotherms of mannitol at 145°C and 150°C, respectively (Figure 7A). These results were comparable to those reported by Telang et al. (2003) (59). Shifting of melting temperatures of the bulk to processed mannitol from 168 °C to 150 °C can be explained by the impact of NaCl on polymorphism of mannitol. The melting point of mannitol when mixed with NaCl was depressed since NaCl had melt miscibility with mannitol (59). The endothermic temperature of CSP7 trifluoroacetate-mannitol (1:70) formulation (145°C) was 5°C lower than that of the reported value of lyophilized mannitol with NaCl. This may be due to the additional effect of salts from DPBS present in the lyophilized sample. For the 1:140 molar ratio formulation, the melting point of the formulation was 150°C since this sample contained a higher amount of β mannitol and less δ

form and amorphous mannitol (Figure 6). It has also been reported that potassium phosphate salts inhibit crystallization of mannitol during the cooling step of lyophilization (63). However, KH_2PO_4 contained in the DPBS buffer used in our study is much less than the NaCl amount. Thus, the crystalline inhibition effect from this salt may be concealed by the effect of the NaCl.

Following exposure to 75% RH for 12 hours, the DSC thermograms of the CSP7 trifluoroacetate-mannitol (1:5) lyophilized formulation showed the broad endothermic event from 90–130°C. This peak probably corresponds to the melting endotherm of crystalline mannitol shifted in the presence of water. The previous study also reported that in the presence of water, the broad endothermic event at temperature lower than the melting point of mannitol crystals was attributed to the temperature-depressed melting of anhydrous crystalline mannitol (55). The endothermic peaks at 148°C were determined in the other two mannitol formulations (1:70 and 1:140). The DSC results confirmed the XRD data, indicating that before and after exposure to 75% RH, moisture induced crystallization of mannitol, observed by XRD (Figure 6). The small amount of the unstable form of crystalline mannitol in the 1:5 was more difficult to detect by DSC.

The DSC curve of bulk lactose monohydrate shows three distinct endothermic peaks at 146°C, 219°C and 245°C, representing the dehydration of lactose monohydrate, melting, and decomposition temperatures of lactose crystalline lattice, respectively (64). After lyophilization, the dehydration endotherm was absent from all CSP7 trifluoroacetate-lactose formulations. DSC thermograms of the CSP7 trifluoroacetate-lactose 1:70 and 1:140 formulations showed a small melting endotherm at ~195°C (Figure 7B), which most likely reflects the DSC-induced lactose recrystallization at elevated temperature. Notably, crystallization of lactose was not detected by XRD without heating the cake (Figure 6B).

After exposure to 75% RH, the CSP7 trifluoroacetate-lactose (1:5) formulation showed a relaxation endotherm from the amorphous lactose at 120°C (65). The 1:70 and 1:140 exhibited two distinct endothermic transitions at about 146°C and a broad endothermic event between 160–210°C, which two distinct endothermic transitions at about 146°C and a broad endothermic event between 160–210°C, which represented the dehydration of lactose monohydrate and the melting lactose (64). These results were in good agreement with the XRD data (Figure 6), which indicated that the physical state of the materials (crystallization) depended on the relative amount of lactose in the formulations.

As shown in the PXRD and DSC data, after lyophilization, all CSP7 trifluoroacetate-lactose formulations were amorphous and in the anhydrous form. Therefore, at the same molar ratio, the lactose-based cakes had greater water uptake than CSP7 trifluoroacetate-mannitol cakes. This was described previously (31), where amorphous lyophilized lactose/peptide cakes had greater moisture affinity than those containing mannitol. The stability of protein/peptide products can be affected by crystallization of excipients during freeze-drying (25). Thus, possible mechanisms of CSP7 trifluoroacetate aggregation as well as properties of aggregates were evaluated next using chemical assay and other analytical methods.

3.7 Moisture-induced aggregation of lyophilized CSP7 trifluoroacetate (mannitol- and lactose-based formulations) is reversible

The formation of aggregates induced by moisture in both mannitol- and lactose-based formulations was investigated. After exposure of lyophilized CSP7 trifluoroacetate formulations to 75% RH for 12 hours followed by reconstitution with SWI, turbid solutions containing aggregates were obtained. The reconstituted solutions of CSP7 trifluoroacetate-mannitol formulations appeared notably more turbid than the lactose-based formulations. The aggregates were isolated by centrifugation and resuspended in DPBS and dissolved by adjusting pH to approximately 8.0 by addition of small amount (less than 1% v/v) of ammonium hydroxide. CSP7 trifluoroacetate in re-dissolved aggregates was characterized by HPLC, and LC/MS and MALDI-TOF MS as described in Materials and Methods. Neither of methods has detected CSP7 multimers proving that the aggregation is reversible and that the soluble aggregates were that the soluble aggregates were converted to soluble monomeric species of the peptide.

Since degradation products of the CSP7 aggregation does not affect chemical stability of CSP7 it has been reported that physical protein/peptide aggregates could be reversible by adjustment of solvent properties such as pH, and/or temperature (66–68). CSP7 trifluoroacetate consists of four threonines, which contain hydroxyl groups, and are considered to be a strong water-binding residue (69). These water-binding sites on the peptide molecule lead to water adsorption when the peptide is exposed to a high moisture environment (70). The amino acids, which contain weak water-binding sites can also contain sorbed water (69). Reversing CSP7 trifluoroacetate aggregation by adjusting pH with ammonium hydroxide can indicate stabilization of aggregates by protonated N-terminal amino group. However, an increase in the ionic strength by adding NaCl up to 2 M did not result in dissolving the aggregates (not shown). Therefore, hydrophobic interactions likely significantly contribute to stabilization of CSP7 trifluoroacetate aggregates. Chemical assay was used next to quantitate CSP7 trifluoroacetate aggregation in moisture exposed formulations with different molar ratios of mannitol and lactose.

3.8 Chemical assay of the aggregates

Two types of clear solutions obtained from the supernatants (1), and the solutions of redissolved aggregates (2) were tested by HPLC and LC/MS in order to determine distribution of CSP7 trifluoroacetate between solution and solid phase after reconstitution of different formulations exposed to 75% RH. Figure 8 shows the results of these analyses as the relative percent of each sample as compared to the freshly prepared samples (100%). The total percent recovery of CSP7 trifluoroacetate from solutions (1) and (2) was nearly 100%, demonstrating that after reconstitution all CSP7 trifluoroacetate is distributed between solution and aggregates and confirming that the moisture-induced aggregates of CSP7 trifluoroacetate are a reversible type. The fraction of aggregates (Figure 8) of CSP7 trifluoroacetate from the mannitol-based formulations (~50%) were substantially greater than that of the lactose-based formulations (~2.4–14.7%). The lyophilized neat CSP7 trifluoroacetate formulation exhibited the highest aggregation (~58%) since there was no stabilizing agent to protect the peptide from aggregation. It indicates that the peptide itself tends to aggregate. The percentage of aggregates in the mannitol containing formulations

was not significantly different. The amount of mannitol in the CSP7 trifluoroacetate-mannitol (1:5) formulation is not sufficient to protect the peptide from aggregation while recrystallization of mannitol in the 1:70 and 1:140 formulations reduced the stabilizing effect of mannitol. Amounts of CSP7 trifluoroacetate aggregates in lactose-based formulations were ranked from high to low as follows: 1:5, 1:140 and 1:70, respectively. Aggregation of CSP7 trifluoroacetate in the 1:70 formulation was lowest since this formulation was amorphous. The CSP7 trifluoroacetate-lactose (1:5) formulation was also amorphous. However, the greater extent of aggregation may result from an insufficient stabilizing property of lactose at this low level. Aggregates of peptide in the 1:140 formulation were attributed to the initial high residual moisture content, which can induce peptide aggregation prior to exposure to 75% RH. Two mechanisms can explain this phenomenon, including physical properties of the bulking agents during lyophilization and interactions between bulking agents and CSP7 trifluoroacetate.

The results agreed with a previous study by Herman et al. (1994), which proposed that the effect of water on amorphous drugs does not depend on the amount of water present, but rather on where it is located. In a lyophilized formulation, mannitol crystallizes either during lyophilization or storage depending on the ratio of mannitol to drug (71). There is not a significant amount of water associated with crystalline mannitol as shown in the sorption/desorption isotherms of bulk mannitol (crystalline) (71). The prior studies also reported similar results; as the crystallinity of mannitol increases, its stabilizing effect is reduced (25,27,72,73). When mannitol recrystallizes with time, water in the lyophilized material redistributes and results in an increase in the free water in the drug microenvironment since mannitol is non-hygroscopic and cannot act as a “sink” for the liberated water (16,25). On the other hand, all lactose formulations remained in their amorphous state after lyophilization. Amorphous lactose may act as an internal desiccant to absorb water into itself therefore, water would not be available to interact with the peptide, resulting in significantly less aggregates formed. Oliyai et al. (31) similarly found that amorphous lactose decreased the number of free water molecules available for chemical reaction with the peptide as compared to the mannitol containing systems. Moreover, lactose also has a much higher T_g than mannitol therefore, it can better retain its glassy state of the formulations with exposure to increased moisture.

An alternative (synergistic) mechanism is interaction between CSP7 trifluoroacetate and bulking agent. CSP7 trifluoroacetate containing a high portion of threonine is also able to form intermolecular hydrogen bonds such as crosslinking (74). Mannitol obviously did not affect aggregation since crystallization of mannitol during lyophilization was not able to form hydrogen bonding to the dried protein (32). In contrast, an increase in the molar excess of lactose almost completely removed aggregation. Interactions of sugars (lactose or trehalose) with dried proteins was investigated (75). The study provided evidence that not only hydrogen bonding occurred between the stabilizing agent-protein, but also that sugar binding is essential for sugar-induced stabilization of protein during lyophilization and rehydration. Moreover, it was conjectured that the hydroxyl groups of bulking agents bond (acting as hydrogen acceptors and donors) with proteins. Therefore, the sugar serves as “water substitutes” to insure conformational stability (76). Finally, lactose, as a reducing sugar, could potentially stabilize peptide in the solution by forming a Schiff base (28,41,77)

due to a reaction with N-terminal amino group of CSP7 trifluoroacetate. Since a Schiff base is reversible, its formation should not affect the biological activity of CSP7 trifluoroacetate, and could serve as a reservoir for the peptide in vivo. Based on these results, it appears that lactose stabilized CSP7 trifluoroacetate against moisture induced aggregation in the solid state to a greater degree than mannitol. In summary, while both pH adjustment and using lactose (1:70 or more) prevented CSP7 trifluoroacetate aggregation, the stabilizing effect of lactose is valuable for using the formulation at physiological pH.

3.9 Effect of nebulization on chemical stability of CSP7 trifluoroacetate

Both CSP7 trifluoroacetate formulations stored without exposure to moisture with mannitol (1:140) and lactose (1:70) easily dissolved in SWI. As compared with the initial bulk solution before lyophilization, the chemical stability of CSP7 trifluoroacetate was not affected by sterile filtration or lyophilization (recovery nearly 100%). Chemical stability of the CSP7 trifluoroacetate from mannitol and lactose containing formulations following nebulization by Aeroneb® Pro nebulizer/EZ Breathe® atomizer were 98.9%/98.2% and 98.7%/98.5%, respectively. These results clearly indicate that efficiency of the vibrating mesh nebulizers; Aeroneb® Pro and the EZ Breathe® was comparable and that nebulization of CSP7 trifluoroacetate solutions were not significantly different. The nebulization conditions (e.g., shear and temperature from the nebulizers) did not affect the chemical stability of CSP7 trifluoroacetate. Stability of the peptide was not significantly decreased by the aerosol cloud collection technique (e.g., peptide did not adsorb onto the surface of the collecting tube) and the degradation products and aggregates of the CSP7 trifluoroacetate were not detected after nebulization.

4. Conclusion

Lyophilized CSP7 trifluoroacetate absorbed moisture, which induced its aggregation. Even though the aggregates are reversible by pH adjustment, once they are formed, it leads to a decrease in chemical stability. Amorphous lactose performed remarkably superior to crystallized mannitol in terms of preventing peptide aggregation. Lactose possibly had a significant interaction with amino acids containing in CSP7 trifluoroacetate exhibiting the stabilizing effect. Long-term stability study indicated the stable formulations and a suitable storage condition for the lyophilized CSP7 trifluoroacetate. While this study determined that 1:70 CSP7 trifluoroacetate:lactose formulation is optimal for long-term shelf storage, possible differences between biological activity of mannitol and lactose formulations will be evaluated in the next step by in vivo comparison of moisture free CSP7 trifluoroacetate formulations. Nebulization using vibrating mesh nebulizers did not affect the potency of the peptide. This stable formulation is useful in an upcoming *in vivo* study. The mechanisms between lactose and CSP7 trifluoroacetate in increasing the stability of the lyophilized peptide cake should be further investigated.

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Figure 1.

Effect of agitation, freeze-thaw cycles and storage temperature on chemical stability of CSP7 trifluoroacetate, $n = 2$

(A) Effect of agitation: solid and dash lines represent the best fit by linear regression for 5°C (blue) and 25°C (red), respectively

(B) Effect of freeze-thaw cycles: solid and dash lines represent the best fit by linear regression for 5°C (blue) and 25°C (red), respectively

(C) Effect of storage temperature on chemical stability of CSP7 trifluoroacetate, $n = 2$

Figure 2. Chemical stability of (A) reconstituted CSP7 trifluoroacetate solutions after storage at 5°C (blue) and 25°C (red) for 8 hours, and (B) of lyophilized CSP7 trifluoroacetate-mannitol (1:140) cakes after storage at 5°C (blue) and 25°C (red) for 4 weeks

Figure 3. Effects of different amounts of mannitol and lactose on stability of CSP7, trifluoroacetate after 10-month storage without exposure to humidity at 5°C, no data of CSP7 trifluoroacetate-mannitol 1:5 presented due to its aggregation, $n = 3$

Figure 4.

Sorption and desorption isotherms of lyophilized neat CSP7 trifluoroacetate and lyophilized CSP7 trifluoroacetate:mannitol formulations (A), and CSP7 trifluoroacetate:lactose formulations (B). The sorption isotherms are shown as dashed lines, and the desorption ones are in solid lines.

Figure 5.
Residual moisture content of the lyophilized CSP7-bulking agent formulations after freshly prepared and after exposure to 75% RH at 25°C for 12 h, n = 3

Figure 6.

PXRD of CSP7 trifluoroacetate:bulking agent; CSP7 trifluoroacetate:mannitol (A) before (a, b, c, e, g, I and k), after exposure to 75% RH (d, f, h and j), CSP7 trifluoroacetate:lactose (B) before (a, b, d, f, and h) and after exposure to 75% RH (c, e, g and i) (The curves were presented in relative scales.)

Figure 7.

DSC thermograms of lyophilized CSP7 trifluoroacetate:bulking agent before (A and C) and after exposure to 75% RH for 12 hours (B and D); mannitol based formulations (A and B) - bulk mannitol (a), α mannitol (b), lyophilized neat CSP7 trifluoroacetate (c), CSP7:mannitol 1:5 (d), CSP7 trifluoroacetate:mannitol 1:70 (e), CSP7 trifluoroacetate:mannitol 1:140 (f) and lactose based formulations (C and D) - bulk lactose monohydrate (a), lyophilized neat CSP7 trifluoroacetate (b), CSP7 trifluoroacetate:lactose 1:5 (c), CSP7 trifluoroacetate:lactose 1:70 (d), and CSP7 trifluoroacetate:lactose 1:140 (e) (The curves were stacked over.)

Figure 8.

Amounts of CSP7 aggregates formed after exposure the lyophilized formulations to 75% RH at room temperature for 12 hours, n = 2

Table 1.

Lyophilized cycle parameters

	Temperature (°C)	Hold time (min)	Pressure (mTorr)	Ramp (°C/min)	Time (min)
Load	5	60	ATM *	-	-
Freeze	-55	120	ATM	0.5	120
Anneal	-15	120	ATM	0.5	80
Freeze	-55	240	ATM	0.5	80
Evacuate	-55	10	100	-	-
Drying					
Primary	-30	660	100	0.1	250
Secondary	30	240	100	0.08	720

* ATM = atmosphere