The Protective Effect of Lactose on Lyophilization of CNK-20402

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ABSTRACT

The goal of this research was to assess the feasibility of using lyophilization to stabilize an exploratory compound, CNK-20402, with a minimal amount of impurity (CNK-20193) formation. A mixed-level full factorial experimental design was used to screen excipients of glycine, mannitol, lactose monohydrate, and povidone K-12. Cryostage microscopy, powder X-ray diffraction, Karl Fischer titration, HPLC, and water vapor sorption were used to assess the formulations' physicochemical properties and stability. Initial physical characterization from powder X-ray diffraction revealed that the mannitol- and glycine-containing formulations were crystalline with the patterns of the pure excipient, whereas the remaining formulations were amorphous in structure. Chemically, the formulations stored at 50°C for 1 month had 2.36%, 1.05%, 0.81%, 0.79%, and 0.49% CNK-20193 for glycine, mannitol, drug alone, povidone K-12, and lactose formulations, respectively. The formulations containing drug-mannitol, drug alone, and drug-lactose were selected for accelerated stability study based on statistical analysis. Recovery of CNK-20193 in these formulations was 1.22%, 1.00%, and 0.55%, respectively, when stored at 40°C/75% relative humidity storage conditions for 3 months. Water vapor sorption analysis revealed weight gains of over 7%, 21%, and 24% for the mannitol, lactose, and drug alone formulations, respectively. Testing formulations with different concentrations of lactose by water vapor sorption indicated that CNK-20402 concentrations as low as 10% (wt/wt) could inhibit the recrystallization of lactose. The lactose-containing formulation exhibited the best stability among the formulations tested. The protective mechanism of lactose on the CNK-20402, based on water vapor sorption studies, is believed to be a result of (1) the drug-lactose interaction, and (2) competition between lactose and drug for the residual water in the formulation.

KEYWORDS: solubility, stability, lactose, mannitol, lyophilization, water vapor sorption

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INTRODUCTION

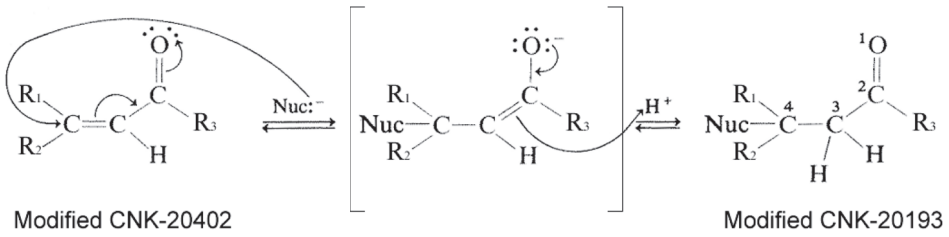
The active pharmaceutical ingredient (API), CNK-20402 (molecular weight ≈480), is an exploratory compound currently under investigation. It exists as a hydrochloride salt with an aqueous solubility of greater than 10 mg/mL. For clinical investigation, CNK-20402 was formulated as a simple aqueous solution with a concentration of 2.5 mg/mL at pH 3 and stored at 5°C. Because of its unique structure, the API is believed to undergo an addition reaction, shown in Scheme 1,[1] to form a major impurity referred to as CNK-20193. The finished product had a shelf life of only 12 months when stored at 5°C.

Preliminary studies indicated that the use of cosolvents could slow down the formation of CNK-20193. However, the lower polarity of the cosolvents triggered the formation of other impurities, making the finished product unacceptable. The objective of this study was to use a lyophilized approach for the development of a commercial product and, furthermore, to investigate the impact of the excipient on the product stability. A mixed-level full factorial experimental design was used to screen various bulking agents and evaluate stability of formulations having different drug loads and drug/excipient ratios. Four commonly used lyophilization fillers were evaluated as potential bulking agents: glycine, mannitol, lactose monohydrate, and povidone K-12.

MATERIALS AND METHODS

Materials

Glycine, mannitol monohydrate, α-lactose monohydrate, hydrochloric acid, and sodium hydroxide were purchased from Sigma (St Louis, MO). Povidone (K-12) was received from BASF (Mount Olive, NJ) as a gift. Water for irrigation was obtained from Baxter (Round Lake, IL). Sterile 0.22-μm polyethersulfone filter systems were obtained from Corning Inc (Corning, NY). All chemicals were either USP or analytical grade and were used as received. Bromobutyl rubber lyophilization stoppers, 20-mm FM257/2 with Omniflex coating, were obtained from Helvoet Pharma (Pennsauken, NJ) and 20-mm, 20-mL USP/EP Type I Borosilicate glass vials were obtained from West Pharmaceutical Services (Lionville, PA). All materials were used as received without further processing.



Scheme 1. Proposed formation mechanism of CNK-20193 where the nucleophile is another functional group on CNH-20402. This scheme is modified from the textbook of *Organic Chemistry*.[1]

Experimental Designs and Data Analysis

Fusion Pro, a statistical software package developed by S-MATRIX (Eureka, CA), was used to create a mixed-level full factorial experimental design. The formulation factors such as drug loading, drug-to-excipient ratio, and type of bulking agent were evaluated at various storage temperatures and time intervals. The initial design matrix is depicted in Figure 1. In total, 18 formulations were prepared and placed in ovens maintained at 25°C and 50°C for the stability screening. Sixteen formulations contained bulking agents and the remaining 2 formulations contained CNK-20402 alone as controls. The data generated from these screening studies were subsequently imported into Fusion Pro to assess the effects of the formulation variables. Initial statistical analysis was based on percent label claim and normalized CNK-20193 content (HPLC % peak area). The optimization feature in the Fusion Pro software package was used to rank formulations according to their ability to minimize CNK-20193 formation and maximize percent parent remaining.

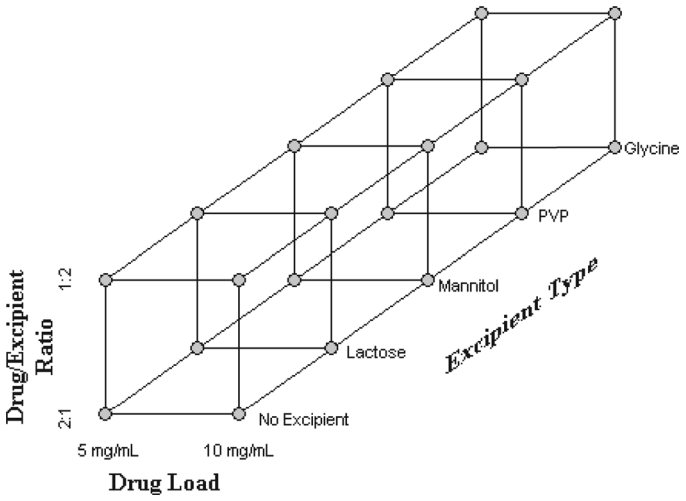


Figure 1. Mixed-level full factorial experimental design for formulation screening.

Analytical Methods

Sub-ambient Characterizations

The collapse or eutectic temperatures of all formulations were assessed using an Olympus BX 50 microscope (Melville, NY) equipped with a cryostage and thermal control system by Linkam (Surrey, England). Liquid nitrogen was used as a coolant and the thermal events were recorded for collapse temperature assessment.

Powder X-ray Diffraction Characterizations

An X-ray powder diffractometer, Rigaku Ultima Plus (Woodlands, TX), with Jade software (Materials Data, Inc., Livermore, CA) was used. XRD patterns were obtained under ambient conditions by exposing the sample to Cu K_α radiation (40 Kv × 40 mA), using a scan rate of 3°/min in the range of 3° to 45°, 2-theta.

Electrochemical Analysis

The moisture content of the lyophilized cakes was determined at ambient conditions using a Mettler-Toledo Karl Fischer (KF) titrator (Toledo, OH). The KF system was calibrated with 3 injections of distilled water to a relative standard deviation of less than 1% before sample analysis.

All pH measurements were performed on an Accumet 60 pH meter equipped with an Accumet Micro glass electrode (Fisher Scientific, Chicago, IL) using a 2-point standardization with pH 1.68 and 4.00 buffers.

HPLC Analysis

Reconstituted or liquid samples were analyzed with an Agilent Technologies 1100 Series liquid chromatography system (Palo Alto, CA) equipped with a Phenomenex LUNA C18(2) column with 5-μm particle size and 250 × 4.6 mm ID (Torrance, CA). Turbochrom software from Perkin Elmer (Shelton, CT) was used for data collection and analysis. The mobile phase was 0.05 M ammonium

acetate:acetonitrile:methanol:triethylamine (40:55:5:0.15). The HPLC was operated isocratically using 22°C column temperature, 1.0 mL/minute flow rate, 254 nm, and 15-μL injection volume. System suitability was evaluated in accordance with USP 621>: system precision of 5 replicate standard preparation injections (RSD $\leq$ 2.0%); resolution between the CNK-20402 and CNK-20193 peaks ($R_s >$ 1.5); number of theoretical plates (N $\geq$ 8000); and tailing factor for the CNK-20402 peak (T $\leq$ 2.0).

Water Vapor Sorption Isotherm Measurements

Water vapor sorption was investigated using a gravimetric sorption analyzer, SGA-100 (VTI Corp, Hialeah, FL). The balance on the analyzer was calibrated using a 20-mg standard weight (National Institute of Standards and Technology grade). Samples were loaded onto a platinum sample holder and equilibrated under dry nitrogen purge for 2 minutes before the start of a run. The relative humidity (RH) was increased sequentially in 10% increments from 10% to 90% RH at 25°C. The equilibration criteria for each step were set for less than 0.02% weight variation over a 5-minute or 3-hour maximum equilibration time.

Formulation Development and Stability Protocols

Screening of Formulations

The compositions of all formulations are depicted in Figure 1. The drug solutions were prepared by dissolving the active and the respective filler in water (water for irrigation grade) followed by adjusting the pH between 3 and 4 with 1N hydrochloric acid. This pH range was previously determined to be optimal for the stability of the API. These formulations were then filtered through a 0.22 -μm filter and 5-mL aliquots were transferred into 20-mL lyophilization vials. These vials were then partially capped with lyophilization stoppers, and freeze-dried using the VirTis freeze dryer (Genesis 35EL with Maestro software, Gardiner, NY). To minimize the impact of the variations in the manufacturing process, all formulations were lyophilized together. Thermocouples were placed into representative samples for monitoring product temperatures. The freeze-drying cycle was programmed based on an average product temperature as described according to the following cycle: (1) cooling and freezing at a shelf temperature of −50°C and maintaining average product temperature at −40°C for 10 hours; (2) primary drying with a heating rate of 0.1 degree per minute to 10°C, at a chamber pressure of 0.1 mm Hg and a condenser temperature of −80°C; (3) secondary drying at a shelf temperature of 50°C for 4 hours after product temperature reached 10°C from primary drying, at a chamber pressure of 0.1 mm Hg and a condenser temperature of −80°C; and (4) fully stoppering vials under nitrogen purge at the end of the cycle. Vials were capped with aluminum flip-top caps using a manual pneumatically powered capper.

Finished lyophilized products were placed in ovens at 25°C and 50°C for stability assessment. Samples were pulled at 0, 1, 2, and 4 weeks for analysis. At the initial time point, all formulations were assayed by HPLC to determine potency and impurities. Formulations with 10 mg/mL drug and 20-mg/mL excipient load were characterized using powder X-ray crystallography, Karl Fischer moisture analyzer, and pH meter (after reconstitution).

Proposed Lead Formulations

Based on the Fusion Pro optimization, the following top 3 formulations from the screen matrices were selected for further development: 10 mg/mL of CNK-20402 alone, 10 mg/mL CNK-20402 with 20 mg/mL of lactose, and 10 mg/mL CNK-20402 with 20 mg/mL of mannitol. New solutions were prepared and all 3 formulations were adjusted to pH 3.0 $\pm$ 0.3 prior to lyophilization. These formulations were freeze-dried simultaneously using the same lyophilization cycle as described above.

Since all formulations were prepared using an unoptimized lyophilization cycle and used unprocessed stoppers, it was critical to test the robustness of the formulations. Some finished products were spiked with water at 3% and 6% (wt/wt) of total cake mass. The additional water was charged into 50-μL centrifuge tubes and these tubes were placed upright in the vials containing the lyophilized cakes. This procedure, modified from Kovalcik and Guillory,[2] prevents the cake from being dissolved by direct contact with the water and, to a certain degree, mimics the equilibrium of water vapor between cake and processed stopper. The finished products, as well as water-spiked samples, were stored in stability chambers maintained at 5°C, 25°C/60% RH, and 40°C/75% RH. Samples were pulled at predetermined intervals for powder X-ray, moisture content, pH, and HPLC analysis.

In addition, 20 mg/mL of lactose placebo, and 1 mg/mL CNK-20402 with 10 mg/mL lactose formulations were prepared for dynamic vapor sorption test purposes. These additional lyophilized samples were prepared under the same conditions as the other lyophilized cakes described above.

RESULTS AND DISCUSSION

The initial physical properties of some representative formulations are listed in Table 1. Since no formulation had a collapse or eutectic temperature lower than −40°C, all

Table 1. Physical Parameters of Initial Screening Formulations*

Formulation	$T_{collapse}$ (°C)	Physical State of Formulation	Moisture Content (%)	pH After Reconstitution
No excipient	−11	Amorphous	3.67	3.59
Mannitol	−3	Crystalline with the features of mannitol	0.91	3.44
Glycine	−12	Crystalline with the features of glycine	3.35	4.09†
PVP	−25	Amorphous	0.95	3.44
Lactose	−27	Amorphous	1.57	3.21

* Only showed high drug and excipient load formulations data for comparison.
† Upward shift of 0.2 pH units from initial solution.

formulations were lyophilized together in a single run to eliminate run-to-run cycle variations. The lyophilized cakes of mannitol and glycine formulations exhibited PXRD features that matched the pure excipient PXRD features. The moisture content of the formulations ranged from 0.9% to 3.7%. All formulations demonstrated short reconstitution times of less than 1 minute. Except for glycine formulations, all other formulations showed no major pH drifts after reconstitution. The glycine formulation exhibited an upward pH shift of ~0.2 units after reconstitution.

Because the formation of CNK-20193 greatly determines the shelf life of the finished product, all stability samples were monitored for CNK-20193. Figure 2 displays the impact of storage temperature on the formation of CNK-20193. For the 5-mg/mL control drug formulation, the 50°C sample showed a higher CNK-20193 content than the 25°C sample. All other formulations had a comparable stability trend.

Figure 3 shows the formation of CNK-20193 after 1 month at 50°C in formulations containing the highest drug (10 mg/mL) and excipient (20 mg/mL) loads. As can be seen from the graph, the lactose formulation has the lowest CNK-20193 formation while the glycine formulation displays the highest CNK-20193 content (about 6 times higher than the lactose formulation). The remaining 3 formulations showed comparable levels of CNK-20193 formation. The formulations with lower drug and excipient loads exhibited similar trends in CNK-20193 formation (data not shown). Although the PVP formulation formed less CNK-20193 than the mannitol formulation, it also had a lower % recovery of parent compound and it was inversely related to the concentration of PVP.

HPLC assay results for the recovered parent compound, CNK-20193 content, and levels of other impurities were used as response factors in the statistical analysis. The statistical analysis suggested that the formation of CNK-20193 was significantly affected by the excipients glycine and lactose, as well as the storage temperature. The amount of CNK-20193 in the formulation increased with increasing temperature, as expected. When compared with the CNK-20402 alone formulation, the glycine-containing formulation had a 5-fold higher level of CNK-20193 ($P < .0001$) when stored at 50°C. Selection of lactose as an excipient decreased the amount of CNK-20193 formation ($P < .0029$) by as much as 30% when compared with the CNK-20402 alone formulation. Although 2-factor interaction

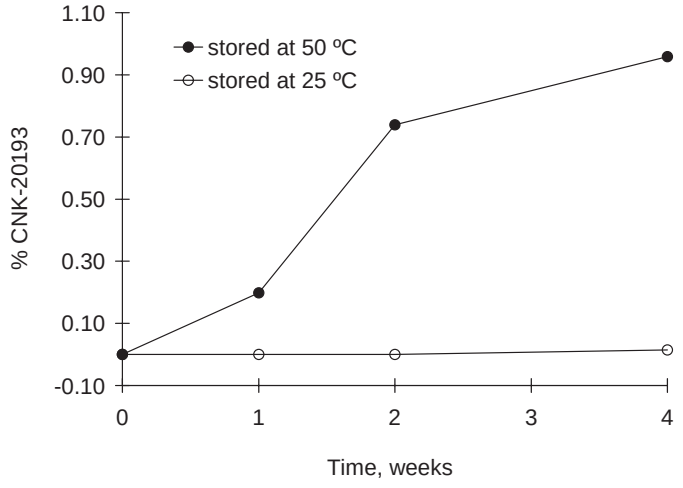


Figure 2. Impact of temperature on CNK-20193 content in 5 mg/mL of CNK-20402 lyophilized cake.

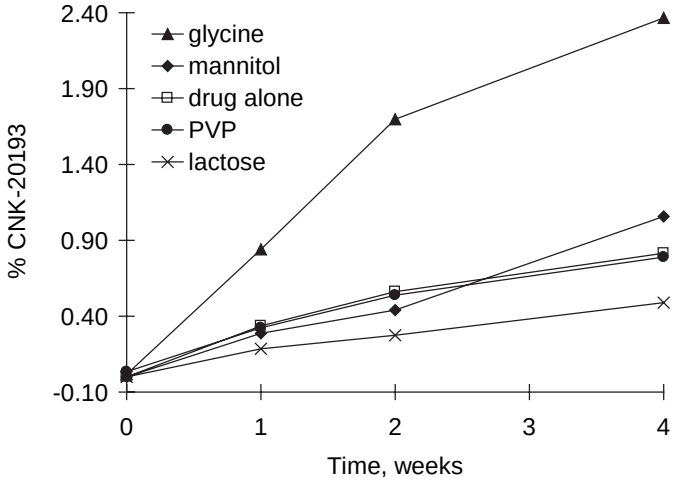


Figure 3. One-month stability on CNK-20193 content from representative formulations stored in a 50°C oven.

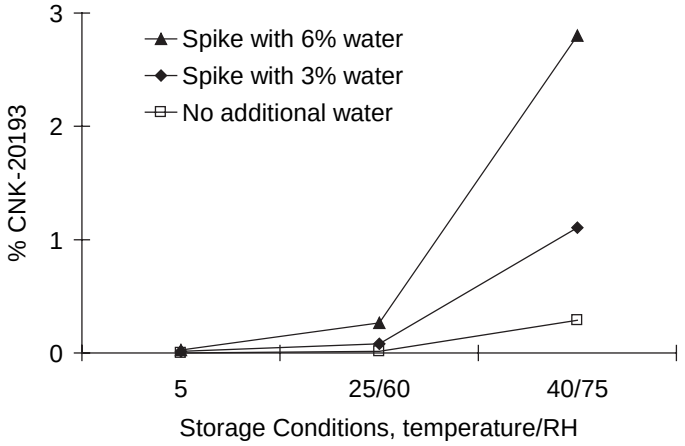


Figure 4. Impact of moisture on the stability of CNK-20402-alone lyophilized formulation.

between storage condition and excipient type exists, the storage temperature is the predominant factor independent of excipient type. Since a mixed-level full factorial design was applied for the initial screening studies, the data were sufficient to generate response surfaces (data not shown) and perform formulation optimization prediction. An optimization package within the Fusion Pro program was used to probe the formulations for their ability to both maximize the amount of parent remaining and minimize the formation of CNK-20193 and other degradants. Based on these results, excipients can be ranked in order of over stability enhancement as follows: lactose $>$ CNK-20402 alone $\approx$ mannitol $>$ PVP $>>$ glycine.

The top 3 formulations were selected for further development based on the statistical analysis described above. These formulations contained 10 mg/mL of active with either 20 mg/mL of lactose, 20 mg/mL of mannitol, or no excipient (control). These formulations were prepared at the same time and lyophilized. Moisture content for CNK-20402 alone, CNK-20402-lactose, and CNK-20402-mannitol formulations were 1.60%, 1.55%, and 2.47%, respectively. The pH values of all formulations reconstituted with water were within the initial range of 3.0 $\pm$ 0.3. By PXRD, both CNK-20402 and CNK-20402-lactose formulations were found to be amorphous. The mannitol-containing formulation was crystalline with the same X-ray features as the filler itself.

Since all formulations were prepared using unprocessed stoppers, which are known to absorb/desorb moisture during the lyophilization process and subsequent storage,[3,4] it was important to evaluate the robustness of the cakes with respect to moisture content. Therefore, cakes that were spiked with additional water were analyzed for chemical content and physical stability. Figure 4 shows the CNK-20193 content for the CNK-20402-alone formulation spiked with 3% and 6% (wt/wt) water after 1 month of storage at 50°C. This plot clearly indicates that the moisture content plays a key role in CNK-20193 formation. Lactose- and mannitol-containing formulations demonstrated similar trends in CNK-20193 formation. The addition of 3% and 6% (wt/wt) water spikes caused no changes in morphology for the CNK-20402-alone or mannitol-containing formulations. However, the lactose-containing formulation collapsed with the addition of 6% (wt/wt) of water when stored at 50°C. The collapse of cake from the lactose formulation could be due to the recrystallization of lactose at 50°C. Although the lactose-containing formulation showed a potential robustness issue with respect to physical stability, processing and rigorous monitoring of stoppers for their moisture content and use of an optimized lyophilization cycle may alleviate this issue.

Figure 5 shows the impact of 3% (wt/wt) water spiking on the formation of CNK-20193 in 3 types of formulations. CNK-20402 alone without water spiking is used as a control reference. It should be noted that lactose-containing formulations show levels of CNK-20193 comparable to the control. This observation suggests that the lactose-containing formulation is more chemically stable (or robust) than the CNK-20402 alone and mannitol-containing formulations.

Three-month accelerated stability data for 3 lyophilized formulations are plotted in Figure 6. The pH values of all reconstituted formulations were within the initial range of 3.0 $\pm$ 0.3. As can be seen from the plot, all 3 formulations showed less than 0.15% CNK-20193 formation at or below ambient storage conditions. Formation of CNK-20193 increased significantly, however, when samples were stored at harsher conditions (40°C/75% RH). The lactose-containing formulation showed better overall stability than the other 2 formulations. This observation is in agreement with the stability data generated from screening and robustness studies presented in Figures 3 and 5.

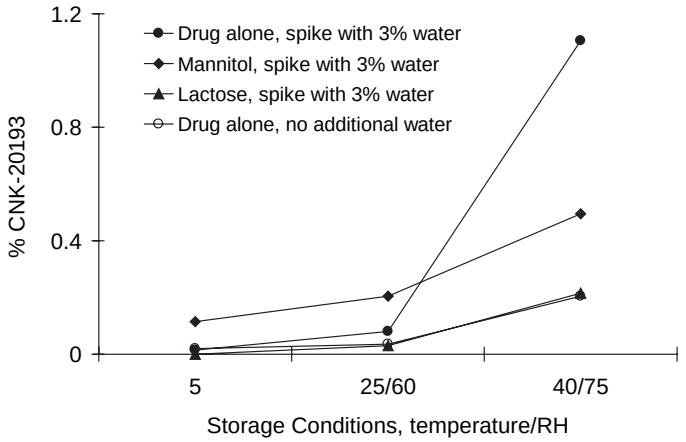


Figure 5. Impact of moisture on the stability of CNK-20402 in different excipient-containing formulations.

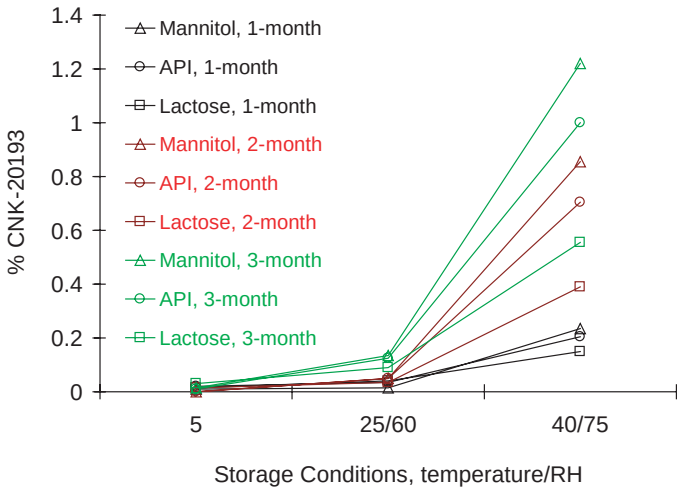


Figure 6. Stability of CNK-20402 formulations under different storage conditions.

The water vapor sorption data of the proposed formulations are displayed in Figure 7. The total weight gains of these formulations can be ranked as CNK-20402 alone > lactose > mannitol. Because VTI samples of lactose- and mannitol-containing formulations contained only 30% of the active compared with the CNK-20402-alone sample, the water sorption data must be normalized to allow proper comparison. If we assume that CNK-20402 is the only component that absorbs water vapor, then the values for the CNK-20402-alone sample should be divided by 3 in order to account for the relative amounts of CNK-20402 in the diluent-containing samples. The normalized data for the CNK-20402-alone sample is displayed as the closed circle symbol in Figure 7. Clearly, the normalized CNK-20402-alone formulation has a nearly identical weight gain as the mannitol-containing formulation suggesting that mannitol absorbs very little or no water vapor, leaving CNK-20402 to absorb the majority of the water vapor. In contrast, the lactose-containing formulation has a substantially higher weight gain than the normalized CNK-20402-alone formulation indicating that both lactose and CNK-20402 absorb water vapor. Consequently, competition for water vapor uptake may occur between CNK-20402 and lactose in the formulation.

To further characterize water uptake, additional water vapor sorption studies were performed on several lactose-containing lyophilized formulations. The resulting vapor sorption data are plotted in Figure 8. Six different water vapor sorption trends are shown in Figure 8: CNK-20402-alone cake, lactose placebo, physical mixture of CNK-20402 and lactose placebo (1:2), CNK-20402-lactose colyophilized cake (1:2), CNK-20402-lactose colyophilized cake (1:10), and predicted CNK-20402-lactose colyophilized cake (1:2). The predicted plot was calculated by the summation of one third of weight gain from the CNK-20402-alone sample and two thirds of weight gain from the lactose placebo sample. As shown in Figure 8, the lactose placebo sample recrystallized after 10% weight gain, followed by a weight loss due to the release of water from the crystalline lactose. A similar water vapor sorption trend for amorphous lactose was reported by Buckton and Darcy.[5] With no drug-excipient interaction, the weight gain trend for the proposed CNK-20402-lactose (1:2) formulation should behave similarly to the predicted sorption curve. As can be seen from the figure, the sorption curve of the physical mixture of CNK-20402 cake and lactose-placebo sample showed the identical trend to that of the predicted sorption curve. However, this trend was not reflected in the colyophilized CNK-20402-lactose (1:2) formulation for which lactose recrystallization was not

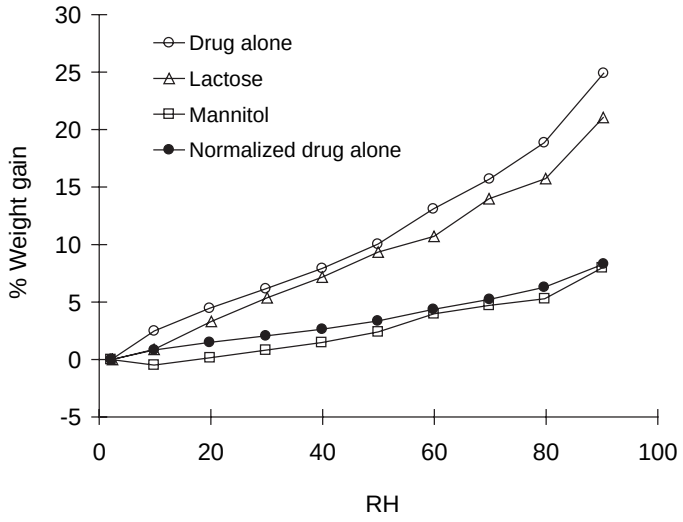


Figure 7. Water vapor sorption vs relative humidity of CNK-20402 in different excipient-containing formulations.

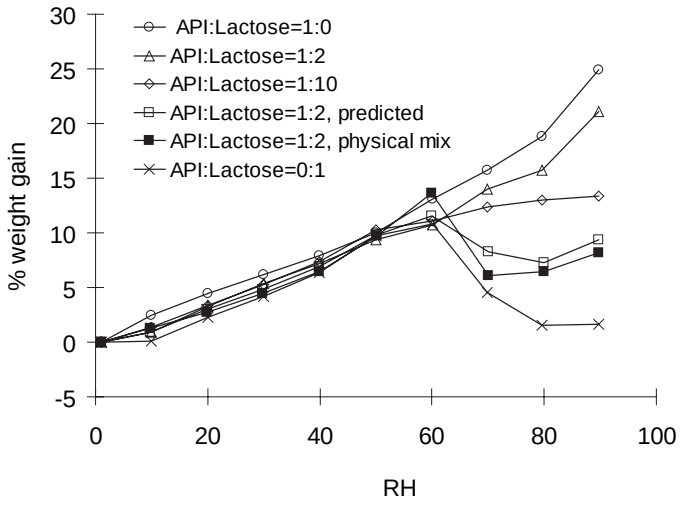


Figure 8. Water vapor sorption vs relative humidity of lactose-containing CNK-20402 formulations.

observed. This discrepancy suggests the potential presence of a drug-excipient interaction similar to that reported by Costantino et al.[6] The presence of CNK-20402 likely hinders or delays the recrystallization process of lactose. This hypothesis is confirmed from the vapor sorption curve of colyophilized CNK-20402-lactose (1:10) samples. While lactose is the predominant species for water sorption in this formulation, the recrystallization of lactose is retarded substantially by CNK-20402. These observations clearly indicate that under current experimental conditions lactose competes with CNK-20402 for water vapor uptake and CNK-20402 interacts with lactose to hinder its recrystallization.

While lactose enhances the stability of the CNK-20402, its protective mechanism is not fully understood. However, based on all of the observed data, the competition for water sorption[7] and the solid phase separation[8,9] between the active and the excipient offer reasonable explanations for the better performance of the lactose formulation. The robustness results from water-spiked studies, and the vapor sorption data presented in Figures 5 and 8 indicate that lactose is likely to compete with CNK-20402 for residual water and any additional moisture introduced to the lyophilized cake (up to 3%). According to the PXRD results, CNK-20402-lactose cakes are amorphous whereas CNK-20402-mannitol cakes are crystalline with the similar PXRD features of mannitol. Therefore, it is likely that there is a phase separation occurring between CNK-20402 and mannitol. Since CNK-20402 remains amorphous within the lyophilized product, it can adsorb more water vapor than the crystalline mannitol[10,11] and subsequently form more CNK-20193 than the lactose-containing formulation. Stability data shown in this investigation agrees with previous finding by Kirsch et al,[8] showing reduced chemical stability of lyophilized formulations in the presence of mannitol. Based on these studies, a lactose-containing formulation was proposed for further development.

CONCLUSION

The stability of lyophilized CNK-20402 was enhanced by the use of lactose. Based on water vapor sorption studies, CNK-20402 was protected by lactose through drug-excipient interactions and competition for residual water in the formulation.

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