Effective Inhibition of Mannitol Crystallization in Frozen Solutions by Sodium Chloride

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Purpose. The purpose of this work was to study the possibility of preventing mannitol crystallization in frozen solutions by using pharmaceutically acceptable additives.

Methods. Differential scanning calorimetry (DSC) and low-temperature X-ray diffractometry (LTXRD) were used to characterize the effect of additives on mannitol crystallization.

Results. DSC screening revealed that salts (sodium chloride, sodium citrate, and sodium acetate) inhibited mannitol crystallization in frozen solutions more effectively than selected surfactants, α-cyclodextrin, polymers, and alditols. This finding prompted further studies of the crystallization in the mannitol-NaCl-water system. Isothermal DSC results indicated that mannitol crystallization in frozen solutions was significantly retarded in the presence of NaCl and that NaCl did not crystallize until mannitol crystallization completed. Low-temperature X-ray diffractometry data showed that when a 10% w/v mannitol solution without additive was cooled at 1°C/min, the crystalline phases emerging after ice crystallization were those of a mannitol hydrate as well as the anhydrous polymorphs. In the presence of NaCl (5% w/v), mannitol crystallization was suppressed during both cooling and warming and occurred only after annealing and rewarming. In the latter case however, mannitol did not crystallize as the hydrate, but as the anhydrous δ polymorph. At a lower NaCl concentration of 1% w/v, the inhibitory effect of NaCl on mannitol crystallization was evident even during annealing at temperatures close to the Tg′ (−40°C). A preliminary lyophilization cycle with polyvinyl pyrrolidone and NaCl as additives rendered mannitol amorphous.

Conclusion. The effectiveness of additives in inhibiting mannitol crystallization in frozen solutions follows the general order: salts > alditols > polyvinyl pyrrolidone > α-cyclodextrin > polysorbate 80 ~ polyethylene glycol ~ poloxamer. The judicious use of additives can retain mannitol amorphous during all the stages of the freeze-drying cycle.

KEY WORDS: Mannitol; sodium chloride; crystallization; DSC; low temperature XRD.

INTRODUCTION

Developers of freeze-dried formulations use mannitol as a crystalline bulking agent because its tendency to crystallize facilitates the formation of elegant product cakes and its high eutectic melting temperature with ice (−1.5°C) enables primary drying at relatively high temperatures, thereby improving process efficiency. Although this use of mannitol is well established, recent studies suggest another potential application of mannitol, namely as a lyoprotectant to proteins and peptides (1–5). To be an effective lyoprotectant, however, mannitol must remain amorphous during processing, which has been achieved in the presence of active ingredients or excipients (2,6–9).

Although a number of lyoprotectants are disaccharides (10,11), mannitol with its structural similarity may serve the same purpose. An advantage of mannitol as a stabilizer is its chemical stability. For instance, unlike disaccharides, mannitol does not undergo hydrolysis at low or high pH. Despite anticipated difficulties with using mannitol as a lyoprotectant, namely its ease of crystallization and low glass transition temperature (6), it seems worthwhile to explore this formulation option for situations where disaccharides are unsuitable.

The objective of this study was to systematically evaluate the influence of pharmaceutically acceptable additives on the tendency of mannitol crystallization in frozen solutions. Although the effect of additives on mannitol crystallization has been studied (2,7,9,12), no studies have systematically examined the relative effectiveness of additives as crystallization inhibitors. We used differential scanning calorimetry (DSC) and low-temperature X-ray diffractometry (LTXRD) to evaluate the effect of additives on mannitol crystallization under freeze-drying conditions. The additives selected for this study included salts, alditols (sugar alcohols), surfactants, and polymers. Salts are used to make parenteral solutions isoosmotic with blood. Alditols are polyols of the general formula $HOCH_2(CHOH)_nCH_2OH$, of which mannitol is a member (n = 4). Owing to their structural similarity, alditols may interfere with the crystallization of mannitol. Polyvinyl pyrrolidone (PVP), a macromolecule effective in inhibiting the crystallization of indomethacin (13) has been used as a vehicle in amorphous drug dispersions. Poly(vinyl alcohol) (PVA), another macromolecule, may exhibit similar properties. α-cyclodextrin was selected for its relatively high Tg′ (−12°C) and its ability to form soluble complexes with drugs. Polysorbate 80 and poloxamer are examples of surfactants sometimes used in freeze-dried formulations and have been observed to affect the polymorphic behavior of mannitol (14).

In the course of this study, salts were found to be especially effective in inhibiting mannitol crystallization from frozen solutions. Further experiments were therefore carried out to characterize the crystallization in the mannitol–NaCl–water system. We report here the results of both the preliminary comparison of additives and a detailed investigation of crystallization from the mannitol-NaCl-water system.

Apart from producing amorphous mannitol, other considerations motivated the present study. It is well known that vials of mannitol-containing formulations occasionally break during freeze-drying (15). This phenomenon is attributed to the crystallization of mannitol when a frozen solution is rewarmed before primary drying (15). Although vial breakage may be minimized through careful design of freeze-drying cycles, additives may offer an alternative solution. Furthermore, a crystalline mannitol hydrate is known to form during freeze-drying and survive the drying process (16). Because the water retained by the mannitol hydrate may be released during shelf storage and cause physical and chemical instability, it is generally held that the mannitol hydrate should be avoided in freeze-drying or removed once formed. Thus, it is worthwhile to examine the effect of additives on the formation of the mannitol hydrate. Finally, an ongoing interest

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exists in understanding the phase transitions in multicomponent solutions during freeze-drying (17). The mannitol-additive combinations studied here have largely been unexplored (18).

MATERIALS AND METHODS

Materials

The following chemicals were purchased from commercial sources and used as received: D-mannitol (99+%, Sigma-Aldrich, St. Louis, MO, USA), NaCl (99+%, Fisher, Fair Lawn, NJ, USA), PVA (98% hydrolyzed, Mw 16,000, ACROS, NJ, USA), polyethylene glycol (PEG; average Mw. 6000, Aldrich), PVP K40, Sigma-Aldrich, MO), polysorbate 80 (Tween 80®, Sigma), poloxamer 188 (Pluronic F-68®, Sigma-Aldrich), α-cyclodextrin (99%, Sigma), D-sorbitol (98+%, Sigma-Aldrich, MO), xylitol (99+%, Sigma), DL-threitol (97%, Aldrich), and D(+)-arabitol (99+%, Sigma).

Differential Scanning Calorimetry (DSC)

A differential scanning calorimeter (Model 2920, TA Instruments), equipped with a refrigerated cooling system was used. Indium and mercury were used for calibration. About 10–15 mg of the solutions were weighed into aluminum pans and hermetically sealed. Three types of experiments were performed. 1) Standard run. The sample was cooled at 1°C/min to −70°C, held for 15 min, and warmed at 2°C/min to 25°C. 2) Cooling with isothermal hold. The sample was cooled at 1°C/min to the desired temperature, held for 120 min, cooled at 1°C/min to −50°C, and heated at 2°C/min to 25°C. 3) Annealing during warming. The sample was cooled at 1°C/min to −70°C, heated at 2°C/min to the annealing temperature, held for the desired time period, cooled back to −70°C, and reheated at 2°C/min to 25°C.

X-Ray Powder Diffractometry (XRD)

An X-ray powder diffractometer (Model XDS 2000, Scintag) with a variable temperature stage (Micristar, Model 828D; working temperature range of −190°C to 300°C) was used. Approximately 100 mg of solution was filled into a copper sample holder, cooled at 1°C/min from room temperature to −70°C, held for 15 min, and heated to room temperature. If annealing was to be performed, the sample was heated to the annealing temperature and held for the desired time period. XRD patterns were obtained with CuKα radiation (45 kV X 40 mA) at a scanning speed of 5°2θ min^{-1} and a step size of 0.03°2θ. During each scan, the sample was maintained at a constant temperature.

RESULTS AND DISCUSSION

Effectiveness of Additives in Inhibiting Mannitol Crystallization: Preliminary Comparison

We began this study by comparing the effect of different additives on mannitol crystallization when the solution was cooled at 1°C/min and subsequently warmed at 2°C/min. Without additives, mannitol crystallizes under these conditions. Because cooling and warming are essential steps of a freeze-drying cycle, producing amorphous mannitol requires inhibiting mannitol crystallization during both cooling and warming. Table I lists the additives used and their concentrations.

Table I. Additives Used and Their Concentration in Solution

Class	Additive compound	MW	Additive concentration (% w/v)
Polymer	PEG	6000	
	PVP K40	40,000	1.25, 2.5, 5
	PVA	16,000	
Surfactant	Polysorbate 80	1309	2.5, 5, 10
	Poloxamer 188	8350	
Cyclodextrin	α-cyclodextrin	972	2.5, 5, 10
Salt	NaCl	58.5	0.5, 1, 2
	Na citrate	258.0	
	Na acetate	82.0	
Alditol	Sorbitol	182.1	
	Xylitol	152.1	2.5, 5, 10
	Threitol	162.0	
	Arabitol	152.0	

Note: The mannitol concentration was 10% w/v in all cases.

Figure 1 shows representative DSC cooling scans of the mannitol solutions studied. The mannitol solution without additive showed three crystallization events (curve a). The first (−15°C) is attributed to ice crystallization while the second (−18°C) and third (−31°C) correspond to the crystallization of mannitol. Although the crystallization of frozen mannitol solutions has been the subject of several investigations (15,19–21), when this study began, the assignment of the −18°C and −31°C events was still unclear (the observation of the −18°C event depends critically on the cooling rate: at high cooling rate, ice crystallization will be postponed so that the event

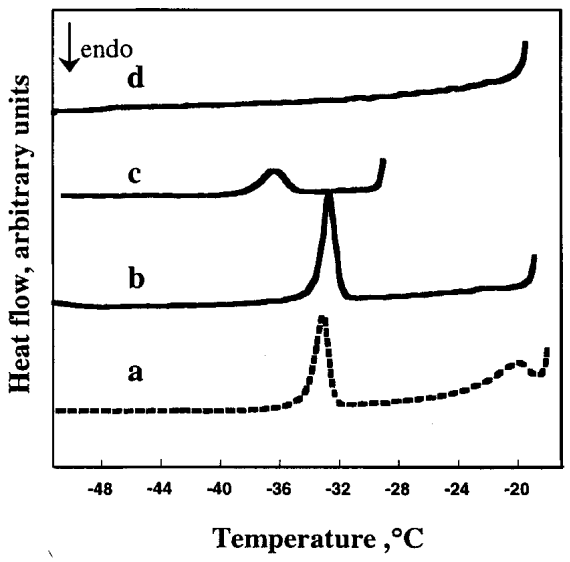

Fig. 1. Representative differential scanning calorimetry profiles of mannitol (10% w/v) solutions with additive (5% w/v) during cooling at 1°C/min. (a) No additive; (b) polyethylene glycol 6000, poloxamer 188, or polysorbate 80; (c) Polyvinyl pyrrolidone, Poly(vinyl alcohol), or α-cyclodextrin; (d) xylitol, threitol, sorbitol, or arabitol. The ice crystallization exotherm is not shown for clarity.

may not be observable; Ref. 19). Based on the new LTXRD data acquired in this study (Fig. 2) these events are attributed to a crystalline mannitol hydrate, which was initially observed by Yu *et al.* (16) and later confirmed by *in situ* LTXRD (21). Figure 2 shows that the crystalline phase appearing at –18°C displayed an XRD peak characteristic of mannitol hydrate at 17.9°2θ. In addition to the mannitol hydrate, some characteristic peaks of anhydrous α- and δ- polymorphs were observed. Because no new peaks emerged as the temperature was decreased below –20°C, the anhydrous polymorphs appeared to crystallize simultaneously with the mannitol hydrate near –18°C. When mannitol solutions were freeze-dried, peaks were observed at similar positions and this issue was discussed previously (16).

Figure 1 shows that in the presence of 5% w/v nonelectrolyte additives, mannitol crystallization during cooling was affected to different degrees. PEG, poloxamer, and polysorbate 80 did not affect the temperature of crystallization (Fig. 1b). PVP, PVA, and α-cyclodextrin delayed the crystallization (Fig. 1c). Alditols (xylitol, threitol, sorbitol, arabitol) completely inhibited mannitol crystallization (Fig. 1d). Compared with the effects of nonelectrolyte additives, the inhibitory effect of salts on mannitol crystallization was markedly stronger. This conclusion was reached from the following observations: 1) at a concentration of 5% w/v, sodium chloride, sodium citrate, and sodium acetate completely suppressed mannitol crystallization; 2) at 1% w/v, these salts inhibited mannitol crystallization, whereas the nonelectrolyte additives did not; 3) at 0.5% w/v, these salts significantly delayed, although did not fully suppress, mannitol crystallization (data not shown).

In the absence of additives, although fast cooling (≥ 20ºC/min) can produce amorphous mannitol, the cooling rate used here (1ºC/min) allowed mannitol to crystallize. Even so, mannitol crystallization during cooling is generally incomplete, allowing additional crystallization to occur during reheating. The crystallization of mannitol during reheating is shown in Fig. 3. The thermal events at –32ºC and at –25ºC (before the crystallization exotherm) were recently attributed to two glass transitions (12).

In general, the effect of the non-electrolyte additives on mannitol crystallization during *warming* was consistent with that during cooling (Fig. 3). We had earlier observed that PEG, poloxamer, and polysorbate 80 did not affect the crystallization of mannitol during cooling. The same was the case during the heating cycle (Fig. 3b). Although α-cyclodextrin delayed mannitol crystallization during cooling (Fig. 1), it had no pronounced effect during warming (Fig. 3c). These additives were therefore deemed ineffective in inhibiting mannitol crystallization. In comparison, the alditols, PVP and PVA significantly delayed the crystallization of mannitol. The peak temperature of the crystallization exotherm was increased by 7.8ºC (± 1.2º) in case of alditols and by 5ºC (± 0.2º) and 2.6ºC (± 1.1º) in case of PVP and PVA, respectively. The influence of PVP on the crystallization of mannitol has been the subject of a detailed investigation (12).

It is noteworthy that with the exception of salts, none of the additives completely inhibited mannitol crystallization during warming, even at the highest concentration used (5% w/v). In comparison, salts at 2% w/v fully suppressed mannitol crystallization during heating (data not shown). Thus, salts effectively inhibited mannitol crystallization both during cooling and warming, outperforming the other additives tested. This conclusion led us to characterize the effect of one of the salts, NaCl, on mannitol crystallization in detail.

Detailed Characterization of the Effect of NaCl on Mannitol Crystallization

We examined the effect of NaCl on mannitol crystallization under three conditions: isothermal hold during cooling, warming after cooling, and rewarming after cooling and annealing.

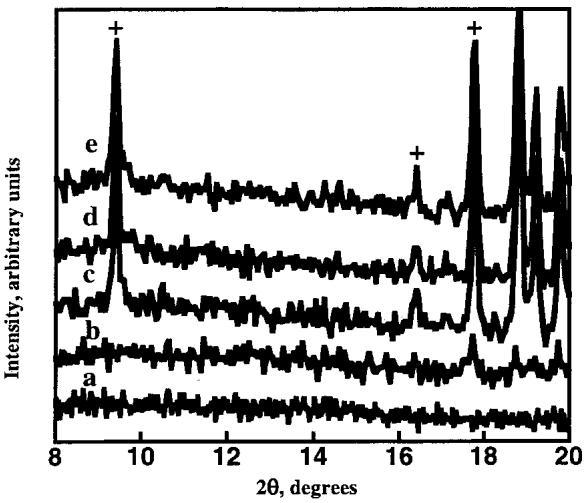

Fig. 2. X-ray powder diffraction patterns obtained during the cooling of mannitol (10% w/v) solution. The solution was cooled from room temperature to –70°C and X-ray powder diffraction patterns were obtained at (a) –15°C, (b) –18°C, (c) –20°C, (d) –22°C, and (e) –32°C. Characteristic peaks of mannitol hydrate were observed at 9.6, 16.5, and 17.9°2θ (+). Peaks of anhydrous δ-(for example at 19.2°2θ) and α- (for example, at 18.8°2θ) polymorphs were also seen.

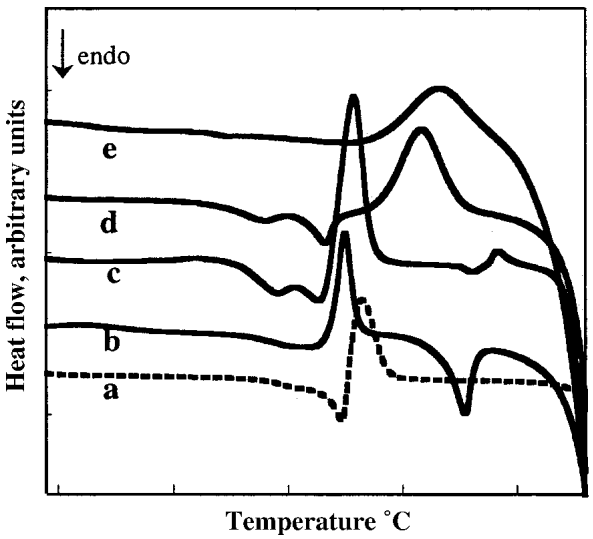

Fig. 3. Representative differential scanning calorimetry heating curves of frozen aqueous mannitol (10% w/v) solutions with additive (5% w/v). (a) No additive; (b) polyethylene glycol 6000, poloxamer 188, or polysorbate 80; (c) cyclodextrin; (d) Polyvinyl pyrrolidone or Poly(vinyl alcohol); (e) sorbitol, xylitol, threitol, or arabitol. Solutions were initially cooled from room temperature to –70°C at 1°C/min, held for 15 min, and then heated at 2°C/min.

Isothermal Hold during Cooling

In this experiment, a mannitol solution containing 0.5% w/v NaCl was cooled at 1°C/min to a temperature $T_{hold,}$ and held isothermally for 120 min. Figure 4 shows the DSC and LTXRD data collected with T_{hold} = –25°C (curve a), –31°C (curve b), and –35°C (curve c). These were chosen to be close to the temperature of the exotherm at –31°C seen in the pure mannitol solution (Fig. 1a). The upper panel of Fig. 4 shows that at T_{hold} = –25 °C, mannitol crystallized in approximately 30 min in the absence of NaCl (broken line), whereas this event was considerably delayed and occurred in approximately 1 h in the presence of NaCl (solid line). LTXRD enabled quantification of the mannitol hydrate crystallization during the isothermal hold (inset). The intensity of the strongest mannitol hydrate peak (2θ = 17.9°) plateaued in approximately 10 min in the absence of NaCl, but in approximately 30 min in its presence. It is noted that the rate of mannitol crystallization in the DSC experiment was generally slower than that in the XRD. This may be caused by the different sample geometry and mass in the two experiments. The DSC sample was approximately 15 mg and enclosed in an aluminum pan, whereas the LTXRD sample was approximately 100 mg and exposed to ambient atmosphere in a copper holder. It is also noteworthy that the integrated intensity of the mannitol hydrate peak (2θ = 17.9°), which reflects the amount of mannitol hydrate in the system, is significantly higher in the absence of NaCl.

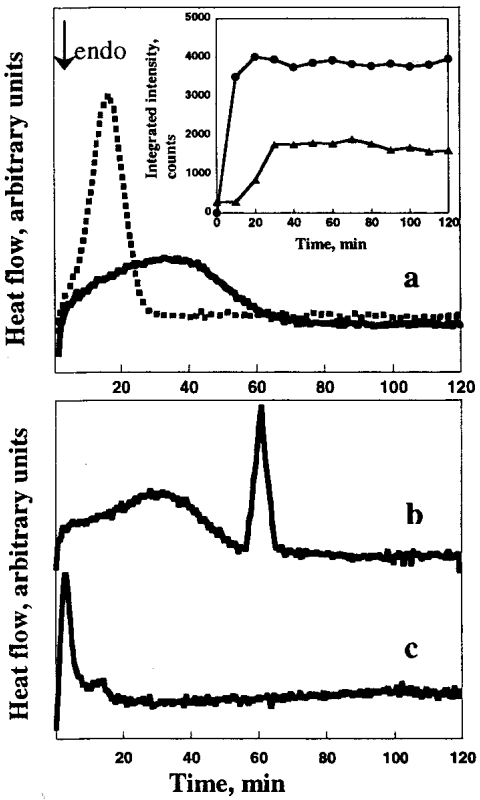

Fig. 4. Isothermal differential scanning calorimetry of mannitol (10% w/v) solution containing NaCl (0.5% w/v). The solutions were cooled at 1°C/min to the holding temperature (T_{hold}) and held isothermally for 120 min. Top, T_{hold} = –25°C. The discontinuous line is the differential scanning calorimetry curve of mannitol in the absence of NaCl. Bottom, T_{hold} = –30°C (b) and –35°C (c). Inset, intensity of the 17.9°2θ peak vs. time during isothermal hold at –25°C. (●) mannitol; (▲) mannitol plus NaCl.

The isothermal crystallization of mannitol at –30°C (Fig. 4b) was similar to that at –25°C (Fig. 4a), except that mannitol crystallization was followed immediately by an exotherm caused by the crystallization of sodium chloride dihydrate ($NaCl \cdot 2H_2O$). Whereas $NaCl \cdot 2H_2O$ crystallization did not occur during the isothermal hold at –25°C, it occurred on subsequent cooling at –39°C (data not shown). In NaCl-water solutions, $NaCl \cdot 2H_2O$ crystallizes at similar temperatures, depending on the degree of supercooling (22). The isothermal hold at –35°C caused immediate crystallization of $NaCl \cdot 2H_2O$ (Fig. 4c). In this case, mannitol had crystallized during cooling the sample to –35°C. It is significant that NaCl crystallization always occurred *after* mannitol crystallization. This order of crystallization is necessary for NaCl to inhibit mannitol crystallization.

Warming after Cooling

The amorphous freeze-concentrate of mannitol displays two glass transitions (–32 and –25ºC) upon rewarming (12). Figure 5 (right panel) shows that NaCl lowered both glass transition temperatures and the degree of depression was concentration dependent. This arises from the low Tg′ of NaCl (< –60ºC) (23). At 0.5% w/v NaCl, mannitol crystallized at a lower temperature i.e., *earlier* during rewarming, in contrast to the *delayed* crystallization of mannitol during cooling. This difference may be attributed to the depressed Tg′ in the presence of NaCl (Fig. 5b).

The depression of glass transition temperatures by NaCl indicates that it is present in the amorphous freeze-concentrate along with mannitol and water. This conclusion is consistent with the absence of the melting of the $NaCl \cdot 2H_2O$-ice eutectic when the frozen solution was warmed. Even at a concentration of 0.5% w/v, NaCl significantly inhibited mannitol crystallization on cooling. If all of this amorphous mannitol crystallized when heated above Tg′, the enthalpy of crystallization is expected to be substantially higher than that in the absence of NaCl. However, the enthalpies of crystallization were approximately the same (13.3 ± 0.5 J/g) in the presence (0.5% w/v) and absence of NaCl, indicating that NaCl, even at a concentration of 0.5% w/v, partially inhibited mannitol crystallization.

Increasing the NaCl concentration to 1% w/v depressed the Tg′ further (Fig. 5c). Interestingly, mannitol crystallization occurred at approximately the same temperature as that in the absence of NaCl. Thus, as the salt concentration increased, so did (Tc – Tg′), where Tc is the peak temperature of crystallization. It is worth recalling that at 1% w/v NaCl, mannitol crystallization was completely inhibited during cooling. At higher NaCl concentrations of 2% and 3% w/v, a crystallization exotherm was not seen and the glass transition temperatures, as expected, were lower (Fig. 5d and e). Only the Tg'_2 was observed and the Tg'_1 was likely to be below the temperature range of the experiment (Fig 5; right panel).

At 5% w/v NaCl, however, a weak exotherm appeared at approximately –35 °C, followed by an endothermic event and then ice melting (Fig. 5f). The exotherm could be a result of devitrification of ice (24). Luyet and Rasmussen observed this

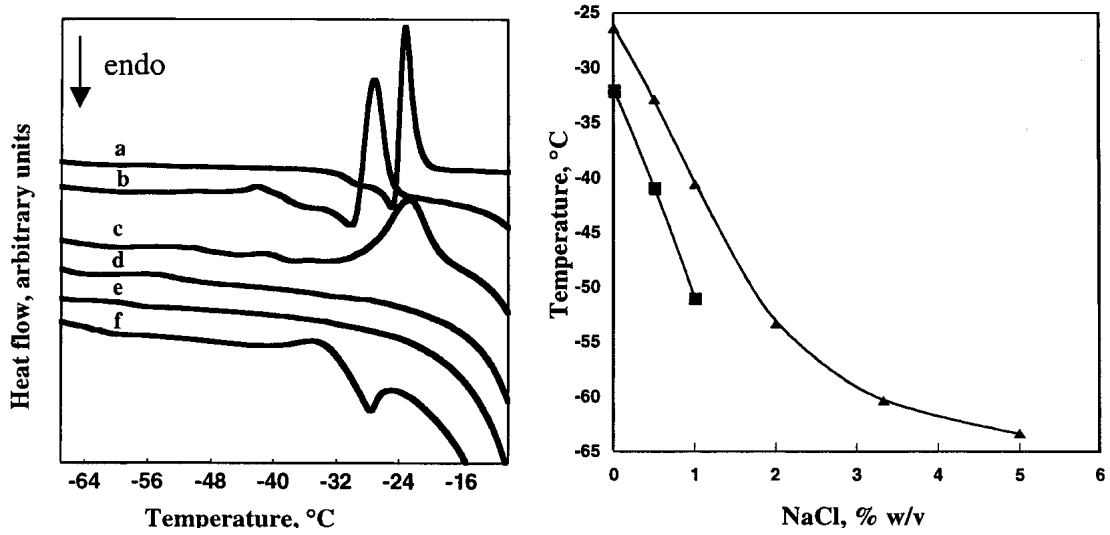

Fig. 5. Effect of NaCl concentration on the crystallization of mannitol. Left, overlaid differential scanning calorimetry heating profiles of frozen aqueous solutions of mannitol (10% w/v) containing different concentrations (% w/v) of NaCl. (a) 0, (b) 0.5, (c) 1.0, (d) 2.0, (e) 3.3, and (f) 5. The solutions were initially cooled at 1°C/min from room temperature to –70°C, held isothermally for 15 min, and then heated at 2°C/min. Right, the glass transition temperatures Tg′1 (■) and Tg′2 (▲) as a function of NaCl concentration.

phenomenon when moderately high to high cooling rates were used (25). Our cooling conditions (1°C/min to –70°C followed by an isothermal hold for 15 min) are unlikely to hinder the formation of ice. However, the amount of unfrozen water associated with amorphous phases will depend on the nature of the solute. Thus, even at this slow cooling rate, given the higher concentration of NaCl that remained amorphous, a larger amount of unfrozen water might be present.

Rewarming after Cooling and Annealing

To understand the thermal events seen at a NaCl concentration of 5% w/v, an annealing experiment was performed at – 40°C, just below the temperature of the exotherm in curve f (Fig. 5). After annealing for different times (30 min to 4 h), the solution was cooled to –70°C at 1 °C/min and rewarmed at 2°C/min. Figure 6 contains the DSC heating curves of the annealed solutions, along with that of an unannealed sample (broken curve) for comparison. After annealing, the exotherm at –35°C disappeared and the –28°C endotherm broadened and increased in enthalpy. Annealing also caused baseline changes at -54°C and -44°C and a new endotherm to appear at –24 °C. The enthalpy of the –24°C endotherm increased with annealing time, whereas the other thermal events were less affected by annealing.

The endotherm at –24°C can be attributed to the melting of $NaCl \cdot 2H_2O$-ice eutectic. In NaCl-water solutions, this eutectic melts at –21.6°C, but the presence of mannitol may lower the temperature. The endotherm at –28°C could be due to the melting of a ternary eutectic. Annealing at –40°C caused $NaCl \cdot 2H_2O$ to crystallize and the amount crystallizing increased with annealing time. The origin of the endotherm at -28°C, seen in both annealed and unannealed samples, is presently unclear.

LTXRD was used to determine the thermal transitions occurring under these conditions. Figure 7 shows the LTXRD data of a 10% w/v mannitol solution containing 5% w/v NaCl after cooling at 1°C/min to –70°C and annealing for 120 min at –40°C. No solute crystallization was detected after cooling to –70°C (curve a). Although warming to –40°C did not cause any changes in the XRD pattern, annealing for 2 h resulted in the crystallization of $NaCl \cdot 2H_2O$, as shown by the peaks at 15.0, 27.3, and 31°2θ (curve b). However, there was no evidence of mannitol crystallization. After the sample was cooled again at 1°C/min to –70°C and gradually rewarmed, no mannitol crystallization was detected up to –40°C (curve c). At –35°C, peaks attributable to δ-mannitol emerged at 9.2 and 20°2θ (curve d). The δ-mannitol peaks grew with increasing temperature (curves e, f). It is noteworthy that in the

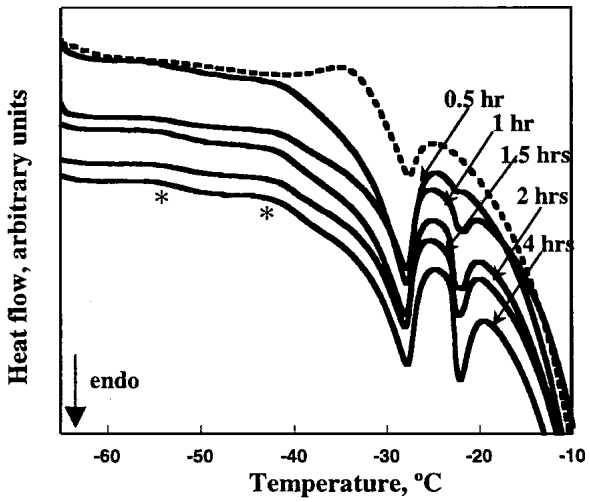

Fig. 6. Effect of annealing on thermal behavior of an aqueous mannitol (10% w/c) solution with 5% w/v NaCl. Solutions were cooled from room temperature to –70°C at 1°C/min, held for 15 min followed by heating to –40°C and annealed for various times as shown on the profiles. The solutions were then cooled back to –70°C and the reheating profiles are shown. The heating rate was 2°C/min. The profile of the unannealed sample seen in Fig. 5 is shown here as a broken curve. The two changes in baseline are indicated as *.

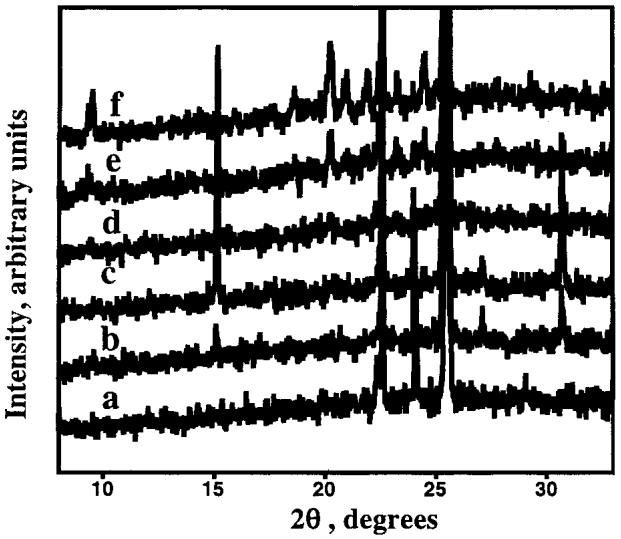

Fig. 7. X-ray powder diffraction patterns of mannitol (10% w/v)-NaCl (5% w/v) frozen solutions after freezing and subsequent warming. The solution was cooled from room temperature to –70°C and its X-ray powder diffraction pattern was obtained (curve a). It was heated to –40°C and annealed for 2 h (curve b). The annealed solution was cooled to –70°C and reheated. The heating and cooling rates were 2 and 1°C/min, respectively. X-ray powder diffraction profiles during warming, (c) –40°C, (d) –35°C, (e) –30°C, and (f) –25°C.

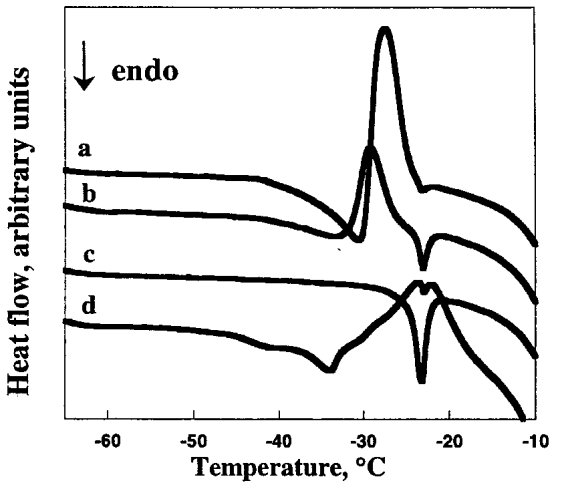

Fig. 8. Effect of annealing on the thermal behavior of aqueous mannitol (10% w/v) solutions containing either 0.5% w/v (curves a–c) or 1% w/v (curve d) NaCl. The annealing conditions were (a) –40°C for 2 h; (b) –40°C for 8 h; (c) –35°C for 2 h; (d) –40°C for 2 h. Solutions were cooled to –70°C, heated to the annealing temperature, cooled, and rewarmed. The rate was 2°C/min in all cases.

presence of 5% w/v NaCl, it is the anhydrous δ-polymorph and *not* the mannitol hydrate that crystallized.

It is instructive to briefly reverse the ongoing analysis of how NaCl affects mannitol crystallization and examine the effect of mannitol on NaCl crystallization. When a binary solution of NaCl and H_2O is cooled, NaCl readily crystallizes as $NaCl \cdot 2H_2O$, as evidenced by the eutectic melting at about –22°C observed in subsequent warming. When ternary solutions of mannitol, NaCl and H_2O were cooled at 1°C/min, NaCl generally did not crystallize, as proven by the missing $NaCl \cdot 2H_2O$/ice eutectic melting in subsequent warming. $NaCl \cdot 2H_2O$ crystallized only after an isothermal hold during cooling (Fig. 4) or warming (Fig. 8c; see later). This effect is in accord with previous findings of suppression of NaCl crystallization by noncrystallizing sugars, such as sucrose and trehalose (22), and by crystallizable solutes, such as mannitol and glycine (18). Hence, in frozen solutions, even the crystallizable sugar mannitol, inhibits the crystallization of NaCl. However, in view of the inhibitory effect of NaCl on mannitol crystallization, this sugar must be retained amorphous in order to inhibit salt crystallization. Chang and Randall have classified NaCl as a partially crystallizing "doubly unstable" glass (23). If such salts are lyophilized without devitrification, the lyophilized product is composed of both a crystalline matrix and a collapsed amorphous fraction. This inability of NaCl to crystallize completely lowers the collapse temperature of the system.

In studies described in Figs. 6 and 7, the NaCl concentration chosen (5% w/v) is relatively high for typical lyophilization process. Figure 8 shows the effect of annealing on the thermal behavior of 10% w/v mannitol solutions containing much lower NaCl concentrations of 0.5 and 1% w/v. In light of the significant molecular mobility of mannitol below its Tg′, the annealing temperature of –40°C was deemed appropriate (26). Annealing for 2 h resulted in a single glass transition at –34°C, followed by the crystallization of mannitol at –28°C (curve a). The crystallization exotherm was followed by a barely perceptible endotherm (–24°C), attributable to the melting of the $NaCl \cdot 2H_2O$–ice eutectic. The magnitude of this endotherm indicates that a substantial fraction of NaCl is amorphous. Increasing the annealing time to 8 h resulted in a weaker glass transition suggesting that a significant fraction of mannitol crystallized during the long annealing (curve b). The pronounced decrease in the enthalpy of mannitol crystallization confirmed its crystallization during annealing. The longer annealing time also facilitated the crystallization of $NaCl \cdot 2H_2O$ resulting in a higher enthalpy value of the $NaCl \cdot 2H_2O$–ice eutectic. The solution was next annealed at –35°C, a temperature close to the Tg'_2, the higher glass transition temperature. Crystallization of mannitol appeared to be complete when annealed at –35°C for 2 h. As a result, only the eutectic melting endotherm was observed when the annealed sample was heated (curve c). The larger enthalpy value indicates that the NaCl also crystallized significantly during annealing.

In the presence of 1% w/v NaCl, 2 h of annealing at –40°C did not cause complete crystallization of either mannitol or $NaCl \cdot 2H_2O$. This is evident from the pronounced crystallization exotherm and the weak $NaCl \cdot 2H_2O$–ice eutectic melting seen at the peak during rewarming (Fig. 8d). We had earlier seen that the Tg'_2 decreased from –33°C to –40°C when the NaCl concentration was raised from 0.5 to 1% w/v (Fig. 5). The annealing temperatures in curves (c) and (d) were selected to be close to the $T_g'_2$ of these two compositions. It is well known that the molecular mobility, and therefore the propensity to crystallize, is proportional to (T_a – Tg′), where T_a is the annealing temperature. For the systems shown in Fig. 8c and 8d, $Ta \approx Tg'_2$. The dramatic inhibitory effect of NaCl is evident from the fact that despite annealing in the vicinity of Tg', an increase in its concentration from 0.5 to 1% w/v retained a considerable fraction of man-

nitol amorphous. The amorphous mannitol recrystallized during warming (curve d).

SIGNIFICANCE

The results of this study demonstrate that sodium chloride, a common excipient in parenteral formulations, effectively inhibited mannitol crystallization in several stages of freeze-drying: cooling, isothermal hold in cooling, warming, and rewarming after annealing. The inhibitory effect of NaCl was observed at concentrations that are of practical interest and significance. These findings raise additional questions: What is the nature of the interactions between mannitol and NaCl? How can this effect be used to prevent mannitol crystallization during freeze-drying?

An ongoing study of the interactions between mannitol and NaCl indicates that despite their dissimilar chemical structures, there exists a slight but significant melt miscibility (ca. 7% w/w NaCl in mannitol). Thus, even though water is essential for holding mannitol and NaCl together to form an amorphous phase that resists crystallization, the melt miscibility of mannitol and NaCl may provide additional cohesive force to inhibit crystallization.

Despite their slight melt miscibility, freeze drying mannitol in the presence of NaCl will not produce any significant amount of amorphous mannitol, because the removal of water from the freeze-concentrate will eventually cause both NaCl and mannitol to crystallize. To produce amorphous mannitol by freeze-drying, we experimented with using a second additive that may prevent the crystallization of mannitol when water is finally removed from the freeze-concentrate (secondary drying). In this approach, NaCl would ensure that mannitol remain amorphous during cooling, annealing, and partially during primary drying, while the second additive would inhibit mannitol crystallization during secondary drying. In our preliminary lyophilization cycle, PVP was used as the second additive. PVP was chosen because of its established use as an amorphous vehicle for drugs and its high Tg′ (–24°C) during freeze-drying. We observed that a 5% w/v mannitol solution freeze-dried in the presence of 2.5% w/v NaCl and 2.5% w/v PVP produced a powder in which mannitol was fully amorphous, even though sodium chloride had crystallized. Thus, a judicious use of additives may retain mannitol amorphous during all the stages of freeze-drying. Occasionally, the active pharmaceutical ingredient may play the role of the second additive (PVP in this case).

In light of the strong inhibitory effect of NaCl on mannitol crystallization, the presence of salt may inadvertently render a fraction of mannitol amorphous. Mannitol in a partially amorphous form can act neither as an effective stabilizer nor a bulking agent (27). It is thus prudent to take appropriate steps to crystallize it completely or stabilize the amorphous form (19). An added complication is the formation of mannitol hydrate, which is seen to survive typical freeze drying cycles (16). The hydrate concentration is variable and appears to be dependent on the processing conditions. This unstable hydrate is likely to dehydrate during product storage and the evolved water can be a source of both physical and chemical instability (16,28). The NaCl-induced inhibition of this hydrate formation is worthy of detailed investigation. Preliminary work in this direction has been initiated in our laboratory.

CONCLUSIONS

Although mannitol, because of its excellent bulking properties, is a widely used excipient in freeze-dried products, its tendency to crystallize reduces its effectiveness as a lyoprotectant. With the overall goal of rendering mannitol amorphous, this investigation focused on a series of additives to inhibit mannitol crystallization in the frozen state. The effectiveness of the additives, in inhibiting mannitol crystallization, can be rank ordered as: salts > alditols > PVP > α-cyclodextrin > polysorbate 80 ~ PEG ~ poloxamer.

NaCl inhibited mannitol crystallization at concentrations as low as 0.5% w/v so long as it was retained in solution. At this NaCl concentration, both mannitol and NaCl can still crystallize after annealing near Tg'. However, raising the NaCl concentration to 1% w/v makes mannitol crystallization significantly more difficult. Preliminary freeze-drying experiments showed that NaCl could be judiciously combined with a second glass-forming additive (or the active pharmaceutical ingredient) to inhibit mannitol crystallization throughout the entire freeze-drying process.

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REFERENCES

1. K. Izutsu, S. Yoshioka, and T. Terao. Decreased protein-stabilizing effects of cryoprotectants due to crystallization. *Pharm. Res.* **10**:1232–1237 (1993).
2. K. Izutsu, S. Yoshioka, and T. Terao. Effect of mannitol crystallinity on the stabilization of enzymes during freeze-drying. *Chem. Pharm. Bull.* **42**:5–8 (1994).
3. K. Izutsu and S. Kojima. Excipient crystallinity and its protein-structure-stabilizing effect during freeze-drying. *J. Pharm. Pharmacol.* **54**:1033–1039 (2002).
4. M. J. Pikal, K. M. Dellerman, M. I. Roy, and R. M. Riggin. The effects of formulation variables on the stability of freeze-dried human growth hormone. *Pharm. Res.* **8**:427–436 (1991).
5. K. Tanaka, T. Takeda, and K. Miyajima. Cryoprotective effect of saccharides on denaturation of catalase by freeze-drying. *Chem. Pharm. Bull.* **39**:1091–1094 (1991).
6. L. Yu, D. S. Mishra, and D. R. Rigsbee. Determination of the glass properties of D-mannitol using sorbitol as an impurity. *J. Pharm. Sci.* **87**:774–777 (1998).
7. A. I. Kim, M. J. Akers, and S. L. Nail. The physical state of mannitol after freeze-drying: effects of mannitol concentration, freezing rate, and a noncrystallizing cosolute. *J. Pharm. Sci.* **87**: 931–935 (1998).
8. T. R. Kovalcik and J. K. Guillory. The stability of cyclophosphamide in lyophilized cakes. Part I. Mannitol, lactose, and sodium bicarbonate as excipients. *J. Parenter. Sci. Technol.* **42**:29–37 (1988).
9. M. G. Fakes, M. V. Dali, T. A. Haby, K. R. Morris, S. A. Varia, and A. T. M. Serajuddin. Moisture sorption behavior of selected bulking agents used in lyophilized products. *PDA J. Pharm. Sci. Technol.* **54**:144–149 (2000).
10. S. D. Allison, B. Chang, T. W. Randolph, and J. F. Carpenter. Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. *Arch. Biochem. Biophys.* **365**:289–298 (1999).
11. M. J. Pikal. Mechanisms of protein stabilization during freeze-drying and storage: the relative importance of thermodynamic stabilization and glassy state relaxation dynamics. *Drugs Pharmaceut. Sci.* **96**:161–198 (1999).
12. R. K. Cavatur, N. M. Vemuri, A. Pyne, Z. Chrzan, D. Toledo-

Velasquez, and R. Suryanarayanan. Crystallization behavior of mannitol in frozen aqueous solutions. *Pharm. Res.* **19**:894–900 (2002).
13. M. Yoshioka, B. C. Hancock, and G. Zografi. Inhibition of indomethacin crystallization in poly(vinylpyrrolidone) coprecipitates. *J. Pharm. Sci.* **84**:983–986 (1995).
14. R. Haikala, R. Eerola, V. P. Tanninen, and J. Yliruusi. Polymorphic changes of mannitol during freeze-drying: effect of surface-active agents. *PDA J. Pharm. Sci. Technol.* **51**:96–101 (1997).
15. N. A. Williams and T. Dean. Vial breakage by frozen mannitol solutions: correlation with thermal characteristics and effect of stereoisomerism, additives, and vial configuration. *J. Parenter. Sci. Technol.* **45**:94–100 (1991).
16. L. Yu, N. Milton, E. G. Groleau, D. S. Mishra, and R. E. Vansickle. Existence of a mannitol hydrate during freeze-drying and practical implications. *J. Pharm. Sci.* **88**:196–198 (1999).
17. E. Y. Shalaev, F. Franks, and P. Echlin. Crystalline and Amorphous Phases in the Ternary System Water-Sucrose-Sodium Chloride. *J. Phys. Chem.* **100**:1144–1152 (1996).
18. K. Ito. Freeze drying of pharmaceuticals. Eutectic temperature and collapse temperature of solute matrix upon freeze drying of three-component systems. *Chem. Pharm. Bull.* **19**:1095–1102 (1971).
19. A. Martini, K. Silvia, M. Crivellente, and R. Artico. Use of subambient differential scanning calorimetry to monitor the frozen state behavior of blends of excipients for freeze-drying. *PDA J. Pharm. Sci. Technol.* **51**:62–67 (1997).
20. P. Meredith, A. M. Donald, and R. S. Payne. Freeze-drying: in situ observations using cryoenvironmental scanning electron microscopy and differential scanning calorimetry. *J. Pharm. Sci.* **85**: 631–637 (1996).
21. R. K. Cavatur and R. Suryanarayanan. Characterization of phase transitions during freeze-drying by *in situ* X-ray powder diffractometry. *Pharm. Dev. Technol.* **3**:579–586 (1998).
22. K. Izutsu, S. Yoshioka, and S. Kojima. Effect of cryoprotectants on the eutectic crystallization of NaCl in frozen solutions studied by differential scanning calorimetry (DSC) and broad-line pulsed NMR. *Chem. Pharm. Bull.* **43**:1804–1806 (1995).
23. B. S. Chang and C. S. Randall. Use of subambient thermal analysis to optimize protein lyophilization. *Cryobiology* **29**:632–656 (1992).
24. S. Ablett, M. J. Izzard, and P. J. Lillford. Differential scanning calorimetric study of frozen sucrose and glycerol solutions. *J. Chem. Soc. Far. Trans.* **88**:789–794 (1992).
25. B. Luyet and D. Rasmussen. Study by differential thermal analysis of the temperature of instability of rapidly cooled solutions of glycerol, ethylene glycol, sucrose and glucose. *Biodynamica* **10**: 1167–1191 (1968).
26. A. Pyne, R. Surana, and R. Suryanarayanan. Crystallization of mannitol below Tg′ during freeze-drying in binary and ternary aqueous systems. *Pharm. Res.* **19**:901–908 (2002).
27. B. D. Herman, B. D. Sinclair, N. Milton, and S. L. Nail. The effect of bulking agent on the solid-state stability of freeze-dried methylprednisolone sodium succinate. *Pharm. Res.* **11**:1467–1473 (1994).
28. C. Ahlneck and G. Zografi. The molecular basis of moisture effects on the physical and chemical stability of drugs in the solid state. *Int. J. Pharm.* **62**:87–95 (1990).