Development of an Efficient Single-Step Freeze-Drying Cycle for Protein Formulations

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Received August 24, 1994; accepted January 20, 1995

Purpose. An efficient freeze-drying cycle for recombinant human interleukin-1 receptor antagonist (rhIL-1ra) formulations, which contained glycine and sucrose as excipients, was developed. *Methods.* Development was based on characterizing the frozen formulations by thermal analysis and by examining the effect of various lyophilization process parameters on the sublimation rate of ice. *Results.* Thermal analysis showed that the metastable glass of glycine in frozen formulation could be devitrified by slowly warming the frozen product to −15°C. During drying, the sublimation rate of ice was increased as a linear function of the difference between the vapor pressure of ice at the product temperature (P_O) and the chamber pressure (P_C). Therefore, the product temperature (T_p) was maintained as high as possible at temperatures below Tg′ of the formulation, in order to maximize the P_O without allowing the collapse of cake. Although various combinations of shelf temperatures and chamber pressures could be used to obtain the same T_p, the combination of higher shelf temperature and lower chamber pressure was used to maximize sublimation rate. *Conclusions.* A single-step drying cycle was developed to take advantage of these observations. The shelf temperature was set for the secondary drying and the product temperature during primary drying was maintained below Tg′ by adjusting the chamber pressure. As the sublimation completed, the product temperature increased naturally to the shelf temperature for the secondary drying. This process resulted in successful drying of 1 ml of rhIL-1ra formulation to 0.4% moisture content within 6 hours.

KEY WORDS: rhIL-1ra; lyophilization; cycle development; sublimation rate; shelf temperature; chamber pressure.

INTRODUCTION

The development of protein-based pharmaceuticals has been a major interest since recombinant DNA technology allowed mass-production of valuable proteins, which have been discovered as mediators of critical human physiological functions. Unfortunately, many purified proteins have a marginal stability in solution. Understanding the nature of denaturation and investigating methods for stabilization have become an important responsibility for formulation scientists, because protein denaturation during storage and delivery renders a protein product unusable. Freeze-drying is considered one of the best ways to preserve proteins, with the potential to yield products that are stable as well as convenient to ship and handle. Water is important for the degradation of proteins because of its role as a medium for the movement of molecules, as an environment fostering conformational changes, and as a reactant for adverse reactions such as hydrolysis. Therefore, many degradation reactions of proteins can be either suppressed or completely eliminated by simply removing water with freeze-drying. Improved stability provides many benefits, such as storage at room temperature, convenient handling, extended shelf-life, and the capacity to distribute the product widely.

However, freeze-drying is known to be a time consuming and expensive process. In fact, in many industries freeze-drying is a rate-limiting process, because of the cost of the instrumentation and long drying cycles. In order to develop an economical drying process, formulations should be optimized to get the maximum allowable temperature during primary drying, which can be either the collapse temperature or the eutectic melting temperature. This will allow the formulations to be freeze-dried at higher temperatures without compromising the appearance of cake (1). A sufficient knowledge of the structural state of the maximally freeze-concentrated matrix surrounding the ice crystals is necessary to optimize the formulation and the drying cycle so that the collapse of cake during the primary drying process is avoided (2, 7, 11). Thermal analysis techniques such as differential scanning calorimetry (DSC) have been successfully used to determine the collapse temperature and to monitor crystallization of constituents (1).

Various process variables affect the efficiency of the freeze-drying. The product temperature is important because the sublimation is generally faster at higher temperatures, but drying at temperatures above the collapse temperature may compromise the appearance of the cake. In addition to visual problems, product in a collapsed cake often does not reconstitute easily. Although several mathematical models have been presented to describe sublimation rate during the primary drying process, very little practical information is available about how to optimize the process parameters. Pikal (11) and Livesley et al. (6) have presented a model in which the difference of chamber pressure and vapor pressure of ice at product temperature is the major driving force. Understanding the relative contribution of these parameters to the sublimation rate is an important step in the optimization of a freeze-drying cycle and is also important to gain theoretical insight into what is important.

The purpose of this study was to find an efficient way to freeze-dry a given rhIL-1ra formulation. Careful investigation of the dynamic relationship between various process parameters allowed us to understand important factors for the freeze-drying cycles. Use of a single drying condition, i.e., single shelf temperature and chamber pressure, to complete both primary drying and secondary drying was proposed to minimize the drying time.

MATERIALS AND METHODS

Materials

rhIL-1ra was produced with *E. coli* using recombinant DNA technology and purified at Synergen, Inc. It was prepared in 2% (w/v) neutral glycine, 1% (w/v) sucrose, and 10

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mM sodium citrate buffer at pH 6.5 (25°C). All the chemicals used for this study were analytical grade or better.

Methods

Thermal analysis of frozen formulations with differential scanning calorimetry (DSC) was carried out with a Perkin Elmer DSC-7. The temperature range of analysis was from −60°C to 25°C. A sample (20 μl) was loaded at 25°C, cooled and frozen to −60°C, with a cooling rate of 10°C/minute. Thermal heat flow was analyzed while the samples were warmed at a rate of 1°C/min. Thermal transition temperatures, such as the glass transition temperature (Tg′), devitrification temperature (Td) and eutectic melting temperature (Te), were analyzed from the heat flow using a Perkin Elmer TAS7 software interfaced with the DSC-7. Glass transition temperatures were determined with the enhanced resolution of derivative thermograms (4). For annealing experiments, the frozen samples were warmed at 1°C/min to the designated temperature, held at that temperature for 5 minutes, and then cooled back to −60°C. The endotherms for melt of the resulting crystals was determined during subsequent warming at 1°C/min.

The rhIL-1ra formulation (1 ml) was placed into 3 ml vial with 13 mm stopper and freeze-dried in a Lyoflex 08 lyophilizer (Edwards High Vacuum, Tonawanda, NY), which was interfaced with a Lyomaster 4000 controller and equipped with a sample extractor. Eight temperature probes were used to monitor temperatures of sample vials at various locations on the shelf. The average temperature of the eight probes was used to determine the product temperature during primary drying. The temperature variation between probes was less than 2.5°C while primary drying was in progress. An Edwards Barocel Capacitance manometer and Pressure Control Pak were used to measure and control the chamber pressure. A Yokogawa HR2400 chart recorder (supplied by Edwards) was used to record the shelf temperature, chamber pressure, condenser temperature, and product temperatures. The shelf temperature was controlled within ±1°C and chamber pressure within ±20 μm Hg during primary drying. The condenser temperature was maintained at −66 ± 4°C during the freeze-drying cycle.

To determine the rate of sublimation, loaded sample vials were extracted during the drying cycle, without disturbing the chamber pressure. The amount of water sublimed (g) was determined by subtracting the weight of the extracted sample for that of the original sample. The residual moisture content of the lyophilized product was determined by Karl Fisher Coulometry using a Mitsubishi Moisture Meter CA06 (Mitsubishi Kasei Co., Tokyo, Japan) as described by May et al. (8).

RESULTS AND DISCUSSION

Calorimetric Analysis of Frozen Formulation

Various physical changes in a frozen protein formulation, such as freezing point depression, eutectic melting, glass transition, and devitrification of metastable glasses, can affect the freeze-drying process. Among various techniques available, differential scanning calorimetry has been successfully used to characterize the frozen protein formulations (1). Glycine was used as a bulking agent in the lyophilized formulation because it has long been used as an isotonicity modifier for parenteral drugs, and it provides a very stable matrix with sucrose after drying (data not shown). When solutions of glycine are frozen, most of the glycine remains amorphous, thus contributing to the mechanical rigidity of the unfrozen fraction. The amorphous glycine has a Tg′ of −37°C (Figure 1), which limits the product temperature during the primary drying process to below −37°C. The amorphous glycine is classified as a metastable glass because it devitrifies during the subsequent heating process. Devitrification is a process by which a metastable glass forms a stable crystalline phase due to heating above its glass transition temperature (6). The first thermogram in Figure 1 shows a glass transition (Tg′) followed by an exothermic peak at Td representing a devitrification or recrystallization of glycine. The result of successful devitrification on the physical properties of a frozen glycine solution is shown in the second calorimeter scan of Figure 1. Compared to the original thermogram (first scan in Figure 1), the thermogram of the devitrified sample shows no obvious thermal transitions at temperatures below the eutectic temperature (Te), which indicates that the metastable glass of glycine has been devitrified by heating. If this type of devitrified formulation is freeze-dried, the strong crystalline network of the glycine will provide a desirable cake. The advantage of allowing glycine to crystallize is that primary drying can be done at higher product temperatures since there is no need to keep product below −37°C, the Tg′ of glycine. In addition to the advantage in primary drying, the crystalline cake will improve the visual appearance of the product. As shown in a second scan of Figure 1, the Tg′ at −37°C disappeared after devitrification.

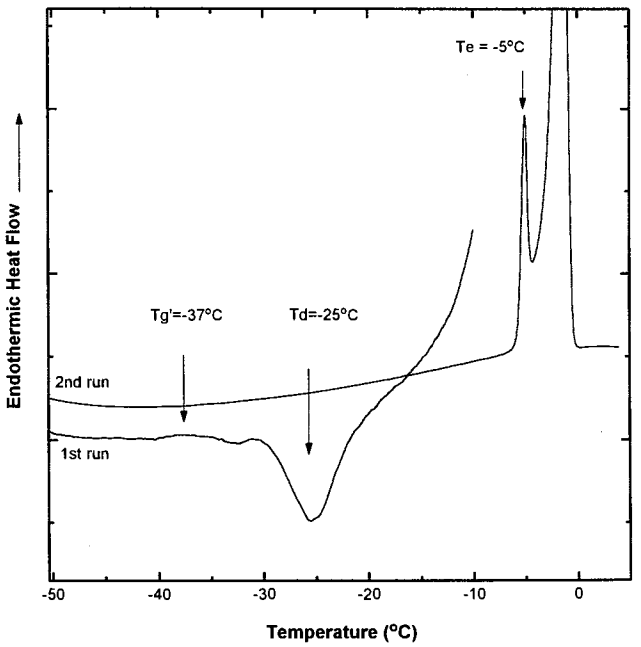

Fig. 1. Thermal analysis of frozen glycine solution. 2% (w/v) glycine was frozen at a cooling rate of 10°C/min. Analysis was carried out while the frozen solution was heated at 1°C/min. First run with 20 μl sample from −50°C to −10°C and second run with 2 μl sample from −50°C to 10°C.

The subambient DSC analysis of complete formulations, i.e., 2% glycine, 1% sucrose, and 10 mM sodium citrate buffer containing various concentrations of rhIL-1ra, is shown in Figure 2. The amorphous glycine fraction, which has Tg′ = −37°C, starts to recrystallize at −27°C, and by the time the temperature gets to −15°C, most glycine has been devitrified (Figure 2). The temperature of the devitrification exotherm increased with increase of protein concentrations. The area of devitrification during the annealing process was also reduced when more rhIL-1ra was included in the formulation (Figure 2). The decrease of devitrification is due to the reduced critical heating rate which is resulting from the increased viscosity by protein (1,3). The lack of a detectable exothermic peak, which suggests lack of devitrification, was found when the rhIL-1ra concentration was 100 mg/ml. A second exothermic peak was observed for 10 mg/ml sample, which is interesting but no explanation is available at this time. The second thermogram after successful devitrification shows no exothermic peak, indicating that the metastable glassy glycine fraction has been crystallized (Figure 1).

Based on DSC results, the freezing protocol used in the lyophilizer was: after freezing at −40°C the temperature of product is increased to −15°C at a rate of 1°C/min., followed by holding at −15°C for 30 minutes to foster the recrystallization of glycine. A formulation with 100 mg/ml of rhIL-1ra was used for the rest of this study.

Development of Drying Cycle

The key variables outlined in the introduction were examined to understand their contribution to the rate of sublimation during primary drying. The temperature of product during the drying process is an important factor. The sublimation will be faster at higher temperature, but the product temperature needs to be controlled below Tg′ to prevent undesirable collapse. Figure 3 shows the effect of shelf temperature and chamber pressure on the product temperature. Both shelf temperature and chamber pressure were major determinants of the product temperature. The product temperature was always lower than the shelf temperature due to the loss of heat by sublimation and thermal resistance between shelf and vials (9). Even when the shelf temperature was raised to a temperature routinely used for secondary drying, e.g., to 30°C, the product temperature remained below −22°C, which is low enough to avoid collapse of this product during primary drying, when the chamber pressure was maintained at 175 μm Hg or lower.

As demonstrated in Figure 3, several combinations of shelf temperature and chamber pressure can produce the same product temperatures during primary drying. The decrease of product temperature when the chamber pressure decreases can be compensated by raising the shelf temperature, and *vice versa*. Using those combinations where the product temperature is kept constant, the effect of shelf temperature and the chamber pressure on the sublimation rate of ice during primary drying can be examined. Such information about the relative role of shelf temperature and chamber pressure in the sublimation rate is critical in order to establish a mathematical model that describes the drying process and can be used to choose the optimum process variables for the maximum rate of sublimation. Figure 4 shows the sublimation rates of the IL-1ra formulation obtained under series of shelf temperature-chamber pressure combinations, each of which led to an identical product temperature of −17°C during primary drying. The sublimation was faster with the combination of higher shelf temperature and lower chamber pressure. The result indicates that the chamber pressure is another important variable that determines the rate of sublimation.

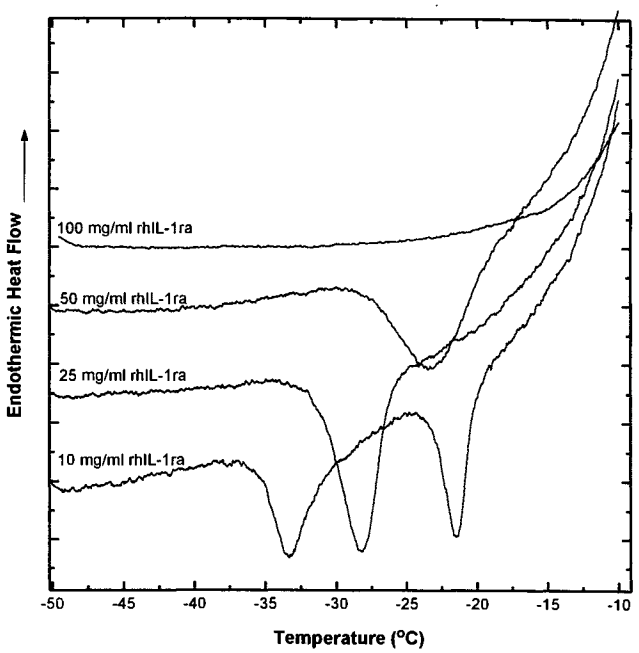

Fig. 2. Thermal analysis of frozen rhIL-1ra formulation. Various rhIL-1ra concentrations were analyzed. See Materials and Methods for the composition of the buffer. Conditions for the analysis were identical as the first run in Figure 1.

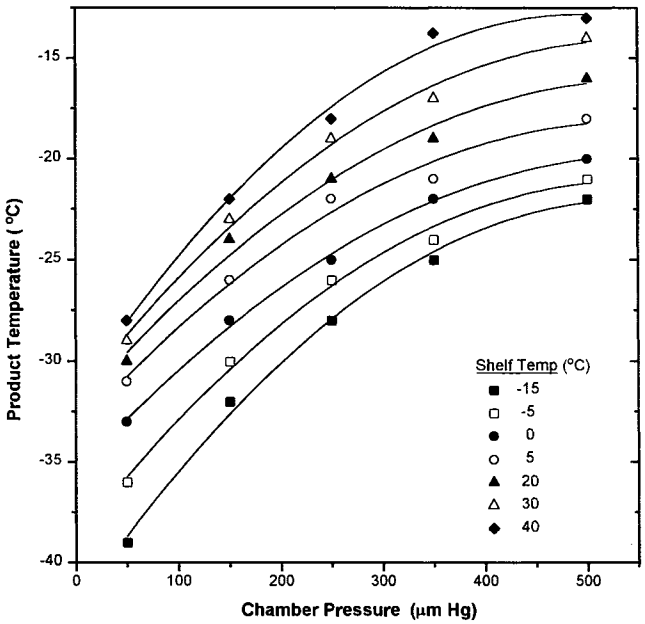

Fig. 3. Effect of chamber pressure on the product temperature of rhIL-1ra formulation (100 mg/ml protein) during primary drying at various shelf temperatures.

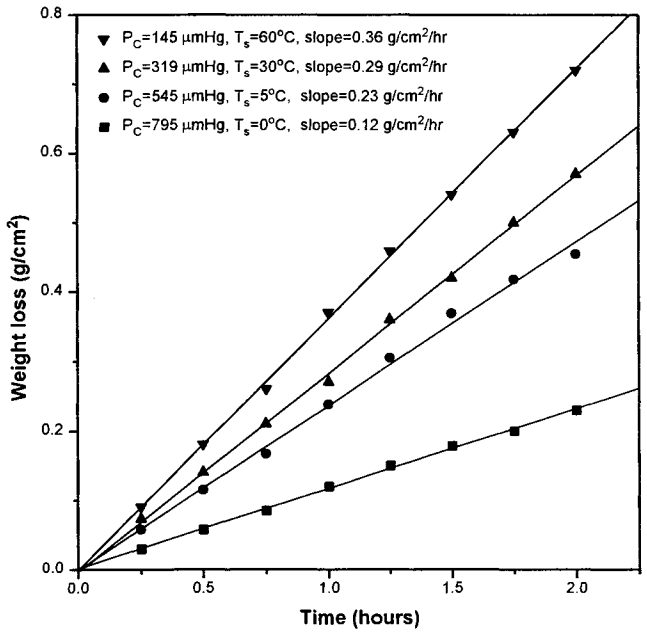

Fig. 4. Determination of sublimation rates by measuring the weight loss during the primary drying of rhIL-1ra formulation (100 mg/ml). Four different shelf temperature-chamber pressure combinations which produce the same product temperature of −17°C were examined. The slope of each curve represents the sublimation rate. Note that the sublimation rate increases as the chamber pressure decreases at a given product temperature.

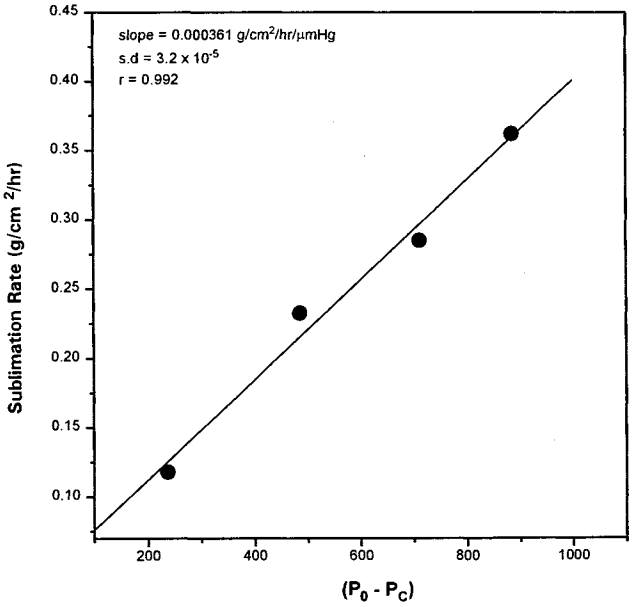

Fig. 5. Sublimation rate of ice during primary drying of rhIL-1ra formulation (100 mg/ml) is a linear function of $(P_O - P_C)$ value. The slope of curve determines the reciprocal value of resistance in Equation (1). Product temperature was −17°C. Data obtained from Figure 4.

To illustrate the observations that chamber pressure is a major determinant of the sublimation rate at a given product temperature, the slope of each curve obtained from Figure 4 was plotted against the pressure difference between vapor pressure of ice at product temperature (P_O) and chamber pressure (P_C). Figure 5 shows that the sublimation rate increases linearly as the pressure difference $(P_O - P_C)$ increases. $(P_O - P_C)$ increases, in turn, when the chamber pressure decreases at a given product temperature. Thus, for a given product temperature maximum sublimation can be achieved by decreasing chamber pressure. This result agrees with Pikal's report (10) that both product temperature, which is expressed as P_O, and chamber pressure (P_C) are major determinants of sublimation rate. The rate of sublimation is given by Equation 1 (10):

$$\frac{dm}{dt} = \frac{(P_O - P_C)}{(R_p + R_s)} \quad (1)$$

where: dm/dt = rate of sublimation, g/cm²/hr, P_O = vapor pressure of ice at the product temperature, μm Hg, P_C = chamber pressure, μm Hg, R_p = product resistance (cm² μm Hg hr g^{-1}), R_s = stopper resistance (cm² μm Hg hr g^{-1}).

The slope of the curve shown in Figure 5 represents the reciprocal value of the product and stopper resistance to sublimation $(R_p + R_s)$ described in Equation (1). In order to examine the temperature dependence of the resistance value, resistance values were calculated at several other temperatures by obtaining sublimation rates under several combinations of chamber pressure and shelf temperatures which generate the same product temperatures, followed by calculating the slopes of curves, as illustrated in Figure 4 followed by Figure 5. As demonstrated in Figure 6, no significant difference in the resistance values was observed between four product temperatures tested. Therefore, the resistance value for the sublimation rate of rhIL-1ra formulation $(1/(R_s + R_p))$ becomes a constant in Equation (1). In other words, the sublimation rate of ice during primary drying is a linear function of $(P_O - P_C)$. Figure 7, which shows the relationship between sublimation rate and $(P_O - P_C)$ for a variety of product temperatures, supports this conclusion

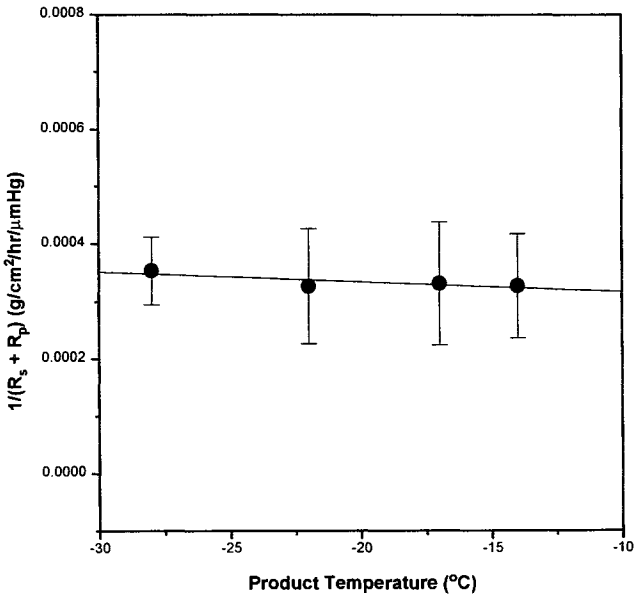

Fig. 6. Resistance value in Equation (1) is not significantly affected by product temperature. Error bars show the standard deviation.

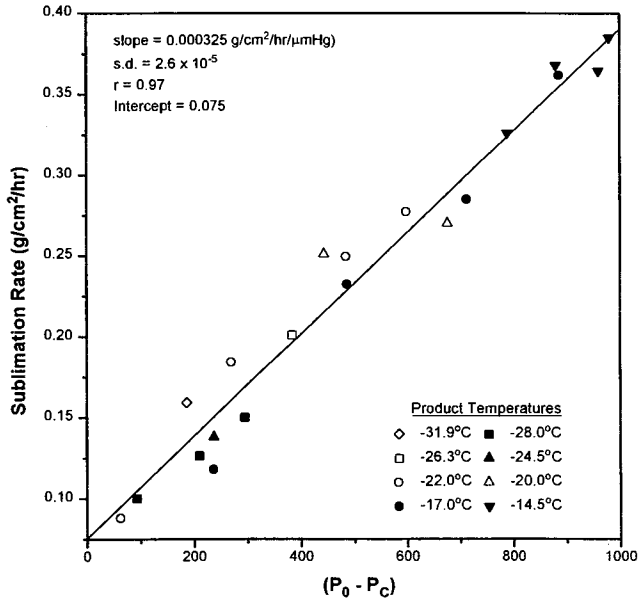

Fig. 7. Linear relationship between sublimation rate and $(P_O - P_C)$ value can be found with all data obtained with various combinations of chamber pressures, shelf temperatures, and product temperatures. The result indicates that $(R_s + R_p)$ value in Equation (1) is constant for the formulation.

because linearity of the curve is maintained at all product temperatures tested. The result suggests that the main parameter to consider for the optimization of primary drying cycle is $(P_O - P_C)$ no matter what combinations of shelf temperature, chamber pressure, and product temperature are used. The constant resistance value $(1/(R_s + R_p))$ for 1 ml fill in 3 ml vial with a 13 mm stopper, which was calculated with all the data shown in Figure 7, was $3.25 \times 10^{-4} \pm 2.64 \times 10^{-5}$ (cm^2 hr μm Hg g^{-1}). Since the curve in Figure 7 does not pass the origin, a slight modification of Equation (1) was made by including the intercept value as shown in Equation (2).

$$\frac{dm}{dt} = 0.000325 \times (P_O - P_C) + 0.0757 \qquad (2)$$

Using the simplified equation, a sublimation rate can be calculated if the product temperature (P_O) and the chamber pressure (P_C) are known. For example, if the combination of shelf temperature at 30°C and chamber pressure of 319 μm Hg maintains the product temperature at −17°C, where the vapor pressure of ice (P_O) is 1031 μm Hg (12), the sublimation rate will be $(1031 - 319) \times 3.25 \times 10^{-4} + 0.0757 = 0.30$ (g/cm^2/hr). With this sublimation rate, 1 ml of formulation in a vial with an inner diameter of 15 mm will complete primary drying within 2 hours.

To maximize the sublimation rate the combination of highest allowable product temperature and lowest chamber pressure should be found, where $(P_O - P_C)$ in Equation (1) is maximum. Figure 8 shows the relationship between chamber pressure, shelf temperature, product temperature, and sublimation rate, which illustrates the rationale to maximize sublimation rate. An effort to increase the sublimation rate simply by reducing chamber pressure to decrease P_C value in Equation (1) will result in decrease of sublimation rate because of concomitant decrease of P_O value as the product temperature decreases. In Figure 8, the sublimation rate of 0.16 (g/cm^2/hr) at condition "A" will decrease to 0.11 if simple reduction of chamber pressure to 140 μm Hg results in the decrease of the product temperature to −25°C (condition "B"). On the other hand, increasing chamber pressure (P_C) at a given shelf temperature results in increase of sublimation rate due to raised product temperature (P_O). Increase of chamber pressure to 420 μm Hg under same shelf temperature resulted in faster sublimation rate of 0.19 due to increased product temperature to −17.5°C (transition to condition "C"). Assuming that −20°C is only slightly below Tg′ of the product, this increase of product temperature may not be desirable. Isothermal curves of product temperature indicate that the combination of high shelf temperature and low chamber pressure provides better sublimation rate than the combination of low shelf temperature and high chamber pressure when maximum allowable product temperature is set. In Figure 8, improvement of drying efficiency without collapsing the product can be found when the process is changed from condition "A" (P_C = 300 μm Hg and shelf temperature = 20°C, sublimation rate = 0.16) to the condition "D" (P_C = 195 μm Hg and shelf temperature = 40°C, sublimation rate = 0.19). The result implies that a very efficient condition can be reached when shelf temperature is set as high as possible within the allowance of system and product stability, and product temperature is maintained below maximum allowable temperature by reducing chamber pressure.

To take advantage of the observations, a single-step drying cycle was developed which can effectively give proper primary and secondary drying. For this cycle, shelf temperature is set for secondary drying and the product temperature for primary drying is controlled by adjusting the chamber pressure. As discussed earlier, product temperature can

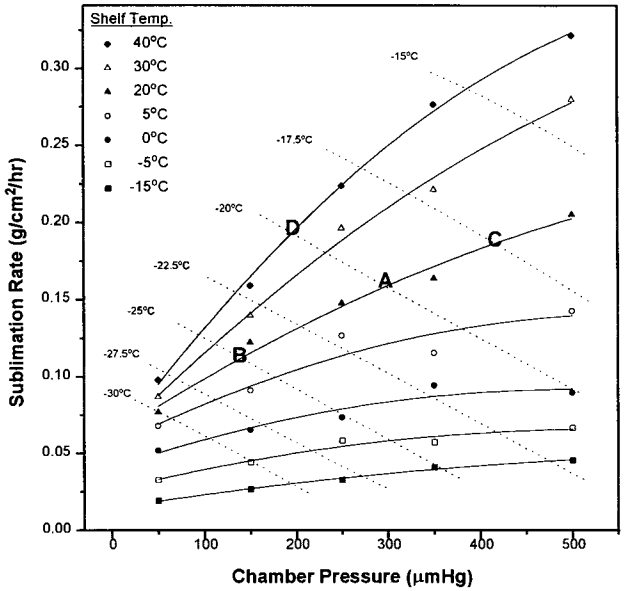

Fig. 8. Effect of chamber pressure on the sublimation rate of rhIL-1ra formulation at given shelf temperatures (solid line). Dotted lines show isothermal curves of product temperature.

be maintained below maximum allowable temperature by properly setting the chamber pressure. As the sublimation of ice completes and the heat loss at the sublimation front diminishes, the product temperature will naturally equilibrate to the shelf temperature, where secondary drying proceeds.

There are numerous advantages to the single-step process. The combination of high shelf temperature and low chamber pressure provides faster sublimation as demonstrated in Equation (1). There is less chance of failure, which may occur with a two-step drying cycle when a transition to secondary drying is forced with incomplete primary drying due to various reasons, because only the completion of primary drying triggers the secondary drying. For the same reason, it is not necessary to wait for the last vial to complete primary drying before starting secondary drying, which eliminates the need for external control for the transition. Since the product temperature is controlled not by the shelf temperature but primarily by the chamber pressure, the single-step process will be useful for containers which have very little thermal transfer from the shelf, e.g., pre-filled syringes. Figure 9 shows an example of the single-step drying cycle. A smooth increase of product temperature after completion of sublimation demonstrates that a secondary drying was naturally continued. Total drying time including freezing process and annealing process was approximately 6 hours for a 1 ml of rhIL-1ra formulation filled in a 3 ml vial with a 13 mm stopper. The moisture content of the lyophilized formulation using the single-step cycle was 0.4%. The lyophilized product had good cake quality and the rhIL-1ra molecule maintained desirable integrity and activity after the single-step drying process.

A precaution for the single-step drying cycle is that decreased thermal transfer from the shelf to the bottom of the vial at low chamber pressure and heat loss from the sublimation front creates a heterogenous temperature distribution throughout the product during a primary drying. The temperature gradient is more significant when larger product volumes are dried and the distance between the sublimation front from vial bottom increases. Figure 10 shows the product temperature at the bottom of the vial is increased as high as −10°C, although the temperature at the drying front is as low as −22°C. The significance of the temperature discrepancy is not clear because the product temperature at the bottom of the vial will decrease to −22°C, which is below the Tg′ of the product, as the sublimation front approaches and the collapse only occurs while ice sublimes (7). This concern may be significant when the temperature at the bottom of vials reaches the eutectic melting or the melting point of the product. No adverse effect of the temperature gradient was observed when 1 ml to 10 ml of the rhIL-1ra formulation was freeze-dried with the same single-step cycle that has been discussed.

It should be noted that in many formulations it is not possible to set the shelf temperature to the secondary drying temperature and maintain the product temperature at a sufficiently low temperature by decreasing chamber pressure. Also the extent to which pressure affects product temperature can be dependent upon the system, such as the contact resistance to heat transfer, the capacity of condenser, and the rate of mass transfer, etc.

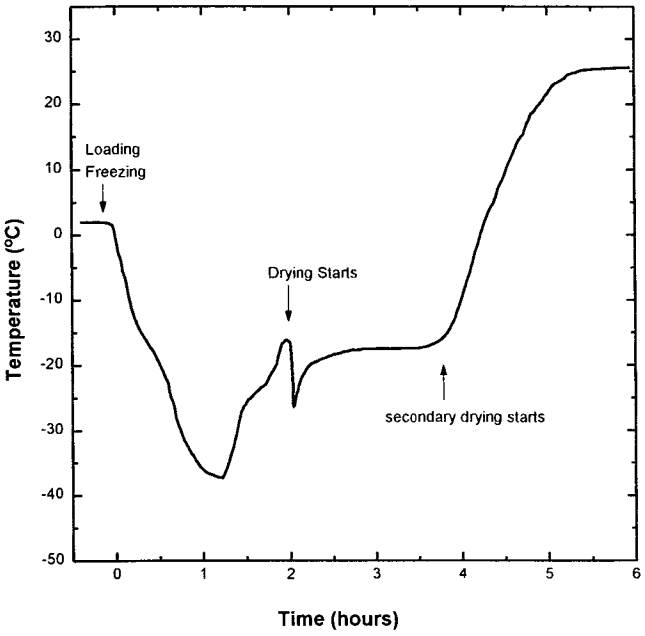

Fig. 9. Examples of a single-step freeze-drying cycle. The chamber pressure was maintained at 325 μm Hg and the shelf temperature was maintained at 30°C for both primary and secondary drying. One ml of rhIL-1ra formulation (100 mg/ml) filled in a 3 ml vial was dried. Product temperature probes were located near the bottom of the vial.

CONCLUSIONS

The major factor determining the rate of sublimation is the difference between chamber pressure and vapor pressure of ice at the sublimation front. Considering this principle, the most efficient cycle can be designed when the shelf temper-

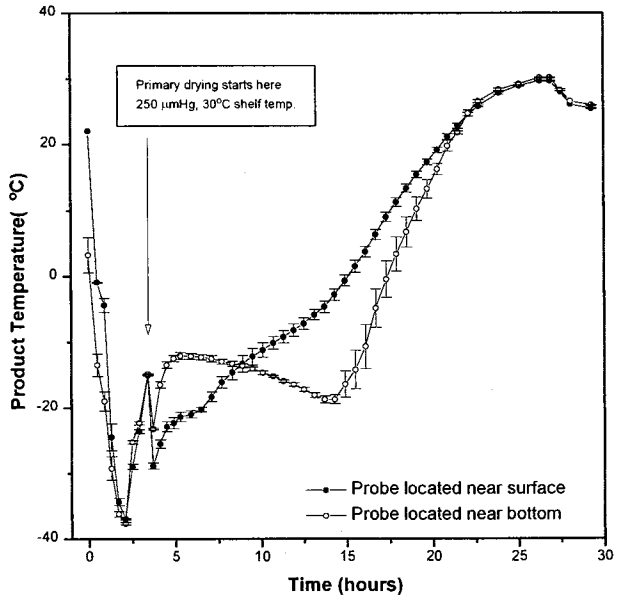

Fig. 10. Variation of product temperature within a vial during a single-step drying cycle. Probes were located near the surface and near the bottom of vials. Ten ml of 100 mg/ml rhIL-1ra formulation filled in 10 ml vials was dried.

ature is set as high as possible within the limitation of safety and the product temperature is maintained below the maximum allowable temperature by adjusting the chamber pressure. The single-step cycle provides an ideal condition for the faster drying with combination of high shelf temperature and low chamber pressure. This study signifies that thorough understanding of freeze-drying can allow one to develop more flexible cycles which require less arbitrary controls.

ACKNOWLEDGMENTS

This manuscript was prepared with the help and guidance of Dr. John F. Carpenter and Dr. James W. Thomson.

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