Human Insulin Microcrystals with Lactose Carriers for Pulmonary Delivery

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Dry powder formulations for pulmonary delivery are attractive because many issues of solubility and stability can be minimized. Human insulin microcrystals with lactose carriers were produced for pulmonary delivery. The average particle diameter was 2.3 μm, with a narrow, monodispersed size distribution. The percentages of high molecular weight proteins (%HMWPs), other insulin-related compounds (%OIRCs), and A-21 desamido insulin (%Dm) were very low throughout the microcrystal preparation process. Administration of the microcrystal powder by intratracheal insufflation significantly reduced the blood glucose levels of Sprague-Dawley rats. The percent minimum reductions of the blood glucose concentration (%MRBG) produced by the insulin microcrystal powder and by an insulin solution reached 40.4% and 33.4% of the initial glucose levels respectively, and their bioavailability relative to subcutaneous injection (F) was 15% and 10% respectively. These results confirm that the insulin microcrystal powder prepared is suitable for pulmonary delivery in an effective dosage form.

Key words: insulin; microcrystal; pulmonary delivery; lactate carriers

The pulmonary tract appears to be the most attractive alternative for non-invasive systemic delivery of peptide and protein drugs.1)10) The lung is naturally permeable to many proteins in the absence of enhancers and has relatively low metabolic enzyme activity.3)–9) Unlike the nasal cavity, which has approximately 180 cm2 of surface area, the lung of an adult offers a large surface area, of approximately 100 m2, for drug absorption.10) The high level of vascularization and the ultra-thin nature of the alveolar epithelium (approximately 0.1–0.5 μm) facilitate rapid drug absorption. Moreover, respiratory delivery avoids the first-pass effect of the gastrointestinal tract.3)–6) However, the combination of insulin analogs and pulmonary administration has not been effective in the control of blood glucose levels because of the short-acting property of insulin analogs and the need for formulations amenable to pulmonary delivery.11,12) Investigators have developed insulin formulations to prolong absorption in the lung, including microencapsulation using a biodegradable polymer, lipid co-administration, and porous particles,13)–15) but there are limitations to these approaches, e.g., accumulation of the biodegradable polymer in the lung,16) loss of insulin activity during the preparation of microspheres,17,18) and the requirement of a large drug mass for human applications.5,6)

Crystallization, as a major technological process of drug formulation in the pharmaceutical industry, plays an important role in enhancing the stability and drug release properties of final dosage forms.1)13) Furthermore, a dry powder aerosol offers the capacity to provide a wide range of single dose per inhalation, low susceptibility to microbial growth, and suitability for both water-soluble and insoluble drugs.20)–22) The thin alveolar epithelium of the lung can be an effective target in delivering drugs as aerosols with mass median aerodynamic diameters of less than 5 μm.1,23) Recently, not only was a unique insulin microcrystallization process using a seed zone method developed, but the bioavailability enhancement and long-acting property of the microcrystals with regard to intrapulmonary inhalation were also confirmed.1,2,5,6) The obtained microcrystals were homogeneous in size (<5 μm almost 90% in volume) with a narrow distribution of sizes, suggesting that preparation of microfine protein crystals suitable for pulmonary application is possible without harsh mechanical processes. In addition, an insulin microcrystal suspension as a long-acting formulation for pulmonary delivery has been studied.5,6)

Interest in dry powder inhalers (DPIs) has been increasing, as the use of chlorofluorocarbons (CFCs) has been prohibited in medical sprays. DPIs result in even better lung deposition of drugs than metered dose inhalers (MDI), which are pressurized. Moreover, coordination of actuation and inhalation is easier with DPIs.24) The smaller insulin microcrystals might be a better model formulation to test the long-acting effect of crystals as a pulmonary formulation.5,6) However, the small particle size necessary for effective lung deposition causes problems in processing (poor flowability) and redispersion (strong agglomeration and adhesion).15,25) Various supplements are typically used in DPIs to aid the flow and dispersion properties of the drugs.27–29) Although many reports have been published on pulmonary delivery of peptide and protein drugs using powder formulations, there have been no studies on a powder formulation containing only carriers without any supplements, e.g., glycerin, mannitol, trehalose, albumin, bile salts, or protease inhibitors, which can enhance the stability or absorption of insulin but can also exhibit many side effects in the lung. The

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Abbreviations: CFCs, chlorofluorocarbons; %CV, coefficient of variation; %Dm, A-21 desamido insulin; DPIs, dry powder inhalers; %HMWP, high molecular weight proteins; MDI, metered dose inhalers; %OIRCs, other insulin-related compounds; SEM, scanning electron microscopy
formulation studied in this research was composed only of insulin microcrystals and lactose carriers. In this study, human insulin microcrystals for pulmonary delivery were attached to commercially available lactose carriers by a drying method, and their size and crystallinity were characterized. The hypoglycemic effect of human insulin microcrystals with lactose carriers was also examined following intratracheal administration to normal rats.

Materials and Methods

Materials. Crystalline recombinant human insulin was purchased from Serological Corporation (Norcross, GA). One commercial batch of lactose monohydrate (Lactochem® crystals, mean diameter 100 μm) was purchased from Borculo Domo Ingredients (Zwolle, Netherlands). All other chemical substances were of analytical grade.

Microcrystallization of human insulin. Insulin microcrystals were prepared using a previously described method with minor modifications.24,30 Crystalline insulin powder and zinc sulfate were completely dissolved in 0.1 N acetic acid (pH 2.0) to generate a 1 mg/ml of insulin solution containing 0.5% zinc ions. The pH of the solution was increased to approximately 6.0 by adding 1 or 10 N NaOH solution. The solution was stirred for 15–30 min at room temperature, followed by overnight stirring at 4 °C to allow the formation of microcrystals. An insulin solution was prepared by dissolving the insulin microcrystals in acidic phosphate buffered saline (PBS, pH 2). The pH of the solution was adjusted to physiological levels (pH 7.2–7.4) with NaOH before use. The samples were stored at room temperature and used within 1 h.

Particle size analysis: characterization of insulin microcrystals. Microcrystals were dispersed in acetic acid (0.1 N, pH 6) and sized by laser diffraction using a CILAS 1064 (CILAS, Orleans, France). The particle size was expressed as volume mean diameter.

Morphology analysis by SEM: characterization of insulin microcrystals. Initial fixation was performed with 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7) for 1 h at 4 °C. Following a brief rinse with PBS (2 times, 5 min), the microcrystals were dehydrated in a graded series of ethanol, replaced with isooamyl acetate, dried directly from hexamethyldisilazane, and sputter-coated with gold palladium before examination in the SEM (JEOL JSM-5300, Tokyo).

Powder preparation of insulin microcrystals with lactose carriers. Insulin microcrystal powder with commercial lactose carriers was prepared as described previously, with minor modifications.30 The microcrystals were dispersed in isopropanol and stirred for 5 min. The lactose carriers were added and stirred for 30 min. The insulin microcrystal/lactose carrier ratios were 1:9, 1:29, and 1:49. The microcrystals-lactose carrier mixtures were dried in a rotary evaporator by rotation under a vacuum for 3 h. The homogeneity of each of the powder mixtures was determined by analyzing the concentration of insulin in the mixtures. Seven samples taken randomly were weighed, dissolved in 0.01 N HCl, and assayed by validated HPLC, as described below (reversed-phase HPLC). The coefficient of variation (%CV) was used to assess the homogeneity of the mixtures. All experiments were performed in triplicate, and variation was expressed as standard error of the mean.

Analysis of product-related impurities during the process. The HPLC system was a Waters Alliance system (Waters Corporation, Milford, MA) equipped with a quaternary pump, a thermostatized column compartment, an autosampler, a Waters M2906 PhotoDiode Array Detector, and data acquisition software, Empower Chromatography Manager. Deionized water prepared with a MilliQ apparatus (Millipore, Billerica, MA) was used. All solvents were filtered with 0.45-μm filters.

Size exclusion HPLC (SE-HPLC). In order to measure high molecular weight proteins (HMWPs), a filtered and degassed mixture of arginine solution, acetonitrile, and glacial acetic acid (65:20:15) was used as a mobile phase. The column temperature was maintained at 40 °C, and the flow rate was 0.5 ml/min. Detection was performed at 276 nm. One hundred microliters of the sample was applied to the column. The percentage of HMWP (%HMWP) was calculated by the following formula:

\[
\%\text{HMWP} = \frac{\Sigma R_H}{\Sigma R_I + \Sigma R_M} \times 100
\]

where ΣR is the sum of the responses for all peaks having retention times less than that of the insulin monomers, and ΣRm is the peak response of the insulin monomers (USP XXVI, 2002).

Reversed-phase HPLC (RP-HPLC). A reversed-phase C-18 column (Vydac, 4.6 × 250 mm, The Separation Group, Hesperia, CA) was used. The solvent was prepared by dissolving 28.4 g of anhydrous sodium sulfate in 1,000 ml of water and adding 2.7 ml of phosphoric acid. The pH of the solvent was adjusted to pH 2.3 with ethanolamine. Filtered and degassed mixtures of the solvent and acetonitrile were used as solution A (82:18) and solution B (50:50). The column temperature was maintained at 40 °C, and the flow rate was about 1 ml per min. Elution peaks were detected at 214 nm. After 20 μl of the sample were applied to the column, a chromatography run was performed. Initially, isocratic elution was performed for about 60 min with a mobile phase consisting of a mixture of 80% solution A and 20% solution B. Following a gradient elution phase of 25 min, during which the ratio of solution A:solution B in the mobile phase progressed from 80:20 to 36:64, the system maintained isocratic elution for 6 min and returned to the initial conditions of 80% solution A and 20% solution B (USP XXVI, 2002). The percentage of insulin, %I, was calculated by the formula

\[
\%I = \frac{R_I}{R_S} \times 100
\]

where RI is the peak response for insulin and RS is the sum of the responses for all the peaks. The percentage of A-21 desamido insulin, %Des, was calculated by the formula

\[
\%\text{Des} = \frac{R_{Des}}{R_S} \times 100
\]

where RDes is the peak response for A-21 desamido insulin. The percentage of other insulin related compounds (%OIRCs) was calculated by the formula

\[
\%\text{OIRCs} = 100 - (\%I + \%\text{Des})
\]

X-Ray diffractometry (XRD). The solid structures of insulin microcrystals, lactose carriers, and their mixtures were determined by X-ray diffraction. After each sample was transferred into a 1-mm glass capillary the capillary tubes were sealed with wax. Diffraction data were collected using a Rigaku R-AXIS IV++* image plate detector (Rigaku International, Tokyo) with a 50 kV, 100 mA rotating copper anode and focusing mirrors, using an oscillation of 240° and 30 min exposure time at a 10 cm material-to-detector distance.

Differential scanning calorimetry (DSC). A differential scanning calorimeter (DSC 6100, Chiba, Japan), equipped with a refrigerated cooling accessory using liquid nitrogen, was operated at a heating rate of 5 °C/min from 40 to 200 °C. The temperature was calibrated with indium. An empty pan was used as the reference.

Animal experiments. Male Sprague-Dawley rats (Tacomin Acmed, Rockville, MD) weighing 200–300 g were kept under a 12-h day-night cycle at 20 °C and a relative humidity of 50% for 7 d. The animals were fasted for 12 h prior to each experiment, but were allowed free access to water. The insulin solution and dry powder were administered by previously described methods,31,32 with minor modifications. The animals were anesthetized by intraperitoneal injection of 60 mg/kg of ketamine and 20 mg/kg of sodium pentobarbital. After the animals were secured on their backs on a slanted board (30° from the vertical)
hanging from the upper incisors, the trachea was subjected to a longitudinal incision along the ventral aspect of the neck. Then 100 μl of insulin solution was injected into the lung through a calibrated 250-μl microsyringe (1725LT 250 μl SYR, Hamilton, Reno, NV). The needle was inserted between the fifth and sixth tracheal rings, and the insulin solution was injected over a period of 1–2 s. The dry powder was placed in the tip of an insufflator and dispersed in the rat trachea by releasing air compressed in a syringe (Fig. 1). The incision in the skin was sutured with a sterile 6/0 silk suture and swabbed with betadine solution. The glucose concentration of blood samples withdrawn from the tail vein at appropriate time intervals was measured with LIFESCAN SureStep™ (Johnson & Johnson, Milpitas, CA).

Data analysis. To estimate pulmonary absorption of insulin, the blood glucose concentration was measured with LIFESCAN SureStep™ (Johnson & Johnson, Milpitas, CA) at the indicated time intervals. The decrease in the blood glucose concentration (D%) was calculated by a modification of the method of Hirai et al.33) by the following equation:

\[
\%D_{Xh} = \left( \frac{AUC_{0-Xh}}{100} \times X\text{hour} \right) \times 100
\]

where \(AUC_{0-13h}\) is the area under the curve for 13 h.

Results and Discussion

Size distribution and morphology of insulin microcrystals

The size distribution of the insulin microcrystals produced in this study was compared to that of a commercial crystalline powder (Fig. 2). The commercial crystalline powder was composed of large particles with a mean diameter of 11.0 μm and a broad size distribution. By contrast, our microcrystals had a narrow, monodispersed size distribution, and their mean diameter was 2.3 μm. The yield of crystallization was maintained at 90% or higher, and almost 90% of the microcrystals were less than 5 μm in diameter.

The morphology of the insulin microcrystals was observed by SEM. While the commercial crystalline powder was very crude, the microcrystals were of homogeneous trigonal forms with some triclinic forms and without aggregates. Therefore, the microcrystals might be optimal for controlling the drug dosage, based on their narrow, monodispersed size distribution, and for effective pulmonary delivery, based on their small size, of 1–5 μm.

Insulin microcrystals attached to lactose carriers

After the drying process, the microcrystals adhered to the surface of commercial lactose carriers, and their size and shape did not change as compared to those prior to the drying process (Fig. 3). Figure 4 shows the %CV (coefficient of variation) of the microcrystals blended with lactose carriers in ratios of 1:9, 1:29, and 1:49 respectively. The %CV decreased with increasing fractions of the lactose carrier, and a satisfactory mixing uniformity with a %CV of less than 3% was observed for the mixture with a ratio of 1:49. It has been found that increasing the elongation ratio of lactose carriers increases the fine particle fraction (FRF) and dispersibility of drug particles after aerosolization of formulations from DPIs.29)

Process-related impurities of insulin microcrystals with lactose carriers

The process-related impurities of insulin produced via the microcrystallization and drying process were investigated by analytical methods approved by the US Pharmacopoeia (USP XXVI, 2002). HMWP analysis using SE-HPLC showed that the percentages of insulin monomers (%I) of the microcrystals both before and after drying, 99.68 ± 0.02% and 99.74 ± 0.03% respectively, were almost the same as that of the insulin powder (99.82 ± 0.01%) that was used as the raw material (Table 1) in generating the crystals. The %HMWPs of the microcrystals before and after micro-
crystallization and drying were 0.32 ± 0.02% and 0.26 ± 0.03% respectively, below the US Pharmacopeia allowable limit of not more than 1.0% (USP XXVI, 2002). In the case of desamido insulin analysis using RP-HPLC, the levels of A-21 desamido insulin (%Des) of the microcrystals before and after microcrystallization and drying (0.59 ± 0.02% and 0.69 ± 0.17%) were almost the same as that of the insulin crystalline powder (0.56 ± 0.03%). These values were much lower than the limit set by the US Pharmacopeia guidelines (not more than 2.0%). The percentage of OIRC (%OIRC) of the microcrystals during processing (0.30 ± 0.01% and 0.16 ± 0.19%) was slightly lower than that of the insulin powder (0.37 ± 0.12%), not enough for consideration. The results demonstrated almost no degradation of insulin during the crystallization and drying process.

Physicochemical properties of insulin microcrystals with lactose carriers

DSC measurements provide qualitative and quantitative information regarding phase transitions in materials that involve endothermic or exothermic processes or changes in heat capacity as a function of time and temperature. A representative DSC thermogram of the microcrystal-lactose carrier mixtures was almost the same as that of the lactose carriers alone (Fig. 5). Both showed a substantial endothermic pattern with increasing temperature, indicating that no transitions occurred during the drying process. These results suggest that there were no changes in the physicochemical properties of the lactose carriers during the drying process.

The crystalline structures of insulin microcrystals, commercial lactose carriers, and microcrystal-lactose carrier mixtures were measured by XRD (Fig. 6). The commercial lactose carriers showed many intensive diffraction peaks, whereas the peaks arising from the microcrystals were weak in comparison (Fig. 6B, D). The diffraction data for the lactose carriers yielded a pattern of well-formed concentric rings of variable intensity (Fig. 6C). As shown in Fig. 6A, the ring pattern of the microcrystals was similar to that of the commercial crystalline powder at low resolution (data not shown), but it was broader at intermediate resolution. It has been found that the diffraction peak and ring pattern are affected by the crystal size and the crystallinity of the particles. In the case of microcrystals, it is likely that the size of the microcrystals led to a reduction in diffraction intensity and a broadening of the rings because the average diameter of the insulin microcrystals (2.3 μm) was about 5 times smaller than that of the commercial crystalline powder (11 μm).

Animal experiments

The blood glucose concentrations following intrapulmonary administration of insulin microcrystal pow-
Table 1. Impurity Analysis of Human Insulin Microcrystals

<table>
<thead>
<tr>
<th></th>
<th>SE-HPLC</th>
<th>RP-HPLC</th>
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<tbody>
<tr>
<td></td>
<td>%Ia</td>
<td>%HMWPb</td>
</tr>
<tr>
<td>Insulin powder (raw material)</td>
<td>99.82 ± 0.03</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td>Microcrystal (after crystallization)</td>
<td>99.68 ± 0.02</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>Microcrystal (after drying)</td>
<td>99.74 ± 0.03</td>
<td>0.26 ± 0.03</td>
</tr>
</tbody>
</table>

a%I, percentage of insulin
b%HMP, percentage of high molecular weight proteins
c%D, percentage of A-21 desamido insulin
d%OIRC, percentage of other insulin related compounds
Each value represents the mean ± S.D. (N = 3).

Fig. 5. Thermograms (obtained by DSC) of Microcrystal and Commercial Lactose Carrier Mixtures (A), and Commercial Lactose Carrier Alone (B). The indicated values are melting temperature (Tm) and enthalpy (ΔH).

Fig. 6. Crystallinity of Human Insulin Microcrystals and Lactose Carriers as Measured by XRD. XRD intensity peaks and ring patterns of microcrystals (A, B), commercial crystalline lactose carriers (C, D), and a mixture of the two (E, F) were compared.

Fig. 7. Blood Glucose Levels after Intratracheal Delivery (IT) and Subcutaneous Injection (SC) of Microcrystal Powder and Insulin Solution. Microcrystal powder by IT 11–12 U/kg (●), insulin solution by IT 20 U/kg (○), and insulin solution by SC 1 U/kg (▼) (N = 4–5) were administered. PBS (▼) was used in the control group. Data points (mean ± S.E.) represent relative values of the initial glucose concentration.

Fig. 8. Blood Glucose Levels after Intratracheal Insufflation of Microcrystal Powder with Lactose Carriers (10–11 U/kg), Commercial Crystalline Insulin Powder (50 U/kg), and Lactose Carriers (N = 3). Sham operations were performed as the control. Data points (mean ± S.E.) represent relative values of the initial glucose concentration.
der (10–12 Unit/kg), solution (20 Unit/kg), and raw material powder (about 50 Unit/kg) are shown in Figs. 7 and 8, and the values of the pharmacodynamic parameters are listed in Table 2. The blood glucose levels in the rats administered with each preparation decreased with time and reached minimum values at the following time points: 40 min (IT raw material powder, SC insulin solution), 2 h (IT microcrystal powder), and 3 h (IT insulin solution). 13 h after treatment, the blood glucose levels in the rats treated with the microcrystal powder with lactose carriers were much lower than in those treated with the insulin raw material powder, and slightly higher than in those treated with the insulin solution (IT) (Figs. 7 and 8). The overall blood glucose concentration (%D) achieved with the microcrystal powder (41.28%) was greater than that with the IT insulin solution (32.51%) and insulin raw material (26.91%) (Table 2). Because different doses were administered to the various groups, the F value, representing bioavailability relative to subcutaneous injection, was used to compare the availabilities for each dosage form. The F value of the microcrystal powder (0.15) was slightly higher than that of the insulin solution (IT) (0.10) and raw material (0.02) (Table 2). These results are in accord with previous reports demonstrating that powder dosage forms of insulin microcrystals have advantages over liquid formulations. There are many barriers to pulmonary delivery of peptides and proteins, including mucociliary clearance, the alveolar lining layer, the alveolar epithelium, the basement membrane, pulmonary enzymes, and macrophages. The improved bioavailability of the microcrystal powder formulation is to be attributed to a different disposition pattern in the lung that influences drug absorption. In the presence of the barriers listed above, insulin molecules dissolved in a solution formulation appear to be more easily degraded by proteases or alveolar macrophages than the insulin microcrystal powder. It is also possible that the dissolution of the powder dosage form in the deep lung epithelium extracts water from the alveolar epithelium, resulting in a widening of the spaces between tight junctions, thus facilitating drug absorption. However, in the case of the insulin raw material powder, the F value was very low (0.021) in spite of a very high dose of insulin (50 U) (Table 2), possibly due to the large particle size (mean diameter, 10.97 μm) and broad size distribution (Fig. 2A). This result is in good agreement with previous reports showing that particles for pulmonary delivery should be less than 5 μm. In addition, it is also been found that in powder dosage forms, the chemical stability of drugs is increased, a preservative is not required, and larger amounts of drugs and excipients can be administered.

### Conclusion

A particle production process for pulmonary delivery of protein drugs was developed, and an insulin microcrystal powder with lactose carriers was prepared. The microcrystals had a homogeneous trigonal shape, with an average diameter of 2.3 μm and a narrow, monodispersed size distribution. A satisfactory mixing uniformity with a %CV of less than 3% was observed in mixtures with a microcrystal powder:lactose carrier ratio of 1:49. The results of HPLC analysis showed no denaturation during the microcrystallization and drying process. After administration of the microcrystal powder to normal SD rats by intratracheal insufflation, blood glucose levels were significantly reduced. The bioavailability of the intratracheally administered microcrystal powder (15%) relative to subcutaneous injection was higher than that of the insulin solution (10%). These results indicate that the administration of insulin microcrystal powder by pulmonary delivery can control blood glucose levels, and does so more effectively than liquid formulations.

### References