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Improvement of stability and absorbability of dry insulin powder for inhalation by powder-combination technique

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Abstract

The effect of pulmonary absorption enhancers on the stability of active ingredients is an important factor for successful inhalation therapy as well as the effect on pharmacological activity and safety. We examined the effect of pulmonary absorption enhancers on the stability of insulin in dry powders prepared by a spray-drying technique. Although the hypoglycemic effect was greatly improved when a dry insulin powder containing citric acid (MIC SD) was administered, insulin in the MIC SD was unstable compared with the other powders examined. Bacitracin and Span 85, which are potent pulmonary absorption enhancers of insulin formulated in solutions, showed no deteriorative effect on the stability of dry insulin powder. However, they did not improve the hypoglycemic effect of insulin in dry powders. We modified the insulin dosage form with citric acid to improve the insulin stability at room temperature without loss of hypoglycemic activity. MIC Mix was formulated as a combination of insulin powder (MI′) and citric acid powder (MC). MIC Mix showed hypoglycemic activity comparable to MIC SD while the insulin stability was much better than that of MIC SD at a 60 °C/dry condition. However, moisture lowered the insulin stability and changed the particle morphology of MIC Mix with time at a 60 °C/75% relative humidity condition, suggesting that a package preventing moisture absorption was necessary for the MIC Mix powder.

Keywords: Dry powder; Insulin; Inhalation; Stability; Absorption enhancer

1. Introduction

Pulmonary administration of drugs is convenient and enables patients to avoid the need for painful injections. It leads to improved patient compliance and quality of life. Recently, the pulmonary route has attracted attention as a noninvasive systemic administration route for peptide and protein drugs. The pulmonary route would be a promising alternative for the delivery of peptide and protein, since many drugs that are poorly absorbed from enteral and other mucosal sites are well absorbed from the lung because of its large absorptive surface area (>100 m^2 in adult human lung) and thin membrane (<1.0 μm). However, pulmonary bioavailability of macromolecules is still below intravenous or subcutaneous bioavailability. The reasons for the low bioavailability of peptides and proteins are attributed to low diffusivity through the epithelial barrier and sensitivity of peptides and proteins to pulmonary enzymes. To overcome this, several chemicals and enzyme inhibitors have been examined as pulmonary absorption enhancers. There have so far appeared many reports on the enhancement of pulmonary absorption of peptides and proteins. In these reports, bile acids, surfactants, fatty acids, citric acid, and protease inhibitors were exam-

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ined (Okumura et al., 1992; Yamamoto et al., 1993; Kobayashi et al., 1994; Todo et al., 2001).

A desirable absorption enhancer should not only have an absorption enhancing effect but also be safe to the epithelium membrane of lungs. Yamamoto et al. intravenously injected Evans Blue and examined its leakage in lung. Although 5 mM *N*-lauryl-β-D-maltopyranoside increased calcitonin activity, it also increased Evans Blue leakage, suggesting lung toxicity. While 1 mM *N*-lauryl-β-D-maltopyranoside, 10 mM glycocholate, and 10 mM mixed micelles of linoleic acid and HCO60 were safe and effective as enhancers (Yamamoto et al., 1997). Recently, we revealed that bacitracin and Span 85 increased pulmonary insulin absorption from solutions in rats but were not effective when formulated in dry powders with insulin. Citric acid is a potent pulmonary absorption enhancer for peptides formulated in dry powders. When an insulin dry powder containing 0.036 mg/dose of citric acid was administered to the rat lung, the lactate dehydrogenase level in the bronchoalveolar lavage was as low as that for saline administration, suggesting citric acid is a safe additive (Todo et al., 2001).

The stability of peptides and proteins in the formulation is another important aspect to be considered in the development of inhalation powders with or without absorption enhancers. In general, a crystalline solid of small molecule drugs is chemically more stable than the amorphous form. In some cases, however, the crystalline state may not be more stable for protein and peptide formulations (Lai and Topp, 1999). The primary degradation pathways of biosynthetic human insulin involve deamidation at the AsnA21 site and covalent dimer formation. When storage stability at 25 and 40 °C at relative humidities between 0 and 75% was assessed, amorphous insulin was far more stable than crystalline insulin under all conditions (Pikal and Rigsbee, 1997).

The hydration state of proteins affects the stability in the solid state. Aggregation of humanized monoclonal antibody and rhDNase in mannitol-formulated spray-dried powders increased with an increase in the storage humidity (Maa et al., 1998). Deamidation at the AsnA21 site of crystalline insulin increased sharply as the moisture content increased, while that of amorphous insulin was almost unaffected by moisture (Pikal and Rigsbee, 1997).

Proteins and peptides are often formulated with sugars, such as lactose, trehalose, and mannitol, to protect them from degradation during the spray-drying process, freeze-drying process, and storage (Broadhead et al., 1994; Labrude et al., 1989). It is known that the chemical stability of proteins in the solid state is enhanced by the presence of some amorphous sugars (Imamura et al., 2001). The activities of the freeze-dried enzymes, L-lactate dehydrogenase, β-galactosidase, and L-asparaginase, depended on the content of amorphous mannitol in the cake (Izutsu et al., 1994).

Despite the accumulated information on the effect and safety of pulmonary absorption enhancers and stability of powders of proteins or peptides, very few studies have been reported on the effect of pulmonary absorption enhancers on the stability of proteins and peptides formulated in powders. In this report, we examined the effect of citric acid, bacitracin, and Span 85, whose pulmonary absorption enhancing activity to insulin in powders and solutions has already been reported (Todo et al., 2001), on the stability of insulin in dry powders prepared by a spray-drying technique. In addition, a combination of insulin powder and citric acid powder was examined to improve the insulin stability.

2. Materials and methods

2.1. Materials

Insulin from the bovine pancreas was supplied by Sigma Chemical Co. (28.0 U/mg; St. Louis, MO). Mannitol, bacitracin, sorbitan trioleate (Span 85), and citric acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ethanol (99.5%) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other reagents used were of analytical grade.

2.2. Preparation of insulin powder using a spray-drying technique

Insulin suspensions and solutions were prepared by adding insulin with or without additives to distilled water. Insulin was suspended by simply adding it to water. The decrease in pH of the insulin suspension

Table 1
Formulation, sprayed volume, and yield of dry powders

Methods	Formulation code	Insulin state[a]	Mannitol (%)	Insulin (%)	Enhancer	Sprayed volume (ml)	Yield[b] (%)
Spray-drying	INS SD	Dissolved	–	1.0	–	100	25.6
	MI SD (susp.)	Suspended	5.0	0.25	–	100	44.2
	MI SD	Dissolved	5.0	0.25	–	100	44.0
	MIC SD	Dissolved	5.0	0.25	0.2% citric acid	100	55.5
	MIB SD	Suspended	5.0	0.25	10 mM bacitracin	100	48.7
	MIS SD	Suspended	5.0	0.25	1.0% Span 85	100	1.79
	MI′	Suspended	5.0	0.50	–	100	40.6
	MC	–	5.0	0	0.4% citric acid	100	53.2

[a] Insulin state in the stock solution.
[b] Yield = amount of powder recovered/amount of ingredients in the sprayed solution.

below the isoelectric point (5.0–5.3) with a 1.0 M HCl solution and the successive increase in the pH to 7.4 with a 1.0 M NaOH solution gave an insulin solution. The addition of citric acid to an insulin suspension resulted in the dissolution of insulin, while it was still suspended after the addition of bacitracin or Span 85.

The preparation of dry insulin powders by a spray-drying technique was reported in our previous report (Todo et al., 2001). Briefly, the following standard operating conditions were used for spray-drying with an SD-1000 spray-drier (EYELA, Tokyo, Japan): an inlet temperature of 90 °C, a drying air flow rate of 0.75 m^3/min, a solution feed rate of 5 ml/min, and an atomizing air pressure of 100 kPa. Operating under these conditions resulted in an outlet temperature 63–69 °C. The code names and compositions of the formulations are listed in Table 1. The dry powder INS SD was prepared from 1.0% insulin solution. The dry powders MI SD (susp.) and MI SD were prepared by spray drying a 0.25% insulin suspension and 0.25% insulin solution, respectively, containing 5.0% mannitol. The dry powder MIC SD were manufactured with 0.25% insulin solution containing 0.20% citric acid and 5.0% mannitol. MIB SD and MIS SD were manufactured with 0.25% insulin suspension containing 10 mM bacitracin and 1.0% Span 85, respectively, and 5.0% mannitol. The dry powder MI′ was prepared by spray drying a 0.5% insulin suspension containing 5.0% mannitol. MC was prepared by spray drying a 0.40% citric acid and 5.0% mannitol solution without insulin.

2.3. In vitro powder characterization

An HPMC capsule (size 2, Shionogi Qualicaps Co., Nara, Japan) with 20 mg of insulin powder was loaded in an inhaler, Jethaler (Hitachi Unisia Automotive, Ltd., Atsugi, Japan). The insulin powder was dispersed into an Andersen Cascade Impactor (Shibata Scientific Technology Ltd., Tokyo, Japan) from the Jethaler for 10 s at an air flow rate of 28.3 l/min. The amount of insulin powder deposited on each stage of the impactor was determined by measuring the difference in the weights of the plate before and after sampling. The insulin powder deposited on the other parts (device, throat, cone) was dissolved with 0.01N HCl and assayed by an HPLC method. The output efficiency (OE) was determined as the percent of total powder mass exiting from the capsule and device. Respirable fraction (RF) of insulin powder was determined by dividing the powder mass recovered from the stages 2–7 (<7.0 μm) of the impactor by the OE value. A plot of the cumulative amount of powder deposited on each stage of the impactor on the probability scale axis against the logarithm of effective cut-off diameter for that stage allowed calculation of the mass median aerodynamic diameter (MMAD) of the particles.

The particle size distribution was also measured with a laser micron sizer LMS-30 (Seishin Enterprise Co., Ltd., Tokyo, Japan) based on laser diffraction. We dispersed the dry powder into a laser beam directly from an apparatus used for intratracheal administration (Todo et al., 2001).

2.4. Evaluation of insulin stability

The dry insulin powders were placed in glass containers and stored at various temperatures in humidified chambers. Various relative humidities (RH) were achieved using the following saturated salt solutions (Young, 1967): NaI for 25% RH at 60 °C, NaBr for 50% RH at 60 °C, and NaCl for 75% RH at 60 °C. A desiccator with silica gel was used for the dry conditions at 40, 50, and 60 °C. The dry insulin powders were stored dry and at 75% RH at 60 °C for 0, 1, 2, 3, 5, and 10 days, while MIC SD and MIC Mix were stored under dry condition and 75% RH for 0, 4, 8, 12, and 24 h and 0, 2, 4, and 8 h, respectively. MI SD (susp.) powder was stored under dry condition at 40 °C for 0, 1, 2, 3, and 4 weeks.

The logarithm of insulin potency was plotted against time and the degradation rate constant was estimated from the slope assuming first-order degradation kinetics.

2.5. X-ray diffractometry (XRD)

X-ray diffraction pattern analysis was performed with a RAD-II VC (Rigaku Co., Tokyo, Japan), using Cu Kα radiation, over a range of (2θ) 5–45° (speed 0.02°/min) at room temperature.

2.6. Evaluation of particle size distribution and particle shape

A scanning electron microscope (SEM) (JSM-T20, JOEL, Tokyo, Japan) was used to observe the particle shape.

2.7. Moisture in the insulin dry powder

The moisture content in the dry insulin powders was analyzed by thermo gravimetric analysis (TGA) using Shimadzu DTG-60. Approximately 20 mg of the powder sample was heated from 25 to 150 °C at a rate of 5 °C/min.

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a 15% SDS-polyacrylamide gel according to Laemmlis method with a constant current of 20 mA for 120 min. Dry insulin powder and insulin standard were dissolved in Tris–HCl buffer (pH 6.8) containing 1% SDS and 1% 2-mercaptoethanol, 20% glycerol and 0.01% bromophenol blue. Samples of 30 μg of insulin were loaded on each lane. Molecular weight standards (Oncogene Research Products, Cambridge, MA) used were ovalbumin (43,000), carbonic anhydrase (29,000), β-lactoglobulin (18,400), lysozyme (14,300), bovine trypsin inhibitor (6200), insulin (α and β chains) (3000). Proteins on the gel were stained using a sliver stain kit (Atto Co., Tokyo, Japan) according to the manufacturer's instructions.

2.9. Intratracheal administration of insulin dry powders in rats

Male Sprague–Dawley rats weighing 300–320 g were anesthetized with pentobarbital (50 mg/kg, i.p.) and secured on their backs on a board during the experiments. The trachea was exposed and a 3.5 cm length of PE-240 polyethylene tubing was inserted to a depth of 0.6 cm through an incision made between the fifth or sixth tracheal rings caudal to the thyroid cartilage according to the method of Enna and Schanker (1972). A PE-50 tubing cannula was placed in the carotid artery for blood sampling.

Insulin dry powder was administered as previously reported (Todo et al., 2001) using an apparatus made with a disposable syringe, three-way stopcock, and disposable tip. The powder taken in the tip was dispersed in the rat lungs through the PE-240 tubing by releasing air compressed in the syringe by opening the three-way stopcock connecting the tip and the syringe. The amount of dry powder administered was calculated by subtracting the tip weight after administration from that before administration.

2.10. Assay of insulin

Insulin was determined using a high performance liquid chromatography (HPLC) system (Shimadzu Co., Kyoto) using the raw insulin bulk drug as the standard (28.0 U/mg). The HPLC system was composed of a pump (LC-10ADvp), diode array detector (SPD-M10Avp), column oven (CTO-10ASvp), and LC work station (CLASS-LC10). The mobile phase

was a 72:28 mixture of 0.1 M ammonium sulfate buffer (pH 2.3) and acetonitrile at a flow rate of 1.2 ml/min. The column was a Shodex Asahipak ODP-50 6D (4.6 mm × 150 mm, 5 μm) (Showa Denko, Ltd., Tokyo) heated at 36 °C. Ultraviolet absorption was measured at 214 nm. The injection volume was 10 μl. The potency of insulin in the powders was defined as the amount of insulin (unit) in 1 mg of the product.

2.11. Assay of blood glucose level

Blood samples (200 μl) were collected before administration and every 30 min up to 360 min after insulin administration and centrifuged at 4 °C to separate plasma. The plasma glucose level was measured with a glucose assay kit, Glucose CII Test Wako (Wako Pure Chemical Industries, Ltd.) based on the mutarotase-GOD method. The change in plasma glucose level (ΔGLC) expressed in %/unit was calculated using the following equation:

$$\Delta\mathrm{GLC} = \left(\frac{\mathrm{GLC}_t - \mathrm{GLC}_0}{\mathrm{GLC}_0}\right) \text{per dose} \times 100 \qquad (1)$$

where GLC_0 and GLC_t are the plasma glucose concentrations at time 0 and at time t, respectively. The change in plasma glucose level was normalized by the dose because the doses were different for each administration.

The area under the curve (AUC) for ΔGLC with respect to time from 0 until 360 min was calculated by the trapezoidal rule.

2.12. Statistical analysis

Statistical differences in insulin absorption of insulin were examined using a one-way analysis of variance (ANOVA) followed by least significant difference test. The significance level was set at $P < 0.05$.

3. Results

3.1. The mass median aerodynamic diameter

The in vitro inhalation performance of spray-dried powders was evaluated using an Andersen Cascade Impactor (Table 2). The mass median aerodynamic diameters of these powders estimated from the impactor data were less than 7.0 μm, suggesting that the spray-dry technique was successful in preparing powders suitable for reaching respiratory regions. INS SD powder had a superior inhalation performance (RF and OE) compared with the other powders.

3.2. Potency of insulin in the dry powder

Fig. 1 shows the effect of absorption enhancers on the stability of dry insulin powders stored at 60 °C and various humidities. Span 85 (MIS SD) and bacitracin (MIB SD) had no influence on the half-life of insulin at the 60 °C/dry condition. However, citric acid (MIC SD), which was a potent pulmonary absorption enhancer for insulin powder, decreased the half-life of insulin (Fig. 1).

Table 2
Physical characteristics of insulin powders[a]

Code	LMS-30 (μm)[a,b]	MMAD (μm)[a,c]	OE (%)[a,d]	RF (%)[a,e]
INS SD	4.27 ± 0.253	3.13 ± 0.033	92.3 ± 1.25	56.7 ± 0.72
MI SD (susp.)	5.71 ± 0.096	4.73 ± 0.120	87.4 ± 0.602	30.6 ± 4.00
MI SD	7.30 ± 0.095	4.33 ± 0.095	84.7 ± 1.62	39.1 ± 1.09
MIC SD	7.24 ± 0.152	4.40 ± 0.115	82.3 ± 0.410	29.9 ± 0.83
MIB SD	5.9	4.3	76.9	36.0
MIS SD	5.1	4.3	30.1	67.9
MC Mix			90.1 ± 2.02	25.8 ± 2.58

[a] Data are represented as mean ± S.E. for three successive measurements except for MIB SD and MIS SD.
[b] Laser micron sizer (LMS-30).
[c] Mass median aerodymanic diameter.
[d] Output efficacy (percent of total powder mass exiting from the capsule and device).
[e] Respirable fraction (percent of the drug mass on the respirable fraction (not more than 7.0 μm) of the impactor divided by emitted dose).

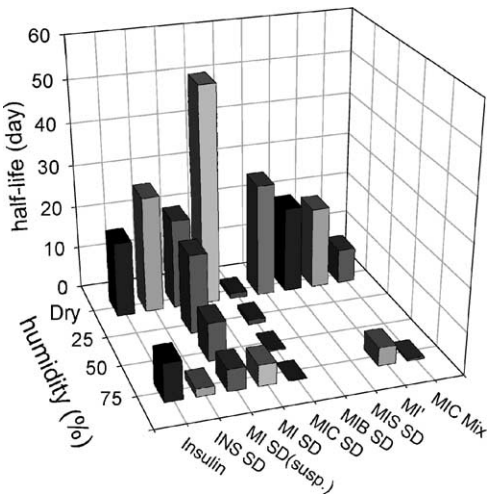

Fig. 1. Half-life of insulin in the dry powders at various humidities ($n = 3$–5, except for MIB SD and MIS SD ($n = 1$)). Coefficients of variation were less than 10% for all the experiments, except for INS SD (12%) and MI SD (12%) at 60 °C/dry and MIC SD (17%) at 60 °C/75% RH.

MI SD (susp.), MI SD, INS SD, insulin bulk drug, and MIC SD were stored at various humidities. The half-life of insulin of these powders was decreased by the increase in relative humidities.

A desirable dry insulin powder for inhalation should be stable enough to allow storage at room temperature. The shelf life (T_{90}), which is defined as the period required for 10% potency loss in this study, of MIC SD at 25 and 15 °C was estimated from an Arrhenius plot (Fig. 2) using the degradation rate constants at 40, 50, 60 °C and dry condition. T_{90} was estimated

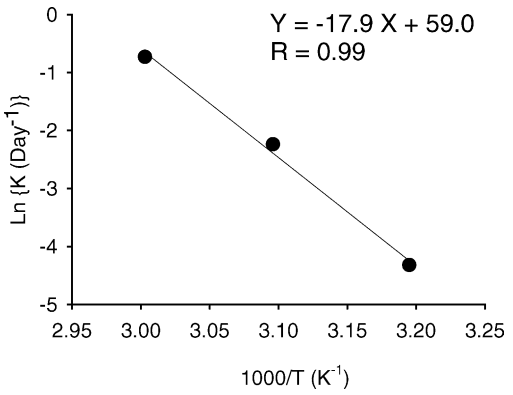

Fig. 2. Arrhenius plot for MIC SD at dry conditions ($n = 3$).

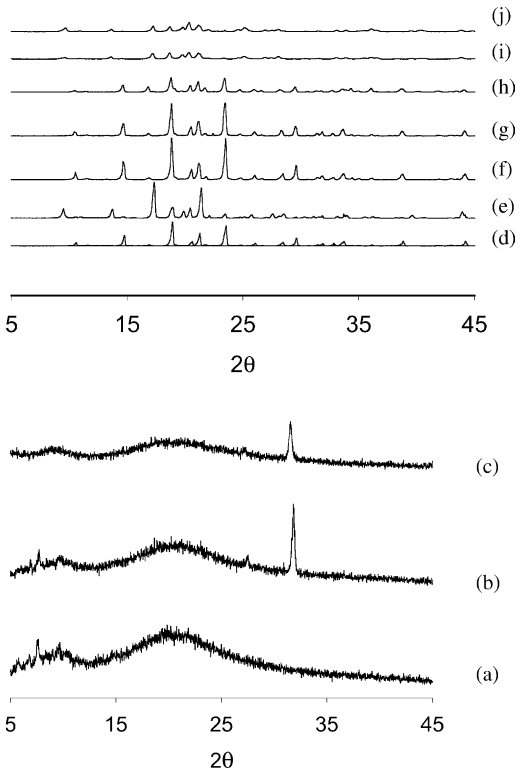

Fig. 3. Powder X-ray diffraction pattern: (a) insulin bulk drug, (b) insulin/NaCl physical mixture, (c) INS SD, (d) mannitol (α form), (e) mannitol (β form), (f) mannitol/insulin physical mixture, (g) mannitol/insulin/citric acid physical mixture, (h) MI SD (susp.), (i) MI SD, and (j) MIC SD.

to be 150 days and 3.6 years in dry condition at 25 and 15 °C, respectively, indicating that the storage in a cold place is recommendable.

3.3. X-ray diffraction

X-ray diffraction patterns of dry insulin powders are shown in Fig. 3. The dry powder containing additives prepared from insulin solution with or without citric acid exhibited β polymorph of mannitol and decreased crystallinity compared with a physical mixture. On the other hand, MI SD (susp.) prepared from insulin suspension exhibited α polymorph of mannitol. X-ray diffraction of INS SD prepared from insulin solution exhibited an intense peak at $2\theta = 31.8°$. This X-ray diffraction pattern agreed with that of an insulin/NaCl physical mixture, which was a mixture of NaCl powder

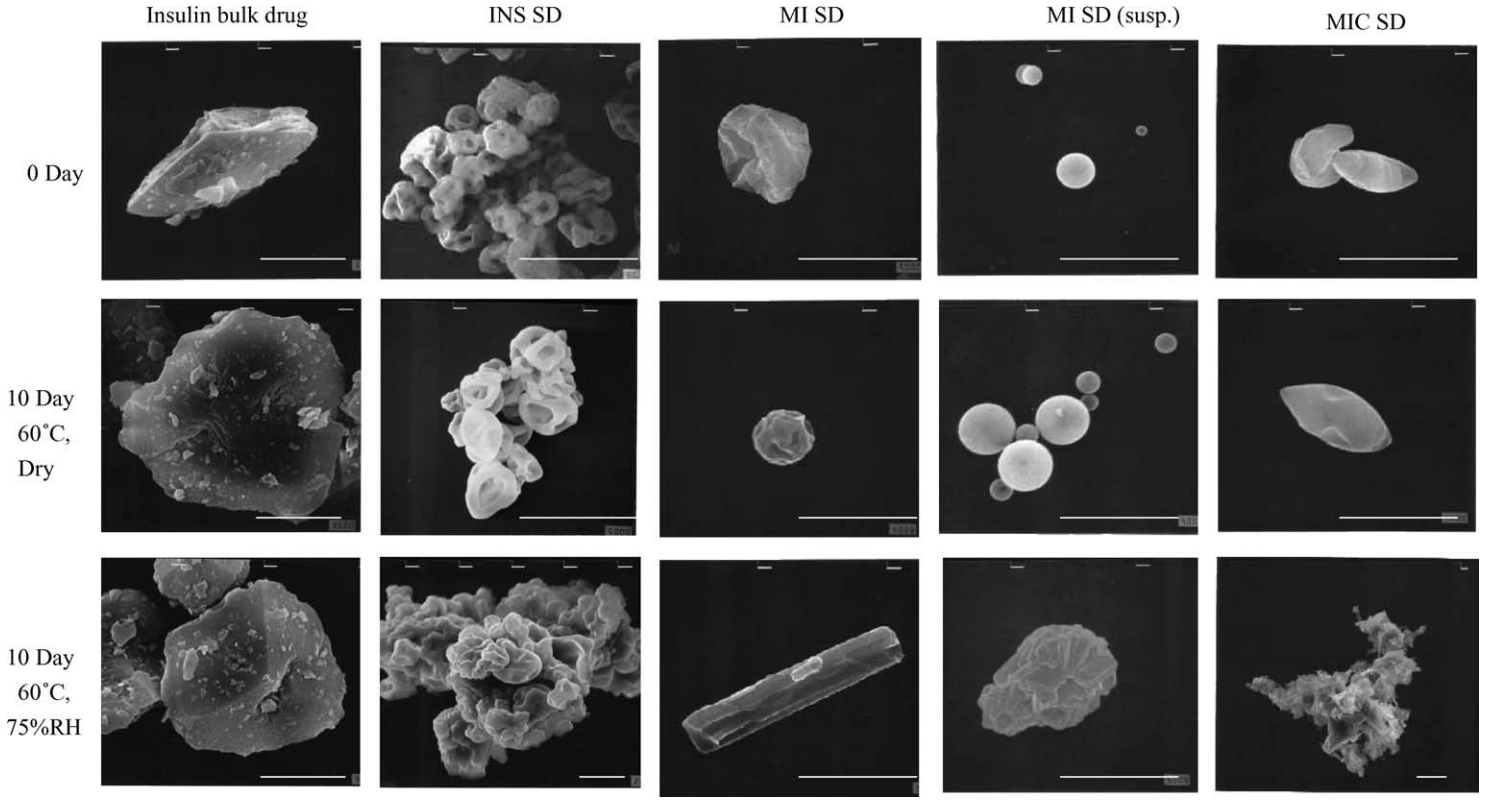

Fig. 4. Scanning electron micrographs of dry insulin powders. Bar: 10 μm.

Table 3
Water content (%) in the dry insulin powders

Formulation code	Day 0	Stored for 10 day	
		60 °C dry	60 °C 75% RH
Insulin bulk drug	4.4	3.2	9.8
INS SD	5.3	4.6	11
MI SD (susp.)	0.42	0.29	0.50
MI SD	0.56	0.52	0.74
MIC SD	0.57	0.58	0.97

prepared by the spray-drying technique from pH 7.4 NaCl solution and the insulin bulk drug.

3.4. Morphological change in dry insulin powder

The morphological change in MIC SD, MI SD (susp.), MI SD, INS SD, and insulin bulk drug was investigated. MI SD (susp.) was prepared from an insulin suspension, MI SD was prepared from an insulin solution, and INS SD and insulin bulk drug were prepared without mannitol or absorption enhancers. MIC SD contained citric acid and was effective to increase insulin absorption from rat lungs. The morphology of these powders changed during storage at dry or 75% RH condition at 60 °C (Fig. 4). The particle size should be not more than 7.0 μm for inhalation therapy in order to deliver the drug deep in the lung (Timsina et al., 1994; Newman et al., 1994; Pavia, 1977). The powder morphology and size did not change after 10-day storage at 60 °C/dry. However, all powders except the insulin bulk drug changed their particle morphology and increased the size after 10-day storage at 60 °C/75% RH.

3.5. Water contents

Table 3 lists the water contents of dry insulin powders after 10-day storage at 60 °C/dry and 60 °C/75% RH. INS SD and insulin bulk drug indicated higher water content than MI SD (susp.) and MI SD. The water content of all dry insulin powders increased after 10-day storage at 60 °C/75% RH.

3.6. Formation of high molecular weight protein in the dry insulin powders

Many different types of chemical and physical changes result in the formation of dimers or aggregations of protein (Lai and Topp, 1999). As for chemical change, temperature, moisture, and formulation excipients affect the solid-state stability of proteins and peptides.

The formation of high molecular weight protein in the dry insulin powders was investigated using SDS–PAGE after the 10-day storage at 60 °C/dry and 3-day storage 60 °C/75% RH (Fig. 5). High molecular weight insulin aggregates were clearly observed for MIC SD to a greater extent than for the other powders examined. This indicates that citric acid tends to denature insulin, which agrees with the rapid decrease in the insulin potency determined by HPLC. The aggregates in the insulin powders prepared from insulin solutions (INS SD, MI SD) were less than those found in the insulin bulk drug stored at dry condition. Contrary to the dry condition, high molecular weight aggregates in the insulin bulk drug were less than those in the other dry insulin powders at 75% RH.

3.7. Improvement of insulin stability with citric acid

Although MIC SD was effective at increasing the pulmonary absorption of insulin, the stability should be improved for storage at room temperature. To improve the stability of insulin formulated with citric acid, we developed an MIC Mix powder, which is a combination of MI′ and MC. The 1:1 combination of MI′ and MC should theoretically have the same composition as MIC SD (Table 1). Fig. 1 shows the stability of insulin in MIC Mix. The stability of MIC Mix was still inferior to that of MI SD; however, the half life of MIC Mix (8.5 ± 1.8 days (mean ± S.D.) at 60 °C/dry and 0.42 ± 0.05 days at 60 °C/75% RH) was significantly improved ($P < 0.05$, Student's t-test) and extended 6.5 and 2.6 times compared with MIC SD (1.3 ± 0.3 days at 60 °C/dry and 0.16 ± 0.05 days at 60 °C/75% RH), respectively.

3.8. Insulin absorption after intratracheal administration of MIC Mix

The hypoglycemic effect of MIC Mix after pulmonary administration to the rat lung was examined and compared with those of INS SD, MI SD, MI SD (susp.), and MIC SD (Fig. 6). MIC Mix showed a rapid onset and elongated hypoglycemic effect compared with INS SD, MI SD (susp.) and MI SD. There

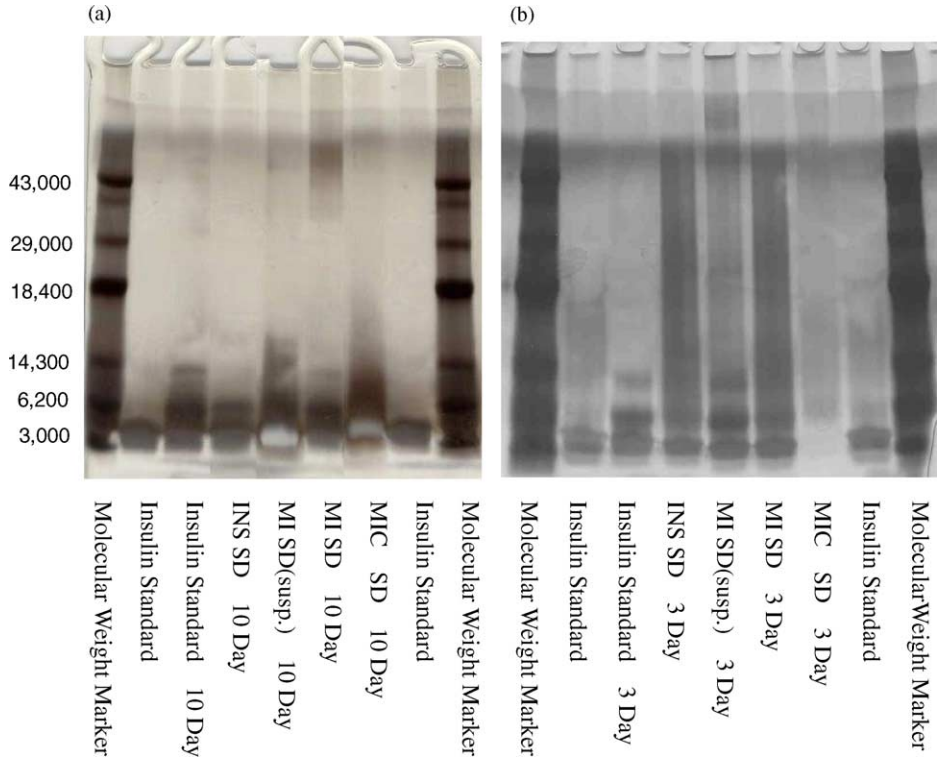

Fig. 5. Gel electrophoresis of dry insulin powder: (a) 60 °C/dry and (b) 60 °C/75% RH.

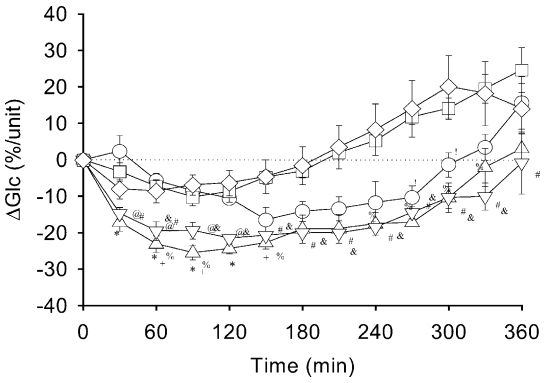

Fig. 6. Profile of the plasma glucose level following intratracheal administration of dry insulin powders in the rat lung. (○) MI SD (susp.); (□) MI SD; (△) MIC SD; (▽) MIC Mix; and (◇) INS SD. Each point represents the mean ± S.E. ($n = 3$–4). Significant differences ($P < 0.01$) were observed for (*) MIC SD vs. MI SD (susp.), ($^{+}$) MIC SD vs. MI SD, (%) MIC SD vs. INS SD, (@) MIC Mix vs. MI SD (susp.), (#) MIC Mix vs. MI SD, (&) MIC Mix vs. INS SD, and (!) MI SD (susp.) vs. INS SD.

was no significant difference observed between MIC SD and MIC Mix regarding the decrease in the plasma glucose level at each time point, suggesting the combination of insulin and citric acid powders was as effective as the dry insulin powder containing citric acid. The AUC values are summarized in Table 4.

4. Discussion

The insulin stability of INS SD at 60 °C/dry was slightly improved compared with the insulin bulk drug. The addition of mannitol to the powder (MI SD) greatly improved the insulin stability. The half-life of MI SD was significantly elongated 3.0 times compared with the insulin bulk drug stored at 60 °C/dry. However, MI SD (susp.) prepared from insulin suspension had an insulin stability almost comparable to that of INS SD despite the addition of mannitol. The water contents in the MI SD and MI SD (susp.) were almost the same. While different X-ray diffraction

Table 4
AUC for intratracheal administration of dry insulin powder in rats

Code	$AUC_{\rightarrow 360} \times 10^{-3}$ (%/unit × min)[a,b]	Insulin dose (unit/rat)[a]	RF × OE × AUC × 10^{-7}
INS SD	1.5 ± 0.49	1.5 ± 0.093	0.80
MI SD (susp.)	2.9 ± 0.74	1.2 ± 0.086	0.76
MI SD	1.1 ± 0.21	1.8 ± 0.14	0.38
MIC SD	6.0 ± 0.50	1.5 ± 0.15	1.5
MIC Mix	5.8 ± 0.30	1.7 ± 0.18	1.3

[a] Mean ± S.E.
[b] Significant differences ($P < 0.05$) were observed for (MI SD (susp.) vs. MI SD). Significant differences ($P < 0.01$) were observed for (MI SD (susp.) vs. MIC SD), (MI SD (susp.) vs. MIC Mix), (MI SD vs. MIC SD), (MIC Mix vs. MIC SD), (MIC SD vs. INS SD), (MIC Mix vs. INS SD).

patterns were observed for MI SD and MI SD (susp.). MI SD (susp.) exhibited α polymorph of mannitol. On the other hand, MI SD exhibited α polymorph of mannitol. It is likely that the polymorph of sugar affects insulin stability in the dry powder.

There have been several studies demonstrating that the mixing ratio of sugar and protein affects the protein stability (Izutsu et al., 1994; Costantino et al., 1998). Costantino et al. (1998) reported that low mannitol contents (10–30%) in the formulation improved the stability of spray-dried rhuMAbE25. We investigated whether mannitol contents in the formulation would influence the insulin stability. The half-life of insulin in a mannitol/insulin = 2/8 (weight ratio) powder was 62 and 3.5 days at 60 °C/dry and 60 °C/75% RH, respectively, which was comparable to the half-life (52 and 5.0 days at 60 °C/dry and 60 °C/75% RH, respectively) of insulin in the MI SD powder (mannitol/insulin = 20:1).

Electrophoresis results showed that insulin in MIC SD was denatured and aggregated in a short time compared with the other powders. Insulin was highly unstable under acidic and basic conditions in solutions (Brange, 1992). Although the HPLC analysis employed in this study was not suitable for detecting insulin aggregates, the increase in insulin aggregates detected by the SDS–PAGE analysis agreed with the decrease in insulin potency determined by the HPLC analysis. Acids such as citric acid seem to deteriorate the stability of insulin in a powder as well as a solution, which made us attempt to improve the stability of MIC SD.

A combination of insulin and citric acid powders is a simple but effective method to improve the stability by physically decreasing the chance of interaction of insulin and citric acid. Another matter of interest regarding MIC Mix may be its hypoglycemic effect, because insulin and citric acid were formulated separately. The intratracheal administration study in rats showed that the MIC Mix is as effective as MIC SD. This suggests that the insulin powders and citric acid powders in MIC Mix would be distributed in the lungs uniformly, making an insulin/citric acid solution available at the absorption sites.

The morphological change in the particles would influence the inhalation performance such as drug emission from a capsule or device and drug deposition in the lung. The present study revealed that humidity caused morphological change. In particular, MIC SD was very susceptible to humidity and its shape changed and expanded in only one day at 60 °C/75% RH (data not shown). The hygroscopicity of citric acid seems to influence the morphological change and moisture content in MIC SD.

Residual water content is often thought to be responsible for protein and peptide chemical instability in the solid state. In general, lyophilized protein formulations are more stable at lower water contents (Costantino, 1994). At a lower water content level, the Maillard reaction is less rapid because the diffusion and mobility of reactants is restricted. As the water contents increase, molecular mobility increases, leading to enhanced reactant mobility, which should facilitate the reaction. The present study also revealed that the increase in water content (Table 3) resulted in the decrease in the stability (Fig. 1).

In order to maximize pulmonary drug absorption, the particle sizes have to be 0.5–7 μm to reach the alveolar region of the lungs (Timsina et al., 1994; Newman et al., 1994; Pavia, 1977). The mass median

aerodynamic diameters of the powders in the present study estimated from the impactor data were less than 7.0 μm (Table 2), suggesting that dry insulin powders were successfully prepared for inhalation. However, a difference in inhalation performance (RF and OE) was observed among the powders examined. INS SD was superior to other dry insulin powders, because irregular the particle surface might result in reduced particle–particle contact and tendency to aggregate. This diminished aggregation means that less energy is required to disperse the particle. So, inhalation performance of INS SD might be better than the other dry insulin powders. On the other hand, we examined the hypoglycemic effect of insulin powders by administering them directly in the rat lung through the trachea and revealed that MIC Mix and MIC SD were much more effective than INS SD. This technique is relatively insensitive to the influence of particle size distribution on their deposition upon inhalation because even the particles which remain in a capsule or on the surface of the mouth or trachea could reach deep into the lung. The therapeutic effect of insulin powders should be evaluated by in vivo absorption study results as well as in vitro powder characterization study results. The OE and RF values (Table 2) and the AUC value (Table 4) are the representative parameters of in vitro and in vivo performance of insulin powders, respectively. The product of them (OE × RF × AUC) would be a useful parameter to estimate the therapeutic effect of the powders, because the product of OE and RF is an estimate for the fraction of powders reaching the alveolar region after inhalation and AUC was an estimate for the bioavailability determined by administering the powders directly into the lungs. MIC Mix indicated high performance compared with the others, suggesting that a combination of insulin and citric acid powders is a very useful method to improve the insulin stability with high therapeutic performance.

5. Conclusion

Although the hypoglycemic effect was greatly improved when the dry insulin powder with citric acid (MIC SD) was administered, insulin in the MIC SD was unstable compared with the other powders. We designed the dosage form to improve the insulin stability without loss of hypoglycemic activity.

MIC Mix was formulated as a combination of insulin powder (MI′) and citric acid powder (MC). MIC Mix showed hypoglycemic activity comparable to MIC SD and improved insulin stability. In this study, moisture affected the insulin stability and particle morphology. It was suggested that a package preventing moisture absorption was necessary for insulin powders prepared with citric acid.

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References

Brange, J., 1992. Chemical stability of insulin. 1. Hydrolytic degradation during storage of pharmaceutical preparations. Pharm. Res. 9, 715–726.

Broadhead, J., Rouan, S.K., Rhodes, C.T., 1994. The effect of process and formulation variables on the properties of spray-dried β-galactosidase. J. Pharm. Pharmacol. 46, 458–467.

Costantino, H.R., 1994. Moisture-induced aggregation of lyophilized insulin. Pharm. Res. 11, 21–29.

Costantino, H.R., Andya, J.D., Nguyen, P.A., Dasovich, N., Sweeney, T.D., Shire, S.J., Hsu, C.C., Maa, Y.F., 1998. Effect of mannitol crystallization on the stability and aerosol performance of a spray-dried pharmaceutical protein recombinant humanized anti-IgE monoclonal antibody. J. Pharm. Sci. 87, 1406–1411.

Enna, S.J., Schanker, L.S., 1972. Absorption of saccharides and urea from the rat lung. Am. J. Physiol. 222, 409–414.

Imamura, K., Iwai, M., Ogawa, T., Sakiyama, T., Nakanishi, K., 2001. Evaluation of hydration states of protein in freeze-dried amorphous sugar matrix. J. Pharm. Sci. 90, 1955–1963.

Izutsu, K., Yoshioka, S., Terao, T., 1994. Effect of mannitol crystallinity on the stabilization of enzymes during freeze-drying. Chem. Pharm. Bull. (Tokyo) 42, 5–8.

Kobayashi, S., Kondo, S., Juni, K., 1994. Study on pulmonary delivery of salmon calcitonin in rats: effects of protease inhibitors and absorption enhancers. Pharm. Res. 11, 1239–1243.

Labrude, P., Rasolomanana, M., Vigneron, C., Thirion, C., Chaillot, B., 1989. Protective effect of sucrose on spray drying of oxyhemoglobin. J. Pharm. Sci. 78, 223–229.

Lai, M.C., Topp, E.M., 1999. Solid-state chemical stability of proteins and peptides. J Pharm. Sci. 88, 489–500.

Maa, Y.F., Nguyen, P.A., Andya, J.D., Dasovich, N., Sweeney, T.D., Shire, S.J., Hsu, C.C., 1998. Effect of spray drying

and subsequent processing conditions on residual moisture content and physical/biochemical stability of protein inhalation powders. Pharm. Res. 15, 768–775.

Newman, S.P., Hollingworth, A., Clark, A.R., 1994. Effect of different modes of inhalation on drug delivery from a dry powder inhaler. Int. J. Pharm. 102, 127–132.

Okumura, K., Iwakawa, S., Yoshida, T., Seki, T., Komada, F., 1992. Intratracheal delivery of insulin absorption from solution and aerosol by rat lung. Int. J. Pharm. 88, 63–73.

Pavia, D., 1977. Effect of lung function and mode of inhalation on penetration of aerosol into the human lung. Thorax 32, 194–197.

Pikal, M.J., Rigsbee, D.R., 1997. The stability of insulin in crystalline and amorphous solids: observation of greater stability for the amorphous form. Pharm. Res. 14, 1379–1387.

Timsina, M.P., Martin, G.P., Marriott, C., Ganderton, D., Yianneskis, M., 1994. Drug delivery to the respiratory tract using dry powder inhalers. Int. J. Pharm. 101, 1–13.

Todo, H., Okamoto, H., Iida, K., Danjo, K., 2001. Effect of additives on insulin absorption from intratracheally administered dry powders in rats. Int. J. Pharm. 220, 101–110.

Yamamoto, A., Umemori, S., Muranishi, S., 1993. Absorption enhancement of intrapulmonary administered insulin by various absorption enhancers and protease inhibitors in rats. J. Pharm. Pharmacol. 46, 14–18.

Yamamoto, A., Okumura, S., Fukuda, Y., Fukui, M., Takahashi, K., Muranishi, S., 1997. Improvement of the pulmonary absorption of (Asu1, 7)-Eel calcitonin by various absorption enhancers and their pulmonary toxicity in rats. J. Pharm. Sci. 86, 1144–1147.

Young, J.F., 1967. Humidity control in the laboratory using salt solutions—a review. J. Appl. Chem. 17, 241–245.