Facilitation of Pulmonary Insulin Absorption by H-MAP: Pharmacokinetics and Pharmacodynamics in Rats

Sandra Suarez,[1] Lucila Garcia-Contreras,[1] Donald Sarubbi,[2] Elizabeth Flanders,[2] Doris O'Toole,[2] John Smart,[2] and Anthony J. Hickey[1,3]

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Purpose. Several low molecular weight amino acids have previously been reported to enable the oral delivery of proteins. In the present studies, the effect of H-MAP (hydroxy methyl amino propionic acid) on the pharmacokinetics (PK) and pharmacodynamics (PD) of porcine insulin delivered to the lungs of rats by spray-instillation (SI) has been determined.

Methods. Aliquots (100 μl) of increasing doses of porcine insulin alone (0.26, 1.3, 2.6, 13, and 26 U/kg) or combined with increasing doses of H-MAP (5, 10, 16, and 25 mg/kg), at pH 7.2–7.6 were administered intratracheally to fasted anesthetized rats using a micro spray-instillator. Blood samples were collected from the jugular vein at specified intervals and the plasma concentrations of insulin and glucose were determined. The PK and PD of porcine insulin alone following subcutaneous (SC) administration of increasing doses were also determined.

Results. The PK of insulin administered either by SI to the lungs or SC injection were absorption rate dependent, resulting in post-peak half-lives 10 to 25-fold greater than the reported intravenous elimination half-life (3 min). The relative bioavailability (F') of insulin administered alone by SI varied from 23.8 to 80% for the lowest and highest insulin dose, respectively. Co-administration of H-MAP and insulin to the lungs significantly changed the PK and PD of insulin in a dose dependent fashion. Maximum PK and PD responses were obtained at an H-MAP dose of 16 mg/kg and an insulin dose of 1.3 U/kg. At this combination, the relative bioavailability of insulin was increased more than 2.5 fold, maximum concentration (C_{max}) increased 2-fold and the minimum plasma glucose concentration (%MPGC) was reduced more than 2-fold with respect to same dose of insulin alone. A greater total reduction in plasma glucose ($\%TRPG_{0\rightarrow t}$) was achieved for H-MAP/insulin combination (66 ± 5 %) compared to insulin alone (47 ± 10 %).

Conclusion. H-MAP has potential for increasing the pulmonary bioavailability of insulin administered through the lungs.

KEY WORDS: insulin; spray-instillation; pulmonary delivery; pharmacokinetics; pharmacodynamics.

INTRODUCTION

The ultimate goal in the treatment of diabetes mellitus is to provide basal and post-prandial blood insulin concentrations concordant with normal glucose blood levels. Subcutaneous (SC) administration of various insulin formulations is employed to achieve this goal. However, SC administration is accompanied by several pharmacokinetic and therapeutic limitations. Injectable insulin can be painful and inconvenient. Absorption occurs slowly and erratically following dilution and subsequent dissociation of the hexameric insulin units into dimers (1,2). Peak plasma insulin concentrations are observed at 60–180 min following SC injection (1) and usually persist for at least 4–6 h (3). Glucose from a meal is usually absorbed and cleared within 2 h. This mismatch between insulin availability and glucose absorption from the gastrointestinal tract may result in hyperglycemic/hypoglycemic episodes with potentially serious long-term side effects. In addition, there is evidence in rats that insulin is, to some extent (15–25%), degraded in the subcutaneous tissue (4).

Several studies in animals and humans (5–7) have shown that insulin administered to the lungs is rapidly absorbed with a time to maximum concentration (T_{max}) and the time to maximum glucodynamic effect significantly shorter than those obtained following subcutaneous administration. However, the bioavailability of insulin after pulmonary administration is relatively low (10–57%) compared to subcutaneous administration (5,6,8,9). The addition of surfactants and/or proteolytic enzyme inhibitors to insulin formulations can significantly increase the bioavailability of insulin (10–12). These compounds however, are highly inefficient and may cause transient to long-lasting membrane damage.

Low molecular weight compounds that facilitate the oral delivery of proteins have been described previously (13–15). Several studies including enzyme inhibitory activity, histopathological examinations (13), and binding affinities (14) have concluded that the observed protein transport cannot be explained through classical enzyme inhibition or general penetration enhancement. Direct measurement of the interaction of these molecules with insulin and other proteins using differential scanning calorimetry, suggests that they reversibly destabilize the native state of the proteins favoring a partially unfolded conformation (14). These data also suggest that the efficiency of these agents in facilitating protein transport is correlated with their affinity toward a partially unfolded form of the protein. In the present studies, the effect of H-MAP on the bioavailability of insulin delivered to the lung of fasted rats by spray-instillation has been assessed. The pharmacokinetics and pharmacodynamics of increasing doses of an aqueous insulin solution at neutral pH administered by spray-instillation or SC injection were compared with that following spray-instillation of insulin combined with increasing doses of H-MAP.

MATERIALS AND METHODS

Chemicals and Reagents

Porcine zinc insulin (25.9 U/mg) was obtained from Novo Nordisk (Copenhagen, Denmark). Sterile saline solution (Abbott Laboratories, North Chicago, IL) was used to replace the blood volume taken during sampling. Sodium heparin injection (1000 USP U/ml, Lyphomed®, Resent, IL) was used after proper dilution with sterile saline (15 U/ml). H-

[1] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599.

[2] Emisphere Technologies, Inc., 765 Old Saw Mill River Rd, Tarrytown, New York 10532.

[3] To whom correspondence should be addressed. (e-mail: ahickey@unc.edu)

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MAP was obtained from Emisphere Technologies, Inc. (Tarrytown, NY).

Animals

The Institutional Animal Care and Use Committee of the University of North Carolina approved all animal procedures. Specific-pathogen free, female Sprague Dawley rats (225–250g) (Charles River, Raleigh, NC) were housed in a constant temperature environment on a 12 h light/dark cycle. Animals were allowed free access to water and food (PROLAB RMH 3000, PMI Nutrition International, Inc., Brentwood, MO), but were fasted 18–24 h before each experiment.

Preparation of Insulin Solution

Crystalline zinc insulin powder was resuspended in distilled water. Aliquots of 0.01 N HCl were added to the suspension until a clear solution was obtained (pH 3). Subsequently, distilled water was added to the clear solution and the final pH adjusted to 7.4 by adding small amounts of 0.01 N NaOH. The solution was adjusted to a final volume of 10 ml using distilled water. An Insulin solution was freshly prepared for every experiment.

Dissolution of H-MAP

H-MAP was dissolved in distilled water, sonicated for 2 min, and its pH adjusted to 7.4 using 0.1N NaOH. Aliquots of insulin solution and H-MAP were combined prior to the spray-instillation on the day of the experiment.

Animal Procedure

Spray Instillation

Each rat was weighed and anesthetized by intraperitoneal injection comprised of 80 mg/kg ketamine: 5 mg/kg xylazine: 2.0 mg/kg acepromazine (Fort Dodge Laboratories Inc. Fort Dodge, Iowa). The anesthetized rat was placed on a heating blanket thermostatically controlled at 37 °C via rectal probe. The right jugular vein of each rat was catheterized using silicone tubing (Helix Medical Silicone Tubing, 0.020''ID/ 0.037''OD) and the patency of the catheter was confirmed by slowly flushing the cannula with 200 μl of heparinized saline with subsequent withdrawal of 100 μl of blood. The cannula was then cleared with 200 μl of heparinized saline solution. The endotracheal tube of the small animal spray-instillator (Penn Century, Philadelphia, PA) was placed at the level of the bifurcation of the trachea using a fiber optic laryngoscope (Custom Manufactured, EPA, Research Triangle Park, NC). Aliquots (100 μl) of increasing doses of either insulin alone (0.26, 1.3, 2.6, 13, and 26 U/kg) or insulin (0.26, 1.3, 2.6 U/kg) combined with H-MAP (5, 10, 16, and 25 mg/kg) were administered into the lower airways using a Hamilton syringe attached to the endotracheal tube of the spray-instillator. Distilled water pH adjusted to 7.4 and H-MAP alone solution (16 mg/kg) were also administered as negative controls. The endotracheal tube was removed following administration and the breathing rate was visually monitored throughout the remainder of the study.

Blood samples (500 μl) were withdrawn at 0 (immediately before drug administration), 10, 30, 60, 90, 120, and 180 min and the cannula was flushed with approximately 200 μl of heparinized saline solution following each sampling. The blood was collected in 1 ml tuberculin syringes (containing 100 μl heparin 20 U/ml) and transferred into microcentrifuge tubes.

Subcutaneous (SC) Administration

Animals were anesthetized and catheterized as above. Increasing doses of insulin in solution (0.26, 1.3, and 2.6 U/kg) were administered subcutaneously to each rat. Blood samples (500 μl) were withdrawn at 0 (immediately before drug administration), 15, 30, 60, 120, 180, 240, and 300 min and the cannula flushed with approximately 200 μl of heparinized saline solution following each sampling.

Blood Samples

Blood samples were immediately centrifuged at 10,300 g for 4 min. Aliquots of approximately 30 μl were transferred to siliconized 1 ml Eppendorf tubes and kept on ice until completion of the study for subsequent glucose determination. The remaining samples were frozen at −70 °C for insulin determination.

Glucose Determination

Determination of fasting plasma glucose levels was performed using the Ektachem DT Slide (GLU) monitor (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY). The analysis is based on the enzyme-catalyzed reaction of glucose with molecular oxygen, followed by a second reaction that produces an intense red color. The color intensity is proportional to the amount of glucose in the sample. This analytical method yielded plasma glucose concentrations in the range of 20–450 mg/dL with ± 2% precision.

Insulin Determination

Plasma insulin concentrations in fasted rats were determined using an ultrasensitive radioimmunoassay (RIA) kit (Linco Research, Inc., St. Charles, MO.). The concentrations of the unknown samples were determined by interpolation from a standard curve ranging from 3 to 240 μU/mL. The cross-reactivity of this kit with rat insulin was less than 1%.

Data Analysis

Pharmacokinetic Analysis

Insulin plasma concentration-time profiles following spray instillation of insulin alone were subjected to standard compartmental and non-compartmental pharmacokinetic analysis. Non-compartmental pharmacokinetic parameters following administration of the insulin/H-MAP combination were determined using WinNonlin (Statistical Consultants, Lexington, KY).

Compartmental Analysis

Average insulin concentration (C_{Ins}) versus time profiles on spray-instillation or subcutaneous administration of porcine insulin alone were fitted to a one-compartment body model, first order absorption using the least-squares nonlin-

ear regression method (PKanalyst, Micromath, Salt Lake City, UT). The evaluation of goodness of fit was accomplished by the respective model selection criteria (MSC) (16).

Non-Compartmental Pharmacokinetic Analysis

The non-compartmental analyses for insulin plasma concentrations over time following spray-instillation of insulin alone and when combined with H-MAP were calculated as follows. The area under the concentration-time curve ($AUC_{0\rightarrow t}$) was calculated by the trapezoidal rule. Terminal rate constants, used for extrapolation to infinity, were determined using nonlinear least-squares regression program, WinNonlin. Mean residence time ($MRT_{0\rightarrow t}$) was calculated as area under the first moment curve (AUMC) divided by the area under the curve ($AUC_{0\rightarrow t}$). $AUMC_{0\rightarrow t}$ was determined using a plot of plasma concentration multiplied by the time (C*t) versus time and calculation of its area under the curve by the trapezoidal rule. MRT from zero to infinity was calculated using the nonlinear regression program WinNonlin. The relative bioavailability (F', %) was calculated using the following equation:

$$F' = \frac{AUC_{0\rightarrow\infty}SI}{AUC_{0\rightarrow\infty}SC} \cdot \frac{Dose_{SC}}{Dose_{SI}} \cdot 100 \qquad \text{(Eq. 1)}$$

Pharmacodynamic Analysis

The percentage minimum plasma glucose concentration (%MPGC) and the time, T, to attain each %MPGC (T%MPGC) were determined from the average plasma glucose levels versus time profiles for the treatments by normalization against controls.

The area above the effect curve (AACE) was calculated as follows:

$$AAC_E = Total\ area - AUC_E \qquad \text{(Eq. 2)}$$

where AUC_E represents the area under the effect curve calculated using the trapezoidal rule.

The percentage total reduction in plasma glucose from 0→t ($\%TRPG_{0\rightarrow t}$) was determined using the following equation:

$$\%TRPG_{0\rightarrow t} = 100 * \left(\frac{AAC_{E0\rightarrow t}}{AUC_{E0\rightarrow t}}\right) \qquad \text{(Eq. 3)}$$

Statistical Analysis

Statistical analysis was performed using the repeated measures mixed-model analysis assuming time behaves as a continuous variable (SAS, Cary, NC). A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Plasma insulin concentration-time profiles following spray-instillation to the lungs of increasing doses of insulin alone are shown in Fig. 1A. Non-linear curve fitting of the average plasma levels indicated that the decrease in plasma concentrations was best described by a monoexponential decline with first order absorption. MSC values ranged from 2.3 to 3.8, indicating that the model was appropriate (16). The average (n = 4 to 7) peak plasma concentrations achieved in these studies were 6.8 ± 2.6 μU/ml (T_{max} = 10 ± 0 min), 87 ± 34 μU/ml (T_{max} = 10 ± 0 min), 152 ± 37μU/ml (T_{max} = 10 ± 0 min), 594 ± 210μU/ml (T_{max} = 30 ± 0 min), and 1926 ± 466 μU/ml (T_{max} = 30 ± 0) for the 0.26, 1.3, 2.6, 13, and 26 U/kg of porcine insulin, respectively. These values are in close agreement with those obtained after curve fitting. The areas under the curve to the last sampling time ($AUC_{0\rightarrow t}$), calculated using the trapezoidal rule, were 484 ± 96, 4147 ± 1108, 9517 ± 3255, 53176 ± 16399 and 174913 ± 17243, μUmin/ml for the 0.26, 1.3, 2.6, 13, and 26 U/kg of porcine insulin, respectively. In addition, the bioavailability relative to SC (1.3 U/kg insulin) ranged from 23.8% to 80% for the lowest and highest doses of insulin, respectively.

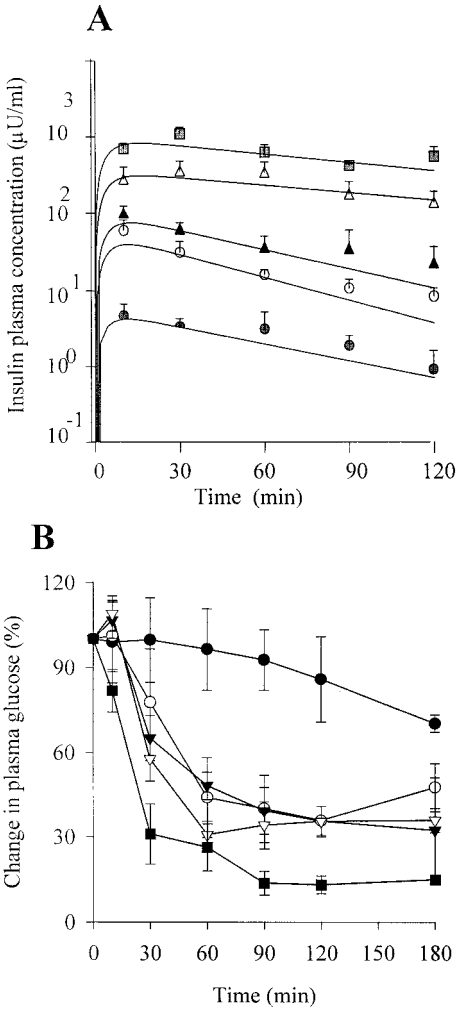

Fig. 1. Insulin plasma concentration-time profiles (A) and glucose plasma concentration-time profiles (B) following lung spray-instillation of increasing doses of insulin alone: 0.26 U/kg (●), 1.3 U/kg (○), 2.6 U/kg (▼), 13 U/kg (∇), and 26 U/kg (■). Bars represent mean ± SD for n = 4 to 5.

Figure 1B shows the glucose concentration-time profiles following spray-instillation of increasing dose of insulin alone. The results show that an increase in dose (from 0.26 to 26 U/kg) produced a significant decrease in the minimum plasma glucose (%MPGC) from 70.2 to 13.1, without changing significantly the time T to reach this minimum (T%MPGC). At the dose range studied, a greater reduction in the total reduction in plasma glucose from 0 to 3 h ($\%TRPG_{0\rightarrow 3hr}$) was achieved from 10.5 to 73.7%, indicating that the bioavailabil-

ity increased with higher insulin doses. The inter-animal variability in the glucose response (CV <30%) was smaller than that observed for plasma insulin (45%). Also, the inter-animal variability observed for both glucose and insulin responses (T_{max}, C_{max}, and AUC) following SC injection was higher than that after SI. Fluctuations in glucose levels were not observed after SI of distilled water pH 7.4 or H-MAP alone solution (16 mg/kg). No insulin was detected in plasma, by RIA, as would be expected since porcine insulin was not administered to these animals.

The pharmacokinetic parameters resulting from the compartmental and non-compartmental analysis of the data obtained following SC injection of 0.26, 1.3, and 2.6, U/kg insulin alone are shown in Table I. T_{max} and $MRT_{0\rightarrow\infty}$ values for equivalent doses were generally longer following SC administration than that after SI, supporting the concept that smaller fluctuations in glucose levels may be obtained on pulmonary insulin administration.

Figure 2A shows the insulin plasma concentration-time profiles following spray-instillation of insulin 0.26 U/kg combined with H-MAP at 16 or 25 mg/kg. A more than 2.5 fold increase in $AUC_{0\rightarrow\infty}$ (from 667.2 ± 125 to 1857 ± 209) was observed following administration of the H-MAP (16 mg/kg)/insulin combination compared to that obtained following the same dose of insulin alone. Likewise, a significant difference in insulin levels was observed between insulin alone and associated with H-MAP ($P < 0.001$). No significant difference in insulin levels was observed between the two doses of H-MAP studied ($P > 0.407$), indicating that the H-MAP-insulin interaction may be saturable. The relative bioavailability increased from 15.2 % to 45.2 % when insulin was combined with H-MAP.

Figure 2B shows the plasma glucose concentration-time profiles and Table II the pharmacodynamic parameters following spray-instillation to the lungs of insulin 0.26 U/kg alone and in combination with H-MAP at 16 mg/kg and 25 mg/kg. The %MPGC for insulin alone was larger than that for insulin combined with H-MAP at either 16 or 25 mg/kg. The time to reach %MPGC decreased from 180 to 90 min for

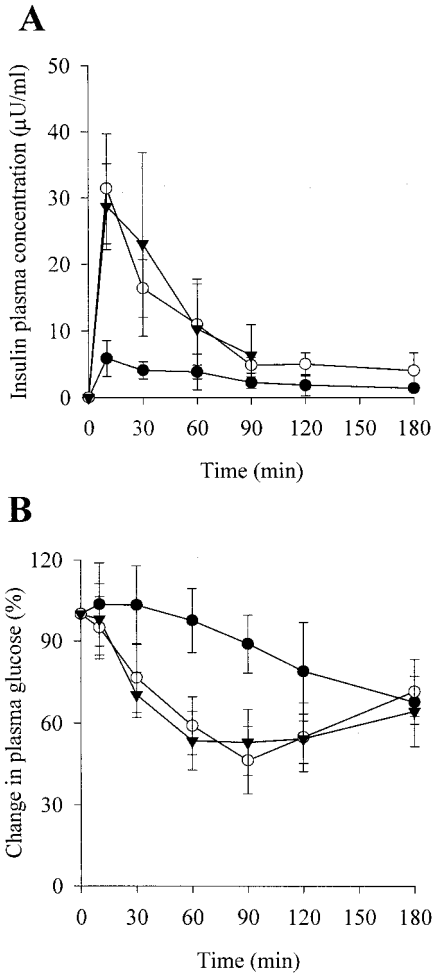

Fig. 2. Insulin plasma concentration-time profiles (A) and glucose plasma concentration-time profiles (B) following lung spray/instillation of insulin alone 0.26 U/kg (●), combined with H-MAP at 16 mg/kg (○),or 25 mg/kg (▼). Bars represent mean ± SD for n = 4 to 5.

Table I. Average Insulin Pharmacokinetic Parameters following Subcutaneous Administration of Escalating Doses of Porcine Insulin to Rats

	Porcine insulin dose (U/kg)		
	0.26	1.3	2.6
Compartmental Analysis (average data)[b]			
C_{max} (μU/ml)	17.9	122	174.4
T_{max} (min)	14	12	38
$AUC_{0\rightarrow t}$ (μU.min/ml)	4088	10349	24112
k_e (min^{-1})[a]	0.3	0.5	0.1
$t_{1/2}$ (elim)(min)	2.3	2.1	23
k_a (min^{-1})	0.005	0.009	0.015
$t_{1/2}$ (abs)(min)	138	77.4	63
Non-compartmental analysis			
C_{max} (μU/ml)	34.8 ± 16.6	100.7 ± 43.4	194.0 ± 74.0[c]
t_{max} (min)	25 ± 8	18 ± 6.7	40.0 ± 15.4
$AUC_{0\rightarrow t}$ (μU.min/ml)	3834 ± 1309	10315 ± 5205	20372 ± 7710[c]
$AUC_{0\rightarrow\infty}$ (μU.min/ml)	4408 ± 1204	13861 ± 4148	23770 ± 9015[c]
$MRT_{0\rightarrow t}$ (min)	120.2 ± 20.0	100.2 ± 11.0	92.3 ± 21.4
$MRT_{0\rightarrow\infty}$ (min)	167.3 ± 25.2	123.6 ± 71	121.2 ± 20.0

[a] The values of k_a and k_e have been exchanged assuming a flip-flop case.

[b] Average data based on n = 4 to 7.

[c] Significantly different with respect to insulin dose 0.26 U/kg ($P < 0.05$).

Table II. Average Pharmacodynamic Parameters following Spray-instillation of Insulin Alone (0.26 U/kg) and Combined with H-MAP at Doses of 16 and 25 mg/kg

	Treatment		
	SI insulin alone dose (U/kg)	SI insulin & H-MAP dose of H-MAP (mg/kg)	
PD parameter	0.26	16	25
%MPGC	70.2 ± 3.2	46.4 ± 12.3[a]	48 ± 10.5[a]
T%MPGC (min)	180 ± 0.0	90 ± 0.0[a]	72 ± 16.4[a]
$\%TRPG_{0\rightarrow 3hr}$	10.5 ± 11.0	35.6 ± 8.5[a]	38.8 ± 6.3[a]
$AAC_{E\ 0\rightarrow t}$ (μU.min/ml)	1890.8 ± 1989.4	6395.3 ± 1609.8[a]	6974.9 ± 1135.1[a]

[a] Significantly different with respect to SI insulin alone ($P < 0.05$). Average data based on n = 4 to 5.

insulin alone and combined with H-MAP, respectively. A significant difference ($p < 0.05$) in the $AACE_{0\rightarrow3}$ in the presence and absence of H-MAP (for both doses) was observed. The $\%TRGP_{0\rightarrow3}$ increased from approximately 11% for insulin alone to 38% for insulin combined with H-MAP, suggesting that higher bioavailability of insulin was attained in the presence of H-MAP. No significant difference in $\%TRGP_{0\rightarrow3}$ was observed between insulin 0.26 U/kg combined with H-MAP at 16 mg/kg and 25 mg/kg.

The plasma insulin concentration-time profiles and pharmacokinetic parameters following spray-instillation to the lungs of 1.3 U/kg insulin combined with increasing doses of H-MAP are shown in Fig. 3A and Table III, respectively. Dose-dependent responses were observed. A maximum insulin absorption was achieved with 16 mg/kg H-MAP. At this dose, C_{max} increased from 87 ± 34 to 158 ± 33 μU/ml, the $AUC_{0\rightarrow\infty}$ increased more than 2 fold (from 4613 ± 949 to 10837 ± 3020 μU.min/mL) and the relative bioavailability increased from 32.9 to 77.4 %, for the insulin alone and the H-MAP/insulin combination, respectively. No significant difference in insulin levels was observed for insulin alone and combined with H-MAP at 5 or 25 mg/kg. A significant difference in insulin levels was observed between insulin alone and the H-MAP/insulin combination at 10 ($p < 0.002$) or 16 mg/kg ($p < 0.0001$).

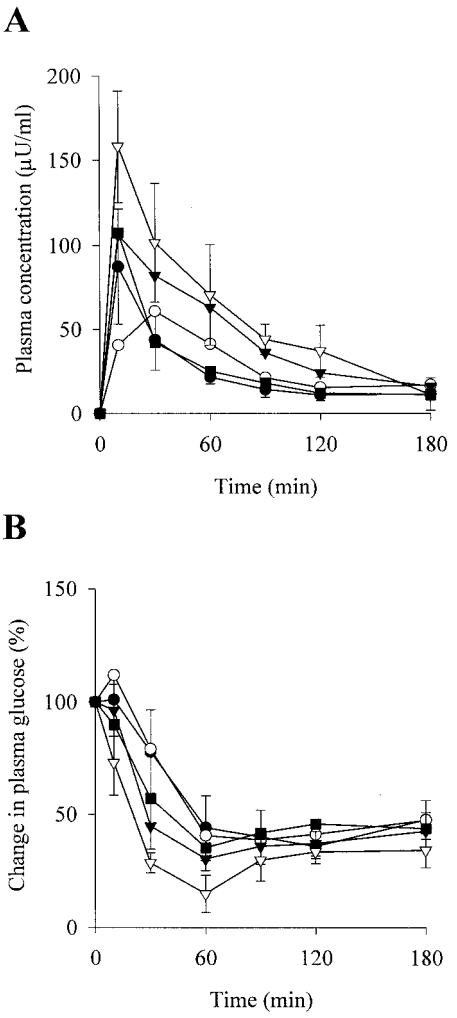

Fig. 3. Insulin plasma concentration-time profiles (A) and glucose plasma concentration-time profiles (B) following lung spray/instillation of insulin alone 1.3 U/kg (●), combined with H-MAP at 5 mg/kg (○), 10 mg/kg (▼), 16 mg/kg (∇), or at 25 mg/kg (■). Bars represent mean ± SD for n = 4 to 5.

Figure 3B and Table IV show the plasma glucose-time profiles and PD parameters following lung spray-instillation of insulin 1.3 U/kg alone and in combination with H-MAP at different doses. No significant difference in glucose levels was obtained for insulin combined with H-MAP at 5, 10, or 25 mg/kg. Statistically significant difference in glucose profiles were observed between insulin alone and combined with H-MAP. The $\%TRGP_{0\rightarrow3}$ increased significantly from 47 ± 10 to 66 ± 5% for insulin combined with H-MAP at 16 mg/kg. This dose-dependence PD relationship found for H-MAP may be explained by the pharmacokinetics of insulin as indicated by the increased bioavailability.

The plasma insulin-time profiles following lung spray-instillation of insulin 2.6 U/kg combined with increasing doses of H-MAP are shown in Fig. 4A. A dose-response relationship was observed achieving a maximum effect at 25 mg/kg of H-MAP. C_{max} increased from 152 ± 37 to 482 ± 151 μU/ml and relative bioavailability increased from 49% to 107.72 %, for the insulin alone and combined with H-MAP, respectively. A significant difference in insulin levels was observed between insulin alone or associated with H-MAP at 25 ($p < 0.0001$) and between insulin combined with H-MAP at 25 mg/kg and at 10 ($p < 0.0001$) or 16 mg/kg ($p < 0.001$).

Fig. 4B shows the insulin concentration-time profiles following spray-instillation of insulin alone 2.6 U/kg and with H-MAP at 10 mg/kg, 16 mg/kg or 25 mg/kg. No difference was observed in glucose levels for insulin with H-MAP at 10 or 16 mg/kg ($P > 0.05$).

DISCUSSION

Pulmonary administration of insulin results in more rapid absorption and clearance than subcutaneous delivery (10). Therefore, insulin may be delivered by this route to closely control fluctuations in circulating glucose concentrations. However, many difficulties have risen in delivering these proteins, which result in poor bioavailability. Among them, precipitation of insulin in the device during nebulization (5–6, 9), poor control in particle size of the aerosol (5–6), losses of dose in the mouth and throat of the animal or patient (7,9). Absolute bioavailabilities of pulmonary insulin in animals have been reported to be 15–50% based on the amount delivered to the lungs (8–9). Whereas in human studies, bioavailability ranged from 20–25% (5–7). Okumura *et al.* (10) reported that the relative bioavailability of insulin after intratracheal instillation (3U/kg) or intratracheal aerosol (7.5U/kg) to rats was 13% and 37.3–98%, respectively, of that obtained after subcutaneous administration (0.3U/kg). These results support the importance of pulmonary delivery efficiency to achieve therapeutic drug concentration at the site of action. The mouth to blood efficiency of insulin delivery of the commercially available inhalation devices is as low as 20–25% (6–7). Therefore, there is an urgent need for new technologies for enhancing the systemic delivery of aerosolized insulin.

Rapid clearance from systemic circulation, closely resembling that of endogenous insulin, may be an advantage of increasing insulin bioavailability by pulmonary administration. Once in circulation, insulin is cleared by the liver and

Table III. Average Insulin Pharmacokinetic Parameters following Subcutaneous Administration (SC) and Spray-Instillation (SI) of Insulin Alone (1.3 U/kg) and Combined with Escalating Doses H-MAP

	Treatment					
	SC insulin alone	SI insulin alone	SI insulin & H-MAP			
	Insulin dose (U/kg)		Dose of H-MAP (mg/kg)			
PK parameter	1.3	1.3	5	10	16	25
C_{max} (μU/ml)	101 ± 43	87 ± 34	67 ± 49	111 ± 51	158 ± 33	107 ± 67
T_{max} (min)	18 ± 7	10 ± 0	33 ± 21	24 ± 22	10 ± 0[a]	10 ± 0[a]
$AUC_{0\to\infty}$ (μU.min/ml)	13861 ± 4148	4945 ± 866	5593 ± 3414	9975 ± 2535	10733 ± 3140	6492 ± 4131
$MRT_{0\to t}$ (min)	100 ± 11	52 ± 8	69 ± 7[a]	58 ± 13[a]	58 ± 4[a]	58 ± 1[a]
Relative bioavailability (%)		36 ± 6	40 ± 25	72 ± 18[b]	77 ± 23[b]	47 ± 30

[a] Significantly different with respect to SC insulin alone.
[b] Significantly different with respect to SI insulin alone.
Average data based on n = 4 to 5.

kidney via a receptor-mediated processes involving the IDE enzyme complex (17–18). Other tissues such as muscle and adipose tissue are also known to metabolize insulin thereby increasing its clearance rate (17).

Numerous attempts to enhance the bioavailability of inhaled insulin have been reported. In general, improvement has focused on the use of penetration enhancers, which may alter membrane permeability in a nonspecific manner or on the use of nonspecific protease inhibitors (10–12,19). These alternatives are highly inefficient and cause varying degrees of membrane damage (10,20).

A microspray-instillator was used in the present studies to circumvent the oropharynx and thereby deliver the drug in solution to the lower airways beyond the first bronchial bifurcation. Gamma scintigraphy studies in which Tc^{99}-labelled lipid DNA complex was spray-instilled into the lungs using this device revealed a uniform distribution of the radiolabelled compound in the central and peripheral regions of the lungs (21). In addition, preliminary studies conducted by our group demonstrated that fluorescein plasma concentrations following spray-instillation of fluorescein in solution into the lungs of anesthetized rats had a coefficient of variation less than 25%, indicating the reproducibility of the method.

In pharmacokinetic studies comparing the intratracheal and aerosol administration of insulin to rabbits, Colthorpe *et al.* (9) found that the intratracheal administration of bovine insulin (5 U/kg) into anesthetized New Zealand rabbits resulted in T_{max} values of 11.3 min and C_{max} values of 60–80 μU/ml. These and the present data suggest that insulin delivery using the microsprayer device is more efficient than intratracheal administration. This is supported by the bioavailability of drugs administered by aerosolization which have been shown to be significantly higher than those obtained following intratracheal administration (9), due most likely to increased distribution to the periphery of the lungs.

In previous studies (10), it was determined that the intratracheal administration of 3.0 U/kg human insulin to rats resulted in an AUC value (mean ± SE) of 15206 ± 2733.5 μU.min/ml and is in close agreement with the AUC value obtained in the present study following spray-instillation of 2.6 U/kg of insulin. These results translated into a significant decrease in plasma glucose and the time to achieve it (Fig. 1B). However, as noted in the results, great inter-animal variability was observed for both glucose and insulin responses, which was higher following SC as compared to SI of insulin solution. These observations can be explained in part by the results reported by Liu *et al.* (22) who studied the *in vitro* biodegradation of insulin in rat lung homogenate. The major metabolite in the lung (des-Phen-$Insulin^{B1}$) retained the same bioactivity of insulin, indicating that the pulmonary route of insulin delivery will not adversely affect its hypoglycemic activity.

In addition to the efficient SI system for insulin delivery directly to the lower airways, described above, H-MAP was

Table IV. Average Pharmacodynamic Parameters following Spray-Instillation of Insulin Alone (1.3 U/kg) and Combined with Escalating Doses of H-MAP

	Treatment				
	SI insulin alone	SI insulin & H-MAP			
	Dose (U/kg)	Dose of H-MAP (mg/kg)			
PD parameter	1.3	5	10	16	25
%MPGC	35.7 ± 5.1	38.6 ± 6.9	30.3 ± 4.1	14.9 ± 8.2[a]	35.2 ± 5.8
T%MPGC (min)	120 ± 0.0	90 ± 0.0[a]	60 ± 0.0[a]	60 ± 0.0[a]	60 ± 0.0[a]
$\%TRPG_{0\to 3hr}$	47.2 ± 9.5	45.7 ± 7.9	58.3 ± 7.0	65.7 ± 4.9[a]	50.4 ± 6.9
$AAC_{E\ 0\to t}$ (μU.min/ml)	8497 ± 1716	8218 ± 1430	10488 ± 1284	11834 ± 872[a]	9067 ± 1248

[a] Significantly different with respect to SI insulin alone ($P < 0.05$). Average data based on n = 4 to 5.

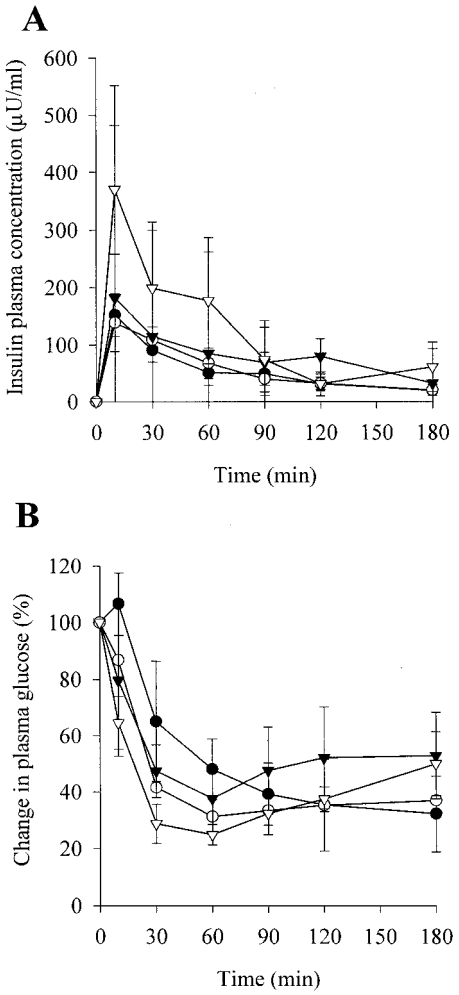

Fig. 4. Insulin plasma concentration-time profiles (A) and glucose plasma concentration-time profiles (B) following lung spray/instillation of insulin alone 2.6 U/kg (●), combined with H-MAP at 10 mg/kg (○), 16 mg/kg (▼), or 25 mg/kg (∇). Bars represent mean ± SD for n = 5 to 7.

co-administered with insulin in solution to improve its bioavailability from the airways. In general, the translocation of proteins across membranes involves different steps including signal-anchor sequences by integral membrane proteins and the transported protein itself, producing a temporal channel until the exit of the transported protein (23–24). Proteins usually remain partly unfolded during transport and once translocated, the protein folds on the trans-side of the membrane with the help of H-MAP or folding enzymes (24). Previous studies conducted using a series of N-acylated-α-amino acids have shown that these compounds facilitate the absorption of different proteins, including insulin, through the gastrointestinal membrane by a protein transport mechanism which cannot be explained through classical protease inhibition or general penetration enhancement (14).

The data reported in the present studies indicate that the H-MAP significantly affects the pharmacokinetics and pharmacodynamics of insulin administered to the lungs of rats by spray-instillation in a fashion which is dependent on the doses of both, H-MAP, and insulin. Coadministration of H-MAP and insulin produced a decrease in plasma glucose significantly greater than that observed following the administration of insulin alone at the same dose. For example, at dose of 0.26 U/kg insulin combined with H-MAP at 16 mg/kg AUC increased 2.5 fold and relative bioavailability almost tripled (Fig. 2A). This resulted in a 33% decrease in %MPGC (from 70.2 to 47%) and the T%MPGC decreasing by half (from 180 to 90 min), which reflected in a tripled increase in the %TRPG (Fig. 2B and Table II). These findings showed the potential of H-MAP to affect not only the plasma glucose levels, but also, and perhaps more importantly, to alter the time to achieve minimum glucose plasma levels. More important, at dose of 1.3 U/kg, insulin administered with H-MAP at 16 mg/kg resulted in maximum pharmacokinetic parameters: Cmax doubled and AUC and relative bioavailability increased more than 2-fold (Fig. 3A and Table III). These reflected into a %TRPG decrease of about 12%.

Control studies demonstrated that the plasma glucose-time profiles following lung spray-instillation of H-MAP at 16 mg/kg administered 15 min before the administration of insulin alone at 0.26 U/kg was consistent with those obtained when insulin was administered alone. In addition, no change in plasma glucose levels was observed following spray instillation of H-MAP alone at 16 mg/kg indicating no direct effect of H-MAP on glucose plasma concentrations.

Interestingly, although insulin at dose of 2.6 U/kg administered with several doses of H-MAP resulted in an increase in some of the pharmacokinetic parameters, this did not translate into differences in glucose levels among the different doses of H-MAP. The apparent absence of an effect may be due to the mechanism of insulin transport which is known to be receptor mediated (17–18,25–26). A possible explanation could be the saturation of insulin receptors in the susceptible tissues (25–26) or saturation of the insulin-H-MAP interaction. A significant difference was achieved when insulin was combined with H-MAP at 25 mg/kg ($p < 0.005$), suggesting that maximum PD effect requires an optimum dose for both, insulin and H-MAP.

These preliminary studies show that H-MAP can significantly increase the bioavailability and improve the pharmacodynamic effect of insulin delivered to the lungs of rats in a dose-dependent fashion for both insulin and H-MAP. However, the effect of multiple doses of insulin-H-MAP on the bioavailability of insulin should be investigated. In addition, specific studies to elucidate the mechanism of action of H-MAP should be conducted. The low efficiency of pulmonary delivery of the commercially available inhalation devices and the relatively low pulmonary bioavailability of insulin represent potential aerosol formulation and delivery barriers. The use of the H-MAP may increase the potential of the lungs as a route of administration for insulin in concentrations required to treat diabetes.

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