articles

molecular pharmaceutics

Diabetes Correction in Pancreatectomized Canines by Orally Absorbable Insulin−Deoxycholate Complex

Sang Kyoon Kim,[†] Seulki Lee,[†] Sunji Jin,[†] Hyun Tae Moon,[‡] Ok Cheol Jeon,[‡] Dong Yun Lee,[*,§] and Youngro Byun[*,†,||]

College of Pharmacy, Seoul National University, Seoul 151-742, South Korea, Mediplex Corporation, Seoul 135-729, South Korea, Department of Bioengineering, College of Engineering, Hanyang University, Seoul 133-791, South Korea, and Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 151-742, South Korea

Received October 27, 2009; Revised Manuscript Received April 1, 2010; Accepted April 2, 2010

Abstract: Oral insulin therapy has great potential benefits over conventional therapy for diabetic patients as well as mimicking the physiological fate of insulin. Here we evaluated the characteristics of insulin and deoxycholate-based synthetic N^{α}-deoxycholyl-L-lysyl-methylester (DCK) complex, and diabetes correction in pancreatectomized canines after oral administration. After the insulin/DCK complexation was made, the insulin's folding structure, stability against digestive enzymes, lipophilicity and permeability to Caco-2 monolayer were evaluated in vitro. Diabetic canines were kept under fasting conditions, and Eudragit-coated gelatin capsules containing insulin or insulin/DCK powder were singly or triply administered. Evaluation of glucodynamics, pharmacokinetics, oral glucose tolerance test (OGTT) and reproducibility were carried out. After complexation with DCK, the folding structure of insulin did not become denatured and the resistance against digestive enzymes was powerfully improved. The lipophilicity and permeability of insulin/DCK (coupling ratio up to 1:10) were also highly increased. The insulin/DCK complex, administered orally into diabetic canines at the doses of 21, 42, and 81 IU/kg, reduced the plasma glucose levels by about 28%, 44% and 67%, respectively, while the plasma insulin concentrations increased. During OGTT, insulin/DCK nearly maintained the normoglycemic state in the diabetic canines, whereas the hyperglycemic state of placebo-treated controls was not corrected. During oral administration of insulin/DCK, it repetitively showed similar therapeutic efficacy in diabetic canines for 3 days. The therapeutic efficacy of insulin/DCK was exhibited in its digestive enzyme resistance, deoxycholate-based lipophilicity for enhancing permeability and intact insulin delivery without chemical modification, providing potential oral therapeutic remedy as an alternative to injectable insulin medication.

Keywords: Insulin; N^{α}-deoxycholyl-L-lysyl-methylester (DCK); pancreatectomized canines; oral absorption; lipophilicity; permeability

Introduction

Injectable insulin therapy is essential for all patients with insulin-dependent diabetes mellitus (IDDM) and for some with non-insulin dependent diabetes mellitus (NIDDM). However, this remedy is painful and uncomfortable as a long-term treatment. Therapy by injection is not an ideal route

* These authors equally contributed as corresponding authors in this work. D.Y.L.: Department of Bioengineering, College of Engineering, Hanyang University, 17 Haengdang-dong, Seongdong-gu, Seoul 133-791, South Korea; tel, +82-2-2220-2348; fax, +82-2-2220-4741; e-mail, dongyunlee@hanyang.ac.kr. Y.B.: Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, and College of Pharmacy, Seoul National University, 599 Gwanak-lo, Sillim-9-dong, Gwanak-gu, Seoul 151-742, South Korea; tel, +82-2-880-7866; fax, +82-2-872-7864; e-mail, yrbyun@snu.ac.kr.

[†] College of Pharmacy, Seoul National University.

[‡] Mediplex Corporation.

[§] Hanyang University.

[||] Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University.

10.1021/mp9002688
Published on Web 04/02/2010

because insulin does not immediately reach the liver following injection, and is also associated with several therapeutic limitations such as hypoglycemia, lipoatrophy, lipohypertrophy, obesity, insulin neuropathy and insulin presbyopia.[1−3] Therefore, the development of alternative insulin medications via oral, buccal, transdermal, intranasal and pulmonary routes has been greatly encouraged.[4−6] Of the alternative routes, oral administration would be highly advantageous because orally absorbed insulin can mimic the physiological fate of insulin and may provide better glucose homeostasis.[7,8] However, successful oral insulin therapy involves overcoming challenges such as degradation by digestive enzymes, epithelial permeability in the gastrointestinal tract and conserving the insulin bioactivity.[9] Currently, several biotechnologies have been introduced to improve oral insulin absorption, overcome barriers and develop safe and effective therapies in attempts to enhance conventional oral insulin therapy.[10−19]

Bile acids, the major cholesterol metabolites synthesized in the liver, are postprandially released into the small intestine, reabsorbed in the ileum, and returned to the liver. Recently, bile acids have been used in oral drug delivery of macromolecules such as insulin, heparin and ceftriaxone because of their amphiphilicity and reabsorption properties as they can be chemically or physically conjugated with target drug and further formulated for enhancing the therapeutic efficacy of drug.[20−28] Previously, we developed a synthetic deoxycholyl-lysine (DCK) having a positive amino residue to make a physical complex with insulin via ion-pair interaction without altering the structure of native insulin, and demonstrated that the insulin/DCK complex in the aqueous formulation could cure hyperglycemia in chemically induced diabetic rodents.[27] DCK is considered to possess

(1) Kennedy, F. P. Recent development in insulin delivery techniques: current status and future potential. *Drugs* **1991**, *42*, 213–227.

(2) Monaco, L.; Gettken, G.; Silverstein, J. H. Accuracy of injection site identification among children with insulin dependent diabetes mellitus: a comparison of traditional and new visual aids. *Clin. Pediatr.* **1996**, *35*, 191–197.

(3) Carlson, M. G.; Campbell, P. J. Intensive insulin therapy and weight gain in IDDM. *Diabetes* **1993**, *42*, 1700.

(4) Owens, D. R. New horizons-alternative routes for insulin therapy. *Nat. Rev. Drug Discovery* **2002**, *1*, 529–540.

(5) Cefalu, W. T. Concepts, strategies, and feasibility of non-invasive insulin delivery. *Diabetes Care* **2004**, *27*, 239–246.

(6) Gordberg, M.; Gomez-Orellana, I. Challenges for the oral delivery of macromolecules. *Nat. Rev. Drug Discovery* **2003**, *4*, 289–295.

(7) Lewis, G. F.; Zinman, B.; Groenewound, Y.; Vranic, M.; Giacca, A. Hepatic glucose production is regulated both by direct hepatic and extrahepatic effects of insulin in humans. *Diabetes* **1996**, *45*, 454–462.

(8) Eaton, R. P.; Allen, R. C.; Schade, D. S.; Standefer, J. C. 'Normal' insulin secretion: the goal of artificial insulin delivery systems. *Diabetes Care* **1980**, *3*, 270–273.

(9) Hamman, J. H.; Enslin, G. M.; Kotze, A. F. Oral delivery of peptide drugs. *Biodrugs* **2005**, *19*, 165–177.

(10) Fasano, S.; Uzzau, S. Modulation of intestinal tight junctions by zonula occludens toxin permits enteral administration of insulin and other macromolecules in an animal model. *J. Clin. Invest.* **1997**, *99*, 1158–1164.

(11) Carino, G. P.; Mathiowitz, E. Oral insulin delivery. *Adv. Drug Delivery Rev.* **1999**, *35*, 249–257.

(12) Mathiowitz, E.; Jacob, J. S.; Jong, Y. S.; Carino, G. P.; Chickering, D. E.; Chaturvedi, P.; Santos, C. A.; Vijayaraghavan, K.; Montgomery, S.; Bassett, M.; Morrell, C. Biologically erodible microspheres as potential oral drug delivery systems. *Nature* **1997**, *386*, 410–414.

(13) Chung, H.; Kim, J.; Um, J. Y.; Kwon, I. C.; Jeong, S. Y. Self-assembled "Nanocubicle" as carrier for oral insulin delivery. *Diabetologia* **2002**, *45*, 448–451.

(14) Damage, C.; Michel, C.; Aprahamian, M.; Couvreur, P. New approach for the oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier. *Diabetes* **1988**, *37*, 246–251.

(15) Onuki, Y.; Morishita, M.; Takayama, K. Formulation optimization of water-in-oil-water multiple emulsion for intestinal insulin delivery. *J. Controlled Release* **2004**, *97*, 91–99.

(16) Cui, F.; Shi, K.; Zhang, L.; Tao, A.; Kawashia, Y. Biodegradable nanoparticles loaded with insulin-phospholipid complex for oral delivery: preparation in vitro characterization and in vivo evaluation. *J. Controlled Release* **2006**, *114*, 242–250.

(17) Tozaki, H.; Komoike, J.; Tada, C.; Maruyama, T.; Terabe, A.; Suzuki, T.; Yamamoto, A.; Muranishi, S. Chitosan capsules for colon-specific drug delivery: improvement of insulin absorption from the rat colon. *J. Pharm. Sci.* **1997**, *89*, 1016–1021.

(18) Wajcberg, E.; Myiazaki, Y.; Triplitt, C.; Cersosimo, E.; De Fronzo, R. A. Dose reponse oeffect of a single dose of orally administered hexyl-insulin monoconjugate (HIM2) in healthy nondiabetic subjects. *Diabetologia* **2003**, *46*, A278.

(19) Musabayane, C. T.; Munjeri, O.; Bwititi, P.; Osim, E. E. Orally administered, insulin-loaded admidated pectin hydrogel beadssustain plasma concentrations of insulin in streptozotocin-diabetic rats. *J. Endocrinol.* **2000**, *164*, 1–6.

(20) Lane, E. M.; O'driscoll, C. M.; Corrigan, O. I. Quantitative estimation of the effects of bile salts surfactant systems on insulin stability and permeability in the rat intestine using a mass balance model. *J. Pharm. Pharmacol.* **2005**, *57*, 169–175.

(21) Lee, Y.; Nam, J. H.; Shin, H. C.; Byun, Y. Conjugation of low-molecular-weight heparin and deoxycholic acid for the development of a new oral anticoagulant agent. *Circulation* **2001**, *104*, 3116–3120.

(22) Lee, S.; Kim, K.; Kumar, T. S.; Lee, J.; Kim, S. K.; Lee, D. Y.; Lee, Y. K.; Byun, Y. Synthesis and biological properties of insulin-deoxycholic acid chemical conjugates. *Bioconjugate Chem.* **2005**, *16*, 615–620.

(23) Lee, D. Y.; Park, K.; Kim, S. K.; Park, R. W.; Kwon, I. C.; Kim, S. Y.; Byun, Y. Antimetastatic effect of an orally active heparin derivative on experimentally induced metastasis. *Clin. Cancer Res.* **2008**, *14*, 2841–2849.

(24) Mesiha, M. S.; Ponnapula, S.; Plakogiannis, F. Oral absorption of insulin encapsulated in artificial chyles of bile salts, palmitic acid and α-tocopherol dispersions. *Int. J. Pharm.* **2002**, *249*, 1–5.

(25) Lee, S.; Kim, S. K.; Lee, D. Y.; Chae, S. Y.; Byun, Y. Pharmacokinetics of a new, orally available ceftriaxone formulation in physical complexation with a cationic analogue of bile acid in rats. *Antimicrob. Agents Chemother.* **2006**, *50*, 1869–1871.

(26) Lee, D. Y.; Lee, J.; Lee, S.; Kim, S. K.; Byun, Y. Liphophilic complexation of heparin based on bile acid for oral delivery. *J. Controlled Release* **2007**, *123*, 39–45.

(27) Lee, S.; Lee, J.; Lee, D. Y.; Kim, S. K.; Lee, Y.; Byun, Y. A new drug carrier, N^{α}-deoxycholyl-L-lysyl-methylester, for enhancing insulin absorption in the intestine. *Diabetologia* **2005**, *48*, 405–411.

various advantages. First, the bound DCK can give insulin high lipophilicity, thereby penetrating the intestinal membrane easily. Second, it can hold monomeric active insulin in place of general hexameric insulin. The third advantage of bound DCK is that it can endow insulin with digestive enzyme resistance by interrupting the recognition of the enzyme. Finally, it can interact with ileal bile acid transporters, thereby enhancing the concentration gradient of insulin across the intestinal wall.

We speculate here about the therapeutic efficacy of insulin/DCK powder form in diabetic canines with respect to potential clinical applications of this new oral medication. To this end, this study aimed to elucidate the physicochemical properties of insulin/DCK for optimum complexation. We also designed a study using total pancreatectomized diabetic canines to assess therapeutic efficacy and susceptibility of the complex after single or multiple oral administration of insulin/DCK powder contained in the Eudragit-coated gelatin capsule.

Experimental Section

Synthesis of N^{α}-Deoxycholyl-L-lysyl-methylester (DCK). Deoxycholic acid (200 mg, 0.5 mmol/L; Sigma-Aldrich, St. Louis, MO) and *N*-hydroxysuccinimide (76 mg, 0.67 mmol/L; Sigma-Aldrich) were dissolved in anhydrous tetrahydrofuran (THF; 20 mL; Sigma-Aldrich). After addition of 1,3-dicyclohexylcarbodiimide (136 mg, 0.67 mmol/L; Sigma-Aldrich), the solution was stirred at 4 °C for 6 h. The urea derivatives produced were filtered. The filtrate was poured into cold *n*-hexane (120 mL), and the precipitates were then dried under vacuum. Then the prepared succinimido deoxycholate (230 mg, 0.48 mmol/L; Sigma-Aldrich) was reacted with the primary amine group of N'-tBOC-Lys-OCH_3 (150 mg, 0.58 mmol/L) and lysine amino acid derivative (Bachem, Bubendorf, Switzerland) in dimethylformamide (DMF; 10 mL; Sigma-Aldrich) containing triethylamine (200 μL, 1.7 mmol/L; Sigma-Aldrich) for 12 h at room temperature. After completion of the reaction, the mixture was diluted with ethyl acetate (30 mL) and washed with 10 mL of 0.5 mol/L HCl, distilled water, 0.5 mol/L NaOH, and distilled water, respectively. The organic phase was dried by adding magnesium sulfate and evaporated. The protected amine group of lysine was deprotected by mixing a trifluoroacetic acid/dichloromethane (1:1 v/v) solution for 2 h at room temperature. After the solvent was evaporated under reduced pressure, cold diethyl ether was then added to induce precipitation, and the mixture was subsequently dried under vacuum. The dried product was dissolved in distilled water and purified by passing the solution through a Sep-Pak C18 column (Waters Co., Milford, MA). Finally, purified DCK was lyophilized and obtained as a white powder. The chemical structure and purity of purified DCK were confirmed by using ^{1}H NMR (JEOL JNM-LA 300 WB FT-NMR, Toyko).

Preparation of Insulin/DCK Complex. The oral insulin/DCK complex was prepared by making a physical complex of human insulin (27 IU/mg; Serologicals, Norcross, GA) with DCK through ion-pair interaction. Briefly, insulin was dissolved in a small volume of 5 mmol/L HCl and the resultant solution was diluted with PBS (10 mmol/L, pH 7.4) to final concentrations of 21, 42, and 81 IU/mL. Different concentrations of DCK (feeding mole ratio 1:0 to 1:30) in PBS were slowly added to the insulin solution while mixing using a vortex. The insulin/DCK complex was lyophilized at −85 °C for in vitro and in vivo experiments. For in vivo study, the lyophilized insulin/DCK powder without any excipients was loaded in a gelatin capsule using a stainless funnel. The capsules were enteric-coated with Eudragit L100 (anionic copolymer based on methacrylic acid and methyl methacrylate) (Degussa, Evonik Inc., Germany) by using tweezers.

Near-UV Circular Dichroism (CD) Spectra. To evaluate the denaturation of the folding structure of insulin after complexation with DCK, the mean residue ellipticity of insulin was determined using a Jasco J-720 spectropolarimeter (Tokyo, Japan). Briefly, different complex ratios of insulin/DCK (0.1 mmol/L) were prepared in 10 mmol/L HCl and filtered through a 0.2 μm syringe filter. In the near-UV wavelength region, the CD spectra of the samples were recorded by scanning five times in 1 cm cuvette cells from 320 to 250 nm with a step size of 0.5 nm and a bandwidth of 1.5 nm, and the final spectrum was acquired on an average. The CD spectra of the buffer were recorded as background and subtracted from the sample spectra. The data was then transformed to the mean residue ellipticity (θ_m) using the expression $\theta_m = (\theta M)/(Cl)$, where θ is the observed ellipticity (mdeg), M is the mean residue molecular weight (g/mol/L), C is the protein concentration (g/mL), and l is the optical path length (cm).

Digestive Enzyme Resistance Assay. Studies using insulin (100 μL, 17.2 μmol/L) or insulin/DCK complexes (1:5, 1:10 and 1:15) were performed in HEPES buffer (50 mmol/L, pH 7.4). After adding α-chymotrypsin (10 μL, 1.5 μg), the solutions were incubated at 37 °C. At each incubation time, aliquots of the solutions were acidified by adding 890 μL of 0.1% trifluoroacetic acid. Each sample was analyzed using reversed-phase HPLC (Shimadzu, Tokyo, Japan) and a C18 Bondapak column (Waters Co.) with a linear gradient of 5−60% solvent B (solvent A, 0.1% trifluoroacetic acid; solvent B, 0.1% trifluoroacetic acid in 95% acetonitrile) over 55 min. The half-life ($t_{1/2}$) of stability was estimated as the time (in min) taken for the degradation of 50% of the insulin administered.

Lipophilicity Measurement. To evaluate the lipophilicity of insulin/DCK in different complex ratios, the partition coefficient of the FITC-labeled insulin/DCK or FITC-labeled insulin (Sigma-Aldrich) in methylene chloride (MC)/water (W) (1:1 v/v) was measured. Briefly, after preparation of a 1 mg/mL aqueous solution of FITC-labeled insulin/DCK or

(28) Kim, S. K.; Lee, D. Y.; Lee, E.; Lee, Y. K.; Kim, C. Y.; Moon, H. T.; Byun, Y. Absorption study of deoxycholic acid-heparin conjugate as a new form of oral anti-coagulant. *J. Controlled Release* **2007**, *120*, 4–10.

FITC-labeled insulin, an equal volume of MC was added to the insulin solution. After shaking for 24 h, the samples were centrifuged for 5 min at 13000*g*. The concentrations of FITC-labeled insulin in the water and MC phases were analyzed using a microplate reader (excitation at 480 nm and emission at 516 nm for FITC-insulin) (synergy HT, BioTek Instruments, Inc., Winooski, VT). The partition coefficient ($P_{MC/W}$) was calculated from $P_{MC/W} = C_{MC}/C_{water}$, where C_{MC} and C_{water} represent the concentrations of FITC-insulin in the respective phase.

Measurement of the Critical Micelle Concentration (CMC) by Fluorometry. DCK will not make a complex with insulin in a concentration over the cmc, because it forms a micelle in this concentration. For measuring the critical micelle concentration of DCK, we used pyrene solution. The desired concentrations of the DCK were prepared by volumetric dilution with 0.1 M phosphate buffer of pH 7.4. To a 50 mL flask containing 15 mL of the DCK, 2 μM of pyrene (Sigma-Aldrich) was added. In the dark condition, it was placed in a shaking bath for 48 h. After this time, an appropriate volume of the clear solution was decanted and the fluorescence spectrum was recorded with 340 nm excitation with water circulating at 25 °C. Fluorescence measurements were performed on a fluorescence spectrofluorometer (RF-1500, Shimadzu, Japan). A shaking water bath (JSSB-50T, JSR, South Korea) was employed to solubilize pyrene in aqueous solution of the DCK.

Permeability Study Using a Caco-2 Cell Transport Model. To study the potential of oral absorption enhancement of DCK, the human colon cancer cell line Caco-2 (American Type Culture Collection, Manasassas, VA) was used as the transport model as previously described.[28] Briefly, Caco-2 cell monolayers with a transepithelial electrical resistance (TEER) value of more than 400 Ω cm^2 were formed in Transwell (Corning Inc., Corning, NY) used for permeability studies. Then Caco-2 cell monolayers were washed twice with cold PBS and preincubated with prewarmed transport media (pH 7.4) supplemented with Hanks balanced salt solution (HBSS; Biowhittaker, Walkersville, MD), 10 mmol/L HEPES (*N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid; Biowhittaker), 25 mmol/L D-glucose (Sigma-Aldrich), and 10 mmol/L metabolic inhibitor sodium azide (NaN_3; Sigma-Aldrich) for 30 min at 37 °C in a 5% CO_2 incubator. After treatment of the FITC-labeled insulin/DCK or FITC-labeled insulin (35 μg/mL; equivalent concentration based on FITC-labeled insulin) in the apical part, the culture medium in the basolateral part was collected at each time (5, 15, 30,45, 60, and 120 min). To confirm that the permeation of insulin or insulin/DCK complex was not occurring by the damage of the Caco-2 cell monolayer after treatment of samples, the TEER value was repetitively measured after treatment of samples. The permeated FITC-insulin concentration was determined using a microplate reader. The apparent permeability coefficients (P_{app}; cm/s × 10^{-7}) for each sample were calculated according to the following equation: $P_{app} = (dQ/dt)/AC_0$; where P_{app} is the apparent permeability coefficient (cm/s), dQ/dt the steady state flux (mol/s), A the surface area of membrane (cm^2), and C_0 the initial concentration in the donor chamber (mol/cm^3).

Total Pancreatectomy of Canine. The animal studies were approved by the animal care and use committee in Korea and conducted according to the NIH Guideline for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985). Healthy male adult beagle canines (2−3 years old; weight ∼10 kg) received a standard diet for 2 weeks prior to surgery. Total pancreatectomy surgery was modified from a general pancreatectomy procedure. Briefly, after an overnight of fasting, the canines were anesthetized by iv injection of ketamine (10 mg/kg) plus xylazine (1 mg/kg) at 10 min after subcutaneous administration of atrophine of 0.05 mg/kg. Once anesthesia was achieved, it was maintained with ketamine and xylazine (5 mg/kg) with checking of vital signs. After receiving anesthesia, only the intact pancreas tissue was exposed through an abdominal incision, clarified by spreading the duodenal mesentery and then carefully dissected from the surrounding tissue after tightly binding the blood vessels with 0.5 cm distance connected to the pancreas without damaging the intestine. After surgery, we monitored the hourly blood glucose levels of the canines and injected regular insulin for maintaining the normal glucose level intramuscularly. They also were supplemented with oral Pancreas (50 mg/kg twice a day; Dae Woong Pharm Co., Seoul, South Korea), which is a food digestion enzyme. All the pancreatectomized canines had high blood glucose levels ranging from 250 to 350 mg/dL, and responded to the insulin treatment.

Oral Absorption Experiment to Pancreatectomized Diabetic Canines. After fasting for 12 h, the capsules having either insulin alone (81 IU/kg) or insulin/DCK (21, 42, or 81 IU/kg) powder were orally administered to diabetic canines, which were randomized according to their average body weights and fasting blood glucose levels. After oral administration of the capsules, the blood samples were collected at each time from the vein of the foot. The blood glucose levels of the fresh samples were determined immediately by using a portable glucometer (Glucocard II; Arkray, Kyoto, Japan). Human insulin concentrations in the plasma were measured using an ELISA kit (human insulin ELISA kit; Linco Research, Inc., St. Charles, MO). To compare with the therapeutic efficacy of insulin/DCK, insulin (0.33 IU/kg) was intravenously injected via tail vein. All the canines were kept in metabolic cages and were given free access to water only during the experiment.

Oral Glucose Tolerance Test (OGTT) after Oral Administration of Insulin. Four hours after oral administration of the capsule having insulin/DCK (42 IU/kg) or placebo (PBS as a vehicle) in advance, glucose solution (0.5 g/kg) in PBS was given orally to each group. Then blood samples were collected and the blood glucose levels were determined at each time interval.

Multiple Administration of Insulin/DCK Complex to the Diabetic Canines. The reproducibility of oral insulin/DCK was evaluated by daily administering the capsules

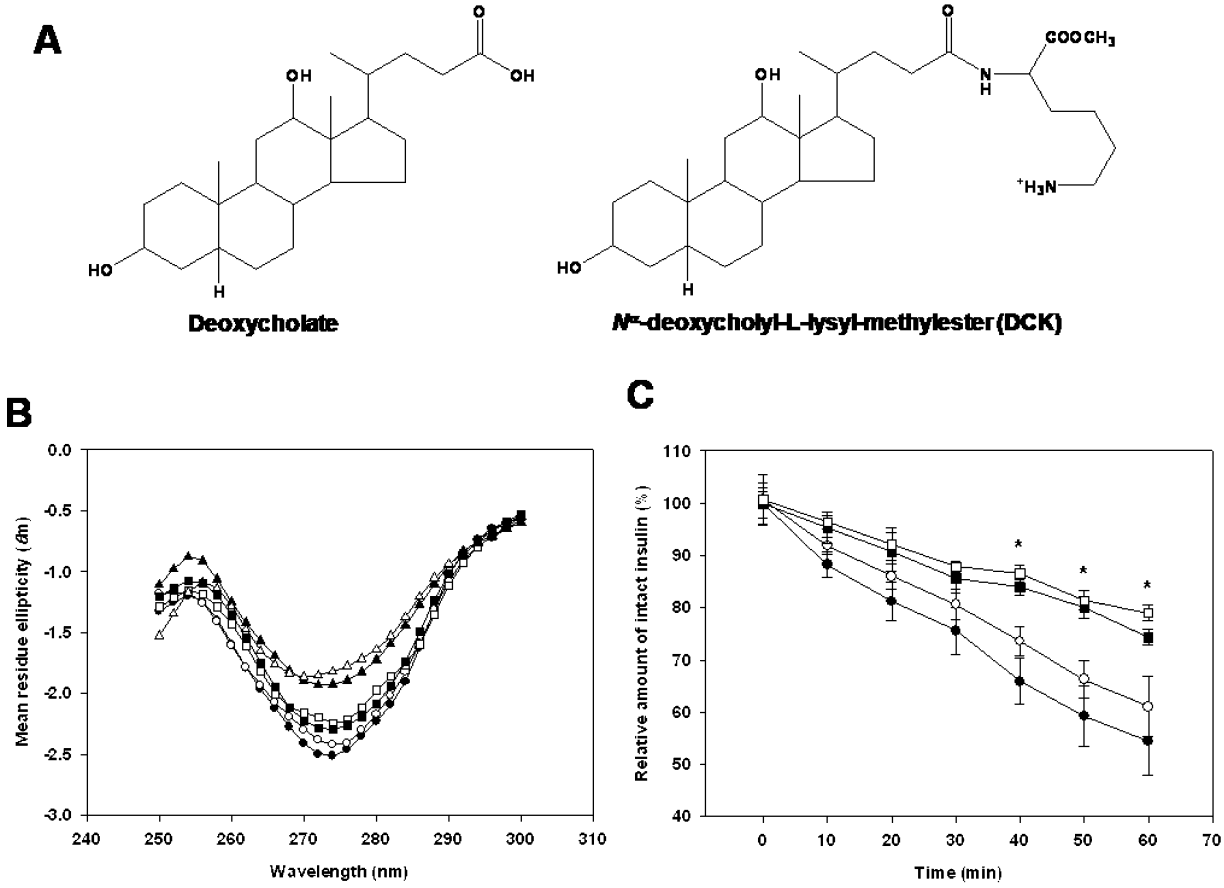

Figure 1. (A) The chemical structure of deoxycholate and N^{α}-deoxycholyl-L-lysyl-methylester (DCK). (B) Mean residue ellipticity (θ_m) of pure insulin and various insulin/DCK complexes by using near-UV circular dichroism (CD) spectra (300−250 nm). Insulin alone (black circles), 1:2 (white circles), 1:4 (black squares), 1:6 (white squares), 1:10 (black triangles) and 1:15 of insulin/DCK complex (white triangles). (C) Digestive enzyme resistance of insulin and various insulin/DCK complexes during incubation of α-chymotrypsin. Insulin alone (black circles), 1:5 insulin/DCK (white circles), 1:10 insulin/DCK (black squares) and 1:15 insulin/DCK (white squares). Data were plotted as means ± SD, $n = 7$. *$P < 0.05$ vs insulin alone at 1:10 and 1:15, respectively.

having insulin/DCK powder into diabetic canines every day for 3 days. After fasting for 12 h, diabetic canines received the capsule having insulin/DCK (42 IU/kg) and their blood glucose levels were measured for 10 h. After the experiment, all the canines were again given free access to feed and water.

Data Analysis. The areas under curves (AUC) were calculated using the linear trapezoidal method. Standard statistical methods were performed using Sigma-plot statistical software (SPSS, Chicago, IL). The data are expressed as mean ± SD. A Mann−Whitney nonparametric test was used to compare groups. P values <0.05 were considered statistically significant.

Results

Characterization of Insulin/DCK Complex. A cationic deoxycholic acid derivative, DCK, that has a cationic residue was synthesized by conjugating a deoxycholic acid (DOCA) precursor and lysine (Figure 1A). The conjugation of lysine and DOCA was confirmed by the existence of lysine and DOCA (data not shown). In the ^{1}H NMR spectra, the peak of the $-CH_2NH_2$ residue of lysine occurred at 2.9 ppm. Also, the triple peak of DOCA occurred at 3.8, 4.2, and 4.5 ppm. The conjugate was obtained with above 99% purity. The critical micelle concentration of DCK was about 7.5 mg/mL. DCK has a hydrophobic part of DOCA and a positive charge of lysine, thereby facilitating the charge-to-charge interactions with the anionic part of insulin. The insulin/DCK complex was easily obtained by electrostatic interactions in neutral pH (pH 7.4) and reversible in accordance with pH. It is possible that the complex would invariably form according to the molar ratio of insulin with DCK. Therefore, we measured the partition coefficient ($P_{MC/W}$) and permeability (P_{app}) values according to the different complexes (Table 1). The partition coefficient and permeability of DCK were increased when the feed mole ratios of DCK ranged from 1:0 to 1:10. At mole ratios of 1:15 and 1:30, however, the partition coefficient and permeability did not increase proportionally. In addition, TEER values before and after treatment of each sample were taken to confirm that the permeability of insulin/DCK complex was not attributed

Table 1. Characterization of the Insulin/DCK Complexes[a]

sample	complex ratio	$P_{MC/W}$[b]	P_{app},[c] cm/s × 10^{-7}
insulin	1:0	0.08	0.30 ± 0.10
insulin/DCK	1:2	0.33	0.26 ± 0.10
	1:5	3.71	1.35 ± 0.56
	1:10	11.64	4.40 ± 0.69
	1:15	6.43	4.55 ± 0.64
	1:30	3.93	1.48 ± 0.2

[a] Data are expressed as means ± SD (n = 7). [b] Partition coefficient ($P_{MC/W}$) as a lipophilicity was calculated from $P_{MC/W} = C_{MC}/C_W$, where C_{MC} and C_W represent the concentrations of insulin in the methylene chloride (MC) and water (W) phases. [c] The apparent permeability coefficients (P_{app}) were measured using the Caco-2 cell monolayer model.

to the damage on the Caco-2 cell monolayer. These results indicated that insulin has an optimum complex ratio with DCK.

To evaluate the denaturation of insulin structure complexed with DCK, the secondary structure of insulin was confirmed by using a near-UV CD spectropolarimeter according to various DCK ratios (Figure 1B). There were no changes in the secondary structure of insulin with DCK: this means that the physically bound DCK cannot affect the insulin secondary structure. In addition, the phase diagram obtained by plotting the ellipticity at 276 nm against that at 251 nm showed that the ellipticity transition can be attributed to the tendency of the hexameric insulin becoming a monomeric form after complexation with DCK. We next investigated the digestive enzyme resistance of insulin/DCK complex (Figure 1C). During incubation with α-chymotrypsin, the native insulin was degraded with a $t_{1/2}$ of 62.3 min. However, the insulin/DCK complexes were resistant to the enzyme and their half-life for stability was increased significantly. For the complex ratios of 1:5, 1:10 and 1:15, each $t_{1/2}$ was 74.3, 114.4, and 128.9 min, respectively. This indicated that the digestive enzyme resistance of insulin could be significantly improved by complexation with DCK.

Glucose-Lowering Effects of Insulin/DCK in Diabetic Canines. The therapeutic efficacies of the capsule having insulin/DCK complex powder in pancreatectomized diabetic canines were evaluated after their oral administration (Figure 2A). Severe fluctuations in the blood glucose level ranging from 300 to 410 mg/dL were observed in the placebo group. In the case of 21, 42, and 81 IU/kg of insulin/DCK, the blood glucose levels were lowered approximately by 21.1 ± 0.3 (from 325.3 ± 51.0 at 0 h to 233.9 ± 2.5 mg/dL at 5 h), 48.1 ± 7.2 (from 273.3 ± 7.4 at 0 h to 153.8 ± 21.3 mg/dL at 5 h) and 66.7 ± 11.2% (from 313.7 ± 37.8 at 0 h to 104.5 ± 35.2 mg/dL at 5 h), respectively, having dose-dependency when compared to each initial value at 0 h. The maximum glucose-lowering effect was observed at 4−5 h after oral administration of the capsule of insulin/DCK, and the blood glucose levels returned slowly to the original levels thereafter. However, for oral administration of the capsule having 81 IU/kg of insulin, the maximum glucose-lowering effect was about 19.2 ± 9.8% (from 309.8 ± 100.8 at 0 h to 250.3 ± 91.0 mg/dL at 5 h).

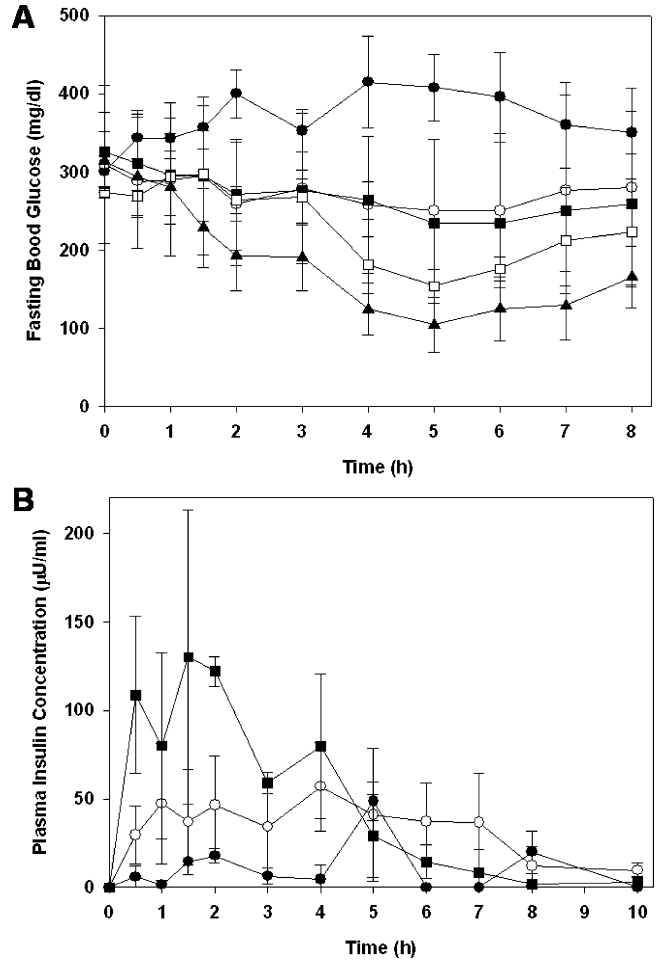

Figure 2. (A) Blood glucose levels of the pancreatectomized diabetic canines after single oral administration of Eudragit-coated gelatin capsule having insulin or various doses of insulin/DCK (1:10 ratio) powder. Vehicle treatment (PBS; black circles); 81 IU/kg insulin alone (white circles); 21 IU/kg (black squares), 42 IU/kg (white squares) or 81 IU/kg (black triangles) of insulin/DCK. Data were plotted as means ± SD, n = 3−4. (B) Plasma insulin levels of the pancreatectomized diabetic canines after single oral administration of Eudragit-coated gelatin capsule having 81 IU/kg insulin alone (white circles), 42 IU/kg (black circles) or 81 IU/kg (black squares) of insulin/DCK (1:10 ratio). Data were plotted as means ± SD, n = 3−4.

Simultaneously with measuring the blood glucose levels, we measured the plasma concentration of human insulin in diabetic canines after oral administration of insulin or insulin/DCK complexes (Figure 2B). The oral administration of 42 and 81 IU/kg insulin/DCK produced a significant increase in the plasma insulin concentrations, whereas plasma insulin was slightly detected in the case of 81 IU/kg of native insulin. The plasma insulin levels observed after the administration of 21 IU/kg of insulin/DCK were similar to those observed with 81 IU/kg insulin (data now shown). The maximum plasma insulin concentrations were observed at 2 to 4 h, and were 48.5 ± 11.0, 60.0 ± 25.0 and 130.1 ± 83.0 μU/mL for 21, 42, and 81 IU/kg, respectively, having dose-

Table 2. Glucodynamic and Pharmacokinetic Parameters after Administration of Insulin (Iv) and Insulin/DCK (Oral)[a]

		insulin/DCK (oral)		
	insulin (iv)			
dose (IU/kg)	0.33	21	42	81
N	3	4	4	4
fasting baseline glucose (mg/dL)	344 ± 20	325 ± 51	273 ± 7	314 ± 38
G_{nadir} (mg/dL)	108 ± 21	234 ± 1	154 ± 21	105 ± 35
glucose decrement (mg/dL)	235 ± 25	92 ± 46	120 ± 20	209 ± 46
T_{nadir} (h)	2	5	5	5
glucose AUC_{0-8h} (mg/dL·h)	1012 ± 101	2124 ± 201	1799 ± 232	1381 ± 334
insulin C_{max} (μU/mL)		48.5 ± 11.0	60.0 ± 25.0	130.1 ± 83.0
insulin AUC_{0-10h} (μU/mL·h)	285.0 ± 88.3	114 ± 22	307 ± 111	448 ± 157

[a] Data are expressed as means ± SD. G_{nadir}, minimum blood glucose levels; T_{nadir}, time at the lowest blood glucose levels; insulin C_{max}, maximum human insulin concentration in the plasma; AUC, area under curve.

dependency with orally administered insulin/DCK doses. In addition, the insulin AUC_{0-10h} (area under curve) values of DCK/insulin were 114 ± 22, 307 ± 111 and 445 ± 152 μU/mL·h for 21, 42, and 81 IU/kg, respectively. The glucodynamic and pharmacokinetic parameters are summarized in Table 2.

We next carried out the oral glucose tolerance test 4 h after administration of the capsule having insulin/DCK complex in diabetic canines (Figure 3). When glucose (0.5 g/kg) was orally administered to the placebo-treated diabetic canines, their blood glucose levels were rapidly increased to 560 mg/dL and maintained at over 400 mg/dL. In contrast, the blood glucose levels in the group treated with the insulin/DCK in a dose of 81 IU/kg were increased slightly and were maintained at approximately 200 mg/dL. Glucose AUC_{0-6h} for nondiabetic normal canines, placebo-treated diabetic and insulin/DCK treated diabetic was 530 ± 26, 2604 ± 236 and 1453 ± 329, respectively (Figure 3B). When compared with that of diabetic canines, insulin/DCK could significantly reduce the blood glucose increment (~44%). From these results, we found that insulin/DCK could attenuate blood glucose in the diabetic canines and still normally control the blood glucose level despite glucose intake.

Reproducibility of Glucose-Lowering Effect of Insulin/DCK in Diabetic Canines. In order to ensure the usefulness of insulin/DCK as a potent oral medication, we carried out a study in multiple oral administrations, as would be needed for a diabetes regimen. The capsule having insulin/DCK complex (42 IU/kg) was orally administered once a day to diabetic canines for 3 days. Figure 4 shows the blood glucose level−time profiles following multiple oral administrations to diabetic canines. The blood glucose levels of the group treated with placebo were never reduced for 3 days, whereas those of the group treated with the capsule having insulin/DCK were significantly reduced and reproducible. For each of the first, second and third administration of insulin/DCK, the reduction of each relative blood glucose level was approximately 30, 27 and 40%, respectively.

Discussion

We report here the physicochemical characterization of orally absorbable insulin/DCK complex, which was physically engineered using ion-pair interaction, and show its therapeutic efficacy in total pancreatectomized diabetic canines after oral administration. Using deoxycholate-based molecules, we developed a new delivery agent that physically interacts with insulin to enable its oral absorption. The synthetic DCK is a positively charged deoxycholate derivative, which is a chemical conjugate of the carboxyl group of deoxycholate and the primary amine group of lysine serving as positive charge, to make a complex with insulin by electrostatic interaction when mixed with insulin in aqueous solution.

In general, a deoxycholate synthesized from cholesterol in the liver is an endogenous substance consisting of a facially amphiphilic steroid, a hydrophobic α-side and a hydrophilic β-side. It is carried via bile duct to the small intestine, from where it returns to the liver by reabsorption through the bile acid transporter. Recently, however, deoxycholates have been widely used in the drug delivery system due to its amphiphilicity and reabsorption properties. In the present study, we developed an orally absorbable insulin/DCK complex by employing the properties of deoxycholate. Our hypothesis is that the hydrophobic part of deoxycholate can be helpful for insulin in penetrating through the intestinal membrane, and the stability of insulin can be protected against the digestive enzymes during cruising the esophagus, stomach and gastrointestinal tract. In addition, the deoxycholate can be recognized by the bile acid transporter, thereby increasing the retention time of insulin in the intestine for making a higher concentration gradient of insulin across the intestinal wall. Actually, when mixed with insulin in aqueous solution, the synthesized DCK readily complexes with insulin, and the lipophilicity ($P_{MC/W}$) and apparent permeability (P_{app}) of the insulin/DCK were also significantly increased. The insulin/DCK complex could attenuate the blood glucose level of the diabetic canines after oral administration.

The plot profile of CD spectra of native insulin or insulin/DCK complex was compared. Despite the increased coupling ratios of DCK, the plot peaks of mean residue ellipticity of insulin were observed around 275 nm and their peak shifts were not significantly altered either, suggesting that the folding structure of insulin was maintained even when bound to DCK. In addition, the peak value of mean residue ellipticity of insulin was significantly reduced at the coupling ratios of 1:10 or 1:15, indicating the different tendencies of hexameric insulin formation. In other words, when insulin forms a complex with DCK, the general hexameric form of insulin is apt to be dissociated to active monomeric insulin. In addition, the insulin/DCK could have a higher digestive enzyme resistance when compared to hexameric insulin. The

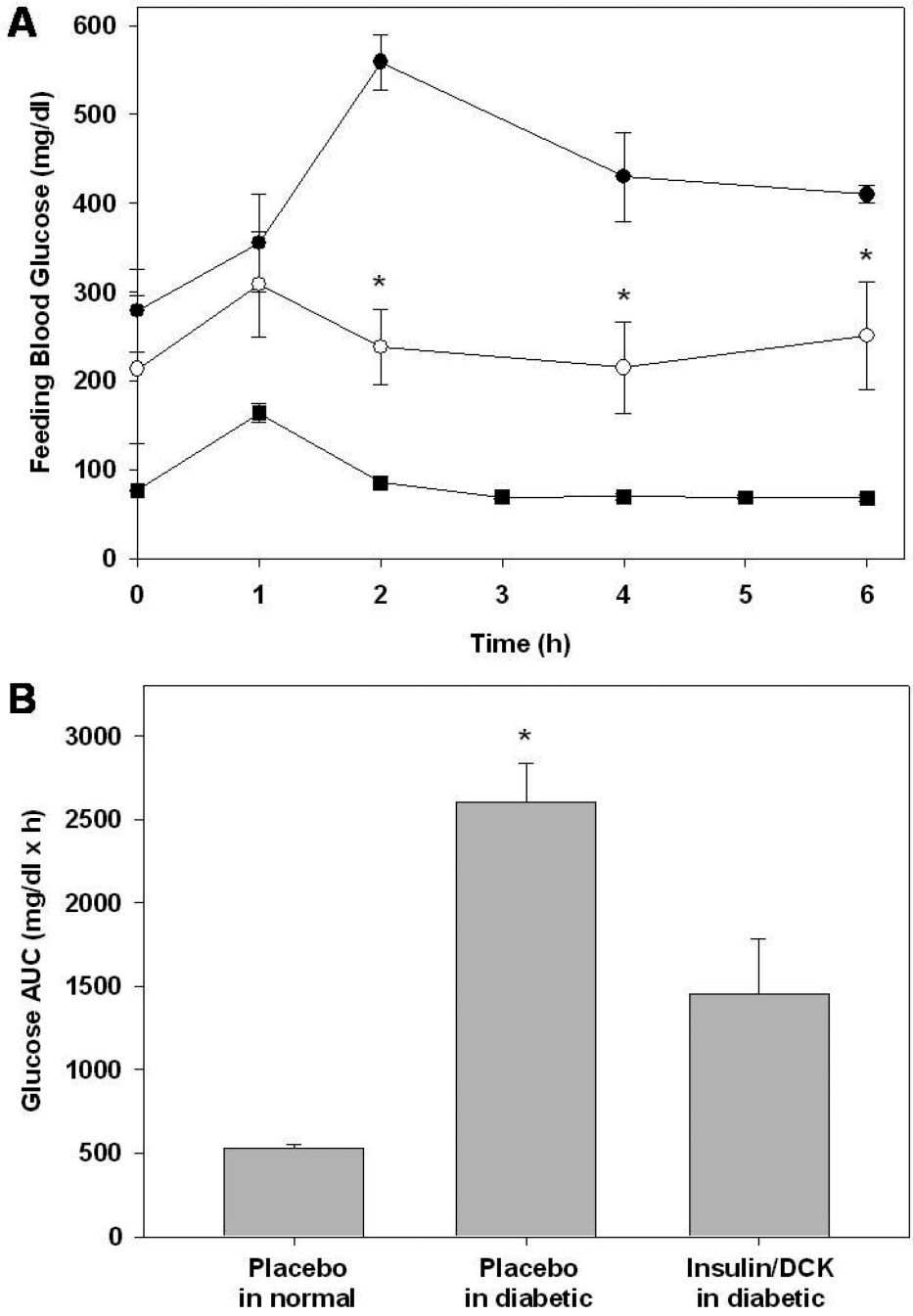

Figure 3. (A) Oral glucose tolerance test (OGTT) curves after single oral administration of placebo (PBS; black circles) or 81 IU/kg of insulin/DCK (1:10 ratio; white circles) to pancreatectomized diabetic canines. As a control, OGTT was also carried out to the nondiabetic normal canines (black squares). Four hours after postadministration of placebo or insulin/DCK orally, 0.5 g/kg of glucose in PBS was orally administered into the diabetic canines. The data was plotted as the mean ± SD, $n = 3$. *$P < 0.05$ vs diabetic canines at each time point. (B) Area under curve (AUC) values of placebo-treated nondiabetic normal canines, placebo-treated diabetic canines or insulin/DCK-treated diabetic canines 6 h after OGTT. *$P < 0.05$ vs normal and insulin/DCK-treated canines.

reason would be a less accessibility of digestive enzymes to insulin due to the bound DCK. On the other hand, the partition coefficient and apparent permeability of the complex were increased but again attenuated above the coupling ratio of 1:15, meaning decrement of forming complexation above 1:15 ratio. This was attributed to the fact that an excess amount of DCK, being a surfactant, would form into self-aggregated particles without insulin or with insulin being entrapped into the hydrophilic cores due to the lysine residue of DCK.[29] Therefore, it is possible that the therapeutic efficacy of insulin might be worse when complexed with an excess amount of DCK.

When we designed this insulin/DCK, it was envisaged that DCK would more easily interact with bile acid transporters

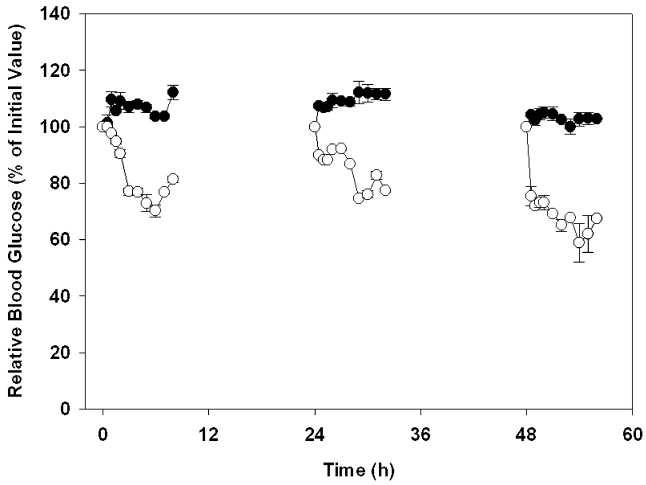

Figure 4. Fluctuation profiles of blood glucose level versus time in diabetic canines after triple oral administration of placebo (PBS; black circle) or insulin/DCK complex (42 IU/kg, 1:10 ratio; white circle). Data were plotted as mean ± SD, $n = 3$.

expressed on the intestinal membrane and that this would increase the absorption of insulin into the intestinal membrane. In our previous studies, we demonstrated that oral absorption of deoxycholate-conjugated heparin occurred mainly in the jejunum and ileum of small intestine, especially in the ileum because bile acid transporters are highly expressed in this region.[26,28,30] In addition, we also experimentally demonstrated the direct interaction between a deoxycholate-conjugated heparin complex and the ileal brush border membrane by using surface plasmon resonance technique.[31] In view of these findings, we can expect that insulin/DCK has sufficient potential to be absorbed through the intestinal membrane because it has a long retention time in the gastrointestinal tract by interacting with bile acid transporters, although we here did not include the direct mechanism study for insulin/DCK.

On the other hand, in the recent literature, it was reported that a semisynthetic bile acid salt, sodium 3α,7α-dihydroxy-12-oxo-5β-cholanate (MKC), could have hypoglycemic activity and synergistic effect with gliclazide pharmacokinetics in healthy and diabetic rats.[32,33] After an oral dose of MKC (4 mg/kg), the decrease in blood glucose concentration was significant, reaching a maximum decrease of 24% from the baseline in healthy rats and 15% in diabetic rats. However, we previously showed that the oral administration of DCK alone (3 mg/kg) did not have the glucose-lowering effect and increased the plasma insulin level.[27] Even though MKC and DCK were made from similar bile acids, namely, cholate and deoxycholate, it is possible that their pharmacological effects can be different due to their metabolite and/or interference with metabolism and glucose transport.

In this study, we orally administered Eudragit-coated gelatin capsules loaded with the powder of insulin/DCK into diabetic canines. After oral administration of these complexes to diabetic canines, the blood glucose levels in the animals began to be corrected within 2 h, with the minimum blood glucose levels (G_{nadir}) observed around 5 h. In the case of oral administration of 42 or 81 IU/kg of insulin/DCK, its therapeutic effect was lengthened up to 8 h. Simultaneously with the blood glucose level change, the plasma human insulin profiles in the canines were also highly increased around 2 to 4 h. From these results, we found that the oral insulin/DCK complex has a delayed therapeutic effect after its oral administration. This might be attributed to the delayed dissolution of capsules having insulin/DCK in the gastrointestinal environment, dissolution of the complex in the bloodstream or late dissociation of the complex. In our previous studies, however, we demonstrated that the aqueous formulation of this bile acid-based physical complexation of macromolecules such as insulin, heparin and ceftriaxone medications could at least rapidly act in a murine model within 30 min.[25–27] Therefore, it is possible that the therapeutic efficacy of oral insulin/DCK can be controlled by differently formulating the insulin/DCK complex.

To evaluate the regulation of postprandial glycemia by insulin/DCK, we carried out the OGTT to diabetic canines 4 h after the administration of the capsule containing insulin/DCK. The orally absorbed insulin/DCK could superbly adjust the blood glucose levels of diabetic canines when compared with the placebo group. However, the fluctuation pattern of blood glucose levels was not completely similar to that of nondiabetic normal canines. This result might be related to the elimination of insulin in the plasma 4 h after administration, as shown in Figure 2B. On the other hand, from the vantage point of long-term-treating therapeutic oral medications, their susceptibility and reproducibility are important elements. Here, we show good reproducibility of the complex after triple oral administration of insulin/DCK in diabetic canines.

(29) Bromberg, L. E.; Klibanov, A. M. Detergent-enabled transport of proteins and nucleic acids through hydrophobic solvents. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 143–147.

(30) Lee, Y.; Kim, S. K.; Lee, D. Y.; Lee, S.; Kim, C. Y.; Shin, H. C.; Moon, H. T.; Byun, Y. Efficacy of orally active chemical conjugate of low molecular weight heparin and deoxycholic acid in rats, mice and monkeys. *J. Controlled Release* **2006**, *111*, 290–298.

(31) Kim, S. K.; Kim, K.; Lee, S.; Park, K.; Park, J. H.; Kwon, I. C.; Choi, K.; Kim, C. Y.; Byun, Y. Evaluation of absorption of heparin-DOCA conjugate on the intestinal wall using a surface Plasmon resonance. *J. Pharm. Biomed. Anal.* **2005**, *39*, 861–870.

(32) Mikov, M.; Boni, N. S.; Al-Salami, H.; Kuhajda, K.; Kevresan, S.; Golocorbin-Kon, S.; Fawcett, J. P. Bioactivity and hypoglycemic activity of the semisynthetic bile acid salt, sodium 3alpha,7alpha-dihydroxy-12-oxo-5beta-cholanate, in healthy and diabetic rats. *Eur. J. Drug Metab. Pharmacokinet.* **2007**, *32*, 7–12.

(33) Mikov, M.; Al-Salami, H.; Golocorbin-Kon, S.; Skrbic, R.; Raskovic, A.; Fawcett, J. P. The influence of 3alpha,7alpha-dihyroxy-12-keto-5beta-cholanate on gliclazide pharmacokinetics and glucose levels in a rat model of diabetes. *Eur. J. Drug Metab. Pharmacokinet.* **2008**, *33*, 137–142.

Therefore, these results suggest that orally treated insulin/DCK can be used for chronic administration in clinical trials as an effective therapeutic medication.

Collectively, the therapeutic efficacy of the newly developed oral insulin/DCK was attributed to the deoxycholate-derived lipophilicity and digestive enzyme resistance, with no chemical modification of insulin as well. Interacting with bile acid transporters in the intestine wall might also enhance its therapeutic effect. It is expected that this new formulation can contribute to an oral therapeutic remedy alternative to injectable insulin, thereby significantly improving patient acceptability and therapeutic effects.

Acknowledgment. This study was supported by a grant from the Next Generation New Technology Development Program (grant no. 10011353) of the Korean Ministry of Commerce, Industry, and Energy (MOCIE), and supported by the World Class University (WCU) program (Grant No. R31-2008-000-10103) and the Converging Research Center Program (2009-0081879) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology, Republic of Korea. The authors affirm that there are no conflicts of interest.

MP9002688