Cyclodextrins as Mucosal Absorption Promoters of Insulin. II. Effects of β-Cyclodextrin Derivatives on α-Chymotryptic Degradation and Enteral Absorption of Insulin in Rats

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The relative effectiveness of two β-cyclodextrin derivatives, i.e., dimethyl-β-cyclodextrin (DMβCD) and hydroxypropyl-β-cyclodextrin (HPβCD), in enhancing enteral absorption of insulin was evaluated in the lower jejunal/upper ileal segments of the rat by means of an in situ closed loop method. The incorporation of 10% (w/v) DMβCD to a 0.5 mg/ml porcine zinc insulin solution dramatically increased insulin bioavailability from a negligible value (≈0.06%) to 5.63%, when administered enteraally at a dose of 20 U/kg. However, addition of 10% (w/v) HPβCD did not improve enteral insulin uptake significantly with a bioavailability of only 0.07%. Similarly, the pharmacodynamic relative efficacy values obtained after the enteral administration of 20 U/kg insulin, 20 U/kg insulin with 10% HPβCD, and 20 U/kg insulin with 10% DMβCD were 0.24%, 0.26%, and 1.75%, respectively. Biodegradation studies of 0.5 mg/ml insulin hexamers by 0.5 µM α-chymotrypsin revealed no inhibitory effect on the enzymatic activity by the two cyclodextrins. On the contrary, the apparent first-order rate constant increased significantly in the presence of 10% DMβCD, suggesting insulin oligomer dissociation by DMβCD. Histopathological examination of the rat intestine was performed to detect tissue damage following enteral administration of the β-cyclodextrin derivatives. Light microscopic inspection indicated no observable tissue damage, thereby arguing direct membrane fluidization as the primary mechanism for enhanced insulin uptake. This study indicates the feasibility of using cyclodextrins as mucosal absorption promoters of proteins and peptide drugs.

KEY WORDS: biodegradation; stability; α-chymotrypsin; cyclodextrins; enteral absorption; histology; insulin.

INTRODUCTION

For noninvasive delivery of insulin, a polypeptide with a molecular weight of 5.7 KD, the oral pathway remains attractive because of direct input of mesenterically absorbed dose to the liver via the portal vein, similar to normal physiologic release and uptake of insulin from the pancreas. However, insulin bioavailability is limited by oligomer formation above 0.1 µM concentration in aqueous solution (1); degradation by luminal and cellular peptides (2,3); and hydrophilicity restricting partitioning across biological membrane barriers (4,5).

In order to improve enteral insulin bioavailability, protease inhibitors, targeted enteral delivery, and facilitated transport by absorption enhancers have been tested (6,7). The application of cyclodextrin derivatives as mucosal drug absorption promoters has been recognized recently (8–13). Physicochemical properties of cyclodextrins enhancing the formulation of macromolecules include solubilization by encapsulation of hydrophobic amino acid side chains to minimize aggregation and polymerization and low permeability through the membrane bilayer thus affording high tissue compatibility.

The relative effectiveness of various cyclodextrins and their derivatives in promoting insulin absorption across the nasal, pulmonary, and rectal mucosa has been studied (8–13). This article characterizes the mechanism and efficacy of cyclodextrins as mucosal insulin absorption enhancers. Two β-cyclodextrin derivatives, i.e., dimethyl-β-cyclodextrin (DMβCD) and hydroxypropyl-β-cyclodextrin (HPβCD), were examined with respect to their effects on insulin degradation by α-chymotrypsin, enteral absorption, and membrane effects. Probable mechanisms of insulin absorption enhancement are further elucidated.

MATERIALS AND METHODS

Materials

Crystalline porcine-zinc insulin (lot #504JR8, potency 26.3 U/mg) was donated by Eli Lilly and Company (Indianapolis, IN). Lysophilized α-chymotrypsin prepared from bovine pancreas (56 units/mg protein) was purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile (HPLC grade) was obtained from Baxter Health care Corporation (Muskegon, MI). Phosphoric acid and triethylamine were obtained from Fisher Scientific (Fairlawn, NJ). Trifluoroacetic acid (TFA), tris(hydroxymethyl)-aminomethane (Tris), and dimethyl-β-cyclodextrin (DMβCD) were procured from Sigma Chemical Co. Hydroxypropyl-β-cyclodextrin (HPβCD) was donated by Pharmatec, Inc. (Alachua, FL). Deionized double-distilled water was used throughout the study. All other chemicals were of analytical reagent grade and were used as received.

HPLC Analysis of Insulin

Insulin analysis was performed on a computer controlled gradient high-pressure liquid chromatographic (HPLC) system (Rainin Instruments, Woburn, MA) equipped with a variable-wavelength ultraviolet/visible detector (Knauer, Germany). The gradient system used in this study consisted of mobile phase A, triethylammonium phosphate (TEAP) solution prepared by adjusting the pH of 0.25 N phosphoric acid to 2.25 with triethylamine, and mobile phase B, 100% acetonitrile. The gradient system was programmed by increasing the proportion of mobile phase B from 26% to 35% over 16 min. Twenty microliters of the sample was injected onto a Rainin reversed-phase C8 Microsorb column (250 x 4.6 mm) connected to a C8 precolumn. The gradient mobile phase was run at a flow rate of 1 ml/min. The ultraviolet/visible detector was set at 220 nm;
the recorded signal was analyzed with an electronic integrator (model 3390 A, Hewlett-Packard Co., Avondale, PA). The chromatographic method as described previously (14) provides baseline separation of insulin from its enzymic degradation products.

α-Chymotryptic Degradation of Porcine-Zinc Insulin

Ten milliliters of 0.5 mg/ml porcine-zinc insulin solution was prepared in a buffer composed of 100 mM Tris and 1 mM CaCl$_2$ adjusted to pH 8.0 at which the enzyme assumes high catalytic activity. The solution was pre-equilibrated at 37°C for 15 min. Just prior to the addition of the enzyme, the solution was vortexed for 2 seconds and a 100 μl sample was immediately taken as the zero time sample. Then 50 μl of enzyme stock solution was added to the insulin solution to generate a final enzyme concentration of 0.5 μM. Aliquots (100 μl) were withdrawn at 1, 2, 5, 10, 15, and 20 min and immediately added to 0.9 ml of 0.2% TFA solution to arrest the reaction. The samples were subsequently stored in a freezer at −20°C until HPLC analyses were performed. Studies were performed in triplicate. This procedure was used throughout the enzymatic degradation study. The cyclodextrin derivative was added to the Tris buffer solution and sonicated for 5 minutes at room temperature prior to the addition of enzyme.

Preparation of Insulin Solutions for Enteral Administration

Crystalline porcine-zinc insulin was dissolved in a few drops of 0.1 N HCl in order to facilitate its solubilization. Diluted phosphate buffered saline, 0.01 M., at pH 7.4 was then added to generate a final insulin concentration of 0.5 mg/ml. The solution was made just prior to use and the cyclodextrin (10%, w/v) was then added. The pH of the final solution was again measured and adjusted to pH 7.4 if necessary.

Enteral Absorption with Closed Loop Technique

Male Sprague-Dawley rats weighing 175–250 g were fasted for 16–20 hours prior to an experiment. Water was allowed ad libitum. The animals were anesthetized by an intraperitoneal injection of a mixture of 90 mg/kg ketamine and 10 mg/kg xylazine. One-third to one-half of the original dose was administered every 45–60 minutes thereafter to maintain anesthesia/analgesia. The core body temperature was maintained close to 37°C by placing the animal on a platform above a 40°C water bath with a 100-watt light bulb and a reflector above.

Cannulation of the right external jugular vein was performed by inserting a 3-inch piece of Silastic® tubing, 0.047 inch O.D. (Dow Corning, Midland, MI). A collar made from a 1-cm piece of PE 200 polyethylene tubing (Becton Dickenson, Parsippany, NJ) was attached to the outer end of the Silastic® tubing. Before insertion, the cannula was filled with saline containing 2 U/ml heparin. Microdissecting scissors were used to cut a small opening in the jugular vein, and one tip of a micro-dissecting forceps, extra delicate, was inserted through the hole to guide the cannula towards the heart. Surgical thread underneath the vein was tied around the collar of the cannula to secure it. A 23-gauge needle with the bevel removed was inserted into the cannula and was attached to a heparinized 1-ml plastic syringe for the removal of blood samples. The sampling times were 0, 15, 30, 60, 90, 120, 150, 180, 210, and 240 minutes following insulin administration.

A mid-abdominal incision was made to expose the small intestine. The distal jejunum/proximal ileum segment with a length of 15 cm, beginning 16 cm above the cecum, was used in this study because a previous report from this laboratory has shown this segment to have higher insulin permeability (5). The segment was washed by perfusing pre-warmed (37°C) normal saline through the lumen via a peristaltic pump (Model 1203, Harvard Apparatus, Millis, MA) to remove any residual gut contents. A total of 30 ml saline was circulated at a rate of 3 ml/min. The segment was then carefully ligated both above and below the incisions to prevent any fluid loss. The distal end of the segment was ligated and appropriate insulin solution (approximately 0.3 ml) was instilled to generate a final insulin dose of 20 U/kg. The concentration of insulin solutions employed was 0.5 mg/ml, or 13.15 U/ml. Finally, the proximal end of the intestinal segment was quickly ligated to form a closed sac which was carefully returned back to its original position inside the peritoneal cavity.

Measurement of Blood Glucose and Plasma Insulin

Blood samples were immediately examined for glucose levels using Chemstrip B® reagent test strips (Boehringer Mannheim Diagnostics, Indianapolis, IN) with an AccuCheck II® blood glucose monitor (Boehringer Mannheim Diagnostics). The sample size consisted approximately of 30 μl whole blood. The precision of the assay was found to be within ±3% and measurable glucose levels ranged within 10 to 300 mg/dl.

The collected whole blood was transferred into a heparinized Natelson® capillary tube (Scientific Products, McGaw Park, IL). It was then centrifuged in a Damon/IEC® CRU-5000 centrifuge for 15 min at 2,500 rpm. The plasma was collected and insulin concentration assayed by a radioimmunoassay procedure using Coat-A-Count® kits purchased from Diagnostic Products Corporation (Los Angeles, CA).

Pharmacokinetics and Pharmacodynamics

Plasma insulin concentrations were normalized by subtracting the endogenous insulin level at time zero. The areas under the plasma insulin concentration curves (AUCs), areas under the first moment curves (AUMCs), and mean absorption times (MATs) were then calculated using Rstrip® software ver. 4.02 (MicroMath Scientific Software, Salt Lake City, Utah) by extrapolating time to infinity. Intravenous injection of 0.2 U/kg insulin was performed through the tail vein and blood samples were collected at 0, 0.5, 2, 4, 6, 10, 15, 20, and 30 min post administration. The absolute bioavailability (F) following enteral administration of insulin in the absence and presence of cyclodextrins was estimated using the following equation:

$$ F = \frac{\text{AUC}_{0-\infty \text{ external}}}{\text{AUC}_{0-\infty \text{ i.v.}}} \times \frac{\text{Dose}_{\text{i.v.}}}{\text{Dose}_{\text{enteral}}} $$

(1)