Regional Differences in Intestinal Spreading and pH Recovery and the Impact on Salmon Calcitonin Absorption in Dogs

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Purpose. To investigate the regional influence of intestinal spreading and pH recovery on the performance of drug and excipient delivery systems and their impact on the oral absorption of a model peptide drug, salmon calcitonin (sCT), in conscious beagle dogs.

Methods. Male beagle dogs were surgically prepared with subdermal Intestinal Access Ports (IAP). The catheter from one port was placed in the duodenum and the other in the ileum. Fluoroscopy and Heidelberg pH capsule studies were performed to characterize intestinal spreading and pH recovery, respectively. Three treatments were performed: (1) a radiopaque dye and citric acid (CA) were infused into the intestinal segments, (2) a radiopaque powder capsule containing CA was given orally, and (3) capsules containing CA and sCT were given orally. Regular blood samples were collected and analyzed by radioimmunoassay (RIA) to determine the absorption characteristics of sCT.

Results. Since sCT is an excellent substrate for the pancreatic serine protease trypsin, the rate of degradation of sCT in the GI lumen is dependent upon the regional pH, activity of digestive enzymes and the concentration of sCT at the site of absorption. Fluoroscopy results clearly showed that when the radiopaque dye was infused into the duodenum and capsule disintegration occurred early, there was significant dilution and spreading of the excipients throughout a large section of the upper small intestine (USI). However, when the radiopaque dye was infused into the ileum and capsule disintegration occurred in the lower small intestine (LSI), the excipients moved along as a bolus (i.e., plug). The pH monitoring results were consistent with the fluoroscopy results. The pH dropped only momentarily and rose quickly in the USI consistent with well-stirred mixing kinetics. In the LSI, dilution and spreading were minimal and the drop in pH was greater and persisted for a longer period of time. Plasma levels of sCT were maximal when disintegration occurred in the LSI.

Conclusions. Since significantly less dilution and spreading occurred in the LSI, the exposure of the intestine to pharmaceutical excipients and sCT was more concentrated resulting in a higher fraction of sCT absorbed. The results of this study demonstrate that intestinal mixing kinetics have a dramatic impact on the ability of pharmaceutical excipients to modulate the oral bioavailability of peptide drugs like sCT.

KEY WORDS: intestinal motility; regional pH; oral absorption; peptide drugs; salmon calcitonin; and IAP dogs.

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INTRODUCTION

Establishing an oral delivery system for peptides and protein drugs is of great importance. During chronic treatment regimens parenteral administration results in poor patient compliance ultimately limiting clinical utility. The clinical development of orally administered peptide drugs, however, has been impeded due to poor absorption across intestinal membranes and rapid proteolytic degradation which typically results in oral bioavailabilities of less than 1–2% (1–3). There are several difficulties associated with delivering peptide drugs in an oral formulation. Although gastric acid and enzymes efficiently degrade peptides, these effects can be minimized by using an enteric coated dosage form to bypass the stomach. Unfortunately, the proteolytic enzymes in the small intestine (SI) are equally proficient at degrading peptide drugs (4–6). Potential approaches to limit the activity of intestinal enzymes include delivery of protease inhibitors, adjusting the pH of the intestinal contents, and maintaining high local drug concentrations (7–9). The successful oral delivery of peptide drugs also depends on regiospecific targeting because of regional differences in the activity of intestinal proteolytic enzymes (6) and intestinal spreading and dilution patterns (10). As a consequence, the oral absorption of peptide drugs may be enhanced by targeting specific regional locations and by manipulating local drug concentrations and pH. Therefore, it is essential to understand if the intestinal microenvironment can be temporarily modified to enhance drug delivery and, if so, to understand how the upper and lower SI respond to changes induced by excipients. Even though calcitonin is an excellent candidate for the development of alternate delivery routes due to its size and wide therapeutic index (11), the preferred route of delivery is oral. Since sCT is an excellent substrate for the pancreatic serine protease trypsin, intestinal stabilization is required for its oral delivery. In our previous report (12), it was demonstrated that the absorption of sCT from the ileum was better than the duodenum and the inclusion of CA significantly improved the oral absorption of sCT in various intestinal regions in IVAP dogs. In this report, radiotelemetric measurements of intestinal pH, radiographic visualization of intestinal flow, and pharmacokinetics were used to investigate how the upper and lower SI respond to pH changes. The results demonstrate the significant impact of regional intestinal dilution and spreading on the oral absorption of a model peptide drug sCT.

MATERIALS AND METHODS

Materials

Recombinant salmon calcitonin (sCT) was obtained from Unigene Laboratories, Fairfield, NJ. sCT antibody (cross reacts less than 1% with mammalian calcitonins) and ^{125}Iodotyrosyl-salmon calcitonin were obtained from Advanced Chem Tech (Louisville, KY). Eudragit L30-D55 was obtained from Huls America Inc. (Sommerset, NJ). Surgical thread, 3-0 vicryl was obtained from Ethicon (Somerville, NJ). Surgical adhesive, Krazy glue was obtained from Berden Inc. (Columbus, OH). IV catheter, 20G Abbocath was obtained from Abbott Labs (North Chicago, IL). Heparinized syringe, Monovette® was obtained from Sarstedt (Newton, NC). The Heidelberg radiotelemetry instrument and the Heidelberg capsules were purchased

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from the Heidelberg International Corporation (Atlanta, GA). The 3 channel mono-crystal antimony pH catheter and portable recorder were purchased from Medtronic Synectics (Shoreview, MN). Intestinal Access Ports (IAP) and 22G Huber needles were obtained from Access Technologies (Skokie, IL). Optiray 320 radiopaque dye was obtained from Mallinckrodt Medical (St. Louis, MO). Dog slings were obtained from Alice King Chatham Medical Arts (Hawthorne, CA). All other materials were obtained from Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical (St. Louis, MO) and were used as received.

Animals

Male beagle dogs weighing 10 to 15 kg were used and fasted over night prior to the study. Water was allowed *ad libitum*. All animal studies were performed under approved protocols (IRB-UCA, Rutgers University and IACUC, University of Medicine and Dentistry of New Jersey) in AAALAC accredited facilities.

Oral Formulations

Enteric coated capsules were manufactured by Unigene Laboratories, Fairfield, NJ (12) and evaluated in these studies. Hard gelatin capsules (#0) containing 1.2 mg sCT, 565 mg CA, and other excipients were coated with approximately 12% (by weight) Eudragit L30-D55 to prevent gastric disintegradation. Enteric coated capsules containing 250 mg of CA and 500 mg of barium sulfate (BS) powder were also prepared with Eudragit L30-D55 in the same way.

Fluoroscopy

Radiographic visualization of gastrointestinal flow patterns was enhanced by the administration of contrast agents. Optiray 320 radiopaque dye or BS are commonly used contrast agents for gastrointestinal contrast radiography. The radiographic techniques use auto voltage adjust in the fluoroscopy mode to maintain the output of the image intensifier at 1 mA (Siemens Medical Systems, Inc., Hoffman Estates, IL). Image intensification fluoroscopy with a television monitor was used to view the segmental contractions. These were recorded on videotape for later analysis.

pH Measuring System

Continuous determination of pH with time was accomplished using a radiotelemetric device, the Heidelberg capsule (HC) (13–15). The device consists of a battery-operated high frequency radio transmitter and a pH electrode housed in a nondigestible acrylic capsule 7 mm in diameter and 20 mm in length. The dogs wear an antenna strapped around the body to receive the radio signal, which is then recorded. The capsule battery was activated with normal saline and calibrated in pH 1 and pH 7 buffer solutions maintained at 37°C. The HC was then tethered to drug capsule using surgical thread (3-0 vicryl) and given orally to dogs. The upper cap of enteric drug capsule was first attached to a 3-0 silk suture with Krazy glue and dried overnight. In the next morning, it was tethered to HC with a suture. Because pH values change with location within the gut and the drug capsule disintegration, alterations in pH should be indicative of the movement of the HC-drug capsule through the different segments until the drug capsule dissolves. Generally, the HC provides readings within ± 0.5 pH unit accuracy with excellent *in vivo* reproducibility in the pH range of 1 to 8 for 22 hr after activation (16).

IAP Dog Studies

Surgery

Male beagle dogs were surgically fitted with IAP. A detailed description of the surgical procedure was given elsewhere (12). Briefly, the ports were implanted along the spine, in the subcutaneous space behind the shoulder blades. Two catheters were tunneled under the skin and through the abdominal wall. The distal end of each catheter was implanted into different portions of the intestinal tract. The first was 10 cm distal to the pyloric sphincter in the duodenum and the second port was placed in the lower third of the small intestine (ileum). The animals were allowed to recover for at least two weeks prior to the initiation of the studies. The ports for IAP infusions were accessed transcutaneously with a 22G Huber needle. The IAP dogs were reused once every 1 or 2 weeks.

Fluoroscopy Study

Three mL of Optiray 320 radiopaque dye was infused into either the duodenum or ileum and contrast radiography was performed to trace intestinal spreading of the radiopaque dye solution for the first 5 min. Momentary recordings were made periodically through the next hour.

Acute pH Study

When an animal was to be euthanized, an acute pH study was performed under general anesthesia with IV administration of 15–20 mg/kg of pentobarbital sodium. The animal was mechanically ventilated with 50% O_2 and 50% air, and anesthesia was maintained by titration with additional pentobarbital sodium. A vertical midline incision was made through the skin and Linea Alba, and the abdominal cavity was entered. A specially designed 3 channel mono-crystal antimony pH catheter (Digitrapper MKIII) was surgically placed in the duodenum or ileum so that the middle sensor (Ch 2) was at the point of infusion, the proximal sensor (Ch 1) was 15 cm proximal (just distal to the pyloric splincter in the case of duodenum), and the distal sensor (Ch 3) was 15 cm distal to the point of infusion. The catheter was attached to a portable recorder (Digitrapper MKIII) which recorded the pH throughout the tests, and the abdominal incision was closed. After allowing the intestine to recover and stabilize for about half an hour, 3 mL of 1 M CA was infused into the ports.

Fluoroscopy Study with Heidelberg Capsule

Enteric coated capsules containing 250 mg of CA and 500 mg of BS powder were tethered to a HC with a 3-0 silk suture and given orally with 10 mL water. Contrast radiography was performed to trace intestinal spreading of radiopaque particles. BS gave a good view of the bowel lumen as long as it was not significantly diluted. Through the belt antenna, the Heidelberg pH capsule machine recorded GI pH. Since the fasted dog has an average small intestinal transit time of 111 ± 17 min (17),

the delay after gastric emptying was used to estimate the location where disintegration takes place. Disintegration that occurs between 10 and 60 min was in the jejunum and after 75 min was an ileal exposure. Due to the vigorous contractions of the duodenum upon gastric emptying, duodenal disintegration is difficult to achieve. In order to observe this, the capsules were tethered with a long suture tied to a collar around the animal's neck in order to hold them in the duodenum until disintegration began.

Normal Dog Studies

Oral Absorption Study with Heidelberg Capsule

An enteric coated capsule containing 565 mg of CA and 1.2 mg of sCT was tethered to a HC with a 3-0 silk suture and given orally to normal dogs with 10 ml water. Blood samples were drawn through a 20G IV catheter with a heparin lock, which was inserted in the brachial vein. The catheter was flushed with heparinized saline (50 units per mL) after each blood draw. Two baseline samples were drawn prior to dosing and another immediately following gastric emptying. From the time the capsules enter the SI, blood samples were taken every 10 min. When the HC showed a drop in pH indicating the disintegration of the test capsule, more frequent blood sampling was performed (3, 6, 9, 12, 15, 20, 30, 45, 60, 75, 90, 120, 150, and 180 min). No more than 25×3 ml samples were taken during a particular study and hematocrit levels were monitored. Transit time was used to estimate the SI location being monitored. The dogs were reused once every 1 or 2 weeks.

sCT Analysis

The concentration of sCT in dog plasma was determined by competitive RIA using sCT antibody and ^{125}Iodotyrosyl-salmon calcitonin as previously described (12,18). The assay was accurate and reproducible over the concentration range of 100–1500 pg/mL of sCT. The lower limit of detection of the assay was 50 pg/mL. Interday coefficients of variation was 7–19% and intraday coefficients of variation was 7–24%. The assay was highly specific with less than 1% cross-reactivity with calcitonin tryptic fragments (19).

Pharmacokinetic Analysis

Plasma concentration-time data were analyzed by non-compartmental pharmacokinetic method (20). The highest observed concentration was defined as C_{max} and the area under the concentration time curve (AUC) was calculated by a trapezoidal method. Gastric emptying (GE) was characterized by the time at which the HC emptied from the stomach (i.e., as indicated by a sharp pH increase from acidic to neutral).

Data Analysis

Among the data of 3–4 replicates, typical fluoroscopy, pH tracing, or plasma sCT concentration are shown in the figures.

RESULTS AND DISCUSSION

To examine the influence of regional intestinal dilution and spreading on peptide bioavailability, preliminary studies were performed in dogs to optimize experimental conditions. Using fluoroscopy, an initial attempt to visualize intestinal motility included infusing 3 mL of 1 M CA with 5% BS into IAP dogs. However, the results of this protocol were unsatisfactory because it was not sufficiently radiopaque resulting in poor visualization of intestinal motility. Optiray 320 radiopaque dye was then used with 1 M CA. Three milliliters was infused into the duodenal port while simultaneously recording the pH of the intestinal lumen contents with a HC. Since the solution quickly dispersed throughout several loops of the USI within 5 min, the correlation with the HC results was no longer useful, however, x-ray images were successfully captured. Still photographs taken from the fluoroscopy video are shown in Fig. 1. In the duodenal infusion, the two frames marked 2 min 30 sec represent a one-second-time lapse demonstrating the capacity of duodenal bulb contractions to cause rapid and thorough mixing of the intestinal contents. After 6 min 30 sec, the dye was significantly diluted and widely spread as to make it difficult to visualize one loop of intestine from another. In the ileum infusion, the initial contractions appeared to mix the infusate but then it traveled without significant spreading. Even after 20 min, most of the dye was contained in a single loop of the LSI (i.e., dilution and spreading were minimal). This result is consistent with a report demonstrating the reduced frequency and velocity of contractions in the LSI (10).

Acute pH studies were performed prior to the euthanization of an animal. Antimony pH sensors were used to monitor the intestinal pH after infusing 3 mL of 1 M CA solution (576 mg) directly into the desired intestinal port. Three antimony pH sensors were spaced at 15 cm intervals, Ch 2 represented the pH at the site of infusion, Ch 1 was 15 cm proximal and Ch 3 was 15 cm distal to Ch 2. When infused into the duodenum, the pH at Ch 2 dropped immediately to below 4.0. The pH in Ch 3 dropped one min later. The pH in Ch 1 remained constant throughout the test. The pH tracings showed the progressive recovery of the intestinal pH to above 5 in 30 min and back to neutral (pH 7.0) in 90 min (Fig. 2A). When infused into the ileum, Ch 1 and Ch 3 sensors were placed 15 cm proximal and distal to the infusion point while the Ch 2 rested at the ileal infusion site, similar to the duodenal study. The pH at Ch 2 dropped immediately and recovered to pH $>$ 5.0 in about 30 min. However, a bolus of acidified chyme was transmitted both antegrade (Ch 3) and retrograde (Ch 1) by a normal peristaltic wave. Other small and/or less acidic boli were also transmitted periodically during the recording (Fig. 2B). Although the surgical procedure and anesthesia may affect intestinal motility patterns, the relative patterns between the USI and LSI should be relatively constant (21). Overall, these results are in agreement with the fluoroscopy results. The duodenum appears to behave like a well mixed tank with significant longitudinal spreading whereas the ileum follows plug flow mixing kinetics.

An enteric capsule containing 250 mg of CA and 500 mg of BS was tethered to a HC with 3-0 silk suture, and given orally to IAP dogs. Capsule integrity was maintained in the stomach and all capsules appeared to disintegrate in the intestine. *In vitro* disintegration study results also demonstrated that capsule integrity was maintained for 2 hr in 0.1 M hydrochloric acid (data not shown). It was observed that the HC always followed the enteric capsule before disintegration, and followed the dissolved contents after disintegration. Fluoroscopy results showed that BS released in the duodenum was widely spread

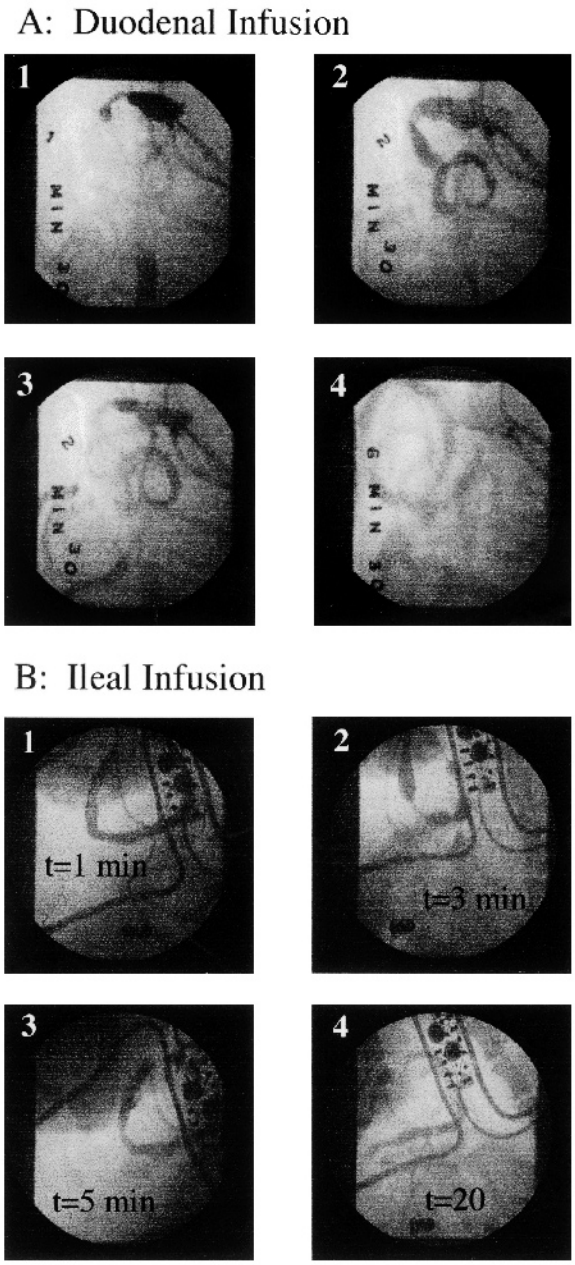

Fig. 1. Radiographic views of intestinal spreading of radiopaque dye after infusion of 3 mL Optiray 320 radiopaque dye into duodenum (A) and ileum (B) in the conscious IAP dogs. A: In duodenum infusion, radiopaque dye is seen as bolus at 1 min 30 sec (1), rapidly spreading at 2 min 30 sec (2, 3), and rapidly diluted by 6 min 30 sec (4). B: In ileum infusion, radiopaque dye is seen as bolus at 1 min (1), spreading as a bolus at 3 min (2) and 5 min (3), and slowly diluted at 20 min (4).

and significantly diluted so that the bowel lumen could not be properly visualized (Fig. 3A). In contract to the USI, when the capsules began to disintegrate in the distal jejunum (Fig. 3B), BS particles remained together and passed with the HC into the ileum. In conscious animals, steady pH lowering or recovery was observed during LSI (distal jejunum/or ileum) disintegrations (Fig. 4B). However, when the capsules disintegrated in the USI, the pH dropped only momentarily and rose quickly back to baseline (Fig. 4A).

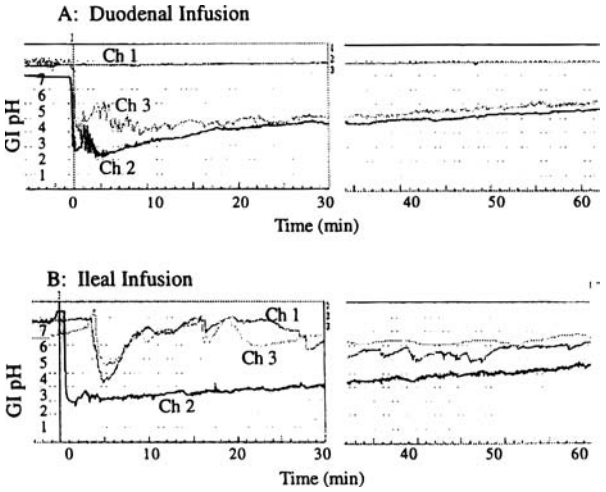

Fig. 2. Regional intestinal pH profiles versus time after infusion of 3 mL of 1 M citric acid into duodenum (A) and ileum (B) in the unconscious IAP dogs. Ch 2 represents the pH at the site of acid infusion, Ch 1 is 15 cm proximal, and Ch 3 is 15 cm distal.

In an effort to correlate the modification of the intestinal environment and the appearance of peptide levels in the blood, enteric capsules containing 565 mg of CA and 1.2 mg of sCT were tethered to a HC with 3-0 silk suture and given orally to normal dogs. Blood samples and pH tracings were taken. Blood sampling was delayed until capsule disintegration was observed. Frequent sampling after that point resulted in a much better characterization of the absorption phase of the sCT plasma level versus time curve. Like all enteric coated dosage forms, the delivery systems prepared for the present studies demonstrated significant performance variability. For example, in Figs. 5 and 6, the same formulation produced different disintegration and absorption curves depending on where the delivery system began to disintegrate. This result indicates that capsule disintegration and dissolution are likely to be significant factors in the large observed variability of sCT bioavailability from various delivery systems (22). Since sCT is protected from enzymatic degradation by the reduced pH and is readily absorbed when local concentrations are high, the best absorption profile occurs when disintegration begins in the LSI. Figure 5 shows disintegration in the duodenum, beginning 5 min after GE (65 min). The initially variable pH momentarily reached pH 3 and soon rose above pH 5. Figure 6 shows a 40 min delay between GE (less than 5 min) and disintegration. The observed pH drop was sharper and steadier with the pH remaining below 5 for nearly 90 min. The C_{max} and AUC were 1.3 ng/ml and 47 ng.min/mL, respectively, for USI disintegration (Fig. 5) and 4.1 ng/ml and 234 ng.min/mL, respectively, for LSI disintegration (Fig. 6). The mean C_{max} and AUC values of sCT were 2.31 ng/mL ($\pm$ 1.07, SD, n = 4) and 110.3 ng.min/mL ($\pm$73.2, SD, n = 4) for LSI, respectively, whereas those were 2–3 fold less for USI. These results clearly show the regiospecific intestinal absorption of sCT and also show a direct relationship between transit time/regional location of disintegration in the small intestine and extent of absorption of sCT. The present findings are consistent with our previous report that the absorption of sCT was better from the ileum than the duodenum in IVAP dogs (12). sCT is an excellent substrate for the pancreatic

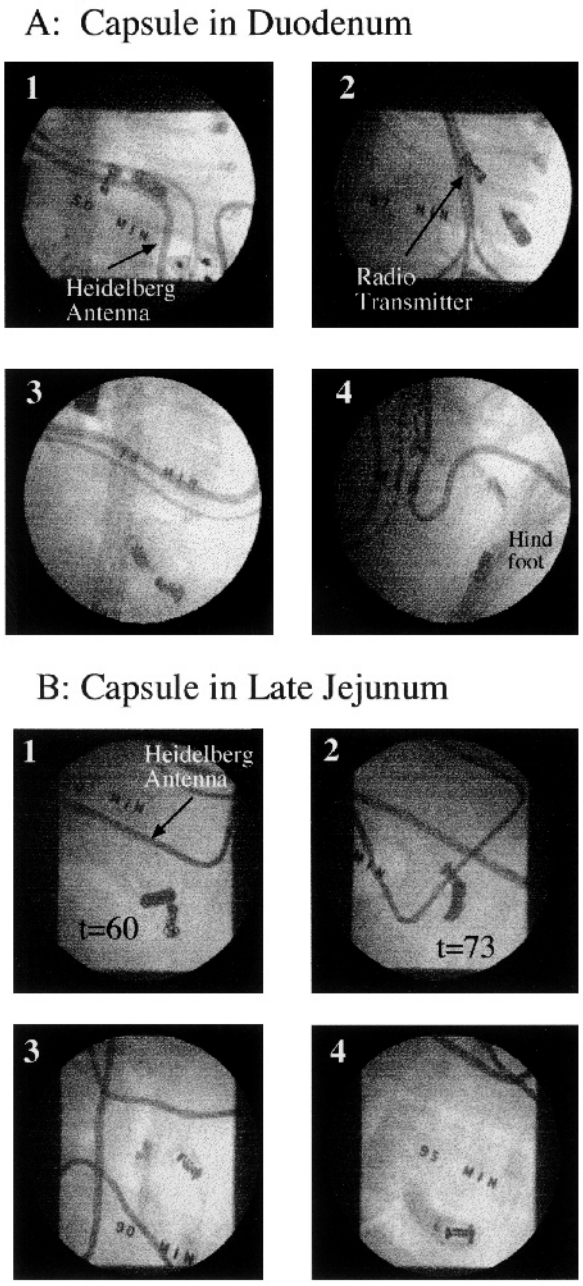

Fig. 3. Radiographic views of intestinal spreading of radiopaque powder after oral administration of capsule containing 250 mg of citric acid and 500 mg of barium sulfate into the conscious IAP dogs. Radiopaque powder capsule is disintegrated in duodenum (A) and late jejunum (B). A: In duodenum disintegration of capsule, the Heidelberg capsule and enteric capsule are tethered in the duodenum at 50 min (1), capsule is disintegrating at 57 min (2) and traveling into the jejunum at 60 min (3), and the disintegrated contents are extensively diluted at 90 min (4). B: In late jejunum disintegration of capsule, the Heidelberg capsule and enteric capsule tether traveled together in the late jejunum at 60 min (gastric emptying was 40 min) (1), capsule is disintegrated at 73 min (2) and slowly spreading at 90 min (3), and the disintegrated contents are slowly diluted at 95 min (4).

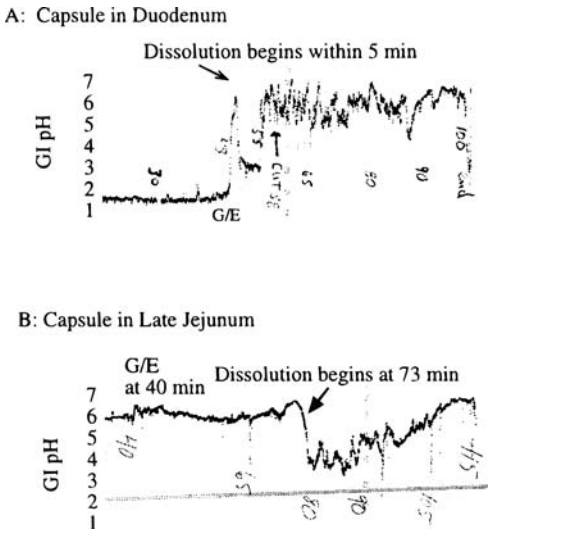

Fig. 4. Regional intestinal pH profiles versus time after oral administration of capsule containing 250 mg of citric acid and 500 mg of barium sulfate into the conscious IAP dogs. Radiopaque powder capsule is disintegrated in duodenum (A) and late jejunum (B). A: In duodenum disintegration, rapid spreading of capsule contents can be seen in the sudden rise of pH prior to cutting the tether at 58 min (gastric emptying was 48 min). B: In late jejunum disintegration, steady drop and gradual rise of pH are seen (capsule disintegration begins at 73 min after gastric emptying at 40 min).

serine protease trypsin, therefore, the rate of degradation of sCT in the GI lumen is dependent upon several factors including the activity of enzymes and the concentration of sCT in the intestinal lumen. Therefore, better absorption of sCT was possible from the LSI because a higher sCT concentration was maintained since dilution and spreading was minimal. Furthermore, the concentration of excipients intended to transiently modify the intestinal environment was not significantly diluted or spread over a large length of intestine. The results of the present study combined with the data from our previous work (22) demonstrate that better absorption of sCT was possible from the LSI because of regional intestinal motility differences and the modification of the intestinal environment. The better

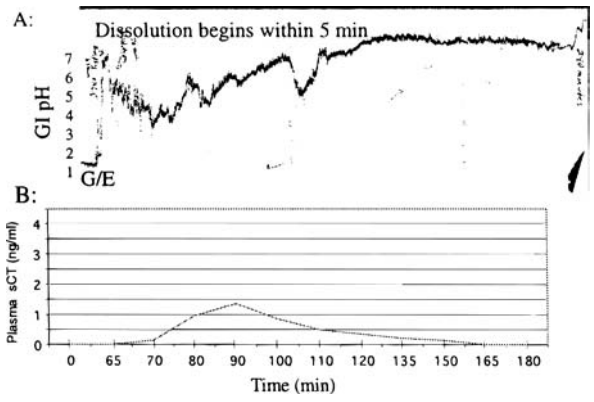

Fig. 5. Duodenal pH recovery (A) and sCT absorption (B) profiles when enteric capsule disintegrated at duodenum after oral administration of sCT formulation containing 565 mg of citric acid and 1.2 mg of sCT into the normal dogs.

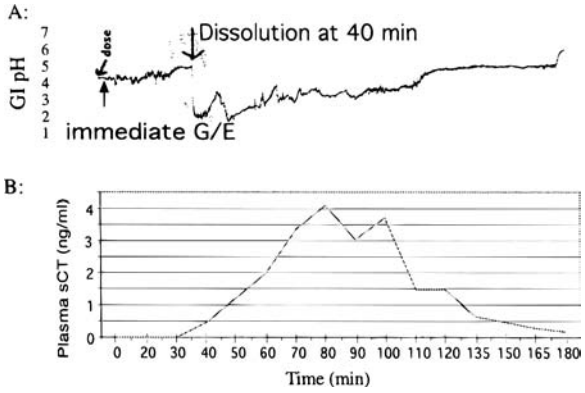

Fig. 6. Late jejunal/or ileal pH recovery (A) and sCT absorption (B) profiles when enteric capsule disintegrated at late jejunum after oral administration of sCT formulation containing 565 mg of citric acid and 1.2 mg of sCT into the normal dogs.

absorption of sCT was also possible from the LSI since proteolytic activity/capacity is low at LSI

When an enteric capsule containing 1.2 mg sCT was administered orally into normal dogs, plasma concentrations of sCT were not observed in formulations where CA was not included (22). An intestinal pH decrease was not observed when CA was not included in the formulation. Therefore, the intestinal pH decrease caused by CA appears to be critical for the oral absorption of sCT. The oral absorption/or enhancement of sCT absorption was directly related to the stabilization of sCT by a reduction of intestinal pH (22). When the rat jejunum and ileum were exposed to 0.2 and 4% of CA, no reduction in transepithelial electrical resistance (TEER) was observed (data not shown), which indicating that CA does not damage tissue at a 4% CA concentration (40 mg/mL).

For most orally administered drugs, three general steps are responsible for the drug reaching the systemic circulation: disintegration, dissolution, and absorption. The process of drug absorption, involves numerous physiological processes such as regional pH differences, motility (intestinal dilution and spreading), permeability, brush border and colonic microflora enzymatic activity that all play an important role in the performance of orally administered dosage forms (23). The processes of disintegration and dissolution are fairly well understood, and efforts to use *in vitro* methods to predict them *in vivo* have been reported. Conversely, in most cases, dispersion processes in the intestine are still not well characterized. Using sCT as a model peptide drug, it was observed that oral absorption was significantly affected by regional intestinal dilution and spreading. Therefore, it is essential to understand how the intestines dilute and spread excipients when developing oral delivery systems for peptide and nonpeptidic drugs. Since this study was performed in the fasting state, the extrapolation of these results to predict food effects is not possible because, in the milieu of intestinal contents, including food, the dilution and spreading behavior may be substantially different. Much research remains to be done to understand the impact of regional intestinal dilution and spreading on the oral absorption of drugs with/or without food in the conscious state. Studies in conscious animal models provide a realistic evaluation of absorption because alterations in GI motility and blood flow brought about by anesthetic regimens are avoided (21).

In conclusion, using fluoroscopy and pH monitoring, the results of this study demonstrate that the disintegration of acidic capsules in the USI resulted in a less effective reduction in the pH of the bowel contents. In the LSI, dilution and spreading were minimal, the pH drop was more significant and steady, and plasma levels of sCT were highest. It appears that, by moving the excipients along as a bolus or plug, each section of the bowel wall received a more stable and concentrated exposure to the excipients and sCT. The results of the present study were successfully used for devising delivery strategies and fabricating oral sCT delivery systems that are currently in clinical testing.

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