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ORIGINAL ARTICLES

Serum Insulin Aspart Concentrations Following High-Dose Insulin Aspart Administered Directly into the Duodenum of Healthy Subjects: An Open-Labeled, Single-Blinded, and Uncontrolled Exploratory Trial

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Abstract

Objective:

The goal of this study was to determine the bioavailability of high-dose insulin aspart administered directly into the duodenum of healthy subjects.

Methods:

In a pilot study, four subjects each received four escalating doses of a 1-ml solution of insulin aspart (100, 300, 600, and 1000 IU, respectively) directly into the duodenum. In the following main study, eight subjects each received two identical doses of insulin aspart of 1000 IU, in 4- and 8-ml solutions, respectively, directly into the duodenum. Subjects in the main study also received an intravenous and a subcutaneous injection of 4 to 6 IU of insulin aspart.

Results:

A considerable number of samples and, in some cases, consecutive samples revealed significantly increased concentrations of serum insulin aspart. Despite the significant serum insulin aspart concentrations, no significant changes of plasma glucose were measured. Moreover, no significant suppression of endogenous insulin secretion was detected, as assessed by the levels of serum human insulin.

Conclusions:

Administration of high-dose insulin aspart directly into the duodenum of healthy subjects resulted in significantly increased serum insulin aspart concentrations in a high number of consecutive samples using a specific enzyme-linked immunosorbent assay. However, no significant changes in the levels of plasma glucose or serum human insulin were observed. Thus, the study did not provide any evidence of biological activity of the original insulin aspart molecule after high-dose administration directly into the duodenum.

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Abbreviations: (ELISA) enzyme-linked immunosorbent assay, (MCA) monoclonal antibodies, (TMB) 3,3′, 5,5′-tetramethylbenzidine

Keywords: absorption, administration, degradation, duodenum, insulin aspart, oral

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Introduction

Over time, numerous attempts have been made to explore alternative routes to subcutaneous insulin administration. So far, no clinically relevant noninvasive insulin administration route has been found. [1–3] Intensive insulin therapy aims to imitate the complex kinetics of physiological insulin secretion. Reproducibility studies of time-action profiles of insulin preparations after subcutaneous injection show considerable intra- and interindividual variations. Furthermore, insulin administered subcutaneously results in peripheral hyperinsulinemia, which is assumed to play a causative role in the development of diabetic micro- and macroangiopatia.[4]

Under physiological conditions, insulin is secreted from pancreatic β cells into the portal circulation in a pulsatile manner showing oscillations of insulin concentrations with a periodicity of 5 to 15 minutes per oscillation. The pulsatile pattern is assumed to have an important impact on insulin action.[5,6] An important physiological characteristic of the β cell is its ability to respond almost instantaneously to glucose stimulation. This response pattern cannot be duplicated by subcutaneous insulin injection.

Assuming that absorption takes place following oral administration, insulin enters the portal circulation from the gastrointestinal tract. The physiological route of endogenous insulin release is thus imitated, which may improve the pharmacodynamic properties of insulin.

Oral formulation of numerous peptide and protein drugs is an area of ongoing intensive research. Peptides and proteins generally have a low bioavailability caused by a poor intrinsic permeability across biological membranes. The inherent low bioavailability is due primarily to the hydrophilic nature and large molecular size, susceptibility to enzymatic attack by intestinal peptidases and proteases, rapid postabsorptive clearance, and chemical instability. There are no efficient membrane carrier systems for the transportation of peptides larger than di- and tripeptides in the gastrointestinal tract. Polypeptides and proteins generally lose their biological activity during the initial stages of the digestive process in the gastrointestinal tract.[7] The bioavailability of orally administered polypeptides and proteins is usually less than 1 to 2%.[8] A limited absorption of proteins after bypassing the acidic environment of the stomach has been reported.[9]

The aim of the present study was to determine the bioavailability of high-dose insulin aspart administered directly into the duodenum of healthy subjects. Insulin aspart was measured by a specific enzyme-linked immunosorbent assay (ELISA), allowing discrimination between insulin aspart and endogenous human insulin.[10]

Methods

The protocol was approved by the Central Denmark Region Committees on Biomedical Research Ethics, the Danish Medicines Agency, and Good Clinical Practice Unit at Aarhus University Hospital, Aarhus, Denmark, and was performed in accordance with the Declaration of Helsinki.

Subjects

Thirteen healthy subjects (01–13) were screened and included in the study. One subject (03) withdrew from the study before the first study day. Thus, a total of 12 subjects participated in and fulfilled the study: four females and eight males, with a mean age of 26.7 ± 4.0 years and a body mass index of 23.9 ± 2.5 kg/m^2.

The insulin aspart dose administered to the eight subjects in the main study was determined in a pilot study including four subjects.

Design

The study was designed as an open-labeled, single-blinded, uncontrolled exploratory trial. Because of safety issues, randomization was not performed.

All subjects reported fasting at the Department of Endocrinology, Aarhus University Hospital, at 08.00. The fasting period was continued until the end of each study day. A gastrointestinal tube coupled to a pH meter was placed in the duodenum just distal to the papilla of Vaterii under X-ray guidance by a trained radiologist at the Department of Radiology, Aarhus University Hospital.

As a precaution against potential hypoglycemia, an indwelling catheter was placed in an antecubital vein for infusion of 20% glucose if needed. A catheter for blood sampling was placed in the contralateral antecubital vein.

Pilot Study

Four subjects each received four escalating doses of a 1-ml solution of insulin aspart (100, 300, 600, and 1000 IU, respectively), with time intervals of 3 hours. Blood sampling for serum insulin aspart, serum human insulin, and plasma glucose was performed every 10 minutes for the first 2 hours and every 20 minutes for the last hour.

Main Study

Eight subjects each received two identical doses of insulin aspart of 1000 IU in 4- and 8-ml solutions, respectively. Time intervals between dose administrations were 3 hours. Blood was sampled with similar intervals as in the pilot study.

On a separate occasion, subjects in the main study received a bolus injection of insulin aspart by intravenous and subcutaneous routes, respectively. Three subjects received 6 IU and five subjects received 4 IU of insulin aspart. Several significant hypoglycemic episodes in the first three subjects prompted the dose reduction.

The intravenous injection was followed by 3 hours of blood sampling. Time intervals between blood samplings were 5 minutes for the first 2 hours and 20 minutes for the last hour. The subcutaneous injection was followed by 6 hours of blood sampling. Time intervals between blood samplings were 10 minutes for the first 2 hours and 20 minutes for the last 4 hours.

Glucose infusion rates required in order to neutralize a potential plasma glucose-lowering effect of the administered insulin were registered during a manual euglucemic clamp aimed at a plasma glucose range between 5.0 and 8.0 mmol/liter.

Assays

Glucose

Plasma glucose concentrations were measured by Accu-Chek Inform, Roche A/S.

Insulin Aspart

Insulin aspart is an analog of human insulin in which proline at position 28 of the β chain is replaced by aspartic acid. For accurate measurement of insulin aspart, it must be separated from endogenously produced human insulin.

Insulin aspart was measured by a specific enzyme-linked immunosorbent assay (ELISA). The assay involves two murine monoclonal antibodies (MCA). One MCA binds to an epitope common for human insulin and insulin aspart and the other binds to an epitope specific for insulin aspart. The monoclonal antibodies X14-6F34 and HUI-018 were developed by hybridoma technology from mice immunized with insulin aspart and human insulin, respectively. The epitope for X14-6F34 is near the C-terminal end of the β chain. The affinity for insulin aspart is 10^7 liter/mol. The antibody does not bind human insulin. The epitope for HUI-018 is centered around the A loop. The affinity for human insulin is 10^8 liter/mol.

Microtiter wells were coated overnight with 100 µl of coating buffer containing 10 mg/liter HUI-018 specific for human and aspart insulin. The coated wells were emptied and blocked with 200 µl of blocking buffer for 1 hour with agitation and washed four times with washing buffer. Insulin determination was performed in duplicate. To each well were added 25 µl of calibrator or serum samples and 75 µl of antibody-detecting buffer with 0.5–2 mg/liter of biotinylated X14-6F34 specific for insulin aspart. All calibrators were prepared in a pool of fasted human serum, which was also used for the dilution of samples with a high insulin aspart concentration.

After 20 to 24 hours of incubation with shaking, the plates were washed four times. After this, 100 µl of 0.5 mg/liter avidin D–horseradish peroxidase in conjugate buffer was applied to each well. After agitated incubation for 30 minutes the plates were washed four times. The enzyme activity in wells was estimated by adding 100 µl/well of 3,3′, 5,5′-tetramethylbenzidine (TMB) in substrate solution. After shaking for 10 minutes, the reaction was terminated by the addition of 100 µl of 4 mol/liter H_3PO_4. Absorbance values were read at 450 nm.

There is no cross-reactivity with human and porcine insulin, human proinsulin, or human C-peptide. The assay is sensitive (limit of quantification = 11.5 pmol/liter), accurate (95 to 107% recovery with human insulin, human plasma, and porcine plasma in the range 16 to 800 pmol/liter), and has a 14.7% total imprecision (sample, assay, and residual) within the entire analytical range. Dilution of samples gives linear results with human serum as the diluents. The calibration curve for the assay follows a logistic function, which includes the zero calibration point.

Human Insulin

The assay used for human insulin analysis of samples is an immunoassay of the sandwich ELISA type.

A monoclonal antibody specific for human insulin is used to capture the analyte from plasma into the wells of a 96-well microtiter plate coated with the antibody. A second, biotinylated monoclonal antibody specific for human insulin and reacting with a different epitope than the capture antibody is used to detect the bound analyte. Plates are developed with conjugated horseradish peroxidase using TMB as the color reagent. The instrument response is obtained using a Tecan Sunrise plate reader controlled by Magellan version 5.03, the instrument responses are exported to DReS release 2.3 where plasma concentrations are quantified, and study tables are generated.

pH Values

The pH probe used was a two-sensor antimony, single-use internal reference pH catheter with a diameter of 2.3 mm connected to a Microdigitrapper (Medtronic Functional Diagnostics A/S). The pH probe had two pH channels placed 5 cm apart.

Duodenal Tubes

Nutritional tubes, length: 120 cm, and diameter: 4.67 mm/14 CH (Flocare, Nutricia).

Insulin Aspart Preparation

Novo Nordisk A/S, Denmark, delivered freeze-dried insulin aspart in 25-ml Mellerud containers to the Pharmacy Department at Aarhus University Hospital. A trained pharmacist prepared the insulin aspart solutions.

Procedure. Approximately 350 mg of insulin aspart was suspended in 7.5 ml of a 20 m*M* phosphate solution under manual mixing. Six hundred microliters of 0.2 *M* NaOH was used to dissolve the insulin aspart. This solution was mixed with sterile water to reach a weight of 10.0 grams. This solution had a concentration of 1000 IU/ml and a pH of approximately 7.4.

NovoRapid® Penfill 100 IE/ml was used for intravenous and subcutaneous injections.

Blood sampling. Two milliliters of blood was drawn at each blood sampling. Blood samples were centrifuged at 3600 revolutions per minute for 10 minutes. The serum component was then distributed into two tubes and labeled. The serum samples were stored in a refrigerator at –20°C. Analyses were performed by a trained laboratory technician at Novo Nordisk A/S, Denmark. Analyses from the pilot study and the main study were performed on 2 separate days.

Statistics

The study was explorative and hypothesis generating. Descriptive statistics was applied.

Results

Pilot Study

Significant serum insulin aspart concentrations were observed in several consecutive samples in subjects 04 and 05 (**Figures 1** and **2**).

For subject 04, significant serum insulin aspart concentrations were measured after the administration of 300, 600, and 1000 IU of insulin aspart, respectively. For subject 05, significant serum insulin aspart concentrations were measured after the administration of 100, 300, 600, and 1000 IU insulin aspart, respectively. For subjects 01 and 02, no significant levels of serum insulin aspart concentrations were detected (**Figures 1** and **2**).

No significant changes in plasma glucose and intravenous glucose infusion were recorded (**Figures 3** and **4**), and no significant changes in serum human insulin were observed (**Figure 5**).

pH values. Channel 1: maximum 8.23 ± 0.21, minimum 1.2 ± 0.08, and mean 6.33 ± 0.12. Channel 2: maximum 8.13 ± 0.34, minimum 1.4 ± 0.12, and mean 6.33 ± 0.22 (**Figure 6**).

All volunteers showed fast and ongoing cyclic changes of pH values throughout the study period.

Main Study

For all subjects, at least one significant serum insulin aspart concentration was measured after the administration of 4- and 8-ml solutions of 1000 IU insulin aspart. For subject 12, no significant serum insulin aspart concentrations were measured after the administration of a solution of 4 ml 1000 IU insulin aspart (**Figures 2** and **7**).

Subjects 06, 07, and 11 displayed the highest levels of serum insulin aspart concentrations after administration of the solutions of 4 and 8 ml 1000 IU insulin aspart. Several of the measurements were consecutive (**Figures 2** and **7**).

No significant changes in plasma glucose and intravenous glucose infusion were recorded (**Figures 3** and **4**), and no significant changes in serum levels of serum human insulin were observed (**Figure 5**).

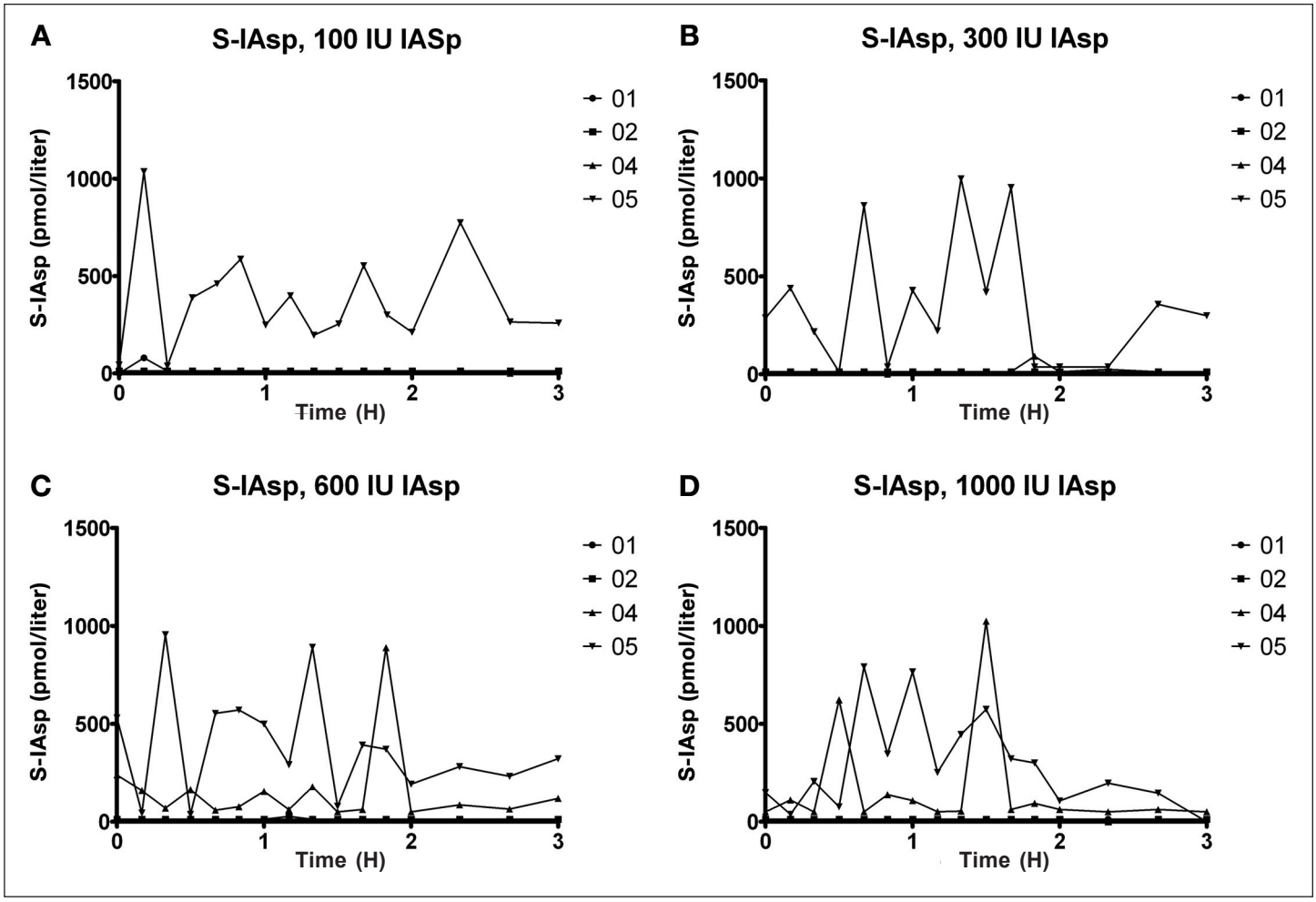

Figure 1. Duodenal insulin administration. Individual serum insulin aspart (S-IAsp) levels for subjects (01, 02, 04, and 05) in the pilot study. **(A)** 100 IU IAsp, **(B)** 300 IU IAsp, **(C)** 600 IU IAsp, and **(D)** 1000 IU IAsp.

pH values. Channel 1: maximum 8.16 ± 0.65, minimum 0.63 ± 0.61, and mean 5.69 ± 0.68. Channel 2: maximum 8.41 ± 0.81, minimum 0.69 ± 0.68, and mean 5.73 ± 0.84 (**Figure 6**).

All subjects showed fast and ongoing cyclic changes of pH values throughout the study period.

Intravenous administration of 4 or 6 IU insulin aspart resulted in a fast and significant elevation of serum insulin aspart concentrations in all subjects, which was followed by a rapid return toward baseline levels (**Figure 8**).

Subcutaneous administration of 4 or 6 IU insulin aspart resulted in a more inhomogeneous response with inter-individual variation in the maximum peak concentration value of serum insulin aspart and time to maximum concentration. Baseline levels were achieved within the observation period in all subjects (**Figure 8**).

Intravenously and subcutaneously administered insulin aspart had the predicted impact upon plasma glucose levels and serum human insulin (data not shown).

Discussion

Optimal glucose homeostasis is crucial in order to reduce the risks of developing complications associated with diabetes mellitus. Oral administration of insulin might mimic the physiological secretion pattern and action of insulin more closely than subcutaneous administration. Another advantage of oral insulin administration is the compliance issues associated with injection therapy.

Several groups have found absorption of insulin across isolated intestinal loops and in animal studies. In these studies the insulin absorption has most commonly been facilitated. Some of the animal studies have shown a glycemic response to the insulin administration.[11] A glucemic response in humans to oral hexyl-insulin monoconjungate 2 has been found.[12–14]

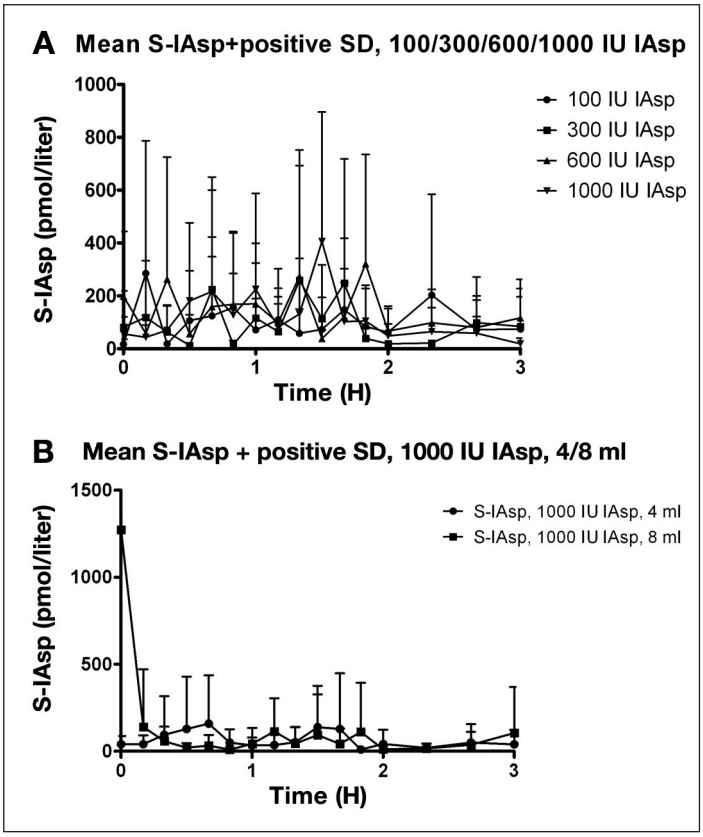

Figure 2. Duodenal insulin administration. Mean serum insulin aspart (S-IAsp) concentrations + positive SD. **(A)** Pilot study and **(B)** main study. Please observe different scale on Y-axes.

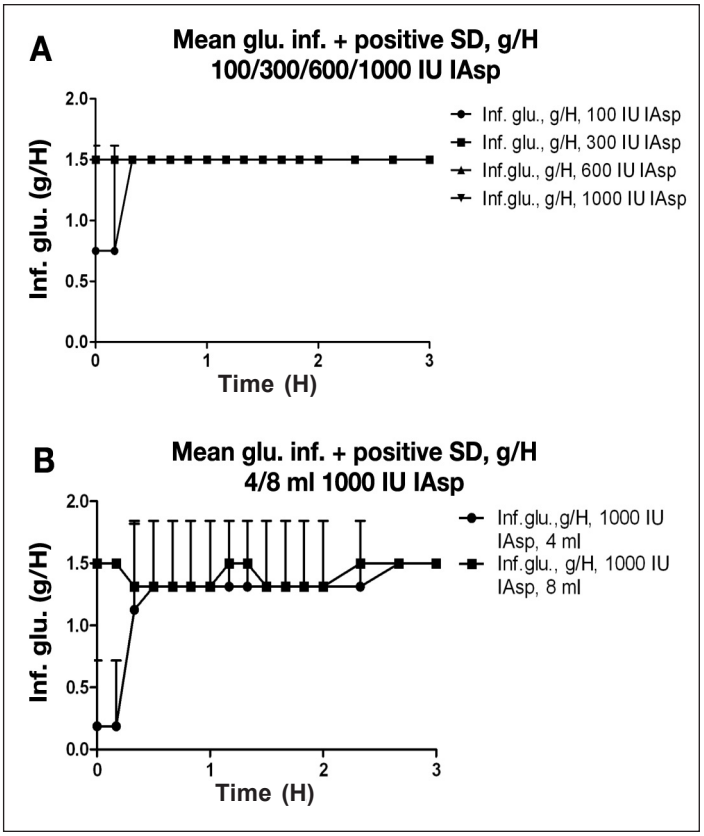

Figure 4. Duodenal insulin administration. Mean intravenous glucose infusion (glu.inf.) g/H + positive SD. **(A)** Pilot study and **(B)** main study.

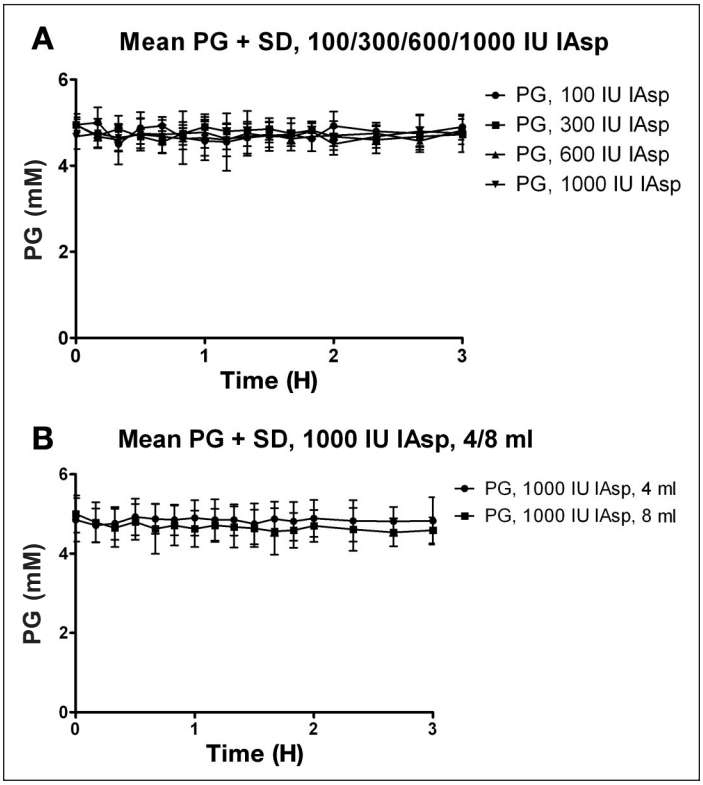

Figure 3. Duodenal insulin administration. Mean plasma glucose (PG) concentrations + SD. **(A)** Pilot study and **(B)** main study.

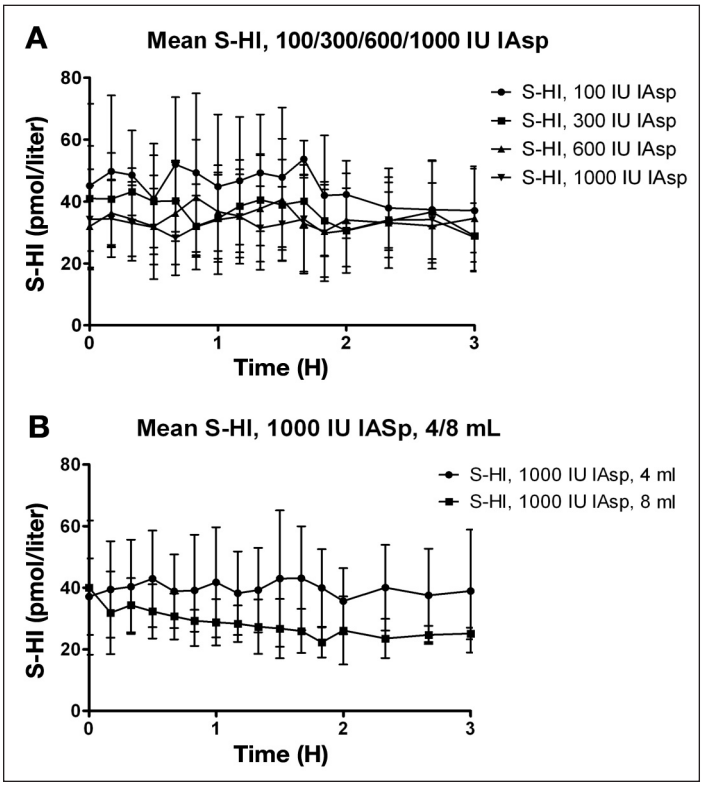

Figure 5. Duodenal insulin administration. Mean serum human insulin (S-HI) concentrations + SD. **(A)** Pilot study and **(B)** main study.

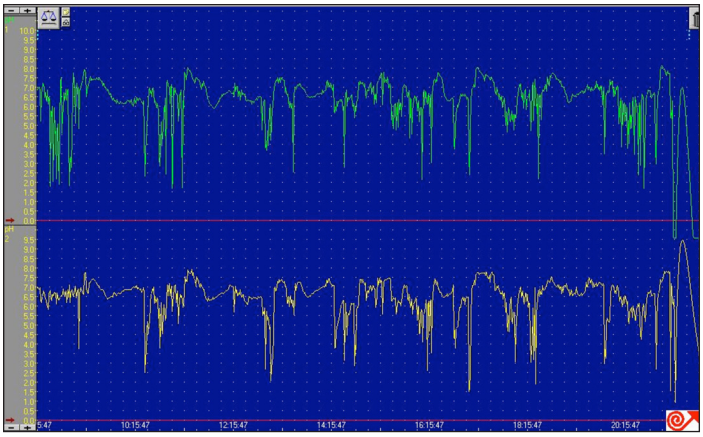

Figure 6. Duodenal insulin administration. pH measurement from subject 01 from the pilot study. pH 1 and pH 2 refer to the two pH channels on the pH meter in the duodenum.

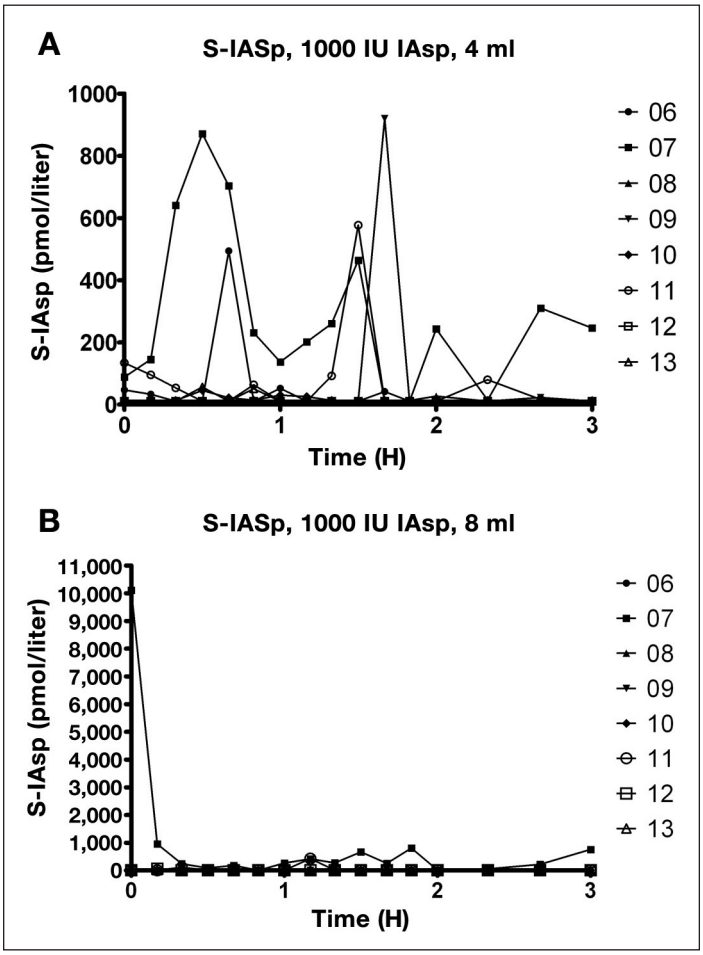

Figure 7. Duodenal insulin administration. Individual serum insulin aspart (S-IAsp) levels for subjects (05 to 13) in the main study. **(A)** 1000 IU IAsp, 4 ml, and **(B)** 1000 IU IAsp, 8 ml. Please observe different scale on *Y* axes.

In the present study we observed several and, in some cases, consecutive samples with significant immunoreactivity against serum insulin aspart. However, despite

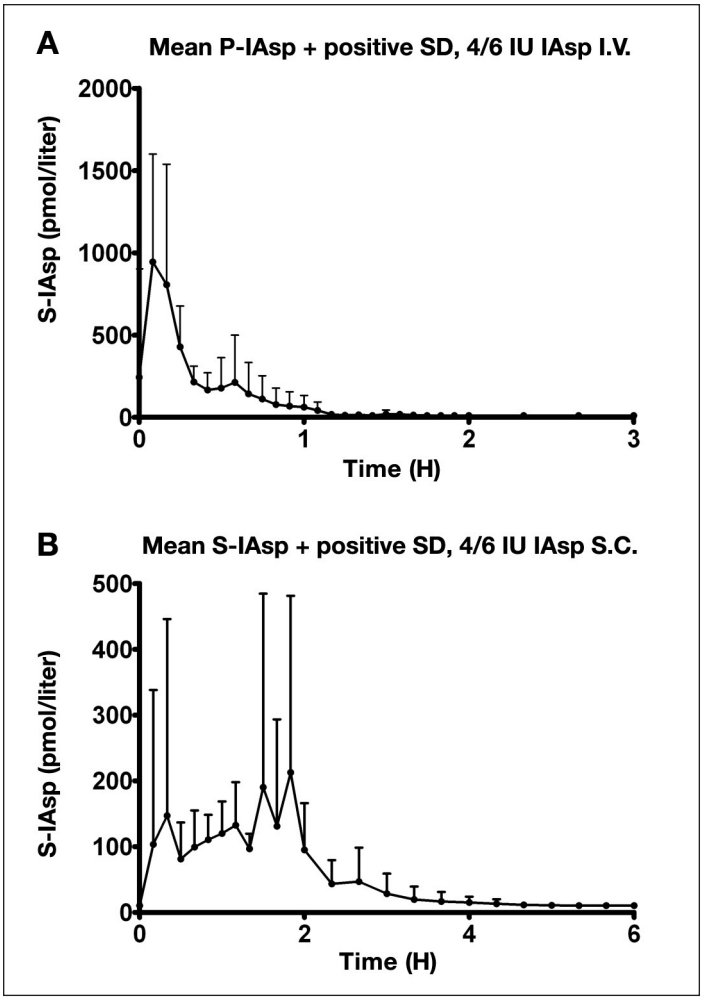

Figure 8. Mean serum insulin aspart (S-IAsp) concentrations + positive SD. **(A)** Intravenous (I.V.) insulin aspart administration, 4/6 IU. **(B)** Subcutaneous (S.C.) insulin aspart administration. Please observe different scale on Y axes.

significant serum insulin aspart concentrations, no significant changes of plasma glucose were measured. Administration of different doses and concentrations of insulin aspart had no significant impact on the observed findings. Furthermore, we observed no impact of duodenal insulin aspart administration upon serum human insulin. Negative feedback of insulin aspart upon endogenous insulin secretion has been demonstrated previously.[15] If the absorbed insulin aspart molecule had been intact with preserved activity, a decline in the serum human insulin concentration would have been expected.

We have found fast and ongoing cyclic changes of pH values throughout the study periods. Several groups have measured pH values in the duodenum. The pH values in the duodenum have been found to show wide, rapid, and frequent fluctuations.[16,17] Thus, our measured

pH values are in correspondence with previous findings. The measured pH values provide no evidence that the absorption of insulin aspart following oral administration is pH dependent.

The discrepancy between apparent pharmacokinetic parameters and pharmacodynamic parameters gives raise to speculations.

Insulin is a polypeptide hormone. The intact insulin, with preserved primary, secondary, and tertiary structures, is important for insulin action. Exactly how the structural and conformational dynamics of insulin elicit its biological responses remains a vigorous area of research.

The degree of denaturation and degradation of serum insulin aspart following absorption from the gastrointestinal tract is not known. The ELISA assay may have measured inactive serum insulin aspart degradation products. The nature of the inactive insulin aspart degradation products has not been defined further as the study was not designed for this purpose. The two epitopes on the insulin aspart molecule recognized in our assay are located near the C-terminal end of the β chain and are centered around the A loop, respectively. Thus, these two epitopes must be present for recognition by the ELISA assay. High-performance liquid chromatography or another assay that may have the ability to further characterize the insulin aspart fragments was not performed. The measurement of insulin aspart degradation products by the specific ELISA assay may be a general source of error in measurements of insulin aspart in subjects with preserved endogenous insulin production.

We found zero-time measurements of serum insulin aspart above the detection level of the assay. This may be explained by the presence of heterophilic antibodies. Heterophilic antibodies can be found in serum/plasma and can bind to immunoglobulins of other species, including the species used to generate the antibodies used as reagents for immunoassays. These antibodies can interfere in immunoassays, causing a spurious elevation of measured values that is independent of the true analyte concentration, thus potentially misclassifying samples.

Heterophilic antibodies show low-affinity competitive-binding kinetics.[18]

Further studies are needed to explain the discrepancy between serum insulin aspart measurements and the pharmacodynamic actions of insulin aspart.

Development of an oral insulin formulation is facing substantial barriers. The poor bioavailability of insulin administered orally must be addressed. Insulin digestion, as with other peptides and proteins, begins in the stomach and is continued by many different enzymes throughout the gastrointestinal tract.

The barriers include inherent poor intrinsic permeability, luminal and enzymatic degradation, and chemical and conformational stability. A number of different strategies have been explored in order to improve the bioavailability of orally administered insulin. The approaches include permeation enhancers, enteric coatings, protease inhibitors, microsphere encapsulation, and combination strategies.[19]

Another strategy is molecular engineering of the insulin molecule. Advances in biotechnology, particularly recombinant protein technology, may be an important step in the development of a modified insulin molecule with clinical relevant bioavailability for oral administration.

A potentially therapeutic role for oral insulin is critically dependent on stable and predictable bioavailability and pharmacodynamic action.

Conclusion

In this study, high-dose insulin aspart was administered directly into the duodenum of healthy subjects. Although several, and often consecutive, significant serum insulin aspart concentrations were recorded using a specific ELISA assay, no significant changes in plasma glucose or serum human insulin were recorded. Thus, no evidence of biological activity with significant pharmacodynamic effects of the original insulin aspart molecule was observed. The measurement of insulin aspart degradation products by the specific ELISA assay might be a general source of error.

Denaturation and degradation of the insulin molecule in the gastrointestinal tract are the greatest barriers to oral administration. This study gives no evidence that the inherent low bioavailability of orally administered insulin can be bypassed by the administration of high-dose insulin aspart directly into the less acidic milieu of the duodenum. The development of insulin delivery systems and/or modifications of the insulin molecule might make oral insulin administration a therapeutic option in the treatment of patients with diabetes mellitus.

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Disclosure:

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