Insulin Nanoparticles: A Novel Formulation Approach for Poorly Water Soluble Zn-Insulin

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Purpose. To determine the feasibility of using wet milling technology to formulate poorly water soluble zinc-insulin as a stable, biologically active, nanoparticulate dispersion.

Methods. The feasibility of formulating zinc-insulin as a nanoparticulate dispersion using wet milling technology was studied. An insulin nanoparticulate formulation was reproducibly obtained after milling zinc-insulin in the presence of F68, sodium deoxycholate and water at neutral pH. The physical and chemical properties of these peptide particles were studied using electron microscopy, laser light scattering, HPLC and SDS-PAGE. To verify efficacy, hyperglycemic rats were dosed subcutaneously and intraduodenally with nanoparticles or solubilized insulin. Glucose and insulin levels were monitored on blood samples collected throughout the study.

Results. Zn-insulin (mean size = 16.162 μm) was processed using milling technology to form an aqueous-based nanoparticle dispersion with a mean particle size of less than 0.150 μm. The formulation was homogeneous and exhibited a unimodal particle size distribution profile using laser light diffraction techniques. Insulin, processed as a peptide-particle dispersion, was shown to be comparable to unprocessed powder using HPLC and SDS-PAGE. In addition, HPLC analyses performed on samples, heat-treated at 70 °C for 100 minutes, demonstrate that under conditions which effect the solubilized peptide, formulated as a peptide-particle dispersion, insulin was chemically stable. Also, when stored refrigerated, the insulin dispersion was chemically and physically stable. Finally, peptide particles of insulin, dosed subcutaneously and intraduodenally, were effective at lowering blood glucose levels of hyperglycemic rats.

Conclusion. Water insoluble Zn-insulin can be formulated as a stable, biologically active nanometer-sized peptide particle dispersion using wet media milling technology.

KEY WORDS: insulin; peptide nanoparticles; poorly water soluble; wet milling technology.

INTRODUCTION

It is generally recognized that the incidence of diabetes is rising and reportedly growing at epidemic proportions. The need for new and improved approaches to treat this disease remains a key focal point for many research and development programs (1–4). Though many approaches for treating the disease have arisen throughout the years and new therapeutic targets are being identified, insulin therapy remains the mainstay of managing diabetes. Being a peptide, insulin therapy is subject to the many liabilities associated with products whose active ingredient is a chemically labile molecule. Over the years, to improve patient compliance and disease management, insulin has been incorporated and studied in conjunction with a variety of drug delivery approaches (5–7). Some of these approaches have been successful and are slowly moving to the clinic and marketplace. Although this area of research has been aggressively pursued, there is a need and an interest in identifying better ways to formulate and deliver therapeutic peptides such as insulin.

One of the well-documented properties of insulin is its concentration/pH dependent self-association phenomenon (8–10). At concentrations <0.1 μM, insulin exists as a monomer. In solution, as a monomer, insulin is biologically active and highly susceptible to enzymatic and non-enzymatic degradation. As the concentration of the peptide increases, monomers dimerize and, at concentrations >2 mM, a hexamer is formed. As the molecule self-associates, biologic activity decreases. However, the association masks and protects highly reactive sites (11,12). In this study, using wet media milling technology (13,14), we describe a physically stable nanoparticle preparation of zinc-insulin that is a water-based system having neutral pH. The insulin nanoparticles appear to be self-associated entities that maintain biologic activity following subcutaneous and intraduodenal dosing.

MATERIALS AND METHODS

Materials

Insulin (noncrystalline) used in this study was purified from bovine pancreas and purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO). Sodium deoxycholate was also a product of Sigma-Aldrich and the pluronic stabilizer F68 (P188) was purchased from BASF (Parsnippany, NJ). Ceramic milling media was purchased from Tosoh Ceramics Division (Bound Brook, NJ).

Insulin Formulations

A nanoparticle insulin formulation was prepared using a low energy wet milling process that was previously described for poorly water-soluble small crystalline molecules (15–17). To generate a physically stable drug particle preparation, stabilizers capable of wetting the material to be processed must be selected (13,15). For insulin, a series of empirical studies were performed to identify a stabilizer or stabilizer system that would provide efficient particle size reduction, maximum stability and be suitable for parenteral and oral administration. For insulin, this was achieved by using a mixture of F68 and sodium deoxycholate. Nanoparticulate insulin dispersions were prepared as follows: Zn-insulin (20 mg/g) was prewetted with an unbuffered-aqueous solution containing 1% F68 and 0.1% sodium deoxycholate. The pH of the crude slurry ranged from 7.0 to 7.5. The crude insulin slurry (3.75 ml) was added to a roller mill jar (28 ml) containing ceramic milling media (7.5 ml volume), placed on the mill (U.S. Stoneware, East Palestine, OH) and processed at room temperature. Processing was performed at 57% critical speed, which is the rotational speed of the grinding vessel when the milling media begins to centrifuge. The aqueous dispersion was assayed for particle size analysis throughout the 16–24 h processing period. After processing, the dispersions were harvested and used for the studies described below.

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Particle Size Determination

Light scattering measurements were performed using the Horiba LA 910 (Horiba Instruments, Inc., Irvine, CA). Prior to use, the instrument was calibrated using NIST traceable polymeric microspheres (0.199 μm ± 0.006 μm) from Duke Scientific Corporation, (Palo Alto, CA) and samples were assayed immediately after being diluted with deionized-distilled water. Light scattering measurements were verified using microscopy. For light microscopy, a wet sample was visualized using a Leica DMR Research Microscope (Leica, Inc., Deerfield, IL) with a 100× (1.3) oil immersion objective and a 3CCD Toshiba Digital camera. For SEM analysis, samples were diluted with deionized-distilled water and an aliquot was dried, sputter coated and visualized using the Topco SM510 (Topcon Technologies Inc., Pleasanton, CA).

HPLC Analysis

For chemical stability analysis, insulin formulations were assayed using a modification of previously described methods (18). The assays were carried out by Impact Analytical (Midland, MI) on a Hitachi Model 7100 U with an Alltech Prosphere C18 (15 cm × 4.6 mm, 5 μm) column using a linear gradient. The gradient was formed using 0.13% trifluoracetic acid (TFA) in water (Solvent A) and 0.1% trifluoroacetic acid (TFA) in acetonitrile (Solvent B) at a flow rate of 1 ml/min. At the initiation of the run, the mobile phase was comprised of 75% (Solvent A) and 25% (Solvent B). At the end of the 24 min run, the mobile phase was 62% (Solvent A) and 38% (Solvent B). Insulin was detected using a diode array detector (Hitachi model L-7455) set at 220 nm. The elution conditions are summarized below:

Time (min)	% Solvent A	% Solvent B	Flow (ml/min)
0.0	75	25	1.0
15.0	62	38	1.0
24.0	62	38	1.0

For a standard solution of bovine insulin (20 μl of a 2 mg/ml solution in 0.01N HCL), the retention time of the major peak 10.88 ± 0.14 min with a minor impurity eluting in 11.64 min ± 0.15 min. To test the stability indicating potential of this method, the elution profiles of a freshly prepared insulin solution in 0.01 N HCL were compared to heat and alkaline treated samples. The chromatograms of the stress-challenged samples differed significantly from those of

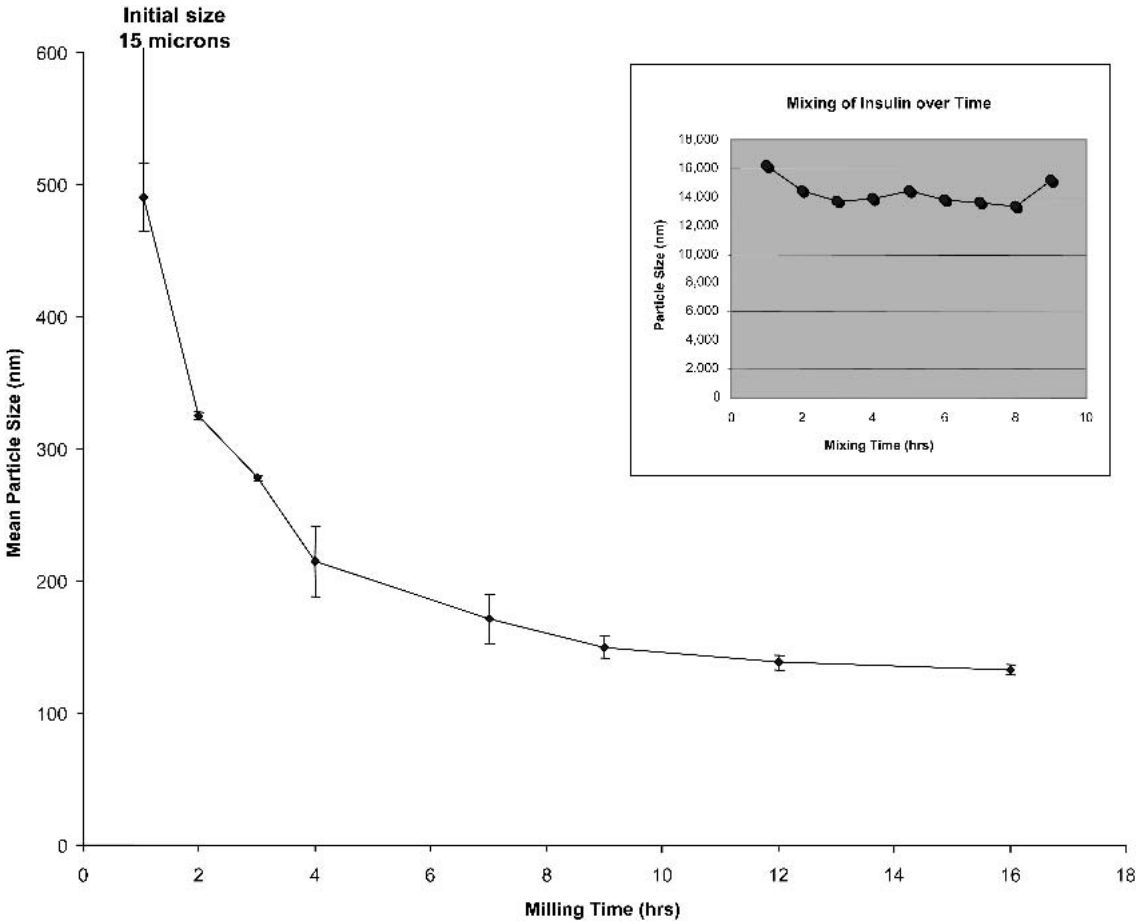

Fig. 1. Particle size reduction/time curve for Zn-insulin processed using a wet media milling process with pluronic F68 plus sodium deoxycholate as a stabilizer system. The average mean particle size plus the standard deviation of three consecutive processing runs are plotted. The inset shows the particle size with time curve for Zn-insulin plus F68 and sodium deoxycholate that was stirred for 16 h rather than processed using a media mill.

freshly prepared material indicating that the assay was capable of resolving insulin from potential degradents (see Fig. 4).

Prior to assaying, the insulin nanoparticle dispersion (2% wt/wt) was solubilized with 0.01 N HCL. The HCL-diluted samples were then assayed. For heat treatment, the undiluted insulin dispersion (2% wt/wt) was heated at 70°C for 100 min. The sample was then diluted with 0.01 N HCL and assayed as described above. Also, solutions of 0.01 N HCL and 1% F68 containing 0.1% sodium deoxycholate were assayed as negative controls.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed to compare the electrophoretic mobility of insulin before and after being formulated as a nanoparticle dispersion. In addition, the nanoparticle preparation was centrifuged at 13,000 × *g* for 15 min to separate the pelleted-particle fraction from the soluble supernatant. The pellet and supernatant were then analyzed using SDS-PAGE to determine relative amounts of insulin associated with each fraction. For electrophoresis, samples were boiled 5min in the presence of sodium dodecylsulfate and β-mercaptoethanol. An aliquot of the samples, equivalent to 10–100 μg of protein, was electrophoresed on a 16% SDS polyacrylamide gel for 1 h at 200 V. Proteins were visualized using Coomassie blue staining which has a detection limit of ~1 μg. Molecular weight protein standards were used to compare the electrophoretic mobility of nanoparticulate and unprocessed insulin.

In vivo Efficacy Studies

Male Wistar rats weighing 250–300 g were used in a randomized non-crossover study design in accordance with institutional guidelines and approval of local ethics authorities. The animals were fasted for 4 h prior to the commencement of the study. Hyperglycemia was induced following an intramuscular injection of ketamine (22.5 mg/animal) and acepromazine (0.75 mg/kg). After 1 h, insulin was administered either subcutaneously or intraduodenally as a solution prepared in Dulbecco's phosphate-buffered saline or as a nanoparticle dispersion. For parenteral studies, insulin was administered at a concentration of 1 IU/0.2 ml. For intraduodenal dosing, the animals were cannulated and insulin was administered at a dose of 150 IU/0.33 ml. Blood samples were collected from the tail vein of the animals at 15 min intervals for a 4 h period. Subsequently, the blood samples were analyzed for insulin using radioimmunoassay (LOD = 3 μU/ml) and for blood glucose levels, the Accutrend alpha glucometer (LOD = 0 to 33 mmol/L).

RESULTS

Nanoparticles of insulin were generated using an aqueous-based media milling process. As described in Methods, water insoluble insulin, at a concentration of 20mg/g, was ball milled in the presence of the pluronic stabilizer F68 containing sodium deoxycholate in water. In Fig. 1, the particle size reduction profile of the noncrystalline insulin powder is shown. The graph shows that within one hour of processing, the mean particle size of the powder is reduced from 16.16 μm to the nanometer size range. Further processing yields a very homogeneous particle preparation with a mean particle size <0.150 μm (Figs. 1 and 2). Using microscopy, the homogeneity and the effectiveness of the media milling process was readily evident. As shown in Fig. 3, in comparison to the unmilled drug substance, the milled insulin was an ultra-fine highly dispersed nanoparticulate preparation. It is interesting to note that the impaction and the shearing forces generated during milling were necessary for achieving the desired results. As shown in the inset (Fig. 1), merely mixing Zn-insulin in the stabilizer solution did not generate nanoparticles. When mixed with pluronic-deoxycholate solution, particle size reduction did not occur. The physical properties of the peptide remained the same throughout the entire processing period. It should be noted that processing was tested using other excipients. However, the combination of F68 and sodium deoxycholate proved to be a superior stabilizer system for insulin providing reproducible particle size reduction, stability and acceptability for both oral and parenteral dosing.

The chemical integrity of the unprocessed and processed peptide was studied using HPLC as shown in Figs. 4 and 5 respectively. In Fig. 4, unprocessed insulin was assayed before and after heat treatment. For insulin in solution (Fig. 4A), the

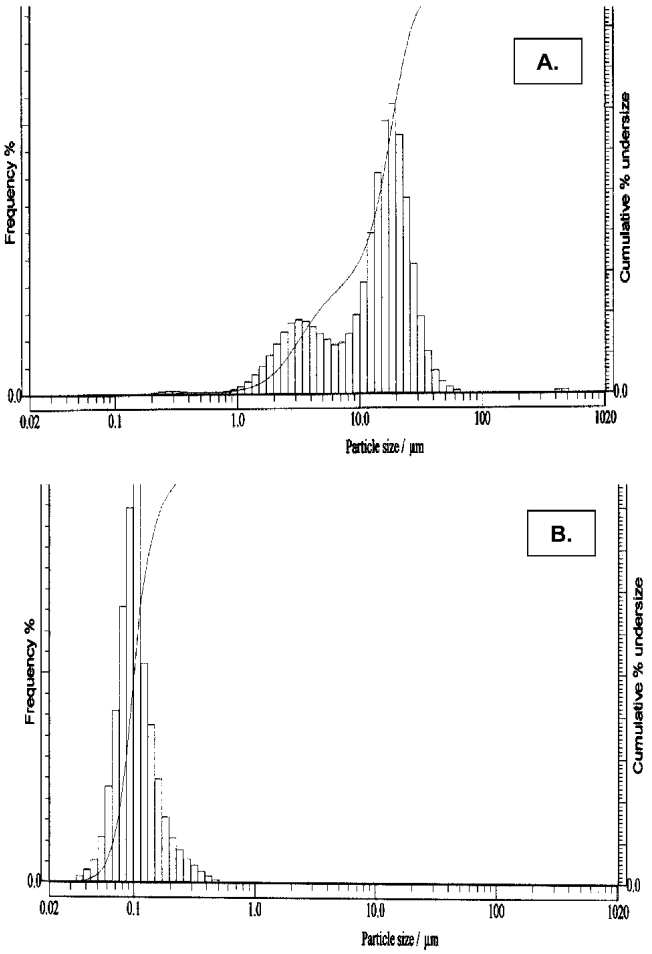

Fig. 2. Particle size distribution profiles of insulin powder using laser light diffraction: (A) unmilled insulin (mean ~16.16 μm) and (B) nanoparticle insulin processed for 18 h (mean ~0.114 μm). The particle size measurements were performed using deionized distilled water as a diluent.

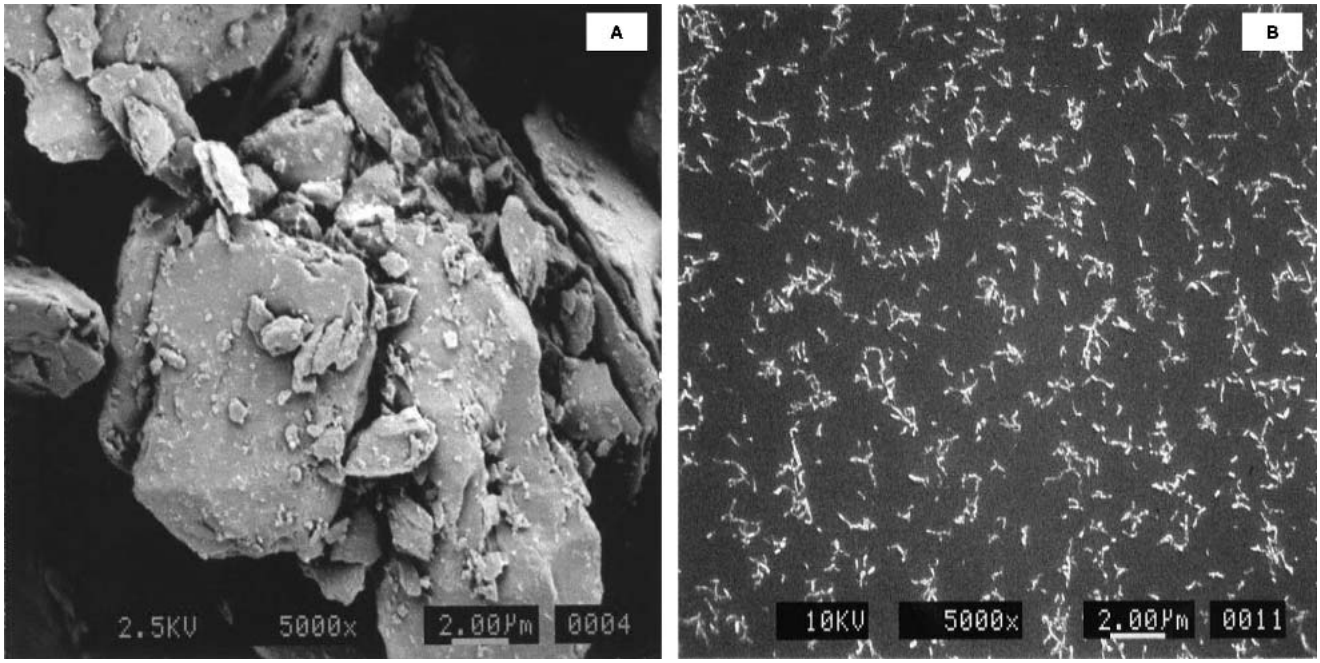

Fig. 3. The micrographs compare unmilled insulin powder (A) and milled nanoparticle insulin (B) using scanning electron microscopy at a magnification ×5000. The measurement bar = 2 μm.

major fraction (97.8%) eluted in 10.88 min with 2.2% of the sample eluting at 11.64 min. When the insulin solution was heat treated for 100 min at 70°C, the fraction of the sample eluting at 10.88 min decreased (72.3%) and the minor peak eluting at 11.64 min increased to 27.7%. The major impurity with a retention time of 11.64 min was not identified in this study. However, the data are consistent with previous studies establishing desamido-insulin as a major degradation product of insulin (19). The milled insulin particles were analyzed by HPLC before and after heat treatment. The HPLC profiles of the milled insulin, shown in Fig. 5, are very consistent with the analysis performed on unprocessed insulin. There is no evidence that the milling process adversely effects the chemical integrity of the peptide. Also, when insulin is formulated as a peptide-particle dispersion, the peptide is more resistant to heat, as evidenced by the lack of significant change in the chromatogram following heat treatment (Fig. 5B). Additionally, the chromatogram of a sample stored refrigerated for two months is identical to that of the standard (Fig. 4A) or a freshly prepared dispersion (Fig. 5A).

The chemical integrity of insulin when processed as a peptide nanoparticle was further verified using SDS-polyacrylamide electrophoresis under reducing conditions.

The electrophoretic mobility of the insulin in the nanoparticle dispersion is identical to that of the unprocessed peptide. In addition, SDS-PAGE provided confirmatory datum regarding the presence of intact peptide in the fraction of the nanoparticulate dispersion that pellets when centrifuged and fractionated into a soluble and particulate component (Fig. 6). Using SDS-PAGE, insulin was not detected in the supernatant or the soluble fraction following centrifugation. Using HPLC to quantify these findings, 97.3% of the peptide was shown to be associated with the particulate fraction with only 2.7% of the sample detected in the supernatant.

As demonstrated in Fig. 7, the insulin nanoparticles generated using this water-based wet milling approach formed a physically stable homogeneous dispersion. The data show the particle size distribution profiles and the corresponding light micrographs of an insulin dispersion assayed immediately after processing (Fig. 7A), and after refrigerated storage as a liquid dispersion for 2 weeks (Fig. 7B) and 34 months (Fig. 7C). Though the mean particle size of this preparation increases slightly during storage from an initial mean particle size of 0.105 μm (Fig. 7A) to 0.137 μm (Fig. 7C), the particle size distribution profile is unimodal with 100% of the particles being less than 0.400 μm. These results were verified using light microscopy. There was no evidence of crystal growth, aggregation and/or flocculation.

To evaluate the biologic activity of the insulin nanoparticulate preparation described above, blood glucose and insulin levels were monitored in a hyperglycemic rat model after dosing with either a freshly prepared insulin solution or with the milled nanoparticulate dispersion. Figure 8 compares the efficacy of the two insulin formulations. The formulations were effective in lowering blood glucose levels throughout the time course of the study. The effects were independent of the route of administration. For animals dosed subcutaneously, the solution and nanoparticle dispersion of insulin elicited a comparable response. In addition, as shown in Table I, pharmacokinetic data for subcutaneously injected insulin are similar for both formulations. Following intraduodenal dosing, the nanoparticle preparation appears to have an advantage. For animals dosed with the insulin nanoparticle preparation, there was a noticeable 50% reduction in blood glucose levels. This reduction in blood glucose levels was maintained throughout the study. In comparison, animals dosed with a solution of insulin experienced a steady rise in blood glucose levels after 1-h post-dosing. At the termination of the study, animals dosed with the solution had significantly higher blood glucose levels in comparison to the initial reading and to val-

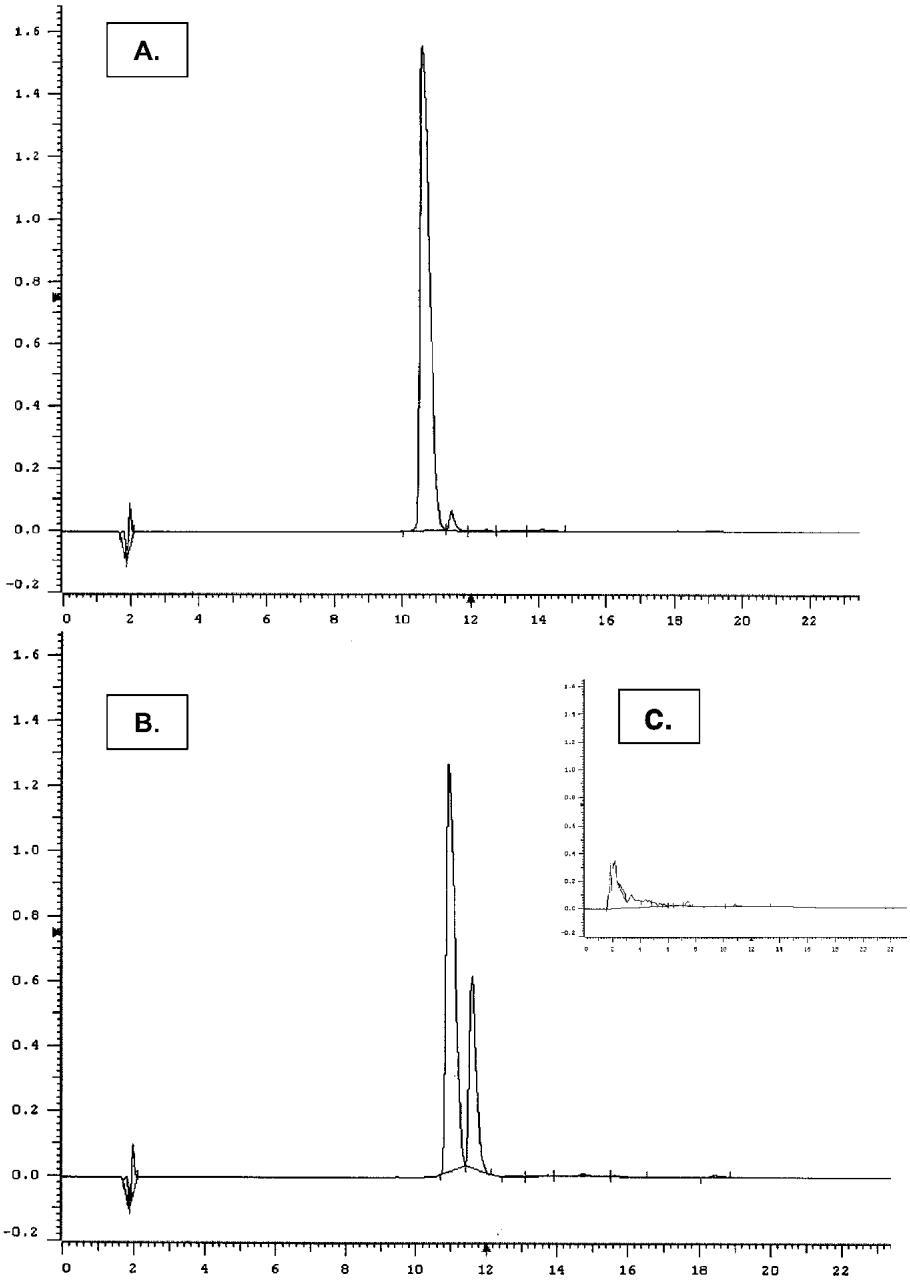

Fig. 4. The HPLC was performed on insulin before (A) and after heat treatment at 70°C for 100 min (B). The heat treatment brings about a significant change in the percent of sample having retention times ~10.88 min (97.8% in "A" to 72.3% in "B") and ~11.64 min (2.2% in "A" to 27.7% in "B"). The insert (C) shows the chromatogram of a sample of insulin solubilized in 1N NaOH and stored for one week at room temperature.

ues observed for the animals dosed with the nanoparticulate preparation. However, as summarized in Table I, the pharmacokinetic data for both formulations dosed intraduodenally were highly variable. Additional studies are needed to address this issue and to establish the actual bioavailability of the nanoparticulate insulin formulations following oral dosing.

DISCUSSION

The objective of this study was to determine if a formulation approach that is applied to poorly water-soluble small molecules would be useful in developing an alternate formulation approach for insoluble peptides. The use of wet milling technology to formulate poorly water-soluble compounds into drug nanoparticles has been applied successfully over the last few years (11, 12, 20). For this process, water, stabilizer and a poorly water-soluble compound (aqueous solubility <1 mg/ml) are wet milled into a fine particle dispersion, wherein, 100% of the particles are <1 μm. The stabilizer is a critical component of the formulation and process. For effective comminution, the stabilizer must wet the drug surface and provide a means to stabilize the particles either through steric or ionic interactions. As a drug delivery platform, nanoparticles have

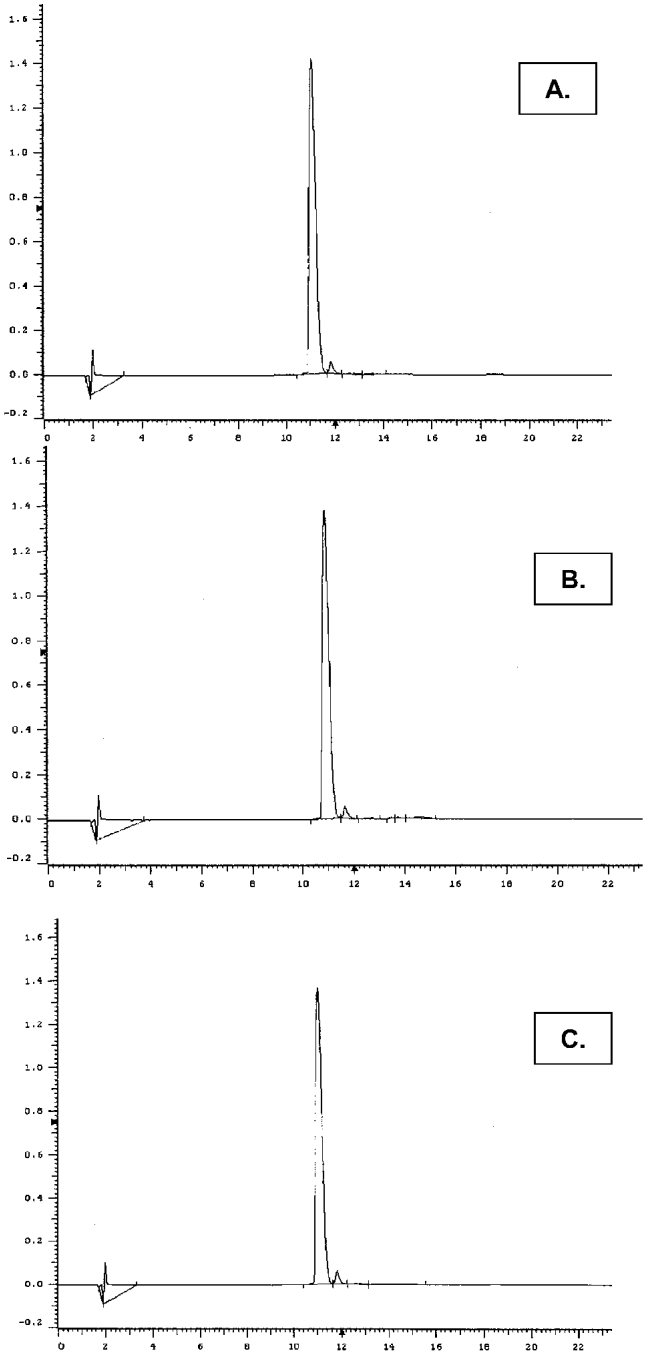

Fig. 5. The HPLC was performed on nanoparticles of insulin before (A) or after being challenged with either a heat treatment at 70°C for 100 min (B) or refrigerator storage for 2 months as a liquid dispersion (C). No significant changes were detected in the chromatograms. For the nanoparticles of insulin, the heat treatment did not shift the percentage of peak area eluting at ~10.88 min (97.6% in "A" to 97.5% in "B") and 11.64 min (2.4% in "A" and 2.5% in "B").

been shown to provide a number of advantages e.g., improved bioavailability, improved stability and a delivery platform acceptable for most commonly used routes of administration. These goals are shared by most drug delivery approaches attempting to improve peptide performance. Therefore, it was of interest to determine if Zn-insulin, being poorly water-soluble, could be processed using media milling technology.

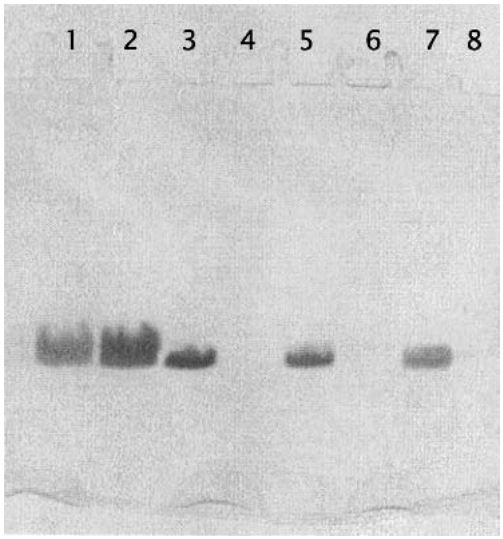

Fig. 6. SDS-PAGE was performed under reducing conditions as described in "Materials and Methods" to determine the amount of insulin associated with the particulate or soluble fraction following centrifugation. The pellet (the particulate fraction in lanes 3, 5, and 7) and the supernatant (soluble fraction in lanes 4, 6, and 8) were analyzed by SDS-PAGE. For comparison, lanes 1 (5 μg/ml) and 2 (10 μg/ml) show the electrophoretic mobility of unprocessed insulin. SDS-PAGE was stained for proteins using Coomassie blue.

Insulin was chosen for this study for a number of reasons. Insulin remains one of the most important and widely used therapeutics developed in the last century and their continues to be interest in identifying new methods for improving patient compliance. Also, of primary importance for this study, insulin complexed with zinc is poorly soluble in water at neutral pH and is a potentially interesting candidate for processing using wet milling technology. As shown in Figs. 1–3, insulin powder containing 0.5% zinc was readily processed into a physically stable, nanoparticulate dispersion. The particle size and processing time were similar to those reported for poorly water-soluble small molecules. The selection of a compatible stabilizer system was performed empirically. Though various stabilizers were tested during the initial phase of this study, pluronic F68 in combination with sodium deoxycholate provided acceptable results. Insulin was processed into a stable nanometer-sized particulate dispersion. It was surprising that nano-insulin was stable when stored refrigerated for 34 months. The particle size distribution profiles and light micrographs shown indicate that following prolonged storage, the insulin nanoparticulate formulation was very homogenous and monodisperse. Also, chemical stability data were acceptable when comparing the HPLC chromatograms of freshly prepared insulin to a nanoparticle formulation stored for 2 months under refrigeration conditions. There were no significant changes detected. In addition, the absence of significant degradation following heat treatment of the nanoparticle formulation, suggests that formulating insulin as a peptide nanoparticle dispersion shields the molecule from insults that could significantly impact its chemical integrity. Though the Zn-insulin used in this study is non-crystalline, wet milling technology is ideally suited for crystalline drug substances. Data generated on poorly water-

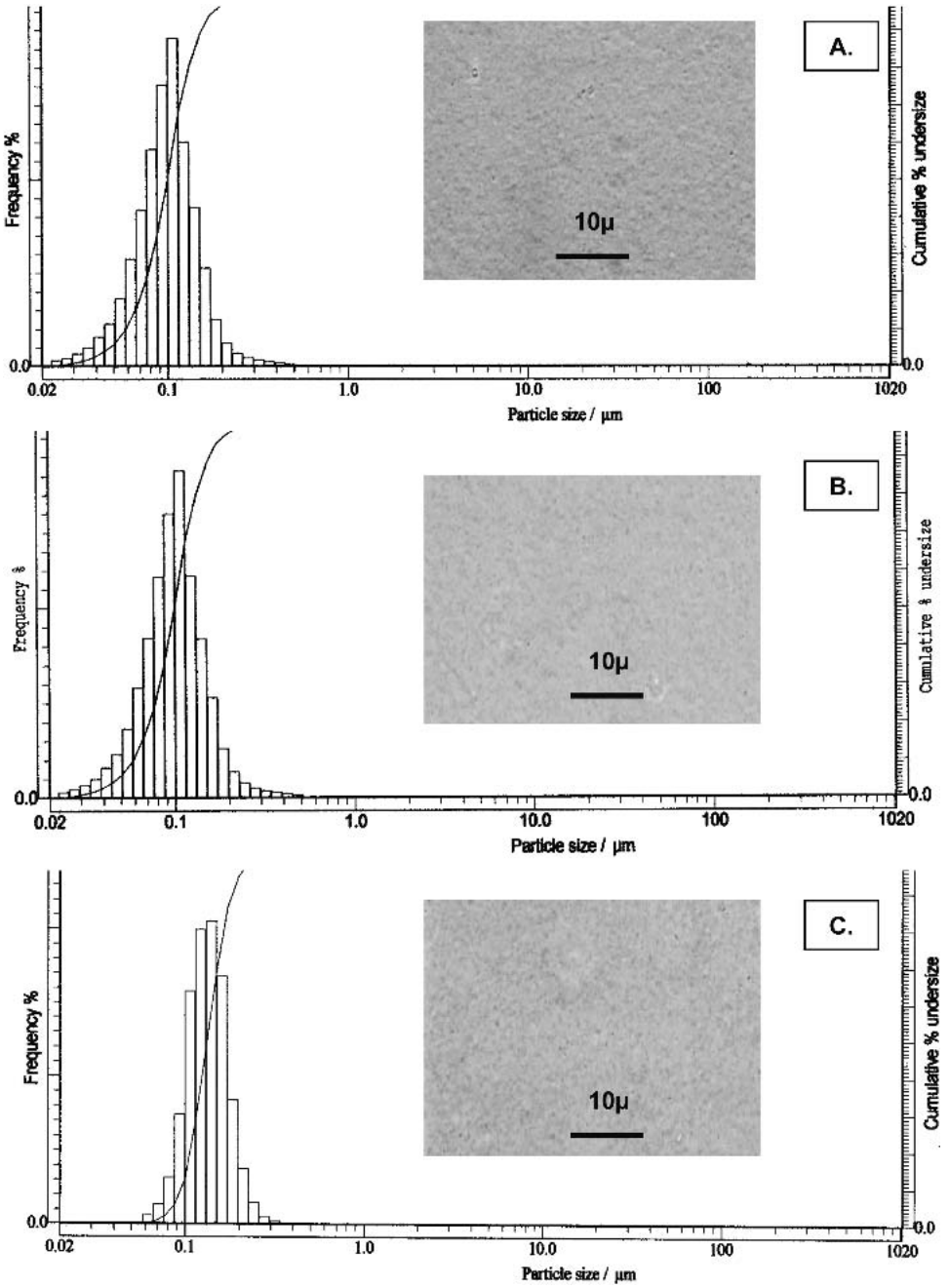

Fig. 7. The physical stability of insulin formulated as a peptide nanoparticle formulation is demonstrated using laser light diffraction and light microscopy. In (A) the dispersion was assayed immediately after processing or after refrigerated storage as a liquid dispersion for 2 weeks (B) or 34 months (C). The mean particle size of the preparations are as follows: (A) = 0.105 μm, (B) = 0.106 μm, and (C) = 0.137 μm.

soluble small molecules (e.g., danazol) show that this process retains the crystalline nature of unprocessed crystalline powder (16). X-ray diffraction studies performed on the milled and unmilled insulin used in this study showed the powder to be non-crystalline before and after processing as anticipated (data not shown).

The nanoparticles of insulin, described in this study are biologically active as demonstrated in Fig. 8. Though more extensive testing will be needed to determine if this approach offers any advantages over other delivery platforms, the data presented are intriguing. The nanoparticles of insulin, in comparison to a solution, have a relatively prolonged hypoglycemic effect and, surprisingly, are effective after intraduodenal dosing. There have been a number of successful approaches for peroral delivery of insulin (21). Since the nanoparticles described in this study do not involve encapsulation technology, the association of insulin in the presence of stabilizers during the milling process produces a particulate entity that must provide some degree of protection to the peptide. Whether the protection is a result of the association of the

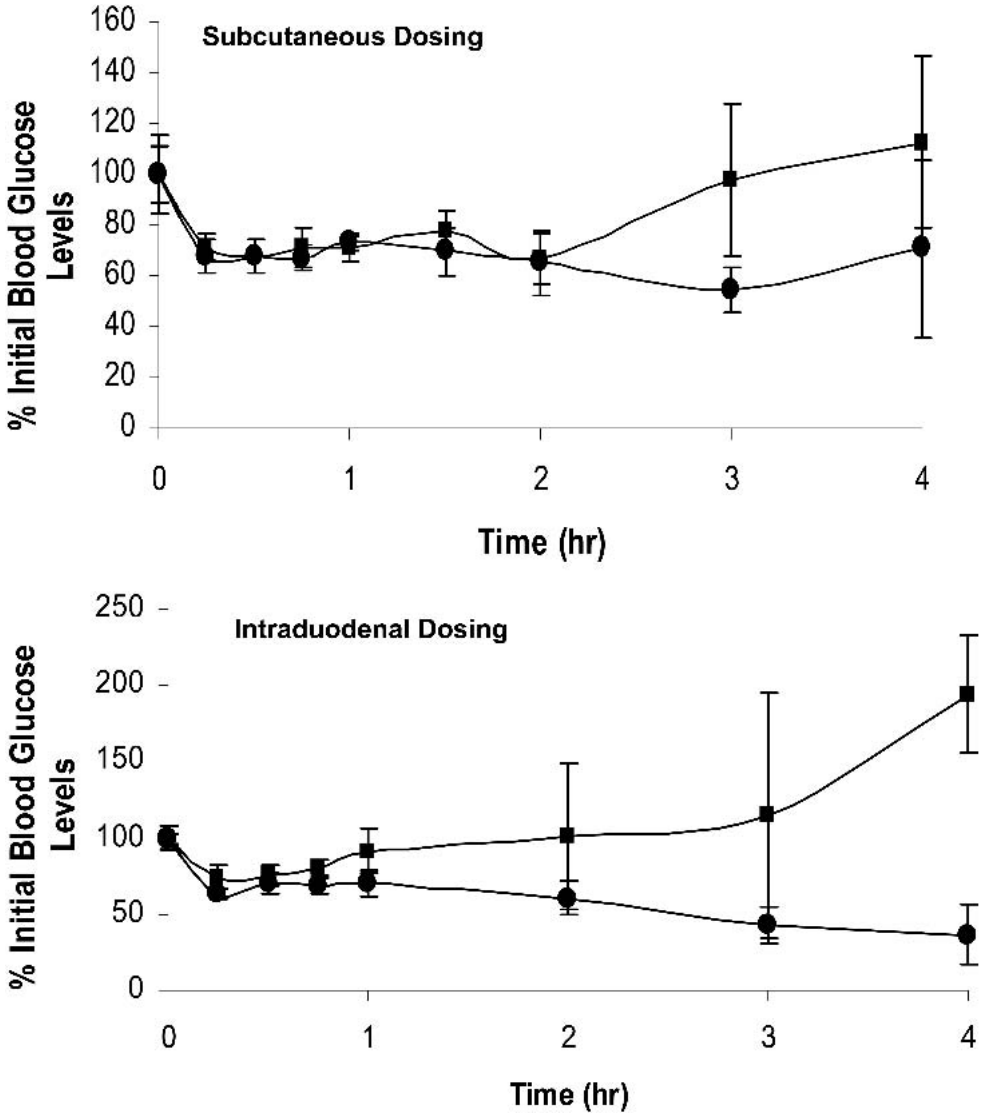

Fig. 8. The percent change in blood glucose levels was monitored with time after dosing hyperglycemic rats with insulin formulated as a nanoparticle dispersion (●) or as a solution (■). Blood glucose levels were monitored throughout the time course of the study for animals dosed subcutaneously (top) or intraduodenally (bottom). Each data point represents the mean results from six animals.

Table I. Pharmacokinetic Parameters of Nanoinsulin Versus Insulin Solution

Route of injection[a]	Nanoinsulin[b]	Insulin solution[c]
Subcutaneous		
AUC (μU/ml · h)	727 ± 187	534 ± 96
C_{max} (μU/ml)	472 ± 133	434 ± 82
T_{max} (h)	0.5 ± 0.2	0.3 ± 0.1
Intraduodenal		
AUC (μU/ml · h)	80 ± 112	0.5 ± 0.2
C_{max} (μU/ml)	31 ± 36	0.2 ± 0.02
T_{max} (h)	0.8 ± 0.7	0.8 ± 1.0

[a] Study was performed as described in "Materials and Methods."
[b] Nanoinsulin is the nanoparticulate insulin dispersion stabilized with F68 and sodium deoxycholate.
[c] Reference: Control insulin administered in PBS as a solution.

insulin monomers and/or involves the adsorption of stabilizer remains to be determined. In addition to protection, in order to maintain biologic activity, the nanoparticles must dissolve or dissociate into free monomers with time after dosing. This more than likely produces the prolonged hypoglycemic effect that is observed. By altering the particle size of the preparation and/or the surface properties of the particles it may be possible to optimize release properties and enhance the therapeutic effectiveness of the molecule. As to how effective this drug delivery platform is relative to other technologies available for peptides and proteins remains to be established. Future studies are being designed to gain further credence to support the potential utility of using this approach for insulin therapy and to determine the general applicability of this approach for other poorly water-soluble peptides and proteins.

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