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Oral delivery of insulin loaded poly(fumaric-co-sebacic) anhydride microspheres

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Abstract

The bioadhesive polymer, poly(fumaric-co-sebacic) anhydride, p(FASA), was used to fabricate small diameter insulin microspheres and evaluate their in vivo performance in a type 1 diabetic rat as well as a type 1 diabetic dog model. The process of phase inversion nanoencapsulation was used to fabricate p(FASA) microspheres containing insulin. Using laser diffraction spectrometry, 90% of the microspheres used in the fed double dose rat experiments were found to have a volumetric diameter of 5.9 μm or smaller. In comparison, 90% of the microspheres used in fed single dose rat experiments were found to have a volumetric diameter of 2.6 μm or smaller while the microspheres used in the diabetic dog experiments were found to have a volumetric diameter of 1.2 μm or smaller. Insulin microspheres administered to diabetic rats in the fed double dose experiment produced a relative bioavailability (RB) of 23.3% while insulin microspheres administered to diabetic rats in the fed single dose experiment produced a RB of 5.5 ± 1.7%. Insulin microspheres administered to fasted diabetic dogs produced a RB of 5.5 ± 3.4%.

Keywords: Insulin; Microsphere; Oral delivery; Polyanhydride

1. Introduction

The oral delivery of insulin remains an elusive goal to which many investigators aspire. However, there are several obstacles that limit the oral bioavailability of peptides and proteins. One of the major obstacles is the harsh environment of the gastrointestinal (GI) tract, which by its very nature, is intended to break down proteins and peptides into their constituent amino acids. In addition to enzymatic barriers, there are also physical barriers which make the oral delivery of a peptide or protein difficult (Lee and Yamamoto, 1989). The lining of the GI tract is composed of a thick wall of epithelial cells covered by a layer of polysaccharides and mucus, which can inhibit the oral delivery of a peptide such as insulin via the hepatic portal vein to the liver.

Several approaches have been taken to improve the oral bioavailability of therapeutic proteins such as insulin. These strategies include the use of protease inhibitors, permeation enhancers, enteric coatings, and microsphere encapsulation (Mathiowitz et al., 1997; Agarwal et al., 2001; Ma et al., 2005; Toorisaka et al., 2005; Katsuma et al., 2006; Morishita et al., 2006; Zhang et al., 2006; Damge et al., 2007). Of these techniques, microsphere encapsulation is the only oral drug delivery vehicle that has the potential to surpass both the enzymatic and physical barriers of the GI tract.

It was demonstrated that lectin modified solid lipid nanoparticles containing insulin orally administered to rats resulted in relative bioavailabilities of between 4.99% and 7.11% (Zhang et al., 2006). Another group successfully maintained plasma glucose levels at pre-diabetic levels for 11 h after the oral administration of chitosan and insulin nanoparticles to diabetic rats (Ma et al., 2005). In addition, microparticles composed of poly(methacrylic acid) and poly(ethylene glycol) and containing insulin that were orally administered to type 1 diabetic rats resulted in suppressed postprandial blood glucose levels (Morishita et al., 2006).

Our group fabricated microspheres utilizing a blend of fumaric acid oligomer and poly(lactide-co-glycolic acid) (p(FAO:PLGA)) and insulin. In a glucose tolerance test, this formulation was fed to normoglycemic rats resulting in blood

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glucose levels which did not increase above fasting levels and which were statistically lower than the saline-fed controls and unencapsulated insulin controls (Carino et al., 2000). In a separate experiment, this formulation was then orally administered to two normoglycemic rats in the fasted state resulting in an oral efficacy of 11.4% calculated from average deviations of plasma glucose levels (Mathiowitz et al., 1997).

In addition, it has also been shown that the nature of the polymer may actually increase the residence time of a drug delivery system within the GI tract. Our group has demonstrated that a drug encapsulated within the hydrophobic bioadhesive polymer p(FASA) can prolong its residence time within the GI tract, possibly resulting in increased intestinal drug absorption (Chickering et al., 1997). However, bioadhesion is only one of the factors to be considered when formulating an oral drug delivery system. If the residence time of the particle within the GI tract can be prolonged through use of a bioadhesive polymer, this may increase the chance of particle uptake by the enterocytes of the small intestine. To increase the likelihood of intestinal particle uptake, it has been shown that microsphere size should not exceed 5 μm in diameter and that particle uptake increases as sphere size decreases (Florence et al., 1995; Carr et al., 1996; Desai et al., 1996).

The goal for the current study is to utilize the bioadhesive polymer, p(FASA), to fabricate small diameter microspheres loaded with insulin and to evaluate their in vivo performance in a type 1 diabetic rat as well as a type 1 diabetic dog model.

2. Material and methods

2.1. Materials

For the fed single dose type 1 diabetic rat and fasted type 1 diabetic dog experiments, 20:80 poly(fumaric-co-sebacic) anhydride p(FA:SA) (MW = 9 K) donated by Spherics, Inc. (Lincoln, RI) was used in the fabrication of microspheres. For the fed double dose type 1 diabetic rat experiments, 20:80 (p(FASA)) (MW = 14.5 K) synthesized by melt polycondensation in our lab was used in the fabrication of microspheres. Bovine zinc insulin (BZI) was obtained from Gibco Life Technologies (Rockville, MD). Insulin was first micronized by a precipitation method resulting in an average particle size of 300 nm (Furtado et al., 2006). Poly(ethylene glycol) (PEG) (MW = 4.5 K) and sorbitan trioleate (Span 85) were obtained from Sigma, Inc.

2.2. Methods

2.2.1. Microsphere fabrication

A phase inversion nanoencapsulation (PIN) technique was used to fabricate insulin containing microspheres (Mathiowitz et al., 1997; Jacobs and Mathiowitz, 2004). While the PIN method of encapsulation was utilized in the fabrication of microspheres for all experiments, slight modifications were made in order to produce microspheres with smaller diameters. For the fed double dose rat experiments, 20:80 p(FA:SA) (MW = 14.5 K) polymer was loaded with 2% bovine zinc insulin (BZI) (w/w) in the following manner. Briefly, the BZI was probe sonicated for 20 s in methylene chloride to form a suspension (solvent phase). The p(FASA) was then added and vortexed until dissolution (∼1 min). The polymer/insulin solution was then quickly poured into petroleum ether (non-solvent phase) at a 1:100 ratio resulting in the spontaneous precipitation of microspheres. The solution was allowed to equilibrate for 2 min and was then filtered under pressure using a 0.22 μm Millipore filter. Finally, the microspheres were collected and stored at −20 °C.

For the fed single dose rat experiments, microspheres were fabricated as described above but in addition, 0.2% Span 85 (v/v) was added to the non-solvent petroleum ether and stirred for 5 min at 500 rpm prior to the addition of the solvent phase. Also, the molecular weight of the p(FASA) used for these microspheres was 9 kDa.

Microspheres fabricated for the type 1 diabetic dog experiments utilized the polymers p(FASA) and PEG. Insulin was loaded into p(FASA) microspheres as described in the fed double dose rat experiments. In addition, a 2% (w/v) solution of PEG (MW = 4.5 K) was dissolved in acetone. Instead of petroleum ether, 2% (v/v) Span 85 was added to the non-solvent pentane in two separate beakers and stirred for several minutes at 500 rpm. The insulin/p(FASA) solution was then added to one of the beakers containing pentane/Span 85 and the PEG solution was added to the other beaker containing pentane/Span 85. In a separate 2 L beaker, 1000 ml of fresh pentane was added prior to adding first the PEG solution and second the insulin/p(FASA) solution. This solution was then filtered utilizing a 0.45 μm Millipore filter. An additional 500 ml of pentane was then used to wash the microspheres prior to collection and subsequent and storage at −20 °C. For all microsphere formulations, the percent loading of insulin was determined as described by Furtado et al. (2006).

2.2.2. Characterization

A scanning electron microscope (Model S-2700 Hitachi Ltd., Tokyo, Japan) was used to visualize microsphere structure and morphology. Microspheres were coated using an Emitech K550 sputter coater for approximately 3 min, which resulted in ∼25 nm layer of gold covering the sample.

A laser diffraction spectrometer (Beckman Coulter, Fullerton, CA) was used to determine the mean diameter of microspheres based on volume statistics. Microspheres were first suspended in 1% hydroxypropylmethyl cellulose (HPMC) (N.F. Grade: 2910; Viscosity Grade: 15 cP): pluronic F127 at a ratio of ∼5 mg/ml. The suspension was then briefly vortexed and bath sonicated to optimize the suspension.

2.2.3. Animal models

Diabetes prone (BBDP) male rats (Biomedical Research Models, Worcester, MA) were utilized for type 1 diabetic rat experiments as described by Furtado et al. (2006). Two female type 1 diabetic beagle dogs (Marshall Farms, North Rose, NY) were utilized for the larger animal model experiments. On non-experimental days, dogs were housed in individual cages and were given food and water *ad libitum*. Blood glucose level testing was performed twice daily in the morning and evening hours by obtaining a blood sample taken via the cephalic vein while the

dogs were conscious. Blood glucose levels (BGL) were determined using a OneTouch® blood glucose meter (LifeScan, Inc., Milpitas, CA). Dogs were maintained on Humulin Regular and Humulin Lente recombinant human insulin obtained from Eli Lilly and Company, USA.

Diabetic rat and diabetic dog experiments followed Institutional Animal Care and Use Committee (IACUC) guidelines for the use and care of laboratory animals.

2.2.4. Diabetic rat experimental protocol

For all diabetic rat experiments, rats were first anesthetized in a 4% isoflurane gas chamber. Rats were then placed on nosecones and maintained on 1–2% isoflurane for the initial blood sample which was taken via tail bleed. For control formulations, blank microspheres (contain p(FASA) only) were suspended at 50–100 mg/ml in 1% HPMC: pluronic F127, vortexed, sonicated and administered to rats by oral gavage. An additional control was performed whereby blood samples were obtained when rats had received neither a control nor an experimental formulation. Two experiments were performed in which insulin containing microspheres were administered by oral gavage to type 1 diabetic rats in the fed state: (1) a single dose of microspheres at a theoretical dose of 250 IU/kg and (2) a double dose of microspheres at a dose of 125 and 75 IU/kg. Experimental formulations were prepared and administered correspondingly to the control formulations. Blood samples were taken from tail bleeds at serial points postoperatively using rat restraint tubes while the rats were conscious. Blood was collected in heparinized tubes, spun down and the plasma recovered for glucose and insulin analysis.

2.2.5. Fasted type 1 diabetic beagle dog experimental protocol

In the following experiments, the dogs were fasted overnight but were allowed access to water prior to and throughout the course of the experiments. In addition, the evening dose of insulin given on the previous non-experimental day was withheld. The last dose of daily maintenance insulin the dogs received was 24 h prior to the start of an experiment. This was short acting insulin which peaked in approximately 5–6 h. Prior to a study beginning, a predose sample of blood was taken via the cephalic vein while the dogs were conscious. Subsequent blood samples were obtained at 0.5, 1.0, 1.5, 3.0, 4.5 and 6.0 h post-initial blood sample. Blood glucose levels (BGL) were determined throughout the experiments using a OneTouch® blood glucose meter (LifeScan, Inc., Milpitas, CA). Dogs were conscious throughout the entirety of all experiments including blood sampling and oral gavaging.

Four experiments were performed. The first experiment was a control whereby blood samples were obtained when dogs had received neither a control nor an experimental formulation. The second experiment was a subcutaneous (s.c.) injection of unencapsulated BZI at a dose of 2.0 IU/kg. The reason for such a seemingly low dose of insulin is due to the fact that the dogs have been fasted; therefore, a dose any higher may have resulted in hypoglycemia and death. This experiment was performed for comparison purposes for relative bioavailability (RB) calculations.

The third and fourth experiments were oral administration studies in which both the unencapsulated and the encapsulated insulin formulation were administered by gastric gavage. For the unencapsulated insulin control, insulin was dissolved in 50 ml of 0.01N hydrochloric acid (HCl) (pH ∼ 1–2) and administered via a feeding tube placed directly into the stomach of the dogs. Tubes were immediately flushed with 10 ml of water to rinse any remaining insulin from the tube into the stomach. For the encapsulated insulin formulation, microspheres were suspended in 50 ml of distilled water by bath sonication at a theoretical dose of 50 IU/kg to one of the dogs and at a theoretical dose of 25 IU/kg to the second dog. Tubes were immediately flushed with 10 ml of water. The insulin containing formulation was repeated several times in each of the dogs due to the fact that there were only two dogs.

2.2.6. Blood analysis

A glucose trinder assay (Diagnostic Chemicals Limited, Oxford, CT) was used to determine plasma glucose levels for the rat experiments. For the detection of exogenous BZI in the plasma, an insulin ELISA was used (Diagnostic Systems Laboratories, Webster, TX).

2.2.7. Relative bioavailability calculations

The relative bioavailability (RB) was calculated by comparing the oral administration of insulin containing microspheres to a s.c. injection of unencapsulated BZI for the same rat according to Eq. (1):

$$\text{relative bioavailability} = \frac{[\text{AUC}]_{\text{PO}}/\text{dose}_{\text{PO}}}{[\text{AUC}]_{\text{s.c.}}/\text{dose}_{\text{s.c.}}} \times 100 \quad (1)$$

where AUC is the area under the curve measured using the trapezoidal rule by plotting average plasma insulin level over time, PO is the insulin containing microsphere formulation administered orally and s.c. is the subcutaneous injection of unencapsulated insulin. For dog experiments, RB was calculated according to Eq. (1) utilizing average deviations of blood glucose levels where AUC is the area under the average glucose level versus time curve.

3. Results and discussion

3.1. Characterization of microspheres

To prepare our microspheres, we utilized the process of phase inversion nanoencapsulation (PIN) which is a very useful encapsulation technique for hydrophobic polymers and hydrophilic drugs (Mathiowitz et al., 1997; Carino et al., 2000; Furtado et al., 2006). Unlike traditional microencapsulation techniques such as solvent removal or solvent evaporation, PIN involves the spontaneous formation of microspheres from a continuous phase. While other scientists (Ma et al., 2005; Damge et al., 2007) have used the process of solvent evaporation or spontaneous formation of microspheres to successfully encapsulate insulin, polyanhydrides such as p(FASA) could not be utilized

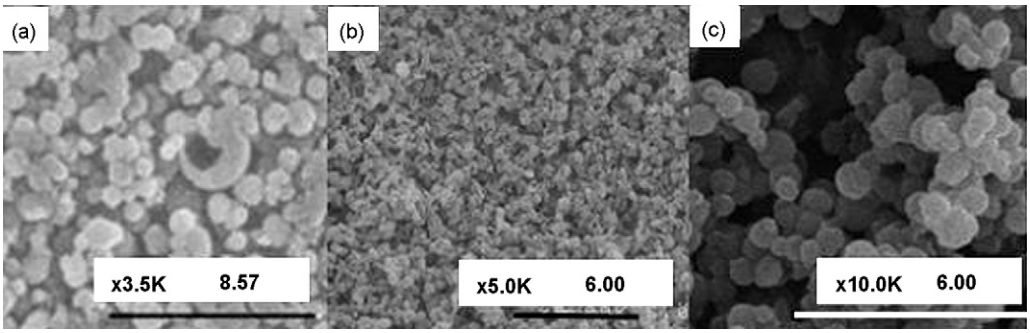

Fig. 1. A SEM of the microspheres used in (a) fed double dose diabetic rat experiment, (b) fed single dose rat experiment and (c) fasted single dose dog experiment.

to fabricate microspheres by either of those methods due to the presence of an aqueous phase.

For the fed double dose rat experiments, the insulin percent loading was determined by dividing the calculated experimental loading by the theoretical load, multiplying by 100 and was determined to be approximately 100% (Furtado et al., 2006). For the fed single dose rat experiments and the fasted diabetic dog experiments, the insulin percent loading was determined to be approximately 50%.

A SEM of a representative sample of the microspheres used in the experiments can be seen in Fig. 1. Fig. 1(a) shows a SEM of the microspheres used in the fed double dose rat experiments, Fig. 1(b) shows a SEM of the microspheres used in the fed single dose rat experiments and Fig. 1(c) shows a SEM of the microspheres used in the fasted dog experiments. Using laser diffraction spectrometry, 90% of the microspheres used in the fed double dose rat experiments were found to have a volumetric diameter of 5.9 μm or smaller. In comparison, 90% of the microspheres used in fed single dose rat experiments were found to have a volumetric diameter of 2.6 μm or smaller while the microspheres used in the diabetic dog experiments were found to have a volumetric diameter of 1.2 μm or smaller. Because it has been shown that microsphere size should not exceed 5 μm in diameter and that particle uptake increases as sphere size decreases (Florence et al., 1995; Carr et al., 1996; Desai et al., 1996), it was the intention of our group to fabricate microspheres with progressively smaller diameters. However, because these size optimizations occurred over a period of time, all formulations were not able to be tested in each group of animals.

3.2. Fed single dose diabetic rat experiments

Fig. 2 is the percent initial plasma glucose concentration (PGC) versus time curve for insulin containing microspheres administered orally at a theoretical dosage of 250 IU/kg ($n = 9$) and for a control in which blood samples were obtained when rats had not been administered unencapsulated insulin nor an experimental formulation ($n = 15$). As can be observed, the rats' PGC steadily increases from initial levels until the 8 h timepoint at which point the PGC attains its maximal value of 153%. Glucose levels only decrease slightly to 143% of initial levels by the final timepoint of the experiment. This is the expected outcome of the experiment as rats did not receive insulin in any form. In addition, rats were allowed access to food *ad libitum* throughout the experiment.

For the insulin containing formulation, PGC decreases to below initial PGC by the first timepoint of the experiment and remains below initial levels through the 6 h timepoint. As can be seen, this in stark contrast to the control experiment in which PGC remains above initial levels throughout the entirety of the experiment. The response to the insulin containing formulation in which there is a slight decrease rather than a large decrease in PGC, more closely resembles the physiological response of a non-diabetic individual in that glucose homeostasis is maintained in the postprandial state (Del Prato, 2003).

Other researchers demonstrated their ability to reduce the plasma glucose levels of fasted diabetic rats after oral administration of insulin containing microparticles. Utilizing the polymers polycaprolactone (PCL) and Eudgragit, Damge et al. were able to reduce the areas under the glycemia versus time curve by 23% and 38% for dosages of 50 and 100 IU/kg, respectively in comparison to controls (Damge et al., 2007). Additionally, Ma et al. achieved pharmacological availabilities of 4.4% and 3.2% for 50 and 100 IU/kg dosages of chitosan–insulin nanoparticles administered to fasted diabetic rats (Ma et al., 2005). While our work demonstrates that slight decreases in initial plasma glucose concentrations are possible, it is important to note that we performed our experiments in the fed

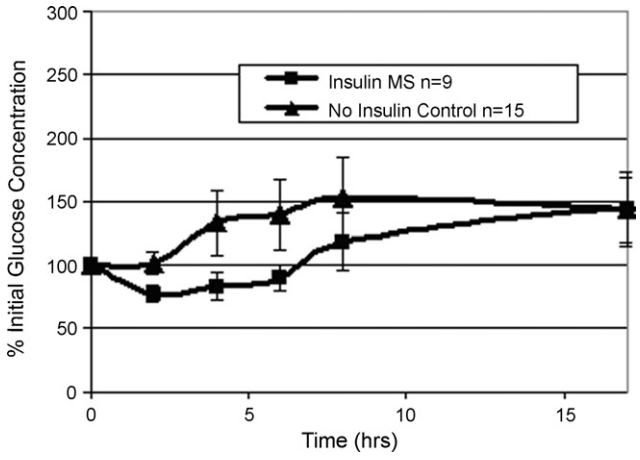

Fig. 2. Percent initial glucose concentration (mean ± S.E.) vs. time curve for insulin microspheres (at a theoretical dose of 250 IU/kg) and a no insulin administration control to diabetic rats in the fed state.

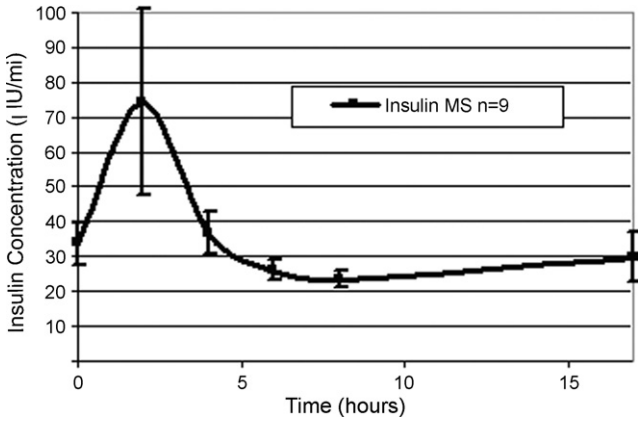

Fig. 3. Plasma insulin concentration (μIU/ml) vs. time curve for insulin containing microspheres administered orally at a theoretical dosage of 250 IU/kg (mean ± S.E.) to diabetic rats in the fed state.

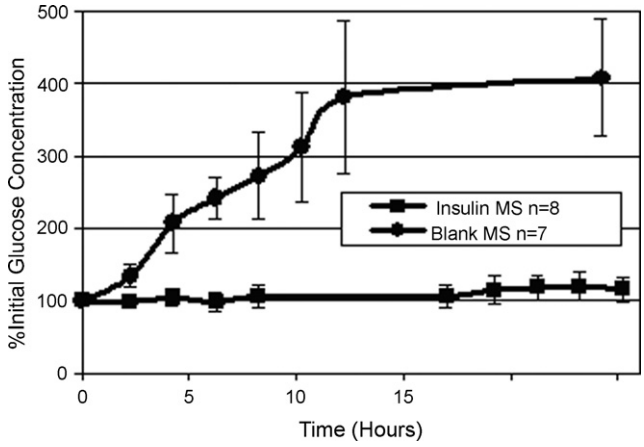

Fig. 4. Percent initial glucose concentration (mean ± S.E.) vs. time curve for blank microspheres (contain p(FASA) only) and insulin containing microspheres administered orally at 125 and 75 IU/kg to diabetic rats in the fed state.

state, that is, diabetic rats were not fasted prior to the oral administration of insulin containing microspheres. Likewise, rats were allowed access to food throughout the experiment.

Fig. 3 is the plasma insulin concentration (μIU/ml) versus time curve for the insulin containing formulation. In the control experiment, insulin was not detected in the plasma (results not shown). This was the expected outcome as insulin was not administered to the rats at any point throughout the experiment. For the insulin containing formulation, insulin was detected in the plasma as evidenced by the insulin ELISA. The peak insulin concentration occurred at the 2 h timepoint and was determined to be 74.2 μIU/ml. Insulin was also detected at the 4 h timepoint and was determined to be 36.6 μIU/ml. The average rat plasma insulin level was used to calculate the relative bioavailability according to Eq. (1) and was determined to be 5.5 ± 1.7%.

While other researchers have demonstrated their ability to detect insulin in the plasma of diabetic rats, values obtained for insulin containing formulations were not different from values obtained from rats receiving control formulations (Ma et al., 2005). Additionally, peak concentrations obtained were between 10 and 20 μIU/ml for diabetic rats receiving insulin containing formulations in the fasted state (Damge et al., 2007) while the peak concentration obtained after the oral administration of our insulin containing formulation in the fed state was 74 μIU/ml.

3.3. Fed double dose diabetic rat experiments

In the following experiment, insulin containing microspheres were administered by oral gavage to diabetic rats ($n = 8$) in the fed state at $t = 0$ and $t = 16.75$ h. Fig. 4 shows the results plotted as percent initial glucose concentration versus time for the blank microsphere control and for the experimental formulation. When blank microspheres are administered orally ($n = 7$), PGC quickly increases to 200% of initial levels by the 4 h timepoint. PGC continues to increase throughout the entirety of the experiment reaching maximum values of almost 400% of initial levels by the final timepoint of the experiment. It can be seen that for the insulin containing formulation, plasma glucose levels remain relatively constant throughout the entirety of the experiment, with only slight increases in initial glucose levels after the administration of the second dose of microspheres at $t = 16.75$ h. These results are encouraging, considering the animal was allowed to eat and drink *ad libitum* throughout the course of the experiment.

Insulin was detected in the plasma of one of the rats which received two doses of insulin containing microspheres in the fed state (results not shown). The curve is typical of what was obtained in other experiments in which insulin was detected in the plasma, whereby a large peak that was short lasting occurs shortly after dosage (see Fig. 3). The peak concentration obtained was 1.01 mIU/ml. If this value is then compared to a s.c. injection of unencapsulated insulin, a relative bioavailability of 23.3% is achieved. Note that this calculation was based on a dosage of 125 IU/kg because the peak occurred before the next dose was administered.

While insulin was detected in the plasma of one of the rats to which a double dose of microspheres had been administered, it is interesting to note that in the single dose experiment, insulin was detected in the plasma of six of the eight rats. The major difference between the single dose and double dose experiments was the size distribution of the two formulations used and this may account for the discrepancy. In the single dose experiment in which 90% of the microspheres used were less than 2.6 μm by volume, insulin was detected in the plasma of the rats more readily than in the double dose experiment in which only 25% of the microspheres were less than 4 μm and 10% of the microspheres were less than 1.6 μm by volume.

Research supports the hypothesis that particle uptake by the enterocytes of the small intestine is size dependent; that is, smaller size particles result in greater uptake than larger size particles (Florence et al., 1995). Because of the large difference in the size distribution of the two formulations, it may be possible that a greater number of microspheres were taken up by the enterocytes of the small intestine with the smaller size formulation. An increase in the uptake of microspheres may lead to more release of insulin into the bloodstream which may

account for the increase in frequency of detection of insulin seen with the smaller size microspheres in the fed double rat experiment.

Nevertheless, it may seem contradictory that a larger insulin peak concentration was obtained in the double dose experiments in which the microspheres were larger than in the single dose experiments. Firstly, the double dose and single dose rat experiments were performed in a different group of diabetic rats; therefore, slight differences in the oral gavage technique may have occurred resulting in more or fewer microspheres reaching the enterocytes of the small intestine. Additionally, because the plasma half-life of insulin is approximately 4.5 min, it is possible that our blood sampling was not frequent enough to detect a higher insulin peak in the single dose rat experiments.

3.4. Fasted type 1 diabetic beagle dog experiments

Because there were only two diabetic dogs, the insulin containing formulation was administered several times to each of the dogs. In addition, each of the two dogs responded slightly differently to experimental and control formulations; therefore, data for each dog is plotted in a separate graph as a change in blood glucose level (BGL) over time.

Fig. 5 shows the results for the two controls and for the experimental formulation. In the control in which glucose levels were monitored when the dog received neither a control nor an experimental formulation ($n = 21$), it can be seen that BGL increases from the beginning of the experiment and remains above initial levels until the end of the experiment. When a dosage of 25 IU/kg of unencapsulated insulin is administered orally ($n = 6$), BGL does not fluctuate significantly from initial levels throughout the entirety of the experiment.

When Dog 1 is given a theoretical dosage of 50 IU/kg of the insulin containing microspheres, there is a gradual decrease in BGL over time; however, the results of the insulin microspheres

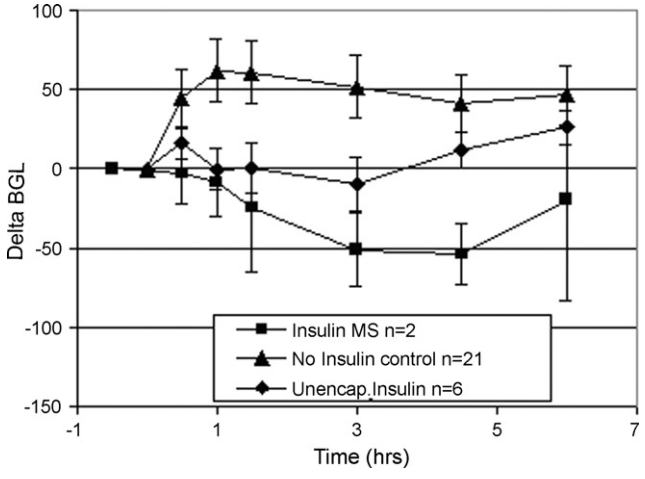

Fig. 5. The change in plasma glucose concentration vs. time curve (mean ± S.E.) for insulin containing microspheres administered orally at theoretical dosage of 50 IU/kg ($n = 2$) to Dog 1 in the fasted state vs. unencapsulated insulin administered orally ($n = 6$) at 25 IU/kg and the no insulin administration control experiment ($n = 21$).

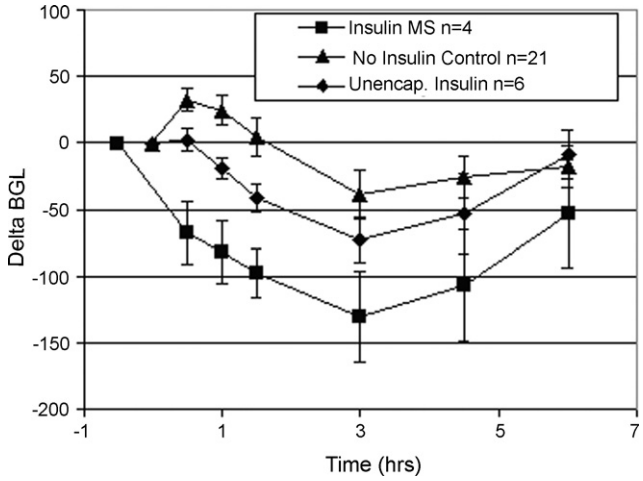

Fig. 6. The change in plasma glucose concentration vs. time curve (mean ± S.E.) for insulin containing microspheres administered orally at a theoretical dosage of 25 IU/kg to Dog 2 in the fasted state vs. unencapsulated insulin administered orally ($n = 6$) at 25 IU/kg and the no insulin administration control experiment ($n = 21$).

do not differ much from the unencapsulated insulin administered orally at least until the 4.5 h timepoint. This is in contrast to the results of the control whereby glucose levels are monitored over the course of the experiment. At no point throughout the experiment do the curves of this control and the experimental formulation overlap.

The results for the controls and insulin containing formulation for Dog 2 can be seen in Fig. 6. The 25 IU/kg dose of unencapsulated insulin administered orally gradually declines before beginning an upward trend at the 4.5 h timepoint. In the control in which BGL was monitored throughout the experiment, BGL initially increases and then decreases at the 3 h timepoint before beginning an upward trend once again. This is in contrast to Dog 1's response to the two controls whereby BGL either remains relatively constant or shows an upward trend. It is possible that for Dog 2, plasma glucose levels decrease due to the fact that the dogs had been fasted for over 24 h; therefore, glycogen stores could have become depleted faster in Dog 2 than Dog 1 resulting in declining plasma glucose levels rather than increasing levels.

Dog 2 had a greater response to the encapsulated insulin microspheres than did Dog 1 and in addition, Dog 2 had a theoretical dosage that was two times smaller than Dog 1. As can be seen from Fig. 6 when a theoretical dose of 25 IU/kg of encapsulated insulin microspheres were administered orally to Dog 2, plasma glucose levels began to decrease by the first timepoint. For the next several timepoints, Dog 2's plasma glucose levels decreased even further. Although glucose levels begin to increase by the 4.5 h timepoint, the change in concentration is still well below initial levels. By the final timepoint of the experiment, Dog 2's plasma glucose concentration is still below initial levels.

The area under the plasma glucose level curve (AUC) for Dog 2 for both the s.c. unencapsulated insulin (results not shown) and the oral insulin formulation were obtained graphically from average deviations of blood glucose levels and were then com-

pared. From this, the relative bioavailability was calculated and determined to be $5.5 \pm 3.4\%$.

Recently, a self-emulsifying insulin emulsion was developed by Ma et al. and orally administered to type 1 diabetic dogs in the fasted state (Ma et al., 2006). At a dosage of 2.5 IU/kg, a relative bioavailability of 15% was achieved. This formulation has recently been approved by the China State Food and Drug Administration to enter clinical trials. While a high relative bioavailability and sustainable insulin release was achieved with this system, similar depressions in plasma glucose levels were obtained after the oral administration of our insulin containing formulation. Additionally, our system was capable of maintaining plasma glucose levels below initial levels throughout the entirety of the experiment.

4. Conclusions

It was demonstrated that insulin containing microspheres could be successfully administered by oral gavage to type 1 diabetic rats and dogs to produce a suppression of blood glucose levels in the fasted and fed state. While other researchers have achieved similar depressions in glucose levels utilizing their insulin containing nanoparticulate systems, the size distribution of their formulations was in the 200–300 nm range (Ma et al., 2005; Damge et al., 2007). While the size distribution of our microspheres was in the 1–5 μm range, relative bioavailabilities of between 5.5% and 23% were achieved in the diabetic rat in the fed state while a relative bioavailability of 5.5% was achieved in the diabetic dog in the fasted state. The advantage of utilizing the PIN method of encapsulation over more traditional methods of encapsulation such as solvent removal or solvent evaporation is that hydrophobic bioerodible polymers such as p(FASA) which are sensitive to aqueous solutions can still be used in the production of microsphere delivery systems. Additionally, as research supports the hypothesis that particle uptake in the GI tract is inversely proportional to the size of the particle, future research will be aimed at optimizing our PIN method of encapsulation to produce particles smaller than 1 μm in diameter. The results achieved with both diabetic dogs and rats (in the fed state), demonstrate the potential of the PIN system for the oral delivery of insulin.

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