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Research Article

Theme: Sterile Products: Advances and Challenges in Formulation, Manufacturing, Devices and Regulatory Aspects
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Controlled Release of Modified Insulin Glargine from Novel Biodegradable Injectable Gels

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Abstract. The objective of this study was to investigate the duration of biological effects of modified insulin glargine released from a novel biodegradable injectable gel in type II diabetic Zucker diabetic fatty (ZDF) rats. Modified insulin glargine was purified from the marketed formulation by process of dialysis followed by freeze-drying, and the purity was confirmed by the single peak, corresponding to insulin glargine in the HPLC chromatogram. To determine and to compare the biological activity of purified insulin glargine with marketed formulation, it was suspended in isotonic saline solutions and administered subcutaneously to ZDF rats at a dose of 10 IU/kg of insulin and the blood glucose levels were measured. The blood glucose levels of ZDF rats after a subcutaneous injection of a suspension of purified insulin glargine decreased below 200 mg/dL within 2 h and remained at this level up to 6 h, then steadily raised above 400 mg/dL in 12 h. Insulin glargine particles were loaded into a novel biodegradable injectable gel formulation prepared from a blend of polylactic-co-glycolic acid (PLGA) and biocompatible plasticizers. Approximately 0.1 mL of insulin glargine-loaded gel prepared with PLGA was administered subcutaneously to the ZDF rats, and blood glucose levels were measured. The PLGA gel formulations prepared with insulin glargine particles had duration of action of 10 days following a single subcutaneous injection. The addition of zinc sulfate to the formulations prepared with purified insulin glargine particles further slowed down the drop in blood glucose concentrations.

KEY WORDS: diabetes mellitus; insulin glargine; novel biodegradable injectable gels; polylactic-co-glycolic acid (PLGA).

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia, which may be due to deficiency or ineffectiveness of metabolic hormone insulin produced by Langerhan's beta cells of pancreas. The form of diabetes characterized by absolute insulin deficiency is known as type 1. The most prevalent form of diabetes is type 2 diabetes mellitus. It accounts for 90% of the cases and is due to ineffectiveness of insulin produced by the pancreas (1–3).

The major objective of diabetes treatment is to achieve normoglycemia by maintaining appropriate concentrations of insulin in the blood throughout the day. Insulin has been complexed with protamine or zinc to delay and extend the release *in vivo* and prolong its effect for 1 day. However, these formulations have been associated with side effects such as hypoglycemia, and inter- and intrasubject variability in absorption, and shorter duration of action than expected, thus leading to poor management of diabetes (1,2,4).

For intensive blood glucose control in people suffering from type 1 and type 2 diabetes, supply of basal insulin (a steady, low concentration of insulin that is constantly present in the circulation to cover postprandial and overnight fasting periods) is very important. Protracted acting insulin preparations supply this basal concentration in clinical settings (5–7). One of the formulations that maintain basal concentration insulin is modified insulin called insulin glargine. It has been altered at two different positions: in A chain, at position 21, where the asparagine has been substituted with glycine, thus imparting more resistance to deamidation in the acidic environment and providing more stability. The C-terminus of the B chain has been elongated by the addition of two arginine molecules. This addition of two positive charges by the addition of arginines shifted the isoelectric point of the modified insulin glargine from pH 5.4 to 6.7. These modifications have made it possible to formulate insulin glargine into slightly acid stable solution, which is easy to inject and improves dose reproducibility. This insulin glargine solution forms stable hexamers

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which precipitate at neutral pH at the subcutaneous site of injection, thus forming a depot of microprecipitates of the modified insulin glargine, which then dissolve at a steady rate for a prolonged period of time (5,6,8).

Insulin and modified insulin like glargine interact with zinc ions to form hexamers. In the presence of zinc, these hexamers get further stabilized. When these hexamers are injected via subcutaneous route, they form a depot at the site of injection because of their inability to cross the capillary barriers. To get absorbed into the blood stream, these hexamers have to dissociate first into dimers and then to monomers. This leads to a delayed and extended effect of insulin and modified insulin glargine (9,10).

A novel biodegradable injectable gel system for controlled release drug delivery has been designed and characterized in our laboratory. It consists of a biodegradable polymer, polylactic-co-glycolic acid (PLGA), biocompatible plasticizer(s), and an active pharmaceutical ingredient (API). To date, several molecules, including antibiotics, opiate analgesics, and narcotic antagonists, and insulin have been successfully incorporated into the gel system, and controlled release of the aforementioned APIs have been achieved in our laboratory.

Therefore, the aim of this study was to develop an injectable biodegradable gel system which can be used to control the release of insulin glargine for one week or longer, after a single subcutaneous injection in type-2 diabetic Zucker diabetic fatty (ZDF) rats.

MATERIALS AND METHODS

Materials

Insulin glargine (Lantus®; Aventis Pharma, Parsippany, NJ) was purchased from Cardinal Health, and insulin glargine particles were extracted using dialysis bags (Spectra/Por® Float-A-Lyzer®, Biotech grade Regenerated Cellulose, 5 mL, 3.5K MWCO, Spectrum labs, Rancho Dominguez, CA). Labconco FreeZone® 12 L Console Freeze Dry System (Labconco, Kansas City, MO) was used for freeze-drying. Recombinant human insulin (insulin) powder (26.9 IU/mg, 0.4% zinc) was purchased from Diosynth France S.A. (Usine St. Charles, France). The HPLC analysis of insulin was performed on a Shimadzu HPLC system (Shimadzu Scientific Instruments, Inc., Columbia, MD). The primary column was a 250×4.6-mm Altech Macrosphere RP 300C18 5 μm column, and the guard column was 7.5×4.6 mm (Altech, Deerfield, IL), packed with same material as the primary column. HPLC grade acetonitrile (ACN), trifluroacetic acid (TFA), and distilled water were used for preparing the mobile phase. The PLGA used in this study was Medisorb® 5050 DL 1A (i.v. 0.09 dL/g) donated by Alkermers Inc. (Cambridge, MA). Triethyl citrate (TEC) and acetyltriethyl citrate (ATEC) donated by Morflex Inc. (Greensboro, NC) were used as plasticizers. Male ZDF (fa/fa) rats (Charles River Laboratories, Inc., Wilmington, MA) weighing 300 to 400 g were used for the *in vivo* studies. Blood glucose was measured using Bayer Ascensia® Breeze blood glucose meter (Bayer Corporation, Elkhart, IN). The concentration of glargine in the plasma or serum was determined using a Mercodia human insulin ELISA kit (Mercodia AB, Uppsala, Sweden). Blood glucose and glargine monitoring in the animals was stopped when the concentration of blood glucose went back to the normally high concentration.

Methods

Extraction of Insulin Glargine Particles

The Spectra/Por® Float-A-Lyzer® (dialysis bags) was removed from the refrigerator and allowed to equilibrate to room temperature. The cap was removed, and the sodium azide solution inside the bag was discarded using a pipette. The dialysis bags were then cleaned with distilled water and placed in a 1-L beaker containing 900 mL of distilled water for 1 h to remove any remaining sodium azide. The bags were removed from the beaker and again washed several times with distilled water. Insulin glargine (Lantus®) solution (5 mL) was loaded into each bag using a pipette. The insulin glargine solution-filled bags were kept in the beaker containing 900 mL of distilled water, and the beaker with the dialysis bags was placed in an ice bath and stirred continuously with a magnetic stirrer for 24 h. The distilled water in the beaker was completely replaced with fresh water after 2, 4, 8, and 12 h. After 24 h, the cap of the dialysis bag was carefully removed and the sample was recovered using a pipette. The recovered sample was placed in a prewashed 20-mL scintillation vial, which was covered with aluminum foil. Holes were punctured in the aluminum foil using an 18-gauge needle. This aluminum foil covered scintillation vial was then placed in a freezer at −80°C for 6 h. The freeze-dryer was then switched on, and vacuum was allowed to come to the maximum concentration. The samples were removed from −80°C and freeze-dried for 12 h. The dried samples were pooled together in a clean 20-mL scintillation vial and sealed with Parafilm, capped and stored at −20°C until used for further studies.

High-Pressure Liquid Chromatography Method of Insulin Glargine

The HPLC analysis of the freeze-dried insulin glargine was performed using a Shimadzu HPLC system (Shimadzu Scientific Instruments, Inc., Columbia, MD) consisting of an SCL-10A vp system controller, an LC-10AD vp pump, a DGU-14A degasser, an SIL-10 Ad vp autoinjector with a cooling system, a CTO-10A vp column oven, an SPD-10AD vp photodiode array detector and a computer loaded with the Shimadzu Class-VP 7.2 software. The primary column was a 250×4.6-mm Altech Macrosphere RP 300C18 5-μm column, and the guard column was 7.5×4.6 mm, packed with same material as the primary column.

The HPLC method was similar to the method reported by Chen Q. for analysis of insulin (11). A gradient elution method was used for analyzing the glargine. Mobile phase A was 0.15% *v/v* TFA in water and mobile phase B was 0.13% *v/v* TFA in 95% *v/v* ACN in water. The mobile phases were filtered through a Whatmann 0.45-μm nylon membrane (Whatmann International Ltd, Maidstone, England) and degassed in an ultrasonicator (Model FS60, Fisher Scientific, Fair Lawn, NJ) for 10 min before pumping it through the HPLC system.

The initial mobile phase composition of 70% solvent A and 30% solvent B was maintained at a flow rate of 1 mL/min for 2 min. Between 2 and 16 min, the percentage of solvent B was increased linearly to 50%. These conditions were maintained from 5 to 21 min. Between 22 and 25 min, the percentage of solvent B was decreased linearly to 30%. The mobile phase was pumped at a flow rate of 1 mL/min. The injection volume of each sample, including the insulin glargine standards, insulin

glargine controls, insulin glargine samples (unknown concentration), and blank samples (0.01 N HCl) without insulin glargine, was 50 μL. The temperature of the HPLC column was maintained at 30°C by using a column oven. The primary detection wavelength of insulin was set to 285 nm.

Preparation of Standard, Control, and Sample Solutions

The standard and control solutions of insulin glargine were prepared from the commercially available Lantus® solution with a concentration of 3.63 mg/mL as the working stock solution. Standard solutions were prepared from the working stock solution by transferring 50, 75, 100, 150, and 200 μL of the working stock solution to five 2-mL volumetric flasks and making up the volume with 0.01 N HCl (pH 2.0).

The insulin glargine controls were also prepared from the working stock solution with a concentration of 100 μg/mL. For sample preparations, approximately 5 mg of freeze-dried insulin glargine particles was weighed on a calibrated Cahn C-31 microbalance (Cahn Instruments, Cerritos, CA) and transferred to a volumetric flask and dissolved in 0.01 N HCl (pH 2.0). The final volume was made with 0.01 N HCl (pH 2.0). The dissolved samples were immediately loaded on the HPLC tray.

Process of Chromatographic Data

After each HPLC injection, the software generated the chromatograph of the sample and integrated the peak area of insulin glargine. The peak area of the standard solutions was then plotted against the concentration of insulin glargine. A linear regression method was used to generate the equation from which the concentration of the unknown sample could be interpolated from the peak area. The concentration of the insulin glargine control was interpolated and compared to the theoretical value. The concentration of insulin glargine samples was determined using the standard curve. The standard curve was linear (r^2, 0.9996) from 72.6 to 363 μg/mL for glargine. The intraday accuracy (in percent) and precision (in percent CV) was from 97.25% to 102.38% and 0.6% to 1.06%, respectively. The interday accuracy (in percent) and precision (in percent CV) was from 96.68% to 105.76% and 0.62% to 3.39%, respectively.

Pharmacodynamics Studies of Insulin and Insulin Glargine in ZDF Rats

All the experimental protocol and procedures for the animal studies were approved by the University of Tennessee Health Science Center's Institutional Animal Care and Use Committee. ZDF, first described in 1961, is an inbred rat model that through genetic mutation and a managed diet of Purina 5008 closely mimic human adult onset diabetes (type 2) and related complications (12,13). When fed a diet of Purina 5008 (14), homozygous recessive males (fa/fa) develop obesity, hyperlipidemia, fasting hyperglycemia, and type 2 diabetes (15).

The ZDF rats were at least 16 weeks old (weighing 300 to 400 g). Before the animals were shipped from Charles River Laboratories, they were fed Purina diet #5008 until they fully developed diabetes (12 weeks old). Hyperglycemia (>400 mg/dL) in the ZDF rats was confirmed by blood glucose measurements. The rats were group housed (three per cage) in the animal facility under a 12-h light/dark cycle. They had access to food and water *ad libitum* throughout the studies. The animals were on regular laboratory rodent food in our facility. The rats were acclimated to the environment for at least 3 days before they were used in the studies.

Insulin (RHI), insulin glargine, and the commercially available formulation of insulin glargine, Lantus®, were administered to the ZDF rats (type 2 diabetes) by a subcutaneous injection. An appropriate amount of insulin or insulin glargine powder was suspended in sterile normal saline to obtain a suspension with a concentration of 40 IU/mL. Lantus® was used as a control and was administered to the animals as is (100 IU/mL).

Blood glucose concentrations in each animal were determined following a subcutaneous injection of each form of insulin at a dose of 10 IU/kg as follows: On each day of the study, the ZDF rats were transferred to the procedure room. The rats were randomly divided into groups, with four to six animals per group. Each group, except the control group, received one dose of a form of insulin. The control group did not receive any insulin injection. Before injection, each rat was placed in an induction chamber and anesthetized by isoflurane (Halocarbon Product Corporation, River Edge, NJ) using a SurgiVet/Anesco ventilator (Waukesha, WI). The body weight of the animal was recorded to determine the volume of the insulin suspensions to be injected. Blood glucose was measured using Bayer Ascensia Breeze blood glucose meter (Bayer Corporation, Elkhart, IN). The predetermined dose of insulin (which was determined based on the animal body weight) was injected subcutaneously at the dorsal neck of the rat using a 0.5 mL Becton Dickinson Lo-Dose™ U-100 insulin syringe with a permanently attached 28-gauge×0.56-in needle (Franklin Lakes, NJ). After injection, the rats were returned to the cages and allowed to recover from anesthesia. At predetermined time intervals, the rats were anesthetized by the aforementioned method and their blood glucose was measured. A plot of blood glucose concentrations *versus* time was generated from the data to describe the glucose-lowering effect of insulin or insulin glargine, from which information such as time of peak action and duration of action could be obtained.

Fabrication of Biodegradable Gels

The gel formulations (Table I) were prepared in two steps: dissolution of polymer in the plasticizer(s) to obtain a blank gel and loading of insulin glargine into the blank gel. The polymer was allowed to come to room temperature in a vacuum desiccator after it was taken out from the freezer. Then an appropriate amount of polymer particles was accurately weighed into a dry, clean glass scintillation vial using a Mettler AE100 analytical balance (Mettler-Toledo, Inc., Columbus, OH). The desired amount of plasticizer(s) was weighed into the scintillation vial containing the polymer. A plastic screw cap with aluminum liner was used to seal the vial. The vial was then vortexed on a vortex mixer to disperse the polymer particles in the plasticizer(s). The vial was placed in a Lab-Line orbital shaker (Model 3527, Lab-Line, Melrose Park, IL), which was maintained at 37°C and shaken at 150 rpm. It took 2 to 3 days for the polymer to completely dissolve in the plasticizer. The resulting blank gel was stored in vacuum desiccators at room temperature until further use (typically 1 to 2 days). One day before the insulin

Table I. Composition of Gel Formulations Used for *In Vivo* Studies

Formulation no.	Concentration of polymer[a] (% *w/w*)	Concentration of ATEC (% *w/w*)	Concentration of TEC (% *w/w*)	Insulin glargine (% *w/w*)	Zinc sulfate (% *w/w*)
OA-G-1	–	–	–	4.0	–
OA-G-2	–	72	24	4.0	–
OA-G-3	5.0	69.75	23.25	2.0	–
OA-G-4	5.0	68.25	22.75	4.0	–
OA-G-5	10.0	64.5	21.5	4.0	–
OA-G-6	5.0	67.88	22.62	4.0	0.50
OA-G-7	5.0	67.9	22.9	4.0	0.25
OA-G-8	5.0	68	22.9	4.0	0.10

OA-G-1 was prepared in PBS
ATEC acetyltriethyl citrate, *TEC* triethyl citrate
[a] Inherent viscosity of the polymer (in deciliters per gram)—0.09

glargine-loaded gel was required, a desired amount of blank gel was transferred to a new glass scintillation vial and an appropriate amount (approximately 80 mg) of insulin glargine particles was accurately weighed and transferred into the blank gel vial. A stainless steel spatula was used to disperse the insulin glargine particles uniformly into the blank gel. Any visible agglomerates of insulin glargine were broken using a spatula, and the insulin glargine-loaded gel was ready to be used for the *in vivo* studies.

In Vivo Studies of Insulin Glargine-Loaded Gel Formulations in ZDF Rats

For the *in vivo* studies, blood glucose concentrations of the diabetic rats were measured before and after injection of insulin glargine-loaded gels. The concentration of insulin glargine of some of the formulations in plasma or serum was also determined, and the data were plotted against time to characterize the release of insulin glargine from the insulin glargine-loaded gel formulations. For gel formulations consisting of insulin glargine and zinc sulfate, an appropriate quantity of zinc sulfate powder (<400 mesh or 37.5 μm) was accurately weighed and added to the insulin glargine-loaded gel.

Typically, a total of 2 g of gel (including insulin glargine and zinc sulfate) was prepared for each gel formulation. On the day of injection, the ZDF rats were transferred from the housing room to the procedure room. The animals were anesthetized by isoflurane. The dorsal neck of each rat (except the animals in the control group that did not receive any injection) was shaved to expose the injection site. Body weight of the rat was recorded. Blood glucose was measured prior to any injection using a glucose meter as described before. The insulin glargine-loaded gel was withdrawn into a barrel of a 1-mL syringe, without any needle. Then a 23-gauge needle (1 in in length) was attached to the syringe, and the volume of the gel in the syringe was adjusted to the desired volume, depending on the dose of insulin for each animal. The shaved area on the dorsal neck of the animal was disinfected with isopropyl swab, and a subcutaneous injection of the insulin glargine-loaded gel was made. The needle was inserted under the skin in such a way that the gel would be injected between the skin and the underlying muscle tissue. The actual procedure of injecting the gel was as follows: At the injection site, the skin was pinched and lifted by the finger and thumb. The needle was inserted under the skin between the finger and thumb. Approximately three quarters of an inch of needle was pierced into the skin to make sure that the injected gel would not ooze out after the needle was removed from the injection site. After injection of the gels, at predetermined time intervals, the rats were anesthetized and blood glucose concentrations were measured. Blood samples (300 to 400 μL) were taken from the tail vein and processed to collect plasma or serum. These plasma or serum samples were stored at −20°C until analysis.

Statistical Analysis

Concentration above the effect curve normalized for baseline glucose levels (AAEC, 16) which is defined as the area below baseline is representative of the effect of the formulation on lowering of blood glucose with greater AAEC implying greater effect. One-way ANOVA within Excel 2007 was utilized to compare AAEC between treatment (N=4) and control (N=4) at 95% confidence level.

Pharmacodynamic Efficiency

Pharmacodynamic efficiency (PDE) as defined by Amidi *et al.* is as follows (16):

$$PDE = AAEC/AUC$$

where AAEC (in days) is the concentration above the effect curve normalized for baseline glucose levels and AUC (in milliunits/liter day) is the area under the plasma insulin concentration curve. PDE (in liters/milli-international units) is a weighted measure of response per unit drug concentration and is particularly useful to assess improvement in drug delivery or formulations (16,17).

RESULTS AND DISCUSSION

Purity of Insulin Glargine Particles

Figure 1 shows the chromatogram of placebo Lantus® formulation (without any insulin glargine). The placebo was made in house, and each milliliter of the formulation contained 2.7 mg of m-cresol, 30 μg of zinc, (from zinc chloride), 20 mg of glycerol 85% *w/w*, and water for injection. This formulation was similar to Lantus® formulation without insulin glargine. It is

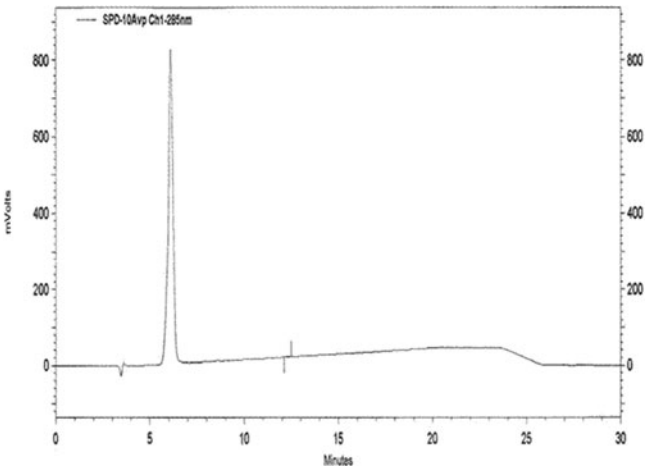

Fig. 1. HPLC chromatogram of placebo of Lantus® formulation

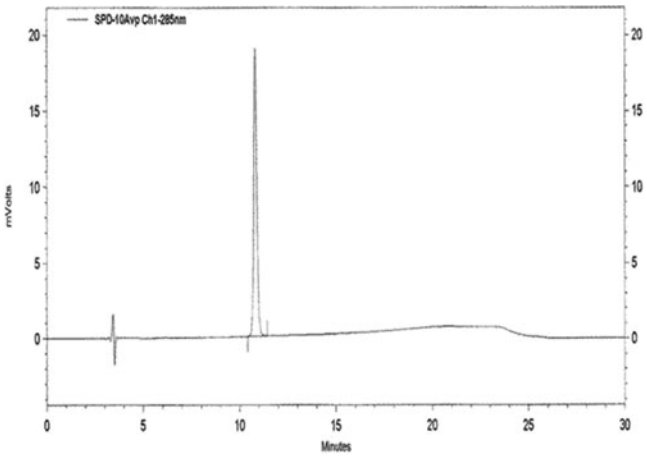

Fig. 3. HPLC chromatogram of insulin glargine particles dissolved in HCl

evident from Fig. 1 that the retention time of the peak of the placebo formulation is approximately 6 min.

Figure 2 shows the chromatogram of Lantus® formulation diluted in 0.01 N HCl. The chromatogram shows two peaks, one at 6 min, which corresponds to the placebo peak, and another peak at 11 min. The peak at 11 min is the peak of insulin glargine or Lantus®. The diluted Lantus® formulations were injected at various concentrations, and the areas under the peaks at 11 min were used to prepare the standard plot.

Figure 3 shows the chromatogram of purified (dialyzed) and freeze-dried particles of insulin glargine dissolved in 0.01 N HCl. The chromatogram shows a single peak at 11 min. It is evident from Fig. 3 that the placebo peak at 6 min is missing, thus indicating that the excipients in the Lantus® formulation were removed using the dialysis and pure insulin glargine particles were obtained after freeze-drying. The calculated purity of these insulin glargine particles was found to be 94.6%.

Pharmacodynamic Studies of Insulin Formulations in ZDF Rats

Insulin and insulin glargine particles were suspended in sterile normal saline (0.9% *w*/*v* NaCl) and administered subcutaneously to ZDF rats to evaluate the biological effect of different forms of insulin on the blood glucose concentrations of the rats. Figure 4 shows the blood glucose concentrations of these pharmacodynamic studies. It is evident from Fig. 4 that the blood glucose concentrations decreased from approximately 452±27.38 to 92.5±4.9 mg/dL within 3 h after a subcutaneous injection of 10 IU/kg dose of RHI in the ZDF rats. Normal blood glucose concentrations (between 80 and 200 mg/dL) were maintained for approximately an hour before reaching the normally high blood glucose concentrations within 6 h. Lantus® was subcutaneously injected at dose of 10 IU/kg (Fig. 4). The blood glucose concentrations after Lantus® injection decreased from approximately 441±17.02 to 229± 45 mg/dL within 4 h postinjection. The blood glucose concentrations after subcutaneous injection of purified glargine particles suspended in saline decreased from approximately 481±16.68 to 136.23±14.56 mg/dL within 2 h (Fig. 4). Normal blood glucose concentrations (between 80 and 200 mg/dL) were maintained for approximately 6 h before reaching the normally high blood glucose concentrations (350 mg/dL) within 12 h. This pharmacodynamic effect of lowering of blood glucose concentrations shows that the insulin glargine particles (purified from the commercially available Lantus® formulation) and suspended in normal saline were bioactive. There was a significant difference between AAEC after subcutaneous injection of insulin glargine particles and control at 95% confidence level (P=0.003) indicating that insulin glargine particles causes significantly greater reduction in blood glucose when compared to control. Moreover, no significant difference was observed between AAEC after subcutaneous injection of insulin glargine particles and Lantus® (P>0.05) at 95% confidence level indicating that the Lantus® is no better than insulin glargine particles in reduction of blood glucose, in ZDF rats.

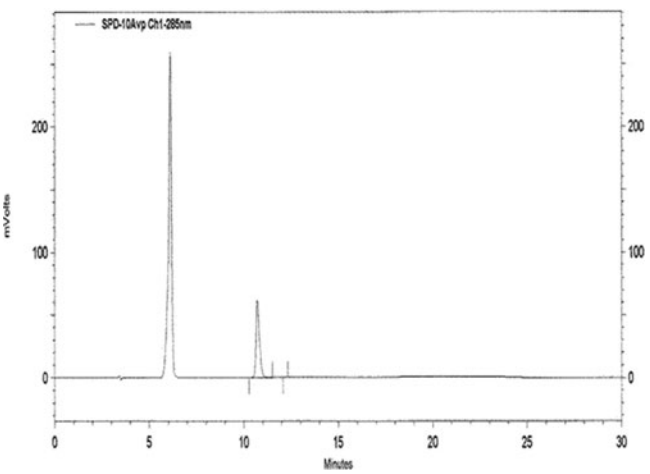

Fig. 2. HPLC chromatogram of Lantus® formulation

In Vivo Studies of Gel Formulations Loaded with Insulin Glargine Particles

Purified and freeze-dried particles of insulin glargine were suspended in phosphate buffer saline (PBS) at 4% loading, and 0.1 mL of this suspension was injected subcutaneously in the ZDF rats. Figure 5 shows the changes in blood glucose concentrations and body weights of ZDF rats after the injection.

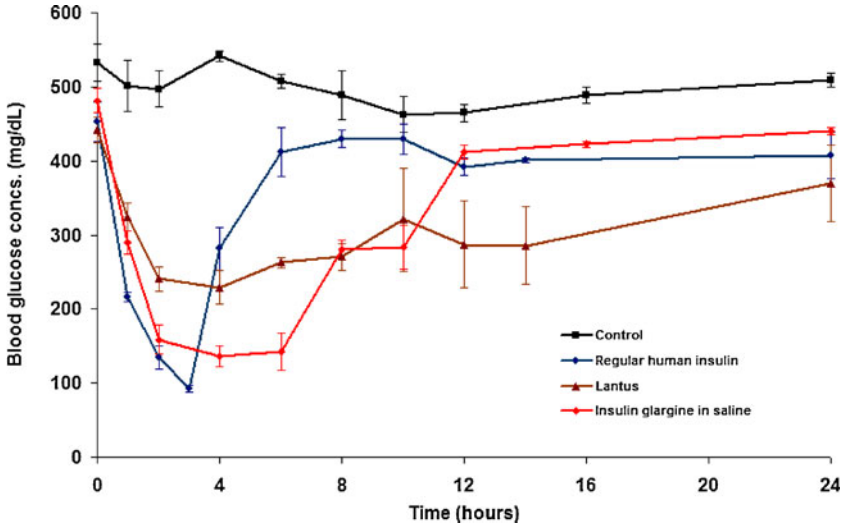

Fig. 4. Blood glucose profiles in ZDF rats after subcutaneous injections of insulin (RHI), Lantus® and insulin glargine particles suspended in saline (dose—10 U/kg; mean±SEM, n=5)

It is evident from the figure that blood glucose concentrations decreased rapidly below 100 mg/dL within 6 h after injection and remained at that concentration until day 3 before rapidly climbing to above 300 mg/dL on day 5.

The rapid decline in blood glucose concentrations is not desirable. Moreover, the rats were nearly hypoglycemic for 3 days. Hence, there was a need to further slow down the release of insulin glargine, which produced a slower and extended effect in the animals. A similar effect in the change in blood glucose concentrations was observed after subcutaneous injection of 0.1 mL of 4% suspension of insulin glargine particles in 3:1 blend of ATEC to TEC plasticizers (Fig. 5). Hence, it is clear from this study that loading insulin glargine particles in a combination of plasticizers alone does not provide extension of biological effect of insulin glargine. However, from Fig. 5, it is clear that as the blood glucose concentrations decreased, the body weights of the rats increased rapidly. Moreover, the body weights of the animals started to decline as the blood glucose concentrations climbed back to the normally high concentrations above 350 mg/dL. This showed that the glargine particles not only produced a lowering in blood glucose concentrations, but also affected the body weights, thus further proving that the purified and freeze-dried particles of insulin glargine were bioactive.

Figure 6 depicts change in blood glucose concentrations in ZDF rats after a subcutaneous injection of PLGA gel formulation (containing 5% or 10% PLGA) loaded with insulin glargine particles (containing 2% or 4% glargine). The subcutaneous injection of a gel formulation (Formulation OA-G-3- 5% PLGA) with a 2% *w*/*w* drug loading (Table I) resulted in the blood glucose concentrations reduction from 425 mg/dL to approximately 100 mg/dL within 24 h after the

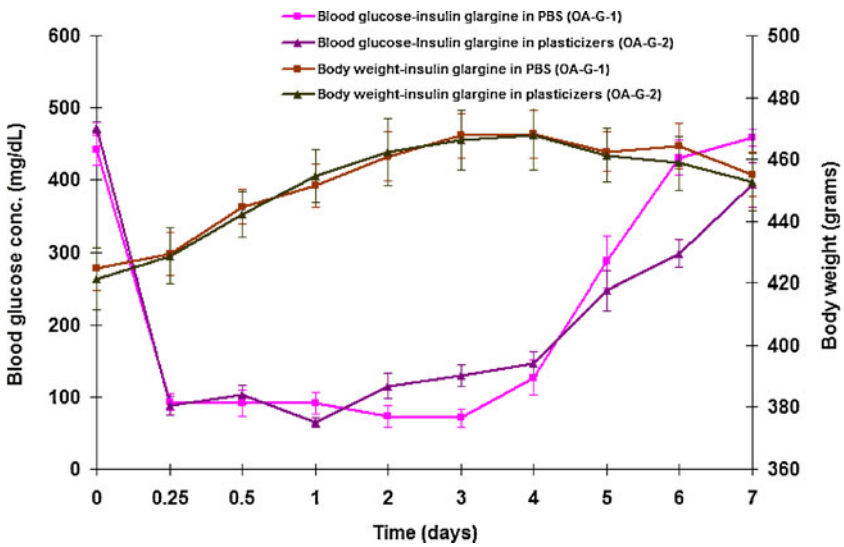

Fig. 5. Changes in blood glucose concentrations and body weights of ZDF rats after subcutaneous injections of 4% suspension of insulin glargine particles in PBS and in blends of ATEC to TEC (3:1; mean±SEM, n=5)

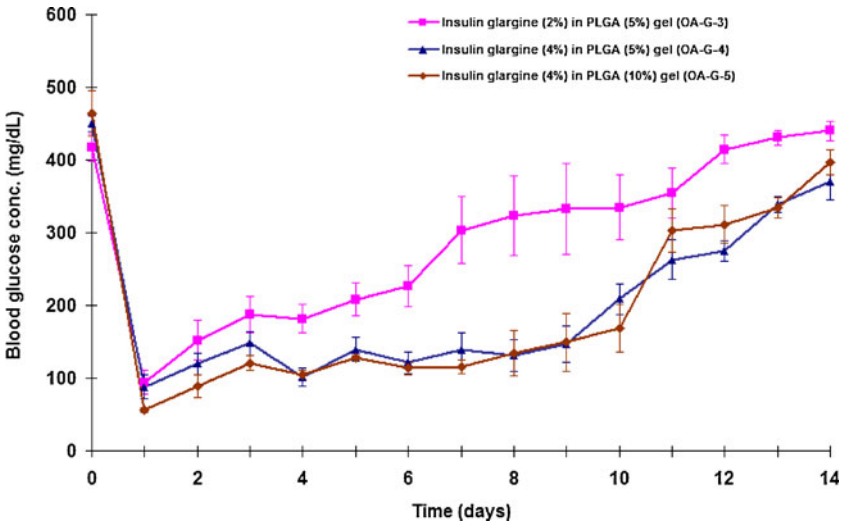

Fig. 6. Changes in blood glucose concentrations and body weights of ZDF rats after a subcutaneous injection of gel (5% or 10 % PLGA) formulation loaded with (2% or 4%) insulin glargine particles (mean±SEM, n=5)

injection and which remained between 100 and 200 mg/dL for 4 days before steadily climbing back up to above 300 mg/dL on day 9. This showed that addition of 5% PLGA to the plasticizer combination of ATEC to TEC (3:1) not only slowed down the *in vivo* effect of insulin, but also extended the duration of action of the drug for approximately 6 days before climbing back up to the normally high concentrations of above 350 mg/dL. It is evident from the figure that the effect of the gel formulation prepared with 5% PLGA and loaded with 4% *w/w* of insulin glargine particles (Formulation OA-G-4) was similar to Formulation OA-G-3 with 2% drug loading. Following the subcutaneous injection of the Formulation OA-G-4, the blood glucose concentrations dropped from 445 to about 100 mg/dL in 24 h, and remained below 150 mg/dL for 9 days, before rising steadily above 300 mg/dL on day 13. Hence, doubling the drug loading from 2% (Formulation OA-G-3) to 4% (Formulation OA-G-4) extended the maintenance of normal blood glucose concentrations from 6 to 9 days, respectively.

To explore the possibility of slower and longer duration of effect of insulin glargine in the ZDF rats, the polymer concentration in the gel formulation was increased from 5% to 10% *w/w*. Figure 6 also shows changes in blood glucose concentrations in ZDF rats after subcutaneous injection of PLGA gel (prepared with 10% PLGA) formulation loaded with 4% insulin glargine particles (Formulation OA-G-5, Table I). The changes in blood glucose concentrations in ZDF rats were similar to Formulation OA-G-4 containing 5% PLGA gel (Fig. 6). Hence, increasing the PLGA from 5% to 10% did not increase the *in vivo* duration of activity of insulin in the ZDF rats. Moreover, the effect of insulin glargine was also seen on the body weights after subcutaneous injection of all the gel formulation loaded with insulin glargine, which steadily increased as the blood glucose concentrations dropped (unpublished data). The body weights remained high and dropped slightly when the blood glucose concentrations began to rise steadily.

Figure 7 depicts the comparison of blood glucose concentrations in ZDF rats after subcutaneous injections of different formulations of insulin glargine with 4% loading with (Formulation OA-G-4) or without PLGA (Formulation OA-G-1 and Formulation OA-G-2). There was a significant difference between AAEC after subcutaneous injection of formulation of insulin glargine with 4% loading with PLGA (Formulation OA-G-4) and control at 95% confidence level (P=0.00001) indicating that insulin glargine with 4% loading with PLGA causes significantly greater reduction in blood glucose when compared to control. Furthermore, there was a significant difference between AAEC after subcutaneous injection of formulation of insulin glargine with 4% loading with PLGA (Formulation OA-G-4) and formulation of insulin glargine with 4% loading without PLGA (Formulation OA-G-1) at 95% confidence level (P=0.0004) indicating that insulin glargine with 4% loading with PLGA causes significantly greater reduction in blood glucose concentrations, when compared to insulin glargine with 4% loading without PLGA.

Moreover, no significant difference was observed between AAEC after subcutaneous injection formulation of insulin glargine with 4% loading without PLGA, *i.e.*, insulin glargine in PBS (Formulation OA-G-1) and insulin glargine in plasticizers (Formulation OA-G-2, P>0.05) at 95% confidence level indicating that the insulin glargine in plasticizers (Formulation OA-G-2) is no better than insulin glargine in PBS (Formulation OA-G-1), in reduction of the blood glucose concentrations. It is clear from the figure that addition of 5% w/w PLGA not only slows down the lowering of blood glucose concentrations, but also increased the duration of action from 3 to 9 days. This extension in the blood glucose-lowering effect of insulin glargine particles is due to presence of PLGA. This effect may be due to formation of a depot of insulin glargine particles encapsulated in the PLGA matrix. This PLGA matrix released the insulin slowly as it underwent erosion with time.

To study the blood concentrations of glargine and its effect on blood glucose concentrations, Formulations OA-G-1 and OA-G-4 were injected into two different groups of seven ZDF rats. The blood glucose concentrations and concentrations of glargine in serum were determined. Figure 8 shows the concentrations of glargine in serum and blood glucose concentrations after a subcutaneous injection of Formulations OA-G-1 and OA-G-4. The concentration of glargine in

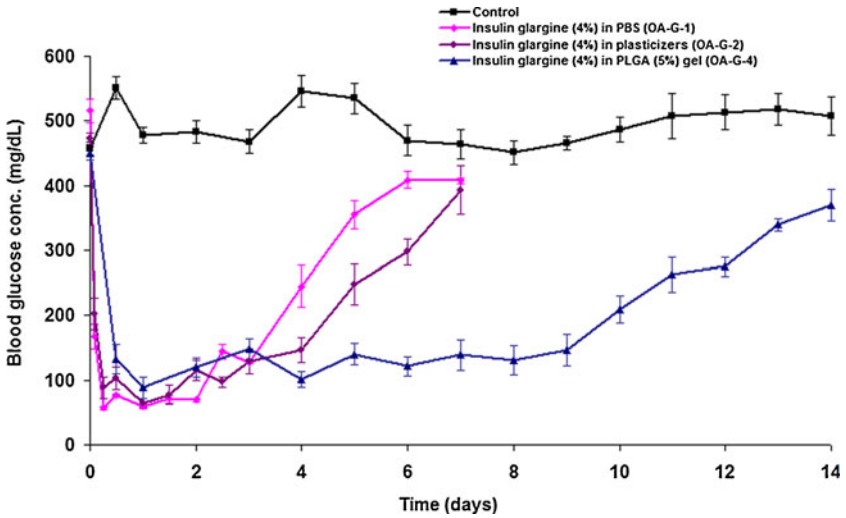

Fig. 7. Comparison of blood glucose concentrations in ZDF rats after subcutaneous injections of insulin glargine (4% loading) in different formulations (mean±SEM, n=5)

serum of the ZDF rats increased very rapidly to 3,563±1,228 mIU/L within 6 h after injection of Formulations OA-G-1 (insulin glargine in PBS). The concentration of glargine remained above 1,500 mIU/L until day 3 and then slowly declined below 100 mIU/L within 6 days after injection. The blood glucose concentrations dropped to a low concentration of 93±31 mg/dL within 6 h after injection, thus corresponding to the burst release of glargine from the formulation. The blood concentrations remained below 100 mg/dL for up to 4 days before it gradually went back up. The blood glucose concentrations went back up to more than 400 mg/dL on day 6 due to the decline in glargine serum concentrations.

Figure 8 also shows the concentration of glargine in serum of the ZDF rats and blood glucose concentrations after a subcutaneous injection of gel Formulation OA-G-4 (insulin glargine in PLGA (5%) gel). The concentration of glargine in serum of the ZDF rats increased to approximately 750 mIU/L within 12 h after injection, and then declined to approximately 500 mIU/L within 24 h after injection. The concentration of glargine was then maintained between 260±134.9 and 188±55.9 mIU/L until day 10. Glargine concentrations decreased to 61.5±16.5 mIU/L on day 11 and then gradually decreased further thereafter. The blood glucose concentrations were well correlated to the concentration of glargine in the serum. The blood glucose concentrations dropped to a low concentration (75±14.5 mg/dL) on the first day after injection and were maintained at the low concentration (<200 mg/dL) for 9 days before it gradually went back up. Blood glucose went back up to more than 400 mg/dL on day 13 and then was constantly maintained at normally high concentrations as seen before the injection.

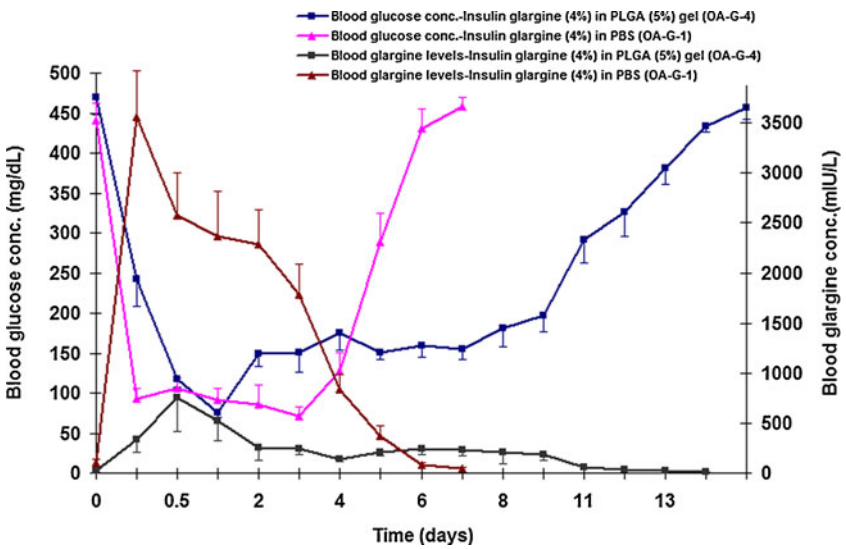

Fig. 8. Blood glucose concentrations and concentrations of insulin glargine in serum of ZDF rats after a single subcutaneous injection of Formulation OA-G-1 (mean±SEM, n=7) and Formulation OA-G-4 (mean±SEM, n=6)

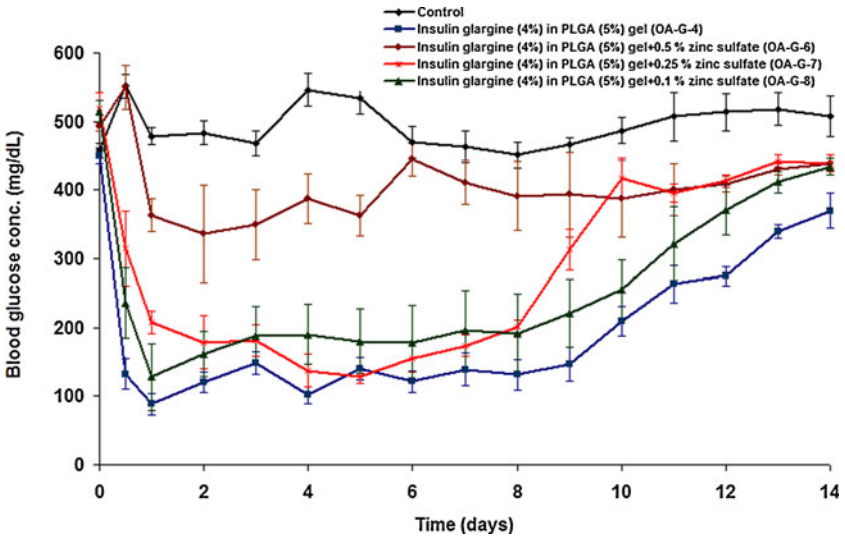

Fig. 9. Comparison of blood glucose concentrations in ZDF rats after injections of insulin glargine (4% loading) in different formulations with various zinc sulfate concentrations (mean±SEM, $n=5$)

There was a significant difference between serum glargine concentrations after subcutaneous injection of formulation of insulin glargine with 4% loading with PLGA (Formulation OA-G-4) and formulation of insulin glargine with 4% loading without PLGA (Formulation OA-G-1) at 95% confidence level ($P=0.001$), indicating that insulin glargine with 4% loading with PLGA causes significantly extension in serum glargine concentrations when compared to insulin glargine with 4% loading without PLGA

The PDE values after subcutaneous injection of the formulation with PLGA (Formulation OA-G-4) and without PLGA (Formulation OA-G-1) were 0.097 and 0.015 L/mIU, respectively, *i.e.*, the PDE for the formulation with insulin glargine in PLGA gel was 6.5-fold higher than the PDE for the formulation with insulin glargine in PBS. The higher PDE after PLGA gel formulation indicates that the glucose response per unit systemic insulin glargine was higher for the PLGA gel formulation than the formulation without the PLGA gel. This may also demonstrate that the biological activity of insulin glargine was not unfavorably affected in the PLGA gel formulation. The higher PDE for the PLGA gel formulation is possibly due to slow release of glargine for extended duration of time.

To further study the effect of presence of zinc sulfate in the PLGA gels loaded with insulin glargine on the biological effect of these gels, three formulations at different concentrations of zinc sulfate were prepared. Figure 9 shows the comparison of blood glucose concentrations in ZDF rats after subcutaneous injections of different formulations loaded with 4% insulin glargine and various zinc sulfate concentrations. It is evident from the figure that addition of zinc sulfate in the formulations lowered the rate of blood glucose-lowering effect. At the highest zinc sulfate concentration of 0.5% (Formulation OA-G-6), the blood glucose concentrations never dropped below 300 mg/dL. Moreover, the blood glucose concentrations remained between 300 to 400 mg/dL for 14 days.

There was a significant difference between AAEC after subcutaneous injection of formulation of insulin glargine with 4% loading with PLGA and zinc sulfate concentration of 0.5% (Formulation OA-G-6) and control at 95% confidence level ($P=0.02$), indicating that insulin glargine with 4% loading with PLGA and zinc sulfate concentration of 0.5% causes significantly greater reduction in blood glucose when compared to control. Furthermore, there was also a significant difference between AAEC after subcutaneous injection of formulation of insulin glargine with 4% loading with PLGA (Formulation OA-G-4) and formulation of insulin glargine with 4% loading with PLGA and zinc sulfate concentration of 0.5% (Formulation OA-G-6) at 95% confidence level ($P=0.002$), indicating that insulin glargine with 4% loading with PLGA causes significantly greater reduction in blood glucose when compared to formulation of insulin glargine with 4% loading with PLGA and zinc sulfate concentration of 0.5% (Formulation OA-G-6).

As the zinc sulfate concentration was reduced from 0.5% to 0.25% (Formulation OA-G-7), the blood glucose concentrations dropped from 520 to about 178 mg/dL in 2 days, and remained below 200 mg/dL for 8 days, before rising steadily above 400 mg/dL on day 10. As the zinc sulfate concentration was further reduced from 0.25% to 0.1% (Formulation OA-G-8), the blood glucose concentrations dropped from 515 to about 128 mg/dL in 24 h, and remained below 200 mg/dL for 8 days, before rising steadily above 400 mg/dL on day 13.

Moreover, no significant difference was observed between AAEC after subcutaneous injection formulation of insulin glargine with 4% loading with PLGA (Formulation OA-G-4) and formulation of insulin glargine with 4% loading with PLGA and zinc sulfate concentration of 0.25% (Formulation OA-G-7, $P>0.05$) and zinc sulfate concentration of 0.1% (Formulation OA-G-8) at 95% confidence level, indicating that formulation of insulin glargine with 4% loading with PLGA (Formulation OA-G-4) is no better than formulation of insulin glargine with 4% loading with PLGA and zinc sulfate concentration of 0.25% (Formulation OA-G-7) and zinc sulfate concentration of 0.1% (Formulation OA-G-8), in reduction of blood glucose concentrations. Hence, zinc sulfate could be used as a release modifier for insulin glargine in the gels. However, the amount of zinc sulfate in the gel needs to be optimized, so that it can provide a prolonged effect *in vivo*.

CONCLUSIONS

Insulin glargine particles were purified and then freeze-dried from commercial Lantus® formulation. The purity of the freeze-dried particles of insulin glargine was determined using HPLC and was found to be 94.6%. These insulin glargine particles were suspended in saline and subcutaneously injected in ZDF rats. The bioactivity, as measured by blood glucose-lowering effect, was found to be similar to regular human insulin and the marketed formulation called Lantus®. The PLGA gel formulations prepared with insulin glargine particles had duration of action of 10 days following a single subcutaneous injection. The concentration of insulin glargine was maintained between 260±134.9 and 188±55.9 mIU/L until day 10 after single subcutaneous injection of some of these formulations, and the corresponding blood glucose concentrations were suppressed from above 400 to below 200 mg/dL from days 1 to 10 postinjection. The addition of zinc sulfate to the formulations prepared with purified insulin glargine particles further slowed down the drop in blood glucose concentrations.

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