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Influence of microencapsulation method and peptide loading on formulation of poly(lactide-co-glycolide) insulin nanoparticles

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Received February 28, 2005, accepted September 12, 2005

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Pharmazie 61: 613–617 (2006)

Insulin stability during microencapsulation and subsequent release is essential for retaining its biological activity. Therefore we investigated a novel solid/oil/water anhydrous encapsulation method with a combination of stabilizers for maintaining the integrity of insulin during formulation and delivery. Two methods were used for preparation of nanoparticles, namely water/oil/water solvent evaporation and s/o/w anhydrous encapsulation to study the influence of the microencapsulation method on nanoparticle characteristics such as size and morphology, drug content, encapsulation efficiency, and *in vitro* and *in vivo* release profile. Poly (lactic-co-glycolic) acid (PLGA) with co-polymer ratio 50 : 50 was selected to prepare drug-loaded nanoparticles. When nanoparticles were prepared by solvent evaporation higher encapsulation efficiencies could be obtained, e.g. 74 ± 13 with 5% target loading, whereas with 12% target loading, encapsulation efficiency was 27 ± 8.6. The s/o/w method has a direct influence on the evaluation parameters where very poor encapsulation efficiencies 11 ± 6.8 (max) were observed. The presence of stabilizers in the nanoparticles resulted in an increase in particle size but a reduction of encapsulation efficiency. Insulin release rate was comparatively higher for the batches prepared by the w/o/w method containing stabilizers than the s/o/w method. Also the presence of stabilizers resulted in sustained release of insulin resulting in prolonged reduction of blood glucose levels in streptozotocin induced diabetic rats. From the *in vitro* and *in vivo* studies, it can be concluded that careful selection of processing conditions and combination of stabilizers also result in beneficial effects without compromising the advantages of these delivery systems.

1. Introduction

There are several reported methods of formulating nanoparticle delivery systems employing PLGA. These methods include w/o/w multiple emulsion systems, s/o/o and s/o/w (anhydrous encapsulation). The w/o/w technique has been widely tested for encapsulation of therapeutic peptides and proteins. However, protein inactivation and aggregation at the water/organic interface (first emulsification) is a detrimental event, which hinders the encapsulation of structurally unperturbed proteins into PLGA nanoparticles (Perez et al. 2002). Insulin is a hydrophilic, unstable peptide susceptible to both chemical and physical degradation. During the formulation of microemulsions, proteins usually tend to migrate to the interface between the aqueous and organic phase where they may unfold resulting in conformational changes (Wang et al. 1998). It has been shown that proteins undergo aggregation and subsequent loss of activity following encapsulation by the w/o/w solvent evaporation technique (Johnson et al. 1991; Chen et al. 1997). Also exposure to various formulation conditions, such as elevated temperature, low pH, organic solvents and agitation, results in biologically inactive linear aggregates (fibrils) of insulin (Isabel et al. 1996). The acidic degradation products of PLGA can interact with positively charged proteins leading to blockade of its release and this exposes the entrapped drug a low pH forming water insoluble non-covalently bound protein aggregates (Brange et al. 1997). This low pH environment is the cause of deamidation at asparaginase A21 of the insulin moiety (Morlock et al. 1997). Thus insulin instability has become a strong obstacle to the development of suitable controlled delivery systems.

To overcome the above pitfalls in insulin delivery, various stabilizers have been used to preserve the integrity of the peptide. The addition of block copolymers of polyethylene-polypropylene glycols such as poloxamers has been shown to stabilize the proteins effectively (Brange et al. 1991b; Grau 1985; Muller et al. 1996; Olbrich et al. 1999). Among different poloxamers, only poloxamer 188 has been found to be relatively efficient in preventing insulin aggregation in solution (Chawla et al. 1985). In addition, poloxamers have been shown to stabilize the primary emulsion and reduce protein-polymer interactions (Nilhant et al. 1994). The emulsification step in the preparation of microparticles results in poor drug encapsulation and stability (Cleland et al. 1997). During the secondary drying

Table 1: Human insulin loaded nanoparticles prepared by the w/o/w method

S.No	Batch code	Method of encapsulation	Mean particle size (nm)	Poly index	% Loading efficiency	% Drug loading	% Yield	Target loading
01	INS-A	Double emulsion solvent evaporation	1145.8	0.246	74.78	3.5	98.75	5%
02	INS-B	Double emulsion solvent evaporation	216.2	0.184	58.83	1.52	82.21	5%
03	INS-C	Double emulsion solvent evaporation	1044	1	8.47	1.68	54.07	12%

phase, the removal of water molecules also contributes to protein aggregation. Addition of the carbohydrate trehalose overcomes these problems and prevents secondary structural changes in the protein (Gribbon et al. 1996; Souillac et al. 2002). The low environmental pH (<3) generated by the degradation of PLGA polymers which triggers the unfolding of proteins has been neutralized by incorporation of salts such as magnesium hydroxide, preventing protein aggregation (Zhu et al. 2000). Based on this hypothesis, we used sodium bicarbonate to neutralize the acidic environment. Recently explored methods of improving the stability and encapsulation of therapeutic proteins are anhydrous encapsulation methods, namely s/o/o and s/o/w. In the s/o/w method, the dehydrated protein powder is suspended in an organic solvent containing the dissolved polymer and this suspension is emulsified in an aqueous solution containing an emulsifying agent. The nanoparticles are then hardened by dissolving or evaporating the organic solvent, after which the particles are finally washed and lyophilized. In this method there is no potentially destabilizing primary w/o emulsion. These techniques also avoid the presence of additional hardening and quench solvents during processing. In the present study nanoparticles of PLGA were made employing the w/o/w and s/o/w methods to study the influence of the microencapsulation method on nanoparticle characteristics such as size and morphology, drug content, encapsulation efficiency, and *in vitro* and *in vivo* release profile. The objective of the present work was to develop insulin loaded nanoparticles using a combination of different stabilizers such as Pluronic F68, trehalose and sodium bicarbonate to improve insulin stability while optimizing nanoparticle characteristics. The effects of the stabilizers and method on nanoparticle properties such as morphology, encapsulation efficiency and *in vitro* release profile were investigated, along with the *in vivo* reduction of blood glucose levels in diabetic rats.

2. Investigations, results and discussion

Insulin loaded nanoparticles with different stabilizers were prepared by the double emulsion evaporation method and the anhydrous encapsulation method using PLGA with copolymer ratio (50 : 50). The influence of various stabilizers and the method on nanoparticle characteristic parameters such as mean particle size, actual loading, encapsulation efficiency and percentage yield are given in Table 1.

Table 2: Human insulin loaded nanoparticles prepared by the s/o/w method

S.No	Batch code	Encapsulation method	Mean particle size (nm)	Poly index	% Loading efficiency	% Drug loading	Target loading
01	P1	S/O/W	223.3	0.159	11.92	1.78	5%
02	P2	S/O/W	243.9	0.266	0.312	0.312	12%
03	P3	S/O/W	238.7	0.37	1.186	1.186	12%

2.1. Structure and particle size

From Tables 1 and 2 it is evident that an increase in the volume of the internal aqueous phase and the target loading of insulin resulted in an increase in mean particle size for the double emulsion solvent evaporation method. The insulin loaded PLGA nanoparticles were examined under the scanning electron microscope. The surface of the nanoparticles prepared with stabilizers was found to be smooth, spherical and non-porous compared to that of nanoparticles without stabilizers (Figs. 1 and 2). In the case of nanoparticles prepared by w/o/w, the drug free nanoparticles had generally smooth and pore-free surfaces, while 5% (w/w) insulin loading resulted in small pores. An increase of the loading from 5% to 12% (w/w) led to larger particles and pores. Nanoparticles prepared by the s/o/w method formed aggregates, which could not be separated by sonication. With increasing insulin concentration, more air may have been incorporated in the

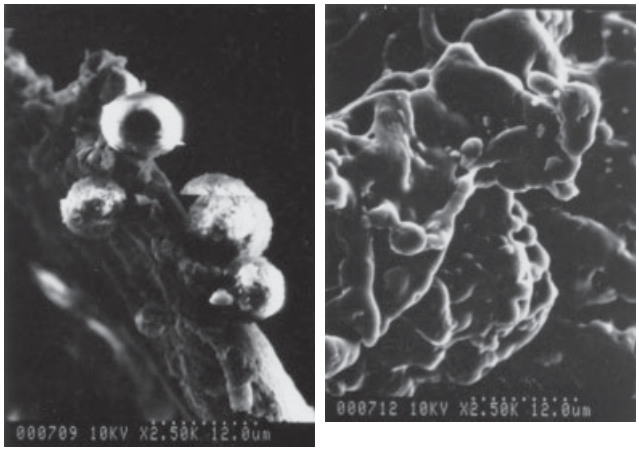

Fig. 1: Scanning electron micrograph of insulin loaded nanoparticles (a) Control 1 (PLGA 50 : 50) (b) S/o/w (PLGA 50 : 50)

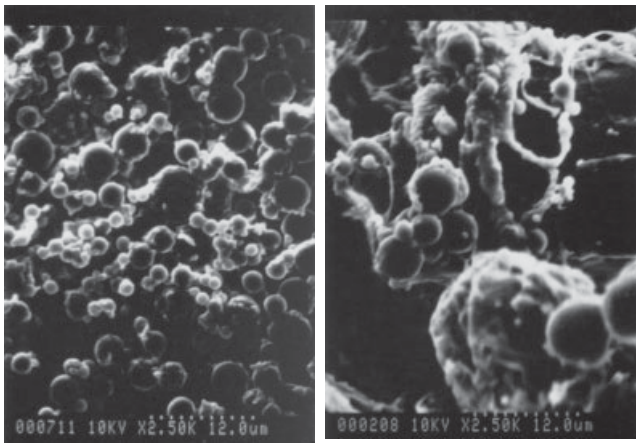

Fig: 2: (c) With stabilizers (PLGA 50 : 50) (d) Without stabilizers (PLGA 50 : 50)

primary emulsion, due to the decreased surface tension, leading to formation of larger pores during the solvent evaporation step. In the case of nanoparticles prepared by the s/o/w method, nanoparticles were found to be discrete and in the form of clusters, unlike those obtained by the w/o/w method, which were free flowing without any aggregates.

2.2. Encapsulation efficiency

The loading efficiency of insulin nanoparticles was found to be $74 \pm 13\%$ at 5% target loading, whereas at 12% target loading, the encapsulation efficiency was $27 \pm 8.6\%$. It was observed that an increase in protein concentration from 5% to 12% resulted in a slight decrease in the encapsulation efficiency. The decrease in encapsulation efficiency with higher protein loadings suggests that there is a limit to polymer encapsulation of protein and beyond that, an increase in protein concentration decreased the encapsulation efficiency of protein in the polymer matrix due to a saturation phenomenon whereby partitioning of peptide into the aqueous phase takes place during the process. Some working groups (Jeffery et al. 1993; Rosa et al. 2000; O'Hagan et al. 1994) reported this effect for ovalbumin loaded nanoparticles. In an attempt to improve the stability and encapsulation efficiency of insulin loaded PLGA nanoparticles we formulated them using s/o/w anhydrous encapsulation. Results were in contrast to the w/o/w double emulsion solvent evaporation method, as even at low loading levels only $11 \pm 6.8\%$ encapsulation efficiency was obtained, and increased loading further lowered the loading efficiency. This may be due to the hydrophilic nature of insulin where partitioning of drug from the inner oil phase suspension to dissolution in the outer aqueous phase occurs in the w/o emulsion step. Our results were in agreement with other reports (Atkins et al. 1997; King et al. 2000; Castellanos et al. 2001) where low encapsulation efficiencies were reported.

2.3. In vitro release

The release profile of polypeptides or proteins from a lipophilic polymer like PLGA is usually described as follows (Mitsuko et al. 2004). In the first phase, protein located at or near the surface of the polymer matrix is released, while during the second phase, the so called 'pore diffusion phase', molecules diffuse through a network of newly generated water filled pores. In the third release phase, protein diffusion from PLGA nanoparticles is controlled by the degradation of the polymeric matrix. In the case of insulin loaded nanoparticles prepared by the w/o/w double-emulsion method, only one release phase could be observed, namely the initial burst (Wang et al. 1998). This observation may be attributed to the formation of aggregates (Jeffery et al. 1993) after water penetration into the matrix of the nanoparticles. These aggregates are no longer available for the release of insulin. To prevent this water-induced aggregation of protein we incorporated Pluronic F68 in the internal aqueous phase to reduce the burst effect. This effect can be clearly seen with *in vitro* release profiles of formulations containing Pluronic F68 in the internal aqueous phase, and those without Pluronic F68. In our study nanoparticles prepared by the s/o/w method failed to show similar release behavior, and addition of Pluronic F68 did not change this general pattern. The amount of insulin released after the initial burst was negligible. The addition of Pluronic F68 to the formulation led to a significant reduction in the initial insulin burst. During the first 24 h, nanoparticles prepared by the w/o/w double-emulsion method with Pluronic F68 showed no burst of encapsulated insulin (Fig. 3). The initial burst seen with particles without Pluronic F68 addition was decreased resulting in to an initial release of only 7%. On the other hand incorporation of $NaHCO_3$ (1%) resulted in significant sustaining of the release of insulin, which is due to the fact that the basic nature of $NaHCO_3$ prevents alterations of the pH microenvironment of the particles which could result in further fast degradation of the polymer and hence increased release of insulin, which is prevented in this case as $NaHCO_3$ neutralizes the acidic pH and so prevents acid induced degradation and formation of porous particles. Formulations control 1 and control 2, without stabilizers, released a significant amount of drug only up to 24 h. The *in vitro* release studies were correlated with the DSC profiles of the formulations with and without stabilizers (Table 3 and Figs. 4 and 5).

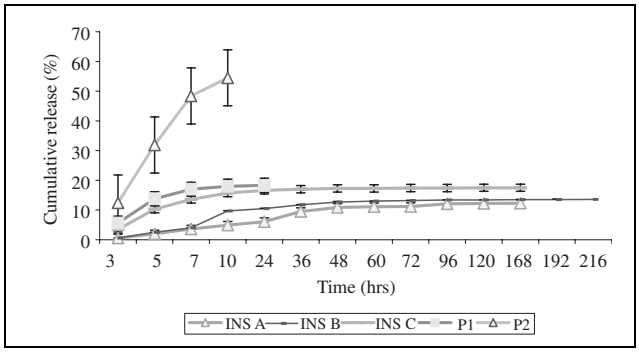

Fig. 3: Comparison of in vitro release profile of insulin loaded PLGA nanoparticles prepared by w/o/w (INS A, INS B, and INS C) and s/o/w (P1, P2) methods. Data are mean s ± s.d., n = 3

Table 3: DSC Profile

S.No	Sample	Glass transition temperature
1	Pure PLGA (50 : 50)	41–50 K
2	Insulin loaded nanoparticles with double emulsion solvent evaporation	61 K
3	Insulin loaded nanoparticles with anhydrous encapsulation	53 K

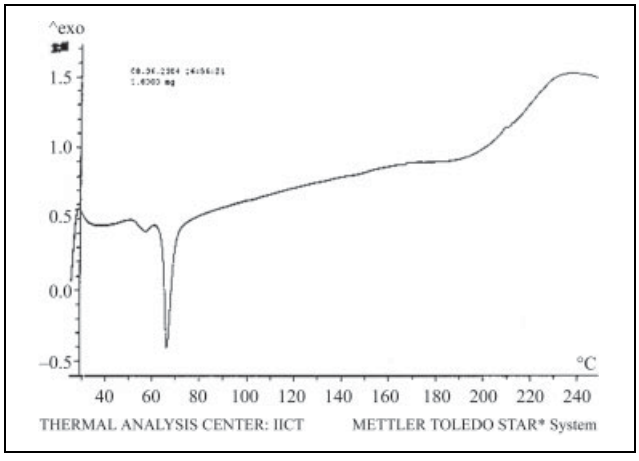

Fig. 4: DSC profile of insulin loaded nanoparticles prepared by s/o/w method

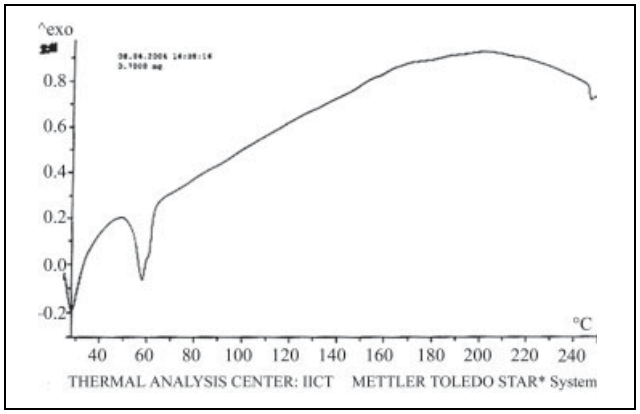

Fig. 5: DSC profile of insulin loaded nanoparticles prepared by w/o/w method

2.4. *In vivo studies*

The results clearly demonstrate the influence of method on the release of insulin from the formulations. Formulations prepared by double emulsion solvent evaporation were found to release insulin in a prolonged manner compared to those prepared by the anhydrous encapsulation method as was evident from the reduction in blood glucose levels. Outcomes clearly show the influence of anhydrous encapsulation on the release of insulin wherein formulation P2 reduced blood glucose levels up to 4 h. Formulations INS A, INS B and INS C, which were prepared by double emulsion solvent evaporation, were found to give prolonged release of insulin, over a period of 72 h (INS C) (Fig. 6). Furthermore the results were found to be statistically significant (Student t-test).

2.5. *Conclusions*

It is evident from the results that formulation of depot microspheres for insulin delivery by the w/o/w multiple solvent evaporation technique produced higher encapsulation efficiencies ensuring stability of insulin from manufacturing stage to release *in vitro* and *in vivo*. Formulation of depot microspheres for insulin delivery by the s/o/w anhydrous encapsulation technique produced negligable encapsulation efficiencies and poor stability of insulin from manufacturing stage to release *in vitro* and *in vivo*. Our *in vitro* outcomes have shown the sustaining effect of PLGA (50 : 50). DSC studies of the encapsulated microspheres have revealed us to correlate some interesting facts and hence led us to correlate these studies with the release profiles of insulin from nanoparticles. With careful selection of polymers, process variables, and sophisticated characterization tools, we can tailor the insulin release in such a way that the release profile of insulin is peakless and without any fluctuations to serve as basal insulin supplementation in cases of type-1-diabetes, with improved patient compliance and reduced cost of the therapy. This study has widened the scope for delivering biopharmaceuticals from days to several months. Further extensive studies in this area may pave the way for scale-up and commercial feasibility.

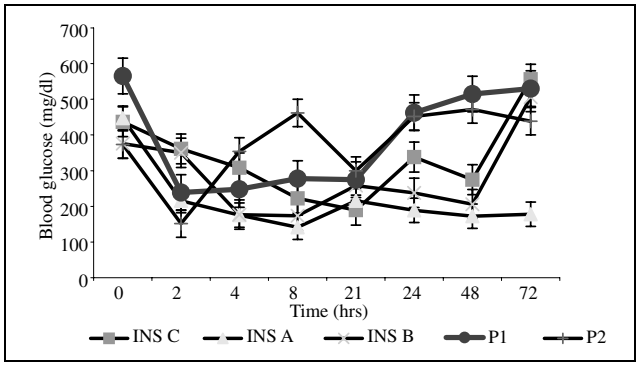

Fig. 6: Plasma blood glucose levels following a single subcutaneous treatment of insulin loaded PLGA nanoparticles to diabetic rats. Data are mean s ± s.d., n = 5

3. Experimental

3.1. *Materials*

Insulin USP-Hu. Recom. Cryst (1 mg = 27.5 IU) was purchased from Serologicals Corporation, Norcross, GA). Poly(DL-lactide-co-glycolide) (50 : 50), Pluronic F68, bicinchinoic acid (BCA) kit, sodium azide, trehalose and streptozotocin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium bicarbonate, polyvinyl alcohol (PVA), sodium hydroxide, disodium hydrogen phosphate, and sodium dihydrogen phosphate were of analytical grade (Loba Chemie, Mumbai, India). Trifluroacetic acid (TFA) was from E. Merck, Mumbai, India. Dichloromethane and acetonitrile were of HPLC grade (Qualigens Fine Chemicals, Mumbai, India).

3.2. *Methods*

3.2.1. *Preparation of insulin loaded nanoparticles*

Insulin loaded nanoparticles were prepared by a modified double emulsion-solvent evaporation technique as reported by Rosa (2000). Briefly, 0.2 ml of aqueous phase containing insulin (5% and 12% theoretical loading) dissolved in 0.01 M hydrochloric acid was poured into 10 ml of dichloromethane containing 100 mg of PLGA and sonicated at 30 W for 20 s (Vibra Cell VC-750, Sonics, USA). The primary emulsion formed was emulsified with 100 ml of 2% w/v aqueous PVA at 60 W for 2 min to form the double emulsion. The emulsion was stirred at room temperature to allow complete evaporation of the organic phase. The nanoparticles were collected by centrifugation, washed with distilled water and lyophilized. When used, 10 mM trehalose, 10% sodium bicarbonate and 1% Pluronic® F68 were added to the internal aqueous phase. The lyophilized nanoparticles were stored at 4 °C in the presence of silica gel. In the s/o/w encapsulation method, lyophilized insulin, trehalose 10 mM and 1% sodium bicarbonate were dispersed in 10 ml dichloromethane containing polymer. The dispersion was sonicated at 30 W for 2 s. To this dispersion 12.5 ml of distilled water and 5 ml of glycerol were added. The resulting solid/oil/water multiple emulsions were rigidized with Pluronic F68 (1%) or polyvinyl alcohol (2%). The organic solvent (dichloromethane) in this double emulsion was evaporated at room temperature and the resulting nanoparticles were centrifuged for 15,000 RPM at 4 °C for 30 min. The resulting nanoparticles were washed repeatedly and were suspended in the minimum quantity of water and lyophilized. The lyophilized nanoparticles were stored at 4 °C in the presence of silica gel.

3.2.2. *Morphology and size*

The surface morphology of the nanoparticles was analyzed by scanning electron microscopy (SEM) (Hitachi S-250, Japan). The nanoparticles were mounted on aluminium stubs, coated with gold under vacuum and observed under SEM.

Size analysis was carried out by the photon correlation spectroscopy technique using a Zeta sizer (300 HS, Malvern Instruments, UK) after dispersing the nanoparticles in distilled water. Particle size is expressed as mean volume diameter in nm ± SD of three batches.

3.2.3. *Insulin analysis by HPLC*

Insulin analysis was carried out by reversed-phase HPLC using a LiChrospher® C8 (250 × 4 mm, 5 m) column at ambient temperature at 214 nm (LC10 ATvp UV-visible detector, Shimadzu, Japan) equipped with an integrator (CR 8A, Shimadzu, Japan). The mobile phase was a mixture of an aqueous solution of 0.15% TFA (pH 2.5) and acetonitrile in the ratio of 65 : 35 run in isocratic mode at a flow rate of 0.7 ml/min.

3.2.4. *Encapsulation efficiency determination*

About 5 mg of insulin loaded nanoparticles were dissolved in 1 ml of acetonitrile to dissolve PLGA and insulin was extracted by adding 2 ml of phosphate buffer saline (PBS). The aqueous phase was separated and filtered through a 0.22 μ nylon membrane filter and a 20 μl aliquot was injected for HPLC analysis. The results are expressed as percentage ratio of actual and theoretical loading.

3.2.5. In vitro release and stability studies

A weighed amount (20 mg) of drug loaded PLGA nanoparticles was suspended in 1 ml PBS containing 0.001% sodium azide as preservative and kept for incubation at 37 °C at 25 RPM (Innova 4230, New Brunswick Scientific, USA). At pre-determined time intervals, 0.5 ml of sample was withdrawn and fresh PBS was added. The samples were filtered and analyzed for insulin content and stability by HPLC using a pure insulin sample as reference. Insulin release is expressed as the cumulative percentage.

3.2.6. In vivo studies in rats

The *in vivo* efficacy of insulin-loaded nanoparticles was carried out in streptozotocin induced diabetic rats. The animal experimentation was carried out in accordance with the guidelines and the protocol was approved by the Institutional Animal Ethics Committee (IAEC, IICT, Hyderabad). Wistar rats of either sex with body weight in the range of 150–180 g received 45 mg/mg of streptozotocin intraperitoneally. Blood glucose levels were monitored daily for a period of three weeks for all the rats until the induction and stabilization of a diabetic state. The diabetic animals were divided into six groups of three animals each and were treated with nanoparticles subcutaneously at a dose of 5 IU/kg. Blood samples were collected at 0, 2, 4, 8, 21, 24, 48, 60 and 72 h after dosing. Serum was separated immediately and blood glucose levels were determined by the glucose oxidase method using an auto blood analyzer (Express Plus, Bayer Corporation, USA). The percentage reduction of blood glucose levels was calculated from the zero hour reading.

Acknowledgements: The author thanks MHRD/AICTE, New Delhi, India for providing financial assistance for his master's project. The author is grateful to the director of the Indian Institute of Chemical Technology (IICT), Hyderabd, A.P, India for providing the necessary facilities for this research project. We thank Ganesh Cherala, and Dr. Kamal S. Jonnalagdda, assistant professor, School of Pharmacy, USIP, Philadelphia for their helpful discussions and technical assistance.

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