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Research paper

Formulation and characterization of an oily-based system for oral delivery of insulin

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ABSTRACT

The present work explored the possibility of formulating an oral insulin delivery system by combining the advantages of nanoencapsulation and the use of oily vehicle. The parameters affecting formulation such as association efficiency were characterized. The preparation was evaluated for its chemical, physical and biological stability. The preparation has unimodal particle size distribution with a mean diameter of 108 ± 9 nm. Insulin was protected from gastric enzymes by incorporation into lipid-based formulation. The results of RP HPLC and ELISA indicated that insulin was able to withstand the preparation procedure. Insulin in the preparations was stable for a period of one month at storage temperatures of 4 and 25 °C. It was also biologically active and stable as demonstrated by the remarkable reduction of blood glucose levels of the STZ-diabetic rats after oral administration of the preparation. Moreover, hypoglycemic effect of nanoparticles administered orally was sustained for a longer period of time compared to the subcutaneous injection. These results clearly evidenced the ability of the nanoparticles to enhance the pharmacological response of insulin when given orally and could be used to deliver other peptides.

1. Introduction

Insulin is an important therapeutic protein due to its role in the treatment of diabetes, which is growing into epidemic proportions in many countries [1]. Despite the advances in development of injectable insulin analogues, the goal of optimal glycemic control has remained elusive. Substantial progress has been made recently in non-invasive methods for insulin administration such as pulmonary, nasal, rectal and oral in order to replace parenteral therapy [2]. The oral route is recognized as the natural and the safest route for drug administration. Generally, oral administration can improve disease management, enhance patient compliance and reduce of long-term complications of diabetes [3]. However, the oral route continues to be a challenge to deliver proteins and various barriers must be overcome to obtain an adequate bioavailability. These barriers are the permeability across gastrointestinal tract (GIT), the enzymatic barriers and protein stability in GIT environment [4].

Different formulation approaches have been investigated to overcome the GIT barriers for the delivery of insulin via oral route such as the use of liposomes, microemulsion, microspheres and nanoparticles. Nanosize carriers have a large specific surface area, and their protection power against gastrointestinal environment is mostly dependent on the degree of protein encapsulation within these nanostuctures. Such nanoparticles are thought to be the most promising solution for oral delivery of peptides and proteins [5]. However, it is well known that proteins and peptides undergo a loss of activity following encapsulation during their processing [6]. A loss of insulin activity was observed following encapsulation in polylactic acid and poly (lactide-co-glycolide) microspheres [7,8]. In addition, the use of some components used in polymerization during nanoparticles preparation may affect biocompatibility of the final formulation [9]. Therefore, researchers recently focused on the use of naturally occurring polymers especially polysaccharide such as chitosan for the preparation of carriers in an aqueous environment avoiding heating, organic solvents and severe mechanical stress [10,11]. Previously, insulin-loaded chitosan nanoparticles were prepared by ionotropic gelation of chitosan with tripolyphosphate anions [12] or by polyelectrolyte complexation with polyanionic polymers such as poly (γ-glutamic acid) and dextran [13,14]. In those studies the in vitro release tests showed a very rapid initial burst effect. The released insulin was likely to be degraded by proteolysis in the GIT resulting in a low pharmacological availability [12].

Recently, polyelectrolyte complexation method was utilized to prepare insulin–trimethyl chitosan nanoparticles for intranasal delivery of insulin [15]. Interaction of chitosan and polyanions leads to spontaneous formation of nanoparticles. However, these polyelectrolyte complexes (PECs) are easily dissociated in acidic medium, because both insulin and chitosan are soluble at lower pH such as that of the stomach. Sadeghi et al. [16] observed a

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prompt release of insulin in 0.01 N HCl from nanoparticles made by PEC method, where the released insulin in the first few minutes was about 70% of the incorporated quantity, while the rest was released after 30 min. In order to protect proteins from the unfavorable environment of the GIT, some researchers proposed the use of oily vehicles [5], where the most promising oily-based preparations are water in oil (w/o) microemulsions. These microemulsions have been proposed to enhance the oral bioavailability of peptides by protecting them from the enzymatic breakdown in the GIT, and increasing their permeability through the intestinal wall [17]. In spite of this when insulin was incorporated into a w/o microemulsion high doses were used (200 IU/kg) to elicit a pharmacological response [18].

In order to overcome the aforementioned shortcomings a new formulation strategy was adopted, where a polyelectrolyte complex of insulin–chitosan was dispersed in a microemulsion. The resultant w/o nanosized system was characterized and tested on diabetic rats.

2. Materials and methods

2.1. Materials

Recombinant human (rh) insulin powder was purchased from Biocon, Bangalore, India. Low molecular weight chitosan was prepared by acidic depolymerization of high molecular weight chitosan HCl (MWT ~ 250 kDa and DDA 93%, Xiamen Xing, Shanghai, China). Oleic acid was obtained from Merck, Germany. Plurol oleique® (polyglyceryl-6-dioleate) and Labrasol® (PEG 8 caprylic/capric glycerides) were purchased from Gattefosse S.A., Priest, France. Streptozotocin, and pepsin were obtained from Sigma–Aldrich, MO, USA.

2.2. Methods

2.2.1. Preparation and characterization of low molecular weight chitosan (LMWC)

2.2.1.1. Depolymerization of high molecular weight chitosan. Ten grams of the high molecular weight chitosan was dissolved in 1 L of 2 M HCl. The solution was heated for 3.5 h under reflux. After cooling, 3 L of ethanol (96%) was added and the precipitate was washed thoroughly with ethanol and freeze dried for 24 h. The resultant chitosan powder was identified by Fourier transform infrared (FTIR) spectroscopy.

2.2.1.2. Determination of molecular weight. The viscosity of chitosan hydrochloride dissolved in water was measured using a viscometer (Vibro viscometer, SV-10, Japan), and the viscosity average molecular weight was deduced using the Mark–Houwink's equation, $[\eta] = k \cdot M^a$, where $[\eta]$ is the intrinsic viscosity, M is the viscosity average molecular weight, and k and a values were 0.00058 and 0.69 based on a previous study [19].

2.2.1.3. Determination of degree of deacetylation. The degree of deacetylation (DDA) was determined according to spectroscopic method of the British Pharmacopoeia (B.P) 2007.

2.2.2. Preparation of insulin–chitosan polyelectrolyte complex (PEC)

2.2.2.1. Insulin–chitosan complex preparation. The low molecular weight chitosan (13 kDa and 99% DDA) was used to prepare the PEC system. Chitosan with volume of 0.5 g was placed in a glass vial, dissolved in 10 mL deionized water and its pH was adjusted to 5.5 using about 4 mL of 0.2 M NaOH, and the final volume was completed to 20 mL using deionized water. In another vial, 100 mg of rh-insulin powder was dissolved in 1 mL of 0.1 M HCl, followed by the addition of 3 mL of 1 M tris (hydroxymethyl)-aminomethane buffer pH 7. Equal volumes of the chitosan and insulin were stirred gently using magnetic stirrer for 15 min at 4 °C.

2.2.2.2. Insulin–chitosan association efficiency (AE). To determine the association efficiency of insulin with chitosan, triplicate batches of insulin–chitosan polyelectrolyte complex were centrifuged at 15000 rpm for 30 min at 15 °C and the insulin content in the supernatant was assayed by reversed phase high pressure liquid chromatography (RP-HPLC) as mentioned earlier [20]. The association efficiency was calculated as described elsewhere [16] using the following equation:

$$\text{Association efficiency (AE)} = \frac{\text{Total amount of insulin} - \text{Free insulin}}{\text{Total amount of insulin}} \times 100$$

To evaluate the factors that might affect the AE, two factors were studied, namely, PEC pH and the ratio of chitosan to insulin in the PEC.

2.2.2.3. Determination of zeta potential of insulin–chitosan complex. The zeta potential measurements were carried out with Zetasizer Nano ZS (Malvern Instruments, UK) at 25 °C. Samples of free chitosan and insulin–chitosan PEC were measured in folded capillary cells integrated with gold electrodes. Three measurements were conducted, and the number of runs in each measurement was automatically determined by the software. Smoluchowski approximation was used and the results were expressed as mean ± SD. The viscosities of samples were measured by Sine wave Vibro viscometer (SV-10, A&D Company, Japan) at 25 °C ± 0.01 in triplicate.

2.2.3. Effect of temperature on insulin stability

2.2.3.1. Determination of melting temperature of insulin. Melting temperature measurements were carried out with Zetasizer Nano ZS (Malvern Instruments, UK) using a 1 °C incremental temperature ramp and a 3 min equilibrium time at each melting temperature measurement. Insulin solutions in tris buffer with the concentration of 1 mg/mL and pH 7 ± 0.1 were prepared and their melting temperature was determined. The marked point where both the size and the intensity start to increase significantly is called melting temperature (T_m).

2.2.3.2. Effect of temperature on the stability of insulin–chitosan PEC. To evaluate the effect of LMWC on the stability of insulin, accelerated stability testing was conducted. PEC suspension with volume of 10 mL was prepared as mentioned in Section 2.3 except that the final concentration of both insulin and chitosan was adjusted to 500 μg/mL. PEC suspension was incubated in a water bath shaker at 55 ± 1 °C with shaking of 100 strokes/min. At predetermined time points (0, 12, 24, 48 h), aliquots were withdrawn and diluted with 0.01 M HCl to dissolve the nanoparticles. The insulin content was determined by RP-HPLC as described earlier. In addition, the stability of a standard insulin solution was assessed under the same testing conditions. All samples were prepared in triplicate.

2.2.4. Preparation and characterization of the nanoparticle dispersion system

In order to obtain a nanoparticle system, insulin–chitosan PEC was mixed with an oily phase and surfactant system. The optimal component concentration range to obtain a transparent system was obtained by the construction of a phase diagram.

2.2.4.1. Construction of a phase diagram. A mixture of 2.5 g of oleic acid/surfactant was accurately weighed into screw-capped glass tubes. Labrasol and plurol oleique were selected as surfactant mix-

ture (S_{mix}), and their weight ratio was fixed at (1:1) w/w. The pseudo-ternary phase diagram was constructed by titration of homogeneous liquid mixtures of oil and surfactants with deionized water at room temperature. The weight ratios of oleic acid/S_{mix} used were 9/1, 8/2, 7/3, 6/4, 5/5, 4/6, 3/7, 2/8 and 1/9. Samples were vortexed for 5 min and then diluted with water in an incremental method, and mixed using vortexer for 3 min to accelerate their equilibration. Following the addition of aliquot of water, the mixture was visually examined for transparency. After water titration, titration of oil surfactant mixtures with PEC was performed in the same manner.

2.2.4.2. Preparation of nanoparticle dispersion system. An aqueous phase and an oily phase were mixed together to prepared the nanoparticle dispersion system.

To prepare the oily phase which is composed of oleic acid/surfactant mixture, plurol oleique® (polyglyceryl-6-dioleate) and labrasol® (PEG 8 caprylic/capric glycerides) were mixed in a ratio of 1:1 w/w using magnetic stirring for 3–5 min. Then, 0.5 g of surfactants' mixture and 2 g of oleic acid were placed in a tube and vortexed vigorously for 5 min.

The aqueous phase (insulin–chitosan PEC) solution was prepared as mentioned in Section 2.2.2.

To prepare the dispersion system, 50 μl of the aqueous phase was added to 2.5 gm of oily phase and vortexed for 1 min at room temperature (25 °C).

2.2.4.3. Particle size determination.

(i) Particle size of insulin–chitosan PEC in oleic acid

The particle size distribution of the dispersed insulin–chitosan PEC (1:1 w/w) particles prepared with oleic acid was assessed by photon correlation spectroscopy using a Malvern Zetasizer Nano-ZS series (Malvern Instruments, UK). Collective 13 readings were performed three times on a sample at 25 °C with a detection angle of 173°. The average and standard deviations were calculated by the instrument built-in software. The viscosity was determined using a Vibro viscometer (SV-10, A&D Company, Japan) at 25 °C. The viscometer was calibrated using standard oil (Viscosity Standard, Poulten Selfe and Lee, UK).

The particle morphology of insulin–chitosan PEC dispersed in oleic acid was assessed by an optical microscope connected to a Camera (Nikon, Japan). Chitosan/insulin was prepared as mentioned previously. Then, 0.5 mL of PEC aqueous solution was mixed vigorously with 25 mL oleic acid resulting in a milky appearance oily dispersion. One drop of the dispersion was placed on a microscopic glass slide and a microscopic photograph was obtained.

(ii) Particle size of the insulin–chitosan PEC in oleic acid/surfactant mixture

The particle size distribution of the dispersed nanoparticles prepared with the surfactant system was assessed by photon correlation spectroscopy, using a Malvern Zetasizer Nano-ZS series (Malvern Instruments, UK). Samples were diluted 1:10 v/v with oleic acid in order to reduce the viscosity of the mixture, so as to correlate the particle size measurement with the viscosity of the solvent, where the viscosity of the solvent (oleic acid) measured at 25 °C was 29 ± 0.34 mPs. The viscosity was determined using a Vibro viscometer (SV-10, A&D Company, Japan) at 25 °C. Particle size and viscosity of the preparation were determined initially and after storage for 1 month at 4 or 25 °C.

The particle morphology was examined by transmission electron microscope (TEM) (Zeiss EM 10 CR, Germany) after precipitating the nanoparticles, where 0.5 mL of the nanoparticle preparation was mixed with 10 mL acetone and placed in a centrifuge at 4000 rpm for 10 min. The precipitated nanoparticles were rinsed several times with acetone prior to redispersing the particles in a 10 mL solution of water: acetone (1:1) v/v. Alternatively, a drop of the solution was added to Formvar coated grid, and left to dry at room temperature to be studied under the TEM.

2.2.4.4. Chemical stability and Immunological activity of the entrapped insulin in the nanoparticle dispersion system. To assess the chemical and immunological stability of insulin following formulation, RP-HPLC and enzyme-linked immunoassay (ELISA) techniques were applied. An extraction fluid composed of methanol and 0.01 M hydrochloric acid with a ratio 2:3 v/v was used to extract rh-insulin for analysis. Two grams of the preparation and 5 mL of extraction fluid were vortexed vigorously for 2 min and were centrifuged immediately at 4000 rpm for 15 min. An aliquot of the separated aqueous phase was used for HPLC analysis of content of rh-insulin in the preparation (HPLC, Thermospectra HPLC using TSP 1000 pump system with TSP 1000 UV–VIS detector and a TSP AS 3000 autosampler, Spectra System, USA) as described previously [20]. The conditions employed were as follows: the column was ACE 5 μm, 250 mm × 5 mm i.d and 300 Å pore size (ACE, Scotland); detection at 214 nm; eluent, acetonitrile-aqueous solution of 0.2 M sodium sulfate acidified with concentrated phosphoric acid to pH 2.3 (volume ratio 27:73); flow rate was 1 mL/min. The HPLC analytical method was validated. A summary of inter and intraday variability using different standard insulin concentrations (0.5, 1, 1.5 and 2 IU/mL) is shown in Table 1, where the coefficient of variation (CV%) within or between runs was below 2%.

Immunological activity of insulin in the formula was analyzed by ELISA (Ins-EASIA, Biosource Europe SA., Rue de l'lndustrie, 8, B-1400 Nivelles, Belgium). Insulin was extracted from the nanoparticles preparation by the method mentioned above, and the extract was assayed according to the instructions of the manufacturer. The results were obtained by reading the optical density at 450 nm using Bio-Rad microplate reader (Bio-Rad, USA).

2.2.4.5. Protection against simulated gastric juice. To assess the protective effect against gastric degradation, 2 g of the preparation was incubated (37 °C) and shaken with 5 mL of simulated gastric fluid pH 1.2 with and without pepsin (SGF USP) for 1 h in a water bath shaker (100 strokes per minute). Enzymatic degradation was achieved by the addition of pepsin (800 U/mg proteins). The enzyme was dissolved in the gastric juice immediately before starting the experiment to prevent loss of activity. The oily phase was separated from the SGF and 1.5 g of the oily phase was mixed with 2 mL of methanol (to terminate the enzyme activity), 3 mL of 0.01 M HCl and insulin was extracted from the nanoparticles preparation as mentioned before. The resultant aqueous phase was separated by centrifugation and analyzed by RP-HPLC. Free insulin solution and insulin–chitosan PEC aqueous suspension with the same concentration as the preparation were also incubated with SGF with and without pepsin, and shaken at 100 strokes per minute and 37 °C for 1 h.

Table 1
HPLC method showing "between" and "within run" variations for insulin measurements ($n = 3$).

Concentration (IU/ml)	Within run CV (%)	Between-run CV (%)
0.5	0.88	0.66
1	1.51	1.38
1.5	0.86	1.63
2	0.31	1.88

CV = coefficient of variation.

2.2.4.6. Chemical stability of insulin nanoparticle preparation. A short-term chemical stability of the nanoparticle preparation was examined at scheduled time intervals over a period of 30 days of storage at 4 °C and at 25 °C by RP-HPLC analysis.

Three independent batches were prepared, and at each time interval two samples were withdrawn and analyzed. Preparation was conducted under sterile conditions in a clean environment Class II Biohazard Safety cabinet (Comfort, PBI, Italy), and autoclaved water was used for the preparation of the batches to minimize the microbial degradation of insulin.

2.2.4.7. Physical stability of insulin nanoparticle preparation. Particle size and viscosity of the preparation were determined initially and after storage for 1 month at 4 and at 25 °C. The viscosity was determined using Vibro viscometer (SV-10, A&D Company, Japan) at 25 °C. The viscometer was calibrated by standard oil (Viscosity Standard, Poulten Selfe and Lee, UK).

2.2.5. In vivo studies on streptozotocin (STZ) diabetic rats

2.2.5.1. Animals. Adult male Wistar rats (250–300 g) were housed in air-conditioned quarters under a photoperiod schedule of 12 h light/12 h dark cycles. The rats received standard laboratory chow and tap water available ad libitum 3 weeks prior to the experiments. All experiments on animals were carried out in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC).

2.2.5.2. Induction of diabetes using STZ. Diabetes was induced in male Wistar rats by intraperitoneal injection of two doses of streptozotocin (80 mg/kg body weight). Streptozotocin was dissolved in 0.1 M citrate buffer (pH 4.5) immediately before use as described previously [18]. Diabetes was confirmed by measuring glucose concentration in a blood sample obtained from the tail vein using a blood glucose meter (One Touch™ Sure Step™, LifeScan, Inc., USA). Only rats with a basal blood glucose levels over 200 mg/dL were considered as diabetic, and used in the present study.

2.2.5.3. Pharmacological activity of the nanoparticle preparation.

(i) STZ-diabetic rats exposed to 12 h fasing/12 h free access to food postdosing

STZ-diabetic rats were used to evaluate the oral pharmacological action of a fresh and a stored preparation. The total duration of the experiment after dosing was 24 h. The animals were fasted from food, but had free access to water during the first 12 h, while in the second 12 h the animals had free access to both water and food. Animals were divided into five groups and 10 rats were used per group. Blood glucose levels were measured at different time intervals after a single oral dose administration (50 IU/kg) of a freshly prepared nanoparticle preparation, nanoparticle preparation stored at 4 °C for 1 month, insulin standard solution and a subcutaneous administration of insulin given in a dose of (1 IU/kg) compared to an oral placebo preparation (without insulin) used as a negative control.

In order to prove the advantage of using insulin–chitosan PEC in microemulsion, another experiment was conducted to compare the pharmacological activity of insulin in an ordinary w/o microemulsion (prepared without the use of chitosan) in comparison with nanoparticle preparation (insulin–chitosan PEC in microemulsion). STZ-diabetic animals were divided into three groups, group one was given the insulin-loaded nanoparticle preparation, group two was given the insulin w/o microemulsion and group three was given a placebo. Blood glucose measurements were performed as mentioned previously.

The relative pharmacological availability (PA%) was calculated using the following equation

$$PA\% = (AAC_{oral}/AAC_{sc}) * (Dose_{sc}/Dose_{oral}) * 100$$

where AAC is the area above the baseline curve calculated from zero time to 24 h. The subscript sc and oral refer to group of rats given subcutaneous injection and oral formulation, respectively.

(ii) STZ-diabetic rats exposed to 24 h free access to food postdosing

In order to evaluate the effect of food on the pharmacological response of oral insulin, the following experiment was carried out. STZ-diabetic rats were fasted overnight for 12 h but had free access to water. At the morning, food was introduced at the time of dosing and continued till the end of the experiment i.e. 24 h. Animals were divided into three groups of 10 rats. Blood glucose level time profiles were constructed after a single oral administration of nanoparticle preparation (50 IU/kg), SC insulin standard injection (1 IU/kg) and a control group of non-fasted diabetic rats.

2.2.6. Statistical treatment

The results were expressed as the mean ± MSE (mean squared error). For group comparison, a one-way analysis of variance (ANOVA) was applied (Microsoft® Office Excel 2003, Microsoft Corporation). A difference was considered statistically significant when the probability value (P) was less than 0.05.

3. Results and discussion

3.1. Depolymerization, molecular weight and degree of deacetylation determination

Depolymerization of high molecular weight chitosan by hydrochloric acid was carried out according to previously reported methods [21–23]. This yielded fractions of low molecular weight chitosans. Identification of theses fractions were carried out by FTIR which showed similar distinct peaks as appeared in the parent compound. This is evidenced that depolymerization process did not change the basic chemical structure.

In this work, a molecular weight of 13 kDa with a degree of deacetylation of 99% was selected for further work due to its suitability as indicated by a previous developmental work [23].

3.2. Preparation of insulin–chitosan PEC

Insulin and chitosan mixture was prepared through the gradual addition of the two solutions containing materials with opposite charges to facilitate the formation of the PEC. However, PEC preparation was previously reported for polymers having ionizable groups and bear opposite charges [24].

3.2.1. Insulin–chitosan PEC association efficiency

3.2.1.1. Effect of pH on AE. Chitosan having low molecular weight and high degree of deacetylation is highly active due to a greater number of amino groups available for interactions with the anionic active sites [25]. Chitosan solution pH was adjusted to 5.5. At this pH about 90% of the amine groups are protonated as it has an apparent pKa of ~6.5 [26]. Such protonation leads to chain repulsion and more extended conformations [11]. This exposes the amine groups to the negatively charged insulin leading to their interaction. Insulin has an apparent isoelectric point (pI) of 6.4 and a charge of −2 at a final pH of ~6.8 [27]. The influence of the final pH on association efficiency was evaluated as presented in Table 2. High AE was obtained at pH 6.5 (about 80%). It was re-

Table 2
Effect of pH and chitosan to insulin weight ratio on AE (average ± standard deviation), $n = 3$.

	AE ± SD
pH	
6.5	78.81 ± 0.68
6.8	30.38 ± 3.71
Ratio (w/w)	
1:1	78.81 ± 0.68
2:1	73.38 ± 3.42
3:1	77.11 ± 3.94

ported that the formation of PEC between the two oppositely charged polymers can only occur at pH values in the vicinity of the pKa interval of the two polymers [24]. The AE fell sharply when the final pH of the complex was adjusted to 6.8 (about 30%). This finding is in agreement with studies of Ma et al. [28] who recorded a sharp change in AE with small pH changes. It seems that chitosan becomes increasingly globular as the pH increases, and starts to precipitate [11].

3.2.1.2. Effect of chitosan to insulin ratio on AE. The AE was not affected when chitosan to insulin ratio was increased from 1: 1 to 3:1 as shown in Table 2. This finding is inconsistent with some previous studies [29,30]. This contradiction may be attributed to the differences in the chitosan molecular weight used and their purity. Chitosan /insulin charge ratio (+/−) was calculated according to the charge and pKa of the components at pH 6.5. It was found 9:1 and 27:1 for chitosan/insulin, 1:1 and 3:1 w/w ratio, respectively. The positive charge of chitosan was present in molar excess, and may partially explain the constant AE at different chitosan to insulin ratios. However, it is not only the charge but chitosan conformation and viscosity were also known to be affected upon the increase in chitosan concentration in solution state. All of these factors could influence the interaction of chitosan and insulin and, hence, affecting the AE. It was reported that chitosan at high concentration adopts a random coil conformation, and the proportions of the charged segments are shielded due to strong intermolecular interactions [31]. In addition, the viscosity of chitosan increases with the increase in chitosan concentration in solution [11]. This may hamper the kinetics and led to a limited binding with insulin.

3.2.1.3. Zeta potential. Zeta potentials of chitosan and of chitosan–insulin (1:1 w/w) complexes were 56.83 ± 0.31 and 29.97 ± 1.65 mV, respectively. This result serves as an evidence for complex formation between chitosan and insulin. As expected the zeta potential of chitosan is positive due to presence of amino groups. All chitosan–insulin complexes displayed a lower positive zeta potential in comparison with the free chitosan. The ratio of negative charge (insulin) to the positive charge of the chitosan remains in the favor of the positive charge of the polymer as only a fraction is neutralized by binding insulin. This indicated that partial neutralization of chitosan charge took place. These results agree well with the results reported by Sadeghi et al. [16].

3.3. Insulin stability

Insulin is a labile protein similar to other proteins. They are affected by formulation components, process and storage temperature [32]. It is well established that all proteins unfold above their melting temperature T_m [32]. In order to assess insulin stability, the temperature used for testing should be far enough from its T_m. Consequently, T_m of insulin should be first measured prior to accelerated stability testing.

3.3.1. Melting point of insulin in tris buffer

Different techniques have been used to determine T_m, such as differential scanning calorimetry, infrared spectroscopy and capillary electrophoresis [33,34]. Recently, dynamic light scattering technique (DLS) was used, when denaturation occurs, the small size of the protein is increased to a value consistent with a random coil polymer of the same molecular weight. In the absence of chaotropic (aggregation prohibiting) agents, inter-polymer hydrophobic interactions may quickly lead to non-specific aggregation of the denatured polypeptide chains. The change in size that accompanies the protein denaturation is easily identified using DLS.

Fig. 1 shows the melting temperature profile for insulin dissolved in tris buffer. At temperatures less than 60 °C, the size and scattering intensity are constant, suggesting a stable tertiary structure. At temperatures higher than 61 °C, both the size and scattering intensity increase exponentially with temperature, indicating the presence of denatured aggregates. The T_m determined by DLS is in agreement with that reported in literature using DSC technique [14]. The average T_m of insulin obtained in this study was 63 ± 1 °C.

3.3.2. Accelerated stability of insulin–chitosan PEC

To illustrate the role of LMWC on stabilization of insulin, insulin–chitosan PEC was subjected to 55 °C temperature which is 8 °C less than the T_m of insulin and was compared with the free insulin solution. It is worth mentioning that other investigators used temperatures ⩾40 °C to study the stability of insulin preparations [35]. After 48 h insulin solution was almost completely degraded as shown in Fig. 2. High temperature causes denaturation of insulin by the rapid formation of bovine insulin fibrils [32]. In addition, high temperature accelerates its chemical degradation. It was reported that formation of insulin oligomers and polymers increases at temperature ⩾25 °C [36], while shaking creates hydrophobic air/water interface and, therefore, initiate aggregation [37]. However, insulin–chitosan PEC protects insulin from degradation for at least 24 h. Although high molecular weight chitosan and chitosan derivatives were found to enhance the thermal stability of insulin [38], the effect of chitosan low molecular weight has yet to be investigated. The protective effect of PEC may be due to the reduction of aggregation and fibrillation of insulin as free insulin solution becomes turbid after 24 h. Other cationic polymers such as poly (ethylene glycol)-*b*-poly (L-histidine) that forms PEC with insulin reduce aggregation of insulin on agitation [39]. Consequently, one can assume that LMWC reduces insulin aggregations. In addition, Mao et al. suggested that higher temperature causes compaction of insulin nanoparticles [15]. However, the influence of LMWC is still wide open for investigation.

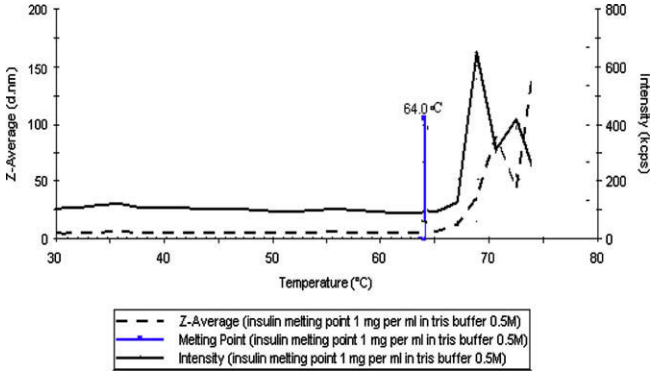

Fig. 1. Melting temperature of insulin dissolved in 0.5 M tris (hydroxymethyl)-aminomethane buffer.

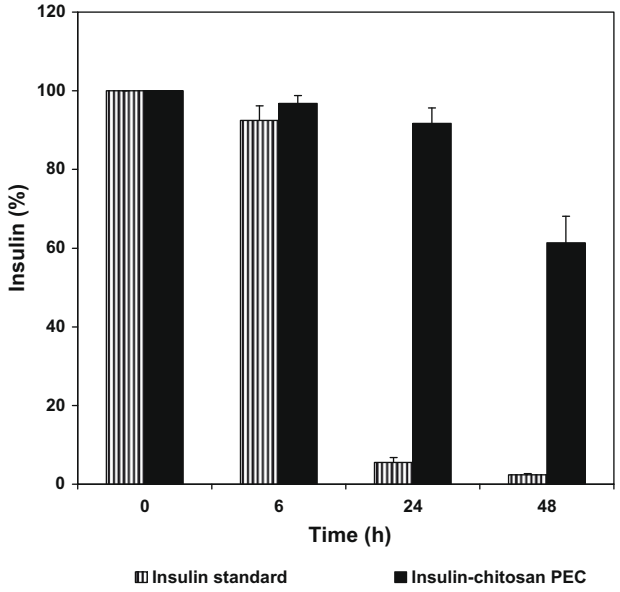

Fig. 2. Chemical stability of free insulin and insulin–chitosan PEC placed on a water bath shaker at a temperature of 55 °C with 100 strokes/min for 48 h.

3.4. Preparation and characterization of nanoparticles

3.4.1. Phase diagram

A pseudo-ternary phase diagram was constructed by titrating the mixtures of oleic acid and surfactants with water. The end point was taken when the system turned into transparent. This was carried out to find the optimal component concentration. The shaded area in the pseudo-ternary phase diagram, as shown in Fig. 3, represents concentrations where w/o microemulsion was formed. The addition of larger volumes of aqueous phase caused turbidity in the solution yielding very limited microemulsion area in the presented phase diagram.

The optimal w/o microemulsion was selected from the transparent area in the phase diagram. It is composed of 20% surfactants mixture, 78% oily phase and 2% aqueous phase as weight percentages. The preparation composition was selected according to offer optimal drug-loading efficiency keeping surfactant mixture in concentration as low as possible to form w/o microemulsion. Generally, surfactants may induce certain mucosal toxicities when used at high concentrations [40]. Furthermore, when the optimal composition was diluted 10-fold with deionized water, phase inversion was not observed. Transparent systems in the phase diagram that were converted to o/w emulsion upon dilution were excluded because of insolubility of insulin in oil. This allows insulin to partition into the external aqueous phase, where it would be subjected ultimately to digestive enzymes upon its dilution with the fluids of the GIT. Such precaution was previously reported [41].

3.4.2. Nanoparticles dispersion system

In the present investigation, it was attempted to formulate an oral insulin delivery system that combines the advantages of nanoencapsulation and the use of an oily vehicle. Nanoparticles are expected to translocate the intestinal epithelium, while the oily vehicle is intended to reduce proteolytic degradation and to improve absorption [42,43]. Watnasirichaikul et al. demonstrated the advantages of dispersion of poly (ethylcyanoacrylate) nanocapsules in w/o microemulsion [44].

The oily vehicle contains known penetration enhancers such as oleic acid and labrasol [45,46]. In addition, LMWC was selected to prepare the nanoparticles as its intestinal absorption is known to be significantly better than the high molecular weight chitosans and showed negligible cytotoxic effect on the Caco-2 cells [47]. Moreover, the interaction between chitosan and oleic acid renders the surface of the particles more hydrophobic, which may promote lymphatic uptake [48]. A previous report illustrates the capability of chitosan to bind various fatty acids to form the corresponding complexes which are stable in the acidic environment of the stomach [49]. The possible advantage for the use of oleic acid as a carrier might be the formation of a protective hydrophobic coating layer at the surface of nanoparticles. This layer is formed due to the interaction between the free chitosan amine groups in the nanoparticles and the adjacent carboxylic acid functional groups of oleic acid as dispersion medium.

The final pH of the aqueous phase was adjusted to 6.5 to ensure high association efficiency. The ratio of chitosan to insulin was selected at 1:1 w/w, since the increase in chitosan did not result in an increase in association efficiency as shown in Table 2.

3.4.3. Particle size and microscopy

Dynamic light scattering of the particles is presented in Fig. 4. The mean diameter of the particles of insulin–chitosan (1:1 w/w)

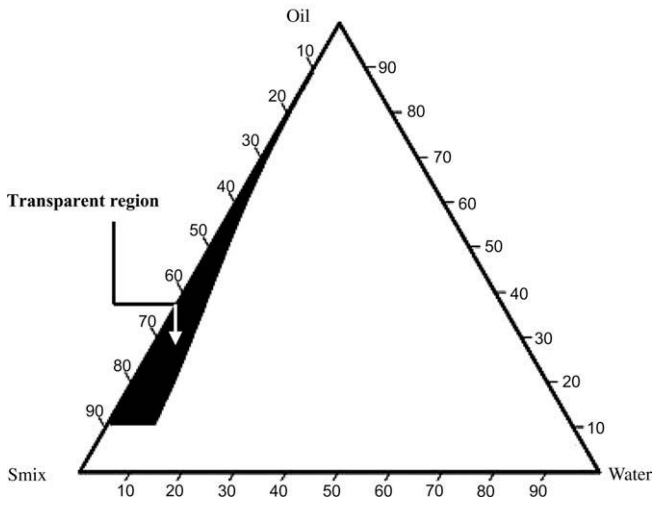

Fig. 3. Pseudo-ternary phase diagram of microemulsion composed of oleic acid (oil), Smix (Labrasol to Plurol-oleique in a ratio of 1:1 w:w) and water. The optimal preparation was selected from the transparent region.

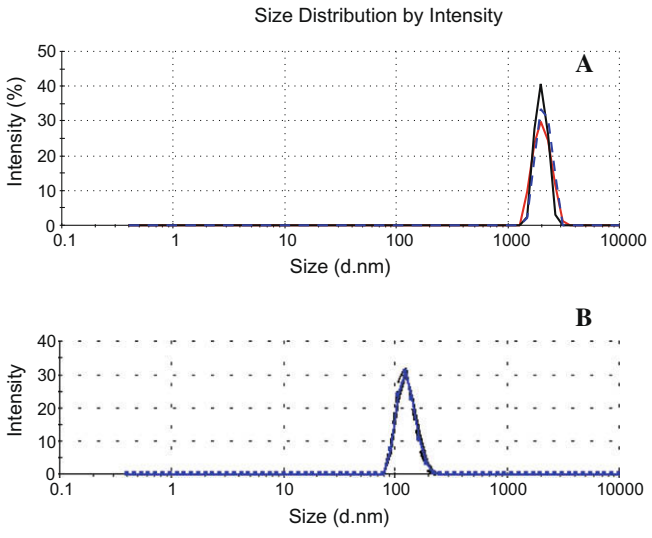

Fig. 4. Particle size distribution of chitosan–insulin PEC particles dispersed in oleic acid (A) and in oleic acid surfactant mixture (B), which shows dramatic reduction in the particle size.

PEC dispersed in oleic acid was 2040 ± 63 nm, while in oleic acid/surfactant mixture was 108 ± 9 nm. This clearly showed the importance of the use of a surfactant mixture with oleic acid in order to produce nanoparticles. The produced nanoparticles had a unimodal size distribution.

It is generally accepted that particle transcytosis increases when the particle diameter decreases. Studies on polystyrene latex revealed that maximal number of absorbed nanoparticles takes place with particles ranging 50–100 nm in diameter, while particle above 1 μm being trapped in the Peyer's patches [41].

Particle shape was assessed using optical microscope and TEM. As shown in Fig. 5, Insulin–chitosan PEC upon dispersing in oleic acid forms relatively large spherical microparticles. The use of a surfactant system mixture has been found to reduce the particle size to the nano-level, while keeping the sphere-like shape as shown by the TEM photos.

The diameter of the nanoparticles obtained from DLS and TEM was estimated to be 108 ± 9 and 100 ± 8 nm, respectively. This confirms the size of the resulted particles.

3.4.4. Chemical stability and immunological activity of the entrapped insulin

One of the challenging tasks in the development of protein pharmaceuticals is dealing with physical and chemical stability of proteins when they are incorporated into drug delivery systems. Both formulation and processing parameters could influence the stability [6]. In the present study, the method of preparation is mild; there is no heat and the mechanical stress is minimal. However, in lipid-based formulations, the protein is exposed to the mixing process of the oil and aqueous phases and to the creation of the oil–water interface [50]. As proteins are amphiphilic, they are susceptible to structural changes when exposed to interfaces created during the preparation [50]. Two different analytical assay procedures (RP-HPLC and ELISA) were used to assess the stability of insulin in formulations. The chemical stability as indicated by RP-HPLC chromatograms demonstrated that insulin was not degraded or aggregated after formulation. The retention times and the shapes of the peaks of freshly prepared insulin solution and insulin extracted from the preparation were identical as shown in Fig. 6, where insulin was recovered with a percentage of 95 ± 3%. In addition, transformation products were not detected. The immunological stabil-

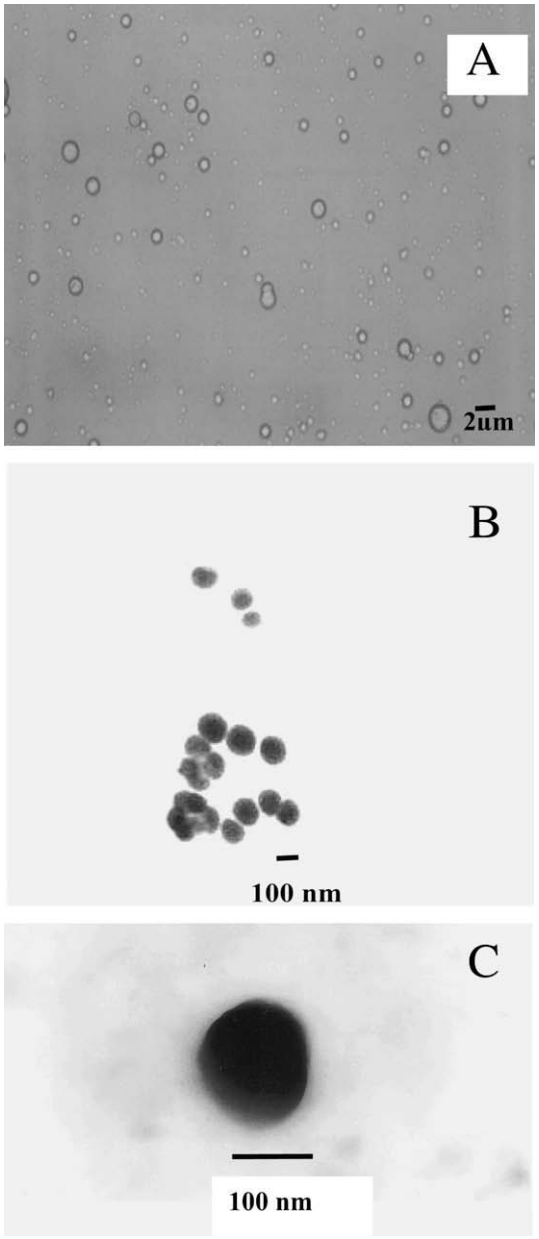

Fig. 5. Optical microscope image of chitosan–insulin microparticles dispersed in oleic acid (A), transmission electron microscope (TEM) images of nanoparticles obtained by precipitation indicating a representative sample of nanoparticles (B) and TEM image of one nanoparticle indicating the shape of a nanoparticle (C). The size of the nanoparticles was confirmed by DLS technique.

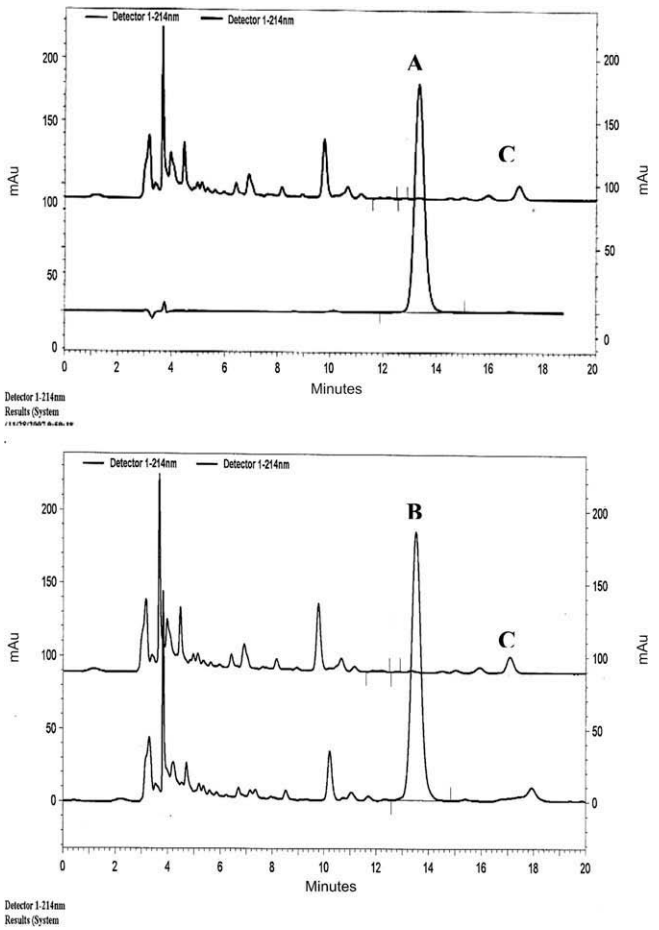

Fig. 6. RP-HPLC chromatograms of (A) for insulin fresh standard, (B) for insulin extracted from the preparation and (C) for the placebo preparation (without insulin).

ity as obtained by the ELISA was also comparable to those obtained from RP-HPLC. The results of these two analytical techniques may indicate that the structure of insulin was able to withstand the preparation process. This may be due to the formation of PEC which prevents free insulin from interacting directly with interfaces. The approach of protecting the protein from the interface via the use of a polymer system was adopted previously [51].

3.4.5. Protection against simulated gastric juice

The protective ability of the preparation for insulin under conditions simulating the gastric environment was evaluated and compared with free insulin and PEC. Free insulin and insulin–chitosan PEC were found to be completely degraded during incubation with pepsin as illustrated in Fig. 7. The degradation of insulin–chitosan PEC might be related to both insulin and chitosan having net positive charges at pH 1.2, the columbic repulsive forces lead to the dissociation of the complex rendering the free insulin subject to degradation.

In the nanoparticles preparation, about 90% of insulin was recovered from the preparation after incubation with pepsin. These results agree well with a previous report in which nanospheres were protected against proteolysis when dispersed in oily vehicle compared to water [52]. These findings illustrate the importance of the external oily phase in the protection of insulin–chitosan PEC from degradation via the stomach enzyme.

3.4.6. Short-term stability of insulin in the nanoparticle dispersion system

Short-term chemical stability of insulin in the nanoparticle preparation was also determined over a period of 1 month storage as shown in Table 3. Results illustrate the absence of significant difference ($P > 0.05$) between the initial assay and the insulin recovered after 30 days storage at 4 and at 25 °C. This indicates that the chemical stability of insulin was maintained for at least 30 days of storage according to HPLC method.

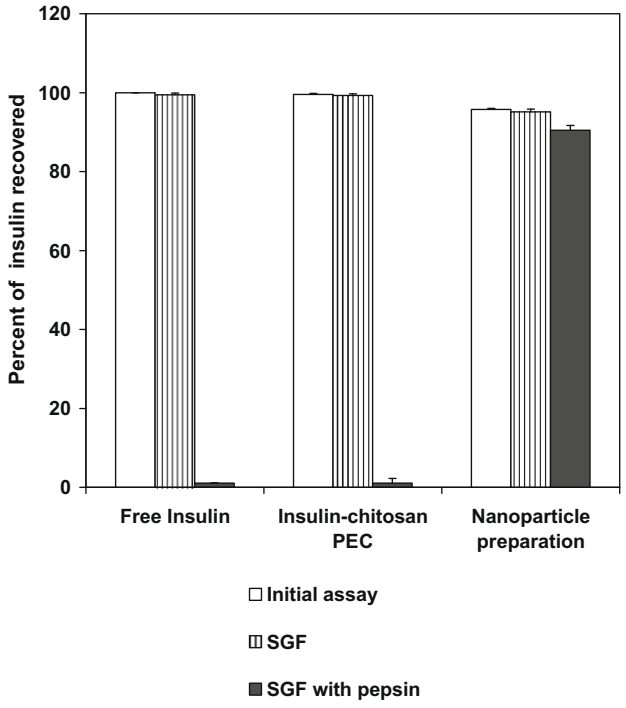

Fig. 7. Percent of insulin recovered (assay) from both insulin/chitosan PEC solution and nanoparticle preparation after incubation for 1 h in SGF with and without pepsin in comparison with a standard free insulin solution.

Table 3
Assay (average ± standard deviation) of the nanoparticle preparation at 4 and 25 °C, $n = 3$.

Intervals	% assay ± SD of insulin in preparation stored at 4 °C	% assay ± SD of insulin in preparation stored at 25 °C
Initial	96.10 ± 0.92	96.10 ± 0.92
3 days	97.90 ± 0.46	96.71 ± 0.60
7 days	95.78 ± 1.08	96.33 ± 0.36
15 days	95.13 ± 0.87	95.17 ± 0.45
21 days	94.42 ± 1.47	95.02 ± 0.74
30 days	94.92 ± 0.35	94 .89 ± 1.29

Table 4
Viscosity and particle size (average ± standard deviation) of the nanoparticles preparation measured initially and after storage at 4 and 25 °C for 1 month, $n = 3$.

	Initial	4 °C	25 °C
Viscosity ± SD (mPa s)	52.25 ± 2.6	53.44 ± 1.56	53.32 ± 2.08
Particle size ± SD (nm)	108 ± 9	118 ± 6	117 ± 6

The viscosity of the preparation and the particle size distribution were also evaluated initially and after storage at 4 and 25 °C as shown in Table 4. No significant change was observed in the viscosity or particle size upon storage ($P > 0.05$). These results illustrate the physical stability of the nanoparticle preparation. Consequently, the physicochemical stability was assumed, and further work on animals was preceded.

3.5. Pharmacological activity of insulin-loaded nanoparticles given orally to STZ-diabetic rats

(i) STZ-diabetic rats exposed to 12 h fasted/12 h free access to food postdosing

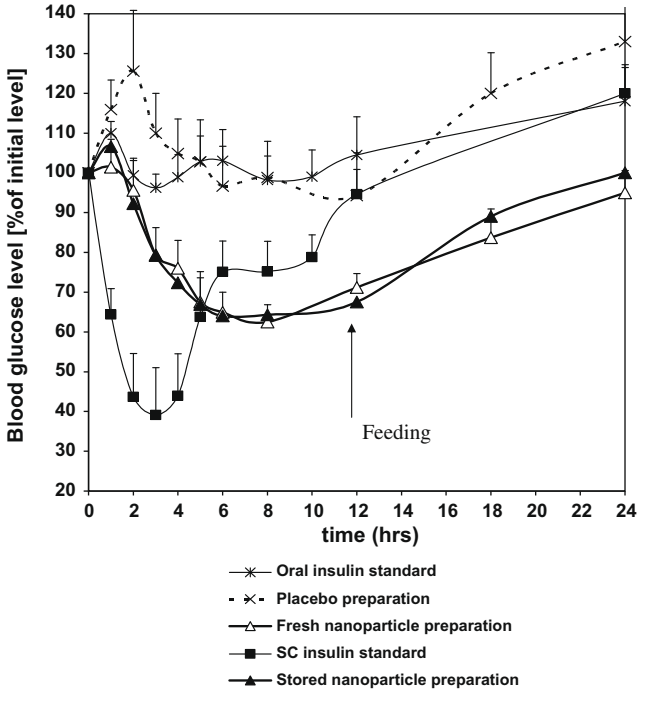

Fig. 8. Blood glucose level versus time profiles after a single oral administration of a freshly prepared nanoparticle preparation (50 IU/kg), nanoparticle preparation stored at 4 °C for 1 month (50 IU/kg), insulin oral standard solution (50 IU/kg) and a subcutaneous administration of insulin (1 IU/kg) to STZ-diabetic rats compared to an oral placebo preparation (without insulin) used as a negative control. Results are expressed as mean ± S.E.M ($n = 10$ per group).

STZ-diabetic rats were used to evaluate the oral pharmacological action of insulin-loaded nanoparticles. Fig. 8 illustrates changes in blood glucose levels after oral administration of nanoparticles. As expected, free insulin oral solution showed no hypoglycemic effect. The blood glucose levels of the rats decreased remarkably after oral administration of chitosan nanoparticles, achieving a significant decrease after 3 h compared to the control group ($P < 0.05$). More interestingly, the hypoglycemic effect was maintained without recovery to the baseline for the first 12 h.

In subcutaneous injection, the minimum glucose concentration was observed 3 h following the injection of free insulin followed by a rapid increase in blood glucose level until it returned to the baseline after about 12 h. In the following 12 h when the rats had free access to food, the rats given the nanoparticle preparation returned slowly to their initial baseline level, and their glucose reduction values were below that of the placebo at all time intervals. This indicates that the hypoglycemic effect of oral insulin nanoparticle preparation was sustained for a longer time compared to subcutaneous injection. This agrees well with the finding by other researchers e.g. Damge et al. [52] observed a sustained effect with poly cyanoacrylate nanoparticles.

The mechanism of absorption of the nanoparticle preparation and the reason for sustained release behavior are still not clear and are currently under investigation in our laboratories.

In the same experiment, another group of STZ-diabetic rats was given insulin-loaded nanoparticle preparation which was stored at 4 °C for 30 days to assess the pharmacological activity of the incorporated insulin after storage. As depicted in Fig. 8 there was no significant difference ($P < 0.05$) between the groups taking the fresh and stored nanoparticle preparation at all time intervals indicating that the biological stability of insulin was maintained at 4 °C. Assessment of biological stability during storage of proteinaceous preparations was not frequently studied. Patel et al. [53] reported a loss of biological activity of insulin in an emulsion formulation between 14 and 28 days.

In order to illustrate the advantage of the presence of insulin–chitosan PEC in microemulsion, two preparations were given orally to STZ-diabetic rats one with a w/o microemulsion (without PEC) and the other w/o microemulsion containing PEC. Insulin microemulsion showed slight reduction in blood glucose level, and this decrease was not significant up to 8 h when compared to a placebo ($P > 0.05$), as shown in Fig. 9. On the other hand, nanoparticle preparation had a significant pharmacological response specifically in the period from 3 to 8 h compared to the control group ($P < 0.05$). The calculated AAC 0–24 for the microemulsion and nanoparticle preparations were 649.06 and 1017.73%h, respectively, as shown in Table 5. The pharmacological availability for STZ-diabetic rats subjected to 12 h fast/12 h fed status postdosing was found to be 4.44 and 7.0% for the microemulsion and nanoparticle preparation when given in an oral dose of 50 IU/kg, respectively. This indicated the higher pharmacological availability of the nanoparticle preparation when compared with the microemulsion which could imply the significance of the presence of chitosan in the nanoparticle preparation.

(ii) STZ-diabetic rats exposed to 24 h free access to food postdosing

The effect of food was evaluated in the STZ-diabetic rats by keeping the animals on free access to food at the time of dosing and until the end of the experiment. Food was found to increase the blood glucose levels as depicted by the increase in glucose levels for the control group even higher than their initial baseline as shown in Fig. 10. The absorbed insulin is believed to exert its short-term pharmacological action by decreasing the glucose level in blood. However, this response depends on the rate and extent of

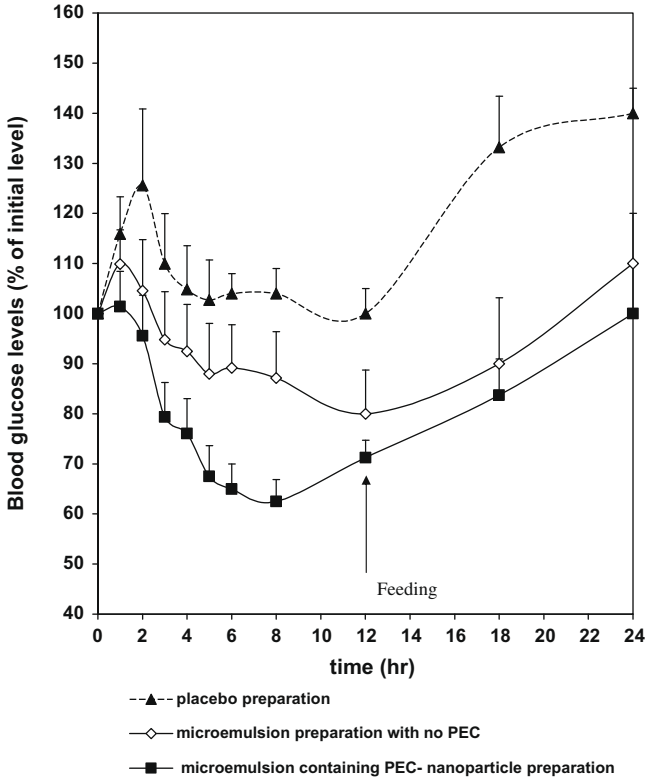

Fig. 9. Blood glucose level versus time profiles after a single oral administration of insulin w/o microemulsion (50 IU/kg), nanoparticle preparation (50 IU/kg) to STZ-diabetic rats compared to an oral placebo preparation (without insulin) used as a negative control. Results are expressed as mean ± S.E.M ($n = 10$ per group).

Table 5
Calculations of area above baseline curve (AAC 0–24) and pharmacological availability for STZ-diabetic rats given an oral insulin dose of 50 IU/kg in form of microemulsion and nanoparticle preparation over a period of 24 h (12 h fasted/12 h fed) postdosing, $n = 10$.

	Microemulsions	Nanoparticles
AAC 0–24 (Mean) (%h)	649.06	1017.73
SD	366.14	452.0
CV%	56.41	44.41
PA%*	4.44	7.0

SD: standard deviation.
CV: coefficient of variation.
PA: Relative pharmacological availability.
PA was calculated based on AAC0-24 of SC insulin standard (291.98%h) given in a dose of 1 IU/kg.
* $P < 0.05$

insulin absorbed. The estimated pharmacological availability was found to be 2.57% for diabetic rats persisted in free access to food for 24 h in comparison with 7% for 12 h fast/12 h fed state. This could indicate that food has a large influence on the pharmacological availability of the oral insulin nanoparticle preparation; when food is administered concurrently with the oral insulin nanoparticle preparation, a significant reduction in pharmacological availability was observed. This could be attributed to the fact that the presence of food in the gastrointestinal tract initiates the process of food digestion and its consequences, where chemical and mechanical stresses take place in the gastrointestinal tract. Insulin nanoparticle preparation is expected to have been mixed with food and digestive enzymes inside the stomach. In addition, the gastric residence time becomes prolonged in the presence of food which could increase the chances of degradation of insulin inside the

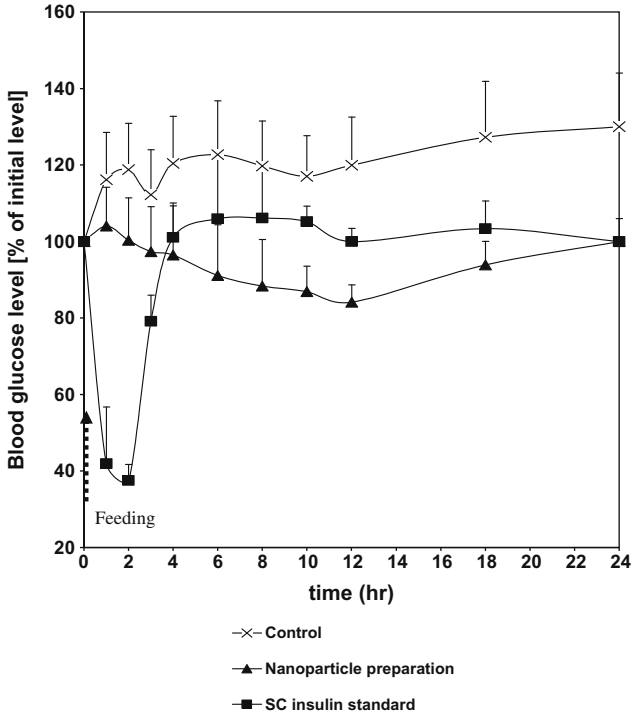

Fig. 10. Blood glucose level time profiles after a single oral administration of nanoparticle preparation (50 IU/kg), SC insulin standard injection (1 IU/kg) and a placebo (control) given to non-fasted STZ-diabetic rats. Results are expressed as mean ± .S.E.M (n = 10 per group).

Table 6
P values calculated based on one-way ANOVA test, and used for the comparison between the insulin nanoparticle preparation and a control under 24 fed state postdosing.

Time (h)	*P*-value
1	0.459
2	0.275
4	0.270
6	0.120
8	0.080
10	0.028
12	0.016

stomach, and could cause an additional delay in insulin pharmacological action [54,55]. In spite of the presence of food, complete insulin nanoparticle deterioration did not occur even at the highest gastrointestinal tract stress [56].

Although, at the time intervals (1–8 h), P value was >0.05 when comparing the nanoparticle preparation with control under fed state, still the trend of P values can be used as an indicator for the significant difference level with time, as shown in Table 6. Decreasing P values may suggest that the there was increase in difference level with time between the preparation and the control groups. Significant difference ($P < 0.05$) between the control and nanoparticles preparation was observed at time periods 10 and 12 h. This could be explained based on the slow rate of oral insulin absorption, where the absorbed insulin exerted its slow pharmacological action to cope with or counteract the glucose that comes from both the absorbed glucose resulted from food digestion and the excessive glucose present in blood due to the induced diabetes. Thus, in the early stage of fed state and under these circumstances, the pharmacological response of insulin was affected and, consequently, the difference between groups which received the insulin nanoparticle preparation and the control groups became less significant to each others. The effect of food as time pass became less significant due to the increase in amount of insulin absorbed which could explain the decrease in P values below 0.05 in the period 10–12 h postdosing.

In SC insulin injection, insulin resulted in a tremendous decrease in the glucose level ($P < 0.05$) for a shorter period of time i.e. 0.5–3 h. This could be attributed to the high amount of insulin absorbed from the injection site (invasive route) which consequently resulted in a rapid decrease in glucose level. Thereafter, the glucose level was increased again and returned to a non-significant level ($P > 0.05$) without observing any trend behavior in comparison with the control.

Similarly, the previous observations during rat feeding after the first 12 h in Figs. 8 and 9 showed that glucose levels were higher in the control groups when compared with the nanoparticle preparation group. Nanoparticle preparation showed higher hypoglycemic effect even in the presence of food in comparison with that of the control group. This may indicate their pharmacological action even in the presence of food.

4. Conclusions

The present investigation explored the possibility of formulating an oral insulin delivery system by combining the advantages of nanoencapsulation and the use of an oily vehicle. The preparation was found to have a unimodal particle size distribution with a mean diameter of 108 ± 9 nm. Insulin was protected from gastric enzymes by incorporation into lipid-based formulation. The results of RP-HPLC and ELISA indicated that insulin was able to withstand the preparation procedure. Insulin in the preparations was stable for a period of 1 month at storage temperatures of 4 and 25 °C. Insulin was also biologically active and stable as demonstrated by the remarkable reduction of blood glucose levels of the STZ-diabetic rats after oral administration of the preparation in fasted and fed states. Moreover, hypoglycemic effect of nanoparticles administered orally was sustained for a longer time compared to subcutaneous injection. These results clearly evidenced the ability of the nanoparticles to enhance the intestinal absorption of insulin and may be used to deliver other peptides.

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