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Oral delivery of insulin using a lipoamino acid nanocarrier system

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Lipoamino acid nanocarriers based on the interactions between L-arginine and oleic acid were formulated with the aid of Tween 80 and explored as a novel carrier for the oral administration of insulin. The interaction between oleic acid and L-arginine was confirmed by FTIR, DSC/TGA, viscosity and surface tension measurements. The pharmacological activity of orally administered insulin-loaded nanocarriers (10, 25 and 50 IU/kg) was evaluated in healthy rabbits. Insulin loaded in lipoamino acid nanocarriers was therapeutically active as demonstrated by the remarkable reduction in blood glucose levels after oral administration of the preparation. A blood glucose lowering of 80% was achieved 4 h after administration of the oral insulin preparation. In addition, insulin-loaded nanocarriers decreased glycemia in a dose dependent manner, with the maximum effect noticed with 50 IU/kg. Moreover, a significant effect observed 1 h after administration suggested that these nanocarriers could be considered as a fast-acting preparation. These results clearly show that these lipoamino acid nanocarriers hold promising potential for oral delivery of insulin.

1. Introduction

Many approaches were evaluated for the oral delivery of proteins; however, nanopaticulates are the most interesting. Nanocarriers could be prepared using polar lipids to form micellar, liposomal or nanovesicle systems. The polar lipids were previously prepared through chemical conjugation of the lipids with polar structures such as amino acids forming lipoamino acids (Infante et al. 1997; Rieux et al. 2006; Yoo et al. 2010). Lipoamino acids and lipopolyamino acids can easily permeate tissues and cellular membranes. Therefore, many synthetic lipoamino acids were prepared through chemical conjugation and used extensively as drug carriers. The inclusion of lipoamino acids with gene delivery carriers has shown positive results for the delivery of an oligonucleotide and protected DNA against nucleases (Coles et al. 2010). Lipoamino acids could also enhance the permeability of biopharmaceuticals. Thyrotropin releasing hormone (TRH) and luteinizing hormone releasing hormone (LHRH) were conjugated with two lipoamino acids. The conjugates developed have been absorbed and detected after oral administration and appeared to be stable for a considerable time *in vivo* (Flinn et al. 1996). Lipoamino acids have been also used in conjugation with liposome in many other previous studies (Babu et al. 1995; Fernandes et al. 1997; Yangi et al. 2000; Riche et al. 2004; Roth et al. 2004).

Due to the presence of many naturally occurring lipoamino acids that act as body regulators and having specific metabolic function, formulation of lipoamino acids as carriers for drug delivery should be taken with caution (Asselineau et al. 1991; Desai and Banat 1997; Brady et al. 2002; Milan et al. 2006). One cannot ignore the fact that the introduction of a new synthetic lipoamino acid could interfere or result into unknown pharmacologic or toxicologic action either through the direct action of the parent lipoamino acid molecule or one of its metabolites. The challenge of measuring toxicity of a new synthetic lipoamino acid could limit their use as drug carriers in the pharmaceutical industry due to the regulatory restriction. However, in this study another approach is going to be adopted based on salt formation between amino acids and the lipid molecules in order to form a nanocarrier with the aid of either emulsifying agents and/or high pressure homogenization. This methodology may require less safety testing for the physically modified excipients (Moreton 1996). The main objective of this study was to develop a novel optimized nanocarrier system which is able to carry protein–based pharmaceuticals such as insulin (INS) through the gastrointestinal tract (GIT) and to increase INS intestinal permeation.

2. Investigations, results and discussion

2.1. Rationale for selection of lipoamino acids nanocarrier components

A physically modified lipoamino acid was developed to encapsulate INS. In previous studies, Elsayed et al. (2009) and other authors prepared lipo-polysaccharide nanocarrires and demonstrated their potential to deliver INS orally (Badwan and Al-Remawi 2008; Badwan et al. 2009; Badwan and Al-Remawi 2009). The replacement of polysaccharide with amino acids could be advantageous in the development of nanocarriers. It is well known that proteins become more stable when mixed with specific amino acids (Baynes 2005; Schneider and Trout 2009). In addition, the lipoamino acids could produce smaller size nanocarriers in comparison to lipo-polysaccharides. This could facilitate nanocarrier penetration in the gastrointestinal tract and thus may increase protein bioavailability. In order to select the suitable amino acid and lipidic structure, it is important

to take into consideration the safety of such materials and their ability to enhance INS role inside the human body. L-Arginine (LAR) was selected as an amino acid due to its unique properties. It has an important impact on glucose metabolism (Schmidt et al. 1992; Thams and Capito 1999; Gannon et al. 2002). In addition, LAR could be useful when mixed with INS since it is effective in suppressing aggregation, assisting refolding of aggregated proteins, and enhancing the solubility of aggregation-prone unfolded molecules *in vitro* (Lyutova et al. 2007). The other additive selected was oleic acid (OA) as the lipid structure due to its favorable penetration enhancing properties and it was widely used to increase drug permeation through the skin and intestine (Baluom et al. 2000; Touito et al. 2002). The combination of LAR and OA could result in the formation of micelles, vesicle-like highly associated aggregates, and lamellar liquid crystals with large amount of water (Kaneko et al. 2005).

2.2. LAR OA interactions

2.2.1. Particle size, zeta potential and viscosity measurements

To get an idea about the nature of systems resulted from the interaction of OA and LAR, the particle size was determined for O/W system composed of different LAR to OA ratios as depicted in Fig. 1. It was observed that the particle size was very small (2.27 ± 0.55 nm) when the ratio of LAR to oleic was 2 to 1 and larger particles were formed when the ratio of LAR to OA was 1 to 1 and 1 to 2 (78.20 ± 8 and 79.23 ± 5.59, respectively). This increment in particle size was not attributed to aggregation of molecules as evidenced by high zeta potential (above -50 mv) recorded at all LAR to OA molar ratios rather a nanovesicle system could be formed. The pH values of LAR to OA 1:2, 1:1 and 2:1 were 7.5, 8.3 and 8.7, respectively. At this high pH, OA with a pKa of 5.02 would be ionized to form oleate. When OA is present in a molar excess compared to LAR then some fatty

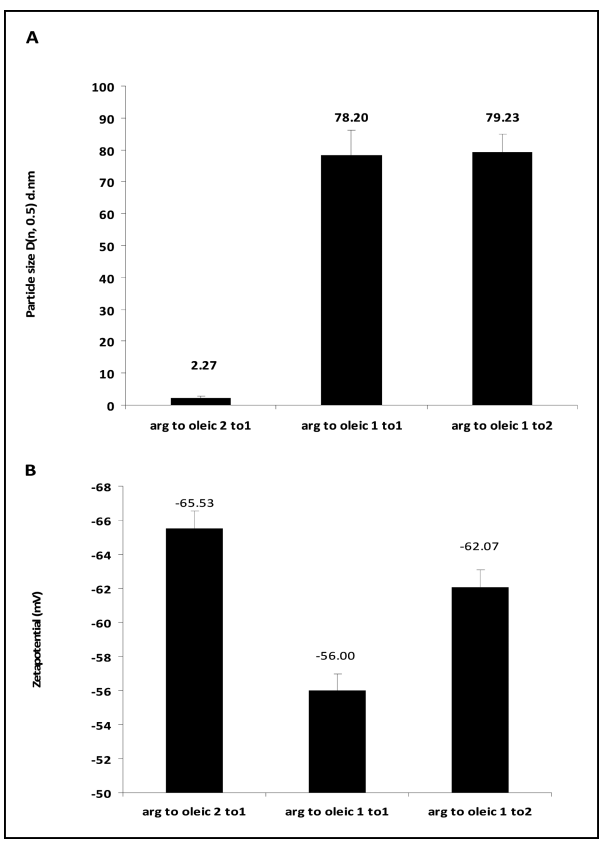

Fig. 1: Particle size (A) and zeta potential (B) of different LAR/OA mixtures.

Table 1: Particle size of OA/LAR (10 mg/ml) mixed in a volume ratio 10 to 1

	PdI	D(n)	D(i)
Median	0.649	1020	1070
stand dev	0.385	496	515

acid molecules will be in the non-ionized or neutral form. Consequently, the ionized form i.e. the negatively charged oleate soap may aggregate and form fatty acid vesicles named ufasomes as demonstrated by other authors. Fatty acid vesicles formed at a particular pH region, where approximately some of the carboxylic groups are ionized (Morigaki and Walde 1988). In this report it is obvious that they are formed at pH values between 7.5 and 8.3 which is consistent with other studies (Cistola et al. 1988; Walde et al. 1994). The particle size of the vesicles was less than 80 nm. Large unilamellar vesicles of OA/oleate were found to have an average size of 100 nm (Morigaki and Walde 1988). LAR with relatively low membrane permeability could stabilize the vesicles by retarding their growth due to its ability to generate pH gradients in OA vesicles. It was observed that the generated pH gradient retarded the extra growth of vesicles due to the energetic cost associated with the transport of the protons into the interior (Morigaki and Walde 1988). The stabilizing effect was also attributed to the adsorption of LAR at the microscopic drop surface, or at the oil/water micro interface through the hydrophobic effect and hydrogen bonds between the carboxyl group of fatty acid and carboxylate of LAR (Hirai et al. 2006). Above a pH of 8.3, the solubilized micellar form may predominate with smaller particle size.

Zeta potential was negative at all ratios as shown in Fig. 1. LAR is a basic amino acid with pKa of about 12.5. This makes the molecule to be protonated i.e. positively charged at the pH range 7.5–8.7. However, the negative charge of OA predominates at pH range 7.5–8.7. These nanovesicles are stable in a narrow pH range and therefore are not suitable for GIT with a pH from about 1–8. In addition, the external phase of the above mentioned nanovesicles is aqueous and INS may degrade easily by the GIT enzymes. To protect INS from the harsh GIT environment, an inverted micellar system with an excess OA would be used. The particle size was measured for a combination of OA/aqueous LAR (10 mg/ml) mixed in a 10:1 volume ratio as indicated in Table 1. The resulted particle size was large reaching the micro-level. The strong binding of LAR cation to anionic oleate micelles may induce the dominant micellar growth as suggested by Hirai et al. (2006).

The change of viscosity upon the addition of LAR solution to OA was determined by a vibro viscometer. Significant increase in viscosity was noticed when LAR solution was added to OA reaching maximum at 30% and then decreases sharply as shown in Fig. 2**.** The increase in viscosity may be associated with the adsorption of LAR at W/O interface and formation of elongated micelles which entangled into a transient network and thereby imparted a high viscosity to the sample (Hirai et al. 2006), whereas, the sharp drop in viscosity could be attributed to the phase inversion of the emulsion. Therefore, the percentage of aqueous phase containing LAR was kept below 30% in the preparation; more precisely it was kept below 10%.

2.2.2. FTIR and DSC/TGA measurements

LAR is a basic amino acid with pKa of about 12.5 at neutral pH. LAR may interact with the adjacent carboxylic acid group of OA. To prove such interactions different analytical methods were investigated. Figure 3 depicts the IR spectra of OA,

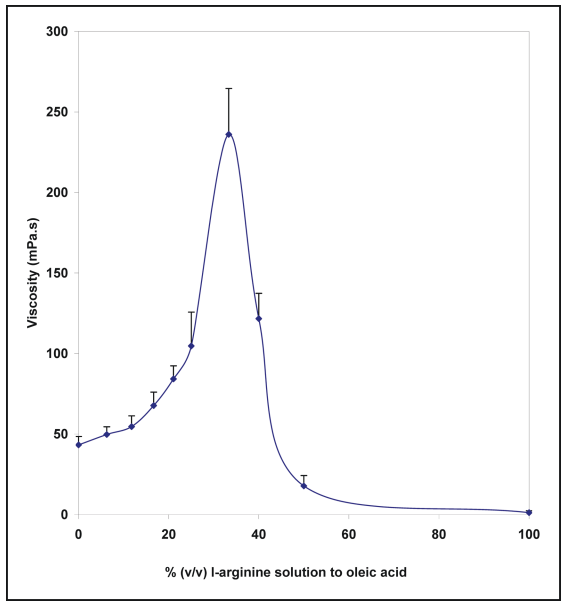

Fig. 2: Change in viscosity upon addition of LAR aqueous solution to OA liquid at different concentrations.

LAR and OA-LAR complex. In the OA spectrum, the C = O stretching band appears at 1714 cm^{-1} (Yang et al. 2010). Mixing LAR with OA may result in the loss of the protons of the carboxylic acid of oleic acid to the basic amine functional groups of LAR. The spectra show the disappearance of the characteristic symmetric (C = O) band of the fatty acid at 1714 cm^{-1} and appearance of a symmetric band (C = O) at about 1550 cm^{-1} typical of carboxylate salt (Chen et al. 2005).

The DSC/TGA of LAR and LAR-OA mixture is shown in Fig. 4. In the DSC trace of LAR, an endothermic peak at about 224 °C corresponds to the melting point of the compound (Chen et al. 2005). This is immediately followed by a sharp endotherm at about 246 °C, coinciding with the major weight loss observed due to the decomposition of LAR as shown in TGA trace. For LAR-OA mixture, the degradation started earlier at about 180 °C due the presence of OA. The degradation of free OA began at 140 °C and ended below 500 °C as stated elsewhere (Vidal-Vidal et al. 2006). The DSC pattern of LAR/ OA mixture was different from that of LAR, characterized by a broad endotherm at about 100 °C. This endotherm was not due to water loss as illustrated by TGA, it may indicate some sort of interaction at a temperature of 50–150 °C.

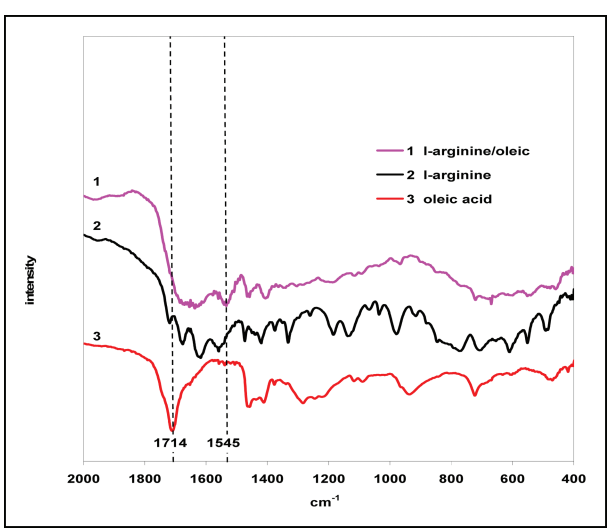

Fig. 3: FTIR spectra of OA, LAR and LAR OA after interaction.

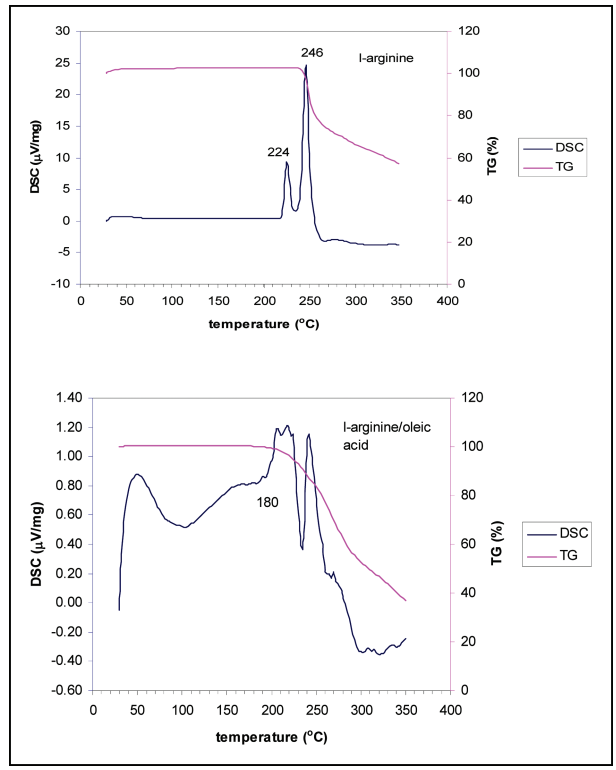

Fig. 4: DSC thermograms of LAR and LAR/OA mixture.

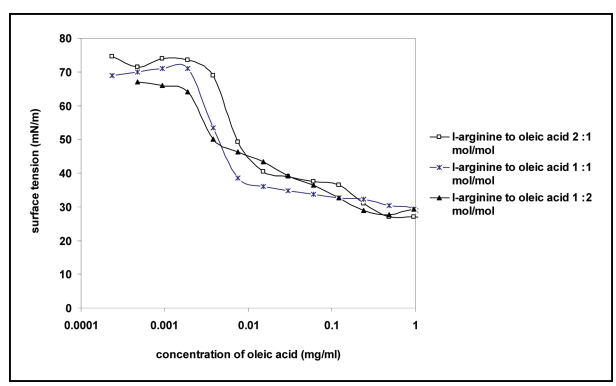

Fig. 5: Surface tension of LAR-OA mixture at different molar ratios.

2.2.3. *Surface tension measurements*

Surface tension *versus* concentration was plotted for different LAR to OA ratios as depicted in Fig. 5. The results revealed that the surface tension decreases with the increase in OA concentration. The presence of break points on the curves indicates the onset of micellization. It was found that oleic acid decreases the surface tension in a pH dependence manner where a minimum was found at pH 9–10 (Paugh and Stenins 1985). However, in this report, the decreasing trend was slightly different for different LAR to OA ratios and was related to non-covalent interactions between LAR and OA that could modify the micellization process.

2.3. *INS-LAR interaction assessment*

2.3.1. *Particle size of INS and LAR*

The median particle sizes by number as D (n, 0.5) and by intensity as D (i, 0.5) of INS, LAR and combination of INS and LAR are summarized in Fig. 6. The particle size of INS was below 5 nm at room temperature and at 50 °C for 2 h. However, size

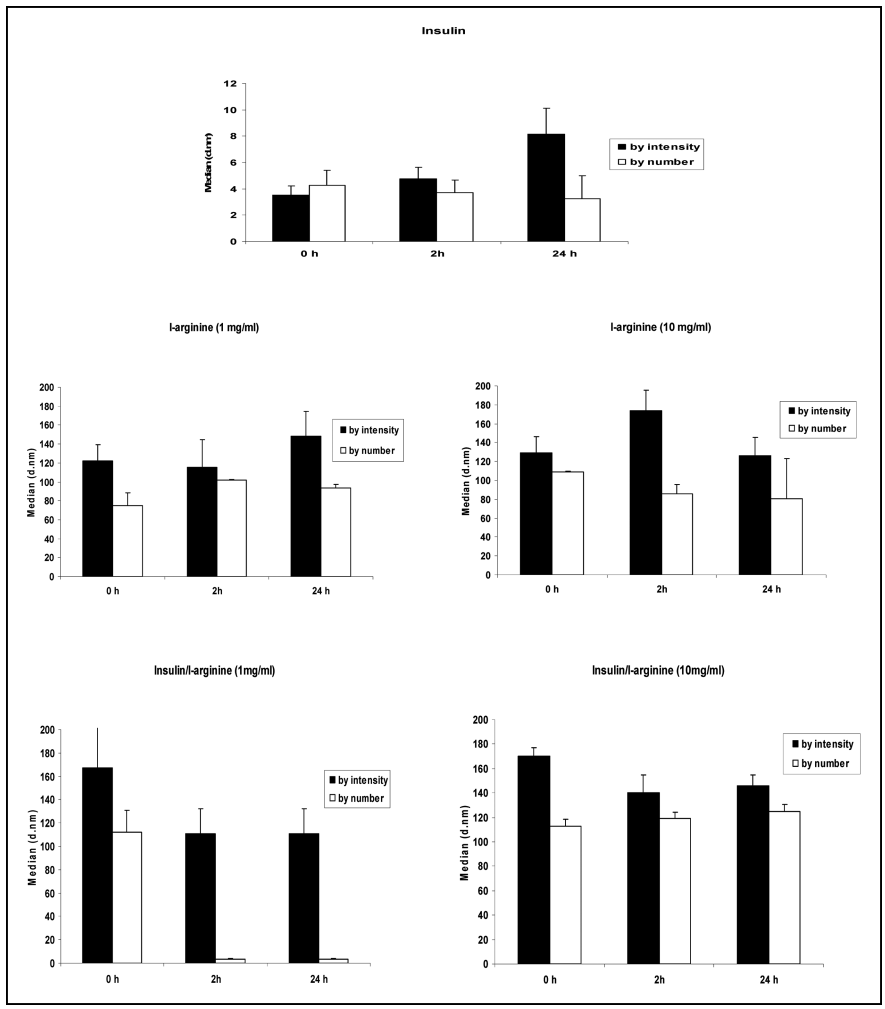

Fig. 6: Median particle size of INS (1 mg/ml), LAR (10 mg/ml), combination of INS (1 IU/ml)/LAR (1 mg/ml) mixed with volume ratio 1:1 and INS (1 IU/ml)/LAR (10 mg/ml) mixed with a volume ratio 1:1 and exposed to 50 °C.

was doubled after 24 h at 50 °C, which may prove that some INS molecules were aggregated as indicated by D (i, 0.5) but large numbers were unchanged as indicated by D (n, 0.5). In addition, it was found that LAR forms larger particles 100–150 nm size than INS in the aqueous solution. The low concentration of LAR (1 mg/ml) results in easily dissociation of INS as free INS especially when the temperature of the preparation was increased to 50 °C as measured by D (n, 0.5), however, this dissociation was not observed when LAR concentration was increased to 10 mg/ml. This may indicate that higher concentrations of LAR were required to prevent free INS dissociation. The interactions between INS and LAR were attributed to the binding of guanidinium group of LAR to aromatic amino acids of INS B-chain and electrostatic forces (Lyutova et al. 2007).

2.3.2. *Zeta potential/pH of INS and LAR*

The pH-dependent behavior of INS-LAR mixture was displayed in Fig. 7. The median diameter of INS-LAR mixture was small below pH 5, began to increase above pH 5 and reached a maximum at about pH 6. The first increase was related to aggregation of INS when the pH was close to INS isoelectric point (*p*I 5.3) and the tremendous increase was attributed to the aggregation of INS-LAR particles at zero zeta potential i.e. at the *p*I of the mixture in this case it was around pH 6. At low pH (1–5) both INS and LAR carry a positive charge (pI LAR 10.8) and therefore zeta potential is positive. At pH 6, the negative charges on INS equal the positive charges on LAR resulting in zero zeta potential (*pI* of the mixture). When the pH is greater than 6, the negative charges of INS gradually increase that superimpose the positive charges of LAR resulting in a negative zeta potential that increases with the increase of pH. Therefore, the pH of the aqueous phase of the preparation was adjusted to 7 to avoid aggregation at the pI of INS-LAR mixture.

2.4. *Preparation of INS-loaded lipoamino acid nanocarriers*

In order to reduce the particle size of LAR-OA microparticles, a non ionic surfactant that is soluble in OA such as Tween 80

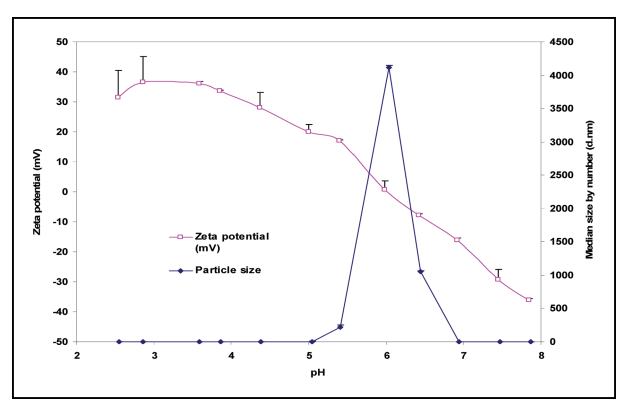

Fig. 7: Median particle size and zeta potential versus pH upon autotitation of INS with LAR.

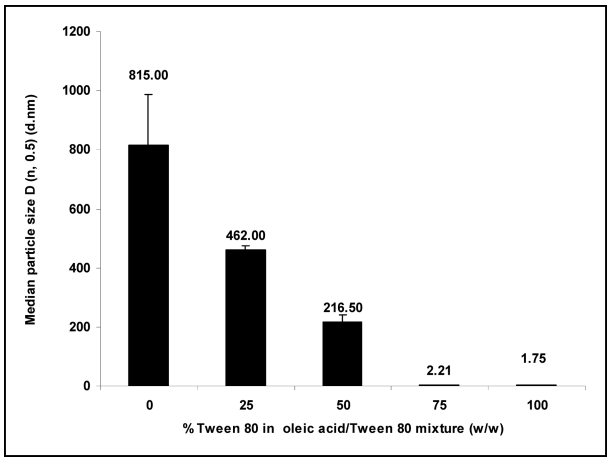

Fig. 8: Median Particle size of INS-LAR dispersed in different OA/Tween 80 mixtures.

was added to the preparation. INS-LAR solution was dropped with stirring in a mixture of OA/Tween 80 and the particle size was evaluated for different Tween 80 percentages as shown in Fig. 8. The particle decreases as the percentage of Tween 80 increases reaching 2.21 nm at 75% Tween 80, where the system was completely in solubilized micellar form that could be easily deteriorated by the GIT enzymes. In contrast, larger size particles having a size of 815 nm were formed when the Tween 80 was not present i.e. 100% oleic acid. This size is considered relatively large enough for intestinal absorption. The optimal nanocarrier size could be obtained at 50% Tween 80. This size could protect INS from intestinal enzymes and could facilitate INS absorption (Rieux et al. 2006).

The physical stability of the particles dispersed in OA-Tween 80 solution composed of different Tween 80 percentages was assessed after centrifugation as shown in Fig. 9. It is obvious that

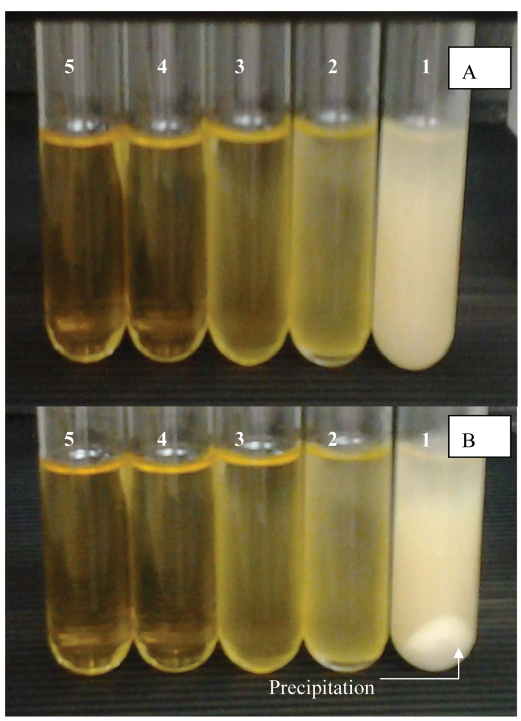

Fig. 9: The oral nanocarrier dispersion in different OA/Tween 80® mixtures before centrifugation (A) and after centrifugation at 5300 rpm for 10 min. Where, percentage of Tween 80 (w/w) is as follows: (1) 0%, (2) 25%, (3) 50%, (4) 75% and (5) 100%.

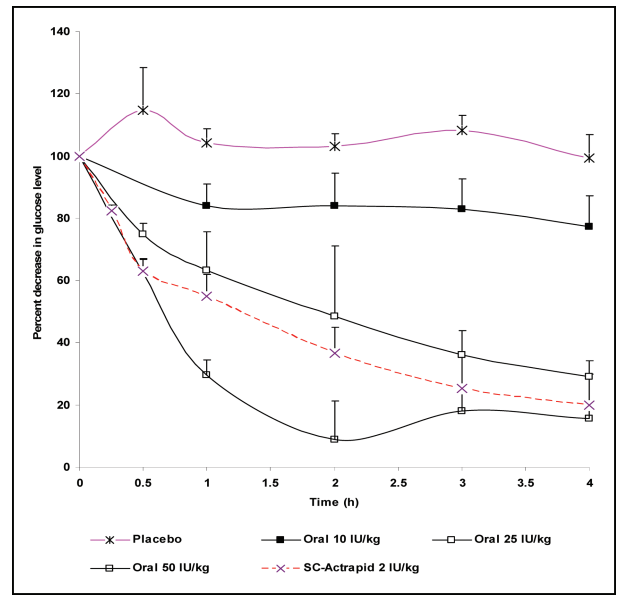

Fig. 10: Percent decrease in glucose level of healthy fasted rabbits given oral nanocarrier preparation (10, 25 and 50 IU/kg) compared to SC Actrapid injection (2 IU/kg). The experiment was discontinued after 4 h because some rabbits were suffering from an extreme hypoglycemia.

preparation 1 (0% Tween 80) was turbid and precipitated easily after centrifugation. The turbidity of the preparations decreased as the percentage of Tween 80 increased which was in accordance with particle size data. When the percentage of Tween 80 was 50% the particles remained dispersed even after subjected to centrifugation at 5300 rpm for 10 min suggesting the physical stability of this preparation and justify the selection of 50% Tween 80 for the *in vivo* studies.

2.5. In vivo response of INS-loaded lipoamino acids nanocarriers given orally to healthy rabbits

So far, there is no standard animal method to study the effects of antidiabetic drugs (Eddouks et al. 2012). Ideally, more than one animal method should be used. However, in this report a proof of the concept approach was adopted to provide evidence about the ability of lipoamino acid nanocarriers to deliver INS orally, an *in vivo* experiment was conducted on healthy fasted rabbits. Normal non-diabetic animals have been used to assess the antidiabetic activity of drugs, plants and insulin-loaded in carriers (Caccetta et al. 2013; Tanko et al. 2008; Zhang et al. 2008). In the normal situation, homeostatic control of blood glucose levels of is achieved mainly through the action of insulin and glucagon. Insulin promotes glucose uptake in the liver, adipose tissue and muscles, whereas glucagon has opposite actions. However, if excessive insulin reached the blood stream, the high insulin level prevents the glycogenolysis necessary to maintain normal blood sugar levels and leads to hypoglycemia (Nelson and Cox 2012). Therefore, healthy animals were used for testing the potential of the nanocarrires to release a predictable amount of insulin that could alter the normal homeostatic control.

Figure 10 illustrates the changes in blood glucose levels after oral administration of INS loaded lipoamino acid nanocarriers at doses of 10, 25 and 50 IU/kg to healthy albino rabbits compared to 2 IU/Kg dose of Actrapid® given subcutaneously and INS free nanocarrier solution administered orally (placebo). The rapid depression in blood glucose levels after subcutaneous administration of Actrapid® injection suggested discontinuation of measuring blood glucose levels 4 h after administration due to rabbit suffering of severe hypoglycemia. INS free preparation (the placebo) showed no hypoglycemic effect as expected.

In contrast, a pronounced drop in blood glucose levels was observed after oral administration of lipoamino acids nanocarriers at all used doses, where a significant hypoglycemic effect was achieved 1, 2, 3 and 4 h after administration ($p < 0.05$) compared to placebo. A blood glucose lowering of 80% at 4 h caused the termination of the experiment due to rabbit suffering. In addition, the decrease in glucose levels was found to be dependent on INS dose. Moreover, a significant hypoglycemic effect was observed 1 h after administration, suggesting that nanocarriers could be considered as a fast-acting preparation. These results obviously demonstrate that INS encapsulated in lipoamino acid nanocarriers was absorbed from the gastrointestinal tract as an intact molecule and elicit a remarkable pharmacological response. Further studies on the mechanism of absorption will be conducted in the future.

In conclusion, the possibility of preparation of insulin carrier from a lipoidal material such as OA and LAR by physical interaction was explored. The conversion into nanocarriers was achieved by addition of surfactant (Tween® 80). This study is a proof of concept that lipoamino acid nanocarriers could enhance the absorption of INS when given orally. Further characterization of the nanocarriers, mechanism of absorption and *in-vivo* studies on diabetic rats are in progress.

3. Experimental

3.1. Materials

Rh-INS and OA were obtained from Sigma Aldrich (Lyon, France). LAR was purchased from Merck KGaA, Darmstadt, Germany. Actrapid HM (100 IU/ml) Novonordisk, Denamrk was purchased from the local market.

3.2. Assessment of interactions between different components of lipoamino acid nanocarriers

3.2.1. OA-LAR Interaction

A fixed amount of OA and LAR were weighed and transferred to a beaker then 50 ml of water was added and sonicated for 5 min using a probe sonicator to form an emulsion (O/W system). The concentrations of OA and LAR were 7.74 mg/ml and 4.8 mg/ml, respectively (OA to LAR in mol ratio 1:1). Additionally, other molar ratios of oleic to LAR (1:2 mol/mol; 7.74:9.6 mg/ml) and (2:1 mol/mol; 15.48: 4.8 mg/ml) were also prepared. 1% LAR aqueous solution was mixed with OA using magnetic stirrer at a 1: 10 volume ratio to evaluate the effect of excess OA.

3.2.1.1. Particle size, zeta potential and viscosity measurements. The particle size and zeta potential measurements were carried out with Zetasizer Nano ZS (Malvern Instruments, UK) at 25 °C. The particle size measurement was evaluated based on low PDI values. Three measurements were conducted and the results were expressed as mean ± SD. The viscosity was measured in triplicate at 2 ± 0.015 °C by Vibro viscometer (SV-10, A&D Company, Japan) for emulsions prepared from different LAR solution: OA volume ratios.

3.2.1.2. Surface tension measurement. Samples of LAR-OA (1 ml) prepared at different molar ratios (1:2, 1:1 and 2:1) were placed in each well of a tensiometer plate and measured at room temperature. The surface tension was determined using (Kibron with KBN 315 sensor head, Finland) surface tensiometer.

3.2.1.3. FTIR Measurements. A sample of 200 mg of LAR was dispersed in few drops (about 3) of distilled water and mixed with 0.5 ml OA to form a slurry. The slurry was triturated with 2 gram KBr. The sample was scanned with a FTIR spectrometer (IR Prestige 21, Shimadzu, Japan). A blank sample was prepared containing KBr and the added water and used as FTIR background. About 5–10 mgs of LAR and OA samples were added separately to 200 mg KBr and measured by FTIR.

3.2.1.4. DSC/TGA Measurements. 10 mg of LAR and 100 mg OA mixture were put in an open DSC cup scanned by a previously calibrated simultaneous thermal analyzer (STA) DSC (STA 449 F1 Jupiter® NETZSCH, Germany). Another 10 mg LAR sample was scanned without OA. The heating rate was 10 °C/min.

3.2.2. INS - LAR Interaction

3.2.2.1. Determination of the particle size of INS and/or LAR. The particle size distribution of two separate solutions of INS (1 U/ml) prepared using 0.1 M HCl and (1 and 10 mg/ml) LAR prepared in distilled water were determined. In addition, a mixture of particles prepared by mixing previous solutions of INS and LAR in 1:1 v/v ratio was assessed by photon correlation spectroscopy using a Malvern Zetasizer Nano-ZS series (Malvern Instruments, UK). The particle size was assessed initially and after keeping the samples in oven at 50 °C for 2 and 24 h.

3.2.2.2. pH autotitration profiles of INS versus LAR. 100 mg of rh-INS was dissolved in 10 ml 0.1 M HCl then distilled water was added to complete the volume up to 100 ml. In another beaker, 1000 mg of LAR was dissolved in 10 ml distilled water. Then, rh INS solution was autotitrated with the positively charged LAR dissolved in distilled water using the pH autotitrator unit attached to Zetasizer Nano-ZS (Malvern Instruments, UK) starting from pH 2.55 until reaching 7.87.

3.3. Preparation of INS loaded- lipo amino acid nanocarriers using non-ionic surfactant and testing physical stability

OA and PEG (20) sorbitan monooleate (Tween 80®) were mixed using a magnetic stirrer for 30 min at different OA percentages w/w (100%, 75%, 50%, 25% and 0) (solution 1). 200 mg rh-INS was dissolved gently in 2 ml 0.1 M HCl, in another test tube 50 mg LAR was dissolved in 1 ml distilled water. The LAR solution was added drop wise with gentle stirring to INS solution until pH reached a value of 7 (solution 2). To the prepared INS powder/LAR solution, 1 ml sodium alginate solution (1% w/v) was added with gentle stirring (solution 2) (used as viscosity increasing agent and emulsifier). Then, 1 ml of solution 2 was added drop wise with mixing to 10 ml of solution 1 with different OA/ Tween 80 mixtures. The median particle size was evaluated using the zetasizer by taking 1 ml of each preparation and diluting with 10 ml OA prior each measurement.

In order to evaluate the vesicles physical stability, 6-ml samples of each preparation were subjected to centrifugation at 5300 rpm for 10 min. The precipitation at the bottom of each test tube was monitored and photographed. The preparation with optimal particle size was selected for the *in vivo* study.

3.4. In vivo pharmacological response of INS-loaded lipo-amino acid nanocarriers given orally to healthy rabbits

The preparation that has been used for the *in vivo* study was prepared according to the previous section with OA to Tween 80 (50:50 w/w).

In all *in vivo* experiments healthy albino rabbits were used to evaluate the pharmacological activity of orally administered INS-loaded nanocarriers. 30 rabbits aged about 18–20 weeks and weighing 1.0–1.53 kg were acclimatized for two weeks. The animals were fed with standard rabbit chow. The rabbits were divided into 5 groups (each 6) and each group got different treatment. The rabbits were fasted for 12 h prior to the test. Group 1 rabbits were given Actrapid® subcutaneous injection (2 IU/kg). Group 2 received oral placebo preparation. Groups 3, 4 and 5 were given INS-loaded nanocarriers at different doses i.e. 10, 25 and 50 IU/kg, respectively. Glucose level was monitored using Glucometer (Bionime GM300, USa Corp., USA) at different time intervals.

3.5. Statistical treatment

One-way ANOVA test was used for comparison. The difference was considered statistically significant when the probability value (P) was less than 0.05.

Conflicts of interest: The authors declare no conflict of interest

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