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Original Article

Oral Insulin Delivery: A New Approach

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ABSTRACT

Oral delivery is the preferred route of administration because it offers several advantages over other routes. However, it is not an effective route for the delivery of peptides and proteins because of so many constraints. The small intestine has been shown to be able to transport the L-forms of amino acids against a concentration gradient and that they compete for the mechanism concerned. So L-Valine was used as a ligand for carrier mediated transport of insulin loaded PLGA nanoparticles. L-valine-conjugated PLGA-nanoparticles were prepared using double emulsion solvent evaporation method. The insulin bearing nanoparticles were also studied for size, drug entrapment efficiency, zeta potential and polydispersity index, *in-vitro* insulin release. *In-vivo* studies were performed on streptozotocin induced diabetic rabbits. Oral suspension of insulin loaded PLGA nanoparticles reduced blood glucose level from 265.4±8.5 to 246.6±2.4 mg/dl within 4 hrs which further decreased to 198.7±7.1 mg/dl value after 8 hrs. Compared with formulation loaded with the drug, the valine conjugated nanoparticles produced a sustained hypoglycaemic response till 12 hrs than 8 hrs. Hence, it is concluded that the L-valine conjugated NPs bearing insulin are the promising carrier for the transportation of insulin across the intestine on oral administration.

1. INTRODUCTION

Diabetes mellitus, often referred to simply as diabetes (ancient greek: “to pass through urine”), is a syndrome of disordered metabolism, usually due to a combination of hereditary and environmental causes, resulting in abnormally high blood sugar levels (hyperglycemia). Diabetes mellitus refers to the group of diseases that lead to high blood glucose levels due to defects in either insulin secretion or insulin action [1-2].

Oral delivery is the preferred route of administration because it offers several advantages over other routes. It is more natural, less invasive, can be self-administered (outside the hospital), and is less expensive. However, it is not an effective route for the delivery of peptides and proteins because of so many constraints. To date, various strategies have been developed to achieve an effective oral insulin delivery system, including co-administration with absorption enhancers [3] or lipid-based carriers as liposomes [4].

Protein drugs, such as insulin, have traditionally been administered by injection because of their large molecular weights and the dependence of their bioactivity on structure. However, the oral administration of protein drugs has major challenges. To meet these challenges, an oral delivery system must protect the drug in the stomach from proteolytic enzymes and acid, but allow absorption of the drug in either the small or large intestine. The target site of absorption is the small intestine which is typically preferred for absorption of hydrophilic proteins due to a more permeable epithelium than the colon [2]. It was also reported that L-valine conjugated drug enhances oral bioavailability of drug implicate the oligopeptide transporter as the carrier involved in absorption of L-valine combined drug.

Hence, it was aimed to develop L-valine combined PLGA nanoparticles bearing insulin for oral administration. The PLGA nanoparticles will protect the insulin from its degradation in gut and L-valine molecules which are attached to the surface

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of nanoparticles will help insulin bearing nanoparticles to transport across intestinal membrane. The present work aimed at the preparation and evaluation of L-valine-combined PLGA-nanoparticles for the oral delivery of insulin using target specific Nanoparticles bearing insulin.

2. MATERIALS AND METHOD

The drug (human recombinant insulin) was a gift sample obtained from Biocon, Bangalore (India). Poly lactic-co-glycolic acid (PLGA) (50:50) polymer was obtained as a gift sample from SPARC (Sun pharmaceuticals advanced research center), Baroda, India. L-valine, Dichloromethane (analytical grade), Arlacel 83 were purchased from Sigma (St. Louis, USA) and PVA and mannitol were purchased from Central Drug House, Mumbai, India.

2.1 Conjugation of polymer with L-valine amino acid

L-valine was conjugated to PLGA using carbodiimide coupling process as reported by Nam and Park (1999). Briefly, one gram of PLGA (50:50) having a carboxylic acid end group was activated by 9 mg of DCC in 3ml of DMSO at room temperature for 2hr. The activated PLGA solution was slowly dropped into 5mL of DMSO containing 100 mg L-valine which had been previously lyophilized at pH 9. The reaction was continued for 3 h under constant magnetic stirring at room temperature. The PLGA-valine conjugate was precipitated by slowly dropping into an excess amount of chilled diethyl ether and centrifuged to remove precipitate then washed with deionized water, and lyophilized. In order to remove completely the residual unconjugated valine fraction, the lyophilized PLGA-valine conjugate was milled, incubated in deionized water at 37°C for a day, centrifuged to precipitate water insoluble conjugate, filtered and then lyophilized washing process was repeated till the complete removal of unconjugated valine [12].

2.2 Characterization of conjugation

2.2.1 IR spectroscopy: Characteristic peaks of valine, PLGA and valine conjugated PLGA were analyzed by FTIR (PerkinElmer, Pyrogon 1000) IR spectra were recorded on a Perkin-Elmer FTIR-1000 spectrometer using KBr pellets. (Infrared spectra are shown in Fig.1)

2.2.2 Conjugation efficiency: Conjugation efficiency was determined. About 10 mg of accurately weighed lyophilized L-Valine conjugated PLGA was dissolved in 2 mL DMSO and incubated for 5 h at room temperature with 10 mL of 0.05 N NaOH / 0.5% sodium dodecyl sulfate (SDS). 1 mL of the resultant solution was mixed with an equal volume of bicinchoninic acid assay (BCA) working solution (prepared by mixing 50 parts of Reagent A with 1 part of Reagent B. Mixed the BCA Working Reagent until it is light green in colour.) at 60°C for 1h and assayed at 562nm spectrophotometrically [9].

$$\text{Conjugation efficiency (\%)} = \frac{\text{Wt. of protein (µg valine)}}{\text{Wt. of lyophilized nanoparticle (mg)}} \times 100$$

2.3 Preparation of insulin loaded Nanoparticles

Nanoparticles were prepared using double emulsion solvent evaporation method. The aqueous primary emulsion was prepared by dissolving 100 mg of Insulin in 2.5mL of 0.01N HCl (pH 2.8) while oil phase was prepared by dissolving 1 g PLGA and 0.25 wt.% Arlacel 83 in a 10 mL mixture solvent of dichloromethane (DCM) and acetone (3:1), then aqueous phase was slowly dispersed in oil phase using sonication (lark Innovation, Chennai) for 30 sec to form w/o emulsion and later this primary emulsion was re-emulsified with 100 mL aqueous phase consisting of stabilizer polyvinyl alcohol (1 g) 70 KDa using sonication for 60 secs. The double emulsion was subjected to magnetic stirring at 100 rpm for 8 h at 25±1°C for complete evaporation of DCM and acetone to obtain concentrated residue which redispersed in distilled water and then purified by ultracentrifugation at 30000 rpm for 15 min to remove PVA. The NPs so formed were collected, washed and freeze dried with cryoprotectants (mannitol) [5-7].

2.4 Characterization of nanoparticles

The prepared nanoparticles were characterized for surface morphology, particle morphology, particle size, entrapment efficiency, zeta potential, and polydispersity.

2.4.1 Shape and surface morphology: Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) were used to characterize the shape and surface morphology of the SLN, respectively. In SEM (Philips XL 30 Scanning Microscope, Philips, Eindhoven, Netherlands), SLN were coated with gold–palladium alloy (150–250 Å) using a sputter coater before analysis. The morphological examination of the nanoparticles was also performed using TEM (JEM-200 CX, JEOL, and Tokyo, Japan). In case of TEM, SLN samples were stained with 2% w/v phosphotungstic acid for 10 s, and excess of the solution was drained off using filter paper. The grid was allowed to thoroughly dry in air and samples were viewed under a transmission electron microscope.

2.4.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS -PAGE): Polyacrylamide gel electrophoresis was performed to compare the electrophoretic mobility of insulin before and after nanoparticles formulation. In addition, the NPs and Con A NPs were centrifuged at 13,000g for 15 min to separate insulin from the pelleted-particle fraction from the soluble supernatant. The pellet and supernatant were analyzed using SDS-PAGE to determine relative amounts of insulin associated with each fraction.[18] For electrophoresis, samples were boiled for 5 min in the presence of sodium dodecyl sulfate and β-mercaptoethanol. An aliquot of the samples, equivalent to 10–100µg of insulin, was electrophoresed on a 16% SDS polyacrylamide gel for 1 hr at 200 V. Insulin was visualized using silver nitrate staining which has a detection limit of 1µg. Molecular weight protein standards were used to compare the electrophoretic mobility of NPs and unprocessed insulin.

2.4.3 Circular Dichroism Study (CD- spectrum): Secondary structure of insulin is necessary for its bioactivity, therefore its secondary structure integrity was analysed by CD Spectrum.

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The extracted insulin was dissolved in isotonic PBS buffer was analyzed for its integrity by CD spectra (Jasco, J-815 Spectropolarimeter, UK) at room temperature with scanning speed of 50 nm/min. The spectra of insulin samples extracted from NPs and Con A NPs with concentrations about 10 mM were compared with pure insulin**.**

2.5 Entrapment Efficiency (EE)

EE was determined using acetonitrile extraction method to analyze the insulin content within the Nanoparticles. Freshly prepared insulin-PLGA-NP suspension (1mL equal to 100 IU or 3.6 mg of insulin) was ultracentrifuged for 1 h at 4ºC at 50,000 rpm (Ultracentrifuge, HEMLA Lab. Germany) in order to isolate the un-entrapped insulin from the entrapped insulin. The supernatant was removed and nanoparticles sediments were resuspended in water. The resuspended solution was ultracentrifuged twice under the same conditions. 200µL Acetonitrile was added into the sediments and the mixture was vortexed for 5 min. Then, 800µL of 0.01M HCl was added and vortexed for 2 min. After centrifugation at 15,000 rpm (centrifuge) at 4ºC for 20 min, supernatant was collected for further RP-HPLC analysis of insulin by the method discussed previously [8,10].

2.6 *In–vitro* release study

2.6.1 Preparation of enteric polymer coated capsules: To protect insulin from harsh environment of stomach, insulin loaded NPs and insulin loaded conjugated NPs were filled separately in hard gelatin capsules (Capsule No. 5) which were coated with enteric polymer Eudragit S 100 using dip coating.

2.6.2 In-vitro drug release: Firstly the dialysis bag (Dialysis membrane MW cut off 12 kda) was treated by heating in distilled water at 60°C for 1 h. One capsule each filled with NPs and conjugated NPs were taken in treated dialysis bag and both ends of bag tied, which was dipped in 100 mL media. The pH of media was changed with respect to time according to following schedule [7]. 0.1N HCl (pH 1.2) Initial two hours. At pH 5.5 - 1h, at pH 6.8 -2h and at pH 7.5- until the end of the test. The final volume in all cases was 100 mL. From the medium 1 mL of the solution was withdrawn after definite time interval then analyzed spectrophotometricaly at 276 nm by UV visible spectrophotometer (GBC Cintra 10) [17].

2.7 Thermal stability of encapsulated drug

2.7.1 Differential Scanning Calorimetry: Differential scanning calorimetry (DSC) represents a thermoanalytical method that measures the difference in the thermal energy required to increase the temperature of a sample and a reference with well-defined heat capacity as a function of the temperature. Ideally, both the sample and the reference are kept at identical temperature, while the temperature increases linearly as a function of time. As soon as the sample undergoes a physical transformation, the heat flow to the sample will be decreased or increased relative to the reference, thus, resulting in a DSC signal [14].

2.8 *In-vivo* studies

Albino rabbits of either sex weighing 2-2.5 kg were chosen for the present studies. All *in-vivo* studies on animals were approved by animal ethical committee of the Dr. H. S. Gour University, Sagar (MP), India constituted under the guidelines of CPCSEA, New Delhi, India through their vide letter no. animal eths. Comm./12/82/35 dated 20/22-05-12.

2.8.1 Induction of diabetes: The diabetes was induced in rabbits with single intravenous administration of streptozotocin (50 mg/Kg). The solution of streptozotocin (50 mg/mL) was made in citrate buffer (pH 4.5). Then, the external marginal ear vein of the rabbits was cleaned with boiled water with the help of the cotton, so that the vein could be visualized easily for i.v. administration. The blood sugar level was first measured before administering streptozotocin solution to animals. The solution of streptozotocin (50 mg/mL) was administered intravenously [16].

After 8-10 days of treatment, rabbits with frequent urination, loss of weight and blood glucose level higher than 250 miligram per deciliter (mg/dL) were selected for study. Animals were housed in groups of three (as per the permission of animal ethical committee) with free access to food and water. Animals were fasted over 24 h before starting the test, but water was provided ad-libitum. Blood glucose level of all animals was checked before starting experiment. During experimentation standard diet was given to the animals of all the groups at 4^{th} h and 12^{th} h [12]. The blood glucose level was monitored using glucose oxidase-peroxidase (GOD-POD) method using calorimeter (J. Mitra & Co Pvt. Ltd., New Delhi, India) with green filter at 510 nm.

2.8.2 Experimental design: Rabbits were divided into five groups and each group consists of 3 animals and all animals were induced diabetes as per the procedure stated above. Group-1: Animals of this group were received only plain water without any drug or formulation (control 1). Group-2: Animals of this group were administered oral insulin powder (20 IU/Kg) (control 2). Group-3: Animals of this group were received insulin (20 IU/Kg) through subcutaneous route. Group-4: Insulin loaded NPs equivalent to (20 IU/Kg) weight of animals were administered to all animals of this group. Group-5: Ligand conjugated NPs bearing insulin equivalent to (20 IU/Kg) weight of animals were administered orally to all animals of group 5.

2.8.3 Sample collection: Blood (1.0 mL) was withdrawn periodically from the marginal ear vein of the rabbits of each group into clean heparinized eppendorf tubes. Blood samples were kept on ice until diagnosis. The same blood samples were used to determine blood glucose level as well as insulin level in the withdrawn samples.

2.9 Insulin plasma level determination

The above withdrawn samples were also assessed for insulin concentration determination using the HPLC method with slight modifications [13].

2.9.1 Instrumentation and chromatographic conditions: The mobile phase consisted of 0.2 M sodium sulphate anhydrous adjusted to pH 2.3 with ortho phosphoric acid and acetonitrile (74:26, v/v), filtered through a 0.45µm Nylon membrane filter

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(Whatman International, Maidstone, UK) under vacuum and degassed prior to use. The analysis was run at a flow rate of 1.2 mL/min. The detector was set at a wavelength of 214 nm. The chromatographic separation of the analyte was achieved at ambient room temperature with HPLC column was C_{18} (4.6 X 250 mm, 5 µm). The injection volume was 20 µL.

2.9.2 Preparation of stock and working standard solutions: A stock standard solution of insulin at 140 µg/mL was prepared from human recombinant insulin in PBS pH (7.4). The working standard solutions were made in the range of 3.5–42 µg/mL. The stock solution of methylparaben, as an internal standard (I.S.) was prepared at a concentration of 500 µg/mL in methanol and was further diluted with the same diluent to give a working concentration of 15 µg/mL. All solutions were stored under refrigeration at 4°C prior to use [5].

2.9.3 Preparation of calibration standards: To 80 µL of blank rabbit plasma, 20 µL of working standard solutions of insulin of an appropriate concentration was added to yield final respective concentrations at range of 50 to 500 ng/mL of insulin in plasma.

2.9.4 Sample extraction procedure: To each of the above mentioned calibration standard, 20 µL of I.S. solution (15 µg/mL), 50 µL of PBS pH 7.4 and 3 mL mixture of dichloromethane and n-hexane (1:1, v/v) extraction solvent were added. The mixture was vortexed for 2 min and centrifuged (Remi, India) at 3,500 rpm for 10 min. The supernatant was transferred to a reactivial placed in a heating block at 40 °C, evaporated to approximately 200 µL under a gentle stream of nitrogen gas, then 200 µL of 0.05 M HCl was added for back extraction, and vortexed for 2 min. Finally, the remaining organic layer was evaporated to dryness under a gentle stream of nitrogen and 20 µL of sample was injected into the HPLC column. The pharmacokinetic parameters were determined using software (kinetical trial version).

3. RESULTS

PLGA was conjugated with L-valine. The molecular conjugation of PLGA with hydrophilic drugs, in particular peptides and proteins, has been scarcely found in the literature. Uncapped and hydrophilic PLGA that has a carboxylic acid group at its terminal end, however, can be readily conjugated to various drug compounds. L-Valine was selected for the conjugation. L-Valine can be molecularly dissolved in DMSO, a polar organic solvent, in which PLGA could also be dissolved. By using DCC as a coupling agent, L-valine was conjugated with PLGA using carbodiimide chemistry where DCC was used as coupling agent.

The conjugation of L-valine with PLGA polymer was proved by FTIR. In PLGA, a peak at 3396 cm^{-1} was found which indicates the bending of free N-H stretching, peak at 1749.3 cm^{-1} represents C=O stretch, peak at 1402.7 cm^{-1} represents N-H bending. In valine-PLGA conjugate, peak at 1718 cm^{-1} represents C=O stretch of valine-PLGA conjugate, peak at 1211,1185 cm^{-1} represents C-O stretch, peak at 1538.3 cm^{-1} represents N-H bending and peak at 3381/3217 cm^{-1} represents N-H stretch which prove the conjugation of NH_2 group of L-valine with COOH group of PLGA.

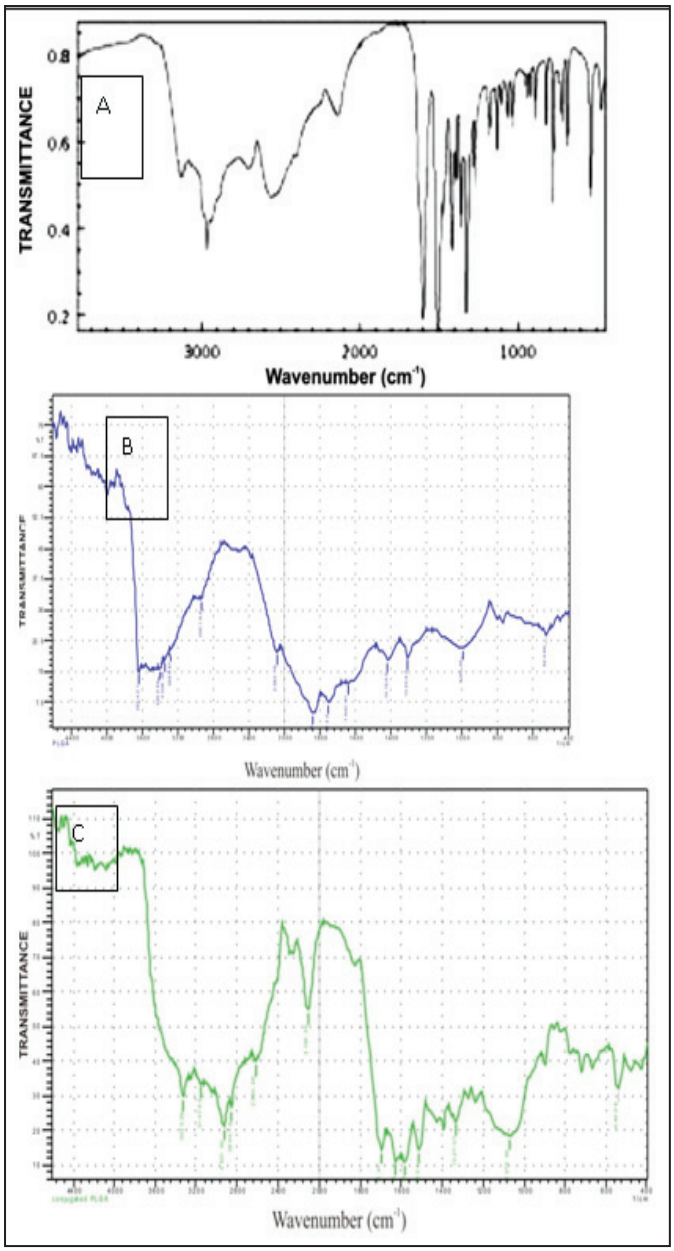

Fig. 1. IR Spectra of (A) valine (B) PLGA and (C) L-valine combined PLGA

PLGA (poly lactic co glycolic acid) was used for the preparation of nanoparticles (NPs). PLGA with 50:50 monomers as ratio exhibits faster degradation (about two months). In addition, polymers that are end-capped with esters (as opposed to the free carboxylic acid) demonstrate longer degradation half-lives and molecular weight 17000 was used in the preparation of nanoparticles. For this study uncapped PLGA was used which is having free carboxylic acid group (COOH) for the conjugation.

3.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS -PAGE)

An aliquot of the samples, equivalent to 10–100µg of insulin, was electrophoresed on a 16% SDS polyacrylamide gel for 1 hr at 200 V. Insulin was visualized using silver nitrate staining

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which has a detection limit of 1µg. Molecular weight protein standards were used to compare the electrophoretic mobility of NPs and unprocessed insulin.

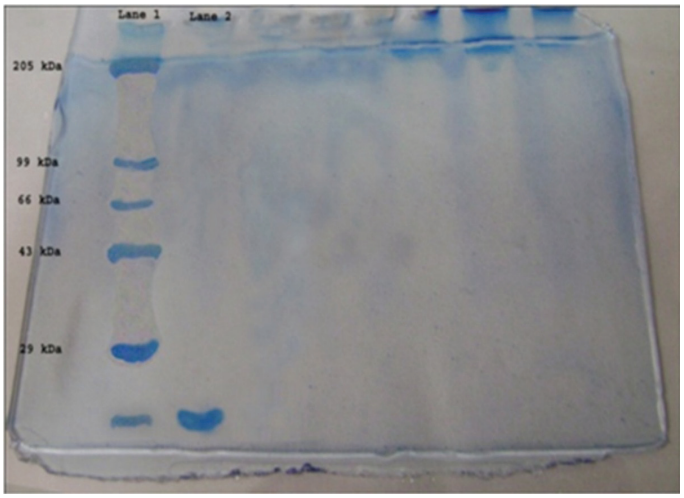


Fig. 2. SDS-PAGE of Insulin. Lane 1: Molecular marker, Lane 2: Insulin.

The CD spectrum of insulin in PBS, at (pH 7.4), shows two minima at 210 and 224 nm. These results were in close agreement with results [15]. There was insignificant deviation of the negative maximum at about 223 nm for insulin from PLGA nanoparticles (Fig.3).

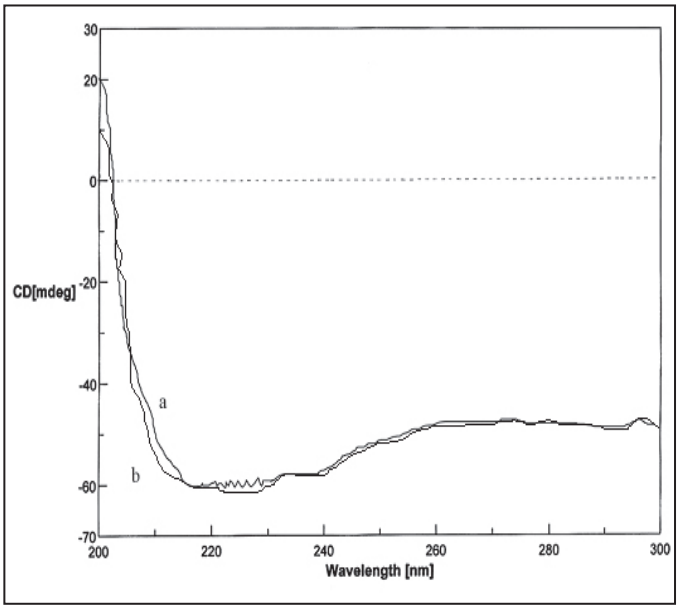


Fig. 3. CD- Spectra of (a) Insulin from conjugated Nanoparticles, (b) Insulin.

It was anticipated from the study of *in-vitro* drug release profile that the formulation gives no detectable release at initial two hours. *In-vitro* release studies of NPs showed a percent cumulative drug release of NPs was 48.58±7.3% after 24 h whereas conjugated nanoparticles showed cumulative drug release of 45.81±8.7% after 24 h (Fig. 4). A significant decline in the percentage cumulative release rate of insulin from conjugated nanoparticles was observed in comparison to NPs.

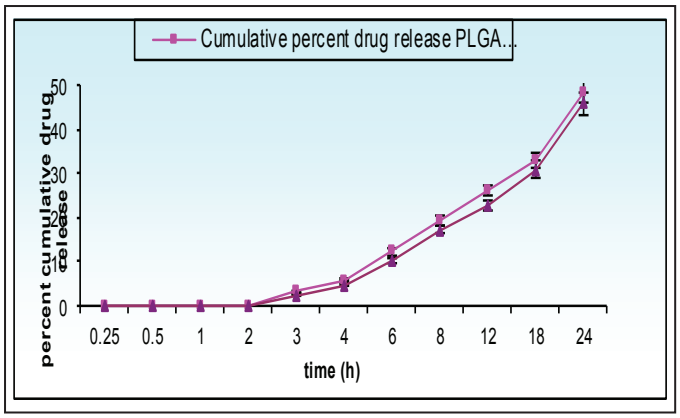
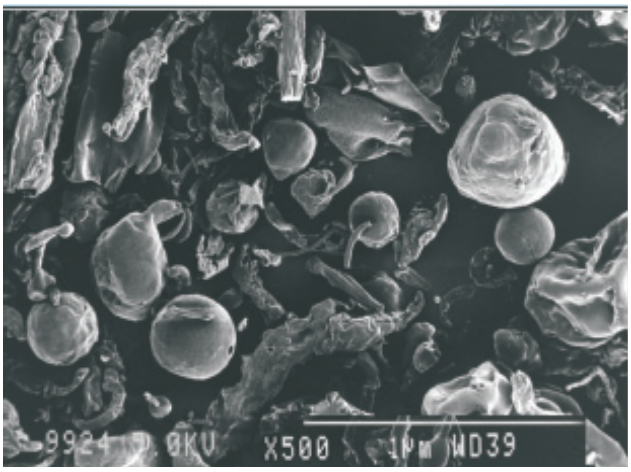
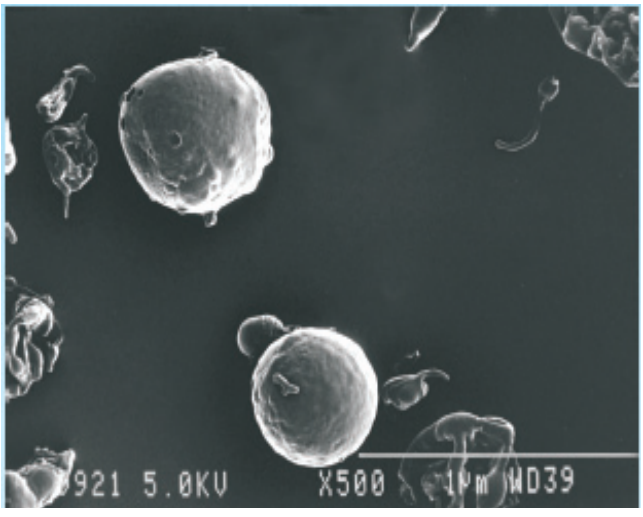


Fig. 4. Cumulative percent drug released at different pH.

The SEM and TEM photomicrographs showed the spherical morphology of the nanoparticles. TEM was used to investigate the shape of NPs. TEM photographs suggested that all NPs and NPs of conjugated polymer were spherical in shape and in nanometric range.



(a) Nanoparticles



(b) Conjugated Nanoparticles

Fig. 5. SEM image of insulin loaded nanoparticles

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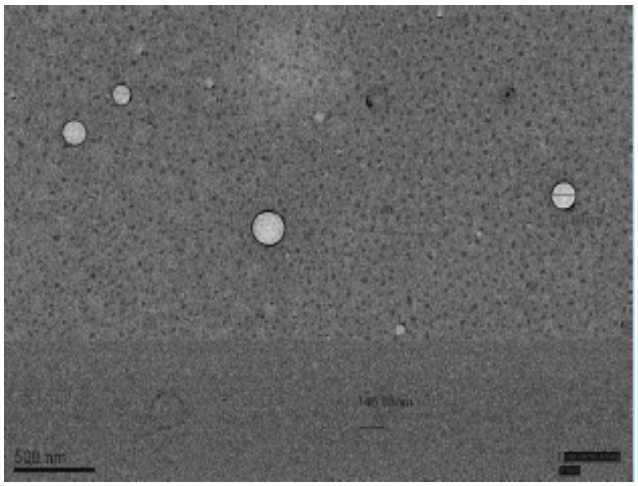
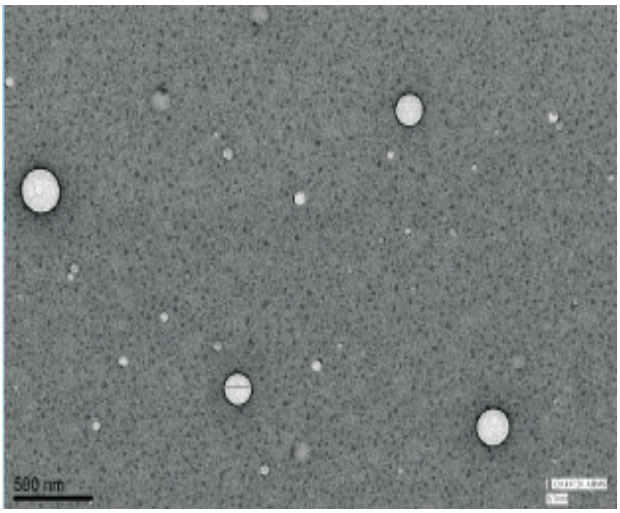


(a) Nanoparticles



(b) Conjugated Nanoparticles

Fig. 6. Photomicrograph is showing TEM image of insulin loaded

The entrapment efficiency of NPs was found to be 50.37±5.9% whereas ligand conjugated NPs showing 52.02±.1.5% drug entrapment efficiency. Particle size was also increased from 209±3.9 nm (NPs) to 213±1.9 nm, (conjugated NPs). The reduction in zeta potential from NPs (−23.03±1.6 mV) to conjugated NPs (−14.4±2.1 mV) was observed. High absolute value of zeta potential indicates higher electric charge on the surface of the drug-loaded NPs, (Table 1).

Table 1. Parameters for formulation of plain and combined Nanoparticles

S. No.	Formulation	Average Particle Size (nm)	PDI	Entrapment efficiency (%)	Zeta potential
1	Plain Nanoparticles	209 ± 3.9	0.342 ± 0.062	50.37 ± 5.9	–23.03 ± 1.6
2	NPs of conjugated polymer	213 ± 1.9	0.318 ± 0.153	52.02 ± 1.9	–14.4 ± 2.1

Mean ± SD (n = 3)

To perform *in-vivo* studies firstly, the NPs and plain insulin powder were filled in hard gelatin capsules (Capsule No. 5) which were coated with enteric polymer Eudragit S 100. The experimental diabetes was induced with streptozotocin in rabbits. As diabetic rabbits were selected for the study which might have died if continuously fasted, so standard feed were given to the animals of all groups at 4, 12 and 24 h of study, which raised BGL values at 6th h and 14th h till 2 h post-feed.

Blood glucose level of group 1 animals showed hyperglycemia consistently throughout the study. While oral administration of insulin to Group 2 animals did not produce significant lowering of blood glucose level.

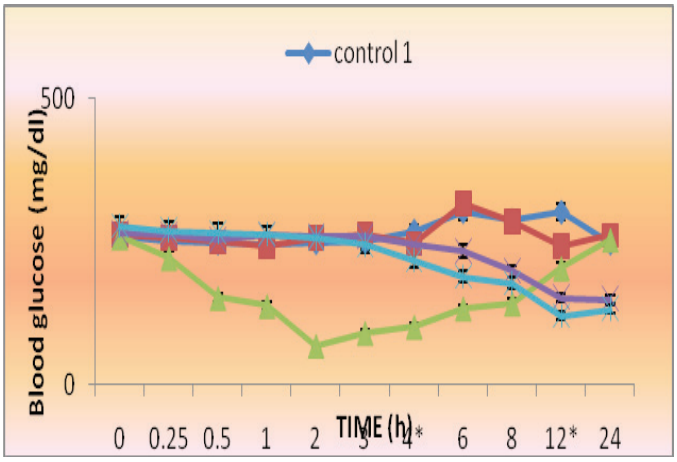


Fig. 7. Blood glucose profile at various time intervals on oral administration of different formulations compared with subcutaneous administration of marketed preparation.

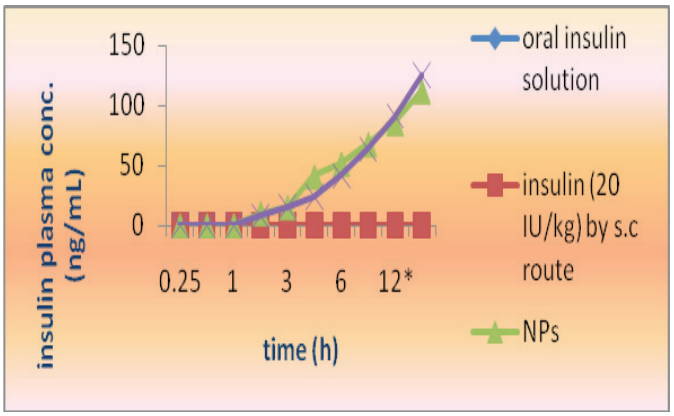


Fig. 8. Insulin plasma level at various time intervals on oral administration of different formulations compared with subcutaneous administration of marketed preparation

*= diet given

Thermal Analysis was performed by DSC. Thermograms were obtained using a Shimadzu DSC-50 system (Shimadzu, Kyoto, Japan). Samples were lyophilized, 2.0 mg of lyophilized powder crimped in a standard aluminium pan and heated from 20 to 350°C at a heating constant rate of 10°C/min under constant purging of nitrogen at 20 mL/min

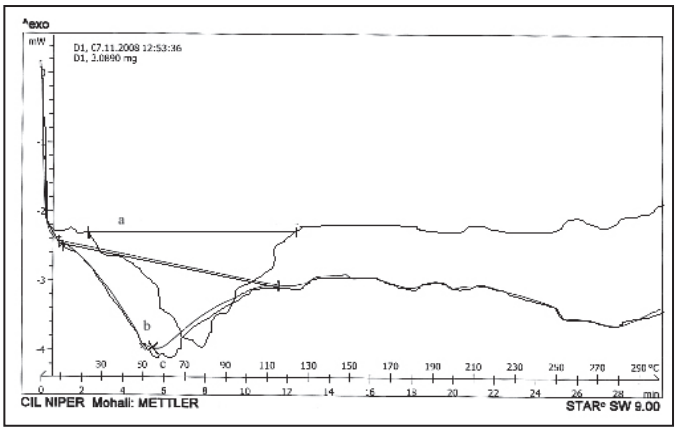


Fig. 9. DSC Thermogram of (a) Pure Insulin, (b) insulin Loaded NPs, (c) insulin Loaded conjugated NPs.

When oral suspension of insulin loaded PLGA nanoparticles was given the blood glucose level is not reduced from 265.4±8.5 mg/dL at initial two hours which further reduced to 246.6±2.4 mg/dL within 4 h, the blood glucose further decreased to 198.7±7.1 mg/dL value at 8th h, depicting the hypoglycaemic effect prolonging for 4 h and this blood glucose level increased to 152.7±5.3 mg/dL after 12 h of administration (Fig.10). The ligand conjugated formulation on oral administration produced hypoglycaemic effect within 4 h of administration, i.e, blood glucose level is reduced from 277.7±5.3 mg/dL at initial two hours further reduced to 216.9±1.9 mg/dL. The hypoglycaemic effect prolonged till 12 h of oral administration. Compared with formulation loaded with the drug, the valine conjugated nanoparticles produced a sustained hypoglycaemic response till 12 h than 8 h. The mean extraction efficiency results of insulin from rabbit plasma at concentrations of 100, 200, 300 and 500 ng/mL were found to be 85.01, 91.51, 84.68 and 90.54%. The mean recovery for I.S. was 85.72 ± 1.62%.

4. DISCUSSION

PLGA was conjugated with L-valine which acts as navigator and enhance the particle uptake. The molecular conjugation of PLGA with hydrophilic drugs, in particular peptides and proteins, has been scarcely found in the literature due to absence of reactive functional groups in the PLGA chain backbone. Uncapped and hydrophilic PLGA that has a carboxylic acid group at its terminal end, however, can be readily conjugated to various drug compounds. The conjugation between PLGA and valine was confirmed by IR spectroscopy.

The conjugation of L-valine with PLGA polymer was proved by FTIR. In PLGA, a peak at 3396 cm^{-1} was found which indicated the bending of free N-H stretching, peak at 1749.3 cm^{-1} represents C=O stretch, peak at 1402.7 cm^{-1} represents N-H bending. In valine-PLGA conjugate, peak at 1718 cm^{-1} represents C=O stretch of valine-PLGA conjugate, peak at 1211,1185 cm^{-1} represents C-O stretch, peak at 1538.3 cm^{-1} represents N-H bending and peak at 3381/3217 cm^{-1} represents N-H stretch which prove the conjugation of NH_2 group of L-valine with COOH group of PLGA.

The single emulsion method is confined to the entrapment of lipophilic compounds; whereas hydrophilic compounds tend to show diffusion and partitioning from the dispersed oil phase into the continuous aqueous phase. Therefore, for an encapsulation of hydrophilic agents such as insulin double emulsion process was used. A buffered aqueous solution of the compound, additionally sonication is added to form the first microfine water-in-oil (w/o) solution. This emulsion was added gently with stirring into a second aqueous phase containing stabilizers (PVA) to form a water-in-oil-in-water (w/o/w) emulsion. The organic solvent is removed by evaporation or extraction. This double emulsion process is best suited to encapsulate hydrophilic drugs like peptides and proteins and also suitable for enhancing protein stability [8].

PLGA (poly lactic co glycolic acid) was used for the preparation of nanoparticles (NPs). It is biodegradable and biocompatible, it degrades by hydrolysis of its ester linkages in the presence of water into two monomers lactic acid and glycolic acid which under normal physiological conditions, are by-products of various metabolic pathways in the body. PLGA with 50:50 monomers as ratio exhibits faster degradation (about two months). In addition, polymers that are end-capped with esters (as opposed to the free carboxylic acid) demonstrate longer degradation half-lives and molecular weight 17000 was used in the preparation of nanoparticles. It has very good mechanical properties and long shelf life. For this study uncapped PLGA was used which is having free carboxylic acid group (COOH) for the conjugation.

The endothermic peak diminishes in the drug loaded preparation indicating the entrapment of drug in the NPs and conjugated NPs. The SEM and TEM photomicrographs showed the spherical morphology of the nanoparticles. TEM was used to investigate the shape of NPs. TEM photographs suggested that all NPs and NPs of conjugated polymer were spherical in shape and in nanometric range.

It was anticipated from the study of *in vitro* drug release profile that the formulation gives no detectable release at initial two hours that must be due to enteric polymer coating. The vitro release studies of NPs showed a percent cumulative drug release of 48.58±7.3% after 24 h whereas conjugated nanoparticles showed cumulative drug release of 45.81±8.7% after 24 h. A significant decline in the percentage cumulative release rate of insulin from conjugated nanoparticles was observed in comparison to NPs that indicated that coupling of valine to PLGA slows down the release of drug from the nanoparticles and thereby imparts a sustained release nature. The NPs and plain insulin powder were filled in hard gelatin capsules (Capsule No. 5) which were coated with enteric polymer Eudragit S 100 to protect drug from the harsh environment of stomach. The experimental diabetes was induced

with streptozotocin in rabbits. As diabetic rabbits were selected for the study which might have died if continuously fasted, so standard feed were given to the animals of all groups at 4, 12 and 24 h of study, which raised BGL values at 6th h and 14th h till 2 h post-feed.

Blood glucose level of group 1 animals showed hyperglycemia consistently throughout the study as they were experimentally induced diabetic and not treated. While oral administration of insulin to Group 2 animals did not produce significant lowering of blood glucose level as the insulin probably degraded in GIT lumen and impermeability of insulin through GIT membrane.

When oral suspension of insulin loaded PLGA nanoparticles was given, the blood glucose level was not reduced at initial two hours due to enteric coated polymer which further reduced within 4 h, the blood glucose further decreased to 198.7±7.1 mg/dL value at 8th h, depicting the hypoglycaemic effect prolonging for 4 h and this blood glucose level increased to 152.7±5.3 mg/dL after 12 h of administration, indicating that the insulin bearing polymeric nanoparticles produced sustained hypoglycaemic response up to 24 hours. The optimum wavelength for detecting insulin with adequate sensitivity was found to be at 214 nm. The elution time of insulin was 16.50 min.

An e-cient recovery of insulin and I.S. from plasma was achieved using a solvent mixture of dichloromethane and n-hexane (1:1 v/v) as an extraction solvent. When dichloromethane was used alone as an extraction solvent, the recovery of insulin was about 60% but with the mixture of dichloromethane and n-hexane, the recovery improved to about 89%.

5. CONCLUSION

L-valine-conjugated PLGA-nanoparticles were prepared using double emulsion solvent evaporation method. The insulin bearing nanoparticles were also studied for FTIR, size, drug entrapment efficiency, zeta potential, SEM, TEM, *in vitro* insulin release, *in vivo* release. The FTIR studies proved the conjugation of NH_2 group of L-valine with COOH group of PLGA. TEM & SEM photographs suggested that all NPs and NPs of conjugated polymer were spherical in shape and in nanometric range. The Entrapment Efficiency was studied using uncapped PLGA was used which is having free carboxylic acid group (COOH) for the conjugation. The endothermic peak diminishes in the drug loaded preparation indicating the entrapment of drug in the NPs and conjugated NPs. The reduction in size of the particle caused a decrease in zeta potential value, as High absolute value of zeta potential indicates higher electric charge on the surface of the drug-loaded NPs. The *in vitro* drug release profile that the formulation showed no detectable release after 24 h which indicated the coupling of valine to PLGA that slows down the release of drug from the nanoparticles and thereby imparts a sustained release nature. An e-cient recovery of insulin and I.S. from plasma was achieved using a solvent mixture of dichloromethane and n-hexane (1:1 v/v) as an extraction solvent. The *in Vivo* studies revealed that, when oral suspension of insulin loaded PLGA Nanoparticles was given, the insulin bearing polymeric Nanoparticles produced sustained hypoglycaemic response up to 24 hours.

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