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Full Length Research Paper

Microspheres of insulin-Eudragit complex: Formulation, characterization and *in vivo* studies

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Insulin, a hormonal drug for the management of diabetes mellitus has continued to attract attention of many researchers and pharmaceutical formulators for more than a decade. However, its low oral bioavailability due to the activities of intestinal enzymes restricts its route of administration to only the parenteral route. This study was designed to evaluate the capacity of mucoadhesive microspheres formulated with varying blends of Eudragit® RL 100 and Eudragit® RS 100 to protect insulin for oral administration. Microspheres containing varying blends of Eudragit® RL 100/RS100 loaded insulin was prepared by solvent evaporation method and were characterized *in vitro* and *in vivo*. Results showed that stable formulation with high encapsulation efficiency, positive zeta potential and high bioadhesion were obtained in all the formulations. *In vitro* release showed a maximum release of 9 and 87% release in pH 1.2 and 7.2, respectively. Single oral studies showed a decreased in blood glucose level comparably equal to that of subcutaneous (sc) administration. The results of this study indicate that insulin-loaded Eudragit RL100/RS100 microspheres could be a promising drug delivery system to improve oral absorption of insulin.

Key words: Insulin, Eudragit®, microspheres, bioactivity, absorption.

INTRODUCTION

The oral route is widely accepted as the most common method of drug administration into the body due to accessibility, convenience, possibility of repeated self-administration by patients and the opportunity to achieve optimum absorption based on the large surface area available in the gastrointestinal (GI) tract (Khafagy et al., 2007). Additionally, oral delivery offers the unique feature of being able to mimic the physiological path that insulin would travel by entering the hepatic portal vein from the intestine and then to the liver (Lewis et al., 1996). In contrast, insulin injected subcutaneously must circulate through the body before reaching the liver. Accordingly, insulin delivered directly to the liver could decrease complications, such as atherosclerosis, which are

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Table 1. Formulation compositions of the microspheres.

Code	Insulin (100 IU/ml)	Eudragit® RS 100 (g)	Eudragit® RL 100 (g)	Magnesium stearate (g)
A1	0.40	2.00	0.00	0.10
A2	0.40	0.00	2.00	0.10
A3	0.40	2.00	2.00	0.10
A4	0.40	2.00	6.00	0.10
A5	0.40	6.00	2.00	0.10

A1- A5 are insulin-loaded microspheres containing varying quantities of polymer ratio of Eudragit® RS100: Eudragit® RL100 of 1:0, 0:1, 1:1, 1:3 and 3:1 respectively.

associated with high concentrations and build-up of insulin in the body. Insulin is intrinsically poorly absorbable through the intestinal membrane owing to their high molecular weight and hydrophilicity. There are two principal factors that are responsible for this low bioavailability: Enzymatic degradation and poor absorption across the epithelial lining of the gastro-intestinal tract (GIT) (Bai et al., 1995; Iyer et al., 2010). Polymeric encapsulation offers a promising method to overcome these obstacles by protecting insulin in the GIT as well as increasing permeability into systemic circulation. Polymeric materials such as poly(glycolic acid), poly(lactic acid), poly(lactic acid-*co*-glycolic acid), poly[lactic acid-*co*-poly(ethylene glycol)], dextran–PEG as well as pH-sensitive polymers like poly(acrylic acid) and poly(methacrylic acid), have been evaluated as carriers for oral insulin delivery (Jiao et al., 2002; Owens et al., 2003; Mundargi et al., 2008,Cadenas-Bailon et al., 2013).

Additionally, entrapment of insulin into polymeric materials has many advantages compared to traditional pharmaceutical dosage forms (Momoh and Adikwu 2008; Owens et al., 2003). The polymeric carrier can maintain the drug in a specific location in the body, have a prolonged duration of contact with the tissue, and increase the treatment efficiency. This is important since localizing the drug at a targeted site of absorption and transporting the drug across the intestinal epithelial layer are two problems associated with the low bioavailability that often plagues oral insulin delivery (Huang et al., 2003; Makhlof et al., 2010). Among these carriers, the pH-sensitive graft polymers of methacrylic acid and polyethylene glycol have been widely investigated for oral delivery of insulin (Chang and Hsiao, 1989; Samir and Sakr, 2003; Ebube and Jone 2004; Damgé et al., 2007). Industrially, acrylate and methacrylate copolymers are commercially available as Eudragit polymers in different ionic forms, and they are widely investigated in the preparation of microspheres to deliver macromolecules (Conti et al., 2007; Damgé et al., 2007; Tuesca, 2008; Damgé et al., 2008). Recently, the capability of Eudragits in protecting peptide and protein drug for oral administration had being re-echoed (Kidane and Bhatt, 2005;Lamprecht et al., 2006; Liu et al., 2006). In addition to biocompatibility (Chen and Langer, 1998; Chernysheva et al., 2003), mucoadhesive properties (Attivi et al., 2005; Zhang et al., 2012), and permeation enhancing effects (Lamprecht et al., 2006), Eudragits-based delivery systems exhibiting reversible formation of inter-polymer complexes that are insoluble at lower gastric pH, but swell in alkaline conditions of the intestine, followed by dissociate of the complexes to release insulin is an added advantage (Morishitta et al., 1998; Morishita and Peppas, 2006;Damgé et al., 2007).

The objective of this study was to develop acid-stable microsphere based on pH-sensitive Eudragit polymer with the capacity to encapsulate, protect and deliver insulin to the mucosal surface of the small intestine. Insulin-loaded Eudragit microspheres were prepared by solvent evaporation technique using Eudragit RL100 and RS-100 and their blends. The prepared microspheres were evaluated *in vitro* for particle size, shape, surface charge, mucoadhesion, insulin loading and release properties. The capabilities of the Eudragit microspheres to protect the bioactivity of insulin were evaluated in an alloxan induced diabetic rats after oral administration.

MATERIALS AND METHODS

Materials use includes insulin, Eudragit® RS100, Eudragit® RL100 (Röhm GmbH, Germany), liquid paraffin (Moko Pharm., Ltd, Lagos, Nigeria), hydrochloric acid, potassium dihydrogen phosphate, magnesium stearate, sodium chloride, polysorbate 60 (Span® 60), acetone, n-hexane (BDH, Poole, England) and distilled water (freshly prepared in Industrial Chemistry Laboratory, University of Nigeria) were used throughout the experiment. All other materials purchased were of analytical grade and were used without further purification.

Preparation of microspheres

Insulin-loaded Eudragit® microspheres were prepared by solvent evaporation technique according to the formula presented in Table 1. A known quantity (2.0 g each alone and 4.0 g blend of different ratios) of Eudragit® RS 100/RL100 was accurately weighed and dissolved at room temperature in a 500 ml beaker containing 12.5 ml of acetone and stirred with glass rod to dissolve the polymers. A known quantity of insulin dissolved in 100 IU and magnesium stearate (100 mg) were also weighed, added to the same beaker containing the Eudragits dissolved in acetone and the contents

further stirred for 10 min. The suspension was homogenized by a magnetic stirrer (Remi Equipments Pvt Ltd, Mumbai) for 5 min at 500 rpm. A 1 % v/v of Span-60 was added to liquid paraffin (125.00 ml) in a 500 ml beaker with continuous stirring and the mixture homogenized using magnetic stirrer for 5 min at 500 rpm. The suspension containing the polymer and drug, was then transferred gradually (drop wise) into the 500 ml beaker containing the liquid paraffin mixture with continuous stirring and the system homogenized using a mechanical stirrer with double blade (4 cm in diameter) (MYP21-250, Henan China (Mainland) at 500 rpm for further 5 min. The resulting solution was stirred at room temperature for 3 h at (450 to 500 rpm) until the acetone evaporated completely. The microspheres were harvested by filtration using filter paper (Whatman No.1), the liquid paraffin and Span-60 was washed off three times with 50 ml of chilled n-hexane. The microspheres were air-dried at room temperature for 24 h, packed in air tight cover bottle and stored at in a refrigerator until further use.

Characterization of the microspheres

Percentage yield

The percentage yield of the microspheres was determined from the ratio of amount of solidified total microspheres to total solid material used in the inner phase.

Morphology and particle size analysis

Particle size analysis was carried out on the microspheres formulation using a digital light microscope (Leica Diestar, Germany) and images captured with Moticam 1000 digital microscope camera (Moticam® models 1000, USA). The morphology and size of the particle was determined based on image analysis of the microspheres.

Particle size distribution and zeta potential

Microparticles were analyzed for their size distribution using dynamic light scattering in a Zetasizer (Malvern Instruments, UK) and for their surface charge using the same instrument. Each sample was measured in triplicate.

Quantitative determination of insulin

The insulin content of the microspheres was determined using a high performance liquid chromatography (HPLC). The chromatographic system consisted of an Agilent 1100 series programmable separating module, quaternary pump G 1311 A (Agilent technology, Geneva, Switzerland), an auto degasser G1322A, and a variable wavelength detector G1314A (Marinfield, Germany). The column was a reverse phase ODS (C-18, 5 mm 4.6 × 250 mm, Supelcosol, Mumbai, India) equipped with a guard. The mobile phase consisted of acetonitrile and water (10:90), perchloric acid was used to adjust the pH to 3. The flow was set at 0.8 ml/min and the chromatogram was recorded at 280 mm.

Insulin entrapment efficiency (IEE%)

A 50 mg quantity of microspheres was dispersed in 10 ml of simulated intestinal fluid (SIF, pH 7.2). The dispersion was allowed to stand for 3 h after which it was mixed with a vortex mixer for 5 min and then centrifuged at 2500 rpm for 20 min. The amount of insulin contained in each batch of the formulations was determined by the HPLC (Builders et al., 2008b). The IEE was then determined according to Equation 1 (Kim and Peppas, 2003; Cui et al., 2006; Sarmento et al., 2007).

$$IEE\ \% = \frac{ADC}{TDC} \times 100 \quad (1)$$

Where TDC is the weight of drug added to the formulation, while ADC is the analyzed weight of the drug in the microspheres.

Measurement of micromeritics properties of microspheres

The flow properties were investigated by measuring the angle of repose of insulin-loaded Eudragit® microspheres using fixed-base cone method. The bulk and tapped densities were measured in a 10-ml graduated measuring cylinder as a measure of packability of the microspheres. The sample contained in the measuring cylinder was tapped mechanically by means of constant velocity rotating cam with the change in its initial bulk density to a final tapped density when it has attained its most stable form (that is, unchanging arrangement). Each experiment was carried out in triplicate.

Bioadhesiveness of the microspheres

This study was based on *in vitro* wash method as described by Attama and Adikwu (1999) with slight modifications. Freshly excised cow ileum was purchased from a local market and used for the bioadhesive study. The ileum was cut into pieces measuring 15 cm (length) × 3.0 cm (internal diameter) and each was gently rinsed with chilled saline to remove intestinal waste materials and quickly pinned unto the polythene support of the developed bioadhesion instrument. A known quantity (200 mg) of the different batches was weighed out, placed on the rough mucus surface and allowed to hydrate for 15 min for microspheres-mucus interaction to take place. A 250 mL of SIF (pH 7.4) contained in a separating funnel was allowed to flow over the hydrated microspheres at a rate of 20 ml/min. The percentage bioadhesion (BD%) of the microspheres adhered to the tissue was calculated from the equation:

$$\mathrm{BD}\ \% = \frac{\mathrm{WAM}}{\mathrm{TWM}} \times 100 \quad (2)$$

Where WAM is the weight of microspheres adhered to the tissue, while TWM is the total weight of microspheres applied to the tissue.

Fourier transfer infrared spectroscopy (FTIR)

Drug-polymer/polymer-polymer interactions were studied by FTIR spectroscopy. The spectra were recorded for pure drug and drug-loaded microspheres using FTIR JASIO (Model No. 410). Samples were prepared in KBr disks (2 mg sample in 200 mg KBr). The scanning range was 400 to 4000 cm^{-1} and the resolution was 2 cm^{-1}.

Differential scanning calorimetry (DSC)

Thermal analysis and changes in heat capacity of the Eudragit® RS100 and Eudragit® RL100 were determined using a calorimeter (DSC) (NETZSCH DSC 204 F1, Germany). Approximately 5.0 mg of the polymer was weighed (Mettler M3 Microbalance, Germany) into an aluminum pan, hermetically sealed, and the thermal behavior determined in the range of 35 to 190°C under a 20 ml/min

Table 2. Some physicochemical and physico-chemical properties of the microspheres.

Batch code	PS (µm)	EE (%)	Yield (%)	ZP (mV)	BD (g/ml)	TD (g/ml)	CI (%)	AR(°)	HR (%)
A1	134.10	94.40	73.10	38.9 ± 0.1	0.37	0.50	26.91	24.10	1.37
A2	111.20	95.20	63.99	29.6 ± 0.3	0.29	0.30	13.43	23.35	1.04
A3	64.00	98.50	64.41	36.4 ± 1.0	0.32	0.40	19.22	20.42	1.24
A4	96.00	98.90	76.53	39.3 ± 0.1	0.59	0.61	13.28	21.41	1.03
A5	87.16	98.70	86.72	38.8 ± 0.1	0.49	0.57	14.58	22.32	1.17

A1 - A5 are polymer ratio (Eudragit® RS100: Eudragit® RL100) of 1:0, 0:1, 1:1, 1:3 and 3:1 respectively; PS = particle sizes, EE = encapsulation efficiency, ZP = zeta potential, TD = Tapped density, CI = Carr's index, AR = angle of repose and HR = Hausner's ratio.

nitrogen flux at a heating rate of 10°C/min. The thermal property of pure insulin was also determined. This determination was extended to the microspheres formulated with or without insulin. The baselines were determined using an empty pan, and all the thermograms were baseline corrected. Reproducibility was checked by running the sample in triplicate.

In vitro release of insulin

The *in vitro* release profiles of the insulin-loaded microspheres were determined quantitatively using a high performance liquid chromatography (HPLC). Briefly, the polycarbonate dialysis membrane (length 10 cm, diameter 3.5 cm, (MWCO 6000 - 8000, Spectrum Labs, Brenda, The Netherlands) used was pre-treated by soaking it in the dissolution medium for 24 h prior to the commencement of each release experiment. In each case, 20 mg of the formulated microspheres was placed in the dialysis membrane containing 3 mL of the dissolution medium, securely tied with a thermo-resistant thread and was then placed in a 250 ml beaker containing 200 ml of phosphate citrate buffer solution (pH 2.2); agitation of the fluid system (60 rpm) was done with a magnetic stirrer (Remi Instruments, Mumbai, India). At predetermined time intervals, 0.5 ml samples were withdrawn and replaced immediately with phosphate citrate buffer solution. After 2 h the pH of the dissolution medium was changed to 7.4 by the addition of 0.1 N sodium hydroxide and further sampling continued for another 6 h. The temperature of the dissolution system and the replacement fluid were maintained at 37 ± 0.5°C. The insulin concentrations of the aliquots were determined by HPLC, and the percentage amount of insulin released from the microspheres was calculated. The percentage of insulin released was plotted against time. Each data point was recorded as mean (± SD) calculated from three measurements.

In vivo hypoglycemic effects

All experiments were carried out in accordance with the Federation of European Laboratory Animal Science Association (FELASA) Guide for the Care and Use of Laboratory Animals and the European Union (Council Directive 86/609/EEC). Male Wistar rats weighing 180 to 200 g were housed in controlled environmental conditions of temperature and relative humidity, maintained at 22 ± 2°C and 45%, respectively. The rats were fed with standard diet feed (MBC, new fields, Nigeria) and tap water provided *ad libitum*. Lighting was on a standard 12 h on /12 h off cycle. Diabetes was induced in rats by a single intraperitoneal injection of streptozotocin (50 mg/mL in pH 4.5 citrate) at 50 mg/kg. After two weeks, rats with fasted blood glucose levels above 250 mg/dL were used for the experiment. The rats were starved for 12 h before experiments and remained starved for 24 h during the experiment, but had free access to water. Forty-eight wistar rats were used for the evaluation of the anti-diabetic effects of the formulations. Rats were divided into nine groups of five animals each and each group of animals was housed in separate metallic cages.

The different formulations of the insulin-loaded microspheres after the solvent were completed evaporated following several washing, they were weight put into hard gelatin capsules (200 mg capacity), with each capsule containing formulated microspheres equivalent to insulin dose of 50 IU/kg body weight for each animal. The capsules containing insulin (experimental) and the unloaded capsule (negative control) were administered orally to the animals in their respective group according to their weight. Group I was orally administered 1.0 ml of distilled water; Group II received unloaded microspheres (no insulin), Group III and IV received oral insulin solution (40 IU/kg) and subcutaneous injection of insulin (40 IU/kg), respectively, as a positive control. The formulated insulin loaded-microspheres (A1-A5) was administered orally to the animals in groups V to IX, respectively according to their body weight. Blood was collected from the tail vein of each rat to obtain a baseline glucose level and, following insulin administration, additional samples were collected at predetermined times intervals: 0, 30 min, 1, 2, 3, 4, 5, 6 and 8 h and blood glucose concentrations were determined using a glucometer (ACCU-Check, USA). The data were corrected by subtracting the baseline glucose for each animal from each data point such that only changes in blood glucose were compared. Results were shown as the mean values (±SD) of basal blood glucose levels of animals of each group.

RESULTS

Percent yield recovery

The percentage of the microspheres recovered from the formulations ranges from 87.3 to 93.5% in the loaded insulin while the unloaded batches shows 75.9% indicates that all the insulin-loaded microspheres had overall higher recovery percentages than the unloaded microspheres.

Morphology and particle size (PS) analysis

The microscopic images of the microspheres are shown in Figure 1. The results indicate that all the microspheres prepared were spherical in shape. However, the ratio of the polymers used in the preparations had no influence on the shape of the microspheres. As shown in Table 2,

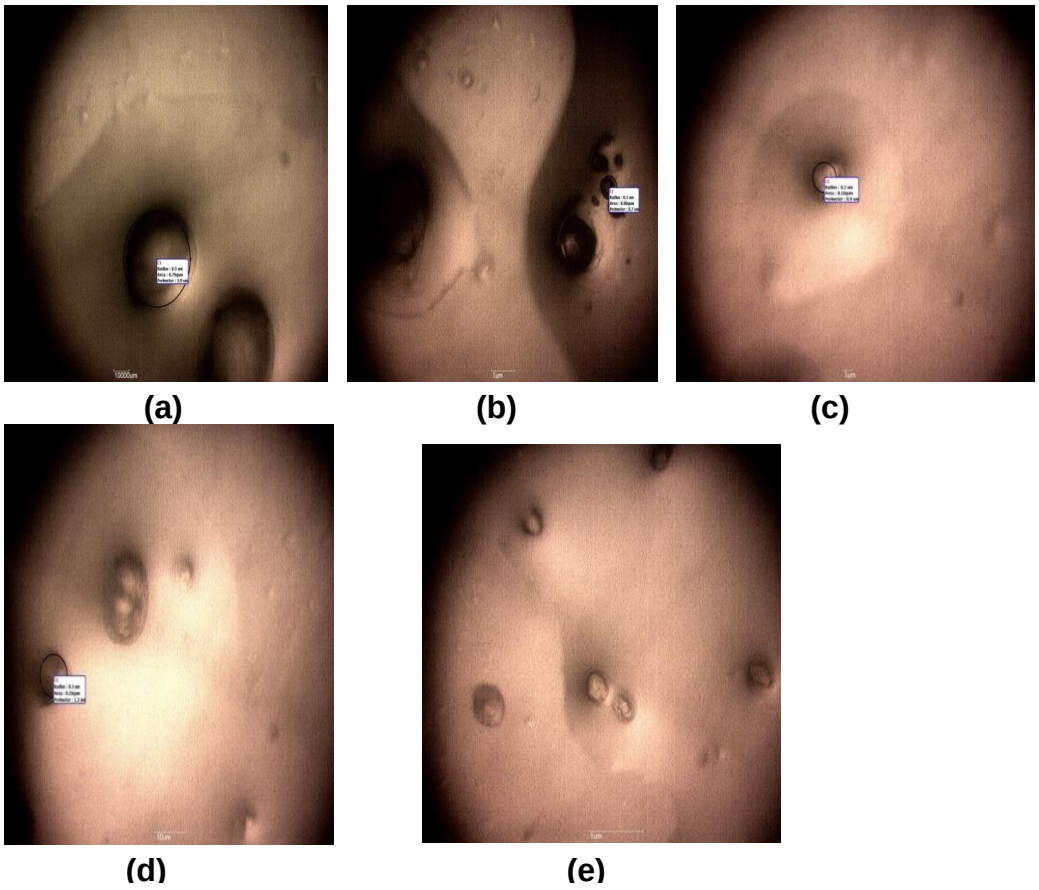


Figure 1. Photomicrograph of insulin-loaded microspheres containing various amounts of Eudragit RS100 and RL100: (a) (A1), (b) (A2), (c) (A3), (d) (A4) and (e) (A5) are insulin-loaded microspheres. Magnification x 65.

the mean diameter of loaded microspheres prepared ranged from 64 to 134 μm. It was observed (Table 1) that the ratio of the polymer used in the formulations affected the size of the microspheres, which is in the range 111.20 to 134.00 μm for the microspheres prepared by individual polymer and 64.00 to 96.00 μm for insulin-loaded blended polymers.

Zeta potential (ZP) measurement

Microspheres containing insulin microparticles prepared with Eudragit® RS100 alone or in combination with Eudragit® RL100 were positively charged (from + 36 to + 44 mV) due to the quaternary ammonium groups of Eudragits. The ZPs were all high as shown in Table 2.

Encapsulation efficiency (EE%)

The results of the IEE% shows that drug IEE% increased when the polymer were blended together as compared to individual polymer, yielding maximum EE% of 95.55, 98.90 and 98.79% for microspheres formulated with Eudragits RS /RL in the ratios of 1:1, 1:3 and 3:1 respectively. Maximum IEE% (98.9) was observed in batch A4, while minimum (94.40) was obtained in batch A1. However, all the batches of the formulations had good encapsulation efficiency above 90% (Table 2).

Micromeritics properties of microspheres

Micromeritics data are shown in Table 2, the value of angle of repose determined ranges between 20.42-24.12°, bulk density (BD) and tapped density (TD) of the formulated microspheres were found to be in the range of 0.2871 - 0.5900 and 0.2973 - 0.6100, respectively. The Carr's index (CI) and Hausner's ratio (HR) was found to be in range of 13.00 - 26.91 and 1.0338 - 1.3682, respectively.

Bioadhesive studies

The bioadhesive strength (Figure 2) of the microspheres

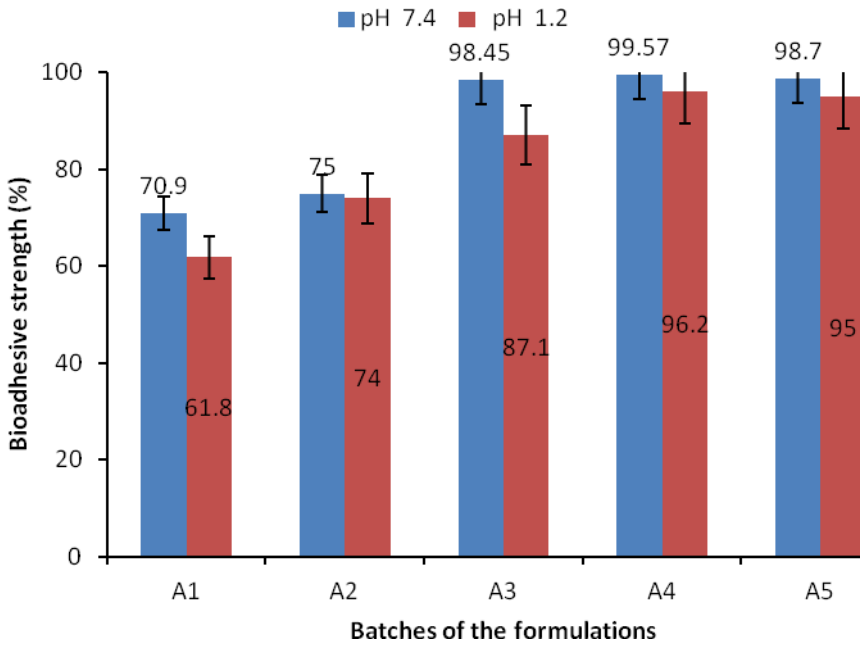


Figure 2. Bioadhesive strength of the various formulations: A1 (inuline+ RS100 alone), A2 (Insulin + RL100), A3 (Insulin + 1:1 of RS:RL), A4 (Insulin + 3:1 of RS:RL) and A5 (Insulin + 1:3 of RS:RL).

Table 3. Thermal properties of the insulin sample and insulin-loaded microspheres.

Code	Melting point (°C)	Enthalpy (mW/mg)	Area (J/g)
Insulin	125.3	-133.0	-186.4
A1	60.9	- 2.263	-37.63
A2	63.6	- 4.777	-170.5
A3	63.2	- 6.729	-132.9
A4	62.3, 83.9	- 6.742, - 6.473	-159.3, - 42.3
A5	65.0, 80.2	- 6.528, - 4.84	- 125.4, - 30.38

A1**,** A2, A3**,** A4 and A5, are insulin loaded polymer ratio of Eudragit® RS100: Eudragit® RL100; 1:0, 0:1, 1:1, 1:3 and 3:1, respectively.

made from the polymers was assessed to determine the bioadhesive effectiveness of blends RS100-RL100 in comparison with its component polymers. The order of bioadhesive strength was A4 > A5 > A3>A2>A1. The adhesion of the polymer discs to the mucus membrane is due to the reduction of the surface energy (interfacial tension) between the membrane and the polymer (Harding, 2003; Guo, 1994).

FT-IR- spectra

FT-IR is a quick and relatively cheap technique for identifying compounds and for detection interactions between drugs excipients and excipients-excipients (Builders et al., 2008a; Builders et al., 2008b). FT-IR measurement was used to identify the polymer and to study the physical and chemical interaction between the polymers as well as drug-polymer interaction. The FT-IR spectra graph is depicted in Figure 3.

Differential scanning calorimetry (DSC)

The thermal heat characteristics as measured by DSC of the insulin-loaded microspheres formulation based on blends polymer of Eudragit RS:RL (1:1, 1:3 and 3:1) are shown in Figure 4c to e whereas the DSC thermograms of Eudragit RS100, RL100 microspheres loaded insulin separately and the unloaded (drug free) polymer blend (1:1) of RS100:RL100 are shown in Figure 4a to b. The thermal properties of the microspheres are shown in Table 3. Thermograms of insulin loaded RS100 and RL100 separately, showed a sharp endothermic peak

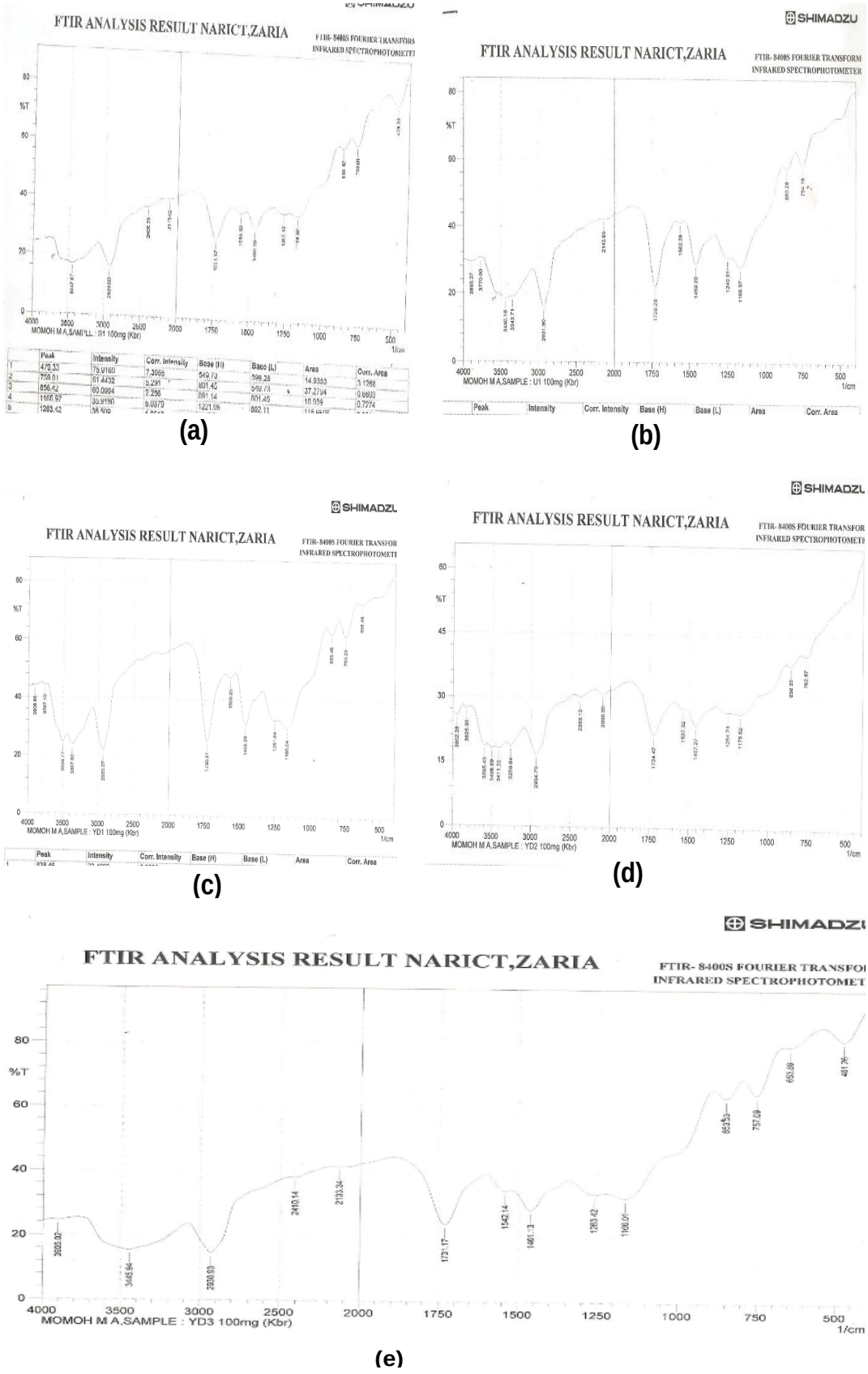


Figure 3. Spectra of A1-A5 of the formulated microsphere. (a) FT-IR spectrum of A1; (b) FT-IR spectrum of A2; (c) FT-IR spectrum of A3; (d) FT-IR spectrum of A4; (e) FT-IR spectrum of A5.

corresponding to melting points at 60.9 and 63.6°C respectively. Thermograms of the drug-loaded polymer blind (A3-A5) showed sharp endothermic peaks at varying degrees (Table 4). It was observed that the DSC traces of the microspheres depend on the polymer ratio. However, the enthalpies of the loaded formulations are much lower than the individual polymers indicating its capability of encapsulating the incorporated drug.

In vitro release of insulin

The *in vitro* release profiles of the insulin-loaded polymeric microspheres in pH 1.2 and 7.4 are shown in Figure 5. This shows that at pH 1.2, the polymer collapsed, whereas at pH 7.4, it swelled to release insulin. At pH 1.2, Eudragit RS 100, RL100 and the various blended polymers showed a maximum insulin release in the range of 2 to 9% within 2 h whereas at pH 7.4, the amount of insulin released ranged from 24.6 to 93.26%. It was observed that formulation A4 has the highest release (93.26%) in about 8 h, while that of A3 has the least release (68.78%), among the blends polymers. However, there was a significant difference ($p < 0.05$) between the release from the polymers blends and individual polymer or unblended polymer. However, the formulations exhibited good and sustained release properties.

In vivo activity of the insulin-loaded microspheres

The results of blood glucose lowering studies shown in Figure 6 show that the formulations exhibited good blood glucose lowering effects. At 30 min post treatment, none of the insulin loaded-microspheres showed any sign of glucose lowering effect, but insulin administered subcutaneously exhibited about 8 to 12% of glucose reduction. At 5 h, the microsphere formulations achieved up to 19 to 25% decrease in blood glucose. At 8 h, up to 30 to 40% of blood glucose reduction was achieved by the batches A4 and A5. However, the insulin loaded into the microspheres was found to show a remarkable decrease in the blood glucose in all the formulations and lasted over 8 h. The decrease in blood glucose levels was comparable to the effect produced by the insulin administered subcutaneously (sc).

DISCUSSION

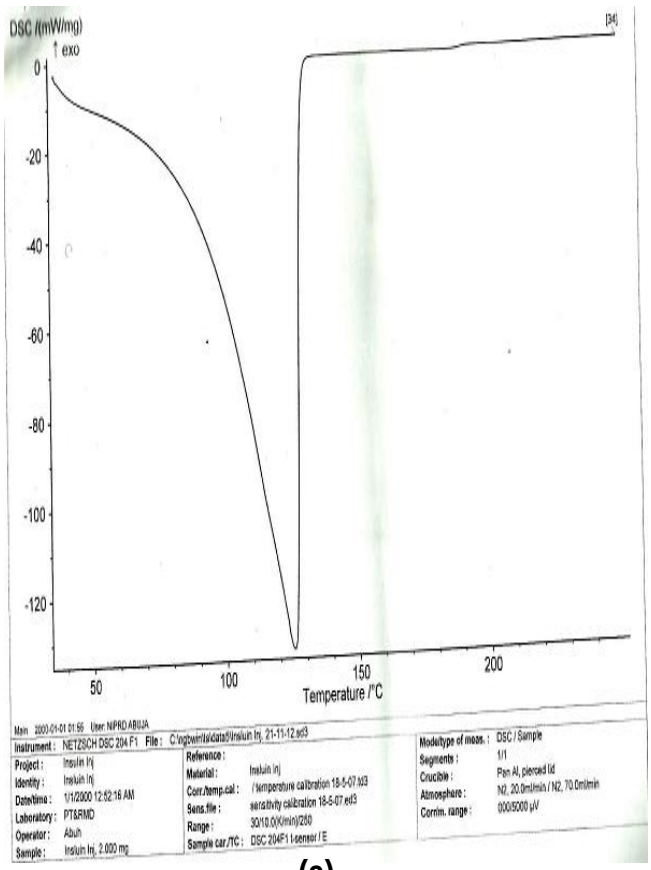
The formulation concept of insulin-loaded microspheres prepared with pH-sensitive polymers such as Eudragit® RS, RL and their blends as carriers was aimed at enhancing the absorption of insulin from the GIT, by providing a protective environment for insulin and improved mucoadhesion of the insulin-loaded microspheres. The process of formulation of the insulin-loaded microspheres was based on emulsification-coacervation technique. This process involves two steps: The first step consists of the formation of the and leads to the formation of droplets of polymers (Eudragit® RS100 and Eudragit® RL100 alone or their blends) dispersed into the organic phase of acetone followed by the water-in-oil-in-oil emulsion obtained by mixing the insulin into a non-aqueous solution of acetone. The second step is the solvent evaporation from the droplets of the second emulsion leading to the precipitation of polymers which are insoluble in water and consequently the solidification of the core of the particles and the entrapment of the drug.

Characterization of polymeric microspheres

High values of the percentage of the microspheres recovered from the formulations are a strong indication that the formulation technique adopted was reliable. The percentage recovery across the batches was generally high. Polymer blends gave the highest yield compared to when the polymers were used alone.

Spherical, free flowing and off white microspheres were successfully prepared with Eudragit RS, RL and their blends in different ratio. The surface morphology of the different formulations of the microspheres was slightly or negligibly affected by the variation in composition of polymer types. Due to their quaternary ammonium groups, Eudragit® RS100 and Eudragit® RL100 have surface active properties able to stabilize the first emulsion, and consequently hampers the coalescence of the droplets, leading to reduction in the diameter of the particles (Chernysheva et al., 2003) and that might be the reason the particle size of the microspheres formulated with Eudragit® RS100 and Eudragit® RL100 blends is less than microspheres of either Eudragit® RS100 or Eudragit® RL100 alone. Also since the particle size is related to a greater extent to the stability of the first emulsion, it can be assumed that some excipients included in the commercial preparation of insulin (Humulin®) could interfere with Eudragit® RS100 and Eudragit® RL100.

The magnitude of the measured zeta potential is an indication of the repulsive forces that are present and can be used to predict the long-term stability of the microspheres (Builders et al., 2008b). When particles have a high negative or positive zeta potential, they tend to repel each other and have no tendency to aggregate. On the contrary, when particles have low absolute zeta potential values, there is no counteracting force to prevent their aggregation and flocculation. Table 1 shows that microspheres prepared with Eudragit RS100 alone or in combination with RL 100 were positively charged (from +36 to +44 mV), due to the predominance of the quaternary ammonium groups of Eudragit RS 100 which


DSC /(mW/mg)
↑ exo
Temperature /°C
Instrument : NETZSCH DSC 204 F1 File : C:\ngbwin\ta\data5\Insulin Inj. 21-11-12.sd3
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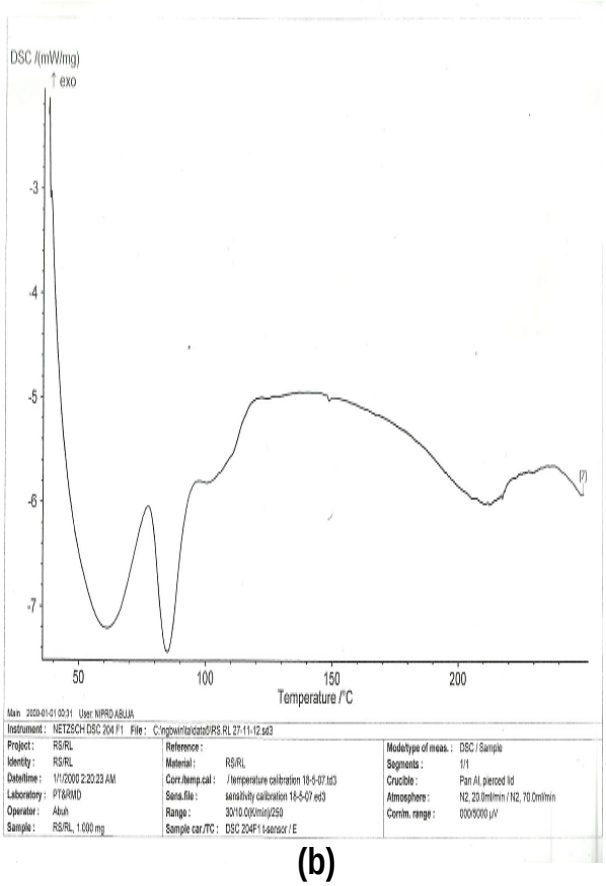
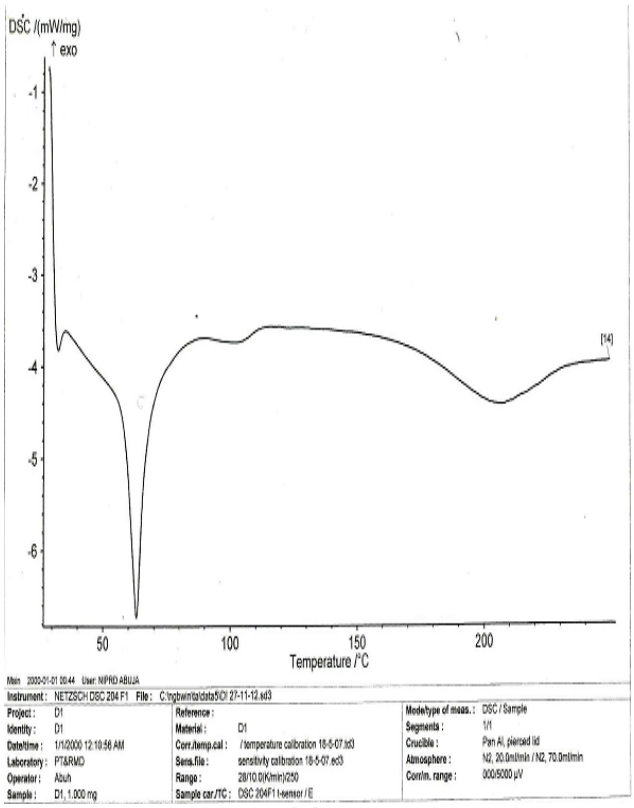
Date/time : 1/1/2000 12:52:16 AM
Laboratory : PT&RMD
Operator : Abuh
Sample : Insulin Inj, 2.000 mg
Material : Insulin inj
Corr./temp.cal : / temperature calibration 18-5-07.td3
Sens.file : sensitivity calibration 18-5-07.ed3
Range : 30/10.0(K/min)/250
Sample car./TC : DSC 204F1 I-sensor / E
Mode/type of meas. : DSC / Sample
Segments : 1/1
Crucible : Pan Al, pierced lid
Atmosphere : N2, 20.0ml/min / N2, 70.0ml/min
Comm. range : 000/5000 µV

(a)


DSC /(mW/mg)
↑ exo
Temperature /°C
Instrument : NETZSCH DSC 204 F1 File : C:\ngbwin\ta\data0\RS.RL 27-11-12.sd3
Project : RS/RL
Identity : RS/RL
Date/time : 1/1/2000 2:20:23 AM
Laboratory : PT&RMD
Operator : Abuh
Sample : RS/RL, 1.000 mg
Material : RS/RL
Corr./temp.cal : / temperature calibration 18-5-07.td3
Sens.file : sensitivity calibration 18-5-07.ed3
Range : 30/10.0(K/min)/250
Sample car./TC : DSC 204F1 I-sensor / E
Mode/type of meas. : DSC / Sample
Segments : 1/1
Crucible : Pan Al, pierced lid
Atmosphere : N2, 20.0ml/min / N2, 70.0ml/min
Comm. range : 000/5000 µV

(b)


DSC /(mW/mg)
↑ exo
Temperature /°C
Instrument : NETZSCH DSC 204 F1 File : C:\ngbwin\ta\data5\D1 27-11-12.sd3
Project : D1
Identity : D1
Date/time : 1/1/2000 12:19:56 AM
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Operator : Abuh
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Material : D1
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Sens.file : sensitivity calibration 18-5-07.ed3
Range : 28/10.0(K/min)/250
Sample car./TC : DSC 204F1 I-sensor / E
Mode/type of meas. : DSC / Sample
Segments : 1/1
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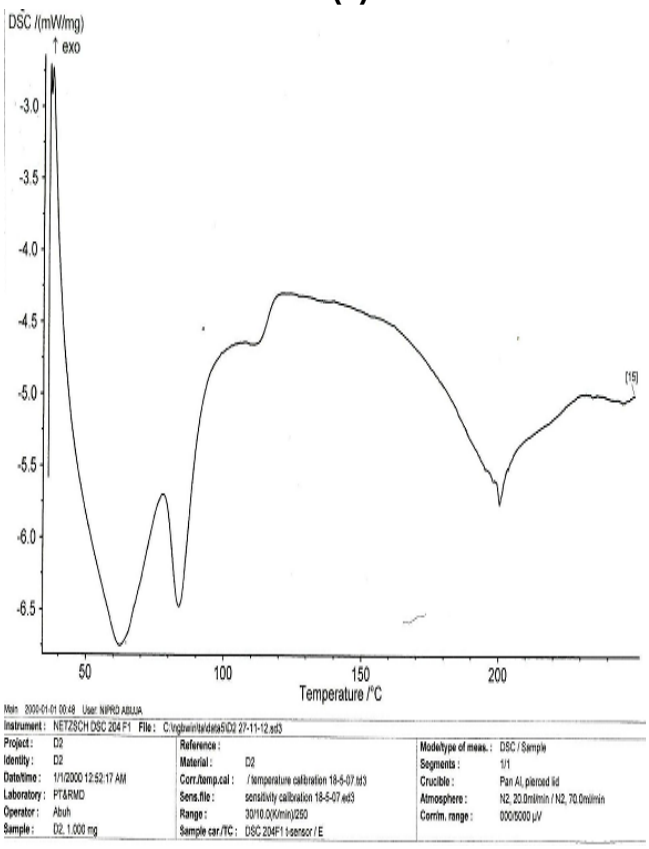
Comm. range : 000/5000 µV

(c)


DSC /(mW/mg)
↑ exo
Temperature /°C
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Identity : D2
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Laboratory : PT&RMD
Operator : Abuh
Sample : D2, 1.000 mg
Material : D2
Corr./temp.cal : / temperature calibration 18-5-07.td3
Sens.file : sensitivity calibration 18-5-07.ed3
Range : 30/10.0(K/min)/250
Sample car./TC : DSC 204F1 I-sensor / E
Mode/type of meas. : DSC / Sample
Segments : 1/1
Crucible : Pan Al, pierced lid
Atmosphere : N2, 20.0ml/min / N2, 70.0ml/min
Comm. range : 000/5000 µV

(d)

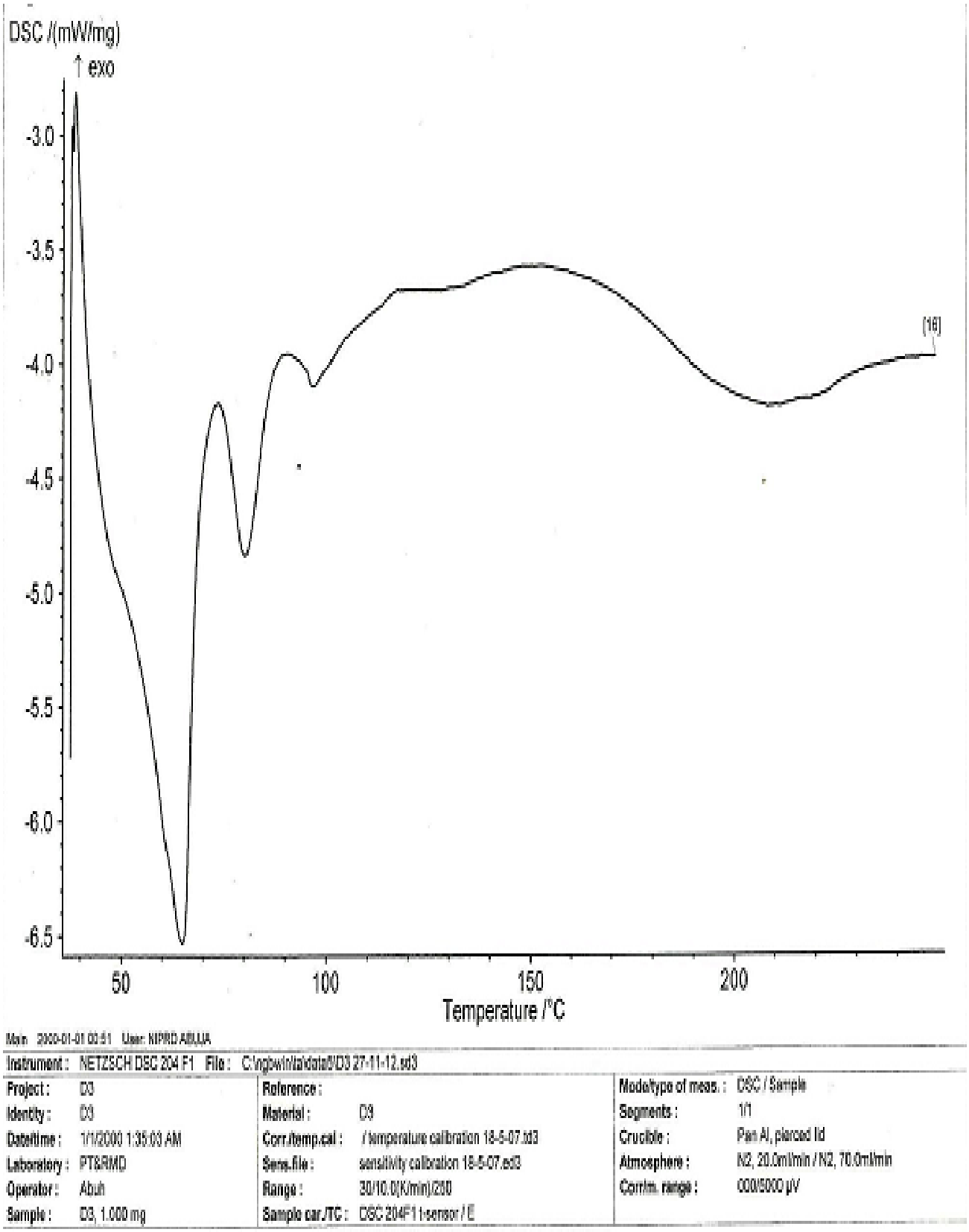

Figure 4. DSC thermograms of Eudragit RS100, RL100 and RS/RL blends at various compositions used in microsphere formulation. (a) Thermogram of insulin inj; (b) Thermogram of polymer ratio; RS/RL; (c) Thermogram of batch A3; (d) Thermogram of Batch A4; (e) Thermogram of Batch A5.

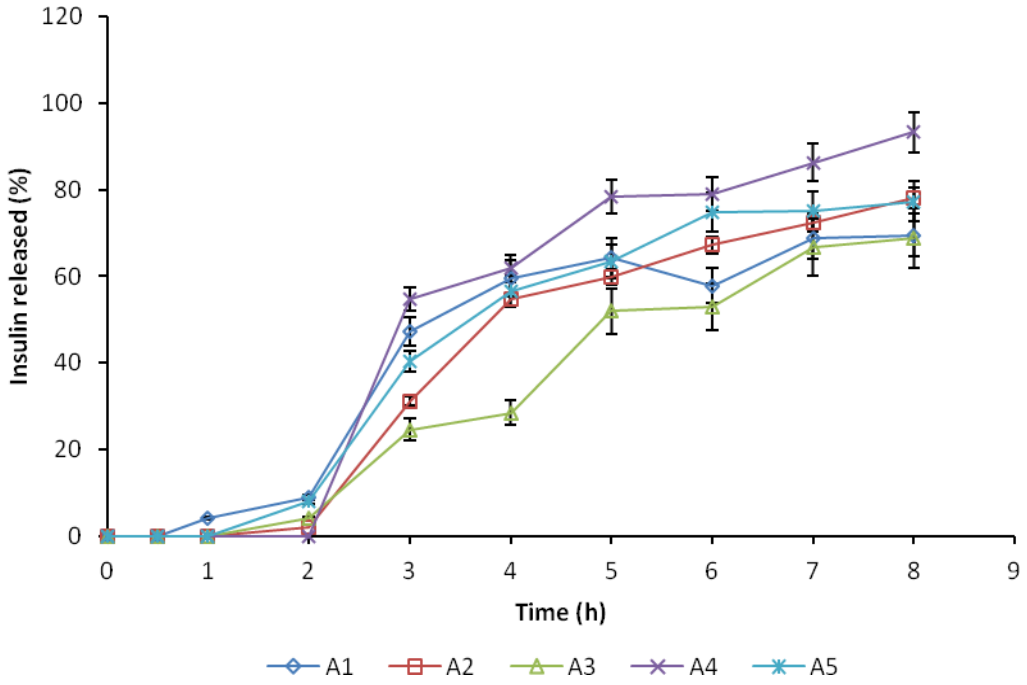

Figure 5. *In vitro* release of insulin-loaded Eudragit RS 100, RL100 and their admixtures RS/RL microspheres at pH 1.2 and 7.4 (*n* = 3). A1-A5 is polymer ratio Eudragit® RS100: Eudragit® RL100 of 1:0, 0:1, 1:1, 1:3 and 3:1 respectively.

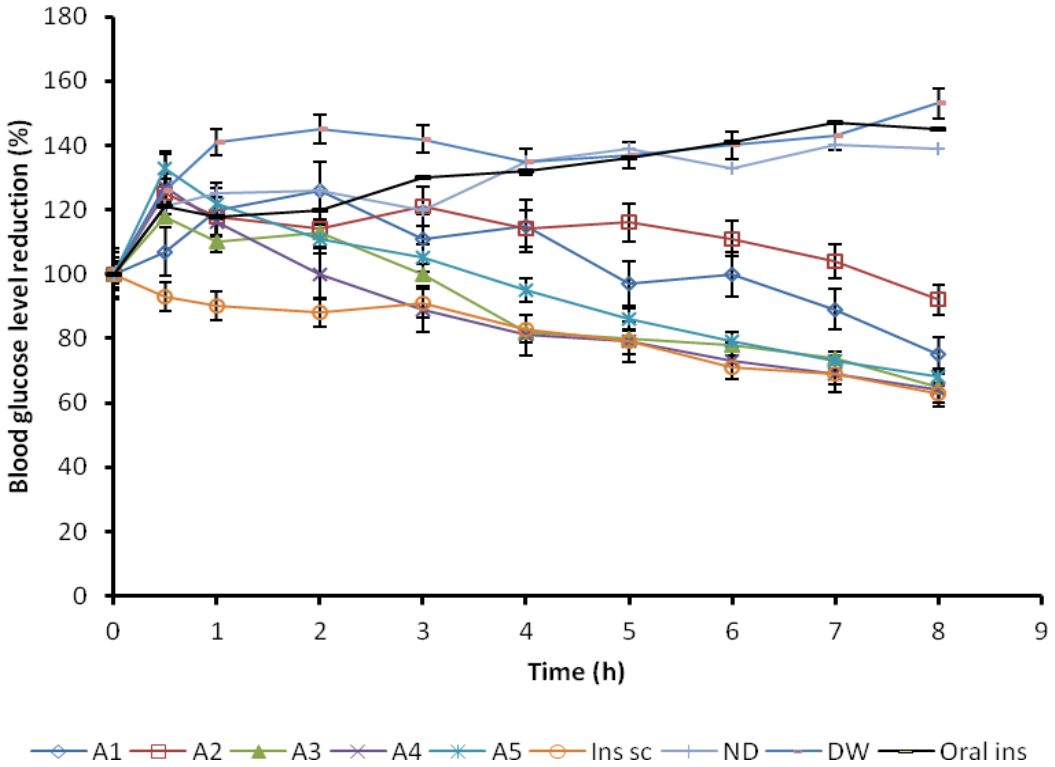

Figure 6. Changes in blood glucose levels after oral administration of control (DW = distilled water), insulin solution (oral ins), insulin-loaded microspheres (A1-A5) and insulin sc administered subcutaneous as a positive control group (mean ± SD, n = 5). A1-A5 is polymer ratio Eudragit® RS100: Eudragit® RL100 of 1:0, 0:1, 1:1, 1:3 and 3:1 respectively, insulin sc; ND = no drug; D/W, oral insulin.

were directed toward the continuous aqueous phase. However, the negative charge of insulin was unable to alter the surface charge of the formulation, an indication that, the insulin was completely encapsulated into the microspheres. Thus, the release data further proves that the insulin was not adhered to the surface of the microparticles as no burst effect was observed (Figure 2). Previous researchers have shown that poor encapsulation of the negatively charged molecule often decreases and alters the surface charge (zeta potential) of the particle (Attama et al.,1999; Bikiaris, 2011). Generally, the magnitude of zeta potential gives an indication of the potential stability of a system. Large negative or large positive zeta potential is required for colloidal dispersion stability.

The general dividing line between stable and unstable formulation is generally taken as either +30 mV or -30 mV. Microspheres prepared with polymer blends (RS/RL) possessed positive zeta potential, with magnitude higher in batches containing more of Eudragit RS (Table 2). Thus, in comparison, the zeta potential obtained when either of the polymers is used alone showed no significant difference ($p < 0.05$) between the values of the particles. The presence of polysorbate 80 in the formulation further improved the surface properties, since polysorbate 80 has been shown to modify the surface properties of microspheres (Attama et al., 2011; Momoh et al., 2012). Surface-modified agents are potential delivery materials due to ability of the fact that, biological macromolecules such as proteins, peptides, and diagnostics could be tethered to the structures formed at the surface and their cellular trafficking improved.

Encapsulation efficiency (%)

The encapsulation efficiency of insulin were high (95.23 - 98.90%), an indication that the polymer blends and the method used in the formulation were able to allow insulin intake and prevent its expulsion during washing. However, electrostatic interactions may also take place during the preparation which led to a low release of the drug and high entrapment efficiency. It was concluded that the encapsulation efficiency may have resulted from the ionic activities of the Eudragit L100 and S100 and the insulin.

FT-IR spectral analyses

FT-IR of pure insulin, various polymers (RS100, RL100 and their blends) and other excipients used in the formulation are depicted Figure 3. No predominant drug interaction was detected between drug and polymers along with the excipients. FT-IR spectrum of insulin-loaded microspheres-based on Eudragit® RL100 (A2) (Figure 3b) showed that peaks of the polymer were observed at wave numbers 2931.90, 1728.28, 1459.20 and 1166.97 cm^{-1} corresponding to C-H stretching, C=O ester vibration, C-H deformation and C-O stretching, respectively. FT-IR of insulin-loaded microspheres based on Eudragit® RS100 (A1) (Figure 2b) showed strong peaks at 2929, 1731.17, 1460.16 and 1166.97 cm^{-1} corresponding to C-H stretching, C=O ester vibration, C-H deformation (CH_3) and C-O stretching respectively. FT-IR spectrum of insulin-loaded microspheres A3 (Figure 3c) (containing parts of Eudragit® RS100 and Eudragit® RL100) showed characteristic peaks at 2929.97, 1730.21, 1458.23 and 1165.04 cm^{-1} corresponding to C-H stretching, C=O ester vibration, C-H deformation (CH_3) and C-O stretching, respectively. FT-IR spectrum of insulin-loaded microsphere A4 (Figure 3d) (containing one part of Eudragit® RS100 and three parts of Eudragit® RL100) showed that principal peaks were observed at wave numbers 2934.79, 1724.42, 1457.27 and 1176.62 cm^{-1} corresponding to C-H deformation (CH_3) and C-H stretching respectively. FT-IR spectrum of insulin-loaded microspheres A5 (Figure 3e) (containing three parts of Eudragit® RS100 and one part of Eudragit RL100) showed characteristic peak of the polymers at 2930.93, 1731.17, 1461.13 and 1166.01 cm^{-1} due to C-H stretching, C=O ester vibration, C-H deformation (CH_3) and C-O stretching respectively. From the FT-IR of insulin-loaded microspheres it can be concluded that there was no interaction between the drug and polymers.

Differential scanning calorimetry (DSC)

The DSC results of the conventional insulin formulation used in this study showed a sharp melting peak of 125.3°, indicating a high level of purity. Also, the DSC results of the pure Eudragits RS100 and RL100, each showed double melting peaks at 60.2, 81.8 and 62.7, 78.2° respectively. The result of DSC of insulin loaded microspheres with individual polymers or their blends produced microspheres with minor changes in the melting enthalpies.

However, the observed peak in pure insulin was slightly altered in the insulin loaded microspheres' an indication that the insulin was completely solubilized in the carrier or was uniformly dispersed in the polymer. The minor shift observed in transition temperature and the enthalpies of the polymer blends (Batch A4 and A5) occurred in accordance with the thermotropic behavior of various polymers ratio.

Studies have shown that the quantity of quaternary ammonium salt present in the polymer has a direct relation with heat changes (Momoh et al., 2008). Thus, this minor increase in the enthalpy is signal of imperfect matrices generated by the polymer due to distortion of crystal arrangement creating more space for drug entrapment as observed in the result of encapsulation efficiency.

Release study

The release profiles of insulin from microspheres varied in accordance to their polymer composition as shown in Figure 5. There was no burst release from insulin-loaded microspheres formulations indicating a very good encapsulation of insulin inside the microspheres. This may have resulted from the method used in formulating the microspheres (Jameela et al., 1997; Kim et al., 2002). As shown in Figure 5, only a fraction of insulin (2-6 %) was released from the formulation in acidic pH of 1.2. The release rate in an acidic medium indicates an increased retardation of insulin release from the microspheres with increase in Eudragit RL100 content relative to Eudragit RS. However, the case is different at pH 7.2 where batch A4 formulation gave the maximum release of 93.26%, while batch A3 gave the least (68. 78%) and batches A5, A1 and A2 released 77.35, 69.55 and 78.12% of insulin respectively at 8 h. There was insignificant difference ($p>0.05$) in the release profiles of the various batches of the microspheres. The drug concentration and the polymeric carrier are some of the main factors affecting drug release (Tozaki et al., 1997). Expectedly, this manner of release is related to the pH responsiveness of Eudragit RL100 (Pignatello et al., 2000; Philip et al., 2010). Similar result was observed when Eudragit RS 100 was used in the formulation of a certain drug molecule (Horoz et al., 2004). In another study by Philip et al. (2010), the author presented a similar effect when a pH responsiveness material such as sodium alginate was used in oral delivery of insulin.

Pharmacodynamic study

The orally administered distilled water (DW), insulin solution (Ins sol) and the subcutaneously (sc) administered insulin solution, all served as controls. The percentage reduction of initial blood glucose level was used as an evidence of insulin absorption (Huang et al., 2003). The mean blood glucose baseline (initial glucose level) value was taken as the 100% level and all other blood glucose level/time data were calculated as a percentage of the baseline. In some of the animals the blood glucose levels were higher than the initial levels within the first 15 to 30 min of administration (Figure 6). This increase could be due to the stress associated with the administration of the microspheres (Huang et al., 2003; Attivi et al., 2005). Rats that received DW continued to have elevated blood glucose levels throughout the 8-h sampling period. This is because there was no insulin in the DW. So the rats remained hyperglycemic all through the period and some even died as a result. To compensate for the effect of drug transport in the GIT, a slightly higher dose (50 iu/kg) of the formulations in the oral evaluation as compare to the classical dose used for subcutaneous injection of insulin (40 iu/kg) in the treatment of diabetes was used. Due to lower absorption figures associated with the oral route, it was deemed necessary to administer a higher dose for the oral route than for the parenteral one.

As shown in Figure 6, the decrease in blood glucose level started 2 h after oral administration. This lag time could be due to the time required for microspheres to reach the site of the gastro-intestinal tract where microspheres or free insulin released from microspheres could be absorbed. The insulin-loaded microspheres prepared with the blends of the polymers produced blood glucose lowering effect higher than those produced by either Eudragit® RS100 or Eudragit® RL100 when used alone. The high blood glucose reduction resulting from insulin-loaded microparticles prepared with polymer blends indicated that there may be synergistic effect between the two polymers in insulin protection or absorption within the GIT. The microspheres prepared by 1:3 of Eudragit® RS100 and Eudragit® RL100 (batch A4) produced maximum blood glucose reduction within 6 to 8 h after oral administration that was equal to that of subcutaneously (sc) administered insulin. The release of insulin from microspheres is first based on the diffusion of the drug through the polymer matrix which takes some time to come into effect. The encapsulation of insulin into polymeric microspheres allowed insulin to be protected against degradation by proteolytic enzymes (that is, trypsin, chymotrypsin), as previously observed (Damgé et al., 1997; Builders et al., 2008a; Builders et al., 2008b) with poly (alkyl cyanoacrylate) nanospheres. The quaternary ammonium groups of Eudragit conferred a global positive zeta potential to microspheres which can interact with the negative charges of intestinal mucus, thereby improves the adhesion of microspheres on the wall of the intestinal barrier, allowing a closer intimacy of contact between drug and mucus membrane at the absorption sites and thus enhancing the permeability as well as reducing the local degradation of the drug. In such case, absorption will be easy and fast as the tight junction will be avoided due the intimacy of the formulation and the absorption site. Previous study has shown that in such intimacy the likely mechanism for drug like insulin to complete its absorption may be either or combination of (i) uptake via a paracellular pathway, (ii) transcytosis or receptor-mediated transcytosis and transport via the epithelial cells of the intestinal mucosa, and (iii) lymphatic uptake via the M-cells of the Peyer's patches mostly abundant in the ileum (Damgé et al., 2008). The importance of cationic microparticles made of Eudragit RS or polycationic of Eudragit RL on the wall of gastrointestinal tract has been evaluated (Jain and Majumdar et al., 2006; Gowthamarajan et al. 2003). Thus, the attachment of the microspheres on the surface of the wall of the GIT which may eventually be replaced by the incorporated drug may further enhance the ease with which this preparation could deliver the active pharmaceutical ingredient (API). Consequently, the free

drug could then be taken up into or transported through the cells thereby eliciting its biological effects.

Conclusion

Oral delivery is a physiological route for insulin administration. Improved disease management, enhancement of patient compliance and reduction of long-term complications of diabetes could be achieved by oral application. However, the challenges for developing oral insulin dosage forms are significant. In this study involving oral delivery of insulin, Eudragit polymers were used as carriers to protect the insulin in acidic conditions and to release it in alkaline medium. Therefore, materials that could change insulin behavior based on the pH of the physiological environment were carefully selected and varied in different ratios. In all cases, the insulin-microspheres formulation showed a prolonged hypoglycemic effect over an 8 h period comparable to intravenous injection of insulin. It can be concluded that insulin bioactivity was preserved by the formulation developed in this study from the enzymatic activities and the harsh acidic environment of the GIT and hence, holds a large promise for an oral delivery of insulin indication that it is an effective alternative for oral delivery of insulin.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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