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Formulation, *in vitro* and *in vivo* evaluation of oral sustained release insulin-loaded microspheres based on Eudragit® RS100

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ABSTRACT

The objective of the study is to formulate oral sustained release insulin-loaded microspheres and to evaluate the *in vitro* and *in vivo* properties. Microspheres were formulated by double emulsion solvent evaporation method using Eudragit® RS 100 as the polymer. The microspheres were characterized based on differential scanning calorimetry, fourier transform infrared spectroscopy, the yield, particle size, micromeritic properties, encapsulation efficiency (EE%), bio-adhesion, *in vitro* drug release and *in vivo* hypoglycemic properties. Insulin-loaded microspheres showed endothermic peaks of 60.9 to 63 °C, maximum EE of 98.89 % and highest percentage bio-adhesion of 95 % and good flowability. Microspheres had good sustained release of insulin with 73.46 at 8 h. Insulin-loaded microspheres had significant blood glucose reduction comparable to the parenteral insulin between 6 to 8 h ($P< 0.05$). In conclusion, insulin-loaded microspheres had good *in vivo* and sustained release properties and could be used orally for the management of diabetes mellitus.

KEYWORDS: *Oral insulin, microspheres, alloxan-induced diabetes, bio-adhesion, sustained release*

INTRODUCTION

Diabetes mellitus is an endocrine disease which is related to the disorders of carbohydrate metabolism brought about by deficiency in insulin secretion, insulin resistance or both [1-3]. About 366 million people have diabetes in 2011 and the number is expected to increase to 552 million by 2030. Diabetes caused 4.6 million deaths in 2011 and at least USD 465 billion dollars in healthcare expenditures in 2011 [4]. The primary mode of treatment of Type 1 diabetes is by exogenous insulin administration mainly by means of subcutaneous route. Most patients need to self-administer at least two injections of insulin daily or three to four injections for best control of blood glucose levels [3, 5], hence the need to develop sustained release oral formulation of this peptide drug in order to enhance patient compliance, improve the efficacy and bioavailability of this drug.

Insulin is a peptide hormone composed of 51 amino acid residues and has a molecular weight of 5808 Da [3, 6]. It is produced in the Islets of Langerhans in the pancreas. It consists of two chains, A (21 residues) and B (30 residues) and is the post translational product of a single chain precursor, namely pro-insulin, which possesses an extra linking peptide of varying lengths depending on the species [6]. The

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three disulphide bonds are essential in maintaining the folding and stability of the protein. Chronic administration of insulin by injection mode brings about various complications such as hypoglycemia, risk of infection, lipoatrophy or lipohypertrophy at the injection sites as well as risk of pump failure or insulin precipitation in pump leading to erroneous insulin delivery [7, 8]. In addition, the sensation of pain has inflicted the quality of life of diabetic patients undergoing the chronic treatment of insulin [3].

The delivery of insulin by non-parenteral routes has gained significant attention over last two decades [9]. The alternate routes explored are ocular [10, 11], nasal [12], buccal [13, 14], rectal [15], pulmonary [16, 17]; and oral [15, 17]. The oral route is recognized as the natural and the safest route for drug administration [18]. Generally, oral administration can improve disease management, enhance patient compliance and reduce of long-term complications of diabetes [18, 19]. However, the oral route continues to be a challenge to deliver proteins and various barriers must be overcome to obtain an adequate bioavailability. These barriers are the permeability across gastrointestinal tract (GIT), the enzymatic barriers and protein stability in GIT environment [19,20]. Much research has been conducted to find an ideal oral insulin delivery system that mimics the physiologic pattern of insulin secretion [21]. To ensure enteric protection, recent advances in pharmaceutical technology have led to a growth of innovative formulations such as liposomes [21, 22], microparticles [14,21], and nanoparticles [23,24].

The success of implementing alternative administration route for insulin delivery is impeded by two major obstacles which include low trans-mucosal absorption capacity and high susceptibility of enzymatic or acid degradation of insulin. In the course of trans-mucosal delivery, it is envisaged that the released peptide or protein will be degraded by peptidases and proteases on the way of dosage form transporting to the interface of absorption membrane and/or within the mucosa [3, 23]. Such degradative metabolism is not preventable by the use of enzyme inhibitors in formulation [3]. It can also be strongly reduced by mucoadhesive formulation via reducing early release of peptide or protein avoiding enzymatic digestion [3, 24]. Adhesion of dosage form onto a specific site of mucosa provides a high drug concentration gradient towards the absorption membrane. Prolonged residence of dosage form on mucosa, leads to an extended period of absorption and improved bioavailability. The acid degradation of insulin is commonly avoided through drug encapsulation in an enteric coat [25].

Microspheres are one of the multiparticulate delivery systems and are prepared to obtain prolonged or controlled drug delivery, to improve bioavailability or stability and to target drug to specific sites. Microspheres can also offer advantages like limiting fluctuation within therapeutic range, reducing side effects, decreasing dosing frequency, and improving patient compliance [26].

Mucoadhesives refers for materials that bind to the mucin layer of a biological membrane [28]. Mucoadhesive drug delivery system utilizes the property of bioadhesion of certain polymers which become adhesive on hydration and can be used to target a drug to a particular region of the body for extended periods of time [27, 28]. Mucoadhesive drug delivery systems have several advantages that arise from localization at a given target site, prolonged residence time at the site of drug absorption and an intensified contact with the mucosa increasing the drug concentration gradient [28]. Hence, uptake and consequently bioavailability of the drug is increased and frequency of dosing reduced, resulting to increase in patient compliance [28]. The aims of the study were to formulate oral sustained release insulin-loaded microspheres and to study the *in vitro* properties including the *in vitro* bio-adhesion potentials of the formulation and the *in vivo* hypoglycemic properties.

MATERIALS AND METHODS

Materials

Eudragit® RS100 (BASF, Germany), insulin (humulin® 70/30 Nordisk A/S, Denmark), polysorbate 60 (span 60), hydrochloric acid, potassium dihydrogen phosphate, magnesium striate, sodium chloride, acetone, n-hexane (BDH Chemicals Ltd., England), liquid paraffin (Moko Pharm, Limited, Nigeria), distilled water (freshly prepared in Biochemistry Lab., UNN, Nigeria). All other materials purchased were analytical grade and were used without further purification.

Preparation of insulin-loaded microparticles

Insulin microspheres were prepared by double emulsion solvent evaporation method. Eudragit® RS100 (ERS 1000) polymer was dissolved in 12.5 ml of acetone in a 250 ml beaker with stirring at room

temperature. Insulin, 0.5 ml (100 IU/ml) and magnesium stearate (0.1 g) were dispersed in the polymer solution. The resulting milky white dispersion was added drop wise into a beaker containing a mixture of liquid paraffin (50 ml) and span 60 (1% v/v) and homogenized using a paddle stirrer at 500 rpm for 1.5 h. The resulting microparticles were harvested by filtration using filter paper (Whatman No. 1) and washed three times with 50 ml of chilled n-hexane until free from oil. Microspheres were air-dried at room temperature for 24 h and then packed in tight cover bottle and stored at 4 ^{o} C in a refrigerator until further use. The process was repeated for the other batches using the materials shown in Table 1.

Composition of insulin-loaded microspheres

Batch Code	Insulin (100 IU/ml)	Eudragit® RS 100 (g)	Drug:polymer ratio	Magnesium stearate (g)	Liquid paraffin (ml)	Span 60 (ml)
S0	0.0	1	0:1	0.1	50.0	1.0
S1	0.5	2	1:4	0.1	50.0	1.0
S2	0.5	3	1:6	0.1	50.0	1.0
S3	0.5	4	1:8	0.1	50.0	1.0

Batches S1, S2 and S3 contain 2, 3 and 4 g of Eudragit® RS 100 (g), S0 is the bland microspheres.

Differential scanning calorimetric analysis

Melting transitions and crystallization behaviours of the microspheres were determined using a calorimeter (Netzsch DSC 204 F1, Germany). About 1 mg of each sample was placed in an aluminum pan, hermetically sealed and the thermal behaviour determined in the range of 30–250 ^{o}C at a heating rate of 10 k/min under a 20 ml/min nitrogen flux. Baselines were determined using an empty pan, and all the thermograms were baseline corrected.

Fourier Transform infrared (FTIR) characterization

Drug-polymer interactions were studied by FTIR spectroscopy. The spectra were recorded for drug-loaded microspheres using FTIR Shimadzu (Model No. 8400S). Samples were prepared in KBr disks (2 mg sample in 100 mg KBr). The scanning range was 500-4000 cm^{-1} and the resolution was 2 cm^{-1}.

Percentage yield of microparticles

The total amount of microparticles obtained was weighed and the percentage yield was calculated for each batch using the formula:

$$\text{Yield (\%)} = \frac{\text{Actual weight of product}}{\text{Total weight of excipient and drug}} \times 100 \quad (1)$$

Microspheres morphology

Microspheres morphology was analyzed using a Hund® binocular microscope (Weltzlar, Germany) attached with a Motic image analyzer (Moticam, China). Samples were dispersed in liquid paraffin, mounted on glass slide and placed on the microscope. The microspheres were observed at a magnification of x 40.

Photon correlation spectroscopy

The particle size distribution and polydispersity index of insulin loaded-microspheres were determined by dynamic light scattering, also known as photon correlation spectroscopy using Malvern Mastersizer 2000MS device (Malvern Instruments, Worcestershire, UK) and laser diffraction. Each sample was measured in triplicate.

Polydispersity index, a parameter calculated from the width of the particle size of the distribution using the equation = D (0.9)-D (0.1)/D (0.5). Where, D (0.9), D (0.5), and D (0.1) are corresponding to particle size immediately above 90%, 50%, and 10% of the sample. The measuring range of the Malvern Mastersizer is from 0.02 µm to 2000 µm.

Zeta potential measurement

The surface charge and electrostatic stabilization of insulin microspheres were measured by laser Doppler electrophoresis using Malvern Zetasizer Nano ZS (Malvern Instruments, UK). The instrument quantifies the electrophoretic mobility of the particles, which was converted into the ZP using the Helmholtz-Smoluchowski equation built into the Malvern Zetasizer software. The measured ZP of particles depends on the dispersion medium; therefore, the surface charge has been measured in deionized distilled water with a conductivity adjusted to 50 µS/cm with sodium chloride.

Determination of encapsulation efficiency

Beer's calibration curve was obtained for insulin in ethanolic buffer at a concentration range of 0.01 to 0.1 mg% at a predetermined wavelength of 276 nm. A 20 mg quantity of microsphere was dissolved in ethanolic buffer, properly diluted and filtered using a non-adsorbent filter paper (Whatman No 1). An aliquot of the filtrate was assayed using spectrophotometer (Jenway 6305 spectrophotometer, UK). The insulin content was determined with reference to the Beer's plot.

Encapsulation efficiency (EE%) was calculated using the following formula:

$$\textbf{Encapsulation Efficiency}\ (\%) = \frac{\textbf{actual loading}}{\textbf{theoretical drug loading}}\ \textbf{x 100} \qquad (2)$$

Micromeritics studies

Bulk and Tapped Densities

A 15 g quantity of the microspheres was placed in a 10 ml measuring cylinder and the volume occupied by the sample was noted as the bulk volume. The bulk density (ℓ_B) was calculated using the equation:

$$\textbf{Bulk density}\ (\boldsymbol{\ell_B}) = \frac{\textbf{Mass of powder (M)}}{\textbf{Bulk volume of powder}\ (\mathbf{V_B})} \qquad (4)$$

The tapped volume was determined by tapping the cylinder on a wooden flat surface from a height of one inch at 2 seconds interval until there was no significant change in volume reduction (Aulton, 2007; Chime *et al*., 2012a). The volume occupied by the sample was then recorded as the tapped volume. The tapped density (ℓ_T) was calculated using the formula:

$$\textbf{Tapped density}\ (\boldsymbol{\ell_T}) = \frac{\textbf{Mass of powder (M)}}{\textbf{Tapped volume of powder}\ (\mathbf{V_T})} \qquad (5)$$

Compressibility index and Hausner's quotient

Carr's compressibility index (%) of the granules was obtained using the formula:

$$\textbf{Carr's index}\ (\%) = \frac{\boldsymbol{\ell_T} - \boldsymbol{\ell_B}}{\boldsymbol{\ell_T}}\ \textbf{x 100} \qquad (6)$$

While Hausner's ratio was obtained using Equation 7:

$$\textbf{Hausner's ratio} = \frac{\boldsymbol{\ell_T}}{\boldsymbol{\ell_B}} \qquad (7)$$

Where ℓ_T and ℓ_B are tapped and bulk density respectively.

In vitro drug release analysis

The USP apparatus type 11 was employed in the study (Veego, India). The dissolution medium consisted of 900 ml of freshly prepared phosphate buffer (pH 7.4) maintained at 37 ± 0.05 °C. The polycarbonate dialysis membrane (MWCO 6000 - 8000, Spectrum Labs, Breda, The Netherlands) selected was pretreated by soaking in the dissolution medium for 24 h prior to use. A 50 mg quantity of the microparticles was introduced into the dialysis membrane containing 5 ml of phosphate buffer and placed in the appropriate chamber of the dissolution chamber. The paddle was rotated at 100 rpm and at intervals; 5 ml portion of dissolution medium was withdrawn using pipette and filtered using a filter paper (Whatman No 1). Sink condition was maintained by replacing the withdrawn volume with a fresh medium. The absorbance readings were obtained using UV spectrophotometer (Jenway 6305 spectrophotometer, UK) at predetermined wavelength of 276 nm. The dissolution test was repeated two times for each batch and the mean values determined at each time.

In vitro release kinetics

The dissolution data from the microspheres were analysed to determine the *in vitro* release kinetic mechanism using three kinetic models including the first order equation, Higuchi square root equation and Ritger-Peppas empirical model.

$$\ln Q_t = \ln Q_0 - K_1 t \qquad (4)$$

$$Q_t = K_2 t^{1/2} \qquad (5)$$

$$M_t/M_\infty = K_3 t^n \qquad (6)$$

where Q_t is the amount of drug released or dissolved at time t, Q_0 is amount of drug released or dissolved at time t = 0, k_1 is first-order release rate constant, k_2 is Higuchi rate constant, M_t/M_∞ is fraction of drug

released at time t, n is diffusion exponent and is indicator of the mechanism of transport of drug through the polymer, k_3 is Ritger-Peppas kinetic constant [29, 30].

Bio-adhesion studies

Bovine ileum was obtained from an abattoir in Nsukka, Nigeria and a 200 mg quantity of microparticles was weighed accurately and placed on a 8.5 cm long bovine ileum. They were allowed to interact and adhere to the surface of the ileum. A separating funnel was clamped to a retort stand and 50 ml of simulated intestinal fluid was poured into it and allowed to run over the bovine ileum treated with the insulin loaded microparticles. The microparticles that detached from the ileum were collected, dried and weighed. This was repeated for all the batches and percent bio-adhesion was calculated using the formula:

Bio-adhesion (%) =

$$\frac{Wo - Wi}{Wo} \times 100 \qquad (3)$$

Where Wo and Wi are mass of microparticles applied and mass of microparticles detached respectively.

Antidiabetic studies

Adult Wistar rats of both sexes weighting 260–280 g were procured from the Biochemistry Department, University of Nigeria, Nsukka and were maintained at standard housing conditions (room temperature, 25 °C) with 12 h light. The animals were left to acclimatize for two days, during which they were fed with a commercial diet (Feeds BC, Nsukka, Nigeria) and water. All animal experimental protocol was in accordance with the Animal Ethics Committee of the Faculty of Pharmaceutical Sciences, University of Nigeria Nsukka and in accordance with the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

Induction of diabetes

The rats were rendered diabetic prior to the study by intravenous injection of 150 mg/kg alloxan in isotonic saline solution. They were considered to be diabetic when the baseline glucose levels were in the range of 115-135 mmol/L. The rats were fasted overnight prior to the induction of diabetes mellitus. Many experimental models have been used for studying diabetes mellitus [31,32]. Chemical induction with alloxan (2,4,5,6-tetraoxypyrimidine; 2,4,5, 6-pyrimidinetetrone), an oxygenated pyrimidine derivative [31], appears to be the easiest, most reliable practical method of inducing diabetes mellitus in rodents [32]. Blood was collected for baseline glucose determination [33]. Fresh solution of alloxan monohydrate (Sigma, USA) was prepared just prior to injection. A stock solution of alloxan monohydrate was made by dissolving alloxan in normal saline (0.9 w/v% NaCl) at a concentration of 100 mg/kg [33]. A volume equivalent to 1 ml of the stock solution was given intra-peritoneally after which the blood glucose levels were measured at regular intervals (i.e. every six hours) four times daily for three days using a glucometer (ACCU-CHECK, Roche, USA). Food consumption was measured in (g), water (ml), and urine volume (ml) on a daily basis. Diabetes was confirmed 3 days post-alloxan administration [33]. They were considered to be diabetic when the baseline glucose levels were in the range of 115-135 mmol/L.

Drug administration

The diabetic rats were randomly divided into nine groups of five animals per group. All animals were fasted for 12 h before the experiment, but had free access to water throughout the whole experiment. The rats in group one received distilled water orally (1 ml/kg), the rats in group two were given the unloaded microsphere (bland microspheres, batch S_0), group three (also a negative control) were given insulin orally (0.54 mg/kg, equivalent to 15 IU/kg), while, the rats in group four received 0.036 mg/kg (equivalent to 1 IU/kg) insulin subcutaneously as a positive control. The rats in groups five received insulin-loaded microspheres reconstituted with 0.5 ml of distilled water (batches S_1, S_2, S_3 and S_4 respectively) equivalent to 0.54 mg/kg of insulin (15 IU/kg) orally using nasogastric tube. The blood glucose was determined using a glucometer (ACC-Chek, Switzeland) and blood glucose test strips at predetermined time intervals of 0, 0.5, 1, 2, 3, 5 and 8 h.

Statistical and data analysis

Data were analyzed using SPSS Version 16.0 (SPSS Inc. Chicago, IL.USA). All values were expressed as mean ± SD. Data were analysed by one-way ANOVA. Differences between means were assessed using student's t-test. $P<0.05$ was considered statistically significant.

RESULTS

Thermal properties of microspheres

The results of the DSC thermograms of the insulin, Eudragit® RS 100, and the microspheres are shown in Fig. 1. Insulin pure sample (Fig. 1a) had melting endothermic peak of 125.3 °C and enthalpy of -133mW/mg, the Eudragit® RS 100 (Fig. 1b) had a melting endothermic temperature of 62. 2 °C and enthalpy of -4. 84 mW/mg. Batch S1 (containing 2 g of Eudragit® RS 100) had melting endothermic peak of 60.9 °C and enthalpy of -2 .26 mW/mg (Fig. 1c). Batch S2 (containing 3 g of Eudragit® RS 100) had endothermic Peak of 63 °C and enthalpy of -4.01 mW/mg (Fig. 1d). The DSC thermograms of the insulin-loaded microspheres formulated with 4 g of Eudragit® RS 100 (batch S3) exhibited melting endothermic peak at 62.8 °C and enthalpy of -9.215 mW/mg (Fig. 1d).

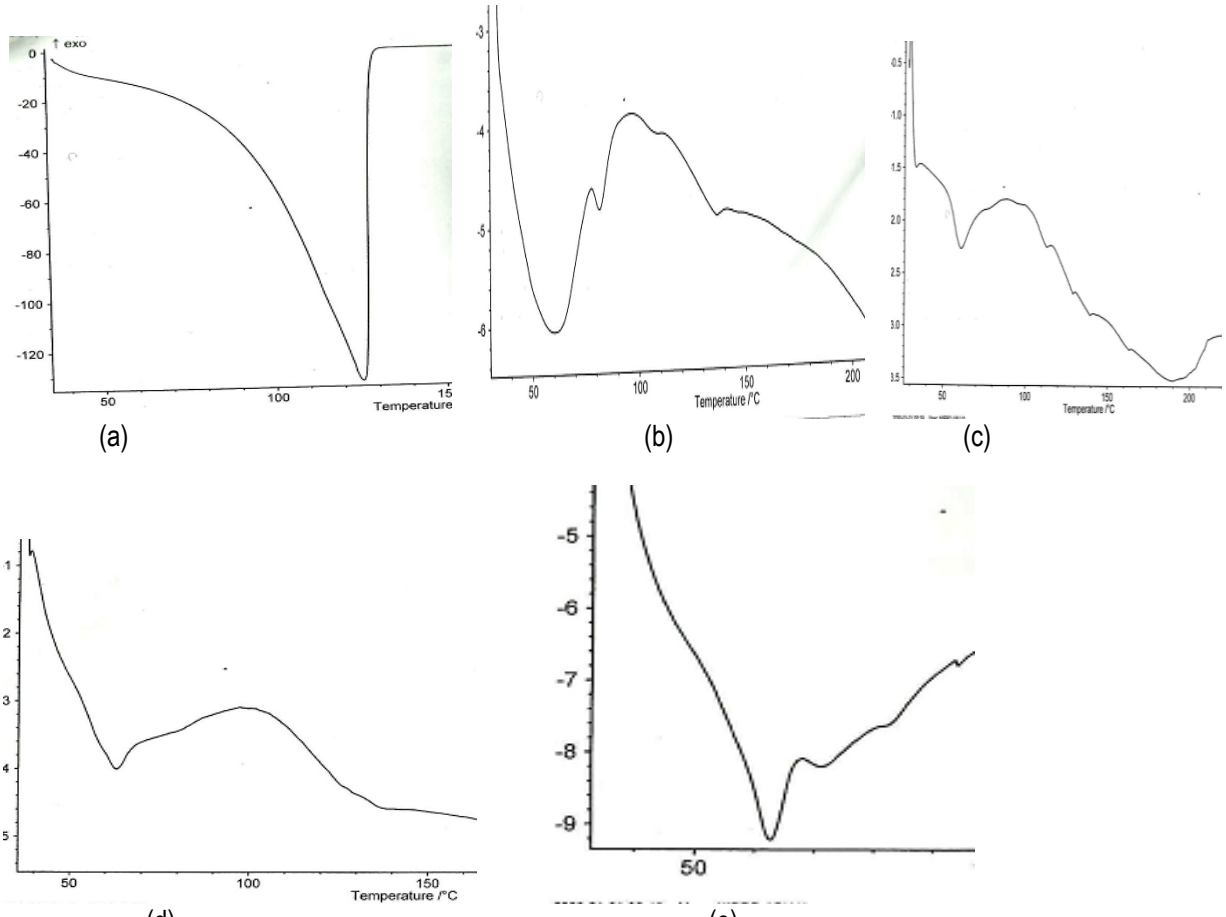


Fig. 1: Differential scanning calorimetry (DSC) thermograms of (a): Insulin, (b): Eudragit RS100, (C): S1, (d): S2, (C): S3. Batches S1, S2 and S3 contain 2, 3 and 4 g of Eudragit® RS 100 (g), S0 is the bland microspheres.

Fourier transform spectroscopy

The results of the Fourier transform infrared spectrums of insulin-loaded microspheres are shown Fig 2. FT-IR spectrum of insulin-loaded microspheres for batch S1,containing 2 g of Eudragit RS 100 (Fig . 2a) showed that principal peaks of the polymer were observed at wave numbers of 2929.00, 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, while the FT-IR spectrum of insulin loaded microspheres containing 4 g (batch S3) of Eudragit RS 100 (Fig. 2c) showed peaks at 2919.36, 1727.31, 1458.23 11773.73 cm^{-1}.

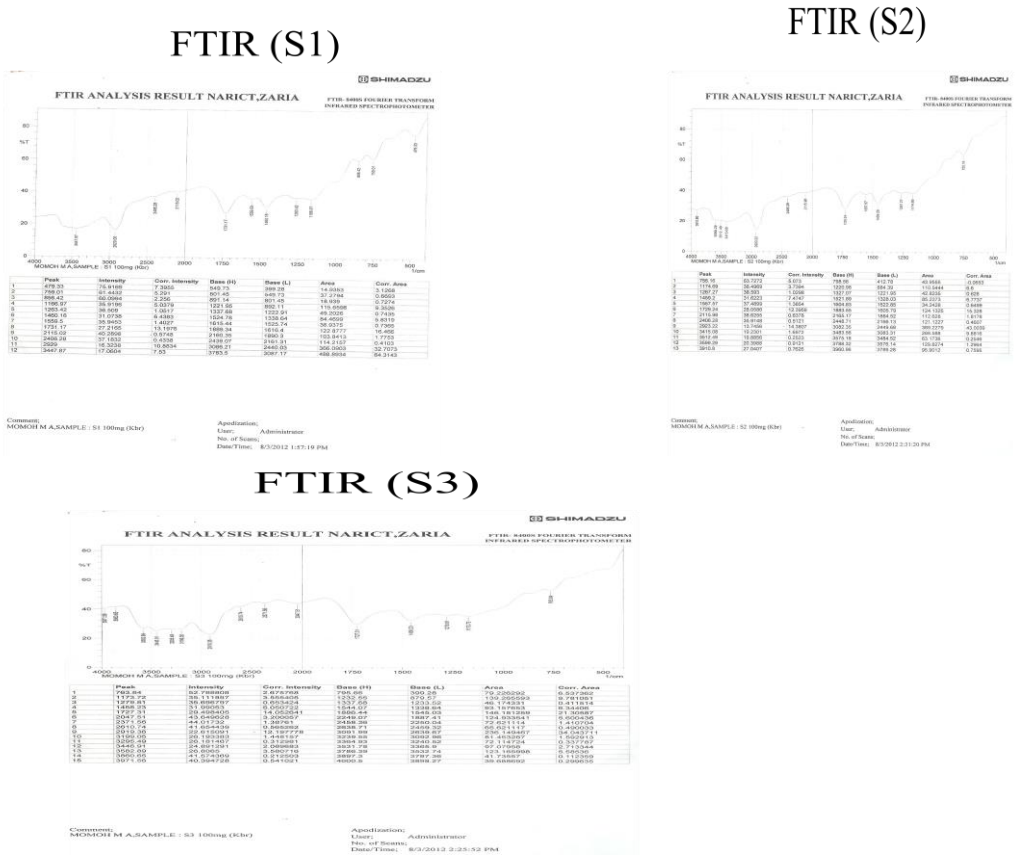


Fig. 2: Fourier transform infrared spectroscopy of insulin-loaded microspheres formulated with Eudragit RS 100.

3.3. Percentage yield of microspheres

The results of the percentage yield of insulin-loaded microspheres are shown in Table 2 and show that batch S2 containing 3 g of the polymer had the highest recovery of 73 .63 %, followed by batch S1 formulated with 2 g of polymer. However, batch S3 containing 4 g of the polymer had the least recovery of 21 %.

Table 2: Physicochemical properties of insulin-loaded microspheres

Formulation Code	Yield (%)	Particle size (µm)*†	Encapsulation efficiency (%)
S1	73.10	22.40 ± 0.11	95.86
S2	73.63	12.64 ± 0.07	96.40
S3	21.69	13.32 ± 0.23	98.89

***Mean ± standard deviation, †n = 100; Batches S1, S2 and S3 contain 2, 3 and 4 g of Eudragit® RS 100 (g), S0 is the bland microspheres.**

Particle size and morphology

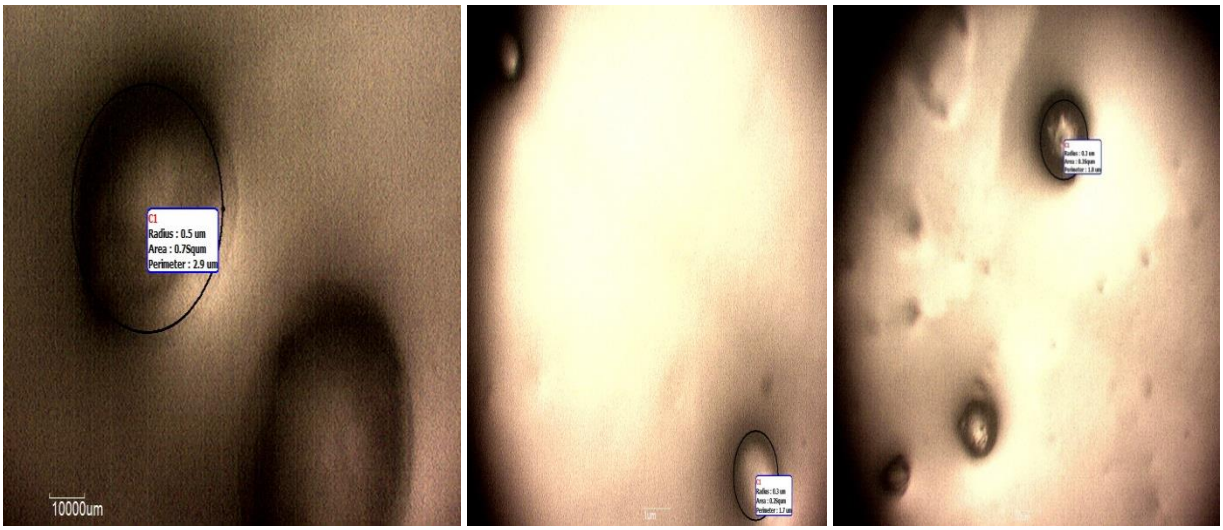
The results of particle size of insulin-loaded microspheres are shown in Table 2 and show that Particle size decreased with increase in polymer concentration. Batch S1 (2 g of ERS 100) had the highest particle size of 22.40 ± 0.11 µm, while S2 (3 g of ERS 100) and S3 (4 g of ERS 100) had 12.64 ± 0.07 and 13.32 ± 0.23 µm respectively. The results of the morphology of the microspheres shown in Fig. 3 revealed that the insulin microspheres were smooth and spherical in shape.

Encapsulation efficiency (EE%)

The results of EE% of insulin-loaded microspheres are shown in T able 2 and show that EE was directly proportional to the polymer concentration. Maximum EE of 98.89 % was obtained for insulin-loaded microspheres formulated with 4 g of ERS 100. Generally, the microspheres had high EE of insulin as shown in Table 2.

Micromeritic properties

The results of the micromeritic properties of insulin-loaded microspheres are shown in Table 3. The results of the bulk and tapped densities, Hausner's quotient and Carr's compressibility indices of the insulin-loaded microspheres fell within the acceptable range for good granule flow. Hausner's ratio ranged from 1.14 to 1.17, while Carr's index ranged from 12.3 to 14.8 % as shown in Table 3.



(S1) (S2) (S3)

Fig. 3: Photomicrographs of insulin-loaded microspheres formulated with 2, 3 and 4 g of Eudragit® RS 100 (S1, S2 and S3) respectively; Batches S1, S2 and S3 contain 2, 3 and 4 g of Eudragit® RS 100 (g), S0 is the bland microsphere

Table 3: Micromeritic properties of the insulin-loaded microspheres

Formulation Code	Bulk density (g/cm³)*†	Tapped density (g/cm³)*†	Hausner's quotient	Carr's index (%)
S1	0.5248 ± 0.009	0.5984 ± 0.0165	1.14	12.30
S2	0.5443 ± 0.0045	0.06275 ± 0.0040	1.15	13.25
S3	0.5450 ± 0.0049	0.6396 ± 0.0126	1.17	14.79

***Mean ± standard deviation, †n = 3; Batches S1, S2 and S3 contain 2, 3 and 4 g of Eudragit® RS 100 (g), S0 is the bland microspheres.**

Bio-adhesive properties

The results of the *in vitro* bio-adhesive properties of insulin-loaded microspheres shown in Fig. 4 show that batch S1 formulated with 2 g of ERS 100 exhibited the highest percentage bio-adhesion of 95 % significantly higher than those of batches S2 (70 %) and S3 (75 %) respectively ($P< 0.05$).

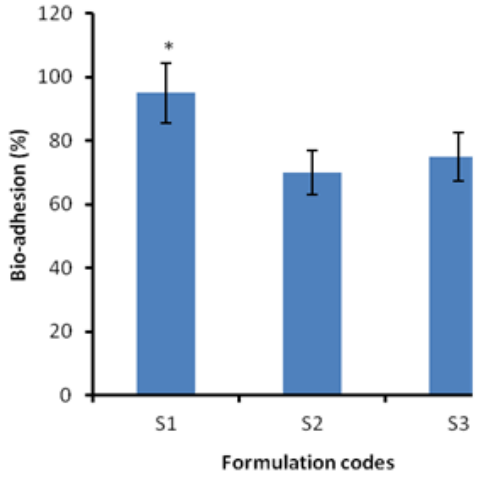


Fig. 4: *In vitro* bio-adhesion properties of insulin-loaded microspheres formulated with 2, 3 and 4 g of Eudragit® RS 100 (S1, S2 and S3) respectively; * significantly different at *P*< 0.05 compared to other formulations.

In vitro drug release

The results of the *in vitro* release of the insulin from the microspheres are shown in Fig. 5 and show that the release was gradual with no form of burst effect. About 27.6, 24.8 and 18.3 % insulin were released at 30 min ($T_{0.5\ h}$) from S1, S2 and S3 microspheres respectively formulated with 2, 3 and 4 g of Eudragit® RS 100. Also, 68.9, 59.5 and 48.5 % of insulin were released at 3 h from S1, S2 and S3 microspheres respectively formulated with 2, 3 and 4 g of Eudragit® RS 100, while at 8 h, 73.5, 64.2 and 58.0 % of insulin were released from S1, S2 and S3 microspheres respectively formulated with 2, 3 and 4 g of Eudragit® RS 100.

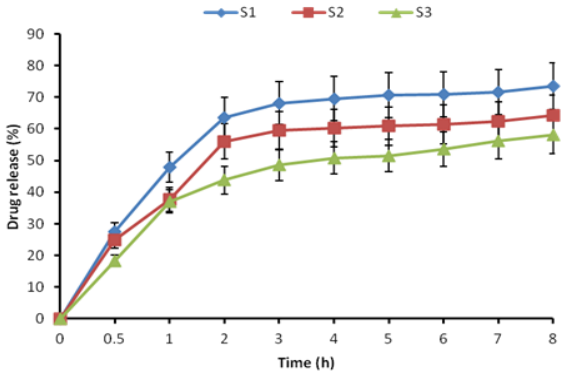


Fig. 5: *In vitro* release of insulin from microspheres formulated with 2, 3 and 4 g of Eudragit® RS 100 (S1, S2 and S3) respectively.

In vitro release kinetics

The results of *in vitro* release kinetics of insulin-loaded microspheres are shown in Table 3. The results show that all the Higuchi plots of amount of drug released versus time were linear (r^2 = 0.9), showing that drug release followed diffusion controlled process. Also the integral form of Higuchi gave slope (n) that ranged from 0.078 to 0.162. The first order plot of amount remaining versus time showed high level of linearity, indicating that drug release followed mixed order kinetic release. Ritger-Peppas models showed good linearity and n values in the range of 0.042 to 0.158.

In vivo antidiabetic properties

The results of *in vivo* antidiabetic studies are shown in Fig. 6 and show that insulin-loaded microspheres for oral administration had good hypoglycemic properties comparable to subcutaneous insulin injection. About 100, 133, 97 and 75 % glucose reduction were obtained for insulin microspheres prepared with 2 g of Eudragit® RS 100 (S1) at 0.5, 3, 5 and 8 h respectively. Also, 143, 101, 89 and 69 % glucose reduction were obtained for insulin microspheres prepared with 3 g of Eudragit® RS 100 (S2) at 0.5, 3, 5 and 8 h respectively. About 132, 122, 99 and 78 % glucose reduction were obtained for insulin microspheres prepared with 4 g of Eudragit® RS 100 (S3) at 0.5, 3, 5 and 8 h respectively, while 115, 88, 79 and 63 % glucose reduction were obtained for the reference, insulin subcutaneous injection at 0.5, 3, 5 and 8 h respectively. The results of the negative controls (groups that received S0, distilled water and

plain oral insulin) used in the study showed that instead of a decrease in the blood glucose of the Wistar rats, there was significant ($p < 0.05$) increase in the blood glucose levels over the study period as shown in Fig. 6.

Table 4: Results of *in vitro* release kinetics of insulin-loaded microspheres

Batch	Higuchi (r^2)	Higuchi (n)	First order (r^2)	Ritger-Peppas (n)	Ritger-Peppas (r^2)
S1	0.906	0.087	0.906	0.051	0.951
S2	0.927	0.078	0.927	0.042	0.974
S3	0.978	0.162	0.978	0.158	0.922

Batches S1, S2 and S3 contain 2, 3 and 4 g of Eudragit® RS 100 (g), S0 is the bland microspheres.

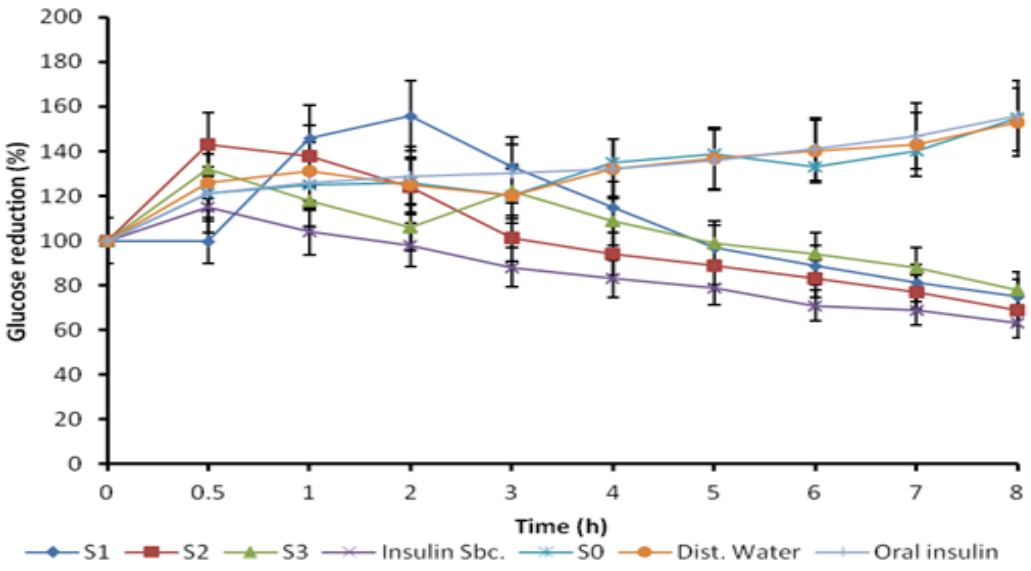


Fig. 6: Antidiabetic properties of insulin-loaded microspheres formulated with Eudragit® RS 100. Batches S0 contain no insulin (negative control), batches S1, S2, S2 are insulin-loaded microspheres formulated with 2, 3, and 4 g of Eudragit® RS 100. **P< 0.05 was considered significant compared to the reference, *P< 0.05 was considered significant compared to the controls.

DISCUSSIONS

The DSC measurements were carried out in order to determine the thermotropic behaviours of insulin, Eudragit® RS 100 and the insulin-loaded microspheres and also to study the solid state properties of insulin in the microspheres. The results of the DSC thermograms of insulin (Fig. 1a) showed a sharp endothermic peak. This sharp endothermic peak indicated the purity of this drug. The endotherms of the polymer, Eudragit® RS 100 had a melting endothermic temperature of 62. 2 ^{o}C (Fig. 1b). The other peaks seen showed that the polymer melted with decomposition. The thermograms of the formulations Fig. 1 (c-e) showed sharp endotherm between 60.9 and 63 ^{o}C. This shows that there was a shift in melting transition of insulin as a result of inclusion of Eudragit® RS 100. Ordinarily, one would expect higher transition temperature due to the melting transition of the insulin used in the study, but the insulin was molecularly dispersed in the polymer with lower melting point, giving sharp endotherms [34]. The lather peak of insulin in the formulation however, agrees with the melting transition of insulin reported by some researchers [19,35]. Lower enthalpy suggests less crystallinity and the possibility of retention of an entrapped drug over time [36, 37]

FT-IR spectrum of insulin-loaded microspheres generally showed C-H stretching (292 9.00 cm^{-1}), C=O ester vibration (1731.17 cm^{-1}), C-H deformation (CH_3) (1460.16 cm^{-1}) and C=O stretching (1166.97 cm^{-1}). FT-IR technique was used to study the physical and /or chemical interaction between the drug and the polymer. FT-IR is a quick and relatively cheap technique for identifying compounds and for detecting excipients interaction. The results (Fig. 2) revealed that there were no changes in major peaks of Eudragit RS 100 as a result of insulin incorporation. The results confirmed that there was no strong interaction between the drug (insulin) and the polymer (Eudragit RS100). Also, the process of microspheres formulation did not affect the integrity of the polymer as shown in the results of the IR spectra.

The results of percentage recovery of the insulin microspheres showed that batches S1 and S2 formulated with 2 and 3 g of the polymer generally had percentage recoveries significantly higher than that of batch S3 formulated with 4 g of polymer ($p < 0.05$). The low yield in some cases could be attributed to the losses occurring during various steps of processing, such as sticking of the polymeric solution to the glass container, loss of microspheres during the washing step, and so forth. Loss because of sticking could be minimized by using an apparatus made of plastic or polyethylene [9]. However, in all the formulations, high percentage of the microspheres recovered from the formulations are strong indications that the formulation method adopted was reliable and could of commercial interest.

The results of particle size and morphology of insulin-loaded microspheres shows that the microspheres were spherical in shape and the size decreased as the concentration of the insulin increased. Particle size of particles is important because they determine the site of administration of the drug and also affect the bioavailability [37,38].

The encapsulation efficiencies of the insulin-loaded microspheres were generally high. Unlike the particle size, EE% increased with increase in polymer concentration. Batch S3 formulated with 4 g of the polymer exhibited the highest EE% of 98.89. Generally, the EE of microspheres could be a function of formulation excipients and the quantities and method of formulation amongst others.

The results of the micromeritic properties of insulin-loaded microspheres show that the results obtained were within the acceptable limit for good powder flow. Bulk and tapped densities determination were necessary in order to access the flowability of the microspheres by indirect method of flow determination. Hausner's ratio and Carr's compressibility index measures the degree of interparticulate fiction and values of Hausner's ratio less than or equal to 1.25 indicates good flow, while values greater than 1.25 indicates poor flow [39]. The results showed that the microspheres had Hausner's ratios that were within the specified limits for good flow. Also, Carr's index of 5 to 16 indicates good flow, while 18 to 23 shows fair flow [39, 40] (Aulton, 2007; Chime *et al*., 2012 a). Therefore, the microspheres exhibited good flowability and consolidation properties.

Besides particle size and surface charge, mucosal adhesion is the next most important characteristic of microspheres drug delivery systems. The rapid clearance of protein drugs from the site of absorption is one of the barriers to insulin absorption [11]. For this reason, prolonging the duration of insulin retention on the mucosa may result in a higher

bioavailability. Eudragit RS100 is a copolymer of acrylic and methacrylic acids containing 0.5–0.8% of a quaternary ammonium compound with a positive charge surface. Its mucoadhesive properties are thought to be mediated by electrostatic interactions between the positively charged D-glucosamine residue and the negatively charged sialic acid residue of mucin. In this study, eudragit microsphere loaded-insulin showed attractive mucosal adhesion (Fig. 4). In this regard therefore, the prolonged duration of in vitro result (Fig. 5) hereby agrees with and confirms the results of mucosal adhesion as well as the biological activity of the entrapped insulin as a function of the blood glucose lowering effect (Fig. 6) re-affirmed the importance of bioadhesive property of the carrier. In all case, the bio-adhesive properties of the insulin-loaded microspheres showed that all the prepared microspheres had good bio-adhesive properties. However, the bio-adhesiveness of the microspheres was affected by the concentration of Eudragit® RS 100 incorporated into the formulations. It was observed that batch S1, formulated with 2 g of Eudragit® RS 100 significantly exhibited higher bio-adhesion than the batches S2 and S3 formulated with 3 and 4 g of polymer respectively ($p < 0.05$). This observation could be attributed to saturation level of the carrier such that further increase in the carrier would have no effect on the bioadhesive activities of the microspheres.

The results of the *in vitro* release of insulin from the microspheres showed that the microspheres exhibited good release of the insulin with no burst effect. The results revealed that the microspheres had good sustained release properties. The formulations being bio-adhesive microspheres, hence the prolonged nature of the release obtained. Contrary to the result of bioadhesive study, the drug release was depending largely on the polymer concentration. In other words, the release of the insulin was delayed more in the batch with highest concentration of polymer (S3 < S2 < S1). This indicates an increased retardation of insulin release from the microspheres with increase in polymer content in the formulation. More so, it can be seen in Fig. 5 that insulin release in vitro underwent a gradual release with no initial burst, suggesting that the bulk of the insulin is encapsulated inside the microspheres. In this way, thus the high encapsulation efficiency observed in all the formulation. Similar behavior has been reported for verapamil microparticles prepared with Eudragit RS100 [41-43].

The *in vitro* release kinetics studied using three kinetic models including the first order, Higuchi and Ritger-Peppas models. The results revealed that the Higuchi plot of amount of drug release against time was linear in all the formulations showing that drug release followed diffusion controlled process. Also the integral form of Higuchi gave n value below 0.5, indicating that diffusion was not the only predominant mechanism of drug release [39]. The first order plots seconds the integral form of Higuchi in the linearity of their plots indicating that drug release also followed first order kinetics. Therefore, the release of insulin from the microspheres followed mixed order of release. The Ritger-Peppas models gave n values significantly lower than 0.45, indicating that drug release followed Fickian diffusion release mechanisms (swellable spherical matrix) [30].

The results of the antidiabetic properties of insulin-loaded microspheres shown in Fig. 6 show that the formulations had good hypoglycemic properties comparable to subcutaneous insulin injection [24]. The percentage reduction of initial glucose level was used as an evidence of the amount of the insulin released and absorbed into the systemic circulation. The mean blood glucose baseline (initial glucose level) value was taken as the 100 % level and others were based on the initial basal blood glucose level. The rats that received normal saline, plain oral insulin and the unloaded microspheres (negative controls) continued to have elevated blood glucose levels throughout the period of study. The plain oral insulin (unformulated) was degraded by the proteolytic enzyme in the gastrointestinal tract (GIT). Insulin-loaded microspheres for oral administration had significant blood glucose reduction comparable to the reference insulin between 6 to 8 h ($P<0.05$), indicating that the microspheres protected the insulin against proteolytic degradation in the GI tract in agreement with earlier reports [9]. The insulin-loaded microspheres and the reference drugs however varied significantly from the controls ($P< 0.05$). The positive control group (reference), insulin subcutaneous injection showed lower percentage reduction in the initial blood glucose level than the formulated microspheres up to 8 h (t_{max}), indicating superior antidiabetic proprerties over the microspheres, consistent with previous report [9]. This suggests that the release of the insulin from the microspheres

stimulated the production of insulin from islet cells of Langerhans in a much more controlled manner than the conventional insulin injection. This also may be due to the controlled release properties of the formulations. Also, the bio-adhesive nature of these formulations reduced early release of peptide or protein avoiding enzymatic digestion [24]. The results also showed that the drug was not denatured by the production process adopted in the study.

CONCLUSIONS

Oral insulin microspheres formulated with Eudragit® RS 100 were formulated and the *in vitro* properties showed that the microspheres exhibited good bio-adhesion properties, high yield of microspheres and encapsulation efficiency. The *in vivo* properties showed that the formulations exhibited good hypoglycemic properties comparable to insulin subcutaneous injections but have some advantages over the parenteral insulin which include ease of administration, patient compliance, possibility of controlled release reducing the frequency of administration and mimicking insulin release from the Islet of the Langerhans, better management of diabetes and improvement in the bioavailability of insulin among others and they prevented degradation of insulin by the GI proteolytic enzymes. Further research into this field is encouraged in order to scale up these formulations and make the product available in the market.

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