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Research Article

ORAL DELIVERY OF INSULIN USING COMPOSITE POLYMERIC MICROPARTICLES

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ABSTRACT

Composite polymeric human insulin loaded microparticles were prepared using triple emulsion solvent evaporation technique with 1% PVA as a stabilizer and 0.1% w/v hydrophilic chitosan in the external aqueous phase, PLGA in internal organic phase and 4-6% w/v Eudragit in external organic phase. The mean diameter of the microparticles was in the range of 1-6 µm. Higher amount of Eudragit polymer provided the maximum drug encapsulation. IVR profile showed that Eudragit L 100 particles have more initial burst release (19.75%) in1 hr. in and % cumulative release (85.15%) in PBS pH 6.8 than Eudragit S 100 particles showing initial burst release (10.31%) in1 hr.and % cumulative release (74.56%). Eudragit L 100: Eudragit S 100 (1:1) particles at pH 6.8 throughout 12 hr. showed more initial burst release (18.45%) in1 hr.and % cumulative release (84.14%) than Eudragit L 100 : Eudragit S 100 (1:1) particles at pH 6.8 in first 1 hr. and remaining 11 hr. at pH 7.4. Eudragit L 100 : Eudragit S 100 (1:1) particles at pH 7.4 throughout 12 hr. showed less initial burst release (10.66%) in 1 hr. and % cumulative release (78.94%). IR spectra suggest the entrapment of insulin in composite polymeric microparticles. CD spectra suggesting maintenance of secondary structure of insulin. SDS-PAGE confirmed that the structural integrity of the drug was maintained during encapsulation. Stability studies results showed that insulin loaded particles stored at 4ºC were more stable than particles stored at 25 ºC and at -20 ºC. In-vitro caco-2 cell line studies show that % viability was more for particles than pure insulin solution. TEER values of caco-2 cell monolayer was found to be more for particles at pH 6.8 than particles at pH 7.4.

Keywords: Insulin, oral, microparticles, Eudragit L 100, Eudragit S 100.

INTRODUCTION

Proteins and peptides play a vital role in all biological processes and have received a growing attention in recent years as drug candidates. The rapid advances in protein and peptidepharmacologyalong with the large-scale production of these compounds by recombinant DNA technology-among other techniques- have fuelled enormous interest in these compounds[2].

Proteins and peptides consist of complex structures. Human insulin is a 51-amino acid polypeptide. It has 2 chains-A and B. A-chain consists of 21 amino acids while B-chain consists of 30 amino acids.[(35)] Both chains are linked by disulphide bonds. The key to the success of proteins as pharmaceuticals is to have in place an efficient drug delivery system that allows the protein drugs to gain access to their target sites at right time and for the proper duration. Four factors that must be considered to fulfil this goal are route of administration, pattern of drug release, method of delivery and fabrication of formulation[1,2].

Insulin can be given by S.C. route but frequent injections (twice daily) are expensive may produce local hypertrophy[24]. It can be given alternatively by nasal[5] oral[11,12], ophthalmic[3,4] buccal,[6,7] pulmonary[9,10] rectal[8] etc. Among these, oral route is the most convenient and provide patient compliance. Indeed, insulin absorbed by the intestinal epithelium reaches the liver through the portal vein and can directly inhibit hepatic glucose output[25]. Oral delivery of insulin can mimic the physiological fate of insulin and may provide better glucose homeostatis. This also will lessen the incidences of peripheral hyperinsulinaemia which is linked to neuropathy, retinoendopathy and so forth.[30] Moreover, it is more natural, less invasive and less expensive[37]. However, oral route has two major challenges

1. Digestion of insulin by proteolytic enzymes like pepsin and chymotrypsin.
2. Lack of lipophilicity leads to poor passage through intestinal membrane to the circulation[20].

To prevent these problems, many protease inhibitors and surfactants were used in insulin formulations. However, protease inhibitors also prevent digestion of important nutrients present in the food[16, (17)]Similarly, surfactants irritate the protective mucous membrane leads to passage of unwanted toxins and pathogens.[16, 17] So, considering these all facts, protease inhibitors and surfactants are not used in the current formulation.

Several researchers have attempted to use polymeric carriers as oral delivery systems for insulin like poly (alkyl cyanoacrylate)[13,34] and poly (lactide-co-glycolide) (PLGA)[12] nanospheres, poly (vinyl alcohol)-gel spheres with protease inhibitor,[14] bioadhesives like hydroxycellulose with permeation enhancers like salicylate[15]. Poly (alkyl cyanoacrylate) nanospheres without the assistance of surfactants (like poloxamer 188 and deoxycholic acid) cannot protect insulin against proteolytic enzymes[13] and PLGA being a non-enteric polymer would have a pH-independent release and released insulin would be degraded by proteolytic enzymes[12]. Poly (vinyl alcohol)-gel microspheres also have a similar drawback[14].

Insulin is better absorbed from the ileum and large intestine as compared to the jejunum[14].Thus a polymer that would release the drug in the ileum or upper intestine has the potential for oral insulin delivery. Eudragit L 100 and Eudragit S 100 are such polymers. These are anionic polymers synthesized from methacrylic acid and methyl methacrylate and have a pH-dependent solubility. Eudragit L 100 would release the drug in the region of gastro-intestine (GIT) of pH (6-6.5) i.e. ileum or upper intestine[16, 35]. Eudragit S 100 would release the drug in the region of gastro- intestine (GIT) of pH > 7 i.e. large intestine or colon[17].

Polymer therapeutics have confirmed that they can satisfy the stringent requirements of both industrial development and regulatory authority approval process[29]. However, only a small fraction of polymeric particles is absorbed by the intestine, because the residence time of these particles is relatively low (4-6 hr.) due to the intestinal fluid flow and peristalsis action of the small intestine[32]. Insulin instability has been regarded as a major obstacle to the development of an insulin oral dosage device aimed at attaining optimal diabetic control. A promising strategy is the use of multifunctional polymers exhibiting gastrointestinal permeation enhancing and mucoadhesive properties. Interest in using natural materials as part of drug delivery protocols has increased in the past two decades[21]. Chitosan is such a polymer. It is partially or fully deacylated derivatives of chitin. The polymer is linear consisting of β (1-> 4) linked D-glucosamine residues with a variable number of randomly located N-acetyl glucosamine groups[33]. It is a non-toxic, biocompatible, safe and efficient intestinal permeation enhancer for absorption of protein drugs and mucoadhesive[18]. PLGA is a polymer which is used for the controlled oral delivery of the drug. It is an anionic, biodegradable, biocompatible and hydrophobic polymer used in oral delivery of proteins [(19),(35),(38)] Many novel polymers including biodegradable polymer backbones, dendritic architectures, block copolymer micelles and polymers containing pendent cyclodextrin are used to prepare second-generation polymer therapeutics[28]. A successful PLGA nanoparticulate system should have a high drug loading capacity as it allows a small quantity of the carrier during a single administration. Eudragit is used for colon targeting[17]. This kind of microparticles not only gives protein a proper microenvironment by hydrophilic protection but also makes the initial release be retarded[26,27]. PLGA is not water soluble and encapsulation of water soluble peptides into PLGA particles requires either double emulsion (w/o/w) solvent evaporation technique or a solvent diffusion/ nanoprecipitation method. [(22)]The preparation of particles from polymers is based on emulsion-diffusion[39]. Triple emulsion solvent evaporation method is used for this purpose.

MATERIALS

PLGA (50:50) was obtained from Birmingham Polymer Inc. USA.Methacrylic acid copolymers

Eudragit L 100 and Eudragit S 100 were supplied as a gift by Rohm Pharma (Weiterstadt, Germany). Hydrophilic chitosan was obtained from Sigma-aldrich Inc., Germany. Recombinant human insulin powder was purchased from Sigma-Aldrich Co., India. Poly vinyl alcohol (PVA, cold, mol.wt. 30-70,000 Da) was obtained from Sigma chemicals Co., USA. Dichloromethane (DCM), Dimethyl sulfoxide (DMSO), Isopropanol (IPA), Ethanol and Disodium hydrogen phosphate were obtained from E.Merck India Ltd.Trypsin (YaxinBio, Japan). Fetal bovine serum (FBS) was obtained from PAA Labs, Germany.MTT (Cayman Chemicals, U.S.).MQ was used throughout the study.

METHODS

Internal aqueous phase (IAP) (100µl.) consists of 20 mg/ml. of recombinant human insulin solution, 2.5 mg BSA, 10 mg sucrose and 2 mg of disodium hydrogen phosphate. Internal organic phase (IOP) (1 ml.) consists of 25 mg of PLGA (50: 50) in 1 ml. of DCM. External aqueous phase (EAP) (3 ml.) consists of 0.1% w/v hydrophilic chitosan with 1% w/v PVA solution and 10% w/v sucrose (cryoprotectant)[23]. External organic phase (EOP) (12 ml.) consists of 3-6% w/v Eudragit solution [L100, S100 and L100:S100 (1:1)] dissolved in ethanol and isopropanol mixture (1:4) because more ratio of ethanol taken in ethanol and isopropanol mixture (2: 1) had resulted in about 20% release of the drug in 0.1 NHCl as performed in the laboratory. Triple emulsion (w/o/w/o) solvent evaporation technique was used. Primary emulsion (IAP/IOP) was prepared by probe sonicator for 1 min. Then secondary emulsion (IAP/IOP/EAP) was prepared by homogenizer at 8,000 rpm for 3 min. Then triple emulsion (IAP/IOP/EAP/EOP) was prepared on magnetic stirring by adding eudragit solution drop by drop by pipette to secondary emulsion. Then organic solvents were removed by overnight magnetic stirring at room temperature.

Abbreviations

IAP**,** Internal aqueous phase; IOP, Internal organic phase; EAP, External aqueous phase; EOP, External organic phase; DCM, Dichloromethane; PVA, Polyvinyl alcohol; PLGA, Poly (lactide- co-glycolide); IPA, Isopropanol; S.C., Subcutaneous; BSA, Bovine serum albumin.

CHARACTERIZATION

Particle size

The freeze-dried microparticles (2mg) were dispersed in 1 ml.MQ and vortexed for 2 min. to bring about disaggregation of the microparticles. Size analysis of microparticles was carried out by laser light scattering using Mastersizer 2000S.

Zeta potential

Surface charge (zeta potential) was measured using Nano Z, Malvern instruments. To determine the zeta potential, suspension of the polymeric particles was diluted with water to ensure that the signal intensity is suitable for the measurement. The suspension is then placed in the electrophoretic cell for measurement.

Microscopic Morphology and Surface Characteristics

Optical Microscopy

20µl. of a suspension of microparticles in MQ was placed on a glass slide covered with a cover slip without an air bubble. Particles were observed at 40 times magnification under inverted microscope, Nikon Eclipse Ti100 and images were recorded by software 'Nis Elements'.

Scanning Electron Microscopy

10-20 µL. of a suspension of microparticles was spread uniformly as a thin film on a round cover-slip mounted on an aluminium stub using a double-sided carbon adhesive tape and lyophilized for 1-2 hours for the complete removal of water. Silver conductive paint was applied between the cover slip and the stub. Stubs were sputter-coated withconductiveGold-Palladium. Samples were viewed under Scanning Electron Microscope (SEM).

Encapsulation Efficiency

Known quantities of microparticles (10 to 15mg) were taken in an eppendorff tube, 1ml of Isopropanol (IPA) was added, vortexed well and centrifuged (13,000rpm, 5 minutes at 4°C). The supernatant was discarded; pellet was resuspended in Isopropanol (IPA) and centrifuged again (13,000rpm, 5 minutes at 4°C). The process was repeated thrice; the pellet finally obtained was dried in the desiccator at room temperature and then resuspended in 1ml. MQ. Insulin content was estimated by micro BCA assay method.

ENTERIC NATURE OF MICROPARTICLES

This test was performed to determine whether the drug would be released in the acidic environment of the stomach (i.e. the pH between 1 and 3). Twenty milligrams of microparticles were soaked in 1 ml. of 0.1N HCl in an eppendorff tube that was put on the incubator shaker at $37^0C \pm 0.5^0C$. After 2 hr., the sample was centrifuged (13,000 rpm, 10 min.,

4^0C) and the insulin content of the supernatant was measured using micro BCA protein assay method.

IN-VITRO DRUG RELEASE

In-vitro release of insulin from microparticles was evaluated in 1X phosphate buffer saline (PBS, pH 6.8) for Eudragit L 100 particles, in 1X phosphate buffer saline (PBS, pH 7.4) for Eudragit S 100 particles and Eudragit L 100: Eudragit S 100 (1:1) particles were evaluated in PBS, pH 6.8 for first one hour which were transferred to PBS, pH 7.4 for remaining 11 hr. Then Eudragit L 100: Eudragit S 100 (1:1) particles were evaluated in PBS, pH 6.8 throghout 12 hr. Then Eudragit L 100: Eudragit S 100 (1:1) particles were evaluated in PBS, pH 7.4 for throghout 12 hr. The pH was adjusted with 2M NaOH or 2M HCl. Insulin microparticles were transferred to the prewarmed dissolution media (100 ml.) and maintained at 37 ± 0.5^0C under stirring at 100 rpm in incubator shaker. Samples were withdrawn at regular intervals (0, 1, 4, 6 and 12 hr.) and volume was replaced by fresh PBS. The sample withdrawn was centrifuged (5,000 rpm, 10 min., 4^0C). Insulin content of the supernatant was estimated by micro BCA protein assay method.

SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS) of human insulin and optimized human insulin loaded microparticles was performed according to the method of Laemmli on a slab gel containing 20% resolving gel and 5% stacking gel. Vertical minigel apparatus (Pharmacia, GE Healthcare, Uppsala, Sweden) was used to check for protein integrity. Samples of insulin released from the microparticles in Tris-HCl buffer, insulin solution and a molecular weight reference marker (m.w. range 10.5 KDa-175 KDa) were dissolved in 1X running buffer containing SDS, Tris-HCl and glycine, loaded onto a vertical slab gel and subjected to electrophoresis at 30 mA. Protein bands were fixed and stained with Coomassie Brilliant Blue R-250 (0.1% w/v) in water: methanol: acetic acid (5:4:1).

CD SPECTROSCOPY

Circular dichroism (CD) spectra were obtained with a Jasco J-720 spectropolarimeter (Tokyo, Japan) equipped with a temperature controller to examine the secondary structure of insulin in particles. Spectra were collected at 25^0C using a 0.1 cm cell over the wavelength range of 200–260 nm. A resolution of 0.2 nm and scanning speed 50 nm /min. with a 4 s response time were employed. Each spectrum obtained represents an average of three consecutive scans. Samples for CD analysis were prepared by dissolution in Tris-HCl buffer. The spectra of insulin samples (insulin loaded microparticles) with concentrations about 169 µg/ml. were compared with that of fresh insulin solutions with same concentration.

FOURIER TRANSFORM INFRARED SPECTROSCOPY

Fourier transform infrared spectra of insulin, polymers and optimized insulin loaded microparicles using different polymers were obtained using ATR -FTIR using a Perkin Elmer, Spectrum BX, FTIR spectrophotometer (Tokyo, Japan) as KBr pellets. Samples were placed on the probe. The scanning range was 4000-400 cm^{-1} and the resolution was 2 cm^{-1}.

STABILITY TESTING

It is the capacity of a drug product to remain within the specifications established to ensure its identity, strength, quality and purity.

Accurately weighed parent insulin (human insulin) and selected batches of insulin microparticles were kept in falcon tubes in self-sealing cover at different temperatures (-20^0C, 4^0C and 25^0 C). They were checked for their drug encapsulation efficiency (% EE). Samples were withdrawn at months 0, 1.5, 3, 6 and 12 and % EE was estimated.

IN-VITRO CELL LINE STUDIES

Caco-2 cell line (colon cell line) was selected for in-vitro cell line studies. Toxicity studies and transepithelial electrical resistance (TEER) of caco-2 cell monolayer were performed.

Caco-2 cell culture

ATCC Caco-2 cells (HTB-37™, were cultured in a37 ^{0}C incubator with a 5% CO2 atmosphere in ATCC-formulatedEagle's Minimum Essential Medium supplemented with 10%fetal bovine serum (FBS) Gold (PAA Laboratories) and 1% penicillin/ streptomycin (Gibco). The cells were seeded at a density of 1 x 10^6 cells per six-well polyester Transwell insert (3 µmpores, Corning Costar) and were cultured to confluence over21 days, Cell culture medium in the upper andlower wells (1.5 and 2.5 mL, respectively, Fig.) was replacedevery 2–3 days and on day 20 of culture. (Fig. 13)

MTT Assay

MTT is (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole). MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce tetrazolium dye, MTT to its insoluble

formazan(purple colour), giving a purple colour in living cells. Dead cells don't stain. MTT assays are done in dark since MTT reagent is sensitive to light. A solubilisation solution (either DMSO, an acidified ethanolic solution or a solution of SDS in dil. HCl) is added to dissolve insoluble purple formazan product into a coloured solution. Absorbance of coloured solution is taken between 500-600nm. When confluency of cell culture has been achieved, added trypsin for withdrawing the adherent cells to the flask (1 ml.), transferred in 15ml. falcon tubespun at 1200 rpm, 4^0C, 5 min.the supernatant was discarded, added fresh 2 ml. media to resuspend the cell culture pellet.To check the density in microscope, withdrawn 20µl.from above pellet in an eppendorff, added 20 µl. Trypan blue dye (1:1) on a particular glass slide and cover slip.
Cell density= cell count x (2 x 10^4)
Then added stock solution diluted with media in all wells of 96-well plate (100µl.)Put the plate in carbon dioxide incubator for 24 hr.(37^0C), added 100 µl. MTT solution in all wells (50µg/ml.). Then withdrawn the remaining media (insoluble formazan dye), added 100 µl. DMSO (to make soluble formazan dye). Then microplate sample reading was taken at 570nm and reference reading at 630 nm.
Then the % viability was calculated using formula give below:

$$\% \text{ viability} = \frac{\text{Test reading}}{\text{Control reading}} \times 100$$

TEER Measurements
Caco-2 cell monolayers were cultured on the tissue-culture –treated polycarbonate filters (diameter23.5mm, growth area 4.9cm^2) in Costar Transwell 6 wells/plates and used for the transport experiments 21 days after seeding. Subsequently, evaluation of the prepared microparticles in enhancing paracellular transport at pH 6.8 and pH 7.4 was investigated in vitro in caco-2 cell monolayers. The change in TEER for the tightness of cell monolayers was measured with a Millicell electrical resistance system connected to a pair of chopstick electrodes.

RESULTS AND DISCUSSION
FORMULATION VARIABLES
The effect of the formulation variables on the encapsulation efficiency and size of chitosan, PLGA, Eudragit L 100 and Eudragit S 100 microparticles prepared by triple emulsion solvent evaporation technique is shown in Table 1. The microparticles prepared with more concentration of Eudragit polymer encapsulated a larger amount of insulin compared with lower concentration of Eudragit polymer. This could be due to more coating of the Eudragit polymer and hence less leakage of the drug occurs due to less number of pores left. It was observed that small volume of IAP and EAP provides the increased encapsulation efficiency and decreased particle size. The mean diameter of the microparticles was in range of 1-6 µm.
It has been reported that the droplet size of a primary emulsion may increase with an increase in the volume of the internal aqueous phase which, in turn may be responsible for the increase in size of particles. Accordingly, during formation of a primary emulsion, sonication of a small volume leads to smaller droplet formation, which could account for a smaller particle size. A higher rate of leaching, poor physical stability of the primary emulsion and an increase in number of pores on the surface of the microparticles may be responsible for the decrease in encapsulation efficiency associated with increase in volume of internal aqueous phase of the primary emulsion. Thus, a smaller volume of the internal aqueous phase is desirable for higher encapsulation efficiency of insulin and particles of smaller size. This increased encapsulation efficiency may be simply due to a relatively greater proportion of polymer with respect to the amount of the drug. The increase in polymer load leads to a shorter time for the composition of the polymer solution to reach the viscous (gelation) boundary, resulting in rapid formation of a film-like membrane on the periphery of the droplets. If the film-like polymeric membrane is quickly solidified, the microparticle structure is more fixed and thus solvent and nonsolvent counter diffusion is delayed. As a consequence, less water may be able to diffuse into the dispersed phase and less drug would be carried by the solvent into the aqueous phase.
Sharma et al.[31] encapsulated papain in Eudragit L 100 and Eudragit S 100 submicron particles using double emulsion solvent evaporation and observed actual drug loading of 2.76% and 2.80% respectively and encapsulation efficiency of 81.17% and 82.35% respectively. In the present experiment, the actual drug loading of human insulin in Eudragit L 100 and Eudragit S 100 microparticles was 0.52% and 0.54% respectively and the corresponding encapsulation efficiency was 78.86% and 81.54%. The lesser drug loading observed in the present study appears to be due to use of a lesser concentration of insulin in the internal aqueous phase compared with papain (20

mg/ml. of insulin against 141.06 mg/ml. of papain).

ENTERIC NATURE OF MICROPARTICLES

Studies of the optimized formulations revealed that the release of insulin from insulin-loaded microparticles was mainly influenced by the nature of the polymer as well as pH of the surrounding media. Complete enteric coating could not be achieved because 6.42%-9.47% of drug release in 0.1NHCl (pH 1.2) in 2 hr. The release of the protein from the microparticles in acidic medium may be due to insufficient coating or film defects created during lyophilization. Since small amounts of chitosan and PLGA were used in the formulation, the particles were only slightly swollen and remained intact in this case. As a result, insulin would remain almost inactive as well as lose its structural integrity at low pH in the stomach (pH 1.2). Hence, a significant amount of insulin wouldn't reach the target site (colon) for digestion of proteins. Thus, use of enteric polymer matrix system for oral delivery of insulin would be satisfactory (because the protein is not released from the optimized formulation in an adequate amount in the stomach) for maintaining the structural integrity of the protein during the transit through the stomach. The microparticles had a negative charge contributed by the carboxylic groups of phthalic acid (pKa about 4.47) and methacrylic acid (pKa about 4.23) residues in the enteric polymer backbone.It is known that the carboxylic groups of anionic particles become protonated if pH is below the pKa of the carboxylic acid, leading to a decrease in the surface charge of the particles. Reduction in the surface charge diminishes the electrostatic repulsion and increases aggregation. Thus, it appears quite natural that the microparticles would aggregate in 0.1NHCl which has a pH 1.2 below the pKa of .phthalic acid (pKa about 4.47) and methacrylic acid (pKa about 4.23). It has been reported that the stomach retains food particles until these are fragmented into particles smaller than 0.5 mm in diameter. Subsequent to gastric emptying, the particles would enter the duodenum where the pH is > 5.0. Accordingly, as the pH of the dispersion of the microparticles in 0.1NHCl was raised to 5.3, deaggregation of the particles was observed due to pH- induced deprotonation of the carboxylic groups and restoration of negative charge. The result suggests that the particles, on being emptied by the stomach into the duodenum, which has a pH > 5.0, would undergo deaggregation, resulting an increase in the effective surface area of the particle.

IN-VITRO DRUG RELEASE

In vitrorelease from all the optimized batches of insulin microparticles showed 6.42%-9.47% of drug release in 0.1NHCl (pH 1.2) in 2 hr. Next, the drug release was studied in PBS at pH 6.8, 7.4 for Eudragit L 100 and Eudragit S100 particles respectively. Eudragit L 100 particles (3 HL III) showed more initial burst release (10.40%) in1 hr.and % cumulative release (75.19%) than Eudragit S100 particles (3 HS III) showing initial burst release (9.32%) in1 hr.and % cumulative release (71.48%). This may be due to pH-dependent release of Eudragit S100 particles at a pH > 7.0 (Fig. 1).

Eudragit L 100 : Eudragit S 100 (1:1) particles (3 HLS III) also showed more initial burst release (10.03%) in1 hr.and % cumulative release (73.87%) than EudragitS100 particles but less than Eudragit L 100 particles (3 HL III).(Thakral, N.K. et al.)

Eudragit L 100 : Eudragit S 100 (1:1) particles at pH 6.8 throughout 12 hr. [3HLS III (I)] showed more initial burst release (10.25%) in1 hr.and % cumulative release (74.46%) than Eudragit L 100 : Eudragit S 100 (1:1) particles at pH 6.8 in first 1 hr. and remaining 11 hr. at pH 7.4 (3 HLS III). Eudragit L 100 : Eudragit S 100 (1:1) particles at pH 7.4 throughout 12 hr. [3HLS III (II)] showed less initial burst release (9.46%) in 1 hr. and % cumulative release (72.86%) than above mentioned particles but showed more initial burst release and % cumulative release than Eudragit S100 particles.

PARTICLE MORPHOLOGY

Scanning electron microscopy was used to observe the morphology of the microparticles. Insulin loaded Eudragit L 100 microparticles, insulin loaded S 100 microparticles, and insulin loaded Eudragit L 100: Eudragit S100 (1:1) microparticles were spherical and had smooth surface. (Fig.2)

CD SPECTROSCOPY

Preservation of the structural integrity of a protein drug after release is crucial for its biological efficacy. Thus, the secondary structure of insulin released from particles was investigated using CD.The circular dichroism spectrum of intact insulin showed ellipticity trough at 212-214 nm (Fig.3).The circular dichroism spectrum of insulin in prepared microparticles was close to that of intact pure insulin (ellipticity trough at 213-217 nm) (Fig.4) demonstrating that the matrix materials and formulation procedure did not significantly influence insulin conformation.

SDS-PAGE

SDS-PAGEanalysis of both insulin and optimized insulin loaded microparticles demonstrated that neither fragmentation nor covalent dimerization occurred during the encapsulation process (Figure 5). There were no additional bands to indicate the presence of aggregates. Hence, the data suggests that the structural integrity (i.e. primary structure) of insulin was not significantly affected during the encapsulation process (and in potentially harsh conditions such as contact with organic solvents and mechanical agitation).

FOURIER TRANSFORM INFRARED SPECTROSCOPY

TheFourier transform infrared spectra of human insulin, Eudragit L 100, Eudragit S100, Eudragit L 100 : Eudragit S100 (1:1), insulin loaded Eudragit L 100 microparticles, insulin loaded Eudragit S 100 microparticles, insulin loaded Eudragit L 100 : Eudragit S100 (1:1) microparticles are shown in Figures (6-12). The Eudragit L 100 and Eudragit S100 polymers contain both carboxylic acid and ester groups. The spectra of Eudragit L 100, Eudragit S100 and Eudragit L 100 : Eudragit S100 (1:1) show the carbonyl stretching vibrations of carboxylic acid group at 1715.37 cm^{-1}, 1713.44 cm^{-1} and 1708.62 cm^{-1} respectively. The spectra of Eudragit L 100, Eudragit S100 and Eudragit L 100: Eudragit S100 (1:1) also show the carboxylate anion stretching vibrations at 1379.82 cm^{-1}, 1375.96 cm^{-1} and 1382.71 cm^{-1} respectively.

Human insulin shows a broad band in the region of 3320-3140 cm^{-1}, having a peak at 3299.61 cm^{-1} respectively due to the N-H stretch of a secondary amide. Human insulin shows a predominant band at 1680-1630 cm^{-1}, having a peak at 1650.77 cm^{-1} respectively due to the carbonyl stretching vibrations of secondary amide. Human insulin shows C-O stretching vibrations due to presence of phenol in tyrosine of insulin in the region of 1410-1310 cm^{-1}, having a peak at 1381.75 cm^{-1} respectively. Human insulin also shows O-H bending vibrations of phenol at 1234.22 cm^{-1} respectively. Human insulin shows C-S stretching of sulfides and disulfides at 662.43 cm^{-1} respectively.

The spectra of insulin loaded polymeric microparticles show peaks at 3364.21cm^{-1}, 3328.53 cm^{-1} and 3447.13 cm^{-1} due to the N-H stretch of a secondary amide. The particles show peaks at 1643.05 cm^{-1}, 1641.13 cm^{-1} and 1644.02 cm^{-1} due to carbonyl stretching of secondary amide. The particles show peaks at 1428.99 cm^{-1}, 1389.46cm^{-1} and 1428.99 cm^{-1} due to carbonyl stretching of phenol and O-H bending of phenol at 1260.25 cm^{-1}, 1260.25 cm^{-1} and 1259.29 cm^{-1}. The particles also show peaks at 599.75 cm^{-1}, 591.08 cm^{-1} and 591.08 cm^{-1} due to C-S stretching of sulfides and disulfides, suggesting entrapment of the drug.

STABILITY STUDIES

Tables 2, 3 and 4 show the results of the stability studies of the optimized insulin loaded microparticles and free insulin formulations. From tables, it is clear that insulin loaded microparticles show more % % EE than free insulin formulations on storage for 12 months. It is clear from tables that all formulations show highest % EE at 4 ^{0}C, lowest at 25 ^{0}C and formulations stored at -20 ^{0}C show higher % EE than formulations stored at 25 ^{0}C but lower than formulations stored at 4 ^{0}C. Thus, the stability of insulin entrapped in microparticles was significantly improved in comparison with the free insulin powder.

IN-VITRO CELL LINE STUDIES

MTT assay

Fig. 14 shows the comparison of % viability of caco-2 cells on addition of insulin solution and insulin loaded particles solution with different concentrations. % viability of caco-2 cells on addition of particles was found to be higher than on addition of pure insulin solution. % viability increases with decreasing concentration of the drug solution.

TEER Measurements

The comparison of TEER valuesof caco-2 cell monolayers on addition of prepared microparticles at pH 6.8 and pH 7.4 with different incubation time was investigated in vitro. (Fig. 15) Results show that TEER values were found to be less formicroparticles at pH 7.4 than for microparticles at pH 6.8. Moreover, particles incubated for longer time periods show lesser TEER values than incubated for shorter time periods.

CONCLUSION

Insulin was successfully entrapped in enteric microparticles by triple emulsion solvent evaporation technique, optimizing the formulation parameters in order to attain the maximum encapsulation efficiency, a spherical shape and *in-vitro* release profile. The release profiles for formulations confirmed their gastroresistance, thus allowing pH-dependent release of insulin in the gastrointestinal tract. The results of in-*vitro* release studies showed that insulinloaded Eudragit L 100 microparticles release more and quicker insulin than insulin

loaded Eudragit S 100 microparticles, and insulin loaded Eudragit L 100: Eudragit S100(1:1) microparticles. Eudragit L 100: Eudragit S100 (1:1) microparticles in PBS pH 6.8 showed quicker and more release than in PBS pH 7.4. Thus, Eudragit S 100, Eudragit L 100 and Eudragit L 100: Eudragit S100 (1:1) microparticles have great potential as oral carriers for delivery of insulin to small and large intestine to facilitate reduction of blood glucose level. These formulations would be an ideal for further in-vivo studies.

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DISCLOSURE

The authors report no conflicts of interest in this work.

Table 1: Characterization of insulin loaded microparticles using combination of 3 polymers

Formulation Code	Actual Loading (µg/mg)	Actual Loading (%)	%EE	Particle Size (d.nm)	Zeta Potential (mV)
3HS I	4.152	0.41	42.60	5958	-32.1
3HS II	5.651	0.56	71.54	4542	-19.1
3HS III	5.414	0.54	81.54	1245	-13.9
3HL I	5.081	0.50	52.13	2659	-28.2
3HL II	5.4568	0.54	69.08	2159	-30.5
3HL III	5.2365	0.52	78.86	3278	-24.2
3HLS I	5.2312	0.52	53.67	1330	-55.9
3HLS II	5.5047	0.55	69.68	1539	-40.8
3HLS III	5.2167	0.52	78.56	2222	-45.5

Abbreviations: H, Human insulin; L, Eudragit L 100; S, Eudragit S 100; LS, Eudragit L 100 : Eudragit S 100 (1: 1); 3, Combination of three polymers; I, 4% w/v Eudragit; II , 5% w/v Eudragit; III, 6% w/v Eudragit; EE, Drug entrapment efficiency

Stability studies

Table 2: % EE remaining at -20^0 C

Formulation code	0 M	1.5 M	3 M	6 M	12 M
HI	84.65%	73.49%	66.65%	60.22%	52.41%
3HS III	81.54%	76.18%	74.98%	72.14%	66.45%
3 HL III	78.86%	74.29%	71.91%	68.11%	62.29%
3HLS III	81.22%	77.41%	76.18%	71.93%	63.38%

Table 3: % EE remaining at 4^0 C

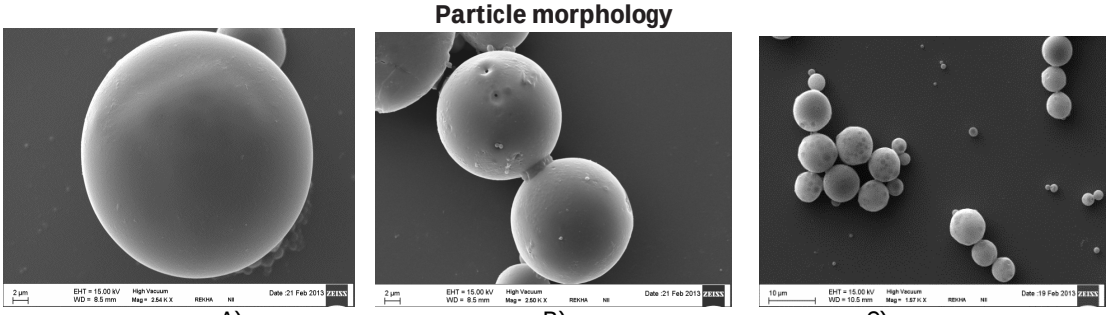
Formulation code	0 M	1.5 M	3 M	6 M	12 M
HI	84.65%	75.29%	68.41%	63.48%	58.14%
3HS III	81.54%	79.44%	76.98%	75.10%	69.11%
3 HL III	78.86%	76.25%	74.20%	71.90%	64.19%
3HLS III	81.22%	79.12%	78.11%	75.41%	66.36%

Table 4: % EE remaining at 25^0 C

Formulation code	0 M	1.5 M	3 M	6 M	12 M
HI	84.65%	71.89%	65.11%	60.19%	51.46%
3HS III	81.54%	73.13%	72.35%	66.22%	61.35%
3 HL III	78.86%	71.36%	68.94%	64.39%	59.12%
3HLS III	81.22%	74.42%	72.24%	66.08%	61.66%

Abbreviations: HI, Human insulin; M, Month

Particle morphology



A) B) C)

Fig. 1: SEM micrographs of optimized insulin loaded A) Eudragit S 100 B) Eudragit L 100 C) Eudragit L 100: Eudragit S100 (1:1) microparticles

***In vitro* release profile**

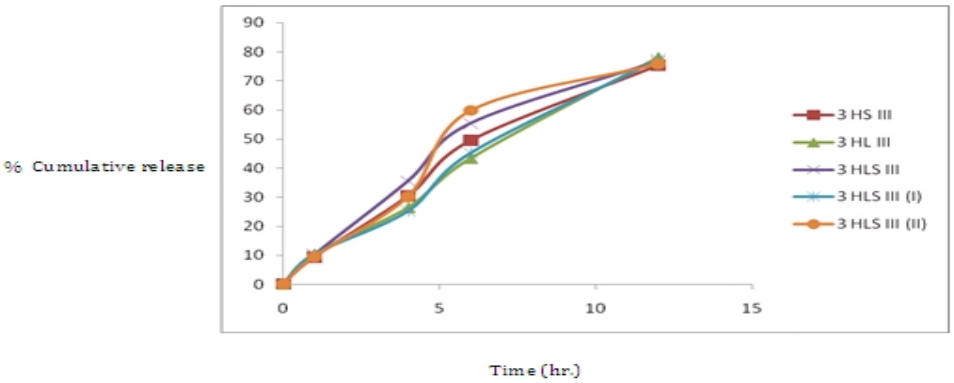


Fig. 2: In *vitro* release profile of the optimized formulation of insulin loaded Eudragit L 100, Eudragit S100 and Eudragit L 100: Eudragit S 100 (1:1) particles at pH 6.8 and 7.4

CD Spectroscopy

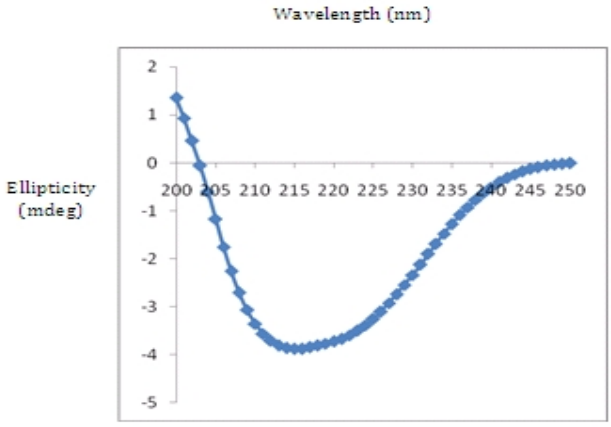


Fig. 3: CD spectra of human insulin solution

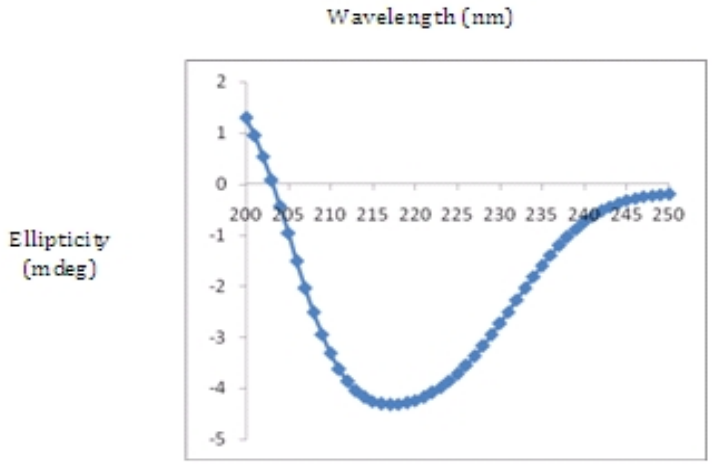


Fig. 4: CD spectra of human insulin loaded microparticles

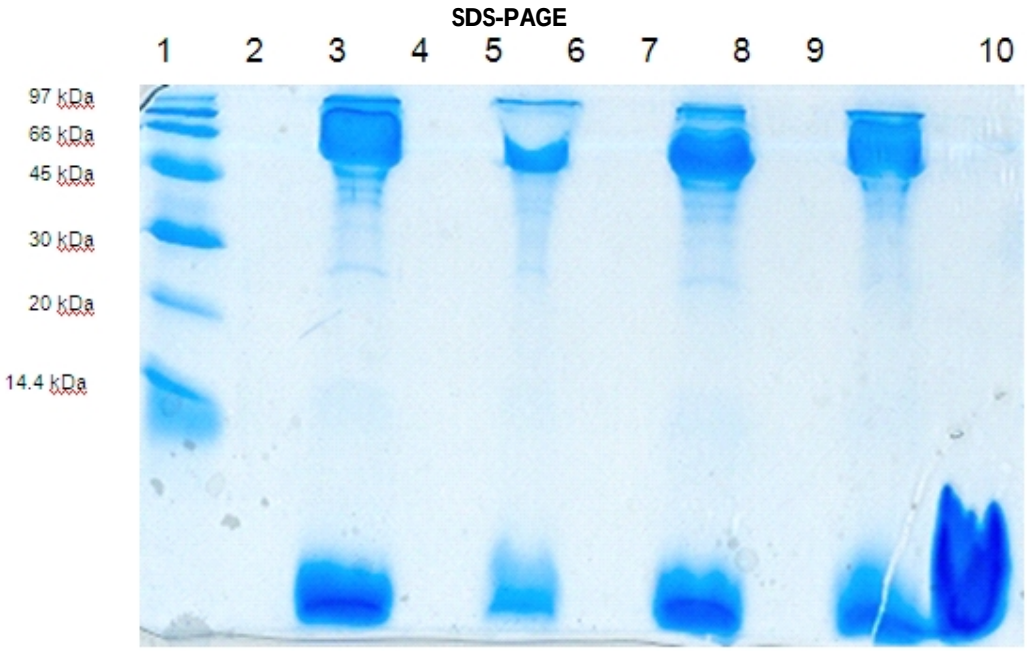


Fig. 5: SDS-PPAGE analysis of insulin and optimized insulin loaded microparticles
Lane-1: M.W. markers; Lane-3: Human insulin loaded Eudragit L 100 microparticles;
Lane-5: Human insulin loaded Eudragit S 100 microparticles;
Lane-7: Human insulin loaded Eudragit L 100 :Eudragit S100 (1:1) microparticles (released in PBS pH 6.8 for 12 hr.)
Lane-9: Human insulin loaded Eudragit L 100 :Eudragit S100 (1:1) microparticles (released in PBS pH 6.8 for first 1 hr. and PBS pH 7.4 for remaining 11 hr.)
Lane-10: Human insulin solution

Fourier transform infrared spectroscopy

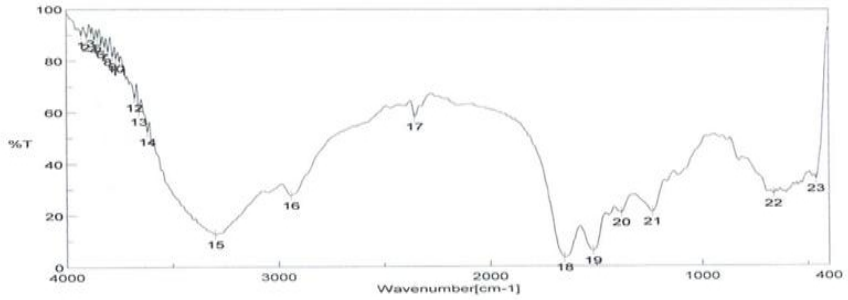


Fig. 6: FT-IR spectra of human insulin

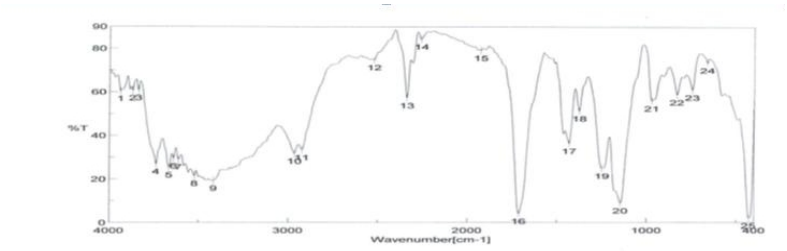


Fig. 7: FT-IR spectra of Eudragit S 100

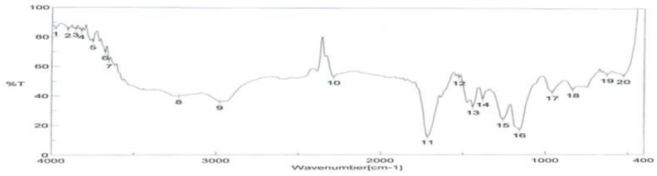


Fig. 8: FT-IR spectra of Eudragit L 100

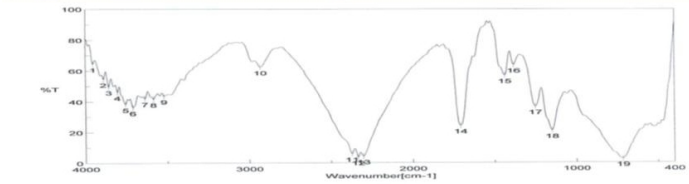


Fig. 9: FT-IR spectra of Eudragit S 100: Eudragit L 100 (1:1)

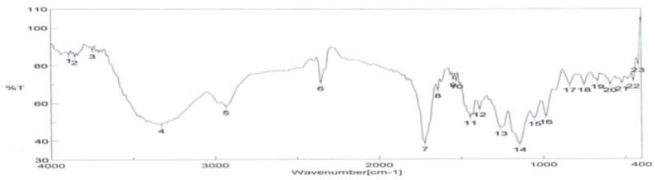


Fig. 10: FT-IR spectra of human insulin loaded Eudragit S 100 particles

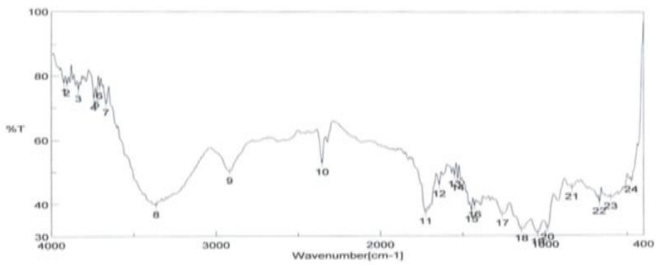


Fig. 11: FT-IR spectra of human insulin loaded Eudragit L 100 particles

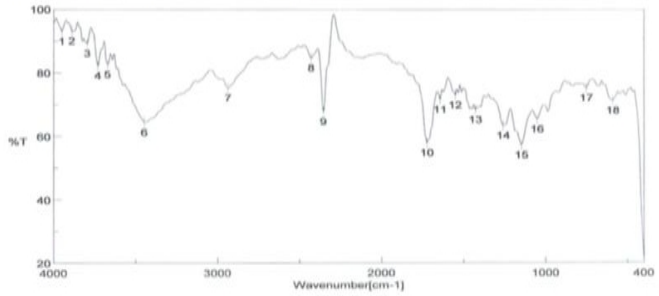


Fig. 12: FT-IR spectra of human insulin loaded Eudragit L 100 :Eudragit S 100 (1: 1) particles

In-vitro cell line studies

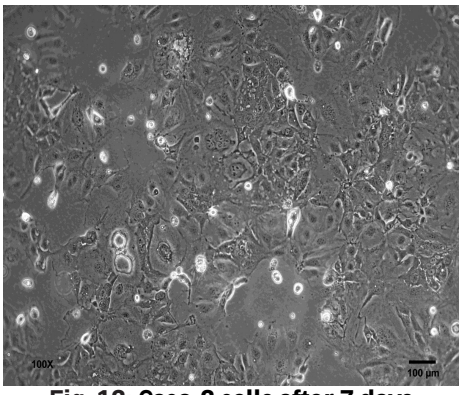


Fig. 13: Caco-2 cells after 7 days

MTT Assay

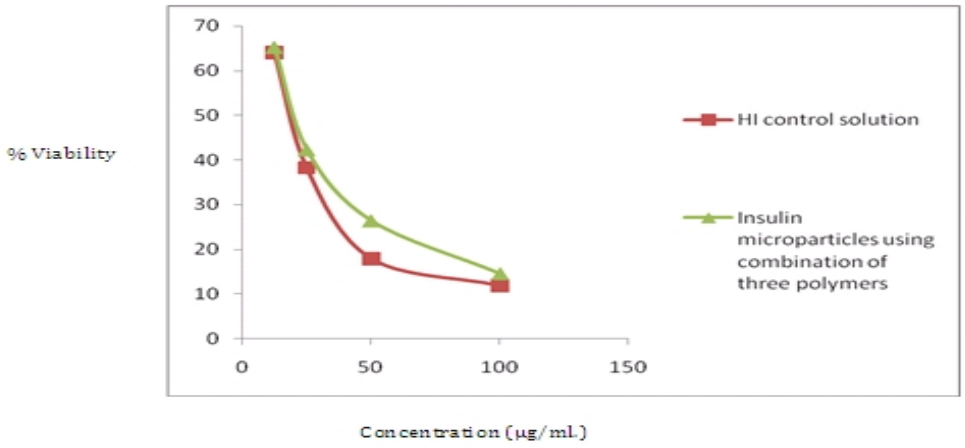


Fig. 14: Comparison of % viability of caco-2 cells on addition of drug and drug loaded particles with different concentrations

TEER Measurements

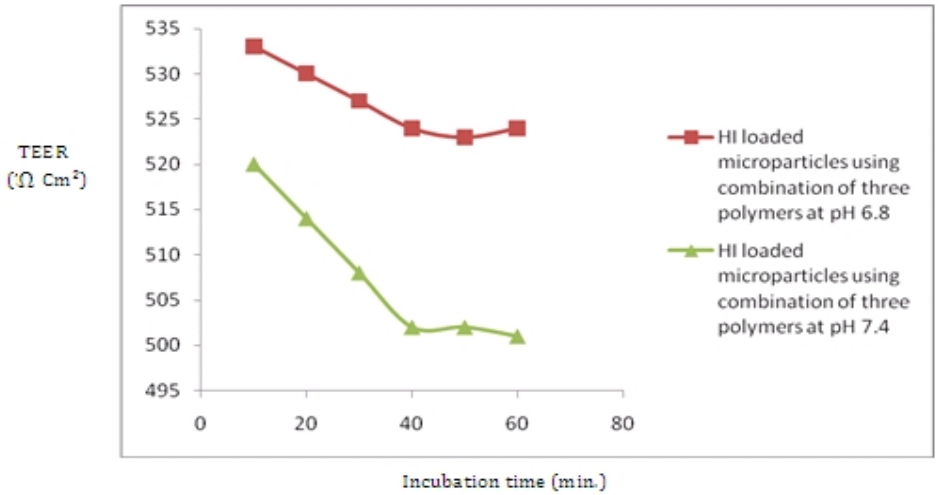


Fig. 15: Comparison of TEER values of caco-2 cell monolayers on addition of insulin particles using combination of three polymers with different incubation time at pH 6.8 and pH 7.4

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