Turk J Pharm Sci 2024;21(5):483-488
DOI: 10.4274/tjps.galenos.2023.10663

ORIGINAL ARTICLE

A Novel Controlled Release Implant of Insulin Based on Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) Polymer Prepared by Extrusion

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ABSTRACT

Objectives: The aim of this study was to develop a biodegradable implant with a slow release of insulin to minimize the amount of repeated drug injections in patients.Developing and designing implants with controlled release of active protein has always been a challenge. To optimize and control the release of insulin in this project, the drug complexing mechanism was used by dextran sulfate sodium (DS) and Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) polymer.

Materials and Methods: The efficacy of drug binding was evaluated under different molecular ratios of DS, and then a thermogravimetric analysis test was done to check the stability of the drug complex in extrusion. In the final stage, rod-shaped implants of complexed insulin were prepared by an extrusion process, and the drug release was evaluated within 32 days. The drug release kinetics were evaluated using mathematical models.

Results: The results showed an increase in insulin binding efficiency percent, up to a ratio of 2.6. The drug release from the implant containing complex insulin was completely controlled. The drug release followed a zero-order release model. Interestingly, the complex form of the drug showed a temperature resistance of 160 ˚C for ten minutes.

Conclusion: In this study, for the first time, a controlled release implant of insulin has been developed based on a PHBV polymer. In this method, the extrusion process has been used, which provides the possibility of preparing implants on an industrial scale in the future. Also, their development appears to be a promising treatment for diabetic patients and leads to the elimination of frequent drug injections and then more adherence of the patients to the continuation of the treatment process.

Keywords: Insulin, PHBV, controlled release, implant, extrusion

INTRODUCTION

Diabetes is a metabolic disease with symptoms of high blood glucose, glycosuria, hyperlipidemia, and nitrogen imbalance that leads to several kidney, eye, vascular, and heart complications. It can be diagnosed in two forms: type 1 and type 2 diabetes. There are about 415 million people with diabetes in the world, almost 1 in 11 people in the population, and the number of people with diabetes worldwide will reach 642 million by 2040.[1-3]

One of the main methods of treatment in patients is insulin injection to reduce blood sugar, but repeated insulin injections in patients are a tiring method and reduce the patient's adherence to continue treatment. Insulin has an *in vivo* short half-life.

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Received: 17.05.2023 , Accepted: 22.08.2023

Nowadays, the development of drug delivery systems has placed promising methods in front of treatment systems. One of these methods is the development of biodegradable implants for the controlled and slow release of insulin in patients. The use of polymeric sustained-release implants increases the half-life of this drug, but the lack of toxicity and biocompatibility of the polymeric compound used in the manufacturing of implants is very important.[1-5]

Biodegradable polymers are an excellent option in medical applications because they are easily destroyed in the environment and then removed from the body. These polymers have various applications in the production of implants, surgical sutures, and drug delivery systems. Many synthetic and natural biodegradable polymers have been evaluated for creating implants. Natural polymers, like collagen, albumin, and gelatin, have been assessed for drug delivery. Nonetheless with their use, their use is limited because of their higher price and purity.[6,7] Polyhydroxyalkanoates are biodegradable polyesters that can be produced through bacterial and synthetic methods.[6,7] The excellent properties of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), like its biological origin, adsorption capacity, and low toxicity, make it an appropriate option for biotechnological applications, like the fabrication of cardiovascular stents and drug delivery systems. It is used in medical packaging and for absorbable surgical sutures, tissue engineering, biosensors, degradable implants, and the construction of porous scaffolds.[8-10]

Implants containing biodegradable polymers can be divided into two categories: matrix systems and reservoir systems. In the matrix system used in this study, the polymer degraded slowly under physiological conditions. Thus, the drug is released via diffusion from the pores of the matrix. In reservoir systems, membrane degradation is slower compared to drug release. In spite of the fact that much research has been done in the field of designing and manufacturing biodegradable implants, only a few of them are in the phase of clinical studies. The most important problem facing biodegradable implants is the problem of designing a formulation with optimal drug release.[11,12]

MATERIALS AND METHODS

Materials

PHBV polymer with 3 *wt.* polyhydroxyvalerate % (PHV) was purchased from Tianan Biologic Materials Ltd., Ningbo (China). Polyethylene glycol (PEG) 6000 was prepared by Sigma-Aldrich (St. Louis, USA). Dextran sulfate sodium (DS) from Leuconostoc *spp*. Mr 5,000 and hydrochloric acid (HCL) were purchased from Merck (Germany). Insulin was prepared from Ronak Daroo (Iran). The Micro BCA assay kit and phosphate-buffered saline (PBS) tablets were obtained from Biobasic (Canada).

Preparation of insulin complexes (Ins.com) using insulin with DS salt at pH 3 and different molar ratios

Insulin (Ronak Daroo company) was used for hydrophobic ion pairing (HIP) complex preparation. Stocks of DS from Leuconostoc *spp.* Mr 5,000 (Merck, Germany), as ion-pairing agent, was prepared in double-distilled water. Briefly, DS in different concentrations was added to the insulin solution with pH 3. To provide more basic amino acid ionization and positive charges on the protein, the insulin solution pH was adjusted with 0.1 N of HCL (Merck, Germany) to the pH value of 3. After mixing two solutions in an optimum ratio by vigorous cortexin, the created HIP complex was centrifuged at 14000 RPM for 15 minutes to isolate the supernatant. The obtained complex was lyophilized into a powder. The micro BCA assay (Biobasic, Canada) was used to measure uncomplexed insulin in the supernatant.

The effect of different molar ratios of DS into the insulin was evaluated. For this purpose, we investigate the impact of different molar ratios -0.88, 1.75, 2.6, 3.5, 5.2, and 8.7- on the binding efficiency percentage. As mentioned above, after mixing insulin solution at pH 3 with the above six molar ratios and vigorous cortexin, centrifugation was performed at 14000 RPM for 15 minutes for supernatant separation, and then, uncomplex insulin was measured by HPLC.

The HIP complexes were washed three times with deionized water and then lyophilized. The insulin complexation efficiency [CE (%)] was calculated by measuring the supernatant levels of insulin using HPLC. Then based on the formula below, CE (%) is calculated, and the levels of insulin in the complex are calculated based on the initial amount of insulin that was added.

$$CE\ (\%) = M_i - M_f / (M_i \times 100\%)$$

Where M_i represents the primary insulin amount that was added to the reaction, while M_f denotes the free insulin amount in the supernatant.

Fourier transform infrared spectroscopy (FTIR) of insulin: DS complexes

FTIR analysis was done by an FTIR spectrophotometer (Tensor 27, Bruker) to investigate the chemical properties of the DS, insulin, and HIP complex separately. The test samples scanned between 500-4000 cm^{-1} in the mid-infrared range. This analysis was completed to determine the molecular modifications induced by the addition of DS and HIP complex production, along with investigating chemical properties on the molecule surface after the complexation reaction. Furthermore, FTIR analysis assessed drug-polymer interaction. For this purpose, the resulting complexes (0.1 mg) were mixed with 1 mL of deionized water and located in a shaker bath at room temperature. Then, 24 hours later, the solution underwent centrifugation at 14000 RPM over 10 minutes; the obtained supernatant passed through a 0.45 µm syringe filter, and the insulin concentration was measured by HPLC.[13]

Preparation of biodegradable insulin implants based on poly(phenylbenzothiazoline-6-sulfonic acid) polymers

For preparation of the rod-shaped implant, 3 *wt.* of PHBV was used. Percent of PHV (Tianan Biologic Materials Ltd., China) and PEG (molecular weight of 600 g/mol) as a pore former (Sigma, Germany) in the ratio of 1:4 (PHBV: PEG) was used.[14,15] The heating process was chosen for the preparation of rod

shaped implant by extruder. In this process, complexed insulin (Ins.com) was mixed with the polymers and extruded at 160 ˚C for 10 minutes.

Release kinetics of the rod implant

HPLC test measured drug release from prepared implant in the PBS medium (pH 7.4) after 48, 96, 192, 384, and 768 hours at 37 ˚C, and then drug release kinetic was reported based on the investigation of different release kinetics equations. Different mathematical models were assessed, including the zero-order Higuchi model, first-order model, Hixson-Crowell model, and Korsmeyer-Peppas model. The final result is reported based on the highest R2 of the regression line.

Statistical analysis

Values are expressed as mean ± standard deviation. Following the evaluation of the variance homogeneity and normal distribution of data, statistical analysis was done via one-way analysis of variance (for more than two groups). Tukey's test was used as a post-hoc test. Statistical analysis was performed using GraphPad Prism version 8.0.2. Values of $p \leq 0.05$ are regarded as significant.

RESULTS

Preparation of insulin complexes with DS salt at different molar ratios

The HIP complex of insulin with dextran sulfate (ion pairing agent) was provided, and its % binding efficiency was assessed. The pKa of the sulfate group in dextran sulfate has been reported to be < 2; thus, it possesses a negative charge above pH 2.[16,17] In this research, the effect of the molar ratio of dextran sulfate to insulin was assessed to obtain maximum binding. The most appropriate molar ratio was selected based on the maximum binding percentage. The effect of 6 molar ratios on the percentage of binding efficiency was investigated. Finally, a molar ratio of 2.6 at pH 3 was considered for drug complexation. The results show an increase in insulin binding efficiency with an increment in molar ratio, but only up to a ratio of 2.6 (Figure 1).

Investigating the characteristics of the complexes

FTIR

To assess the nature of the interaction between the sulfate group of DS, and the amino group of amino acids of insulin, an FTIR test was performed (Figure 2).

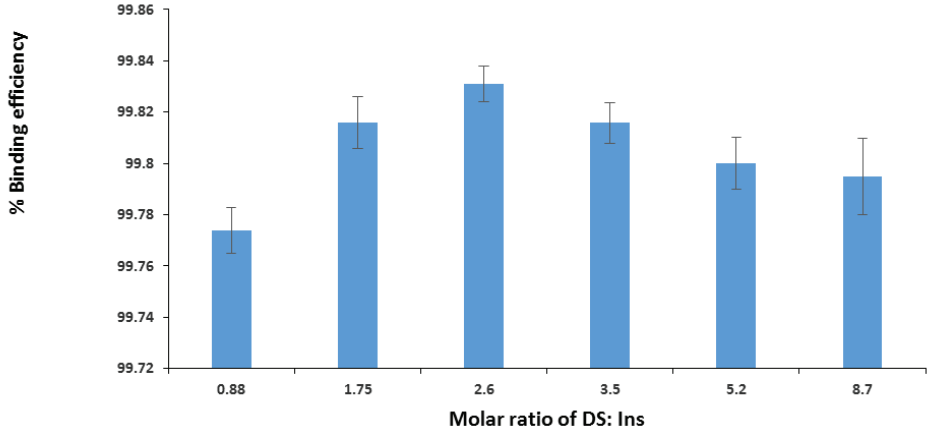

Figure 1. The % binding efficiency of ins. in different molar ratios of DS (ins). Values were represented as mean ± SD, n= 3
DS: Dextran sulfate sodium, Ins.: Insulin, SD: Standard deviation

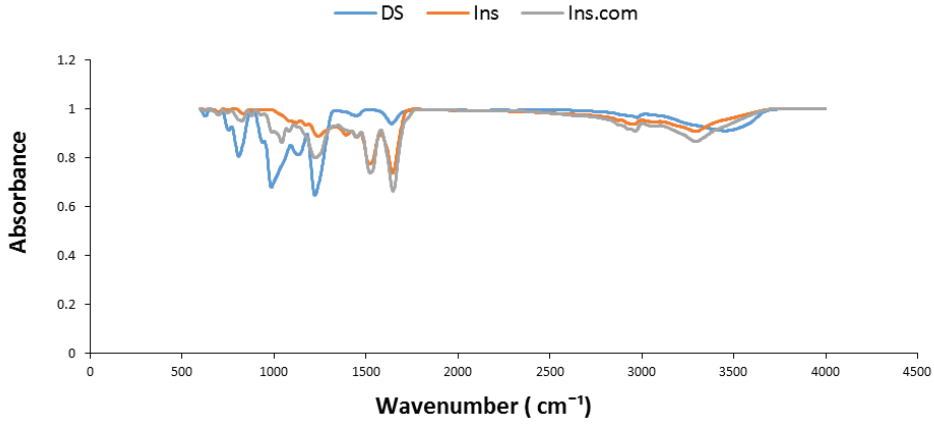

Figure 2. FTIR results of ins., DS, and Ins.com
DS: Dextran sulfate sodium, Ins.: Insulin, Ins.com: Insulin complex

Thermogravimetric analysis (TGA)

This test was performed to examine weight changes in insulin at 160 ˚C for 10 minutes. Weight changes were observed with a TGA instrument SDT Q600 V20.9 Build 20 at 30 to 160 ˚C. This test was done for evaluation of the terminal stability of insulin used in prepared implant and showed weight loss that probably is due to loss of water because this change is about 5 percent; on the other hand, the changes in weight loss have reached a relatively stable state before reaching the temperature of 160 ˚C (Figure 3).

Release kinetics of the rod implant

The drug release kinetic and dissolution behavior of implant -obtained data from *in vitro* drug release- were determined as drug cumulative percentage, drug log cumulative percentage, and remaining drug log cumulative percentage (Figure 4). The curves were then constructed according to various kinetic factors. For the interpretation of release kinetic, the R-square value was determined, and then their comparison helped us to choose the best kinetic model.[18] According to the counted R^2, the best fit kinetic model is the zero-order model (R2: 0.9942 for insulin implant), which shows the drug release at a constant rate.

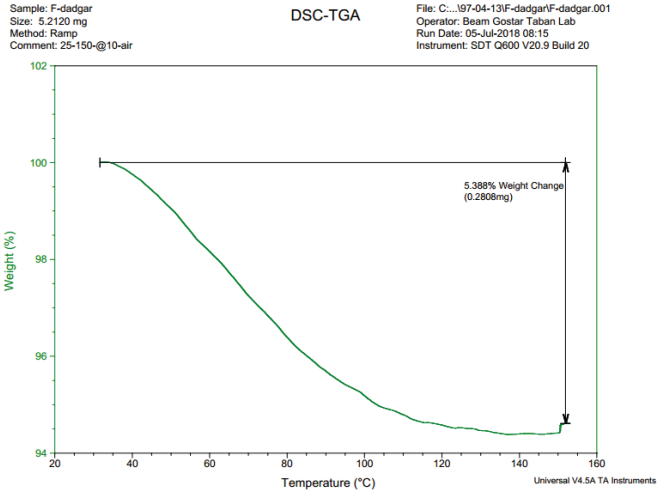

Figure 3. TGA of ins

Ins.: Insulin, TGA: Thermogravimetric analysis, DSC: Differential scanning calorimetry

DISCUSSION

The isoelectric pH (pI) of insulin is 5.3, and the protein surface charge at pH below pI will be positive and, thus, bind with the negatively charged dextran sulfate as ionic interactions. Hence, following the decrease in pH, the protein surface charge increases, and, based on Figure 1, % binding efficiency increases with a decrease in pH. The maximum % binding efficiency of insulin with DS is found when the dextran sulfate to the protein surface charge ratio is approximately 1:1.[19] In fact, at the molar ratio of 2.6, the surface charge in dextran sulfate and protein is nearly similar, and maximum binding is observed. Another point to note is that, depending on the drug's molecular weight, it is better to select the nearest molecular weight of the HIP agent to achieve the optimal molar ratio. Therefore, DS is chosen for this study.

About FTIR results, As mentioned in the literature, the characteristic peaks for sulfate groups of DS in the FTIR are 804.31 cm^{-1} (S-O-S vibration), 983.6 cm^{-1} (symmetric SOO-stretching vibration), and 1226.7 cm^{-1} (asymmetric SOO-stretching vibration). The ionic interactions that occur between amino and sulfate groups can lead to attenuation in IR peaks for the sulfate group or their shift. Due to such interactions, observed IR peaks for sulfate groups in DS showed attenuation in our investigation.In addition, this analysis was used to evaluate the stability of the secondary protein structure. The amide I and II bands are the two main bands to characterize protein secondary structure.[19] In the infrared spectroscopy (spectrum) of insulin, it can be determined that the amide I band (1698 cm^{-1}) and the amide II band (1550 cm^{-1}) of the complex insulin and its pure form are similar. Therefore, the protein's secondary structure is retained in the complex form.

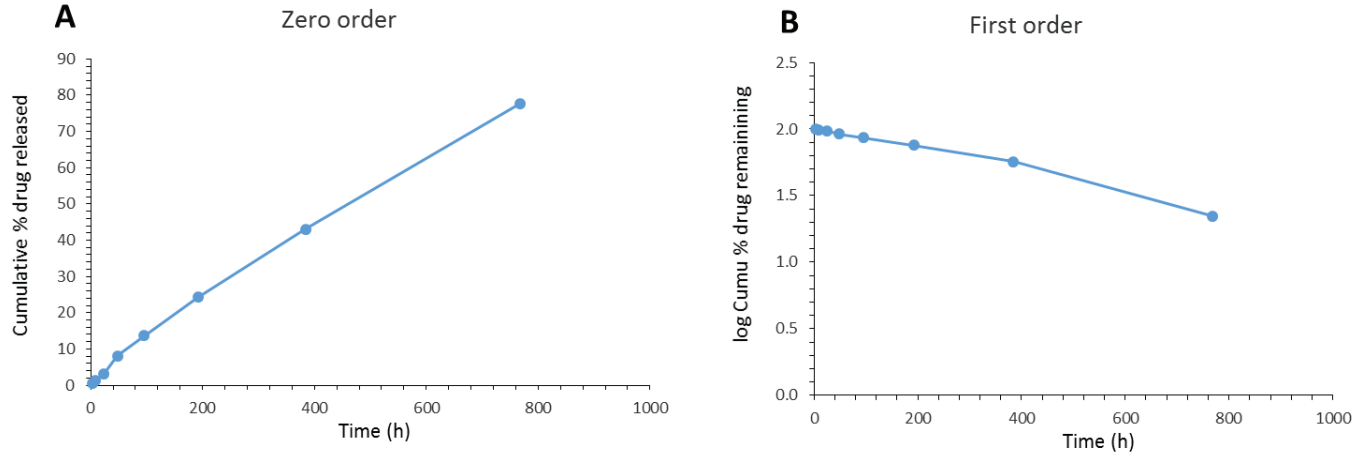

Figure 4. The release kinetics of insulin from prepared implant a protein drug: (A) zero-order model; and (B) first-order model. The error bars lie within the points (values were represented as mean ± SD, n= 3)

SD: Standard deviation

Recent research shows that protein complexing increases the heat resistance of the drug; therefore, according to these results, it is predicted that after complexing insulin with sodium dextran sulfate, the heat resistance of the drug will increase in the stages of implant preparation by the extrusion method. However, additional animal studies are required to measure the functional activity of the drug.[20,21]

The results of drug release kinetic investigation demonstrate that our formulation is sustained-release. The calculated R^2 for zero order kinetic is very close to the R^2 of the first order kinetic, indicating that the prepared implant is also a controlled release formulation that follows zero-order kinetics. Regarding the first-order model, the release profile is associated with the drug concentration in pharmaceutical formulations. It is applicable for the dissolution of water-soluble insulin in a porous matrix created from PHBV/PEG in this implant.[20-22]

CONCLUSION

Insulin implants were effectively obtained by the melting approach using PHBV and PEG-6000 as the pore former. The results of recent research projects show that by complexation, protein activity can be highly preserved under melting conditions and is applicable to developing implants containing protein drug using an extruder in research and also industry. Therefore, in this project, insulin-drug complexation was performed with the aim of the controlled release of insulin. The results show that very few weight changes occur in the insulin drug at 160 °C temperature, and probably the biological activity of the drug will be maintained to a large extent. Moreover, PHBV polymer is applicable for the preparation of sustained-released implants, including protein drugs like insulin. Due to its controlled release property, insulin implants can be a promising treatment for diabetics and lead to the elimination of frequent drug injections in patients and, as a result, more patient adherence to treatment. Optimization of formulations, and also *in vivo* studies, are needed for the production of an effective dose form of this implant for application in patients.

Acknowledgments

Research grants from the Tehran University of Medical Sciences, Deputy of Research (grant number: 35574-30-03-96), and Iran National Science Foundation (grant number: 96004866) supported this research. The authors greatly appreciate this financial support.

Ethics

Ethics Committee Approval: There is no requirement for ethical approval.

Informed Consent: There is no requirement.

Authorship Contributions

Surgical and Medical Practices: F.D.P., J.D.P., A.N., Concept: F.D.P., J.D.P., A.N., A.P., M.H.N., F.A.D., Design: F.D.P., J.D.P., A.N., A.P., M.H.N., F.A.D., Data Collection or Processing: F.D.P., J.D.P., A.N., A.P., M.H.N., F.A.D., Analysis or Interpretation: F.D.P., J.D.P., A.N., A.P., M.H.N., F.A.D., Literature Search: F.D.P., J.D.P., A.N., A.P., M.H.N., F.A.D., Writing: F.D.P., J.D.P., A.N., A.P., M.H.N., F.A.D.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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