ORIGINAL ARTICLE
DOI: 10.4274/tjps.galenos.2023.10663

A novel controlled release implant of insulin based on Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) polymer prepared by extrusion process

Short title: A controlled release implant of insulin

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17.05.2023
22.08.2023
28.08.2023

Abstract

Introduction

Developing and designing an implant with a controlled release of active protein drugs has always been a challenge. In order to optimize and control the release of insulin in this project, the drug complexing mechanism was used by dextran sulfate sodium and PHBV (Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)) polymer.

Methods

For this purpose, initially, the efficacy of drug binding was evaluated under different molecular ratios of Dextran sulfate sodium, and then a TGA test was done to check the stability of the drug complex in extrusion. In the final stage, rod shape implants of complexed insulin were prepared by an extrusion process, and the drug release was evaluated within 32 days. The kinetics of drug release were evaluated based on mathematical models.

Results

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

Discussion and 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, and also, their development appears to be a promising treatment for diabetic patients and lead 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

1. 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 seen 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, 2]

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. On the other hand, insulin has an in vivo short half-life. Nowadays, the development of drug delivery systems has put 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. In the case of reservoir systems, these systems must be removed after complete release of the drug. [1,2,3,4]

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 and 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, their use is limited because of their higher price and purity. Polyhydroxyalkanoates are biodegradable polyesters that can be produced through bacterial and synthetic methods.[5,6] 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 absorbable surgical sutures, tissue engineering, biosensors, degradable implants, and the construction of porous scaffolds.[7-17, 24]

Implants that contain biodegradable polymers are divided into two categories: matrix systems and reservoir systems. In the matrix system used in this project, the polymer degraded slowly under physiological conditions. Thus, the drug is released by the diffusion process from the pores of the matrix. In reservoir systems, membrane degradation is slower compared to drug release. In spite of the fact that many researches have 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.[18, 19]

In this project, the goal was to develop a biodegradable implant with a slow release of insulin to minimize the amount of repeated drug injections in patients.

1. Materials and methods

1.1 Materials

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

1.2 Preparation of insulin complex (Ins.com) by insulin with dextran sulphate sodium salt at pH 3 and different molar ratios

Insulin (Ronak Daroo company) was used for Hydrophobic ion pairing (HIP) complex preparation. Stocks of dextran sulphate sodium (DS) from Leuconostoc spp. Mr 5,000 (Merck, Germany), as ion- pairing agent, was prepared in double- distilled water (DDW). Briefly, dextran sulphate sodium 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 vortexing, created HIP complex was centrifuged at 14000 RPM for 15 min to isolate the supernatant. The obtained complex was

lyophilized into powder. The micro BCA assay (Biobasic, Canada) was used to measure uncomplexed insulin in the supernatant.

The effect of different molar ratios of dexteran sulphate sodium 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, 8.7— on the binding efficiency percentage. As mentioned above, after mixing insulin solution at pH 3 with above six molar ratios and vigorous vortexing, centrifugation was performed at 14000 RPM for 15 min for supernatant separation, and then, uncomplex insulin was measured by HPLC.

The HIP complexes were washed three times with deionized water and lyophilized. The insulin complexation efficiency (CE%) was calculated by measuring 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 insulin primary amount that was added to the reaction, while M_f denotes the free insulin amount in the supernatant.

1.3 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 dextran sulphate sodium, insulin, and HIP complex separately. The test samples scanned between 500–4000 cm^{-1} in the mid-infrared (mid-IR) range. This analysis was completed to determine the molecular modifications induced by the addition of dextran sulphate sodium 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 min; the obtained supernatant passed through a 0.45 μm syringe filter, and the insulin concentration was measured by HPLC. [22]

1.4 Preparation of biodegradable insulin implant based on PHBV polymer

For preparation of the rod shaped implant, PHBV with 3 wt. % of PHV (Tianan Biologic Materials Ltd, China) and polyethylene glycol (PEG, molecular weight of 600 g/mol) as a pore former (Sigma, Germany) in the ratio of 1:4 (PHBV: PEG) was used. [28, 29] 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 extrude at 160 °C for 10 min.

1.5 Release kinetics of obtained rod shape 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 model, Higuchi model, first-order model, Hixson-Crowell model, and Korsmeyer-Peppas model. The final result was reported based on the highest R-squared of the regression line.

2. Results and discussion

2.1 Preparation of insulin complexes with dextran sulphate sodium salt at different molar ratios

The HIP complex of insulin with dextran sulphate (ion pairing agent) was provided, and its % binding efficiency was assessed. The pKa of sulphate group in dextran sulphate has been reported to be <2; thus, it possesses a negative charge above pH 2. [22, 23] In this research, the effect of the molar ratio of dextran sulphate to insulin was assessed to obtain maximum binding. The most appropriate molar ratio was chosen based on the maximum binding percentage. For this purpose, 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 (Fig. 1). The isoelectric pH (pI) of insulin is 5.3 [21], 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 of pH, the protein surface charge increases, and, based on Figure 1, % binding efficiency increases with a decrease in pH. The results show an increase in insulin binding efficiency with increment in molar ratio, but only up to a ratio of 2.6 (Fig. 1). The maximum % binding efficiency of insulin with DS is found when the dextran sulphate to the protein surface charge ratio is approximately 1:1.[20, 22] In fact, at the molar ratio of 2.6, the surface charge in dextran sulphate 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. [20, 22] Therefore, DS is chosen for this study.

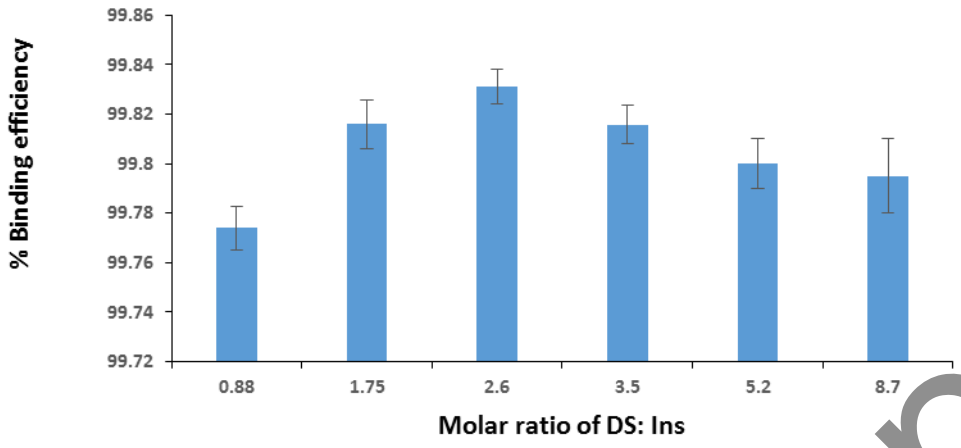

Fig. 1 The % binding efficiency of insulin in different molar ratios of dextran sulfate sodium (DS): Insulin (Ins). Values were represented as mean ± SD, n = 3.

2.2 Investigating the characteristics of performed complexes

2.2.1 FTIR

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

As mentioned in the literature, the characteristic peaks for sulphate 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 sulphate groups can lead to attenuation in IR peaks for sulphate group or their shift. [20, 22] Due to such interactions, observed IR peaks for sulphate groups in DS showed attenuation in our investigation.

In addition, this analysis was also used to evaluate the secondary protein structure stability. The amide I and II bands are two main bands to characterize protein secondary structure. [20, 27] In the IR 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 is similar. Therefore, the protein's secondary structure is retained in the complex form.

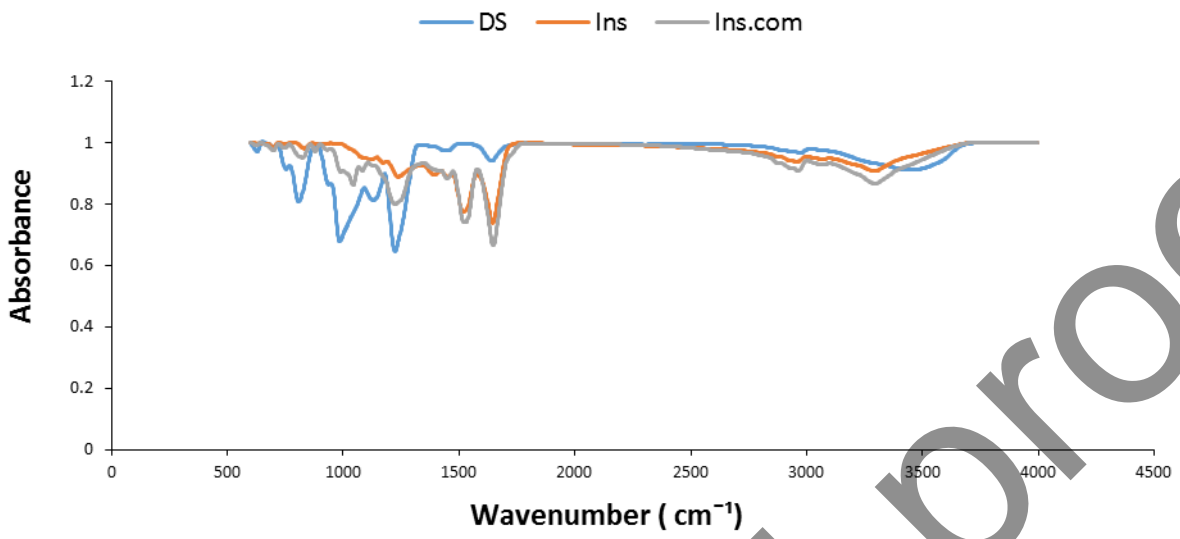

Fig. 2 FTIR results of insulin (Ins), dextran sulphate (DS), and insulin complex (Ins.com)

2.2.2 Thermogravimetric analysis (TGA)

This test was performed to examine the weight changes of insulin at 160 ° C for ten 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. Recent researches show that complexing proteins increases the heat resistance of the drug, and 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 extrusion method. However, additional animal studies are needed to measure the functional activity of the drug.[20]

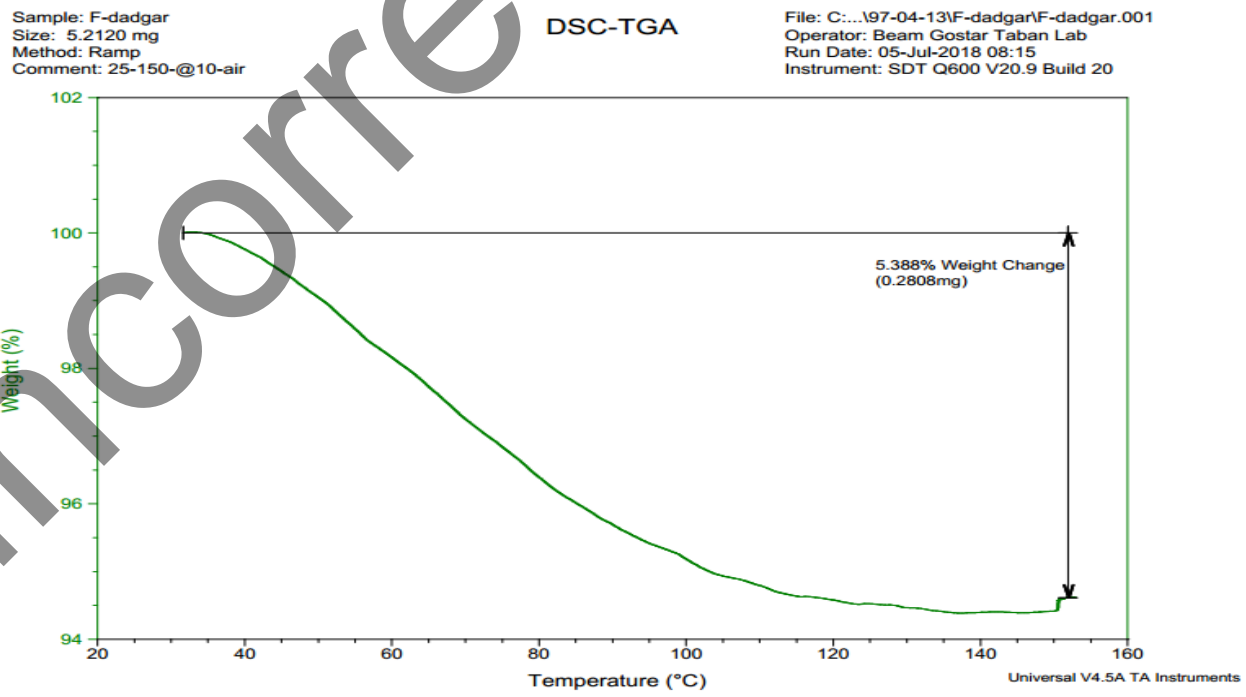

Fig. 3 Thermogravimetric analysis (TGA) of insulin.

3. Release kinetics of obtained rod shape 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 (Fig. 4). Then, their curves were 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.[25] According to the counted R^2, the best fit kinetic model is zero order model (R2: 0.9942 for insulin implant), which shows the drug release at a constant rate. This demonstrates 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 and is applicable for the dissolution of water-soluble insulin in a porous matrix created from PHBV/PEG in this implant.[26]

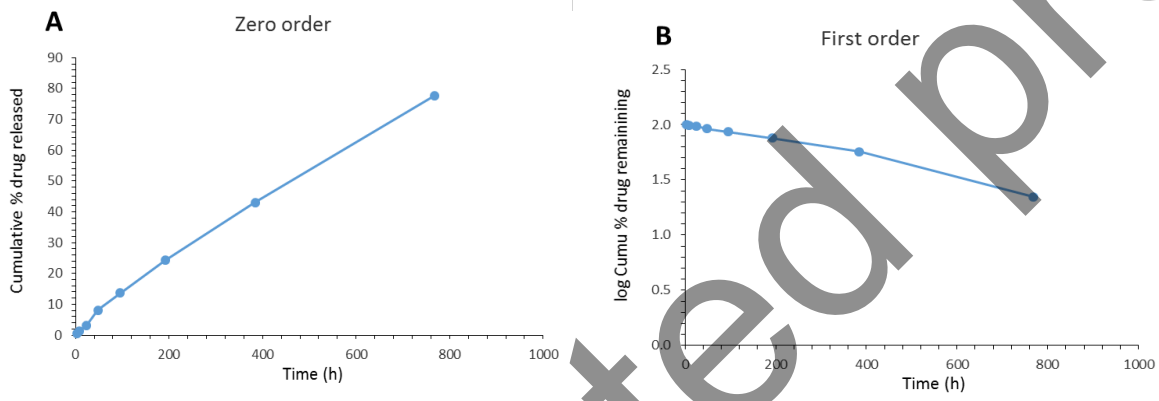

Fig. 4 The release kinetics of insulin from prepared implant a protein drug: (A) zero-order model; and (B) first-order model. The error bars lie within the points (Values were represented as mean ± SD, n = 3).

4. Statistical analysis

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

5. Conclusion

Insulin implant was effectively obtained by melting approach with PHBV and PEG-6000 as 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 develop implants containing protein drug using an extruder in research and also industry.[20] Therefore, in this project, insulin drug complexation was done with the aim of 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 implant, including protein drugs like insulin. Due to its controlled release property, insulin implant 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 production of effective dose form of this implant for application in patients.

6. Acknowledgments

Research grants from 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 backing.

7. Conflict of interest

We ensure that no conflict of interest exists with any funding institutions in regard to the materials examined in this paper.

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