Influence of crosslinker concentration on the characteristics of erythropoietin-alginate microspheres

[Influencia de la concentración de reticulante en las características de las microesferas de eritropoyetina y alginato]

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Abstract

Context: Microspheres have several advantages including protecting proteins from degradation and clearance after administration and produce a long-term therapeutic effect. Erythropoietin as a neuroprotectant agent has protein-like properties which are susceptible to degradation and have low in vivo bioavailability. Microspheres formulations is one of potential drug delivery system for erythropoietin.

Aims: To evaluate the effect of CaCl2 concentration on the characteristics (particle size, morphology, swelling index, and yield) of erythropoietin-alginate microspheres.

Methods: Erythropoietin-alginate microspheres prepared by ionotropic gelation method with aerosolization technique using sodium alginate as polymer and CaCl2 as crosslinker. The concentrations of alginate used were 2%, and CaCl2 concentrations were 0.5 M (F1), 0.75 M (F2) and 1 M (F3).

Results: Results showed smooth and spherical microspheres for all formula with average particle size were 3.23 ± 0.05 μm (F1); 2.99 ± 0.07 μm (F2); and 2.86 ± 0.03 μm (F3). Mass swelling index at 24 h were 1.25 ± 0.10 (F1), 1.18 ± 0.11 (F2), and 1.11 ± 0.10 (F3); at 30 h were 2.00 ± 1.25 (F1), 1.85 ± 0.14 (F2), and 1.72 ± 0.15 (F3) while particle size swelling index at 24 h were 1.15 ± 0.10 (F1), 1.11 ± 0.10 (F2), and 0.97 ± 0.10 (F3); at 30 h were 1.81 ± 0.09 (F1), 1.73 ± 0.15 (F2), and 1.54 ± 0.14 (F3). Respectively yield percentages were 77.76 ± 6.49, 80.01 ± 3.53, and 82.97 ± 4.22. By using One Way ANOVA, it was found that there were significantly differences between three formulas.

Conclusions: The particle size of formulas decreased by increasing concentration of CaCl2, whereas no significant difference on swelling index and yield from microspheres with increasing CaCl2 concentration simultaneously.

Keywords: Ca-alginate microspheres; characterization; erythropoietin; ionotropic gelation.

Resumen

Contexto: Las microesferas tienen varias ventajas, incluyendo la protección de las proteínas contra la degradación y el aclaramiento después de la administración, y producen un efecto terapéutico a largo plazo. La eritropoyetina como un agente neuroprotector tiene propiedades tipo proteína, que son susceptibles de degradación y tienen baja biodisponibilidad in vivo. Las formulaciones de microesferas es uno de los posibles sistemas de administración de fármacos para la eritropoyetina.

Objetivos: Evaluar el efecto de la concentración de CaCl2 sobre las características (tamaño de partícula, morfología, índice de hinchamiento y rendimiento) de las microesferas de eritropoyetina y alginato.

Métodos: Las microesferas de eritropoyetina-alginato preparadas por el método de gelificación ionotrópica con técnica de aerosolización que usa alginato de sodio como polímero y CaCl2 como reticulante se secaron usando el método de lisificación con maltodextrina como lioprotector. Las concentraciones de alginato usadas fueron del 2%, y las concentraciones de CaCl2 fueron 0.5 M (F1), 0.75 M (F2) y 1 M (F3).

Resultados: Los resultados mostraron que las microesferas lisas y esféricas para todas las fórmulas con un tamaño de partícula promedio eran 3.23 ± 0.05 μm (F1); 2.99 ± 0.07 μm (F2); y 2.86 ± 0.03 μm (F3). El índice de hinchamiento masivo a las 24 h fue 1.25 ± 0.10 (F1), 1.18 ± 0.11 (F2), y 1.11 ± 0.10 (F3); a las 30 h fue 2.00 ± 1.25 (F1), 1.85 ± 0.14 (F2) y 1.72 ± 0.15 (F3) mientras que el índice de hinchamiento del tamaño de partícula a las 24 h fue de 1.15 ± 0.10 (F1), 1.11 ± 0.10 (F2) y 0.97 ± 0.10 (F3); a las 30 h fue 1.81 ± 0.09 (F1), 1.73 ± 0.15 (F2), y 1.54 ± 0.14 (F3). Respectivamente los porcentajes de rendimiento fueron de 77.76 ± 6.49, 80.01 ± 3.53 y 82.97 ± 4.22. Los valores presentaron diferencias estadísticamente significativas entre las tres fórmulas.

Conclusiones: El tamaño de partícula de las fórmulas disminuyó al aumentar la concentración de CaCl2, mientras que no hubo una diferencia significativa en el índice de hinchamiento y el rendimiento de las microesferas con el aumento de la concentración de CaCl2 simultáneamente.

Palabras Clave: caracterización; eritropoyetina; gelificación ionotrópica; microesferas de alginato de Ca.
INTRODUCTION

Erythropoietin is a 30.4 kDa molecular weight glycoprotein hormone consisting of 165 amino acids and has a carbohydrate content of 40% of the total erythropoietin molecule (Jelkman, 2004). Erythropoietin has a short half-life in the body, which is 4-9 h (intravenous) and >24 h (subcutaneously), requiring a frequent administration (Wolfgang, 2013). Erythropoietin has protein-like properties because their structures are similar to proteins. Proteins are susceptible to degradation during storage and have low in vivo bioavailability (Wang et al., 2013).

To overcome the problem, a microsphere delivery system was created. A microsphere is a 1-1000 μm structure made of one or more polymers mixed with molecularly or macroscopically-distributed drug particles (Dumitriu, 2002; Munmaya, 2016). Microspheres have several advantages, including protecting proteins from degradation and clearance after administration, may produce a long-term therapeutic effect, unlike conventional injections that can raise drug content in the blood immediately after administration but that which will quickly decrease (Wang et al., 2013). One of the polymers that can be used in a microsphere system is sodium alginate.

Natural polymers such as alginate are used as microsphere matrices due to their non-toxic, biodegradable, and biocompatible properties. The biodegradable polymers can degrade in the body into non-toxic degradants and thus do not cause problems with toxicity (Munmaya, 2016). To form a gel, a crosslinker of divalent cations such as Ca²⁺, Sr²⁺, or Ba²⁺ is needed. Ca²⁺ ion does not have the highest affinity with alginate, but Ca²⁺ ion is often used because of its low toxicity and because it is cheaper (Zhai, 2012). The mechanism of formation of Ca-alginate gel involves the dimerization of two units of G (guluronate) from opposite directions due to the addition of Ca²⁺ ion. This arrangement forms a diamond-like hole composed of hydrophilic cavities that bind Ca²⁺ ions through the oxygen atoms of the carboxylate group of G alginate units. As a result of this polymer interaction, there are many crossing areas, resulting in an egg-box shape. In the egg-box area, each calcium cation binds 4 units of G (Ching et al., 2015).

The microsphere manufacture method used was ionotropic gelation with aerosolization technique. The ionotropic gelation method has been widely used because the process is easy, simple, and can maintain the bioactivity of proteins contained in microspheres (Wang et al., 2013). In addition, this method does not use organic solvents and high temperatures, so protein integrity can be better maintained (Sapana et al., 2014). The aerosolization technique was chosen because they can produce smaller and more uniform particle sizes.

Microspheric characteristics include particle size, morphology, swelling index, and yield. The ideal microsphere characteristics are: having particle size of less than 5 μm (parenteral injection), spherical microsphere morphology with smooth and flat surface and high yield. The characteristics of the microspheres are influenced by the concentration of polymers and drug materials, time of manufacture of microspheres, temperature, and presence of additives such as crosslinking and surfactant stabilizers (Koukaras et al., 2012; Patil et al., 2012).

If the concentration of alginate is fixed and CaCl₂ concentration increases, as long as alginate is sufficient, then the crosslinking interaction with the polymer will increase and the more egg-box structures are formed so that the microspheres formed are more compact. This affects microspheres characteristics, such as microspheres size, spherical and flat microspheres morphology, a decrease the swelling index, and an increase in yield.

This study will observe effect of different CaCl₂ crosslinker concentrations of 0.5 M, 0.75 M, and 1 M on erythropoietin-alginate microspheres characteristics, including particle size, morphology, swelling index, and yield.

MATERIAL AND METHODS

Materials

Recombinant human erythropoietin (Daewoong Inc.), pharmaceutical grade sodium alginate (Sigma-Aldrich Inc.), pharmaceutical grade of CaCl₂·2H₂O, sodium citrate, Na₂HPO₄, KH₂PO₄, NaCl, NaOH, and maltodextrin were purchased from PT Bratachem, and demineralized water.
Instrumentation

Analytical balance (Chyo Balance Serial 51347), FT-IR spectrophotometer (Perkin Elmer Instrument), differential thermal apparatus/DTA (Mettler Toledo FT 900 Thermal System), sprayer with 35 μm holes (spray), centrifuge (Rotofix-32) stirring plate (Dragon Lab MS-Pro), magnetic stirrer, freeze dryer (Eyela FD-8), optical microscope (Axioscope 40 Zeiss), pH meter (Eutech Instrument pH 700), Scanning Electron Microscope (SEM, (Hitachi TM3000, Japan)), moisture analyzer (Mettler Toledo HB43-S).

Microspheres formula

The microspheres formula was shown in the Table 1.

<table>
<thead>
<tr>
<th>Material</th>
<th>Function</th>
<th>Microsphere formula</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
</tr>
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<tbody>
<tr>
<td>Erythropoietin</td>
<td>Active agent</td>
<td>5000 IU</td>
<td>5000</td>
<td>5000</td>
<td></td>
</tr>
<tr>
<td>Na Alginate</td>
<td>Polymer</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
<td></td>
</tr>
<tr>
<td>CaCl₂ solution</td>
<td>Crosslinker</td>
<td>0.5 M</td>
<td>0.75 M</td>
<td>1 M</td>
<td></td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>Lyoprotectant</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
<td></td>
</tr>
</tbody>
</table>

The values are written as value of concentration of each formula. Concentration is defined as international unit for erythropoietin; percentage for natrium alginate and maltodextrin and molar for CaCl₂.

Production of erythropoietin-alginate microspheres

Sodium alginate were dissolved in 100 ml demineralized water erythropoietin was dispersed into alginate solution and stirred until homogeneous. Erythropoietin-alginate solution was sprayed using aerosol spray with a hole size of 35 μm, constant pressure of 40 psi, and spray distance of 8 cm into 200 mL CaCl₂ solution (as per formula) and was continuously stirred for 30 min at a speed of 1000 rpm. Microspheres that were formed in centrifuges at a speed of 4000 rpm for 6 min were then washed using distilled water 2-3 times. The washed microspheres were suspended in a 5% maltodextrin solution, then dried with freeze drying at -45°C for 30 hours.

Evaluation of erythropoietin-alginate microspheres

FTIR spectrophotometry

The FTIR spectrophotometric evaluation was performed by the KBr pellet technique, by means of 1 mg of microspheres crushed with 300 mg of dried KBr powder, then compressed with a hydraulic press equipped with a steam enhancer to obtain a thin, light-permeable thin plate. Then, observations were performed at wave numbers 4000-500 cm⁻¹. The infrared spectra of the obtained microsphere formulas were compared with the infrared spectra of erythropoietin, sodium alginate, maltodextrin, and blank-alginate microspheres.

Thermal analysis

The thermal analysis of the microsphere was performed through DTA (Differential Thermal Analysis). Samples 3-5 mg were measured, then inserted into sample pan with a crucible aluminum type which has a maximum temperature of 350°C and closed. Then, the sample pan was inserted into the sample holder. Thermal analysis began with preliminary testing with a wide range (room temperature to decomposition temperature) at high heating rates (10°C up to 20°C/min) to uncover any unusual effects. Then, a re-test was performed with a narrower range at low heating rate (5°C/min).

Moisture content

The moisture content of the microspheres is determined using a moisture analyzer. This evaluation was done by weighing 0.5 grams of microspheres and insert them into the moisture analyzer tool to heat the sample and evaporate the moisture content of the sample. The heated microspheres were weighed. The moisture content was calculated using the following formula:

\[ MC(\%) = \frac{\text{initial microsphere mass} - \text{final microsphere mass}}{\text{final microsphere mass}} \times 100\% \]

Microspheres size distribution

The observation of erythropoietin-alginate mi-
Microsphere size distribution was performed on wet microspheres by optical microscope. The particle diameter of the microsphere of each formula was measured on 300 particles. Then, the average diameters were calculated using the following formula:

\[
(d_{vs}) = \frac{\sum nd^3}{\sum nd^2}
\]

Microsphere size distribution was determined by calculating PDI (polydispersity index) with the following formula:

\[
M_w = \frac{\sum (d^2 \times n)}{\sum (d \times n)}
\]

\[
M_n = \frac{\sum (d \times n)}{\sum n}
\]

\[
PDI = \frac{M_w}{M_n}
\]

Where \( n \) is the number of microspheres observed and \( d \) the microsphere diameter.

Microspheres morphology

To observe the morphology of erythropoietin-alginate, SEM was used.

Swelling index

The determination of swelling index was performed based on mass and particle size. This was performed by weighing 50 mg microspheres and then adding a 5 mL PBS pH 7.4 medium. Samples were measured in terms weight and particle size at 24 and 30 hours. The microspheres were filtered and stirred at 37°C for 2 h to remove the excess media on the surface of the microsphere. The swelling index was calculated using the following formula.

\[
\text{Swelling index based on mass:}
\]

\[
\text{Swelling index based on particle size:}
\]

Determination of yield

The yield value is determined by the ratio of the total weight of the dry microspheres obtained to the amount of weight of erythropoietin, sodium alginate, and maltodextrin. The formula for yield calculation is as follows:

\[
\text{Yield(%) = \frac{\text{total microspheres weight (mg)}}{\text{weight of erythropoietin + sodium alginate + maltodextrin (mg)}} \times 100}.
\]

Statistical analysis

To determine the effect of crosslinker concentration of CaCl₂ on the characteristics of erythropoietin-alginate microspheres, each data from particle size, swelling index and yield data were statistically analyzed using the one-way ANOVA through the IBM SPSS Statistics 23.0 program with a 95% confidence degree (\( \alpha = 0.05 \)). Then, the analysis proceeded with Tukey HSD (Honest Significant Difference) test to find out the formula that has significant difference.

RESULTS AND DISCUSSION

An examination using FTIR spectrophotometry was performed to determine the interaction between the drug, polymer, and other materials in the microsphere formula. The result of spectral observation of the three formulas in Fig. 1 shows the interaction between alginate polymer, CaCl₂ crosslinking solution, and maltodextrin. The absorption of specific group of O-H stretching of sodium alginate (3452.18 cm⁻¹) and maltodextrin (3432.15 cm⁻¹) fused into 3398.3 cm⁻¹ (F1), 3418.34 cm⁻¹ (F2), and 3398.8 cm⁻¹ (F3), indicating an interaction between sodium alginate and maltodextrin. This corresponds to the maltodextrin mechanism, which can stabilize the microsphere during the drying process by means of the hydroxyl group maltodextrin forming a hydrogen bond with the polar group on the surface of the microsphere at the end of the drying process and replacing the water position on the microsphere surface, maintaining the spherical structure of the microsphere (Like et al., 2015).

Two specific erythropoietin absorbances, namely C≡C alkyne and C=O stretching amide were still observed in all three formulas, showing that eryth-
Erythropoietin is absorbed in the microsphere system. In the spectra of the three formulas, the specific absorbance of the symmetric carboxylic salt group (1615.22 cm⁻¹) and guluronate fingerprint (948.31-820.29 cm⁻¹) disappear. The loss of absorption in carboxylic salt group and guluronate fingerprint was due to the formation of microspheres that occurred due to crosslinking interactions between alginate and CaCl₂ crosslinker, which involves the exchange of ions on the oxygen atom of the carboxylic group of guluronic acid alginate with Ca²⁺ ion of the crosslinker (Ching et al., 2015).

The evaluation of thermal analysis with DTA aims to determine the interaction of materials in the microsphere formulas characterized by the change in DTA thermogram profile. The results of the thermal analysis are shown in Table 2 and Fig. 2.

**Figure 1.** FTIR spectra of (A) erythropoietin, (B) sodium alginate, (C) maltodextrin, erythropoietin-alginate microspheres with different concentrations of CaCl₂ (D) 0.5 M, (E) 0.75 M, and (F) 1 M.
Table 2. Thermal analysis results (DTA).

<table>
<thead>
<tr>
<th>Melting point of raw materials (°C)</th>
<th>Formula</th>
<th>Melting temperature (°C)</th>
<th>ΔH (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na-alginate</td>
<td>Erythropoietin</td>
<td>CaCl₂</td>
<td>F₁</td>
</tr>
<tr>
<td>238.7</td>
<td>53</td>
<td>176.5</td>
<td>F₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F₃</td>
</tr>
</tbody>
</table>

The values are written as value of melting point of active agent, ingredient and formulas.

Erythropoietin has a melting point of 53°C (Christensen et al., 2016). Sodium alginate is a polysaccharide containing galactose and mannose sugar units. In the sodium alginate thermogram, a widening endothermic peak is visible, followed by a sharp exothermic peak at 238.7°C. The endothermic peak signifies the process of loss of water content in the hydrophilic polymer group, whereas the exothermic peak signifies the degradation process of sodium alginate (Tripathi and Mishra, 2012). The CaCl₂ thermogram results showed a sharp peak at 176.5°C, indicating the melting point of CaCl₂·2H₂O. The micro-

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sphere thermograms of the three formulas show the peak shift (endothermic) of the alginate thermogram and CaCl₂, namely to 158.2°C (F₁), 170.3°C (F₂), and 163.5°C (F₃) indicating that a microsphere has been formed (Table 2).

The moisture content is one of the parameters that need to be controlled. High moisture content in microspheres can cause particle agglomeration, thereby decreasing the stability of the active ingredient in the microspheres and forming particles with large sizes (Müller et al., 2014; Shan et al., 2016).

The results of determination of moisture content were 9.53 ± 0.18% (F₁), 9.17 ± 0.17% (F₂), and 9.45 ± 0.32% (F₃), indicating that the moisture content of the three formulas were less than 10%, which still satisfies the microsphere criteria.

The result of determination of mean diameter and polydispersity index is shown in Table 3. Blank microspheres or microspheres no medicinal ingredients are smaller in size than the formula microspheres due to the absence of additional erythropoietin during the microsphere manufacture process. Increased concentration of CaCl₂, crosslinker from formula F₁ (0.5 M) to F₃ (1 M) yielded smaller particle size and showed a significant difference between formulas (p = 0.000), with p values between the formulas as follows: F₁:F₂ (p = 0.003); F₁:F₃ (p = 0.000); F₂:F₃ (p = 0.050) (Hariyadi et al., 2014).

The decrease in particle size occurs as more CaCl₂ crosslinkers penetrate into the droplet and interact with the alginate polymer to form a more compact egg-box structure so that the particle size decreases. The PDI (polydispersity index) of the three formulas approached 1, indicating a uniformly shaped microsphere (De La Vega et al., 2013).

The mean diameters and polydispersity indices of erythropoietin-alginate microspheres show in the Table 3.

The morphological observation results of erythropoietin-alginate microspheres were obtained SEM and shown in Fig. 3. The microspheres produced in the three formulas are spherical with smooth and even surfaces.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Average diameter (μm)</th>
<th>Polydispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank F₁</td>
<td>3.01 ± 0.02</td>
<td>1.06 ± 0.08</td>
</tr>
<tr>
<td>Blank F₂</td>
<td>2.71 ± 0.10</td>
<td>1.07 ± 0.05</td>
</tr>
<tr>
<td>Blank F₃</td>
<td>2.34 ± 0.10</td>
<td>1.06 ± 0.02</td>
</tr>
<tr>
<td>F₁</td>
<td>3.23 ± 0.05</td>
<td>1.08 ± 0.02</td>
</tr>
<tr>
<td>F₂</td>
<td>2.99 ± 0.07</td>
<td>1.04 ± 0.01</td>
</tr>
<tr>
<td>F₃</td>
<td>2.86 ± 0.03</td>
<td>1.05 ± 0.01</td>
</tr>
</tbody>
</table>

The values are written as average index value mean ± SD of diameter particle size and polydispersity index of three formulas. Significant difference in particle size were found between formulas. Increased crosslinker concentration from formula F₁ to F₃, decreased the particle size. However, no significant difference in polydispersity index of all formulas. The significant values of average diameter size between the formulas is represented as follows: F₁:F₂ (p = 0.003); F₁:F₃ (p = 0.000); F₂:F₃ (p = 0.050).

Swelling index describes the capacity of the microsphere to absorb water and inflate (Shivhare et al., 2014). There are several parameters that influence swelling index, namely: molecular weight of polymer, polymer concentration, and pH of media (Andhariya and Burgess, 2016).

In this study, the alginate polymer used was of low molecular weight with a concentration of 2%, and the medium used was PBS pH 7.4, which described the subcutaneous pH of the site of administration. The swelling index was observed at 24 and 30 h to observe the microsphere swelling process for a long duration according to the target sustained release of the erythropoietin-alginate microspheres. Swelling index is determined by mass and particle size after swelling. Swelling index results are shown in Table 4. In the process of swelling of microspheres, there is a substitution of divalent cations of crosslinkers of microspheres by Na⁺ ions present on PBS pH 7.4 (Hariyadi et al., 2014).

Theoretically, increasing the concentration of crosslinking solution can decrease the swelling index as more egg-box structure is formed so that the crosslinker cation turnover of Na⁺ ions from the PBS medium is not as high at lower crosslinker concentrations (Kaur, 2013). However, statistically the
increase of CaCl₂ from 0.5 M to 1 M did not give significant difference to swelling index with \( p = 0.328 \) (swelling index at 24 h); \( p = 0.120 \) (swelling index at 30 h) based on mass and \( p = 0.144 \) (swelling index at 24 h); \( p = 0.099 \) (swelling index at 30 h) based on particle sizes. The result of determination of swelling index of erythropoietin-alginate microspheres show in Table 4.

At neutral pH, alginate polymers have a swelling drug release mechanism followed by erosion (Liang et al., 2015). When the swelling process reaches the maximum, the alginate will experience erosion. Based on the observation of swelling index at 24 to 30 h the microspheres still experience an increase of swelling index, which indicates that the swelling process is still happening, so it is expected the drug material can still be released slowly and a sustained release effect is achieved.

The yield values of the microspheres were 77.76 ± 6.49% (F1); 80.01 ± 3.53% (F2); and 82.97 ± 4.22% (F3) (Table 5). Increased concentration of CaCl₂ crosslinker can increase the yield value, because the higher the CaCl₂ the more alginate interacts with CaCl₂ to form egg-box structures which results in higher microspheres (Hariyadi et al., 2014). However, statistically the yield increase of the formula F1 to F3 is not significant with \( p=0.473 \). The result of yield show in the Table 5.

**CONCLUSIONS**

Erythropoietin-alginate microspheres were successfully prepared by the ionotropic gelation method of aerosolization technique with increased concentration of CaCl₂ from 0.5 M to 1 M. Influence of increasing CaCl₂ concentration on the characteristics of erythropoietin-alginate microspheres were shown such as a decreased of particle size and spherical microspheres with smooth surfaces were formed. However, it was found that no significant effect of the crosslinker concentration on the swelling index and yield values.
Table 4. Swelling index of erythropoietin-alginate microspheres.

<table>
<thead>
<tr>
<th>Formula</th>
<th>t = 24 h (± SD)</th>
<th>t = 30 h (± SD)</th>
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</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.25 ± 0.10</td>
<td>2.00 ± 1.25</td>
</tr>
<tr>
<td>F2</td>
<td>1.18 ± 0.11</td>
<td>1.85 ± 0.14</td>
</tr>
<tr>
<td>F3</td>
<td>1.11 ± 0.10</td>
<td>1.72 ± 0.15</td>
</tr>
</tbody>
</table>

The values are written as index value mean ± SD of swelling index of three formulas. No significant difference in swelling index of formula 1 compared to formula 2 and formula 3. All formulas produced as same as swelling index both based on mass and size.

Table 5. Yield of erythropoietin-alginate microspheres.

<table>
<thead>
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<th>Formula</th>
<th>Yield (%)</th>
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<tr>
<td>F1</td>
<td>77.76 ± 6.49</td>
</tr>
<tr>
<td>F2</td>
<td>80.01 ± 3.53</td>
</tr>
<tr>
<td>F3</td>
<td>82.97 ± 4.22</td>
</tr>
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</table>

The values are written as percentage mean ± SD of yield of three formulas. No significant difference in yield of formula 1 compared to formula 2 and formula 3. All formulas produced as same as yield.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contribution:

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