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Formulation and evaluation of erythropoietin-alginate microspheres at different amount of drug

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ABSTRACT

This research formulate erythropoietin-alginate microspheres and to evaluate characteristics of erythropoietin-alginate microspheres at different amount of drug using aerosolization. Amount of erythropoietinare 10,000 IU (F1); 20,000 IU (F2); 60,000 IU (F3). The mixture of erythropoietin-alginate was sprayed into $CaCl_2$and was stirred at 1000 rpm for 30 minutes. Formulas resulted spherical shape of microspheres. The size of microspheres was 2.77 µm for F1; 3.89µm for F2; and 4.42µm for F3. The results of swelling index showed that swelling index of microspheres increased by increasing the concentration of erythropoietin. The results were in accordance with the size of the microspheres that increased with increasing concentration of drug. The yields of microspheres obtained were respectively 91.92%; 87.53%; 86.50% for F1, F2 and F3. It can be concluded that the particle size of microspheres, swelling index increased by increasing concentration of erythropoietin. In contrast, yield of microspheres decreased by increasing drug concentration. In conclusion, formulas of microspheres were potential in terms of characteristics and may recommend for further in vivo study.

Keywords: erythropoietin, alginate microspheres, drug concentration, aerosolization

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INTRODUCTION

Erythropoietin is a 30.4 kDa glycoprotein hormone which is the main regulator of red blood cell production (Prenggono, 2015). Erythropoietin has a protective role against tissue ischaemia (Pachos, 2008). Erythropoietin, which is a protein, has several weaknesses, such as unstable in biological fluids because of easily digested by enzymes, cannot be fully absorbed from the digestive tract due to a high molecular weight resulting to low bioavailability, and has short half-life (Wang *et al*., 2013). Erythropoietin is stable at pH 4-<9 and has the highest activity at pH 7-8 (Chang *et al*., 2013). Erythropoietin is also stable at -20°C or -70°C (Chang *et al.*, 2013). At pH 9 and 40 °C, erythropoietin will undergo dimerization to form large molecules that can induce the body's immune response, which can harm patients (Chang *et al*., 2013). Some alternative is by formulatemicrospheressystem to release the drug in a sustained release manner (Chaudhary *et al*., 2010).

Microspheres are a drug delivery system in which the drug is diluted or dispersed homogeneously in the polymer matrix (Kumar *et al*., 2011). The advantage of a microsphere delivery system is that the drug's release properties in the body occur gradually, making it suitable for carrying the necessary drugs into the body with a fixed amount. In addition, the microspheres can also protect peptide/protein drugs from hydrolytic enzyme attacks (Takada, 2008).

The manufacture of erythropoietin microspheres by ionic gelation method and aerosolization technique requires polymers and crosslinking solutions. One of the most commonly used natural polymers is alginate.

Alginate is a polysaccharide which is a salt of alginic acid, comprising a monomer (1-4)-β-D-manuronic acid (unit M) and α-L-guluronic acid (unit G) that varies in quantity and distribution along its polymer chain (Pal *et al.*, 2013). Na alginate is widely applied for biomedical purposes as a drug delivery system due to its biocompatibility, biodegradablity, low toxicity, relatively low cost, and lighter gelation by the addition of divalent cations such as Ca^{2+} (Hariyadi *et al*., 2014).

The frequently used crosslinking cations are divalent and trivalent cations (Ahirrao *et al*, 2014). The addition of monovalent metal ions to the alginate will form a soluble salt (Lee and Money, 2012). Pb, Cu, Cd cations are rarely used because they have some toxicity (Zhai, 2012). The use of Ba and Sr cations should be in low concentration as they are slightly toxic (Zhai, 2012). Ca^{2+}cations are often used because of their low toxicity and lack of high affinity for alginates (Zhai, 2012).

The ionic gelation method is based on the ability of the polyelectrolyte to be crosslinked in the presence of crosslinking ions forming a hydrogel. This method is prepared by dripping the drug-polymer solution into a cation polivalent solution. The cation diffuses into the droplets of a polymer solution, forming a three-dimensional crosslinked formation (Patil *et al*., 2012). The ionic gelation method is suitable for the active ingredients of proteins because of the simple and light process, which is greatly helpful for maintaining the bioactivity of the proteins (Koppolu *et al*., 2014). The advantage of the ionic gelation method is that all polyelectrolytes are diluted in the water so that the protein can be encapsulated without the use of organic solvents and the ionic gelation method does not use high temperatures so that protein integrity is maintained. In addition, this method is considered simple, fast, cost-effective, and has a small particle size diameter (Hariyadi *et al*., 2014).

Microspheres that have been formed will undergo the drying process. The drying process of microspheres in this research was the freeze drying technique, because it is able to maintain protein integrity during the drying process (Abdelwahed *et al*., 2006). The freeze drying process requires pressure during the freezing and drying processes, requiring a lyoprotectant (Abdelwahed *et al*., 2006). A lyoprotectant can stabilize the microsphere through the mechanism of hydrogen bond formation with polar groups on the surface of the microsphere at the end of the drying process in order to maintain the microsphere structure and replace the water's position on the microsphere surface (Abdelwahed *et al*., 2006). One of the usable lyoprotectants is maltodextrin, because it protects protein stability during the freezing and drying (Hariyadi *et al*., 2014)

Factors that may affect the characteristics of the resulting microsphere are the number of polymers, the number of crosslinkers, the amount of drug ingredients used, and the crosslinking time

(Jin *et al*., 2009). The higher the concentration of the drug, the higher the microspheres size that is obtained (Wang *et al.*, 2013). In a study conducted by Balasubramaniam *et al*. (2007) on the effect of drug concentration on microsphere size using the emulsification technique, it was found that the greater the concentration of drug used, the greater the microsphere size that is obtained. This was because the higher the concentration of the drug, the greater the droplet size produced, resulting in the larger size of the formed microsphere (Balasubramaniam *et al*., 2007). The microspheres size range generated from the study was 47-50 μm (Balasubramaniam *et al*., 2007). In addition, increasing the concentrations of medicinal ingredients may also increase the efficiency of encapsulation (Balasubramaniam *et al*., 2007). The differences of the research conducted by Balasubramaniam *et al*. (2007) with this research is on aerosolization technique used in method of produced microspheres, therefore it is expected that the microsphere particle size formed is <5 μm. The determination of target size of microspheres of <5 μm is adjusted to the particle size requirement for suspensions (Patel, 2010).

This study was conducted to determine the effect of erythropoietin amount(10,000, 20,000, 60,000 IU) on erythropoietin-alginate microspheres characteristics using ionotropic gelation method and aerosolization technique.

MATERIALS AND METHODS

Materials

Pharmaceutical grade Erythropoietin (Daewoong Inc.), Sodium alginate (Sigma-Aldrich Inc.), $CaCl_2.2H_2O$ (Solvay Chemicals International), demineralized water, pro-analysis Na_2HPO_4 (Merck), KH_2PO_4 (Merck), NaCl (Merck), NaOH (Merck), and pharmaceutical grade Maltodextrin (PT Bratachem).

Research Method

Microspheres formula

Sodium alginate (2 gram) were dissolved into 100 mL of demineralized water. Erythropoietin was dispersed into 100 mL of alginate solution based on drug concentration. After that, $CaCl_2$ $solution_2$ 1M was made in 200 ml aqua demineralized by stirring homogeneously. The formed erythropoietin-alginate solution was then sprayed into $CaCl_2$ solution using aerosol spray with a 35 μmhole size and a constant pressure of 40 psi while being continuously stirred using a magnetic stirrer at 1000 rpm for 30 minutes. The microspheres suspension that had been formed was centrifuged at 4000 rpm for 10 minutes, then washed with demineralized water 3 times to separate the microspheres from the $CaCl_2$ solution. The washed microspheres were collected and dried using the freeze drying technique at -26°C for 38 hours.

Table I. Erythropoietin-alginate microspheres formulas

Materials	Function	F1	F2	F3
Erythropoietin	Active agent	10.000 IU	20.000 IU	60.000 IU
Na Alginate	Polymer	2% (in 100ml)	2% (in 100ml)	2% (in 100ml)
$CaCl_2$	Crosslinker	1 M(in 200 ml)	1 M (in 200 ml)	1 M (in 200ml)
Maltodextrin	Lyoprotectant	5% (from wet microspheres weight)	5% (from wet microspheres weight)	5% (from wet microspheres weight)

Evaluation of erythropoetin-alginate microspheres

Particle size distribution

The evaluation of erythropoietin-alginate microsphere size distribution was performed using 400x optical microscope magnification. The diameter of erythropoietin-alginate microspheres was observed with a 400x magnification of 300 particles. Then, the mean diameter was determined and the microsphere size distribution curve was made. The average diameter was calculated using the formula:

$$dvs = \frac{nd^3}{nd^2}$$

Microspheres shape and surface morphology

The morphological evaluation of erythropoietin-alginate microspheres and surface forms was performed using a Scanning Electron Microscope (SEM). This evaluation was performed by placing a sample on the handle of the preparation with an adhesive material containing metal grains, such as the metal Pt. The gold in the Chamber was evaporated so that the gold steam coated the entire surface of the microparticles. The surface of the gold-coated microparticles was observed using SEM to observe the morphology of the shape and surface of the microspheres.

Moisture content determination

The moisture content determination on erythropoietin alginate microspheres can be determined using the moisture balance. This evaluation can be performed by weighing 0.5 grams of microspheres and inserting them into the moisture balance. The tool would work for 10 minutes. The moisture content (MC) was calculated by the equation: $MC = \frac{Initial\ weight - Final\ weight}{Final\ weight}\ x\ 100\%$

Determination of Swelling Index (SI)

The determination of swelling index was performed by weighing 50 mg microspheres and then adding 5 ml PBS pH 7.4 in a vial. The swelling index determination was performed at 24 and 30 hours. After the observation time had been reached, the wet microspheres were filtered using filter paper. After no PBS was dripped from the filter paper, the wet microspheres were transferred to a dry filter pad and then the microspheres were flipped continuously to remove the adsorbed water on the microsphere surface until the filter paper became not too wet. Afterwards, the wet microspheres were stirred at 37^{o}C for 2 hours or until the microspheres weight became constant. The microspheres was weighed as the swelling weight. The swelling index (SI) value was calculated using the formula:

$$SI = \frac{Swelling\ weight - Dry\ weight}{Dry\ weight}$$

The swelling index observation was also performed based on the microsphere ssize. A little amount of microsphere were taken to be observed for microspheres size during swelling using an optical microscope. The swelling index (SI) value was calculated using the formula:

$$\frac{Swelling\ microsphere\ size - Dry\ microsphere\ size}{Dry\ microsphere\ size}$$

Yield determination

The yield value was 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 yield value can be calculated by the formula:

$$Yield(\%) = \frac{total\ microspheres\ weight\ (mg)}{alginate + erythropoietin + maltodextrin\ weight\ (mg)} \times 100\%$$

The data from each examination were compared with each of the formulas. The data analysis was performed using SPSS 23 statistical program using the One Way ANOVA method.

RESULTS AND DISCUSSION

The mean diameter for blank microspheres was 2.31 µm and the mean diameters of F1 microspheres, F2, F3 using 400 times magnification were 2.77 ± 0.08 µm; 3.89 ± 0.12 µm; and 4.42 ± 0.06µmrespectively (Figure 1). These results indicated that blank microspheres were smaller than the three formulas microspheres with erythropoietin. The observation also showed an increase in the size of the microsphere as the levels concentration of erythropoietin from F1 to F3 increased with the values of 10,000, 20,000, and 60,000 IU. The average microspheres diameter result met the specification of <5µm, where the size is considered good for a parenteral suspension (Patel, 2010).

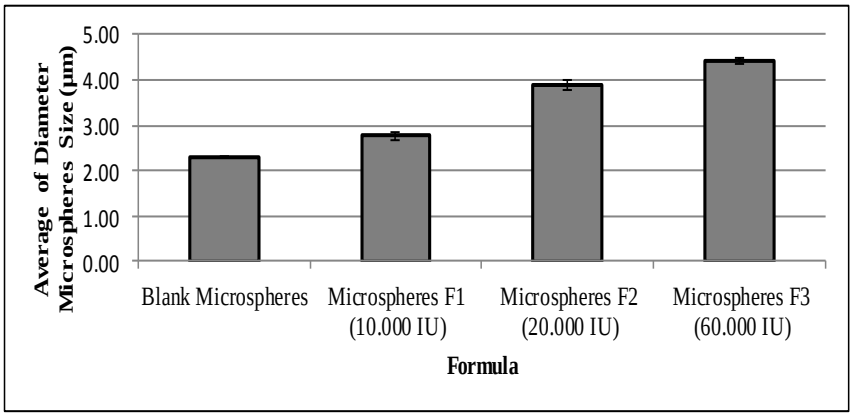
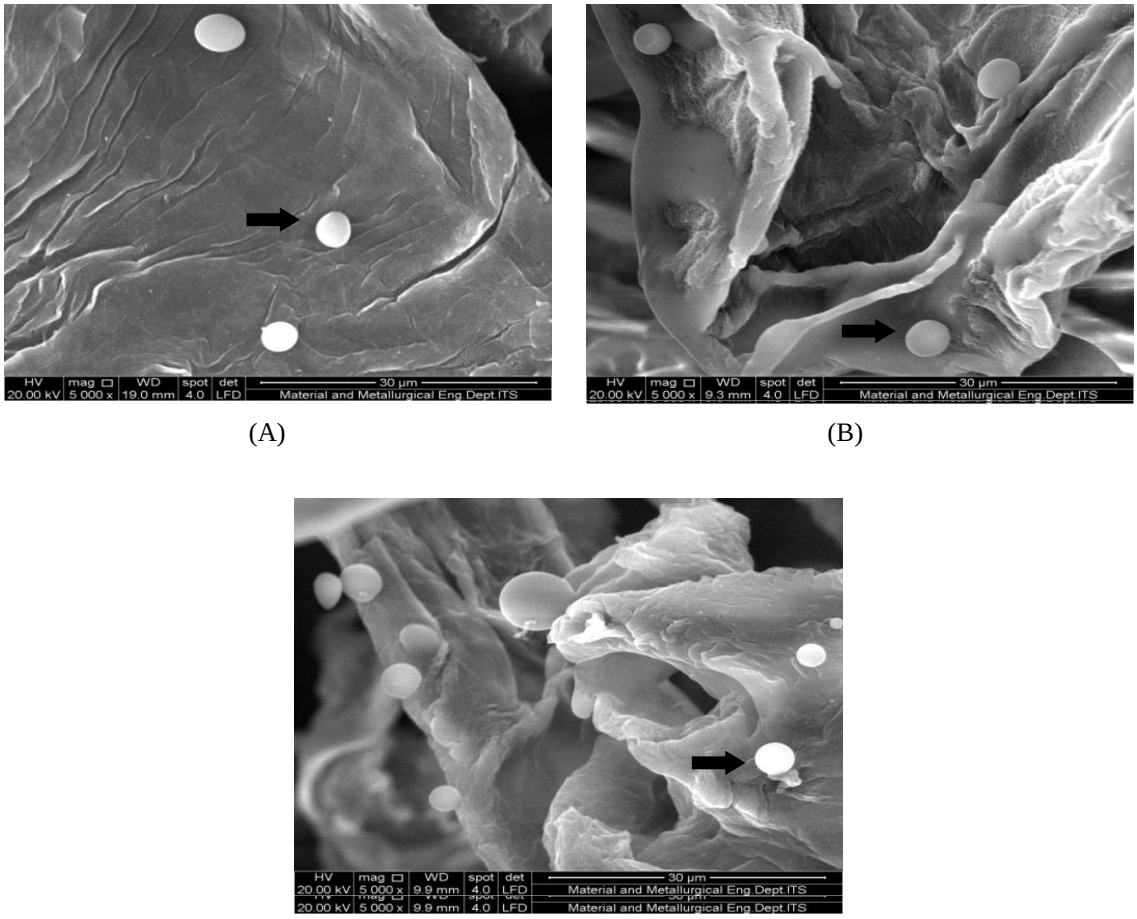


Figure 1.Histogram of diameters of microspheres sizes of blank microspheres and microspheres of F1, F2 and F3

The microspheres size increased by addition of erythropoietin levels was due to two factors. First, the higher the amount of erythropoietin, the more erythropoietin can filled the remaining empty egg-box structures. Second, there was a repulsive force between erythropoietin and alginate. Alginate as a bioelectrolyte can caused repulsive force with erythropoietin. The repulsive force occurred between the COO^-group of alginate with the COO^- group of erythropoietin. The larger the erythropoietin, the greater the repulsive force that occurs with alginate. The existence of such repulsive forces may increase the viscosity, resulting in increased microsphere size (Chaudhari *et al*., 2015).

In the analysis of the effect of erythropoietin levels on microspheres, sig values of 0.000<0.05 for F1:F2; 0.000<0.05 for F1:F3; and 0.001 <0.05 for F2:F3 were obtained. The three comparisons of the formula showed sig values of <0.05, so we can say there is a significant difference in microsphere size between F1, F2, and F3.

The morphological observations of the shape and surface of the microspheres using the Scanning Electron Microscope (SEM) of the F1, F2, F3resulted in spherical and smooth shapes (Figure 2). These results can be obtained due to the addition of maltodextrin as a lyoprotectant which made the form of the microsphere to be spherical with a smooth and flat surface. The maltodextrin would filled the cavity on the surface of the microsphere and form the hydrogen bond with the polar group on the microsphere surface during the freeze drying process (Abdelwahed *et al*., 2006).



(A) (B)

(C)

Figure 2. The shape and surface morphology of the erythropoietin microspheres (A) F1, (B) F2, (C) F3 observed using the ScanningElectron Microscopy at 5000x magnification

The result of MC determination showed that the average of water content in the three formulas were 5.85 ± 0.10%; 5.08 ± 0.13%; and 8.08 ± 0.21% as seen in Table I. The MC of three formulas corresponded to specifications of MC of <10%. The high moisture content can decreased the stability of the active ingredients in the microspheres due to the formation of particle agglomeration (Shan *et al.*, 2016).

Table II. Moisture content of microspheres formula

Microspheres Formula	MC (%) ± SD	CV (%)
F1	5.85 ± 0.10	1.71
F2	5.08 ± 0.13	2.56
F3	8.08 ± 0.21	2.60

From the determination of swelling index, the average swelling index at 24 hours based on the weights of microspheres in formula F1, F2 and F3 were 1.300 ± 0.071; 1.463 ± 0.046; 1.749 ± 0.044, while during the observation at 30 hours, the average swelling index based on the weight of the microsphere in the formula F1, F2, and F3 were 1.867 ± 0.056; 2.272 ± 0.154; 2.475 ± 0.189. The average swelling indices at 24 hours based on microspheres sizes in the formula F1, F2, and F3 were 1.13 ± 0.10; 1.37 ± 0.05; 1.67 ± 0.04, respectively. Meanwhile, during the observation at 30 hours, the

average swelling indices based on the microspheres size in formulas F1, F2, and F3 were 1.85 ± 0.04; 2.07 ± 0.12; 2.28 ± 0.15.

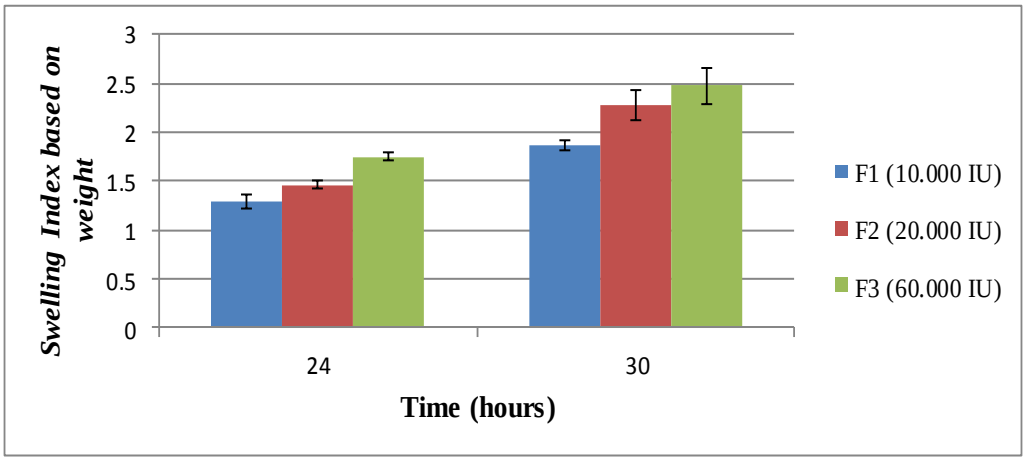


Figure 3. The swelling index histogram based on weight of microspheres F1, F2, F3

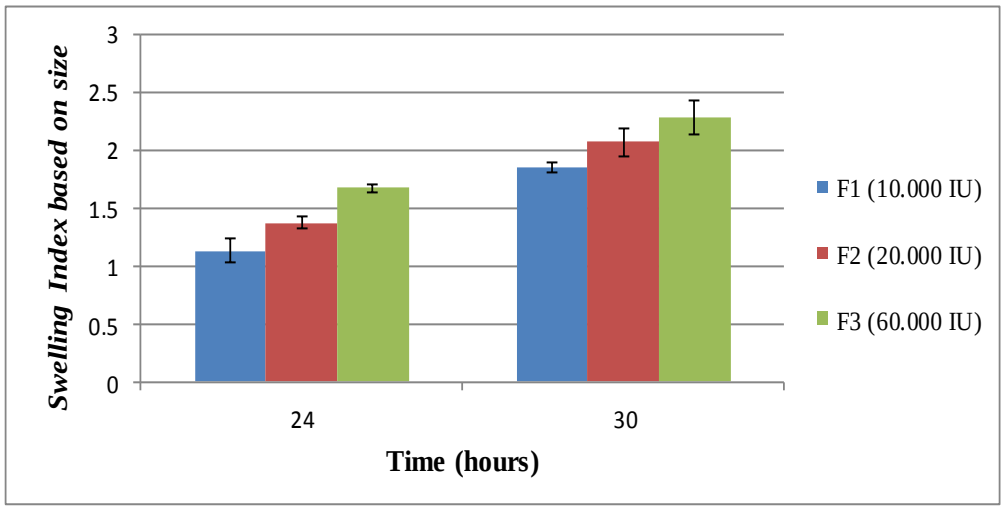


Figure 4. The swelling index histogram based on the size of microspheres of F1, F2, F3

The average swelling indices based on microsphere weight at 24 hours and 30 hours yielded gave greater result than swelling indices based on microspheres sizes. However, if it was tested statistically using the independent t-test, the difference of swelling indices between microsphere weight and microsphere size was not significant due to the 2-tailed sig value > 0.05. The increase of swelling index values at 24 to 30 hours based on the weight and size of microspheres can be said to differ significantly because the independent t-test results showed that the 2-tailed sig value < 0.05.

When we looked at the average swelling index results, both based on microsphere weight and microsphere size, it seems that both yielded the same resulted that the swelling index value increased with the increased of the ingredients added in the formula. This corresponded to the average yield of microspheres in which microsphere sizes increased with the ingredients added in the formula. The swelling index results based on microsphere weight and microspheres size were also in accordance

with the theory, where the higher the observation time, the higher the swelling index value was obtained (Navneet *et al*., 2011).

The swelling index evaluation was used to ensure the polymer can expand, then it can released drug. Alginate, in releasing drug substances, had diffusion, swelling, and erosion mechanisms (Fernanda *et al.*, 2014). The results of the evaluation have been found to be in accordance with the theory in which alginate can expand, as evidenced by the addition of weight and size of microspheres after being put aside for 24 and 30 hours.

From the result of yield determination, the average yields obtained in formula F1, F2, and F3 were 91.92 ± 3.33%; 87.53 ± 6.01%; 86.50 ± 4.59%. From these results, it is known that the value of the yield fallen along with the increase of the ingredient content of the drug added in the formula. This was because microspheres have entrapment capacities in entrapping drug ingredients (Rastogi *et al*., 2007). The increasing yield values indicated that erythropoietin with a level of >10,000 IU is not entirely entrapped in the microspheres and was dissolved when washed with water since erythropoietin was also soluble in water. The results of statistical analysis showed that the effect of erythropoietin content on yield was mas determined by sig values of 0.534>0.05 for F1: F2; 0.403> 0.05 for F2: F3; and 0.963> 0.05 for F2: F3. The three comparisons of the formula showed sig values of < 0.05, so we can say there is a significant difference in microspheres size between F1, F2, and F3.

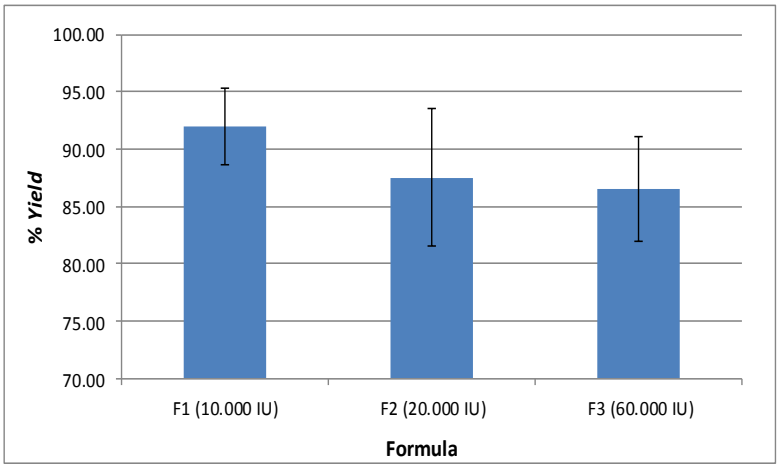


Figure 5. Histogram of % yield of microspheres of F1, F2, F3

CONCLUSION

The formulation of erythropoietin-alginate microspheres by the ionic gelation method and aerosolization technique with different erythropoietin amount (10,000, 20,000, 60,000 IU) resulted in microspheres which were spherical and smooth. Increasing levels of erythropoietin increased the size of the microspheres and swelling index significantly, but did not have a significant effect on yield.

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