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Effects of Sonicator Usage Time on Entrapment Efficiency of Liposome Magnesium Ascorbyl Phosphate Made by Thin Layer Hydration Method.

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ABSTRACT

Magnesium Ascorbyl Phosphate (MAP) is one of derivatives of vitamin C which is more stable in water. MAP hydrophilic nature makes it difficult to penetrate the stratum corneum. Therefore, to increase the penetration, MAP is encapsulated in a liposome system. The sonication can be used to regulated the vesicles size and increase the entrapment efficiency. The purpose of this research was to investigate the effects of sonicator usage time on entrapment efficiency. The thin layer hydration method was used to prepare MAP liposomes with a ratio of lecithin and cholesterol in 30: 1, respectively. The variations of sonicator usage time were 10,15 and 20 minutes. Characterization of liposomes was observed by morphology, vesicle size and drug entrapment studies. The results showed that MAP entrapment efficiency percentage consecutively was 63.4705%; 70.4261%; and 78.0869%; while the size obtained is 466.9 nm; 298.6 nm; and 179.4 nm. One-way ANOVA test results showed a significant difference in each treatment. It can be concluded that the increasing sonicator usage time can decrease vesicle size and increase entrapment efficiency of MAP liposomes.

Keywords: Liposomes, Magnesium Ascorbyl Phosphate, Sonication, Sonicator

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INTRODUCTION

Drug delivery system is an important part of the drug development and treatment. Some examples of targeted particulate drug delivery systems, among others, microparticles, nanoparticles, microcapsules, microspheres, micelles, lipoproteins, and liposomes [1]. However, from some of these delivery systems, liposomes can only provide preparation characteristic similar to biological membranes of the body, to localize the drug at the target, and can improve delivery efficiency into the cytoplasm [2]. Almost all the drug characters can be encapsulated in liposomes to make modifications on the preparation [3].

Drug delivery systems such as liposomes, may affect the drug in the body since during the delivery to the workplaces, drugs are often confronted with obstacles and interference thereby reducing the efficacy [4]. Vitamin C has the primary efficacy as an antioxidant, vitamin C is a hydrophilic compound so that its penetration ability is very low. Vitamin C formulated into the liposome system aims to increase the penetration into cells. Magnesium Ascorbyl Phosphate is a derivative of vitamin C which is soluble and stable in the water [5].

In previous studies, the best formula of lecithin and cholesterol was combination 30: 1, which produces liposome in the form of spheric vesicles with the size of 70-800 nm [3]. Thin layer hydration method is the most widely used method in the preparation of liposome system, it is related to the evaporation certainty of organic solvents and its simpler method compared to other methods. Liposome size and entrapment efficiency have an important role in the achievement of the drug into the target. There are several ways that can be used to reduce the liposome size and narrowed size distribution ranges, for example by extrusion through a carbonate filter membrane, high speed mixing or by ultrasonication [6]. Based on this background, the research about MAP liposomes was conducted by thin layer hydration method using the best formula as the previous study. Based on the formula, variation of the sonicator usage time can decrease vesicle size and increase entrapment efficiency of MAP liposomes.

MATERIALS AND METHODS

The instruments were Rotary Evaporator (Eyela n-1000), Spectrophotometer UV-Vis (Shimadzu), Analytical Balance (Mettler Toledo), Particle Size Analyzer (Delsa Max), Sonicator (LAB. Companion), vortex mixer, Microcentrifugation refrigerator (BioLion XC-HR20), SEM (Zeiss EVO MA10), and glass tools.

The materials were lecithin (Natural Sourching), cholesterol (Kanto chemical), magnesium ascorbyl phosphate (Spec-Chem Ind.), nitrogen gas, diethyl ether (Merck), sodium hydroxide (Asahimas chemical), potassium hydrogen phosphate (Merck), ethanol absolute (Merck), and distilled water.

EXPERIMENTAL

Preparation of Phosphate Buffer solution pH 7.0 and pH 7.4 [7]

Phosphate buffer solution pH 7.0 was prepared by mixing 50 ml of 0.2 M potassium hydrogen phosphate with 29.10 ml of 0.2 N sodium hydroxide in 200 ml volumetric flask, then the volume was fixed little by little with CO_2-free distilled water to 200 ml.

Phosphate buffer solution pH 7.4 was prepared by mixing 50 ml of 0.2 M potassium hydrogen phosphate with 39.10 ml of 0.2 N sodium hydroxide in 200 ml volumetric flask, then the volume fixed little by little with CO_2-free distilled water to 200 ml. Previously, potassium dihydrogen phosphate 0.2 M was made by weighing 6.8045 g of potassium dihydrogen phosphate powder, then dissolved with CO_2-free distilled water in a 250 ml volumetric flask, and the volume was fixed little by little until the volumetric flask limit. In the preparation of 0.2 N sodium hydroxide, sodium hydroxide was weighed as much as 8.0 g and then dissolved in 1000 ml of CO_2-free distilled water.

Determination of MAP Maximum Wavelength in a Phosphate Buffer solution pH 7.0

Magnesium ascorbyl phosphate was weighed as much as 100 mg and dissolved in phosphate buffer pH 7.0 to 100 ml, this solution was referred to as a standard solution. Pipetted as much as 1 ml of standard solution, then diluted with phosphate buffer solution pH 7.0 to 50 ml, then MAP standard solution with a concentration of 20 ppm was obtained. Spectrophotometer was set at a wavelength of 200-400 nm [6]. Standard solution created previously then was put in a cuvette to read the spectrum, in order to obtain the maximum wavelength of MAP at 258.4 nm.

Preparation of MAP solution calibration curve in phosphate buffer solution pH 7.0

Spectrophotometer was set at the maximum wavelength of 258.4 nm. MAP solution prepared in phosphate buffer with five different concentrations that was 6, 12, 18, 24 and 30 ppm. Each MAP solution was measured its absorbance alternately by a spectrophotometer at a wavelength of 258.4 nm. Absorbance obtained from five concentration of MAP solution then recorded and the calibration curve was made.

Preparation of Liposomes Using Thin Layer Hydration Method and Evaluation

Table 1. Magnesium Ascorbyl Phosphate Liposome Formula

Ingredient	Formula	Function
Magnesium Ascorbyl Phosphate (MAP)	50 mg	Active Pharmaceutical Ingredient
Lesitin	600 mg	Lipid Phase
Kolesterol	20 mg	Lipid Phase
Dietil Eter	7.5 ml	Organic Solvent
Phosphate buffer	10 ml	Water Phase

Liposomes was prepared by using thin layer hydration method. Weighed the lecithin and cholesterol then dissolved in diethyl ether 7.5. Evaporation of diethyl ether solution by rotary evaporator tool at 45°C with 120 rpm and vacuum condition to vaporize diethyl ether until a thin layer is formed [8]. Flowed the nitrogen gas in the thin layer formed on the round flask [9] let it for 24 hours. Dissolved the MAP in phosphate buffer solution at pH 7.4. Hydrate the thin layers in the flask with 10 ml phosphate buffer pH 7.4 containing of MAF until all thin layers exfoliated from the flask wall, with rotary evaporator temperature at 60° C for 1-2 hours to form a yellow suspension. Homogenized the suspension by inserting it in the vortex tube with a speed of 100 rpm for 2 minutes [10].

Liposomes was as much as 50 ml for each treatment (sample). The first sample inserted in the sonicator for 10 minutes (Treatment I), the second sample was inserted in a sonicator for 15 minutes (Treatment II), and the third sample was inserted in the sonicator for 20 minutes (Treatment III) with the aim to minimize and uniform liposome size. After that the evaluation of liposomes containing Magnesium Ascorbyl Phosphate (MAP) was conducted.

Determining Liposomes Morphology or Form

The morphology or physical form of the liposomes was determined by using Scanning Electron Microscope (SEM). For the use of SEM, the samples were prepared by means of freeze dry process, it aimed to dry the sample. After the samples were dry, the samples coated with Au metal as a conductor. Further, the samples installed into the specimen room. After that the samples were analyzed. A picture would appear automatically after the specimen was set in the specimen room and the vacuum had been ready [11].

Determination of Liposomes Size Distribution

The particle size distribution was determined by the particle size analyzer. The tool was on and waited until all the tool parameters were ready for use had been fulfilled. Samples were inserted into the cuvette, and

the cuvette is inserted into the particle size analyzer tool. The tool was set for sample reading for 3 minutes, then running the tool till the results was read on a computer monitor. Then, the data obtained was stored [12].

Liposomes purification and entrapment efficiency calculation

The liposomes was purified by multilevel centrifugation with microcentrifugator to separate the entrapped and not entrapped active substances at a speed of 18,000 rpm for 30 minutes at a temperature of 4ºC in a vacuum [10], then the supernatant and precipitates were separated. Supernatants then were recentrifuged for 30 minutes, the supernatant and precipitates were separated again. Supernatant obtained then was recentrifuged for 15 minutes. Through the centrifugation process a separation between the supernatant and the precipitate was formed.

Supernatant part then was diluted the concentration. Dilution was done by adding a phosphate buffer pH 7.0 in a 10 ml flask until the absorbance obtained from the solutions was in the range of 0.15 to 0.85. After dilution was completed, then the absorbance was measured alternately by UV-Vis spectrophotometer at a wavelength of 258.4. Precipitate part was dissolved in absolute ethanol and then inserted in the sonicator for 10 minutes [13] it aimed to pull out the entrapped MAP from liposomes vesicles, then the concentration was diluted with a solvent concentration phosphate buffer pH 7.0 in a 10 ml flask. The absorption obtained was inserted into the equation of the calibration curve to obtain the concentration of magnesium ascorbyl phosphate entrapped within liposomes. The entrapment efficiency was calculated as [14] :

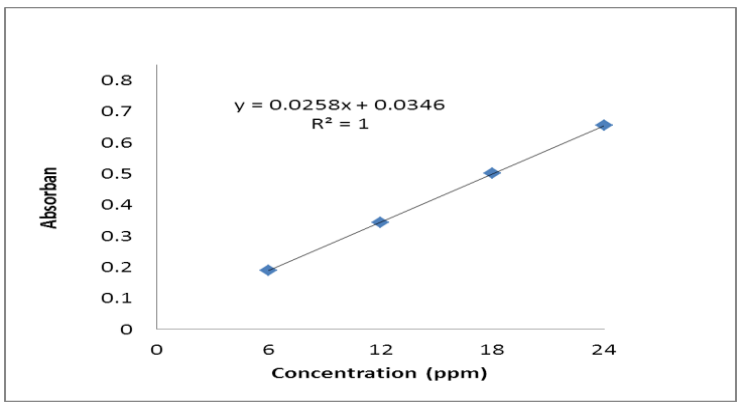
$$\% EP = (xj/xt) X 100\% \quad \text{..... (1)}$$

EP **:** entrapment efficiency
xj : entrapped drugs
xt **:** total drug

The entrapment efficiency data and the vesicles size were analyzed using one-way variance analysis test (ANOVA) by comparing the entrapment efficiency and the vesicle size to the sonicator usage time at each treatment. Then followed by Tukey test to see the significant differences between each treatment period of sonicator usage.

RESULTS AND DISCUSSION

Based on the determination of the maximum wavelength of MAP in phosphate buffer solution pH 7.0 at a concentration of 20 ppm, the maximum wavelength of 258.4 nm with absorbance 0.5394 is obtained. After the determination of MAP wavelength, the calibration curve of MAP in phosphate buffer solution pH 7.0 is determined. The solution is prepared by 5 different concentrations i.e., 6 ppm, 12 ppm, 18 ppm, 24 ppm and 30 ppm. Absorbance is measured using a UV-VIS spectrophotometer at a wavelength of 258.4 nm and the absorbance obtained sequentially by 0.189; 0.343; 0.501; 0.655; and 0.807. Based on the relationship between concentration and absorbance, then a linear line with a correlation coefficient (r) close to 1 is 0.9999 and the linear regression equation y = 0.0346 + 0.0258 x are obtained.



Figur 1. Graph calibrating curve of MAP in phosphat buffer solution pH 7.0

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In this research, the formula is made by using the lecithin and cholesterol combination by 30:1. Each formula is made as much as 50 ml, after that each vesicle size of the formula is set by using sonicator with different sonicator usage time. The method used to create liposomes is thin layer hydration. There are two steps taken in this method which is the thin layer formation. Speed used is 120 rpm, the temperature used for the preparation of liposomes is 45^{o}C, it aims to keep the stability of the drug substance and liposome components. At the thin layer formation, the heat effect plays a role in the formation of the fat double lamellar system on the flask base wall and help diethyl ether evaporation. During the hydration process, thin layer exfoliation is helped by inserting glass beads into the flask. In the hydration process used pH 7.4 phosphate buffer solution containing magnesium ascorbyl phosphate at a concentration of 5000 ppm. Hydration process is done by using a rotary evaporator at a speed of 150 rpm at 60° C. This fairly high speed is necessary so that the lipid thin layer around the round flask is more easily hydrated, while the temperature of 60^{o}C intended to reach the lipid bilayer transition temperature (56-62^{o}C) so that the bilayer phospholipid fragmentation which is the vesicles composer can be formed. It is based on the basic nature of the phospholipids that in an aqueous medium at a transitions temperature will be exposed and form vesicles [15].

Liposomes size is resized using a sonicator with the length of sonicator usage time in accordance with the treatment of each sample. Physically, the result formed is a yellow suspension with a lecithin distinctive odor. Sonicator used in regulating the liposomes size is the bath sonicator type, this is because the other types namely probe tip sonicator type has the disadvantage that is deliver high energy input to the suspension of fat but the excessive heat makes the suspension degraded. Tools used here have the ultrasonic waves of 40 kHz.

Data obtained after the samples size is set using the sonicator for 10 minutes has a diameter of 466.9 ± 18.09 nm; for 15 minutes has a diameter of 298.6 ± 15.91 nm; and for 20 minutes has a diameter of 179.4 ± 13.96 nm. As for the sample before the use of sonicator has a average diameter of 572.4 nm. The magnification of the size of the vesicles due to the process of freeze dried and the use of contrast agents is gold that can affect the size of the vesicles of liposomes [16]

The data show that the use of sonicator can reduce the liposome vesicles diameter produced, and the longer the sonicator usage time, the smaller the vesicles diameter produced. This relates to the length of time of the ultrasonic energy interaction to the liposome bilayer fragments. Liposome bilayer fragments that interact with ultrasonic energy, the stability will be affected, in addition aggregation and fusion are occurred, consequently there is also a change in the vesicles size [17].

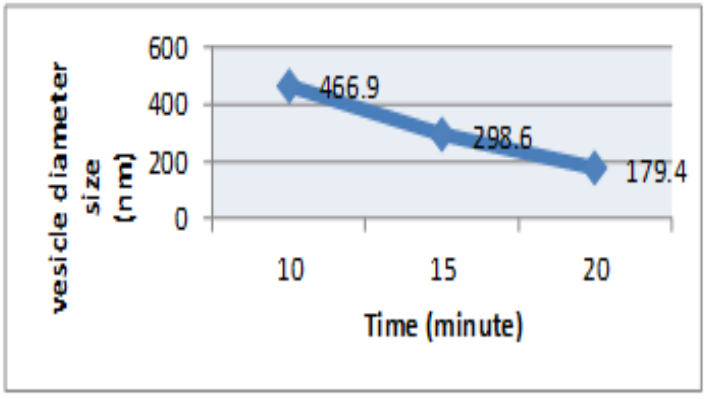

Figure 2. Graph vesicle size distribution of liposome after used sonicator measurenment result with the particle size analyzer

Liposomes morphology evaluation is done by using Scanning Electron Microscope (SEM). The results obtained from the samples evaluation using SEM with a magnification of 1500 and 18000 times show liposome globules formation in the suspension. The existence of larger several globules than the others caused by the merger of several liposome globules.

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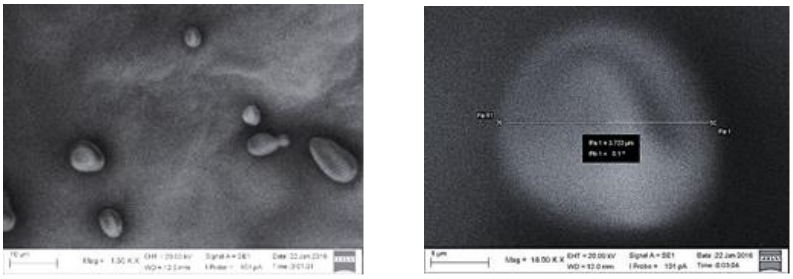

(a) (b)

Figure 3. The results SEM morphology with a magnification of 1500 (a) and 18000 times (b)

Purification is done by microcentrifugation speed of 18,000 rpm at 4°C to separate the supernatant and precipitate. Centrifugation done by means of multilevel. The results of this separation are then read the absorption using UV-Vis spectrophotometer.

The percentage of MAP entrapment within liposomes with sonicator usage time for 10 minutes is 63.4705% ± 1.06; for 15 minutes is 70.4261% ± 0.78; and for 20 minutes is 78.0869% ± 1.33. The entrapment efficiency calculation is based on a theory proposed by Barenholz and Crommelin which is, (% EP = (xj/xt) x 100%) with xj is entrapped drugs and xt is total drug. The results of three of sonicator usage time treatment, time which produces the best entrapment efficiency percentage is the use of sonicator for 20 minutes, to see the diagram of the effect of sonicator usage time on entrapment efficiency can be seen in Figure 3.

Absorption changes which resulting change in the liposome entrapment percentage can occur because of sonication affect the stability of the phospholipid bilayer fragments which are the liposomes composer. The stability of the bilayer membrane as a result of the ultrasonic waves causes the vesicles to be able to open and shut so the entrapped MAF can be free or otherwise the free MAP can be entrapped.

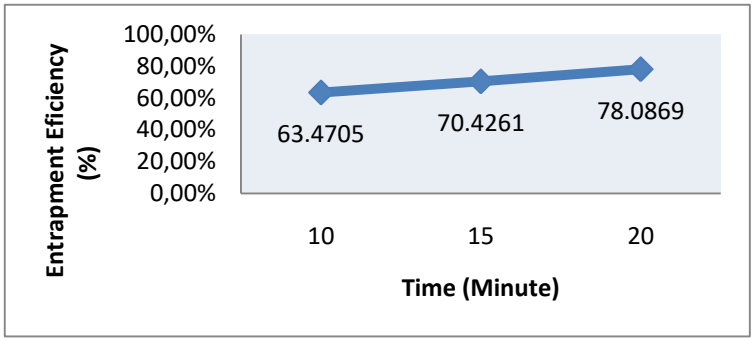

Figure 4. Graph the percentage of entrapment efficiency MAP

The MAP determination results which is entrapped in the liposomes at 3 time intervals of sonicator usage shows the different entrapment percentage. In general, it appears that the length of sonicator usage time impacts significant to the entrapment efficiency.

CONCLUSION

Based on the results of the research that has been conducted by using sonicator for 10 minutes, 15 minutes and 20 minutes, it is found that they can increase the percentage of entrapment efficiency and reduce the size of the vesicles liposomes produced. The results show that the MAP entrapment efficiency percentage consecutively is 63.4705% ± 1.06; 70.4261% ± 0.78; and 78.0869% ± 1.33, while the vesicles size obtained is 466.9 ±18.09 nm; 298.6 ± 15.91 nm; and 179.4 ± 13.96 nm.

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