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Research Article

PREPARATION AND CHARACTERIZATION OF ANTI-ACNE ETHOSOMES USING COLD AND THIN-LAYER HYDRATION METHODS

RACHMAWATI RAMADHANA MUSTOFA, ISKANDARSYAH*

Laboratory of Physical Pharmacy, Faculty of Pharmacy, Universitas Indonesia, Depok, 16424, Indonesia. Email: aya_2803@yahoo.com

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ABSTRACT

Objective: This study aimed to prepare and characterize anti-acne ethosomes using the cold- and thin-layer hydration methods.

Methods: A sonication step was included during ethosome preparation to improve the quality of the cold method. Azelaic acid, Phospholipon 90G, ethanol, propylene glycol, and phosphate buffer (pH 7.4) were used in the procedures. Prepared ethosomal suspensions were characterized using transmission electron microscopy, particle-size analysis, and spectrophotometry.

Results: Ethosomes prepared using the thin-layer hydration method (F1) had small unilamellar vesicles, while those prepared using the cold method with 15-min sonication (F4) showed spherical, elliptical, unilamellar, and multilamellar vesicles. F1 ethosomes had a D_{mean} volume of 648.57±231.26, whereas those prepared using the cold method with 5- (F2), 10- (F3), and 15-min (F4) sonication had D_{mean} volumes of 2734.04±231.49 nm, 948.90±394.52 nm, and 931.69±471.84 nm, respectively. Polydispersity indices of F2, F3, and F4 ethosomes were 0.74±0.21, 0.86±0.05, and 0.91±0.03, respectively, with a poor particle-size distribution, compared to that of F1 (0.39±0.01). Zeta potentials of F1–F4 ethosomes were −38.27±1.72 mV, −23.53±1.04 mV, −31.4±1.04 mV, and −34.3±1.61 mV, respectively. Entrapment efficiencies of F1–F4 ethosomes were 90.71±0.11%, 53.84±3.16%, 72.56±0.28%, and 75.11±1.42%, respectively.

Conclusion: Anti-acne ethosomes produced using the thin-layer hydration method had superior properties than those produced using the cold method with 15-min sonication.

Keywords: Azelaic acid, Characterization, Cold method, Ethosome, Preparation, Sonication, Thin-layer hydration method.

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INTRODUCTION

Azelaic acid is a compound effective in treating mild-to-moderate acne when formulated in 20% w/w cream and applied topically. Azelaic acid works by preventing the growth of skin bacteria causing acne, *Propionibacterium acnes* and *Staphylococcus epidermis*, due to its bactericidal properties. In addition, it has bacteriostatic properties against some aerobic microorganisms [1]. Azelaic acid is soluble in ethanol and slightly soluble in water [2] and, therefore, is compatible with ethosome formulations. Ethosomes are lipid vesicles with a high ethanol concentration. They consist of a phospholipid bilayer with hydrophilic heads and hydrophobic tails enabling entrapment of lipophilic or hydrophilic drugs.

Ethosomes are special type of ultra deformable vesicles. Consisting essentially of phospholipids, water, and a high quantity of ethanol [3]. They are smaller, softer, and more flexible than liposomes. These properties result in higher drug penetration and transdermal absorption [4]. Soya or egg phospholipids are usually used for ethosome preparation (i.e., phosphatidylcholine and phosphatidylinositol) at concentrations of 0.5–10% w/v. Ethanol is usually used at 20–45% v/v concentrations as a penetration enhancer [3].

There are several methods of preparing ethosomes, including hot, cold, and thin-layer hydration methods. In the thin-layer hydration method, phospholipids are dissolved in an organic solvent, and a thin-layer lipid film is formed using a rotary vacuum evaporator. Organic solvents and rotary vacuum evaporation are not needed in the cold method, making it more practical, time-saving, and economical than thin-layer hydration. Moreover, thermodynamically unstable active compounds cannot be formulated using thin-layer hydration because the process requires higher temperatures (below lipid transition temperatures) to evaporate the organic solvents. However, if the drug is thermostable and its solubility increases with temperature, thin-layer hydration should be used because it can increase drug entrapment efficiency. Mistry and Ravikumar reported that the cold method produces azelaic ethosomes with an entrapment efficiency of 35–43% which was lower than that resulting from the thin-layer hydration method (55–88%) [5]. Mbah *et al*. prepared metronidazole ethosomes using the thin-layer hydration method with an entrapment efficiency of 50.31±3.38%, average particle size of 179.9 nm, and a polydispersity index (PDI) of 0.338 [6]. Linoleic acid ethosomes prepared using the cold method had an entrapment efficiency of 64.37±5.91%, average particle size of 343.10±32.49 nm, and a PDI of 0.105±0.012 [7]. Ethosome formulations and the preparation methods affect the quality of ethosomes produced.

The size and lamellarity of ethosomes are very crucial in topical and transdermal drug delivery. The vesicle size should be <200 or 300 nm. In both the cold and thin-layer hydration methods, ethosomal suspensions are extruded or sonicated to obtain the required size and lamellarity. The extrusion process is performed by extruding the suspension under low pressure for certain cycles through polycarbonate membranes (50–300 nm). Sonication can be performed using either a probe sonicator or an ultrasonic bath. Sonication and extrusion will produce small unilamellar vesicle (SUVs). Maestrelli *et al*. showed that the vesicle size produced through sonication (189.3±13.7 nm) was smaller than that via extrusion (295.8±33.2 nm) [8]. Chen *et al*. reported that entrapment efficiency of triptolide ethosomes increased with increase in sonication time until a specific limit; however, the entrapment efficiency of extrusion was higher than that of sonication [9]. In addition to formulations and preparation methods, size reduction processes also affect ethosome properties significantly. Therefore, further studies are needed to observe how such processes can improve the quality of ethosomes.

In the present study, preparations of anti-acne ethosomes containing azelaic acid were done using two different methods, the cold- and thin-layer hydration methods. The inclusion of a sonication step in the cold method was done to increase the quality of the ethosomes produced. Then, the particle-size analysis, transmission electron microscopy (TEM), and ultraviolet and visible (UV-Vis) spectrophotometry were used to characterize the suspensions.

MATERIALS AND METHODS

Materials

Azelaic acid (Sigma-Aldrich, Jerman), ethanol (Brataco, Indonesia), Phospholipon 90G (Lipoid GmbH, Jerman), dichloromethane (Brataco, Indonesia), methanol (Brataco, Indonesia), propylene glycol (Brataco, Indonesia), demineralized water (Brataco, Indonesia), sodium hydroxide (Brataco, Indonesia), and sodium dihydrogen phosphate monobasic (Brataco, Indonesia) were of high-performance liquid chromatography grade. All the other materials were of analytical grade.

Thermal analysis

Thermal analysis was performed using differential scanning calorimetry (DSC) to observe the transition temperature of Phospholipon 90G with STA 6000 (Perkin Elmer, USA). 35.528 mg Phospholipon 90G was placed in a closed plate and heated from 30.00°C to 1000.00°C at a rate of 10.00°C/min and constant nitrogen flow (20 mL/min).

Preparation of anti-acne ethosomes

Thin-layer hydration method

Table 1 shows the formulations of the cold- and thin-layer hydration methods. The procedure followed a method developed by Wirarti [10] with slight modifications. For 25 mL of ethosomal suspension, 500 mg Phospholipon 90G was dissolved in 5 mL dichloromethane and added into a clean and dry round bottom flask. The organic solvent was removed using a rotary vacuum evaporator at a temperature below the lipid phase transition temperature (52±2°C). The rotation speed was set at 25°rpm and increased by 25 rpm every 10 min until 150 rpm. Nitrogen was then supplied for 3°min primarily to avoid phospholipid oxidation and to remove the residual solvent in the flask. The flask was closed tightly and kept in the refrigerator (4°C). After 24 h, the thin layer was hydrated using a solution containing 500 mg azelaic acid, 8.75 mL ethanol, 100 mg propylene glycol, and 15.15 mL phosphate buffer (pH 7.4) at a temperature of 40±2°C and 150 rpm. The colloidal suspension obtained (F1) was subjected to sonication for 5 min (3 cycles) using the ultrasonic bath Bransonic 3200 (47±6 kHz), and then stored in the refrigerator at 4°C.

Cold method

About 500 mg Phospholipon 90G was dissolved in 3 mL ethanol (solution 1). Azelaic acid was dissolved in 5.75 mL ethanol (solution 2). Solution 2 was added to solution 1 and stirred with a magnetic stirrer. Propylene glycol was added and the solution was stirred again (organic phase). The preparation was done at room temperature (25±2°C). The aqueous phase (phosphate buffer pH 7.4) was added dropwise to the organic phase under constant mixing at 700 rpm; the stirring was continued for another 30 min [11]. The ethosomal suspensions were sonicated in 3 cycles for 5 (F2), 10 (F3), or 15 (F4) min, and stored in the refrigerator at 4°C.

Table 1: Ethosome formulations in the cold- and thin-layer hydration methods

Components	Concentration (%)
Azelaic acid	2
Ethanol	35
Phospholipon 90G	2
Propylene glycol	0.4
Phosphate buffer pH 7.4	100

Characterization of ethosomes

Morphology

TEM was used to observe the morphology of ethosomes. A drop of the sample was placed on a carbon-coated copper grid. Filtration paper was used to remove the excess.

Particle-size distribution and zeta potential

Ethosome size distribution and zeta potential were determined using dynamic light scattering (DLS) using a Malvern Zetasizer at a scattering angle of 173°C. The software Zetasizer was used to process the data.

Entrapment efficiency

Azelaic acid stock solution (1000 ppm) was prepared by dissolving azelaic acid in pH 6.8 phosphate buffer and then diluted to concentrations of 150, 200, 250, 300, 450, and 600 ppm. The analysis was conducted at the maximum absorption wavelength of azelaic acid in pH 6.8 phosphate buffer at 204 nm [12]. The absorbance values obtained were plotted against concentrations to create a calibration curve. The regression equation was y=0.0012x+0.0407 with a correlation efficient (r) of 0.999.

Each of the formulations was placed in a centrifuge tube (Vivaspin centrifugal concentrator) with a membrane (5000 kDa) and centrifuged at 4000 rpm for 6 h in a LABSCO centrifuge. The supernatant (filtrate) was collected and diluted using pH 6.8 phosphate buffer to determine the free drug concentration. Total azelaic concentration was determined by analyzing diluted samples. The ethosomal suspension and methanol (1:1) were added to a 1.5 mL vial, vortexed for 3 min and further diluted. Methanol was used to disintegrate the vesicles to obtain more accurate readings. A Shimadzu UV-Vis spectrophotometer at 204 nm was used to analyze the azelaic acid concentration. The entrapment efficiency was calculated by dividing (T–S) with T then times 100% in which EE=Entrapment efficiency (%), T=Total azelaic acid concentration in the original suspension, and S=Azelaic acid concentration in the supernatant [13].

RESULTS AND DISCUSSION

Thermal analysis

DSC was used to do the thermal analysis. The DSC thermogram (Fig. 1) showed that the Phospholipon 90G transition temperature was at 55.08°C.

Ethosome characterization

Morphology

TEM analysis was conducted to observe ethosome morphology. Due to a technical issue, the samples could not be analyzed using the same instrument. F1 ethosomes were analyzed using a JEOL JEM1400 with 2% uranyl acetate, and F4 ethosomes were analyzed using the microscope Tecnai 200 kV D2360 SuperTwin. Ethosomes produced using the thin-layer hydration method had SUVs and had a spherical shape. In theory, the brighter inner part contains ethanol, phosphate buffer, and azelaic acid. As seen in Fig. 2, the darker outer part consists of a phospholipid bilayer and azelaic acid. As Fig. 3 shows, F4 ethosomes had multilamellar and SUV.

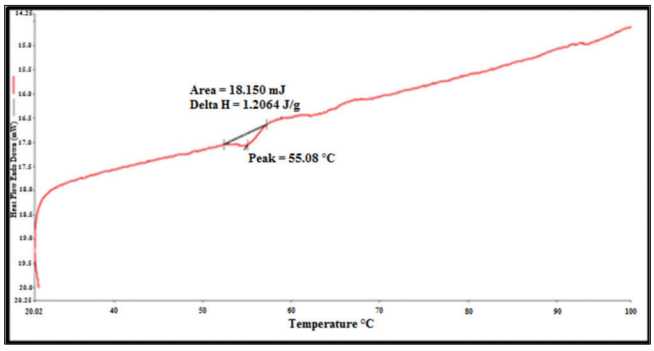

Fig. 1: Differential scanning calorimetry thermogram

Particle-size distribution and zeta potential

Table 2 shows the particle-size distributions. As much as 10% of the total particles in F1 ethosomes had sizes smaller than 74.57±38.50 nm, 50% had sizes smaller than 285±62.64 nm, and 90% had sizes smaller than 765.67±127.21 nm. Similar data were obtained for F2, F3, and F4 ethosomes. From the data acquired, the D_{mean} volume value was used to describe the particle size. Table 2 shows that F2, F3, and F4 ethosomes had D_{mean} volumes of 2734.04±231.49, 948.90±394.52, and 931.69±471.84 nm, respectively. The PDI values of F2, F3, and F4 ethosomes were higher than 0.5 (Table 3), whereas the PDI of F1 ethosomes was 0.39±0.01. F1 ethosomes had a higher zeta potential (−38.27±1.27 mV) than all those prepared using the cold method. Out of the three cold method formulations, F2 ethosomes had the lowest value at −23.53±1.04 mV, while F4 ethosomes had the highest value (−34.3±1.60 mV).

Entrapment efficiency

The entrapment efficiency indicates the amount of drug trapped inside the vesicles. The results in Table 4 show that F1 ethosomes had the highest entrapment efficiency with a value of 90.71±0.11%. The entrapment efficiency of F2 ethosomes was 53.8±3.16% that of F2 ethosomes was 72.56±0.89% and that of F4 ethosomes was 75.50±1.25%, making F4 ethosomes the most efficient in entrapment among all the cold method formulations.

Discussion

Thermal analysis

The transition temperature is the point where phospholipids transform from a gel phase to a liquid crystalline phase. Membrane fluidity increases during the liquid crystalline phase and the quality of vesicles produced decreases because they are susceptible to leakage [14]. Phosphatidylcholine is likely to assemble good vesicles under its transition temperature. Therefore, 52±2°C was chosen as the temperature used in the thin-layer formation method.

Ethosome characterization

Morphology

The vesicles produced using the thin-layer hydration method can be categorized as SUVs because they had been sonicated during the formation process. A high ethanol concentration (35% v/v) caused the formation of thin phospholipid bilayers [11]. F4 ethosomes were chosen to be evaluated for the next stage because they had the best characteristics compared to F2 and F3 ethosomes. As Fig. 3 shows, F4 ethosomes had multilamellar and unilamellar vesicles. Kasetvatin, Rujivipat and Tiyaboonchai showed that vesicles do not always have a perfect spherical shape; therefore, the vesicles formed (Fig. 3) could still be referred to as ethosomes although they were elliptical in shape. The elliptical form can be caused by the high concentration of ethanol which increases membrane fluidity. Cholesterol can be added to decrease the fluidity [15-18] and produce more spherical vesicles. Spherical SUVs were found inside multilamellar vesicles due to sonication, which may have disturbed the multilamellar structure and created unilamellar vesicles [19]. The inner part of the vesicle in Fig. 3 is not as visible as that in Fig. 2 because the sample in Fig. 3 was not stained.

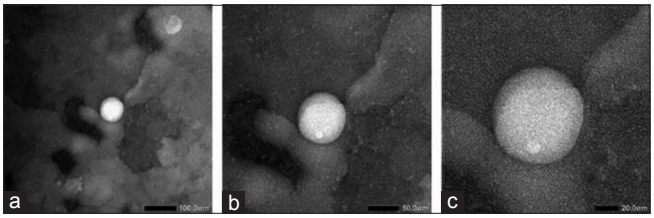

Fig. 2: (a-c) Inner and outer part of ethosomes

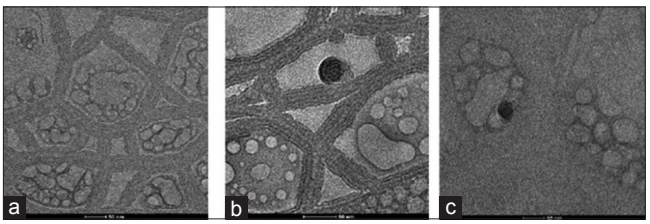

Fig. 3. (a-c) Vesicles of ethosomes

Particle-size distribution and zeta potential

Particle size and zeta potential affect the stability of the ethosomal suspension. DLS is a technique used to measure particle size based on the velocity of Brownian motion. Brownian motion occurs due to the collision of particles with surrounding solvents. The smaller the particle size, the greater the Brownian motion [20].

Volume diameter was used in the present study because it shows the actual particle size and is more representative and specific (Table 2). Table 2 shows that there was a decrease in D_{mean} volume of F2, F3, and F4 ethosomes. Therefore, it can be concluded that the addition of sonication to the cold method decreased the particle size of ethosomes.

The PDI value should be <0.5. The degree of heterogeneity of particle-size distribution is lower when the PDI value is closer to zero [17]. The PDI values of F2, F3, and F4 ethosomes were higher than 0.5 (Table 3) which indicated rather polydisperse (heterogeneous) suspensions and poor particle-size distribution; particle-size distribution curves with abnormal distributions and more than one peak. In addition, the TEM images (Fig. 3) support the finding that F4 ethosomes vary in particle size. The heterogeneity might be due to the sonication time not being optimal, resulting in particles with decreased and also increased sizes. The PDI of F1 ethosomes was 0.39±0.01 indicating a rather homogeneous suspension and a better particle-size distribution than other formulations.

During sonication, sound waves are transmitted through the medium and create high and low pressure alternately at a rate depending on the frequency. Cavitation occurs during low pressure with high-intensity ultrasonic waves forming microscopic vacuum bubbles in the medium. These bubbles burst and release high amounts of energy when no more energy can be absorbed [19]. This energy causes the breakdown of multilamellar layers resulting in a smaller size of unilamellar vesicles.

The particle size of vesicles depends on the intensity of the ultrasonic waves and the sonication time. The higher the intensity, the greater the cavitation energy released. The longer the sonication time, the longer the particles will be exposed to the energy, resulting in a smaller particle size. However, if the sonication is too long (beyond the optimum time), it will cause agglomeration and increased particle size. Ethosomes prepared using thin-layer hydration had bigger particle sizes than those prepared using the cold method. F1 ethosomes had an average particle-size diameter of 648.57±231.26 nm. F1 ethosomes was prepared by making a thin-layer of phospholipid followed by hydration. The thin layer was made at a temperature below the transition temperature and then cooled for 24 h at

Table 2: Particle-size distribution of anti-acne ethosomes

Formulation	Average particle-size distribution±SD (nm)			
	Dv10	Dv50	Dv90	D_{mean} volume
F1	74.57±38.50	285±62.64	765.67±127.21	648.57±231.26
F2	333.33±40.27	1600.67±577.13	5990±20	2734.04±231.49
F3	120.67±8.08	501.67±50.29	2334.33±2672.40	948.90±394.52
F4	146.33 ± 40.67	486 ± 55.03	3604.667 ± 2567.55	931.69 ± 471.84

SD: Standard deviation, Dv: Diameter volume

Table 3: PDI and zeta potential of anti-acne ethosomes

Formulation	PDI±SD	Zeta potential±SD (mV)
F1	0.39±0.00	−38.27±1.72
F2	0.74±0.21	−23.53±1.04
F3	0.86±0.05	−31.4±1.04
F4	0.91 ± 0.03	−34.3 ± 1.61

SD: Standard deviation, PDI: Polydispersity index

Table 4: Entrapment efficiency of anti-acne ethosomes

Formulation	Entrapment efficiency±SD (%)
F1	90.71±0.11
F2	53.84±3.16
F3	72.56±0.89
F4	75.14 ± 0.66

SD: Standard deviation

4°C. After hydration at 40±2°C and sonication, the suspension was stored at 4°C. These heating and cooling processes made the vesicles formed more rigid and less likely to agglomerate. In the cold method, the phospholipids were prepared at room temperature (25±2°C). The phospholipids will immediately return into a spherical shape in an organic medium and, therefore, the vesicles were already formed during the preparation. These vesicles are prone to agglomeration, resulting in a greater particle size.

The zeta potential value represents suspension stability. It represents the repulsive and attractive forces between the particles. Suspensions are known to have better stability when the zeta potential is more negative than −30 mV or more positive than +30 mV. F1 ethosomes had the highest stability because they had the highest zeta potential (−38.27±1.27 mV) compared to ethosomes prepared using the cold method. Among the three cold method formulations, F2 ethosomes had the lowest value at −23.53±1.04 mV, while F4 ethosomes had the highest value (−34.3±1.60 mV). The presence of ethanol resulted in a negative surface charge on the vesicles and a negative zeta potential, indicating electrostatic repulsion between particles and preventing vesicle aggregation [21].

Entrapment efficiency

The entrapment efficiency of F1 ethosomes was 90.71±0.11%, whereas that of F4 ethosomes was 75.50±1.25%, showing that thin-layer hydration resulted in the highest entrapment efficiency compared to all the cold methods used. The hydration process was done at 40±2°C increasing the solubility of azelaic acid, resulting in greater drug trapping. However, a sonication time up to 15 min increased the cold method entrapment efficiency from 53.84±3.16% to 75.50±1.25%. Sonication can increase azelaic acid solubility and breaks down multilamellar vesicles, creating unilamellar vesicles. During this process, cavities or pores may be formed causing azelaic acid to move into and out of the vesicles, increasing entrapment efficiency. Even though azelaic acid may be trapped in both the vesicle sac and the phospholipid bilayer, the vesicle sac is bigger in volume, resulting in more azelaic acid entrapment in SUVs than in small multilamellar vesicles. The vesicles in Fig. 2 were unilamellar while those in Fig. 3 were multilamellar.

CONCLUSION

Sonication during vesicle preparation for 15 min in the cold method produced spherical, elliptical, multilamellar, and unilamellar anti-acne ethosomes with a D_{mean} volume of 931.69±471.84 nm, a PDI of 0.91±0.03, a zeta potential of −34.3±0.13 mV, and entrapment efficiency of 75.11±0.66%. The thin-layer hydration method resulted in better characteristics, producing spherical unilamellar ethosomes with a D_{mean} volume of 648.57±231.26 nm, a PDI of 0.38±0.01, a zeta potential of 38.27±1.72 mV, and an entrapment efficiency of 90.71±0.11%.

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CONFLICTS OF INTEREST

All authors have none to declare.

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