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Original Article

IMPROVED CHARACTERISTICS OF GLIBENCLAMIDE AS TRANSETHOSOME VESICULAR SYSTEM: PHYSICOCHEMICAL, SOLUBILITY AND *IN VITRO* PERMEATION STUDY

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ABSTRACT

Objective: Transethosome as a vesicular system offers high skin permeation; therefore, it is expected to improve the solubility and permeability of the poorly soluble drug glibenclamide. The study aimed to optimize the effect of lipid and surfactant concentration as well as sonication time on the physical characteristics of glibenclamide-loaded transethosomes.

Methods: The transethosomes were prepared by solvent evaporation method. An experimental Box-Behnken design optimized the formula by assessing particle size, polydispersity index, zeta potential, and entrapment efficiency as response parameters. Further characterizations were conducted by determining the morphology by TEM, chemical interaction by FTIR, thermal behavior by DSC, as well as solubility improvement by using *in vitro* drug release and permeation study.

Results: The result showed that the optimal formula was that with the lipid composition of 75 mg of soya lecithin, 5 mg of tween 80 as surfactant at a sonication time of 18.79 min. The responses were particle size of 166.8±5.3 nm, polydispersity index of 0.463±0.1, zeta potential of-44.7±2.2 mV, and entrapment efficiency as much as 87.18±3.8%. Glibenclamide-loaded transethosomes exhibited a spherical morphology with no visible aggregation. FTIR study revealed no chemical interactions between Glibenclamide and the excipients. Solubility and *in vitro* drug release tests showed a significant increase of Glibenclamide from transethosome ($p<0.05$) compared with that as a bulk powder.

Conclusion: Overall, the optimized glibenclamide-loaded transethosomes designed with Box Behnken resulted in improved physicochemical characteristics and increased solubility and drug release compared with that from ethosomes and bulk powder comparison, which will be promising for Glibenclamide to be formulated as transdermal drug delivery.

Keywords: Glibenclamide, Transethosome, Box-behnken, Transdermal

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INTRODUCTION

Glibenclamide is a second-generation sulfonylurea used as an oral antidiabetic for hyperglycemic patients that increases endogenous insulin release and lowers serum glycogen levels. According to the Biopharmaceutical Classification System (BCS), Glibenclamide is classified as class II with low solubility in water with good permeability [1, 2]. Glibenclamide has a plasma half-life ($t^{1/2}$) of about 4-6 h, with the first cross-metabolism in the liver as much as 50% [3]. Patient adherence becomes very important in using Glibenclamide due to its long consumption period. Mishra *et al.* showed that transdermal administration of Glibenclamide microcapsules increased the bioavailability and provided longer hypoglycemic effects than oral administration [4].

Transdermal would become a promising alternative route of administration for BCS II drugs [4]. It provides significant advantages, such as controlled drug release, reduced dose frequency, and improved patient adherence. It is also reported to improve bioavailability, reduce side effects, and avoid first-hand metabolism [5-7]. Accordingly, the limitation of the transdermal system is the ability of drug molecules to penetrate and reach the action site due to the presence of stratum corneum (SC) as the outermost layer of the skin, which acts as the main barrier for drug delivery into the skin [8, 9].

The vesicular system has been developed to overcome the stratum corneum's barrier effect. This system works by mechanisms of vesicular fusion, disrupting the lipid stratum corneum or increasing lipid fluidity between cells [10]. Conventional vesicular systems such as liposomes have been the potential approaches to achieve better skin penetration due to the encapsulation of various bioactive compounds, thereby increasing physicochemical stability, reducing skin irritation side effects, and increasing topical absorption [8, 10-13]. It has been considered to increase tissue permeation despite their limited penetration into the systemic circulation [14-16]. When liposomes do not penetrate the skin deeply, they remain confined to the upper layer of the stratum corneum [17–20]. One way to overcome this problem is by utilizing the concept of drug carriers or manipulating the vesicles to increase the penetration [21].

Transethosome, first introduced by Song *et al.*, is derived from liposomes characterized by a high ethanol content and an edge activator in the formulation. A transethosome is a new vesicle system containing ethanol, surfactant, and phospholipids, equal to the transfersome and ethosomal systems [22]. Transfersome is a vesicular system that can deform after skin penetration [23]. It offers the ability to overcome the difficulty of drug penetration into the skin by changing its shape to pass through the intracellular stratum corneum [24]. The ethosomes, first developed by Touitou *et al.*, represent the third generation of elastic lipid vesicular carriers with relatively high ethanol content [25]. Transethosomes have the advantage of a combination between transfersomes and ethosomes. Ethanol in transethosomes stabilizes the vesicles and increases their softness and the ability to load lipophilic drugs [25]. In the transethosome system, edge activators, such as surfactants, also implement ethanol composition, which increases drug entrapment efficiency and penetration potential. Another advantage of transethosomes is their better stability [26].

Based on this background, the optimum formulation of Glibenclamide transethosome for developing transdermal dosage forms is interesting to create. The influence of phospholipids, surfactants, and sonication time on glibenclamide transethosomes' physicochemical properties had been studied and optimized using Box Behnken design to obtain the model for desirable physicochemical characteristics. Hypotheses on improved Glibenclamide's solubility and permeability were also studied to prove that transethosomes could be developed into transdermal formulations.

MATERIALS AND METHODS

Materials

Chloroform (Smart-Lab, Indonesia), disodium hydrogen phosphate (Multi Usaha Mandiri, Indonesia), ethanol 70% (Multi Usaha Mandiri, Indonesia), glibenclamide (Prudence Pharma Chem, India), iodine (Multi Usaha Mandiri, Indonesia), methanol (Merck, India), sodium dihydrogen phosphate (Multi Usaha Mandiri, Indonesia), soya lecithin (Multi Usaha Mandiri, Indonesia), tween 80 (Multi Usaha Mandiri, Indonesia).

Experimental methods

Determination of minimum concentration and critical micelle concentration (CMC) of surfactants

Determination of the minimum surfactant concentration was carried out by calculating the amount of tween 80 as a surfactant using the formula;

$$Qs = \frac{6(\frac{\rho s}{\rho})}{10 - 0{,}5\,.\,RHLB} + \frac{4Q}{1000} \ldots\ldots\ldots (1)$$

Qs = the amount of mixed surfactant in grams required to form an emulsion

ρs = mixture of surfactant density (g/ml)

ρ = density of the dispersed phase (g/ml)

Q = dispersing phase

RHLB = required hydrophilic-lipophilic balance

Determination of the Critical Micelle Concentration (CMC) of surfactants was carried out by placing 2.5 ml of a saturated aqueous solution of iodine in several stoppered and calibrated test tubes. Various amounts of surfactant solution were added and were made up to a volume of 10 ml by water. Their absorbances were measured at 286, 346, and 460 nm. The maximum absorbance (λmax) of I_2 in each solution with increasing concentration of the nonionic surfactant (Tween 80), which showed a blue shift from 460 nm, was measured [27].

Design of experiment by box behnken design

The effect of various process parameters on the physical properties of transethosomes was investigated by the Design of Experiment (DOE) approach, as shown in table 1. The total experiments to be run were designed by Design-Expert software (version 12.0.3.0, State-Ease Inc., Minneapolis, MN) [20, 28].

Table 1: **Formulation variables of glibenclamide transethosome**

Independent variables (Factors)	Level	
	-1	+1
A= Soya lecithin (mg)	75	95
B= Tween 80 (mg)	2	5
C= Sonication time (min)	15	35
Dependent variables (Responses)		
Y_1 = Particle size (nm)	10	600
Y_2 = Polydispersity index (PDI)	0	1
Y_3 = Zeta potential (mV)	Less than-30	Greater than 30
Y_4 = Entrapment efficiency (%)	50	100

Preparation of glibenclamide-loaded transethosomes

Conventional thin layer evaporation followed by sonication technique had been used to prepare transethosome [23, 29-31]. As much as 20 mg of Glibenclamide, 75–95 mg of soya lecithin, and 2–5 mg of tween 80 were dissolved in 50 ml mixture of methanol: chloroform (1:2, v/v) in a round bottom flask. The thin lipid film was obtained after the solvent was evaporated at 40 °C, 75 rpm, under reduced pressure on a rotary evaporator (IKA, China). The film was hydrated with 20 ml of ethanol 70% by rotation at 75 rpm for 30 min at 40 °C. The suspension was sonicated for 15-35 min with a titanium probe ultrasonicator (Ultrasonicator, 200 Watts, 24 kHz, Hielscher Ultrasonics GmbH, Berlin, Germany) in an ice bucket to produce transethosomes [29]. Glibenclamide ethosomes had been prepared using the same formula and method, except that the Tween 80 as a surfactant was missing.

Characterization of glibenclamide-loaded transethosomes

The particle size and polydispersity index (PDI) were measured by (Dynamic Light Scattering) method by 10 times dilution with aqueous media at room temperature. Zeta potential determination (Horiba SZ-100, Horiba Ltd. Japan) was employed in all formulations. Transethosome suspension (0.5 ml) was diluted into 10 ml and was illuminated with laser light. Therefore, the vesicles scattered the light. All the measurements were conducted in triplicate. Zeta potential was determined to analyze the physical stability of a suspension, including Glibenclamide loaded transethosome, blank transethosome, and glibenclamide ethosome for comparison study [32, 33].

Entrapment efficiency

The sample (1.5 ml) was subjected to cold centrifugation at 13500 rpm for 30 min at 4 °C. The supernatant (0.25 ml) was diluted with 5 ml methanol, and the absorbance was measured at 229 nm using UV-visible spectroscopy (UV-200 Specord). Entrapment efficiency percentage of glibenclamide-loaded transethosomes, as well as glibenclamide-loaded ethosome for comparison, were calculated using the formula:

$$\text{Entrapment efficiency} = \frac{\text{Total ammount drug} - \text{Free amount drug}}{\text{Total ammount drug}} \text{x} 100\%$$

Transmission electron microscopy (TEM)

Transmission electron microscopy was carried out to determine the morphology of the glibenclamide transethosomes using JEOL JEM-1400 series 120kV (JEOL USA, Inc.). The sample was placed in the specimen, and 400 mesh TEM Grid was put onto the example for one minute. The application of 10 µl uranil acetate negatively stained the grid and let air dry. The ArrayGrid was placed on a regular holder, and the picture of the vesicle was recorded at a proper magnification [23, 34-35].

Fourier transform infrared spectroscopy (FTIR)

The sample in the form of vesicle suspension was dried at room temperature. The powdered sample was obtained easily due to the quick evaporation of ethanol in the hydration media. Next, potassium bromide was added, and then the sample was analyzed using Perkin Elmer's Fourier Transform Infrared Spectroscopy

(FTIR) (RXIFT-IR system, USA). The data were collected in the spectral range of 450-4000 cm^{-1} [34, 36].

Differential scanning calorimetry (DSC)

The samples in the form of suspension were dried at room temperature. The DSC studies were performed by heating ~3–6 mg (solid) sample at a heating rate of 10 °C/min from 20 °C to 400 °C in a covered sample pan under nitrogen gas flow (60 ml/min) [37–39].

Solubility test

0,25 ml, the suspension samples (glibenclamide transethosome, blank transethosome, and Glibenclamide ethosomes) were dissolved in 5 ml methanol and were mixed with a bath sonicator, filtered by using a 0.45 µm membrane filter, and were analyzed by the UV–Vis spectrophotometer at λmax 229 nm. The analysis was performed in triplicate [40].

Permeation study

Permeation studies were performed on the formula of glibenclamide transethosomes having the best physical characteristics, glibenclamide ethosomes, and glibenclamide bulk powder. The shaved dorsal skin of male Wistar rats was used as a membrane after repeatedly washing with distilled water, soaked in saline water for 5 min, and kept at −18 °C until used [39]. The surface area of the membrane for the study was 1.5 cm^2 and was kept in contact with the receptor compartment containing phosphate buffer (pH 7.4), which was stirred at 100 rpm at 37 °C±0.5 °C. The diffusion cells were loaded with 2 ml sample equal to 2 mg glibenclamide. The sink condition was maintained throughout the experiments. The samples were taken at a specified time, and the media were replaced with fresh phosphate buffer Aliquots (2 ml). The percentage of drug released was determined by spectrophotometric analysis at 229 nm. The research had been performed in triplicate, and statistical analysis by Anova had been conducted. In comparison, drug release of glibenclamide bulk powder and glibenclamide ethosomes were also evaluated [17, 28, 41]. All steps were approved by the Research Ethics Committee in the Universitas Padjadjaran Bandung. All animal experiments were carried out following the scientific procedures 208/UN6. KEP/EC/2022 for animal experiments.

***In vivo* drug release study**

Animal setup

The animals used for *in vivo* experiments were Wistar rats (6-8 w) weighing 200-250 g. The rats were kept at 25±1 °C and 45–55% relative humidity with a 12-hour light/dark light cycle.

Induction of diabetes mellitus and hypoglycemic activity in diabetic rats

Male Wistar rats were fasted overnight before being treated. Rats were induced to become diabetic by intraperitoneal injection of alloxan 125 mg/kg BW suspended in NaCl (0.9% w/v). After 72 h, rat blood glucose was determined using Onetouch Ultra Plus Flex Glucose Meter (The LifeScan Inc., India). Rats with blood glucose levels>250 mg/dl were selected for further *in vivo* drug release study [42].

Glucose tolerance test

Mice were fasted overnight and divided into 4 groups. Group 1 was a negative control and was given a patch without the active substance. Group 2 served as a positive control and was given oral glibenclamide suspension (5 mg/kgBB), Group 3 was given a patch containing glibenclamide bulk powder, and Group 4 was given glibenclamide transethosome patch (3 mg), which was applied with an area of 2.25 cm^2. Blood glucose was monitored at time sampling of baseline 0, 2, 4, 6, 8, 10, and 24 h.

RESULTS

Critical micelle concentration (CMC) of surfactant

Critical Micelle Concentration (CMC) of surfactant needed to stabilize the vesicular system in this experiment was observed as the fracture in the curve of surfactant concentration. The lowest concentration of Tween 80 as surfactant used in the experiment was 2 mg, calculated by Equation (1). The absorbance at 286, 346, and 460 nm of the mixture of 2.5 ml iodine with various concentrations of Tween 80 showed that there were fractures of the graph at a concentration of 5 mg (fig. 1). It can be concluded that the concentration of Tween 80 as a surfactant above 5 mg was assumed to be above the Critical Micelle Concentration (CMC).

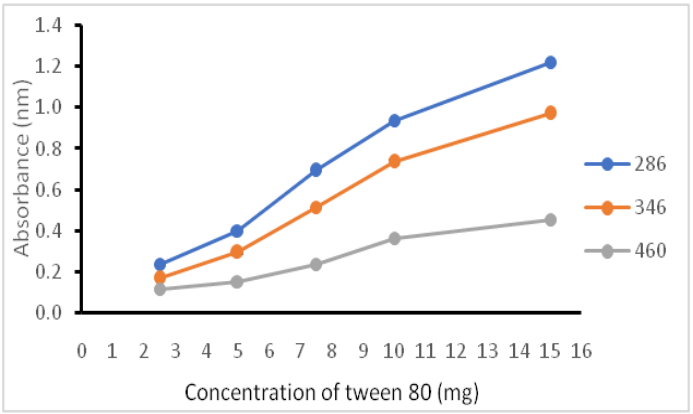


Fig. 1: The absorbance of different concentrations of surfactant tween 80 with the addition of iodine at an additional lambda maximum, (n=5)

The fracture could be due to the aggregation following the micelle formation in the mixture [27]. This experiment's CMC determination revealed that the minimum concentration of tween as surfactant was below 5 mg. The result was then used for further investigations in determining tween 80 concentration to be used in transethosome formulations.

Transethosome optimization by using box behnken design

To evaluate the affecting parameters on the properties of the transethosome, including the concentration of phospholipid (soya lecithin) and surfactant (tween 80) as well as sonication time in the formulation process, a 3-factors, 4-responses–Behnken design had been employed [40, 43].

Based on Box Behnken Design's optimization, 17 experimental runs had been constructed (Design-Expert software ver. 12.0.3.0, State-Ease Inc., Minneapolis, MN). The three-factors, four-responses Box-Behnken design was used to explore the quadratic response surfaces and construct second-order polynomial models. The 4 levels of responses used were particle size, polydispersity index, zeta potential, and Entrapment efficiency (table 2).

Table 2: Observed response in box–behnken design on optimization of transethosome glibenclamide predicted values generated by design expert software (n=17)

Run	Independent variables			Dependent variables			
	Soya lecithin (mg)	Tween 80 (mg)	Sonication time (min)	Particle size (Y1) (nm)	Polydispersity index (Y2)	Zeta potential (Y3) (mV)	Entrapment efficiency (Y4) (%)
1	85	3.5	25	269.5	0.596	-44.9	91.08
2	75	2	25	277.9	0.653	-48.6	45.86
3	95	3.5	35	199.1	0.066	-50.6	86.76
4	95	5	25	263.1	0.39	-50.8	59.36
5	85	3.5	25	185.5	0.154	-51	83.1
6	85	3.5	25	200.5	0.628	-45.5	75.11
7	85	5	35	196	0.424	-52.9	81.08
8	85	3.5	25	223.8	0.245	-47.5	80.33
9	85	2	35	265	0.352	-42.9	81.4
10	85	5	15	733.5	0.889	-44.6	88.95
11	75	3.5	15	232.3	0.576	-45.7	90.89
12	85	2	15	310.4	0.876	-55.6	85.94
13	75	5	25	252.7	0.407	-39.5	92.56
14	85	3.5	25	318.1	0.669	-48.6	87.54
15	95	3.5	15	646.4	0.732	-49.2	86.2
16	75	3.5	35	197.8	0.1	-53.8	84.03
17	95	2	25	261.7	0.622	-46.3	55.59
Mean				296.08	0.49	-48.12	79.75
Std. Dev.				100.51	0.18	2.99	8.47
F-value				4.61	5.82	3.46	3.71
p-value				0.017	0.01	0.04	0.04

Data are expressed as mean±SD, n=17

The regression analysis was performed using Design Expert software on the data obtained to obtain the Y_1, Y_2, Y_3, and Y_4 equations. The results of the study of variance showed that the model F values of Y_1, Y_2, Y_3, and Y_4 were 4.61, 5.82, 3.46, and 3.71, which indicates that the model was significant ($p<0.05$) to predict the optimal conditions of independent variables. The significance ($p<0.05$) of each variable was considered to form the quadratic polynomial equation of Y_1, Y_2, Y_3, and Y_4.

Effect on particle size (Y_1)

Based on the optimization results, the effect of the parameter process on particle size can be explained as follows:

$$Y_1 = +296.76+51.2A+41.2875B-133.088C+6.65AB-103.2\ AC-123.025BC \quad \ldots\ldots (2)$$

Y_1 = Particle size

It can be concluded that Variable A (soya lecithin) and B (tween 80) is a determining factor on particle size, while C (sonication time) is a dependent variable that significantly affects negative interaction. BC (combination of tween and sonication time) gave significant negative interaction.

Effect on polydispersity index (Y_2)

The effect of the parameter process on the polydispersity index can be explained as follows:

$$Y_2 = +0.492882+0.00925A-0.049125B-0.266375C \quad \ldots\ldots\ldots (3)$$

Y_2 = polydispersity index

It can be concluded that Variable A (soya lecithin) is a determining factor on the polydispersity index, while C (sonication time) is the dependent variable, which significantly affects negative interaction.

Effect on zeta potential (Y_3)

The effect of the parameter process on Zeta Potential can be explained as follows:

$$Y_3 = -48.1176-1.1625A+0.7B-0.6375C-3.4AB+1.675AC-5.25BC \ldots (4)$$

Y_3 = zeta potential

The equation revealed that Variable B (tween 80) is a determining factor on zeta potential, while AB (combination of soya lecithin and tween 80) and BC (combination of tween 80 and sonication time) show significant negative interaction.

Effect on entrapment efficiency (Y_4)

The effect of the parameter process on Entrapment Efficiency can be explained as follows:

$$Y_4 = +83.432-3.17875A+6.645B-2.33875C-10.7325AB+1.8555AC-0.8325BC-8.731A^2-11.3585B^2+12.269C^2 \ldots\ldots (5)$$

Y_4 = entrapment efficiency

The equation revealed that Variable B (tween 80) is a determining factor on entrapment efficiency (EE), while AB (combination of soya lecithin and tween 80) significantly affects the EE with negative interaction.

Validation of experimental design and optimization of formulation

From the mathematical model, the optimum formulation of glibenclamide-loaded transethosome with particle size (Y_1), polydispersity index (Y_2), zeta potential (Y_3), and entrapment efficiency (Y_4) can be determined using the point prediction of Design Expert software. The optimal levels of the parameter were determined as follows: X_1 (soya lecithin) = 75 mg, X_2 (tween 80) = 5 mg, and X_3 (sonication time) = 18.79 min. The appropriate predictive response values were: particle size (Y_1) = 374.52 nm, polydispersity index (Y_2) = 0.6, zeta potential (Y_3) =-38.15 mv, and entrapment efficiency (Y_4) = 91.76%. The desirability value obtained was 0.833. Three verification experiments used optimal formulation parameters to validate the predicted values generated according to the design results. As the results, Glibenclamide loaded transethosome with a mean particle size of 166.8±5.3 nm, polydispersity index of 0.463±0.1, zeta potential of-44.7±2.2 mV, and entrapment efficiency as much as 87.18±3.8% had been obtained. The measurement data revealed that the polydispersity index, zeta potential, and entrapment efficiency agreed with the theoretical prediction.

Response surface analysis through polynomial models on glibenclamide transethosomes

Particle size

In topical and transdermal drug delivery, particle size plays an essential role in determining the pharmacologic properties of the product. Particle size also affects the rate, depth, and absorption pathway through the skin [44, 45].

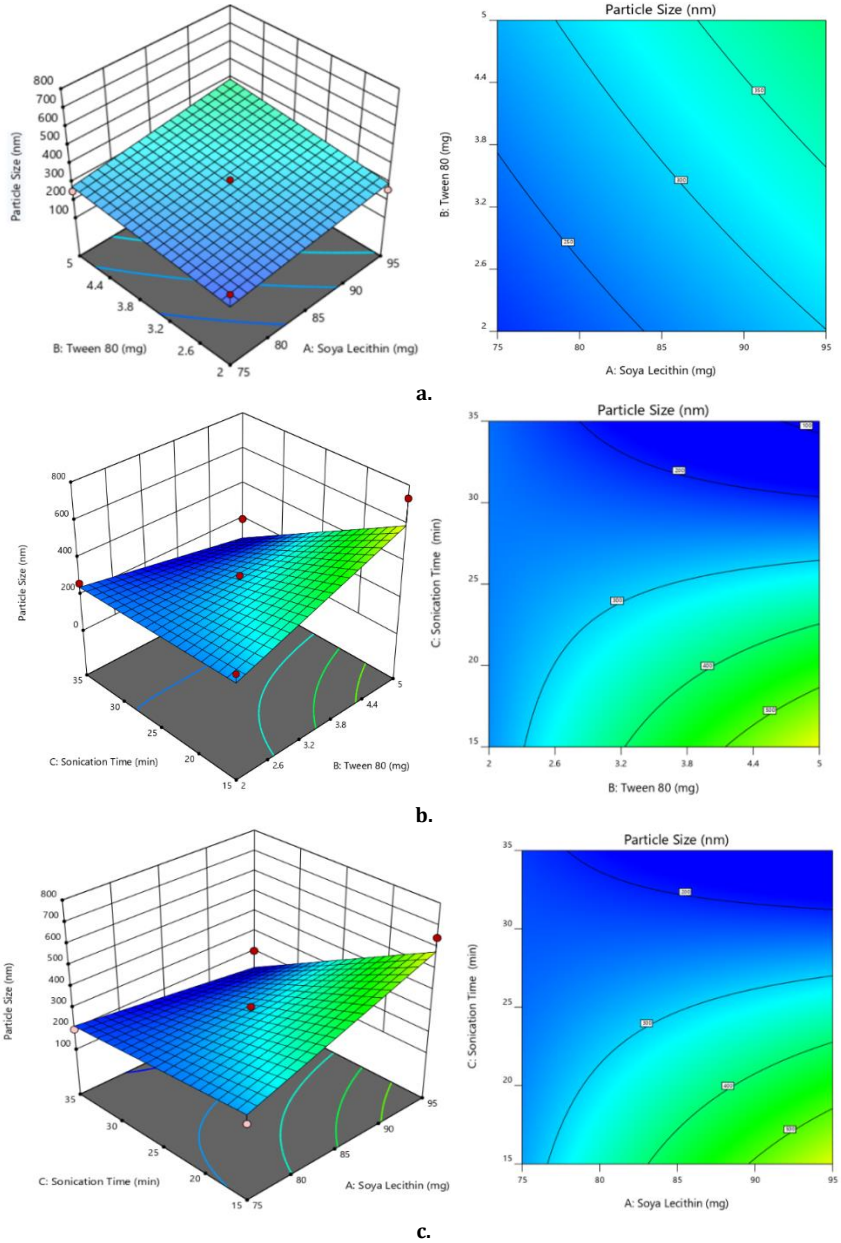
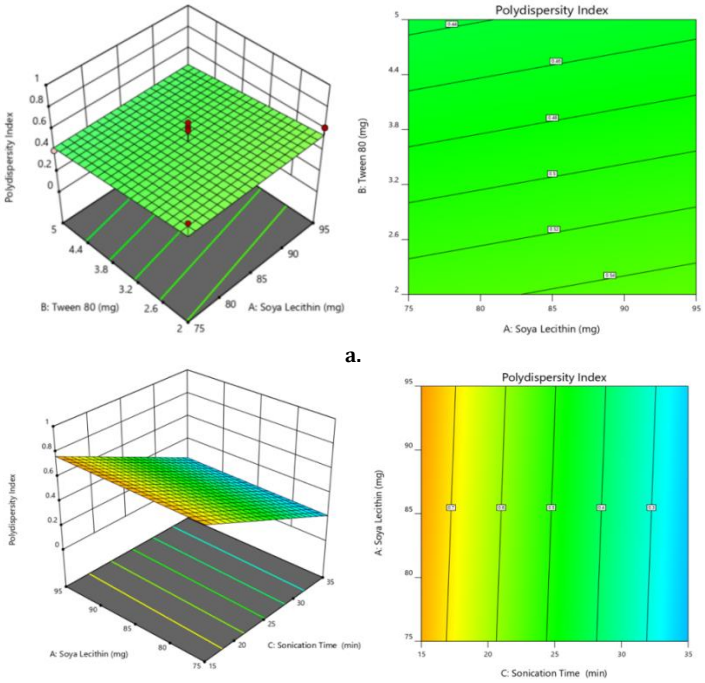


Fig. 2: Effect of independent variables on particle size of glibenclamide transethosomes (n=17)

Based on fig. 2, the lower amount of tween 80 and soy lecithin resulted in smaller particle size of transethosomes (fig. 2a). In general, a combination of high concentration of Tween 80 and soya lecithin in formulation resulted in larger particle size due to high concentration of phospholipid. By increasing the concentration of surfactant or phospholipids, the particle size decreased (fig. 2b, 2c). Despite the increasing concentration of surfactant, sonication time showed a significant effect, either increasing (<25 min.) or decreasing the particle size (>25 min.) As predicted in Eq. (2), sonication time is the dependent factor for particle size, to which at some point (25 min.) gives the negative interaction, thus decreasing the particle size. The effect of sonication time and Tween 80 on the particle size of transethosomes showed that a low concentration of Tween 80, with shorter sonication time, decreased particle size. This can be explained by the effect of concentration below the CMC of Tween 80 [46]. At shorter sonication time with increased concentration of Tween 80, it had been observed that the particle size increased. In contrast, a reduction in particle size was obtained by employing a sonication time of more than 25 min with the increase in Tween 80 concentration due to better vesicle individualization. In conclusion, higher sonication time in the formulation decreased the particle size of the transethosome, which is in line with the theoretical concept that increasing stirring speed would reduce the particle size. The study on the effect of minor soya lecithin concentration on particle size showed that by employing the decreased soya lecithin, the particle size of the transethosomes decreased. However, a high amount of soya lecithin with a shorter sonication time gave a larger particle size.

Polydispersity index (PDI)

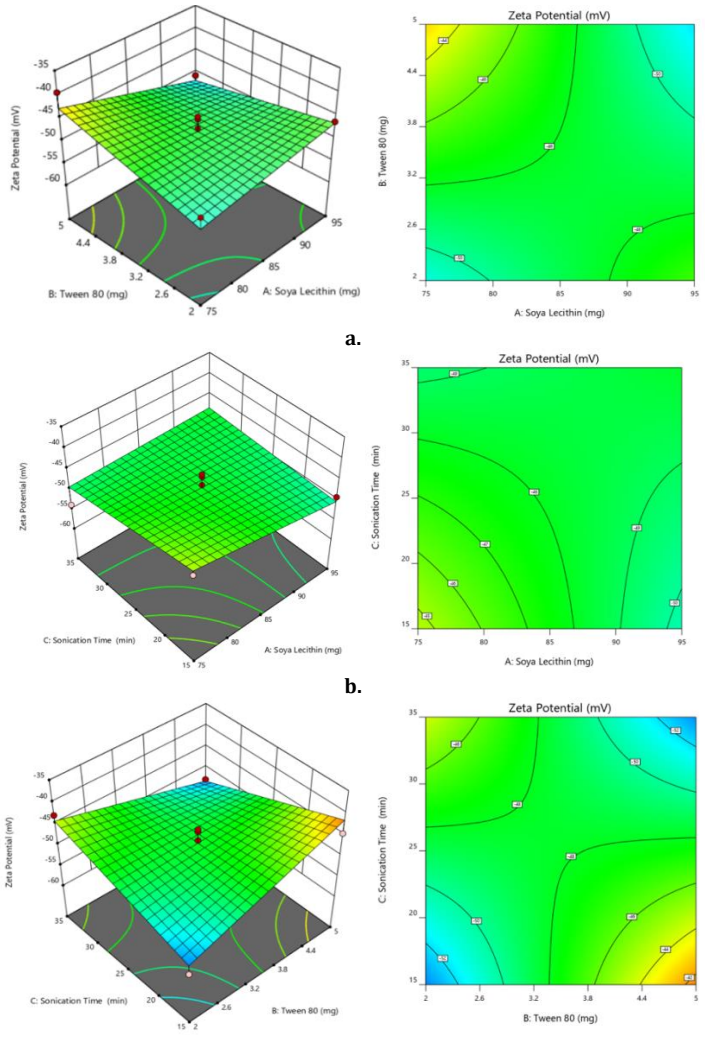
PDI represents the distribution of size populations within a given sample. Also known as the heterogeneity index, PDI is a number calculated from a two-parameter fit to the correlation data (the cumulative analysis) [47]. The results (fig. 3) showed that a low concentration of Tween 80 decreased the polydispersity index as expected from Eq. (3) prediction, the amount of Tween 80 as surfactant has a determining effect on PDI while the amount of soy lecithin predicted to have no significant impact on PDI and sonication time also affected the PDI value as the dependent variable with negative interaction. Higher sonication time has resulted in a more excellent PDI value due to better vesicle individualization (fig. 3b).



a.

b.

Fig. 3: **Effect of surfactant tween 80, sonication time and phospholipid amount on polydispersity index (n=17)**



a.

b.

c.

Fig. 4: Effect of independent variables on zeta potential (n=17)

Zeta potential

Zeta potential was used to quantitate the surface charge of these vesicles. The zeta potential of nanoparticles is an important influence factor on a particle's stability.

Based on fig. 4, studies on the effect of tween 80 and soya lecithin on zeta potential showed that a combination of the low amount of tween 80 and low amount of soya lecithin resulted in smaller zeta potential value or more stable vesicles. This result was in agreement with the prediction given by Eq. (4). However, a high concentration of tween 80 with low soya lecithin showed a high potential zeta value (close to 0) or decreased the amount of charge around the vesicle (fig. 4a), hence less stabile suspension which may due to aggregate formation above critical CMC value [46]. Studies on the effect of sonication time and soy lecithin amount on zeta potency showed that longer sonication time with lower soya lecithin amount increased the zeta potential value (fig. 4b), leading to reduced particle loading caused by larger particle sizes. Studies on the effect of sonication time and tween 80 on zeta potential showed that faster sonication time with a high amount of tween 80, decreased the zeta potential (fig. 4c). However, when the sonication time is increased with a decrease in the concentration of tween 80 it results in a more significant increase in the zeta potential value (close to 0). This allows aggregates to form due to large amounts of surfactant.

Entrapment efficiency

The entrapment efficiency is the amount of drug entrapped in the nanoparticles. A good nanoparticle system is a nanoparticle that has a high entrapment efficiency. The high entrapment efficiency is advantageous because it can transport enough drugs to the target cell.

A study on the effect of tween 80 and soya lecithin on entrapment efficiency showed that formulation using a low amount of tween 80 and soya lecithin revealed the lowest entrapment efficiency (fig. 5a). However, when a high amount of tween 80 with low soya lecithin been employed, the highest entrapment efficiency had been obtained. The properties of surfactants can explain this phenomenon. Surfactants are reported to affect the percent efficiency of drugs in the vesicles significantly. Surfactant monomers will combine through the lipid bilayer at low concentrations, thereby limiting vesicle growth. An increase in surfactant concentration promotes an increase in the fluidity of the vesicles through the membrane, which increases drug entrapping [48].

The effect of sonication time under soya lecithin concentration on entrapment efficiency showed that a high percentage of entrapment efficiency can be obtained by employing either sonication below 20 or above 30 min with soya lecithin concentration in the 80-90 mg range.

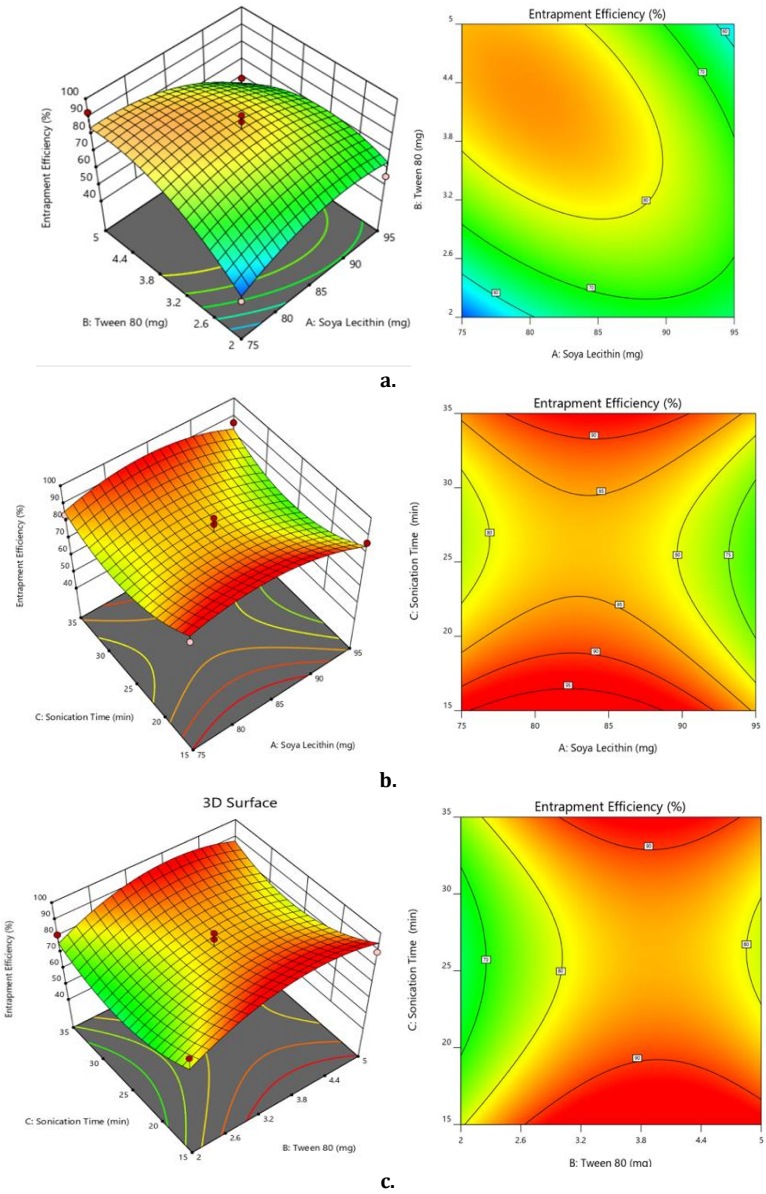


Fig. 5: Effect of independent variables on entrapment efficiency (n=17)

The effect of sonication time and tween 80 concentration on entrapment efficiency shows that high entrapment efficiency values can be obtained when the sonication time is above 30 or below 20 min with an increase in the concentration of tween 80. A sonication time of more than 30 min will allow damage to the vesicles during the formation of the vesicles. Therefore, drug diffusion across the vesicle membrane is also affected. The mixing process with a sonication time of under 20 min will result in imperfect vesicle formation, so the trapped drug will also be low.

Characterization of glibenclamide-loaded transethosomes

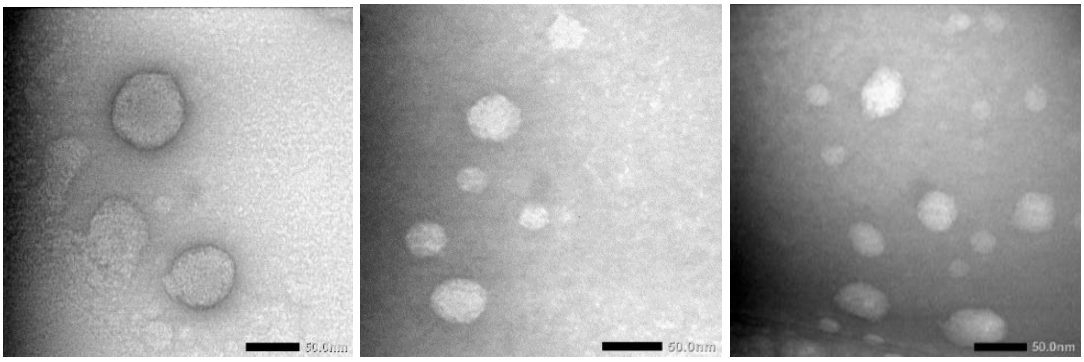
Based on the Box Behnken Design approach, the optimal formula for glibenclamide-loaded transethosome was 75 mg soya lecithin, 5 mg tween 80, and a sonication time of 18.79 min. The corresponding predicted response values were Y_1 (particle size) = 374.5 nm, Y_2 (polydispersity index) = 0.6, Y_3 (zeta potensial) =-38.16 mV, and Y_4 (entrapment efficiency) = 91.76%. According to Box Behnken design, a confirmation test on the optimum of the Glibenclamide transetosome formula was conducted, and the results were as follows (table 3). It can be concluded that the predicted optimum formula of transethosome revealed physicochemical characteristics in the range of expected values and gave better performances compared with those from glibenclamide ethosomes.

The morphology of the vesicles had been studied by employing Transmission electron microscopy (TEM). It is a microscopy technique whereby a beam of electrons is transmitted through an ultrathin specimen, interacting with the specimen as it passes through. An image is formed by the interaction of the electrons transmitted through the specimen; the idea is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or to be detected by a sensor, such as a camera [49].

Table 3: Physicochemical characteristics of the optimum formula of glibenclamide as transethosomes compared with ethosomes

Formulation	Particle size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)
Blank Transethosomes	236±7.6	0.378±0.1	-67.1±2.4	-
Glibenclamide Transethosomes	166.8±5.3	0.463±0.1	-44.7±2.2	87.18±3.8
Glibenclamide Ethosomes	291.7±5.8	0.872±0.4	-64.4±3.0	74.73±2.0

Data are expressed as mean±SD, n=3



(a) (b) (c)

Fig. 6: Morphology of glibenclamide vesicles with transmission electron microscopy at 80000 magnification as (a) ethosomes (b) transethosomes, and (c) blank transethosomes as comparison

The Glibenclamide transethosomes vesicles have a well-identified spherical shape as individualized vesicles with sharp boundaries. The morphology also showed that the particle size was in the nanometric range (fig. 6b). Likewise, transethosome vesicles without Glibenclamide (blank transethosomes, fig. 6c) were spherical as individualized nanometric-sized vesicles. Glibenclamide ethosomes, as the vesicles without surfactant addition, have bigger particle sizes than spheric individualized vesicles. This result agreed with those from particle size measurements (table 3). This also explains the advantage of the transethosomal system, which owing better skin permeation due to either a more flexible structure from surfactant property in the formula or because of the smaller particle size of the vesicles.

During the formulation process, chemical interaction could happen between Glibenclamide and another excipient in the vesicular system. This possible interaction was studied by employing FTIR spectroscopy to Glibenclamide in bulk, transethosome, ethosome, and blank transethosome. The FTIR method relies on physicochemical principles that underlie the ability of chemical bonds to absorb energy from incident light at characteristic energies/wavelengths. The molecular bonds stretch upon absorption of this energy, referred to as molecular vibrations. During these vibrations, the distribution of electrons in the molecule (electron density) changes as the bonds expand and contract. Therefore, the vibrations arising from the absorption of incident energy can change the relative distance between the positive and negative charges in the molecule, known as the dipole moment [50].

FTIR analyses were performed on Glibenclamide, the excipients (soya lecithin and tween 80), and Glibenclamide formulated as either transethosome or ethosome. Observations were also performed on blank transethosomes as vesicles without Glibenclamide for comparison. The spectrum was overlaid and compared to assess any possible interaction (fig. 7). The FTIR spectra of Glibenclamide as bulk powder showed characteristic peaks of secondary NH at wave numbers 3310-3500 cm^{-1} and CH at 2853-2962 cm^{-1}. In comparison, FTIR spectra of glibenclamide transethosome have a similar peak to that of glibenclamide bulk powder, indicating no change in the IR peak of Glibenclamide after being formulated into transethosomes. Thus it can be concluded that no chemical interactions occurred. Accordingly, the FTIR spectra of transethosomes without Glibenclamide (blank transethosome) showed the same peak as the vesicle-forming component (phospholipids) in the 2853-2962 cm-1 range. Glibenclamide vesicles without surfactant (glibenclamide ethosome) also showed similar peaks with bulk Glibenclamide.

To study possible physicochemical interactions between glibenclamid with excipients in vesicular system with regard to the thermal behavior, analysis by Differential Scanning Calorimetry (DSC) was performed. This method is one of the critical techniques to characterize the melting and crystallization behavior of crystalline materials [51].

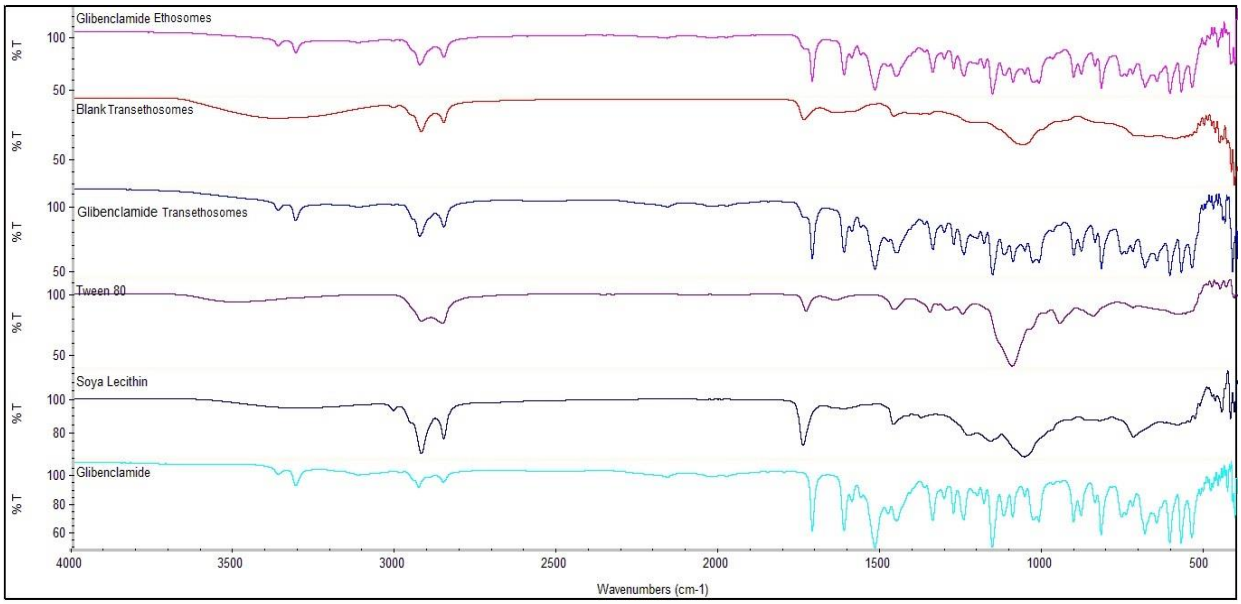


Fig. 7: FTIR spectra of glibenclamide, soya lecithin, tween 80, glibenclamide transethosome, blank transethosome, and glibenclamide ethosome

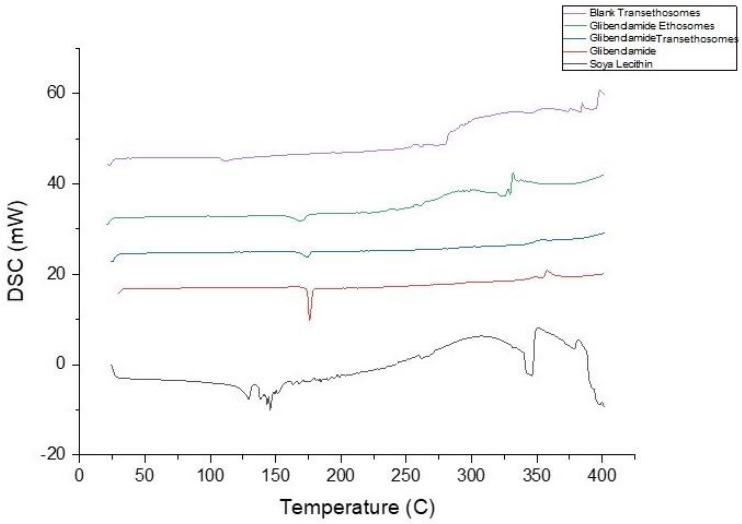


Fig. 8: Diffractogram of glibenclamide, soya lecithin, glibenclamide ethosomes, blank transethosome, glibenclamide transethosomes

The results of characterization using Differential Scanning Calorimetry showed that there was no shifting in the melting point of the glibenclamide-loaded transethosomes compared to that of Glibenclamide (176,01 °C to 173.77 °C) (fig. 8).

Transethosomes can overcome the low permeability of drugs due to their ability to deform; hence, they can pass through the intracellular stratum corneum and give better skin penetration [18]. As the transethosome was developed for transdermal preparation, skin penetration may be the key to success for the next step in the formulation. Preliminary research on skin penetration was conducted by performing a solubility test of the transethosomes. A solubility test is usually carried out to determine a compound's ability to dissolve in a solvent, generally some liquid [52]. Formulation study of glibenclamide transethosome in this research revealed that the solubility test on the drug-loaded transethosome had been significantly increased ($p<0.005$) to 86.05±8.0% compared with that of glibenclamide bulk powder and glibenclamide ethosomes (fig. 9a). It can be concluded that transethosomes could increase the solubility of Glibenclamide. The increase in solubility offers an excellent opportunity for Glibenclamide to be formulated as transdermal preparation. This hypothesis was confirmed by *in vitro* permeation study [48], which was the final confirmation of the research on developing glibenclamide transethosome for transdermal preparation.

It was found that the drug release of glibenclamide-loaded transethosomes was 91.65%, while that of glibenclamide ethosomes was 81.40% (fig. 9b). As a comparison, the glibenclamide bulk powder showed poor drug release to which 1.70% within 8 h (480 min). The release of glibenclamide transethosome showed a significant increase ($p<0.001$) as well as that from ethosomes ($p>0.05$). Despite a slight rise of glibenclamide release from transethosomes compared with those from ethosomes, physical stability issues of transethosome (measured by particle size, PDI, zeta potential as well as entrapment efficiency), which was better than ethosome (table 3) should be taken into consideration.

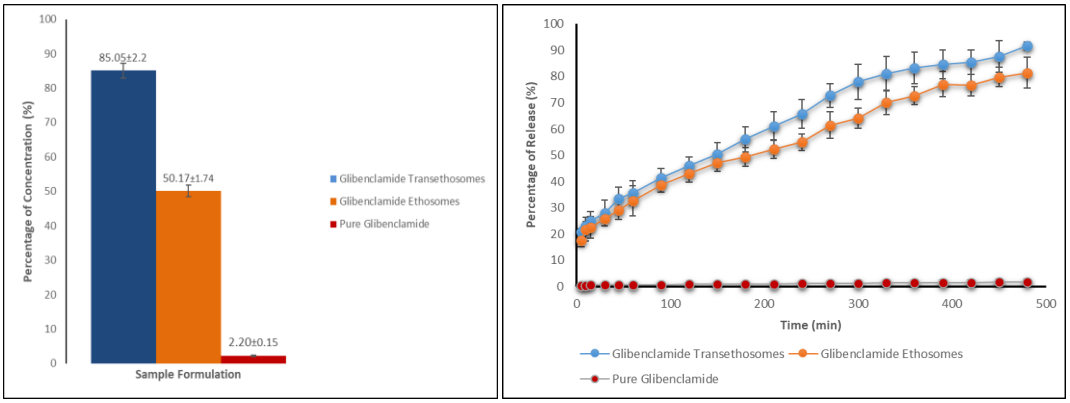


Fig. 9: Improved physical characteristics of glibenclamide as transethosomes, ethosomes, and pure drug powder measured as (a) solubility and (b) *in vitro* permeation study (n=3)

***In vivo* release study of glibenclamide transethosome from patches**

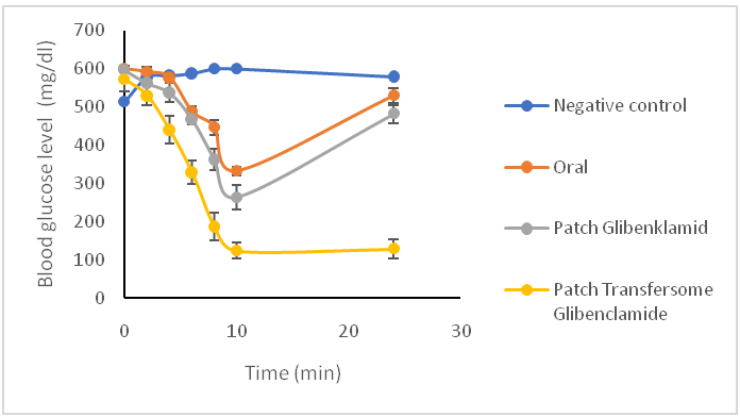


Fig. 10: *In vivo* drug release study of glibenclamide (n=6)

In vivo, glibenclamide drug release was conducted using Wistar rats divided into four groups. The animals were treated with carboxymethyl cellulose suspension in the negative control group. There was no significant change in glucose level after 24 h. The group that was treated with Glibenclamide either in oral or transdermal route in the form of a patch showed a decrease in glucose level. The group with glibenclamide transethosome patch had a significant difference ($p<0.05$) compared with the others.

DISCUSSION

Preliminary research on formulating glibenclamide transethosomes was the determination of Critical Micelle concentration (CMC) of Tween 80 as a surfactant to prevent the presence of micelles formed during the testing process. The results revealed that the minimum concentration of tween 80 as surfactant was below 5 mg. The maximum concentration for the use of surfactants needs to be known to avoid the formation of aggregates originating from micelles [27, 53-54]. Micelle is an aggregate of surfactant molecules dispersed in a liquid, forming a colloidal suspension. Micelles are included only when the surfactant concentration exceeds the critical micelle concentration (CMC). It can be determined by measuring the surface tension at different concentrations. The method used in this experiment was based on the donor-acceptor interaction method with iodine [27]. The absorbance of the mixture of iodine with various concentrations of Tween 80 showed a fracture of the graphs at a concentration of 5 mg. This value had been used as the maximum concentration of surfactants in the formulation of glibenclamide transethosomes.

The optimal transetosome formulation was determined using the Behnken Box design to predict desired physicochemical characteristics. This design is part of the Response Surface Methodology (RSM), an effective mathematical method and statistical technique for optimizing a complex process. It is advantageous since it does not contain points at the ends of the cubic region created by the combination of two-level factorial levels, which will be expensive to test or unsupportive due to physical constraints in the experiment [54].

Based on the results of the Box–Behnken Design, it was found that the largest particle size of glibenclamide transethosome was 733.5 nm coming from the formulation with 85 mg of soya, 5 mg tween 80, at a sonication time of 15 min. The increase in phospholipid concentration resulted in increased particle size of the transethosomes (fig. 2) due to increased viscosity. The particle size of the transethosome increased due to the swollen core of the particles loaded with liquid phospholipid [55]. Phospholipids as components of the vesicular system have several advantages, such as increased efficacy and therapeutic index, increased stability of encapsulated drugs, and reduced toxicity [56]. Soya lecithin is a type of phospholipid that is the main component of biological membranes. Based on these experiments, the concentration of soya lecithin as phospholipid directly affected vesicle particle size. The increase of phospholipid concentrations in the aqueous phase enhanced the viscosity of the dispersion as well as the particle size. The result agreed with the previous study on liposomes, which reported that particle size decreases with decreasing phospholipid concentration [57]. Liposomes with low amounts of phospholipid (phosphatidylcholine 50%), were smaller in size than those with higher amounts of phospholipid (phosphatidylcholine 95%) [58].

Surfactants, a unique class of surface active molecules, have the remarkable ability to control crystal growth as well as the shape and

size of the molecules. Tween 80, as a surfactant, is an edge activator that can improve vesicle permeability. The surfactant molecules were taken up into the liposome membrane at low concentrations without breaking up the vesicle. As the concentration increases, the proportion of surfactant molecules in the membrane increases until a critical value. Above this threshold value, rupture of membrane parts is induced [57]. High surfactant concentration allowed micelles to form; thus, more giant-size vesicles were obtained [59]. The desired morphological adjustment can be achieved by controlling surfactant architecture and self-assembly behavior, such as surfactant concentration. Therefore, the concentrations of surfactants need to be carefully selected to ascertain vesicle formation. At specific concentrations, tween 80 in vesicles caused a decrease in particle size due to steric repulsion rendered by surfactant molecules, which prevents or minimizes the aggregation of the vesicles [57]. Surfactants interact with the surface of transethosome, and depending upon the property of the head and tail groups of surfactants and their molar equivalent ratio to the precursors, they affect transethosome's vesicle growth. The presence of surfactant maintained the stability of transethosome size [57].

Studies on the effect of sonication showed that particle size decreased when the time of sonication increased due to more incredible energy released to the system, which led to the rapid formation of polymeric dispersion of inorganic phase as nanodroplets, thus smaller particle size. Increasing the sonication time will also increase the specific surface area of the prepared samples [60]. Accordingly, a high concentration of tween 80 combined with a shorter sonication time caused the increase in particle size due to aggregate formation. Using surfactant in concentration beyond the CMC with low stirring speed promoted big droplets of transethosome during the vesicle formation process.

According to Danaei, *et al.* (2018), the particle size range for transdermal are 10-600 nm [47, 61]. It has to be noted that vesicles with an average diameter of 600 nm or beyond will not be able to penetrate the skin layer and will mostly remain on the surface of the stratum corneum. Based on the results, it can be concluded that the transethosomes vesicular system offers a promising possibility of Glibenclamide being formulated as transdermal delivery.

Polydispersity characterization is essential in nanoparticle applications, as it is challenging to control sample-wide uniformity with surface conjugation chemistry due to aggregation of particles [62]. Based on the requirement, the range of numerical value of the particle size distribution should be between 0.0 (for a perfectly uniform sample) and 1.0 (for a highly polydispersed sample with multiple particle size populations) [47]. Based on fig. 3, it was found that the most extensive particle size distribution was from experiments using 85 mg soy lecithin, 5 mg tween 80, at a sonication time of 15 min. An increase in tween 80 concentration as surfactant made the particle size distribution larger. Sonication time it also affected the polydispersity index (fig. 3). By employing the decrease in sonication time, the polydispersity index values tend to be greater due to more heterogeneous dispersion during the vesicle formation process. This result was in line with a former study that found that longer sonication times could make the dispersion more homogeneous and monodispersed [63].

Zeta potential indicates the degree of repulsion between intensely charged particles deployment. High zeta potential implies highly charged particles, which prevent aggregation particles due to electrical repulsion [64]. To have a high degree of stability, the required zeta potential value should be greater than+30 mV or less than-30 mV. The sample has sufficient repulsion force in those conditions to achieve a colloidal system with better physical stability [64–66]. From the results, it can be observed that the formula having zeta potential value close to the requirement was that with 75 mg of soya lecithin, 5 mg of tween 80, and a sonication time of 25 min.

The effect of tween 80 on zeta potential is due to the surfactant mechanism. Surfactants are crucial excipients in the synthesis of nanovesicles. They are amphiphilic molecules characterized by a hydrophilic head group (ionic or nonionic) and a hydrophobic tail. The amphiphilic nature of surfactants will stabilize the hydrophobic nanomaterials in aqueous media. Hydrophobic regions interact with nanovesicle surfaces, while hydrophilic ones will interact with water, thus providing colloidal stability and improving dispersion stability by preventing nanovesicle aggregation [67, 68]). The addition of surfactant decreases interfacial energy in the system and eliminates the hydrophobic groups in contact with the water. Hence, the system's free power shrinks. When surfactant concentration increases, excess surfactant molecules begin to agglomerate with others and form micelles. An increase in the surfactant beyond the CMC value results in more micelle formation [69]. As many studies revealed, more positive or negative zeta potential values of nanovesicles indicate good physical stability due to the electrostatic repulsion of individual particles. On the other hand, zeta potential value close to zero can result in particle aggregation and flocculation due to van der Waals attractive forces [70].

Drug entrapment efficiency (EE) is also known to be influenced by several factors, namely the composition of the vesicles and the manufacturing process. A study on the effect of sonication time on entrapment efficiency of transethosomal glibenclamide system showed that a high value of EE can be obtained when sonication time was above 30 or below 20 min with Tween 80 concentration between 2-5 mg. Over time, sonication (higher than 30 min) destroyed the vesicle during self-assembly of the vesicle; therefore, drug diffusion on the vesicle membrane was also affected. Mixing process by sonication below 20 min promoted the uncomplete vesicle formation; thus, the entrapped drug would also be low. A study on the effect of tween 80 concentration as a surfactant on entrapment efficiency showed that increasing the amount of tween 80 had increased drug entrapment efficiency. This may be explained by increased drug solubility in the lipid system due to increasing surfactant concentration [55]. Surfactants are also reported to affect the percent efficiency of drug absorption in vesicles [48]. A small surfactant concentration affects the percentage of entrapment efficiency due to the nature of surfactant monomers, which combine through the lipid bilayer. Hence, it will limit the vesicle growth. The increase in surfactant concentration also promotes the increase in the fluidity of the vesicle in the permeation process through a membrane, which leads to the rise in the entrapped drug [48].

Characterization on particle size, polydispersity index, zeta potential, and entrapment efficiency of Glibenclamide transethosome were compared with those from transethosome without Glibenclamide (blank transethosome) and glibenclamide-loaded ethosome (table 3). Glibenclamide ethosome formulation had the same phospholipid and ethanol as well as parameter production processes despite the absence of tween 80 as the surfactant in the formulation. The particle size of the glibenclamide ethosome was more significant than those of transethosomes and blank transethosomes due to the presence of surfactant. Likewise, the polydispersity index of glibenclamide ethosome was higher, reflecting the possibility of the vesicle being more heterogeneous than Glibenclamide as transethosomes. The polydispersity index is a measurement of a sample's heterogeneity based on size. Surfactants play a role in forming vesicles that lead to differences in particle size. The presence of surfactants in the transethosome causes modification of the vesicle surface charge, affecting vesicle characteristics such as particle size [64]. The presence of surfactant in transethosomes formulation promoted a more permeable membrane for the drug to be absorbed in the membrane layer, thus leading to higher entrapment efficiency than in ethosomes.

The results of TEM images also showed that the vesicles were not aggregated, which is also in line with the development from zeta potential and PDI measurement (table 3). Likewise, the FTIR results showed that glibenclamide transetosome did not reveal any functional group formation compared to those from bulk glibenclamide powder. Based on the data, it can be concluded that after formulation into either transethosome or ethosome, glibenclamide as an active drug did not undergo any chemical interaction with the excipients.

Characterization of the thermal behavior of glibenclamide transethosomes by DSC revealed that the widening of the peaks that occurred and the reduction of peak intensity in glibenclamide transethosomes was due to an increase of the amorphous form in the vesicular product, which leads to the increase in solubility [51, 64].

The transethosomal system of glibenclamide has proven to increase *in vitro* drug permeation compared with those from the ethosomal system and especially when compared with the original glibenclamide bulk powder (fig. 9). The mechanism of permeation of drug compounds involves the disruption of dense lipids that fill the extracellular space of the stratum corneum. Glibenclamide transethosomes contain soya lecithin as a phospholipid component, which allows modification of the structure of the stratum corneum. In addition, the surfactant in glibenclamide transethosomes formulation provided the possibility to undergo deformation, thus enhancing the vesicles to be more easily penetrated [71, 72].

The glibenclamide patch containing glibenclamide in a transethosome system revealed a significant decrease in rats' blood glucose levels. The presence of surfactants and ethanol in the transetosome vesicles had proven to increase the permeation of glibenclamide into the skin, thus increasing its effectivity as antidiabetic [18].

CONCLUSION

Development of glibenclamide-loaded transethosome using surfactant as edge activator through optimization with Box Behnken Design resulted in transethosome composition as ultra-deformable vesicles that met the physical characteristics, increasing solubility and permeability of glibenclamide. Increased permeability of glibenclamide transethosome offered the potential to be developed as transdermal preparations to overcome the low permeability of glibenclamide.

AUTHORS CONTRIBUTIONS

All authors contributed to this article. Nurul Arfiyanti Yusuf contributed to the design, definition of intellectual content, literature search, clinical trial, experimental studies, data acquisition, data analysis, statistical analysis, and manuscript preparation of this article. Marline Abdassah contributed to concepts or ideas, design, definition of intellectual content, experimental studies, data acquisition, and manuscript preparation of this article. Iyan Sopyan contributed to experimental studies, data acquisition, data analysis, and statistical analysis of this article. Rachmat Mauludin contributed to concepts or ideas, design, definition of intellectual content, manuscript preparation, and manuscript editing of this article. I Made Joni contributed to concepts or ideas, design, definition of intellectual content, literature search, manuscript preparation, and manuscript editing of this article. Anis Yohana Chaerunisaa contributed to concepts or ideas, design, the definition of intellectual content, data acquisition, data analysis, manuscript preparation, and manuscript editing of this article. They also approve the journal to which the article will be submitted, give final approval for the version to be published and agree to be accountable for all parts of the work.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The author reports no conflicts of interest in this work.

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