








Article

Vesicular Emulgel Based System for Transdermal Delivery of Insulin: Factorial Design and in Vivo Evaluation

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Abstract: Transdermal delivery of insulin is a great challenge due to its poor permeability through the skin. The aim of the current investigation was to evaluate the prospective of insulin loaded niosome emulgel as a noninvasive delivery system for its transdermal therapy. A 2³ full-factorial design was used to optimize the insulin niosome emulgel by assessing the effect of independent variables (concentration of paraffin oil, Tween 80 and sodium carboxymethyl cellulose) on dependent variables (in vitro release, viscosity and in vitro permeation). The physical characteristics of the prepared formulations were carried out by determining viscosity, particle size, entrapment efficiency, drug loading, drug release and kinetics. In vitro permeation studies were carried out using rat skin membrane. Hypoglycemic activity of prepared formulations was assessed in diabetic-induced rats. It was observed that the independent variables influenced the dependent variables. A significant difference ($p < 0.05$) in viscosity was noticed between the prepared gels, which in turn influenced the insulin release. The order of permeation is: insulin niosome emulgel > insulin niosome gel > insulin emulgel > insulin gel > insulin niosomes > insulin solution. The enhancement in transdermal flux in insulin niosome emulgel was 10-fold higher than the control (insulin solution). In vivo data significantly demonstrated reduction ($p < 0.05$) of plasma glucose level (at six hours) by insulin niosome emulgel than other formulations tested. The results suggest that the developed insulin niosome emulgel could be an efficient carrier for the transdermal delivery of insulin.

Keywords: niosome; optimization; emulgel; insulin; skin permeation

1. Introduction

It is well known fact that transdermal delivery is considered an alternative route to both oral and parenteral drug administration [1]. The transdermal route has the advantages of avoiding liver metabolism, providing constant plasma drug concentration and avoids GIT irritation than oral route

and shows better patient compliance than parenteral administration [2,3]. However, extremely low permeability of the skin to water soluble molecules secondary to the formidable barrier property of the lipophilic stratum corneum has been an obstacle to its use [4,5]. The barrier effect of the skin further makes it difficult to prepare transdermal systems capable to effectively deliver drugs in high concentrations into the blood. Therefore, transdermal systems with enhancing skin permeability and increasing drug delivery are of great interest nowadays. Transdermal drug-delivery systems including emulsions, gels and their combination (emulgels) have been found to increase skin permeability for drugs and thereby improve systemic absorption and drug effectiveness [6,7]. Due to its greaseless and thixotropic behavior, emulgel demonstrates an enhanced drug release profile, facilitates spreadability on the skin and increases absorption while preventing typical stability problems associated with emulsions, such as phase separation, creaming and coalescence [6]. In addition, novel drug-carrier systems such as liposomes, niosomes, and microemulsions have demonstrated encouraging results in skin, cutaneous and transdermal delivery of various drugs [8–10]. On the other hand, transdermal delivery of protein pharmaceuticals has also been extensively studied [11,12].

Incorporation of nanovesicles into transdermal vehicles has proved to enhance percutaneous drug absorption [13]. Niosomes are liquid dispersions that require gelling agents to be suitable for transdermal application. The backbone of this system is the non-ionic surfactant. Being amphiphilic in nature, niosomes can accommodate drug molecules with diverse physicochemical characteristics. They may increase the fluidity of the skin membranes through their interaction with the lipophilic layer—which reduces membrane resistance to drug permeation [14]. Moreover, several studies indicated that the niosomes may diffuse deep in the skin [15] and can deliver drugs into the systemic circulation [16,17]. The combination of niosomes with emulgels has resulted in the emergence of new drug-delivery system called the niosome emulgel. This system demonstrates superior transdermal perfusion compared to niosomes, which may be attributed to the dual effect of surfactants presented in niosome formulation and emulsion preparation. Moreover, they have the ability to diffuse across the narrow pores in the skin [6]. Recently, our group succeeded in enhancing the transdermal permeability of ketorolac tromethamine (water soluble drug) by novel niosome emulgel combination [6]. In the same context, the purpose of the current investigation was to maximize the transdermal permeability of insulin via utilizing niosome emulgel combination.

Insulin is a well-known therapy for treatment of insulin-dependent diabetes mellitus. Insulin is a hormone formed of two peptide chains and has a molecular weight about 5.7 kDa [18]. Subcutaneous administration is considered as the main route for insulin delivery into the human body; however it has several drawbacks [19]. Literature suggests that various skin-permeation approaches—including chemical enhancers, nano or micro vesicles, low electric current, ultrasound, jet injection and micro needles—have been assessed to enhance the delivery of insulin [20,21]. In the last two decades, several research groups have successfully developed various devices capable of biosensing and deliver insulin through the skin [22]. In the current investigation, an optimized insulin-loaded niosome emulgel combination was investigated as a safe, painless and high-patient compliant drug-delivery system. To our knowledge, no previous study has assessed the potential of the niosome–emulgel combination as a drug-delivery system for the transdermal therapy of insulin.

2. Methodology

2.1. Materials

Human recombinant insulin was donated by the Saudi Pharmaceutical Industries & Medical Appliances Corporation (SPIMACO), Saudi Arabia. Sorbitan monostearate (Span 60), sodium azide and cholesterol (>99%) were procured from Sigma chemical Co., St. Louis, MO, USA. Sodium carboxymethyl cellulose was gift from Delta pharm Co., Egypt. All other chemicals and solvents used were of analytical reagent grade and were procured from local vendors.

2.2. Preparation of Niosome Emulgel

The schematic representation of prepared gels and emulgels of insulin are presented in Figure 1. The compositions of various formulations are summarized in Table 1. Niosomes were primarily prepared according to the method reported in our earlier study [23] with certain modifications. Accurately weighed amounts of Span 60 were mixed with a fixed ratio of cholesterol (1:1) in chloroform in a round bottom flask to obtain a final concentration of 100 μmol , total lipid. The previous mixture was evaporated in vacuum rotary evaporator (Cole-Parmer T-1602-21, Japan), for 15-min until a dry thin film was formed. The thin lipid film was hydrated with phosphate buffer (pH 7.4) containing insulin (1.4 mg/mL), then the flask was securely closed and heated in a thermostatic water bath (55–60 °C) for 5 min until to form niosome suspension. Subsequently, the resultant niosomes were sonicated at 150 W (probe sonicator, Qsonica, LLC, Misonix, Farmingdale, NY, USA) for about 15 min at 20 °C in cycles of 3 min sonication followed by 2 min stop of sonication to prevent heating of the niosome–insulin suspension. The gels were formulated by sprinkling adequate quantity of gelling agent evenly over the surface of the water containing insulin and blending for 10 min until a homogenous gel was obtained. In case of emulgel, insulin was dissolved in aqueous phase containing the surfactant (Tween 80), and then the oily phase (paraffin oil) was added gradually. The mixture was homogenized at 12,000 rpm for 5 min. The required amount of sodium carboxymethyl cellulose was dusted gradually over the surface of the drug-loaded emulsion and homogenized at the same rate for 10 min until a homogenous emulgel was obtained [6]. In case of niosome gel or emulgel combinations, an equivalent amount of insulin solution was replaced by insulin-encapsulated niosome preparations and the formulations was developed as mentioned before.

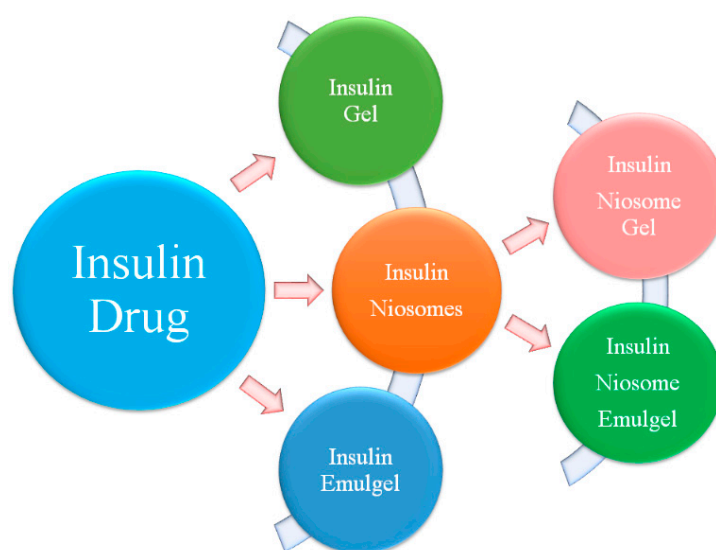


Figure 1. Schematic representation of prepared gels and emulgels of insulin.

Table 1. Composition of prepared gels and emulgels of insulin.

Materials	Insulin Gel	Insulin Niosome	Insulin Niosome Gel	Insulin Emulgel	Insulin Niosome Emulgel
Insulin (mL) *	4	0	0	4	0
Sodium carboxymethyl cellulose (g)	0.4	–	0.4	0.4	0.4
Liquid paraffin (mL)	0	0	0	1.25	1.25
Tween 80 (mL)	0	0	0	0.125	0.125
Niosomes-loaded insulin (mL) *	0	4	4	0	4
Buffer pH 7.4 Q.S. to (g)	10	10	10	10	10

* Each mL contained 1.4 mg of insulin.

2.3. Experimental Design

To enhance the effect of insulin niosome for transdermal delivery, it was decided to develop a niosome gel as well as a combination approach of niosome emulgel system. Based on preliminary studies of batches containing insulin gel, insulin niosome, insulin niosome gel, insulin emulgel and insulin niosome emulgel, it was observed that niosome emulgel formulation shows better percutaneous permeation than other formulations. Hence, for further optimization, in experimental design study, niosome emulgel formulations were taken into consideration. By looking at the composition of niosome emulgel and data of preliminary batches, it was found that the concentration of paraffin oil, Tween 80 and sodium carboxymethyl cellulose have significant effect on formulation viscosity, drug release and permeation. This information helped us to select a 2^3 full-factorial design—an ideal method for optimizing various factors with fewer but enough runs when the number of variables are two to four. The full-factorial design creates experimental points using all the possible combinations of the levels of the factors in each complete trial or replication of the experiments. It can vary all factors simultaneously at a limited number of factor levels. Three factors were considered at two levels for further optimization as shown in Table 2. The niosomes were prepared as per method mentioned earlier and utilized for experimental design study. Design expert software (DX 11 Software, StatEase Design Expert, Statistical software, Minneapolis, MN, USA) was used for optimization of insulin loaded niosome emulgel, the effect of 3 and 2 leveled numeric variable (2^3 full-factorial design) was selected for evaluation using contour plot and 3D response surface methodology. For this optimization process, concentration of paraffin oil, Tween 80 and sodium carboxymethyl cellulose were taken as independent variables and in vitro release, viscosity and in vitro permeation were chosen as dependent variables. According to factorial design, 08 runs were designed to perform the best close-fitting model.

$$Y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_{12}x_1x_2 + \beta_{23}x_2x_3 + \beta_{13}x_1x_3 + \beta_{123}x_1x_2x_3 + C \quad (1)$$

where, Y is response, β_0 is intercept, $\beta_{1,2,3}$ are the main effect of the variables x_1 , x_2 and x_3 , β_{123} is the interaction effect of all three variables and C is standard error of design.

Table 2. 2^3 Full-factorial design study with independent variables.

Formulation No.	Independent Variables in Coded and Actual Values					
	X ₁ (Conc. of Paraffin Oil (mL))		X ₂ (Conc. of Paraffin Oil (mL))		X ₃ (Conc. of Sodium Carboxymethyl Cellulose (g))	
	Coded	Actual	Coded	Actual	Coded	Actual
F1	1	2.5	−1	0.125	−1	0.4
F2	−1	1.25	−1	0.125	1	0.6
F3	1	2.5	1	0.25	−1	0.4
F4	−1	1.25	1	0.25	−1	0.4
F5	1	2.5	1	0.25	1	0.6
F6	−1	1.25	−1	0.125	−1	0.4
F7	1	2.5	−1	0.125	1	0.6
F8	−1	1.25	1	0.25	1	0.6

2.4. Evaluation

2.4.1. Viscosity

The formulations were tested at room temperature using Brookfield viscometer (Ametek GB LTD T/A Brookfield, UK). The dynamic viscosity measurements were performed employing spindle R5 at 0.5 rpm.

2.4.2. Particle Size

Particle size, size distribution and polydispersity index (PDI) of various niosome formulations were analyzed by Zetasizer Nano ZS90 (Malvern Instruments, Ltd., Worcestershire, UK) at 25 °C [24].

2.4.3. Entrapment Efficiency

Samples of niosomes (1 mL) were placed in Eppendorf tubes and centrifuged ($14,269\times g$) for 40 min at 4 °C. Niosome pellets were suspended in phosphate buffer (pH 7.4) and the procedure was repeated twice to recover any untrapped drug exists between void volumes of niosomes. The supernatant was isolated each time from niosome pellets and quantified for free insulin by measuring the absorbance using UV-Vis spectrophotometer (Jenway, 6205, spectrophotometer, UK) at 214 nm [25]. Similarly, percentage of drug loading was estimated by the equation [26]; Percent drug load = (Amount of drug in niosomes)/Amount of surfactants incorporated + Amount of drug incorporated) \times 100.

2.4.4. Scanning Electron Microscopy

The morphology of niosome emulgel formulation was determined using scanning electron microscope (JEOL JSM-5510LV, Tokyo, Japan). The niosome emulgel was diluted with water (1:10). Few drops of sample was mounted on a stub covered with double adhesive tape and dried and further coated with gold for visualization.

2.5. Drug Release and Kinetics

The in vitro drug release study was performed using a customized horizontal glass diffusion tube and cellulose dialysis membrane (MWCO 12–14 kDa, Spectra/por® Spectrum Laboratories, Inc., Rancho Dominguez, Berkeley, CA, USA). The tubes with 0.5 g of gel were attached to the dissolution apparatus and was allowed to stir at 100 rpm in 250 mL of phosphate buffer (pH 7.4) maintained at 37 ± 0.5 °C. Samples were collected at predetermined time intervals (5, 15, 30, 45, 60, 120, 180, 240, 300 and 360 min) and replaced with equal volumes of fresh buffer solution. Samples were filtered using syringe membrane filter (0.2 μ m, Millipore Corporation, Bedford, MA, USA) and readily analyzed by UV-Vis spectrophotometer (Jenway, 6205, spectrophotometer, UK) at 214 nm. A blank experiment was carried out under similar experimental condition without the drug [25]. A control experiment was carried out with insulin solution.

The in vitro release percentages were examined using different kinetic models in order to explain the mechanism of drug release from prepared formulations. The following equations for Zero order, First order and Higuchi diffusion model were applied to find out the mechanism of insulin release [27]. If R^2 is the greatest for a certain model, then release kinetics is best fitting to this model.

Zero order “A plot of drug concentration% against time”.

$$C = K_0 t \quad (2)$$

where C is the insulin concentration released at time t and K_0 is the zero order release constant.

First-order “A plot of log drug concentration% against time”.

$$\text{Log } C = \text{Log } C_0 - k_1 t / 2.303 \quad (3)$$

where, C_0 is the initial insulin concentration and K_1 is the first-order release rate constant.

Higuchi diffusion model “A plot of drug concentration against square root of time”.

$$Q = K_H t^{0.5} \quad (4)$$

where, Q is the cumulative amount of the drug, K_H is the Higuchi dissolution constant and $t^{0.5}$ is the square root of the time.

2.6. Preparation of Rat Skin

Wistar rats (250–300 g) were obtained from the animal breeding center, Faculty of Science, King Faisal University, Saudi Arabia. Animals were treated according to the guidelines of Ethical Committee

of Animal Handling in Zagazig University, Egypt (ZU/FP/282015; dated 8 February 2015). Hair was removed from the abdominal skin using an electric clipper with adequate care in order to avoid damage to the surface of the skin. Rats were sacrificed by the administration of excess chloroform inhalation to get the full thickness abdominal skin membrane. The subdermal tissues of the skin were subsequently removed by rubbing with a cotton swab. Before the permeation study, the skin samples were hydrated in phosphate buffer (pH 7.4) (containing 0.02% sodium azide as a preservative) at 4 °C overnight in a refrigerator.

2.7. Permeation of Insulin

The skin permeation of insulin from prepared formulations was measured using the previously described diffusion cell [28]. The rat skin membranes were mounted between the two compartments with the stratum corneum side facing the drug-loaded system and the dermal side is in contact with the receptor compartment containing 250 mL phosphate buffer (pH 7.4) and 0.02% sodium azide as preservative (to maintain skin integrity) and temperature was maintained at 37 ± 0.5 °C [29]. The test formulations were individually placed into membrane holders and fixed to the glass tubes. The gel was covered with Parafilm (American National Can Company, Chicago, IL, USA) to avoid water evaporation and rotated at 100 rpm. Control experiment was carried out with drug solution. At 5, 15, 30, 45, 60, 120, 180, 240, 300 and 360 min after starting the experiment, 3-mL aliquots were withdrawn from the receptor compartment and replaced with the fresh buffer. Samples were analyzed by UV-Vis spectrophotometer at 214 nm using samples from drug-free systems as a blank. To evaluate the influence of composition on permeation rate, parameters such as steady state transdermal flux (SSTF) and enhancement ratio (ER) were determined for various formulations as follows [30]:

$$\text{SSTF} = [\text{amount of permeated drug}/(\text{time} \times \text{area of permeation membrane})]: (Q/(t \times A)) \quad (5)$$

$$\text{ER} = \text{SSTF from test}/\text{SSTF from control} \quad (6)$$

2.8. In Vivo Antidiabetic Activity

2.8.1. Animals

Antidiabetic activities were performed in male Wistar rats (200 ± 20 g, 6–8 weeks) placed in an animal house maintained temperature 25 ± 1 °C and relative humidity $55\% \pm 5\%$ with a 12-h light/dark cycle. The guidelines of the Institutional Animal Ethics Committee were strictly followed while performing experiments (ZU/FP/282015). The animals were given unlimited access to food and water for 24 h.

2.8.2. Antidiabetic Study

Antidiabetic activity was evaluated utilizing diabetic rats. Animals were rendered diabetic by intraperitoneal injection of streptozotocin (50 mg/kg) freshly dissolved in 0.1-M citrate buffer at pH 4.5. Confirmed diabetes induction occurred 14 days following streptozotocin injection. Animals with plasma glucose level more than 250 mg/dL were selected for the study. Rats were randomly divided into 6 groups of each containing six animals. Five groups of animals were treated with different formulation while the control group remains untreated. The animal's hair was removed and calculated amounts of different formulations were loaded into Teflon rings (1.5×2 cm i.d.) which fixed to the dorsal side of rat body firmly. Blood samples were withdrawn from animal tail and assessed for blood glucose levels at specified time intervals after glucose challenge by using Accu-Chek® active laboratory equipment supplied with Accu-Chek® active strips Code 970 (Roche, Mannheim, Germany) [31].

2.9. Statistics

For comparison, a one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) as a post hoc test was applied, using (SPSS 23, SPSS, Inc., Chicago, IL, USA). The statistical differences between values showing $p < 0.05$ was considered as significant.

3. Results and Discussion

Different formulations, namely insulin gel, insulin niosome, insulin niosome gel, insulin emulgel and insulin niosome emulgel (Figure 1) were successfully formulated to assess their feasibility to deliver insulin through the skin. The prospective of these formulations to transport insulin by transdermal delivery was evaluated at various phases of the study.

3.1. Physical Characteristics of the Prepared Formulations

Prepared insulin niosome emulgel, insulin niosome gel, insulin emulgel and insulin gel were homogenous in nature. Higher encapsulation efficiency ($42.5\% \pm 2\%$), good drug loading ($13.5\% \pm 3\%$) with nanometric particle size (885 ± 45 nm) were observed in prepared insulin niosomes. Interestingly, insulin is soluble in phosphate buffer (pH 7.4) and also in the lipid mixture. Hence, the high entrapment efficiency and drug loading of insulin is due to its solubility in surfactant/cholesterol lipid phase and also the internal aqueous compartment of niosome vesicles. In addition, the higher encapsulation efficiency observed in insulin niosomes could be attributed to the high cholesterol concentrations included in niosomes. Increase in cholesterol concentration can presumably reorganize the chain order of the liquid-state bilayer and thereby enhances the strength of the nonpolar tail of nonionic surfactant. It was reported previously that, increasing the cholesterol percentage to 50% with the surfactant resulted in increasing the niosome particle size and subsequently increasing its encapsulation efficiency [23]. A representative size distribution curve of insulin niosome is shown in Figure 2. The figure demonstrates narrow distribution (PDI < 0.3) of niosome particles in the formulation while the data in Table 3 signifies average vesicles size of 885 nm (insulin niosome). The measurement of the formulation viscosities are illustrated in Table 3. It is clear that, the viscosity increased from 6550 cP into 12,250 cP for insulin gel and insulin niosome emulgel, respectively. The increasing of the viscosity could be explained according to the formulation ingredients. Simple gel formulation showed low viscosity, while insulin emulgel and insulin niosome emulgel showed higher viscosity.

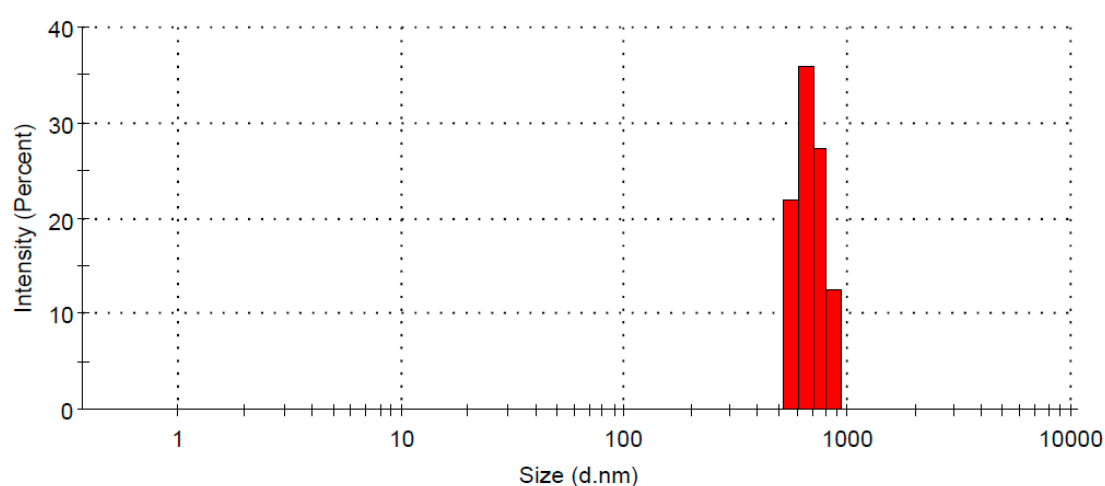


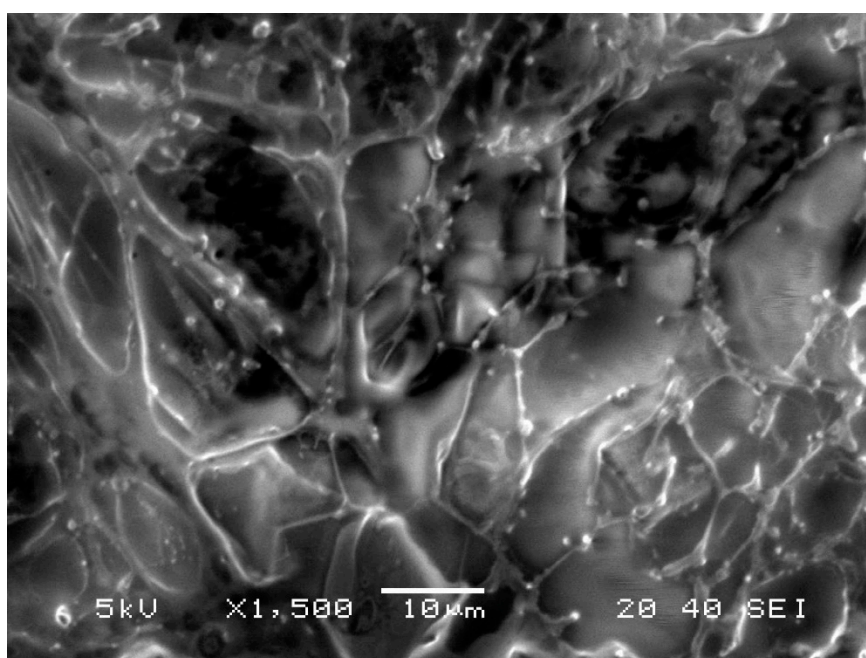
Figure 2. Representative size distribution curve of insulin niosome.

Table 3. Viscosity of the prepared formulations.

Property	Insulin Gel	Insulin Niosome	Insulin Niosome Gel	Insulin Emulgel	Insulin Niosome Emulgel
Viscosity (cP)	6550 ± 50	2150 ± 75	8220 ± 80	11,050 ± 50	12,250 ± 70

Results represented as means ± SD (n = 6).

A representative scanning electron microscopy image of prepared insulin niosome emulgel is shown in Figure 3. Figure indicated small spherical vesicles distributed through the network of macromolecular polymer used. No marked crystals of insulin observed in the formulation indicating a very good solubility of the drug.

**Figure 3.** Representative scanning electron micrograph of insulin niosome emulgel.

3.2. Analysis of 2³ Factorial Design

Based on 2³ full-factorial design studies, it was observed that the concentration of paraffin oil, Tween 80 and sodium carboxymethyl cellulose have significant effect on in vitro drug release, in vitro permeation and viscosity of the formulations. These results support the selection of independent variables in the current study. It was reported that non-ionic surfactants with long ethylene oxide chain lengths like Tween 80 influences the formulation of niosomes as well as enhance transdermal penetration of drug molecules [32,33]. Similarly, the effect of sodium carboxymethyl cellulose on the viscosity, drug release and permeation of drugs from gel formulations are also demonstrated [28,34]. The effect of various independent variables on dependent parameters is shown and discussed below.

3.3. Effect of Independent Variables on Viscosity and In Vitro Release Studies

The percentages of insulin released across cellulose membranes from prepared gels are shown in Figure 4. It is evident from Figure 4 that about 96% of insulin was released after 1 h from solution form (control). On the other hand, 33.44%, 58.56%, 66.33%, 74.89% and 94.22% of the drug was released after 6 h from insulin niosome emulgel, insulin emulgel, insulin niosome gel, insulin niosome and insulin gel, respectively. The difference ($p < 0.05$) in the drug release could be related to the viscosity for the four insulin preparations containing gelling agent. Gel consistency plays an important role as it ensures adequate retention and also releases the drug in controlled manner [35]. It is well known fact

that, the increase in viscosity can cause increase in resistance to drug diffusion, mobility and rate of dissolution [6]. Emulgel composed of paraffin oil, gelling agent (sodium carboxymethyl cellulose) and surfactant (Tween 80) which contribute to higher viscosities and hence showed lower percentage of drug release.

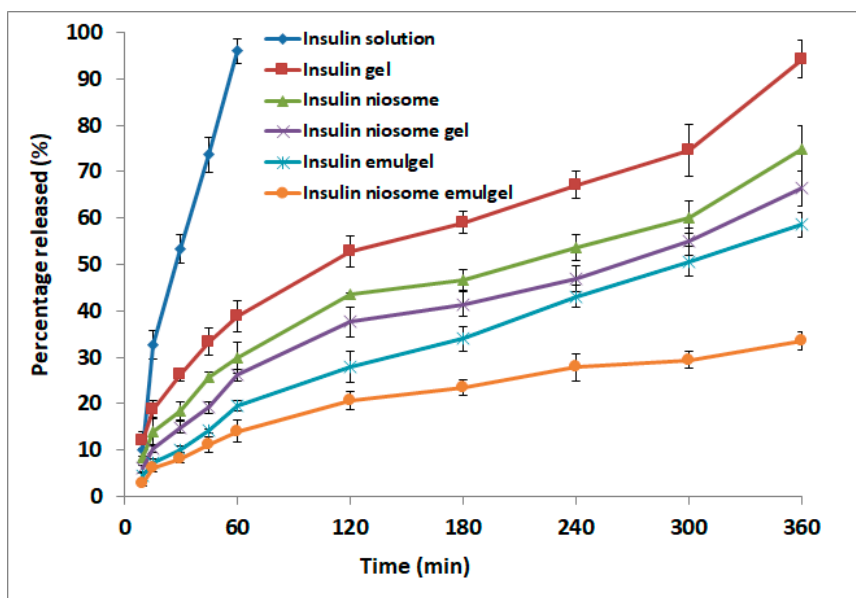


Figure 4. In vitro release profile of insulin from different formulations and control (insulin solution) across cellophane membrane into phosphate buffer (pH 7.4) at 37 ± 0.5 °C. Data represented as mean \pm SD (n = 6).

The 3D response surface plot and contour plot (Figure 5) shows the effect of independent variables on viscosity of niosome emulgel. It is evident from the 3D response surface plot that as concentration of paraffin oil and sodium carboxymethyl cellulose increase, the viscosity of the formulation increase. There is no significant effect of Tween 80 observed on viscosity. It is also apparent from contour plot that sodium carboxymethyl cellulose and paraffin oil have significant effect ($p < 0.005$) on the viscosity of formulations.

The fitted polynomial equation for viscosity is given below:

$$Y = 9.74 + 0.0737x_1 + 0.0266x_2 + 0.2383x_3 + 0.0044x_1x_2 + 0.0051x_1x_3 + 0.0061x_2x_3 + 0.0080 \quad (7)$$

Therefore, minimum concentration of sodium carboxymethyl cellulose and paraffin oil compared to Tween 80 was found suitable for controlled in vitro release of drug. This was illustrated in the 3D response surface plot and contour plot (Figure 6). It was evident from the 3D response surface plot that as concentration of sodium carboxymethyl cellulose increased, in vitro release decreased. Sodium carboxymethyl cellulose concentration had a significant effect on in vitro drug release as discussed earlier. It was also apparent from contour plot that paraffin oil was having inverse effect on in vitro release. The fitted polynomial equation shown as:

$$Y = 3.26 - 0.0771x_1 - 0.0243x_2 - 0.1661x_3 - 0.0069x_1x_2 - 0.0115x_1x_3 - 0.0021x_2x_3 + 0.0027 \quad (8)$$

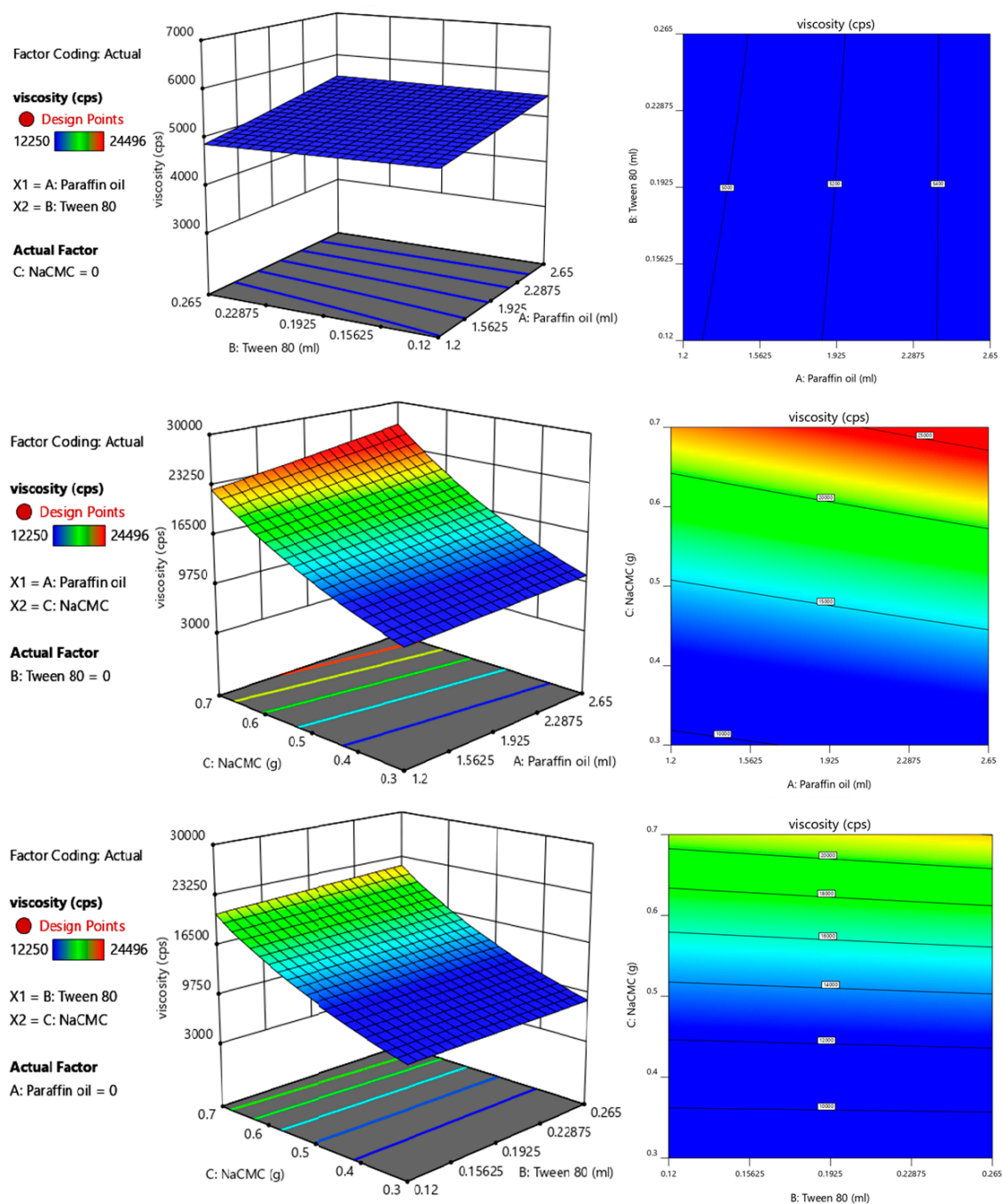


Figure 5. Surface response plot and contour plot showing the influence of three independent variables (concentration of paraffin oil, Tween 80 and sodium carboxymethyl cellulose) on viscosity of insulin loaded niosome emulgel.

For the check point batches F9*, the predicted values were in good agreement with the observed values (Table 4). Thus, it could be concluded that the evolved equation could be used for prediction of responses using 2^3 full-factorial design.

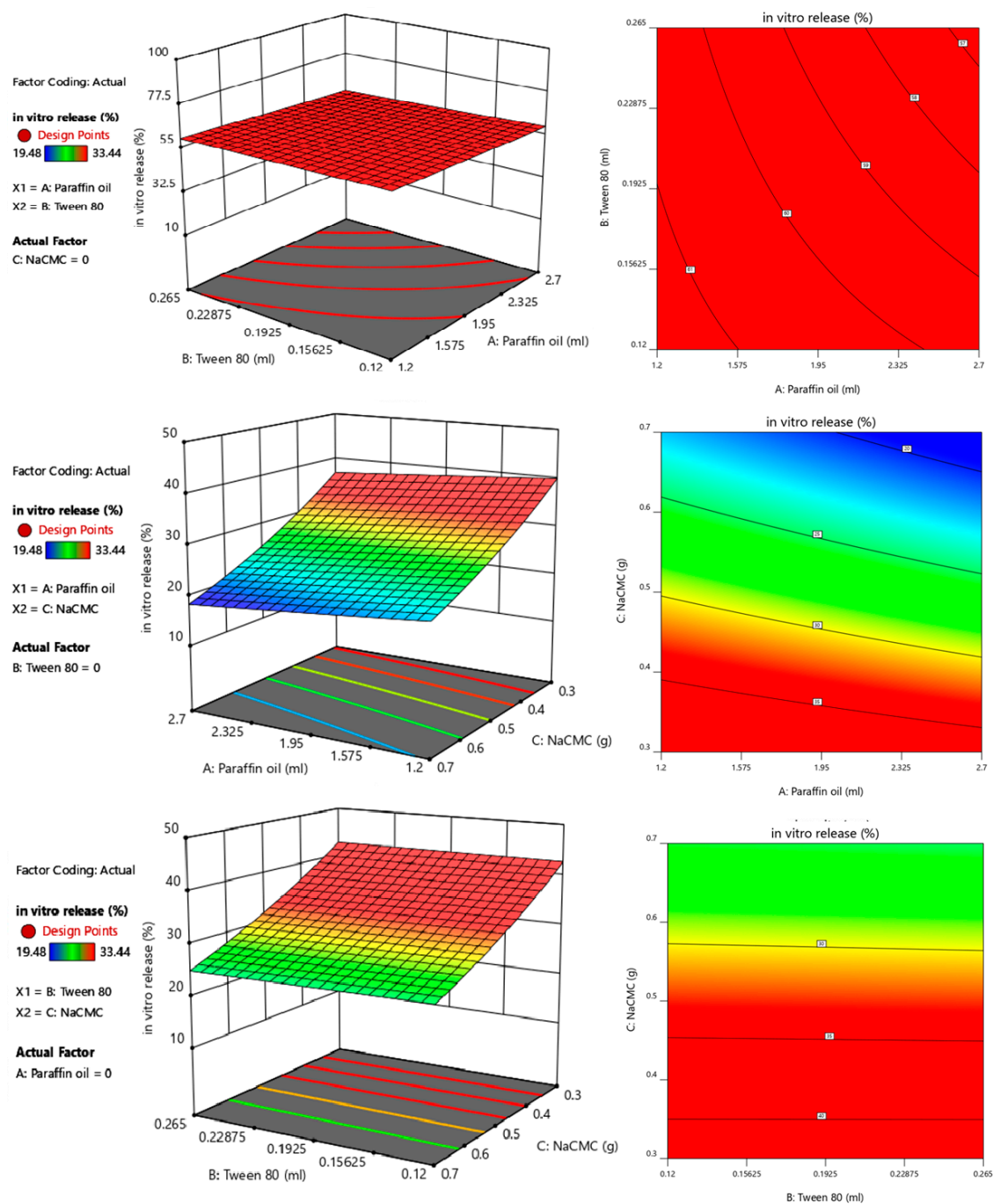


Figure 6. Surface response plot and contour plot showing the influence of three independent variables (concentration of paraffin oil, Tween 80 and sodium carboxymethyl cellulose) on in vitro release of insulin from niosome emulgel.

Table 4. Comparison of the observed value with predicted value of check point batch.

Check Point Batch	Responses					
	In Vitro Release (%)		Viscosity (cP)		Cumulative Amount Permeated ($\mu\text{g}/\text{cm}^2$)	
	Predicted	Observed	Predicted	Observed	Predicted	Observed
F9	26.79	27.38	18,397	18,529	82.85	83.43

The normal gels were of lower viscosities compared to the emulgel formulations, hence showed higher percentages of insulin release. Interestingly, when comparing the percentages of insulin released from emulgels with that released from niosome gels after 6 h, there were no statistical differences. However, insulin niosome emulgels showed a significant reduction in insulin diffusion across cellulose membranes ($p < 0.05$). This could be ascribed to the resistance of niosome lipid membrane to release the trapped insulin that represent additional barrier for drug diffusion in addition to high emulgels viscosity [36]. The gelling effect was clear on insulin release in gel formulations when compared to control (gel solution). The kinetic analysis (Table 5) showed that the mechanism of insulin release across cellulose semipermeable membrane from all formulations was obeying Higuchi diffusion model except solution form of insulin that followed zero order kinetics. This result excludes the effect of cellulose semipermeable membrane on the amount of insulin released from niosomes, gels and emulgels niosome/emulgel combinations. Hence, the vehicle in which insulin was dispersed was the sole factor that affected insulin release across cellulose membrane.

Table 5. Release kinetics of insulin formulations across cellulose membrane.

Formulation	Zero Order (R ²)	First-Order (R ²)	Higuchi Model (R ²)	Order
Insulin solution	0.9819	0.7157	0.9130	Zero
Insulin gel	0.9242	0.4780	0.9896	Higuchi
Insulin niosome	0.9282	0.5246	0.9850	Higuchi
Insulin niosome gel	0.9421	0.5786	0.9866	Higuchi
Insulin emulgel	0.9482	0.6694	0.9838	Higuchi
Insulin niosome emulgel	0.9236	0.6288	0.9918	Higuchi

3.4. Permeation

The permeation of drug across biologic membranes is influenced by various factors which include physicochemical properties of drug, physiological properties of the barrier and its composition, as well as transport route selected for the drug permeation [4,12]. The release results obtained using cellophane membrane not always fits to those results obtained using natural skin. The skin composition with hair and sweat glands and its thickness is greatly affecting the drug diffusion [12]. Moreover, the skin interacts with the drug vehicles that could disrupt its barrier function and enhance drug diffusion. Figure 7 shows the order of insulin permeation across the rat skin which was found highly influenced by the vehicle used as follows: insulin niosome emulgel > insulin niosome gel > insulin emulgel > insulin gel > insulin niosomes > insulin solution. However, comparison of permeation data with release data (Figure 4) signifies that there is no correlation between them. Insulin permeated across the rat skin was significantly lower compared with that released using cellulose membrane. This may be probably due to the lipophilic barrier of the skin that hindered insulin diffusion [37]. These data also suggest that the insulin diffusion happens mainly from the carriers.

Careful examination of Figures 4 and 7 shows that prepared niosomes could enhance the insulin permeability by only 1.45-fold (Table 6). The SSTF of insulin from niosome preparation was only 13.17 $\mu\text{g}/\text{cm}^2/\text{h}$ compared to 9 $\mu\text{g}/\text{cm}^2/\text{h}$ from insulin solution. The possible reasons for greater permeation by niosomes could be due to its potential to extract skin lipids or disruption of corneocytes [38]. On the other hand, gel prepared using sodium carboxymethyl cellulose gave SSTF of 18.39 $\mu\text{g}/\text{cm}^2/\text{h}$ with 2-fold enhancement ratio which could be due to the colloidal properties of the gel and its enhanced surface area contact to the skin [17]. Insulin emulgel on the other hand showed 3.17-fold enhancements for drug permeability and is likely due to its surfactant contents that act as permeability enhancer in addition to its colloidal properties [28]. In addition, the in vitro permeability study showed superior enhancement for insulin diffusion across the rat skin for both niosome emulgel and niosome gel by 10 and 5.86-fold, respectively. Greater flux observed with niosome emulgel could be attributed to the dual effect of surfactants present in niosome formulation and emulsion preparation, which has the ability to diffuse through narrow pores [6], hence giving 10-fold increase in permeability.

However, niosomes only interact with skin by enhancing drug permeability via surfactant effect [38] so it gives lower permeability compared to niosome emulgels.

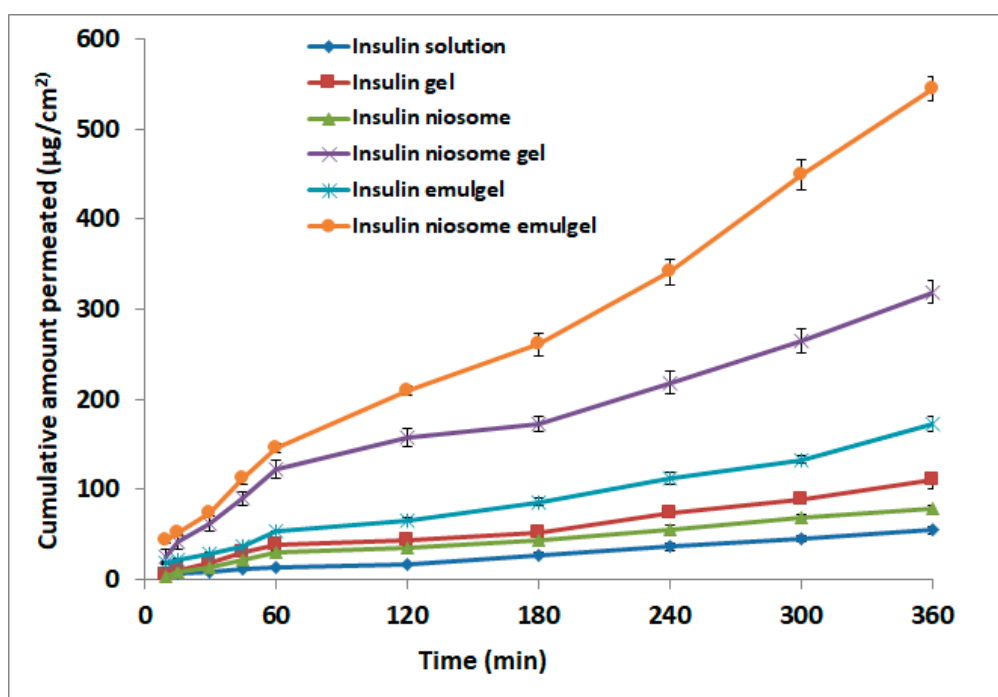


Figure 7. Permeability profiles of insulin from different formulations and control (insulin solution) across abdominal rat skin. Data represented as mean \pm SD ($n = 6$).

Table 6. Permeation parameters of the prepared insulin formulations.

Time	Insulin Solution	Insulin Gel	Insulin Niosome	Insulin Niosome Gel	Insulin Emulgel	Insulin Niosome Emulgel
SSTF ($\mu\text{g}/\text{cm}^2/\text{h}$)	9.06	18.39	13.17	53.11	28.72	90.78
ER	1.00	2.03	1.45	5.86	3.17	10.02
Mechanism	Zero	Zero	Higuchi	Higuchi	Zero	Zero
R^2	0.986	0.967	0.973	0.976	0.982	0.989

Insulin solution was taken as control for calculations of enhancement ratio (ER).

The designed batches were also evaluated for the amount of insulin permeated through the skin membrane from prepared niosome emulgels and the effect of three independent variables on in vitro permeation of insulin is shown in Figure 8 in the form of 3D surface response plot and contour plot. It is evident from the 3D surface response plot that as the concentration of sodium carboxymethyl cellulose increased, the permeation of drug decreased while paraffin oil and Tween 80 had no significant effect on drug permeation. It was also apparent from contour plot that sodium carboxymethyl cellulose concentration had significant effect on drug permeation. The percentage drug release and amount of drug permeation were found to be significantly higher only when sodium carboxymethyl cellulose content was very low.

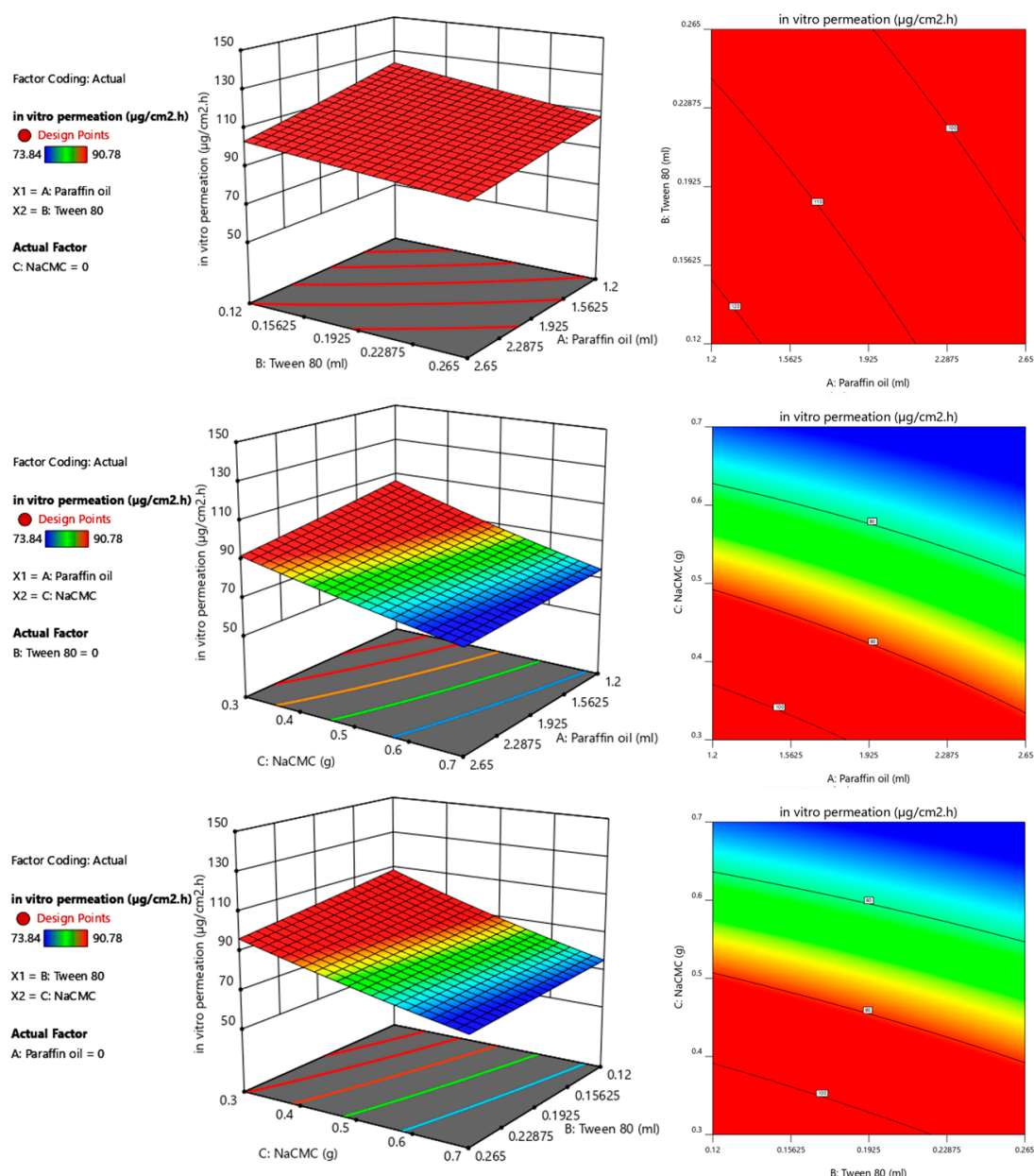


Figure 8. Surface response plot and contour plot showing the influence of three independent variables (concentration of paraffin oil, Tween 80 and sodium carboxymethyl cellulose) on in vitro permeation of insulin from niosome emulgel.

The fitted polynomial equation for in vitro permeation is given below:

$$Y = 4.38 - 0.0269x_1 - 0.02x_2 - 0.0585x_3 + 0.0060x_1x_2 + 0.0085x_1x_3 + 0.0064x_2x_3 + 0.0022 \quad (9)$$

Increase in insulin diffusion is apparently due to the interaction between the nonionic surfactant (span 60) and the rat skin lipids. It has been disclosed that, the surfactant could increase the fluidity of skin membranes leading to a faster drug diffusion rate across natural membranes [39]. Niosomes can diffuse across the dermis also and release antidiabetic drugs directly inside the skin [20]. Moreover, from the results in Table 6, it is obvious that the niosome vesicles combined with emulgels could have synergistically enhanced insulin skin penetration. In a previous study, we have shown that there may be a plausible merging between niosomes and emulsion droplets resulting in new structured vesicles resembling transfersomes [6]. This could be the major determining factor that resulted in the greatest

SSTF for insulin from niosome emulgel. Conversely, as shown in Table 6, emulgels yielded higher SSTF than gels; however, emulgels are of greater viscosity as shown in Table 3. This result could be explained by the fact that emulgels contain the nonionic surfactant and hydrocarbon oil that had been reported as penetration enhancers [40]. Moreover, kinetic analysis revealed that the mechanism of insulin permeability from niosome suspension and niosomes in gel was according to Higuchi diffusion model that excludes the effect of rat skin on insulin permeability (Table 6). In contrary, all other vehicles including emulgels, sodium carboxymethyl cellulose gel, niosome/emulgel and solution are showing zero order release kinetics that confirms the vehicle/skin interactions.

3.5. In Vivo

The in vivo antidiabetic investigations were carried out in diabetic-induced Wistar rats and the plasma glucose level was measured over a period of 6 h. Optimized insulin niosome emulgel (F6) was selected for in vivo studies because this formulation shown the highest permeation ($90.78 \mu\text{g}/\text{cm}^2/\text{h}$) in the in vitro studies (Table 6) and was compared with other formulations prepared. The blood glucose level of all animals was measured before the study and was over 250 mg/dL. The requirement of insulin in human varies as the dose is based on individual's glucose level, which range between 0.5 and 1 unit/kg/day in multiple doses. In rats (250–300 g), the requirement of insulin is 5–5.5 unit/kg/day, according to the equation described in the literature [41]. However, this dose is administered by subcutaneous route. On the other hand, the dose of transdermal system primarily depends on the skin in put rate (flux), which is also influenced by the drug concentration in the formulation [42]. Therefore, a higher dose is generally used in transdermal formulations to achieve adequate skin in put rate. Hence, a higher dose of 0.54 mg (equals 15.56 IU of insulin) was incorporated in all formulations and were applied into animals. Figure 9 showed the results of the percentage of plasma glucose level reduction. Significant reduction ($p < 0.05$) of plasma glucose level was observed in insulin niosome emulgel preparation during 6 h investigation compared to all formulation under investigation except with insulin niosome gel, the plasma glucose level was only significant at 4 and 6 h ($p < 0.05$). Indeed, the blood glucose monitoring showed that the applied dose effectively reduced blood glucose by more than 30% for insulin niosome emulgel formulation. On the other hand, the percentage of plasma glucose level remains same in untreated control group. The results suggested that, the insulin niosome emulgel preparation could be considered as efficient transdermal delivery system for insulin.

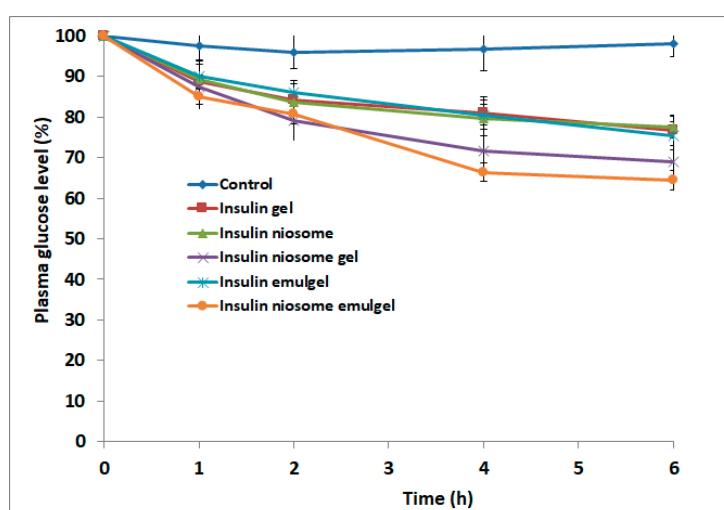


Figure 9. Percentage of plasma glucose level of initial-time profile following transdermal applications of different formulations in diabetic rats. Data represented as mean \pm SD ($n = 6$).

4. Conclusions

Insulin as a large peptide has very difficult transdermal absorption process because of the formidable barrier function imparted by the lipophilic stratum corneum of the skin. Combination of niosomes and emulgel could successfully enhance the insulin percutaneous absorption. The antidiabetic activity of insulin niosome emulgel showed highest plasma glucose level reduction among all formulations tested. Further studies in human are necessary to validate this data. In addition, frequency distribution study in humans are required to know the exact quantity to be applied on the skin surface for individuals according to the glucose level. Thus, patients can use an applicator of suitable size for applying required amount of niosome emulgel. In conclusion, the incorporation of insulin into niosome emulgel could represent a new era for noninvasive insulin administration which would offer higher patient compliance.

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