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Research Article

PREPARATION AND CHARACTERIZATION OF PHENYTOIN SODIUM NIOSOMES FOR ENHANCED CLOSURE OF SKIN INJURIES

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ABSTRACT

Objective: Niosomal gel formulations containing phenytoin sodium have been prepared for enhancing skin wound healing.

Methods: Solvent evaporation-film hydration methodology was adopted for noisome formation. Different compositions of phenytoin, surfactant and cholesterol were tested. The prepared niosomes were evaluated for size of vesicles, drug entrapment efficiency and release profiles.

Results: Niosome micrographs obtained by scanning electron microscopy indicated well-defined and spherically shaped vesicles. Niosomes's size and zeta potential indicated a smallest average size (74.4 nm), large polydispersity index (0.85) and optimum zeta potential (-58.9 mV) from niosomes containing Span 60 and Pluronic F127 at 1:1 ratio. Niosomes also enabled sustained release of phenytoin sodium from niosomal vesicles which dependedon the type of surfactant used. Formulation F2 containing Span 20 released more than 70 % and 100 % after 14 and 18 hours, respectively. Other formulations containing span 60 alone or mixed with other surfactants sustained the release for more than 24 hours. In-vivo evaluation performed on artificially injured guinea pig skin indicated significant differences ($P < 0.05$) between healing times for treated group which completely healed within less than 9 days upon using phenytoin sodium niosomal gel formulations compared to placebo gel counterparts which lasted more than 17 days.

Conclusion: These findings indicate that niosomes are considered highly effective carriers and skin penetration enhancers for phenytoin sodium. The effects were mainly due to their high content of surfactants and cholesterol combined with collagen proliferation benefits of phenytoin both led to successful and rapid wound healing when employed topically.

Keywords: Phenytoin; Niosomes; Polydispersity index; Film hydration; Skin injury; Wound healing

INTRODUCTION

Skin integrity is considered a strong barrier against injury or invasion of microorganisms and parasites. Wounds and skin burns constitute a major risk to human body during daily life and following accidents. Topical application of antibiotic creams, ointments and dressings always takes long time for complete recovery and healing of injured skin. Phenytoin sodium is an antiepileptic drug with a common side effect of gingival hyperplasia. This effect was evaluated for acceleration of gingival wound healing [1]. Phenytoin was tested for enhancement of topical healing of chronic skin ulcers in 75 patients, where rapid wound area reduction with new granulation tissue were observed in presence of phenytoin. The healing rate was faster than in control patients [2]. A recent study on phenytoin sodium applied topically to rat skin burns indicated shorter period of time needed for developing new epithelial tissue and wound contraction in affected mice than the control or other treatment groups given sulphadiazine, silver or dexamethazone [3]. The mechanism of action by which phenytoin accelerates wound healing is exactly not known. However its strong effects on collagen proliferation is considered the main cause as it is well known that fibroblasts deposit large mass of collagen matrix in the wound during the healing process [4]. Niosomes are special type of the vesicular drug delivery systems based on nonionic surfactants and cholesterol forming microscopic lamellar structures. These upon subsequent hydration in aqueous media, sonication and/or homogenization give rise to small unilamellar vesicles [5].

In these vesicular systems nonionic surfactants have replaced the phospholipid component in liposomes [6]. Niosomes can be used for entrapping both hydrophilic and hydrophobic drugs, in the aqueous layer or in the vesicular membrane made up of lipid materials respectively [7]. Niosomes have many advantages common to all vesicular systems such as prolongation of the circulation of entrapped drugs, possible targeting to special organs and tissues and controlled release of entrapped drugs and being biodegradable and non immunogenic [8].

Niosomes are more stable than liposomes [9]. Their content of cholesterol tends to increase the entrapment efficiency. Unlike liposomes, niosomes do not require low temperature or inert atmosphere for storage and the relatively cheap materials used in their manufacture [10]. When applied locally to the eye, niosomal formulations controlled ocular delivery and protected against drug metabolism by enzymes present in the lachrymal fluid [11]. Niosomes have been reported by many researchers to easily penetrate skin layers by virtue of its cholesterol lipoidal membrane and their high content of surfactants [12, 13] as well as the presence of other ingredients that act as skin permeation co-enhancers [14]. Variables affecting formulation of stable niosomes include: type and HLB of surfactant [15], ratio between drug, surfactant and cholesterol, ultra-sonication time, film hydration temperature and presence or absence of a surface charged stabilizer [16]. Until now, no research work involved niosomal vesicles for preparation or evaluation of the effects of phenytoin sodium on injured or wounded skin. Therefore, in this research, niosomes were selected and evaluated for entrapment efficiency and controlling the release of phenytoin sodium to be applied topically for accelerating wound healing.

MATERIALS AND METHODS

Materials

Phenytoin sodium and cholesterol were purchased from Sigma-Aldrich (through the Egyptian International Center for Import Ltd. (Cairo, Egypt). Span 60 (sorbitan monostearte), Span 20 (sorbitan monolaurate) and Pluronic F 127 (poloxamer 407) were purchased from BASF (Ludwigshafen, Germany).

Cremophor EL 35 (polyoxyl-35 castor oil), Tween 40 (polyoxyethylene sorbitan monopalmitate) and sodium alginate were gifted from Delta-pharm (Cairo, Egypt). Methanol (HPLC grade) was purchased from Fisher Scientific Ltd. (Loughborough, UK). All other reagents were of analytical grade and were purchased from El-Nasr chemical Company (Cairo, Egypt).

The animal test protocol was approved by the ethics review committee for animal experimentation of Minia University, Egypt. Statistical analysis of the data was undertaken using Student t-test and the level of significance was set at $P < 0.05$.

Preparation of niosome formulations

The niosomes were prepared using film hydration technique. Firstly used for liposomes preparation [17] and then adopted by other researchers for niosomes [18]. In a flask of a rotary evaporator (Barloworld Scientific Ltd., Stone, Staffordshire, ST15 0SA, UK), the weighed amounts (mg) of surfactants, drug and cholesterol were dissolved in a solvent mixture consisting of 6 mL carbon tetrachloride and 3 mL methanol (see Table 1). Then the previous solution was completely dried in a rotary evaporator at a rotating speed of 50 rpm and water bath maintained at 50 º C. The dry film formed on the walls of the rotating flask, was then rehydrated with 15 mL pH 6.5 freshly prepared ultrapure distilled water [7]. The resulting suspension was subjected to sonication in a water bath sonicator (Ney Instruments Co. Ltd, Ultrasonic Cleaner Model 57 H, USA) at 60 °C for 1 hr then left to cool to room temperature until a complete niosomal suspension is formed. The suspension was then transferred overnight to a refrigerator (2-8 ºC) for complete annealing of niosomes. Centrifugation using a cooling ultracentrifuge (Sigma 3-30 K, Rotor 19776, Eingangssicherung, Germany) at speed of 14000 rpm and a temperature of 4 ºC was used for concentrating the nanosuspension and separation of the niosomal mass. The prepared niosomal suspension formulations were then examined microscopically.

Table 1: Composition of the prepared niosomal formulations

Formula	Phenytoin sodium (%w/w)	Surfactants		Cholesterol (%w/w)	Ratio[a]	Organic solvent (mL)	Rehydration fluid (%w/w)	Gelling agent[b]
		Type	(%w/w)					
F1	0.32	Span 60	0.64	0.32	1:2:1	9	96.30	2.42%
F2	0.32	Span 20	0.64	0.32	1:2:1	9	96.30	2.42%
F3	0.32	Span 60/Span 20	0.32/0.32	0.32	1:2:1	9	96.30	2.42%
F4	0.32	Span 60/ Pluronic F127	0.32/0.32	0.32	1:2:1	9	96.30	2.42%
F5	0.32	Span 60/Tween 40	0.32/0.32	0.32	1:2:1	9	96.30	2.42%
F6	0.32	Span 60/Cremophor El 35	0.32/0.32	0.32	1:2:1	9	96.30	2.42%

a: Phenytoin: Surfactant: Cholesterol, b: 2.42 % (w/w) sodium alginate

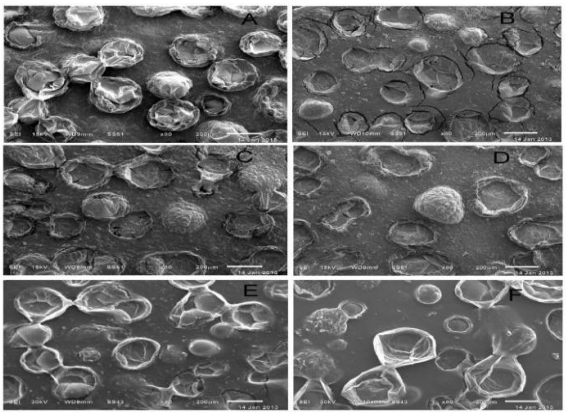

Fig. 1: Scanning electron micrographs showing niosome formulations; F1 (A), F2 (B), F3 (C), F4 (D), F5 (E) and F6 (F)

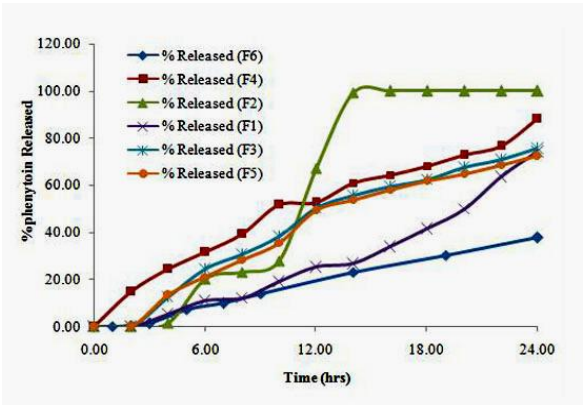

Fig. 2: Phenytoin sodium release profiles from prepared niosomal formulations

Morphological characterization

Samples of diluted niosomal suspension were examined under a light microscope connected to an ht-TVR video camera (Honest Tech. Ltd., Austin. USA).

Scanning electron microscope (SEM) JEOL-JSM-6510LA Analytical Scanning Microscope (JEOL Ltd., Japan) was also used for detailed morphological characterization of niosomal vesicles after coating with a gold sputter (SPI-Module Sputter Coater, SPI Supplies Inc., USA) see Fig. 1.

Evaluation of particle size and zeta potential

Niosomes size and size distribution were measured using laser light scattering technique (Mastersizer-2000, Malvern, UK). One milliliter of the diluted niosomal suspension was vortex mixed for 5 minutes then placed in the cell of the master sizer and the size and polydispersity index (PI) were determined. The Zeta potential was determined with photon correlation spectroscopy (PCS) using Malvern Zetasizer (Malvern, UK) see Table 2.

Determination of drug encapsulation efficiency (% EE)

The amount of drug entrapped into niosomal vesicles was determined by separating the vesicles from the supernatant using ultracentrifugation. In this method 1 mL of the niosomal suspension was placed into 2 mL microcentrifuge tubes and ultracentrifuged for one hour at 4 ºC. Samples of the separated niosomes (50 µL each) were mixed with 1 ml of isopropyl alcohol [19] to disrupt the niosomal membrane, the volumes were then completed to 10 mL with the mobile phase. The resulting solution was measured by HPLC using an isocratic pump (Model LC-10 As, Shimadzu, Japan), C18 Column (3.9 X 150 mm Bonda Pack 10 µm (Water associate, USA) and an ultraviolet wave length detector (Model SpD-10 A, Shimadzu, Japan). The mobile phase consisted of 0.05 M phosphate buffer adjusted to pH 2.8 with 2 M orthophosphoric acid and methanol in a ratio of (65:35). The flow rate was kept at 1.2 ml/min and the detection was carried out at a wave length of 220 nm [20]. The % EE was calculated according to the following equation [7]:

% EE = {amount of phenytoin analyzed/ amount used in preparation of vesicles} X 100

Table 2: Size and zeta potential of prepared niosomal formulations

Formula	HLB	Average niosome size (nm) ± SE[a]	Peaks in nm (% No. of Particles)			Polydispersity Index (PI)	Zeta potential ± S. D[b] (mV)
			Peak 1	Peak 2	Peak 3		
F1	4.70	317.62 ± 33.92	1216.0 (10.7)	197.2 (89.3)	0.00 (0.0)	0.948	-84.66 ± 24
F2	8.60	312.96 ± 30.58	956.7 (16.8)	5160.0 (0.0)	190.9 (83.1)	0.624	-76.80 ± 15.2
F3	6.65	145.15 ± 11.91	584.7 (4.8)	117.7 (95.2)	0.00 (0.0)	0.687	-77.45 ± 19.3
F4	9.50	74.38 ± 1.36	615.1 (1.5)	72.87 (98.5)	0.00 (0.0)	0.851	-58.93 ± 25.2
F5	10.15	201.04 ± 22.97	858.0 (10.6)	129.3 (89.4)	0.00 (0.0)	0.721	-65.16 ± 16.2
F6	8.85	570.10 ± 29.32	227.2 (22.8)	665.6 (77.2)	0.00 (0.0)	0.472	-56.4 ± 12.9

a: Standard Error and b: Standard Deviation

In-vitro release studies

The drug release from the prepared formulations was determined using a modified Franz diffusion cell technique [21]. In this method 1 mL of the prepared niosomal suspension was placed inside a cut tube through the open end; the other end was closed with a dialysis membrane (SERVAPOR® dialysis tubing, MWCO 12 000 - 14 000) with 25 Å pore diameter (SERVA Electrophoresis GmbH D-69115 Heidelberg, Germany). The tube was then placed on the surface of the release medium (pH 5.5 phosphate buffer) and fixed to the walls of the dissolution vessel with a piece of cork. The membrane end is touching the 100 mL media surface. The normal sink conditions were followed by replacing each 5 mL withdrawn samples with fresh ones. The dissolution medium was kept at 37 ºC ± 0.5 with rotating paddle speed of 50 rpm inside a flask of a USP dissolution tester apparatus 1(paddle method; Hanson Research, SR 8 plus model, U.S.A). The experiment was carried out for each formulation using 6 flasks and the average values were recorded (see Table 3). The release mechanism was evaluated according to zero, first and Higuchi kinetics.

Evaluation of pharmacological activity on skin wounds

The topical pharmacological activity of phenytoin (accelerated wound healing) was evaluated using twelve male guinea pigs with artificial wounds made using 10 mm diameter punch on the shaved skin of the back. The average weight of healthy animals was 350-400 g and aged two months at the time of the study. The animals were bred locally and housed individually per cage in a room with natural light cycle and constant temperature (25 ± 2 ˚C) with food and water available at all times. The animals were anesthetized by intra-peritoenal injection (75 mg/kg) of pentobarbital immediately before wound induction [22]. After shaving the backs of the animals, a modified punch was used for excising lesions (10 mm diameter) over skin of the back. Animals were randomly divided into 2 groups; control group and treated group. The control group received vehicle (drug free niosomal gel), while the treated group received topical niosomal gel containing 1% phenytoin once daily from the beginning of experiments until complete wound closure. Wounds were photographed using a digital camera (Canon PowerShot SD790 IS, Japan) on day 0, day 3, day 6 and on day 9. All photographs were taken from a fixed distance of the wound (see Fig. 3).

Results and discussion

Morphological characterization

The scanning electron micrographs of the prepared niosomes shown in Fig.1 demonstrated formation of defined shape and detached vesicles from Formula F1 (Fig.1A). Formula F2 and F3 showed smaller vesicles with collapsed surface (Fig. 1B and 1C). The smallest vesicles are given in Fig. 1D by formula number F4 containing Pluronic F127 followed by Formula F3 (containing Span 20) and F5 (containing Tween 40) in addition to equal amount of Span 60 in each of them. This may be due to hydrogen bonding association between the straight-chain structures of Pluronic F127 (although it has a larger Molecular weight (M. wt) of 11500) and Span 60 during formation of noisome vesicles. This interaction seemed to occur at lower extent between Tween 40 (M. wt = 1277) containing a tetrahydrofurane ring attached to 3 branches of polyoxyethylene chains and Span 60 (M. wt = 430.62) which also contains a tetrahydrofurane ring. The same comparison applies for Span 20 (M. wt = 346.46) with Span 60 leading to formation of relatively larger vesicles compared to F4. Formula F6 containing Cremophor EL 35 showed the largest vesicles which may be also due to difficulty in interaction or association between Cremophor EL 35 (containing high proportion of hydrophobic triricinoleate molecules) and Span 60 through H-bonding leading to formation of bulky structures. Formulations F1 and F2 showed intermediate sizes of 317 and 312 nm respectively, as each contained a single type surfactant, Span 60 and Span 20 respectively (Fig.1 and Table 1).

Vesicle size and zeta potential

The average size and zeta potential of niosomes are shown in Table 2. Formulation F1 showed the highest zeta potential and largest polydispersity index with average size of 317.62 nm. Formulation F2 showed similar average sized vesicles to F1 (312.45 nm), lower polydispersity index and comparable zeta potential. When a mixture of Span 60 and Span 20 was used (F3), it was observed that the size of the vesicles decreased to 145 nm. However, the zeta potential and polydispersity indices were maintained as those observed for F2. The decrease in size may be attributed to reduction of the proportion of Span 60 (having higher M. wt). When poloxamer 407 was used with span 60 in a ratio of 1:1 as the surfactant (F4) both noisome size and zeta potential were decreased (74 nm and -58.93 mV) relative to other formulations. These results are in good agreement with the literature [23], regarding stability of small niosomes with optimum zeta potential < -60 mV. It also indicates the advantage of using Pluronic F127/Span 60 mixture (1:1) in producing smaller and physically stable niosomes. Incorporation of Tween 40 with Span 60 in formulation F5 resulted also in slightly larger but acceptable noisome size (201 nm) and zeta potential (-65 mV). The largest vesicle size (570 nm) was obtained from niosomes containing a surfactant mixture composed of Span 60 and Cremophor EL35 (F6), which also demonstrated the lowest zeta potential and the smallest polydispersity index. This behavior was expected and may be attributed to the branched structure and relative bulkiness of the Cremophor molecules leading to increased size of the vesicles.

Encapsulation efficiency

The data obtained from calculated percentage encapsulation efficiency indicated that most of the formulations were able to entrap the drug into the vesicles with high efficiency. The results are shown in Table 3. The constant content of cholesterol in all formulations and the fixed ratio between phenytoin sodium: surfactant and cholesterol to 1:2:1 helped in formulating rigid vesicles which were able to encapsulate the drug efficiently. Formula F1 showed maximum encapsulation (95 %) followed by F4 (91%). However, formula F2 demonstrated the lowest entrapment efficiency (49 %) which may be due to delicate wall of the vesicles that might have undergone some leakage of the drug with time.

In-vitro release of phenytoin from niosome formulations

The release data of phenytoin sodium from prepared niosomes indicated that the drug is released according to Higuchi diffusion model, when percentage drug released was plotted against the square root of time high values for the squared linear correlation coefficient (r^2 = 0.998) were obtained, compared to other kinetic models. All tested formulations were able to sustain the release of phenytoin sodium for up to 24 hours (see Fig. 2). The rapid release behavior of F2 can be attributed to the presence of Span 20 as the only surfactant which formed with cholesterol delicate vesicles with thin wall that was subjected to the burst effect. This behavior of F2 was confirmed by its poor encapsulation efficiency relative to other formulations. The optimum release rate was obtained from formulations F1, F3, F4 and F5, where more than 70-88 % of phenytoin content was released in 24 hours.

These results are in good agreement with the average sizes and polydispersity indices of these formulations being smaller in average size and have large size distribution.

The release profile of formulation F3 showed a similar behavior to F5 (released > 50 % in 12 hours and >75 % in 24 hrs) due to smaller average vesicle size (145 nm for F3 and 201 nm for F5 respectively).

Table 3: Percentage encapsulation efficiency and drug released from niosomal formulations

1.	Formula	2.	% Encapsulation ± SD	3.	percentage released (mcg) after Q (hrs) ± SD 4. Q 6	5.	Q 12	6.	Q 24
7.	F1	8.	95.46 ± 0.54	9.	10.95 ± 0.01	10.	25.20 ± 0.03	11.	74.08 ± 0.21
12.	F2	13.	49.14 ± 0.43	14.	20.16 ± 0.02	15.	67.06 ± 0.04	16.	100.00 ± 0.14
17.	F3	18.	82.43 ± 0.25	19.	24.53 ± 0.04	20.	50.32 ± 0.05	21.	75.63 ± 0.32
22.	F4	23.	91.49 ± 0.35	24.	31.65 ± 0.05	25.	52.72 ± 0.12	26.	88.11 ± 0.23
27.	F5	28.	70.33 ± 0.40	29.	21.28 ± 0.12	30.	49.45 ± 0.03	31.	72.43 ± 0.25
32.	F6	33.	75.21 ± 0.22	34.	8.57 ± 0.01	35.	18.54 ± 0.02	36.	37.76 ± 0.45

Formulation F4 showed the optimum sustained release between other niosomal formulations which can be attributed to the smaller vesicular size and the presence of poloxamer 407 block copolymer molecules on the surface of the vesicles facilitating H-bonding interaction with water and creation of channels in vesicle membrane. Other formulations, however containing surfactants with similar HLB, yet the hydrophobic portions were bulky and might have shared in formation of larger vesicles with relatively thick hydrophobic membrane (F6). Therefore slower interactions of these vesicles with water in the dissolution medium were observed. The slowest release rate was demonstrated by formulation F6 which released only 37.76 % after 24 hours. This result can be explained by the effect of average size and size distribution of niosomes in formulation F6 being 570 nm (the largest vesicles with thick wall) and also the lowest polydispersity index (0.47) both helped in entrapping large amount of the drug in approximately similar sized vesicles and the release rate will be slower under these two limiting variables. Due to the optimum release characteristics (>88 % in 24 hrs), zeta potential (-58.93 mV) and smallest vesicle size (74 nm), formula F4 was selected for the in-vivo study.

Pharmacological activity

At the end of the treatment period (9 days), niosome-treated lesions were completely healed. For the control group (vehicle treated), there was no evidence for complete healing of the lesions within the 9 days treatment period (see Fig. 3). The data obtained visually from the photographs of the healed wounds also indicated that the progress of healing started from the first day as the diameter of the wound started to shrink and the color of the affected area also changed to the buff color rather than the red color of the control group. Statistical t-test analysis of the data (diameter of wound and number of days required for complete healing) carried out between treated group and the control group indicated that, there was a significant difference ($P < 0.0001$) between the wound diameter on days 3, 6 and 9 between both groups (see Table 4). The same significance was detected for the total number of days required for complete healing in both of control and treated groups.

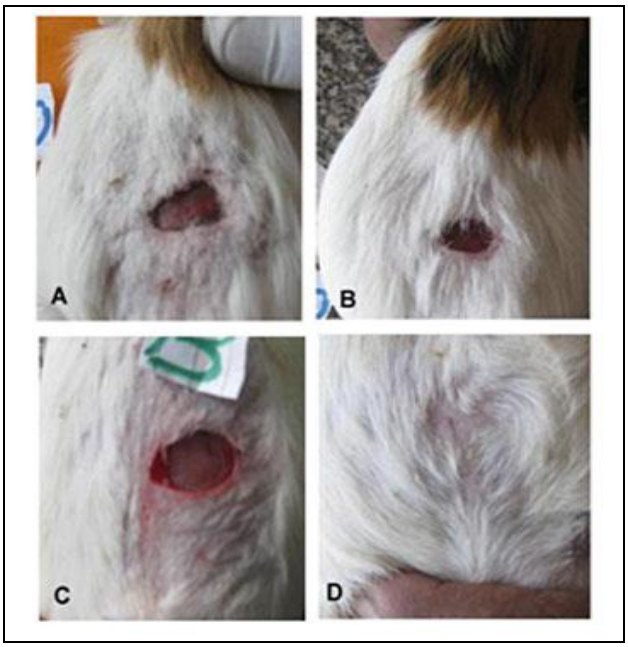

Fig. 3 Photographic shots of wounds in control group; A (day 0), B (day 9) and treatment group; C (day 0) and D (day 9)

Table 4: Statistical analysis of the effect of phenytoin sodium niosomes on wound diameter and healing time in control and drug-treated animals

Animal	Control group				Treatment group			
	Average wound diameter (mm)			Days for complete healing	Average wound diameter (mm)			Days for complete healing
	Day 3	Day 6	Day 9		Day 3	Day 6	Day 9	
1	9.0	8.5	7.0	19.0	6.8	3.6	0.0	8.0
2	8.9	8.2	6.3	18.0	6.7	3.5	0.0	8.0
3	9.0	8.7	6.6	17.0	6.9	3.4	0.1	9.0
4	8.8	8.0	6.4	17.0	6.6	3.2	0.0	7.0
5	8.9	7.8	6.2	17.0	6.5	3.1	0.0	8.0
6	8.7	7.8	6.3	18.0	6.6	3.3	0.0	7.0
St. dev.	-	-	-	-	0.13	0.29	0.21	0.78
Calculated t-value	-	-	-	-	28.70	28.30	53.20	21.70
P – value	-	-	-	-	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*

: statistically significant at $P < 0.05$

CONCLUSION

Topical delivery of medications largely depends on selection of the appropriate carrier which can easily deliver the drug into the stratum corneum. Niosomes or lipid-membrane vesicles become the first choice for this purpose especially if prolonged treatment is required as in ulcers, burns and wounds. In this work niosomes made up of mixed surfactants proved to be effective carriers and

skin penetration enhancers for delivery of phenytoin through artificially injured skin.

The niosomal gel formulations remained attached to the skin and sustained the release of the drug from the reservoir vesicles for more than 24 hours. Phenytoin sodium containing niosomes can be considered a better aid for rapid healing of skin injuries due to its high drug partitioning into skin cells and prolonged stimulation of collagen formation effect. Therefore, niosmoes containing phenytoin sodium helped in acceleration of complete wound healing in a shorter time compared to placebo formulations.

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