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Research paper

Novel sugar esters proniosomes for transdermal delivery of vinpocetine: Preclinical and clinical studies

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ABSTRACT

Vinpocetine (Vin) existing oral formulations suffer poor bioavailability (~7%) since Vin undergoes a marked first-pass effect (~75%) and its absorption is dissolution rate-limited. In this study, a novel sustained release proniosomal system was designed using sugar esters (SEs) as non-ionic surfactants in which proniosomes were converted to niosomes upon skin water hydration following topical application under occlusive conditions. Different in vitro aspects (encapsulation efficiency, vesicle size and shape, effect of occlusion, in vitro release, skin permeation and stability) were studied leading to an optimized formula that was assessed clinically for transdermal pharmacokinetics and skin irritation.

All formulae exhibited high entrapment efficiencies, regardless of the surfactant HLB. Vesicle size analysis showed that all vesicles were in the range from 0.63 μm to 2.52 μm which favored efficient transdermal delivery. The extent of drug permeation through the skin from the optimized formula – containing laurate SE with shorter fatty acid chain length and high HLB – was quite high (91%) after 48 h under occlusive conditions. The extent of absorption of Vin from proniosomes was larger when compared to the oral tablet with a relative bioavailability (F_{rel}) of 206%. Histopathological evaluation revealed only moderate skin irritation when using SEs compared to skin inflammation when using Tween 80. Sugar esters proniosomes may be a promising carrier for vinpocetine, especially due to their simple scaling up and their ability to control drug release.

1. Introduction

Since the first introduction of liposomes in the early 1960s, it was encouraging to observe the outbreak of both theoretical and practical applications, which this versatile drug delivery system has invoked. No doubt that drug delivery systems using colloidal particulate carriers such as liposomes or niosomes have proved to possess distinct advantages over conventional dosage forms because the particles can act as drug reservoirs, can carry both hydrophilic drugs by encapsulation or hydrophobic drugs by partitioning of these drugs into hydrophobic domains and modification of the particle composition or surface can adjust the drug release rate and/or the affinity for the target site [1].

Liposomes are unilamellar or multilamellar spheroid structures composed of lipid molecules, often phospholipids, assembled into bilayers. Because of their ability to carry a variety of drugs, liposomes have been extensively investigated for their potential application in pharmaceutics, such as, drug delivery [2–4], for drug targeting [5], for controlled release [6], or for increasing solubility [7]. However, there remain significant problems in the general application of liposomes for drug delivery. In a dispersed aqueous system, liposomes may suffer some chemical problems associated with degradation by hydrolysis or oxidation [8], as well as physical problems as sedimentation, aggregation, or fusion of liposomes during storage [9].

Two approaches were adopted in dealing with these problems; the first was to address the problems with the physical stability of aqueous suspensions of liposomes. In 1986, Payne et al. [10] introduced 'proliposomes', a dry free-flowing granular product which could be hydrated immediately before use. Proliposomes are composed of water-soluble porous powder as a carrier upon which one may load phospholipids and drugs dissolved in organic solvent. Proliposomes can be stored in a dry state and dispersed/dissolved to form an isotonic multilamellar liposomal suspension by the addition of water as needed. It was reported that amphotericin B proliposomes could be stored for 9 months without significant changes in distribution of vesicle size, and for at least 6 months without loss of pharmacological activity. Even though proliposomal formulations are an improvement over conventional liposome dispersions in terms of the physical stability of the preparation,

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chemical instability is still present and therefore a vacuum or nitrogen atmosphere is recommended during preparation and storage to prevent the oxidation of phospholipids [11].

The second approach was to address the chemical problems by using alternatives to phospholipids in preparing vesicles. One alternative involves the formation of 'niosomes' from hydrated mixtures of cholesterol and non-ionic surfactant [12]. These 'niosomes' are quite stable, require no special conditions, such as low temperature or inert atmosphere for production or storage and the relatively low cost of the materials that form them make niosomes more attractive than liposomes for industrial manufacturing [12]. However, even though niosomes exhibit good chemical stability during storage, aqueous suspensions of niosomes may exhibit problems of physical instability such as aggregation, fusion, leaking of entrapped drugs, or hydrolysis of encapsulated drugs, thus limiting their shelf life [11].

The latest approach in the field of vesicular delivery is to combine the two previously mentioned techniques by extending the pro-vesicular approach to niosomes through the formation of "proniosomes" which are converted to niosomes upon hydration. Proniosomes avoid many of the problems associated with aqueous niosome dispersions, and problems of physical stability (aggregation, fusion, leaking) could be minimized. The additional convenience of the transportation, distribution, storage, and dosing would make 'dry niosomes' a promising industrial product [11]. Proniosomes offer a versatile vesicle delivery concept with potential for delivering drugs via transdermal route. This is based on proniosomes ability to form niosomes upon hydration with water from skin following topical application under occlusive conditions [13].

Yet, and to the best of our knowledge, a very few number of research activities have been done to explore the full potential of these systems specially with transdermal delivery and published work have mainly used sorbitan esters (Spans) and polyoxyethylene sorbitan esters (Tweens) as non-ionic surfactants together with a limited amounts of lecithin and cholesterol [13–16]. In this study, a novel approach was developed that utilizes food-grade sugar esters as non-ionic surfactants in formulating proniosomal systems.

Sugar esters (SEs) are non-ionic surfactants having a sugar substituent, sucrose, as the polar head group and fatty acids as non-polar groups. These esters contain different fatty acids (stearic, palmitic, myristic and lauric acid) in different ratios. The type of fatty acid and the degree of esterification determine the hydrophilic lipophilic balance (HLB) value and the melting point of these materials. They have HLB values from 1 to 16, and therefore they can be applied in many areas of pharmaceutical and cosmetical technology as emulsifiers, solubilizing agents, lubricants, penetrating enhancers and pore forming agents [17]. Possible future utilization of these surfactants in more developed systems is expected because they have low toxicity, biocompatibility, and excellent biodegradability [18].

Vinpocetine [Vin], a poorly water-soluble vincamine derivative, is widely used for the treatment of disorders arising from cerebrovascular and cerebral degenerative diseases. Although it has been shown to increase the cerebral flow in the ischemic patients with cerebrovascular disease, to increase red cell deformability in stroke patients and to have neuroprotective abilities against brain ischemia [19], the clinical use of its marketed commonly used oral formulations is limited by its poor absorption, extensive first pass metabolism and its extreme slow dissolution rate that results in very low bioavailability (~7%). This poor oral bioavailability together with the small $t_{1/2}$ implies the necessity of frequent drug dosing (three times daily), a situation that is inconvenient for patients of dementia and results in poor compliance [20,21].

Based on these considerations and to overcome all these problems, the current work has aimed to develop a novel vinpocetine proniosomal controlled transdermal strategy, in which sugar esters were incorporated as permeation and absorption enhancers. In this way, smooth and continuous vinpocetine delivery through the skin into the bloodstream could be ensured thus avoiding its first pass metabolism and produce stable plasma concentrations over a long period. The influence of HLB of different SEs and their fatty acid type on the in vitro characters of the formula (encapsulation efficiency, vesicle size and shape, drug release, drug permeation across hairless rat skin and stability) were investigated. Skin irritation induced by SE patch was evaluated by measuring skin conductivity, skinfold thickening and by histopathological evaluation. The second objective of this work was to select the best formulation for clinical study where in vivo Vin plasma levels were measured after SE proniosomal patch application and compared to plasma levels produced by an existing Vin marketed product in healthy volunteers.

2. Materials and methods

2.1. Materials and animals

Vinpocetine (Vin) was kindly provided by Medical Union Pharmaceutical Co. (Cairo, Egypt). Vinporal® was purchased from Amriya Pharmaceutical Co. (Alexandria, Egypt). Different grades of sugar esters: sucrose stearate S-1670 (HLB = 16), S-970 (HLB = 9), S-370 (HLB = 3), sucrose palmitate P-1670 (HLB = 16), sucrose myristate, M-1695 (HLB = 16), and sucrose laurate L-1695 (HLB = 16), were kindly donated by Mitsubishi-Kagaku Foods Corporation (Tokyo, Japan). Hydrogenated lecithin S75-3N (stored at −5 °C) was kindly donated by Lipoid (Ludwigshafen, Germany). Cholesterol (stored at −5 °C), Span 80 (Sorbitan monooleate; HLB = 4.3), Span 20 (Sorbitan monolaurate; HLB = 8.6), Tween 80 (Polyoxyethylene (20) sorbitan monooleate; HLB = 15) and Tween 20 (Polyoxyethylene (20) sorbitan monolaurate; HLB = 16.2) were purchased from Oxford Chemicals (Mumbai, India). Methanol and glacial acetic acid HPLC grade were purchased from Sigma Chemicals (St. Louis, MO, USA). Spectra Por® cellophane dialyzing membrane, molecular weight cut-off of 14,000 was purchased from Spectrum Medical Inc. (Los Angeles, CA, USA). Occlusive film was purchased from Transpaseal, Porcupine Canvas (Ontario, Canada). All other chemicals were of analytical reagent grade and were obtained from EL-Nasr Company (Cairo, Egypt).

Male healthy New Zealand Albino rabbits weighing between (2.0–3.0 kg) were obtained from the German University in Cairo Animal House, Cairo, Egypt. All studies performed in this work were approved by the University Protection for Animal Care and Use Committee, and the protocol was compliant with the "Principles of Laboratory Animal Care" [NIH Publication # 85-23, revised 1985].

2.2. Methods

2.2.1. Preparation of proniosomes

Proniosomal formulae were prepared by a method reported by Fang et al. [13] using different types of non-ionic surfactants, lecithin and cholesterol and evaluated in a "one factor at a time" fashion. Appropriate amounts of proniosomal components together with vinpocetine were mixed with 125 ml absolute ethanol in a wide-mouth glass tube. The open end of the glass tube was covered with a lid and warmed in a water bath at 65 ± 3 °C for 5 min. Eighty microliters of pH 7.4 phosphate buffer was added and warming was continued on the water bath for about 2 min till a clear solution was observed. The mixture was allowed to cool down at room temperature till the dispersion was converted to proniosomal gel.

The exact mechanism of conversion to the gel state is described in details by Vora et al. [14]. The effects of surfactant type, amount of surfactant, lecithin and cholesterol as well as drug load were studied. The composition of different formulations (F1–F18) is listed in Table 1.

2.2.2. Vinpocetine entrapment efficiency% (EE%)

Proniosomal gel (0.2 g) was reconstituted with 10 ml of pH 7.4 phosphate buffer in a glass tube. The aqueous suspension was sonicated in a sonicator bath (MU-8 Clifton, NJ, USA) for 30 min. The vinpocetine-containing niosomes were separated from untrapped drug by centrifuging at 14,000 rpm at 4 °C for 30 min (Ultra centrifuge 5417R, eppendorf, Hamburg, Germany). The supernatant was taken and diluted with methanol, and the vinpocetine concentration in the resulting solution was assayed by HPLC method. The percentage of drug encapsulation was calculated by the following equation [13,15]:

$$EE(\%) = [(Ct - Cf)/Ct] \times 100$$

where Ct is the concentration of total vinpocetine, and Cf is the concentration of free vinpocetine.

2.2.3. Vesicle size analysis

The vesicle size was determined using Mastersizer S – laser diffractometer (Malvern Instruments, Malvern, Worcestershire, UK). In a glass tube, 0.2 g proniosome gel was diluted with 10 ml of pH 7.4 phosphate buffer. The vesicle measurements were done at temperature 25 ± 0.5 °C using a lens (with a laser range of 300 mm that measure particle size range from 0.5 to 900 μm and a beam length of 2.4 mm was attached to a measuring cell). The obscuration level was kept at 10% at a stable count rate. Three replicates were taken for each sample, and polystyrene beads were used as a standard to check instrument performance [22].

The polydispersity index (PI) was determined as a measure of homogeneity. Small values of PI (<0.1) indicate a homogeneous population, while PI values >0.3 indicate high heterogeneity [23].

2.2.4. Microscopical examination

2.2.4.1. Optical microscope. In a glass tube, 0.2 g proniosome gel of F5, F17 and F18 having different drug loads were diluted with 10 ml of pH 7.4 phosphate buffer; a few drops of the formed niosomal dispersion were spread on a glass slide and examined for the presence of insoluble drug crystals using ordinary light microscope with varied magnification powers (10× and 40×). Photomicrographs were taken using a digital camera (Sony Cybershot DSC-w55 7.2 megapixel, Tokyo, Japan).

2.2.4.2. Scanning electron microscopy (SEM). In an attempt to illustrate the role of cholesterol in vesicle formation, the morphological differences in shape and surface characteristics of the prepared proniosome-derived niosomes of formulae F5 and F14 having different cholesterol contents were examined using a scanning electron microscope. In a glass tube, 0.2 g proniosome gel was diluted with 10 ml of pH 7.4 phosphate buffer; the dispersion was sprinkled and fixed on a SEM holder with double-sided adhesive tape and coated with a layer of gold of 150 Å for 2 min using a Sputter coater (Edwards, S-150A, England) working in a vacuum of (3×10^{-1} atm) of Argon gas. The samples were examined using a scanning electron microscope [22] (Jeol, JSM T20, Tokyo, Japan).

2.2.5. Differential scanning calorimetry (DSC)

To study the possible interactions between Vin and vesicle ingredients, F8 of highest EE% was chosen and samples of 4 mg of each of Vin, sugar ester S-370, empty and drug loaded proniosomes-derived niosomes were submitted to DSC analysis using

Table 1
Composition (mg), EE% and vesicle size of various proniosomal formulae.

Formula no.	Formula description		Vinpocetine	Cholesterol	Lecithin	Span 20	Span 80	Tween 20	Tween 80	SE L-1695[c]	SE M-1695[c]	SE P-1670[c]	SE S-370[d]	SE S-970[c]	SE S-1670[c]	Entrapment Efficiency % (EE%)[a]	Vesicle size (μm)[a] VS ± SD	Polydispersity index (PI)[b]
F1	Different type of surfactant		5	20	180	180	–	–	–	–	–	–	–	–	–	98.25 ± 0.32	0.63 ± 0.12	0.14
F2			5	20	180	–	180	–	–	–	–	–	–	–	–	98.34 ± 0.9	0.84 ± 0.12	0.19
F3			5	20	180	–	–	180	–	–	–	–	–	–	–	81.75 ± 0.25	1.26 ± 0.24	0.01
F4			5	20	180	–	–	–	180	–	–	–	–	–	–	87.17 ± 0.33	1.36 ± 0.01	0.19
F5			5	20	180	–	–	–	–	180	–	–	–	–	–	85.37 ± 0.16	0.97 ± 0.26	0.27
F6			5	20	180	–	–	–	–	–	180	–	–	–	–	89.02 ± 0.23	1.13 ± 0.18	0.16
F7			5	20	180	–	–	–	–	–	–	180	–	–	–	92.14 ± 0.34	1.39 ± 0.19	0.19
F8			5	20	180	–	–	–	–	–	–	–	180	–	–	98.99 ± 0.28	0.63 ± 0.13	0.2
F9			5	20	180	–	–	–	–	–	–	–	–	180	–	98.33 ± 0.47	0.82 ± 0.01	0.01
F10			5	20	180	–	–	–	–	–	–	–	–	–	180	98.51 ± 0.52	1.65 ± 0.18	0.14
F11	Different surfactant amounts	Low	5	20	180	–	–	–	–	90	–	–	–	–	–	76.29 ± 0.65	0.81 ± 0.09	0.11
F12		High	5	20	180	–	–	–	–	270	–	–	–	–	–	97.25 ± 1.6	1.93 ± 0.19	0.1
F13	Different cholesterol amounts	Low	5	10	180	–	–	–	–	180	–	–	–	–	–	74.25 ± 1.18	1.14 ± 0.18	0.16
F14		High	5	30	180	–	–	–	–	180	–	–	–	–	–	79.91 ± 0.69	0.84 ± 0.08	0.1
F15	Different Lecithin amounts	Low	5	20	90	–	–	–	–	180	–	–	–	–	–	78.36 ± 0.61	0.69 ± 0.12	0.17
F16		High	5	20	270	–	–	–	–	180	–	–	–	–	–	91.98 ± 0.88	2.52 ± 0.22	0.09
F17	Different drug loads	Low	3	20	180	–	–	–	–	180	–	–	–	–	–	99.2 ± 0.4	0.65 ± 0.12	0.18
F18		High	7	20	180	–	–	–	–	180	–	–	–	–	–	71.06 ± 0.9	1.68 ± 0.01	0.01

[a] Each value represents the mean ± SD ($n = 3$).
[b] Polydispersity index is obtained as PI = (standard deviation (SD)/vesicle size (VS)).[14].
[c] Melting points of SE L-1695, SE M-1695, SE P-1670, SE S-970 and SE S-1670 are 47 °C, 40 °C, 48 °C, 56 °C and 56 °C respectively.
[d] SE S-370 has a dual melting point at 58, 69 °C.

differential scanning calorimeter (Shimadzu DSC-60, Kyoto, Japan). Each sample was sealed in a standard aluminum pan and scanned between zero and 200 °C while another empty pan was used as a reference. The Thermograms were obtained at a scanning rate of 5 °C/min. The heat flow calibration was performed with indium.

2.2.6. In vitro release test

The release of vinpocetine from different proniosomal formulations were determined using USP dissolution tester (Apparatus I) (USP Dissolution tester DT 800-LH, Erweka, Heusenstamm, Germany). Proniosomal gels containing the equivalent of 5 mg vinpocetine (with exception to F17 having an equivalent of 3 mg and F18 having an equivalent of 7 mg) were placed in glass cylindrical tubes (2.5 cm in diameter and 6 cm in length). Each tube was tightly covered with a soaked Spectra por© molecular porous membrane tubing (molecular weight cut-off of 14,000) from one end and attached to the shafts of the USP Dissolution tester apparatus, instead of the baskets, from the other end.

The shafts were then lowered to the vessels of the dissolution apparatus so that the dissolution medium outside and the proniosomal preparation inside were adjusted at the same level. The release medium was 500 ml pH 5.6 of 70:30 (%v/v) citrate buffer:ethanol to mimic the pH of the skin. Ethanol was added to maintain sink conditions due to the extreme poor solubility of vinpocetine in water [13], and the vessel was covered during the test to minimize ethanol evaporation. The release study was carried out at 32 ± 0.5 °C, and the stirring shafts were rotated at a speed of 100 rpm. The experimental conditions were set following the FIP/AAPS Guidelines to dissolution/in vitro release testing of transdermal formulations [24].

Samples of five milliliters were withdrawn periodically at predetermined time intervals of 1, 2, 3, 4, 6, 8, 12, 24 and finally at 48 h. Every withdrawal was followed by immediate replacement with fresh medium to maintain a constant volume. Samples were centrifuged at 14,000 rpm at 4 °C for 30 min to precipitate any present niosomal vesicles [25], and supernatant was analyzed using HPLC method illustrated in Section 2.2.12.2. All release experiments were done in triplicates.

In order to understand the barrier presented by the dialysis membrane, the in vitro release study of a plain drug solution in ethanol (5 mg/5 ml) was carried out in a similar manner. The obtained release data were subjected to kinetic treatment according to zero, first and Higuchi diffusion models [26]. The correlation coefficient (r), the order of release pattern and $t_{50\%}$ value were determined in each case. The obtained $t_{50\%}$ values were subjected to statistical evaluation using ANOVA at a 0.05 level of significance.

2.2.7. Ex vivo permeation

Permeation studies give an important insight into the drug behavior in vivo, since the amount of drug permeated dictates the amount of drug available for absorption into systemic circulation [14]. The skin permeation of vinpocetine from optimized proniosomal formulae F19 and F20 was determined using vertical Franz diffusion cell. Permeation of vinpocetine (equivalent to 5 mg) dispersed in carbopol 934 gel was used as a control.

Newly born Wistar albino rats weighing 80 ± 20 g were sacrificed and the full thickness skin, free of bites and scratches were excised. The dermal surface was carefully cleaned to remove subcutaneous tissues without damaging the epidermal surface. When not used immediately, the skin was kept refrigerated (2–5 °C) and was used within 3 days.

A Franz diffusion cell was first filled with 20 ml of 30%:70% (v:v) ethanol:pH 7.4 phosphate buffer. Ethanol was added to maintain sink conditions due to the extreme poor solubility of vinpocetine in water [13]. The skin with a surface area of 2.8 cm^2 was placed across the ground glass joint with the stratum corneum facing the donor compartment.

To simulate the in vivo conditions where proniosomes are applied onto the surface of skin under occlusive conditions, proniosomal formulae were spread evenly on skin surface and covered tightly with an occlusive film. The circular rim of the dosing site was sealed with paraffin wax in order to ensure occlusion and to prevent leakage of the tested proniosomal formula during permeation study [27]. No buffer was added to the donor compartment. Finally, the Franz cell compartments were clamped together.

The temperature of the receptor compartment was maintained at 37 ± 0.5 °C with an external constant temperature circulator water bath, and the receiver medium was continuously stirred with a small magnetic bar in order to prevent any boundary layer effects.

At predetermined time intervals, namely 1, 2, 3, 4, 6, 8, 12, 24 and 48 h, samples of 1 ml were taken from the receptor compartment and the cell was refilled with an equivalent amount of fresh receptor solution. Samples were analyzed by HPLC. Each permeation experiment was replicated three times and from the concentration of vinpocetine in the receiving solution the amount permeated through the skin membrane was calculated.

The cumulative amount of vinpocetine permeated into the receptor compartment was plotted against time to obtain a percentage permeation profile. The steady state flux, J_{ss} (μg/cm^2/h), was calculated from the slope of the linear portion of the plot of the cumulative amount permeated versus time and expressed as

$$J_{ss} = dM/dt$$

where M is the cumulative amount of vinpocetine permeated through skin per unit area in (μg/cm^2) in experimental time t in (h) [28,29]

The apparent permeability coefficients (P_{app}) were calculated according to the following equation:

$$P_{app} = J_{ss}/C_{donor}$$

where P_{app} in (cm h^{-1}) is the permeability coefficient of vinpocetine through the membrane. C_{donor} is the concentration of vinpocetine in the donor chamber in (μg/cm^3) [30].

Enhancement ratios were calculated according to the following expression:

$$ER = J_{enh}/J_{ctrl}$$

where J_{enh} is the enhanced flux with application of proniosomal formulation and J_{ctrl} is the flux of drug from the control formula [13].

2.2.8. Effect of occlusion on permeation

Proniosomes are converted to niosomes by hydration with water; thus, the application of proniosomal gels under occlusion is recommended. To study the role of occlusion, skin permeation of the optimized formula F20 under occlusive and non-occlusive conditions were studied and compared. Therefore, the same experimental technique mentioned previously in Section 2.2.7 was adopted but without covering the formula with an occlusive film.

2.2.9. Stability

The optimized proniosomal formulae F19 and F20 (Table 2) were sealed in 30 ml clear glass vials and stored at refrigeration temperature (2–8 °C). After 90 days, hydration step was carried out and the entrapment efficiency as well as the mean particle size of each sample was determined and compared to the freshly prepared proniosomes-derived niosomes.

2.2.10. Irritation studies

2.2.10.1. Measurement of skinfold thickening. Skin swelling and thickening are major signs of skin irritation and inflammation

Table 2
Composition of optimized formulae F19 and F20 (mg).

Formula no.	Formula Description	Vinpocetine	Cholesterol	Lecithin	Tween 20	L-1965
F19	Optimized formulae	5	10	90	270	–
F20		5	10	90	–	270

[31]. Six male healthy Albino rabbits weighing between 1.50 and 2.0 kg were used in this study to test the irritation potential by measuring the skinfold thickening [32]. The study performed in this section was approved by the university protection for animals care and use committee and the protocol complied with "the Principles of Laboratory Animal Care" [NIH publication # 85-23, revised 1985]. Right and left flanks were carefully shaved 3 h before the experiment using razor with no apparent lesions or wounds. One gram of each medicated formulation was applied topically over a surface area of 2×2 cm^2, where the skinfold thickness was measured out before and after 48 h of application using a micrometer (Altraco MM-22, Sausalito, CA, USA) and the changes were plotted versus time [32].

2.2.10.2. Histopathological evaluation. To confirm previous irritation results, a histopathological study was carried out using nine healthy Albino rabbits. Back skin was carefully shaved 12 h before the experiment using razor with no apparent lesions or wounds. One gram of each medicated formulation was applied topically on the rabbit's dorsal skin over a surface area of 2×2 cm^2.

Rabbits were divided into three groups each of three animals, which were treated with the following:

Group I: control group.
Group II: multiple topical applications of F19 once daily for 7 days.
Group III: multiple topical applications of F20 once daily for 7 days.

On the 8th day, animals were killed, samples of 2 cm^2 of the applied skin areas were taken, flattened and preserved in 4% buffered formaldehyde. Biopsies were embedded in paraffin, sectioned at 5 μm using cryostat microtome and stained with haematoxylin and eosin. Finally, sections were investigated under light microscope, photomicrographed and compared relative to the control group [33].

2.2.11. In vivo absorption study

2.2.11.1. Study design. Based on previous investigations, one proniosomal formulation (F20) was selected to be tested in vivo. The in vivo study was carried out to compare the pharmacokinetics of vinpocetine from F20 proniosomal patch containing 5 mg vinpocetine (treatment A) to an oral commercial tablet (Vinporal® – Amriya Pharmaceutical Co., Alexandria, Egypt) containing the same dose of vinpocetine (treatment B) using a non-blind, two treatment, two-period, randomized, crossover design.

Twelve healthy non-smoking male volunteers (26–37 years, 78–96 kg) participated in the study and were randomly assigned to one of the two treatment groups of equal size. Each subject read, understood and signed an informed written consent and was well informed about the risks and objectives of the study.

All subjects showed no clinically relevant positive findings from standard physical examination at entry, normal clinical and laboratory tests within 24 h prior to the start of the study. No history of cardiovascular, renal or hepatic disease and no history of hypersensitivity, asthma, urticaria or other allergic symptoms.

The study protocol was approved by German University in Cairo (GUC) protection of Human Subjects Ethical Committee, and the protocol complies with the declarations of Helsinki and Tokyo for humans.

The study was performed on two phases. In Phase I, half the number of volunteers received treatment A and the remainder received treatment B which is considered as a standard. A washout period of 1 week separated the phases. In Phase II, the reverse of randomization took place.

For the transdermal absorption study, proniosomal patch (treatment A) was prepared by equally spreading an amount of proniosomal gel (F20) containing 5 mg vinpocetine on an occlusive plastic backing layer; this system was covered by a non-irritating adhesive sheet and applied to the forearm of the volunteers. While for oral administration, one Vinporal® tablet (treatment B) was administered to subjects of group 2 fasted for at least 10 h before the study day [34].

Each group was supervised by a physician who was also responsible for their safety and collection of samples during the trial. At 9:00 am the assigned treatment was given and no food was allowed for 4 h after dosing.

Venous blood samples (5 ml) were withdrawn and collected into heparinized tubes at the following time points: 0 (predose), 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 48 and 72 h after the administration of a treatment. Plasma was obtained by centrifugation at 6000 rpm for 10 min (Hettich EBA20 Centrifuge C2002, Djblabcare, Buckinghamshire, UK). The plasma was pipetted into glass tubes and then frozen at −22 °C until ready to be analyzed using HPLC.

2.2.11.2. Plasma analysis. The plasma obtained after receiving treatment A and treatment B was assayed using HPLC method described in Section 2.2.12.3 without further addition of vinpocetine. As an exception, for low concentration plasma samples, the samples were spiked with known concentrations of vinpocetine before analysis.

2.2.11.3. Pharmacokinetic analysis. Pharmacokinetic characteristics from plasma data following the two treatments were estimated for each subject by using a computer program, WinNonlin (version 4, Scientific consulting, Inc., Cary, NC, USA). Non-compartmental analysis was used.

C_{max} (ng ml^{-1}) and t_{max} (h) were the observed maximal drug concentration and its time, respectively. The area under the curve, $AUC_{(0-t)}$ (ng h ml^{-1}), was calculated using the trapezoidal rule from zero time to the last time of blood sample. K (h^{-1}) the terminal elimination rate constant was estimated by log-linear regression analysis and apparent terminal elimination half-life $t_{1/2}$ (h) was calculated as $t_{1/2} = 0.693/k$. The relative bioavailability F for transdermal drug delivery was calculated by comparing the transdermal and oral AUCs.

2.2.12. HPLC analysis of vinpocetine

2.2.12.1. Chromatographic conditions. A modified HPLC method for vinpocetine determination was adopted [35]. The HPLC system (Spectra System®, Thermo Fisher Scientific, Waltham, USA) consisted of a Spectra System solution degasser SCM 1000, Spectra System Pump P2000, Thermo C18 reverse-phase column (5 μm particle diameter, 4.6×250 mm), maintained at room temperature and a guard column C18 Thermo (4.6×20 mm). The mobile phase was composed of a mixture of methanol:water (80:20 (v/

v)), containing 0.1% w/w triethylamine and adjusted to pH 7 using glacial acetic acid. The mobile phase was filtered through 0.2 μm membrane filter and delivered at a flow rate of 1 ml/min. Samples were injected using Spectra System Auto sampler AS3000, and effluents were monitored at 274 nm using Spectra System Detector UV 3000 and the sensitivity was set at 0.0001 AUFS.

2.2.12.2. In vitro standard calibration curve. Aliquot portions of vinpocetine stock solution (10 μg/ml) were transferred into 10 ml volumetric flasks. To each flask, 0.1 ml of ketotifen (internal standard) stock solution (10 μg/ml) was added. Methanol was added to give final concentrations of vinpocetine ranging from 50 to 3000 ng/ml. A 20 μl aliquot from each of the previous solutions was analyzed by HPLC. A curve was constructed by plotting the ratio of the peak area of the drug to that of the internal standard against concentration, and the curve was used in the determination of in vitro concentrations. All assays were performed in triplicate.

During the assay of the samples, the intra-batch precision and accuracy of the analytical procedure were evaluated after replicate analysis ($n = 3$) of control samples spiked at three concentration levels: 100, 500 and 2000 ng/ml. The lower limit of quantification was 20 ng/ml with a linear response across the full range of concentrations from 50 to 3000 ng/ml ($R^2 = 0.998$).

2.2.12.3. In vivo standard calibration curve. In a 10 ml glass centrifuge tube, 1 ml of blank plasma was spiked with vinpocetine stock solution (10 μg/ml) to contain 50, 75, 100, 150, 200, 250, 300 ng/ml. Fifty microliters of ketotifen (10 μg/ml) was added to each sample as an internal standard and the tube was vortexed for 30 s. Two consecutive portions of 3 ml *n*-hexane were added and the tube was shaken manually for 5 min; the organic phase after each addition was transferred into another dry tube and finally evaporated till dryness by gentle warming. The residues were reconstituted in 100 μl of mobile phase and 20 μl of the resulting solution was injected onto the HPLC column [36]. A plasma sample, without the addition of vinpocetine, was also treated in the same way.

A standard curve was constructed by plotting the ratio of the peak area of the drug to that of the internal standard against concentration, and the curve was used in the determination of in vivo concentrations. All assays were performed in triplicate, and the percent drug recovery after plasma treatment was 95.36 ± 1.76. During the assay of the samples, the intra-batch precision and accuracy of the analytical procedure were evaluated after replicate analysis ($n = 3$) of control samples spiked at three concentration levels: 50, 150 and 300 ng/ml. The lower limit of quantification was 20 ng/ml, and a linear response across the full range of concentrations from 50 to 300 ng/ml ($R^2 = 0.993$) was obtained.

2.2.13. Statistical analysis

The data obtained from different formulations were analyzed for statistical significance by one-way analysis of variance (ANOVA) adopting SPSS statistics program (version 16, SPSS Inc., Chicago, USA) followed by post hoc multiple comparisons using the least square difference (LSD). Differences between series were considered to be significant at $p \leqslant 0.05$.

3. Results and discussion

3.1. Entrapment efficiency% (EE%)

The entrapment efficiency is one of the important parameters in the design of vesicular formulations. Vesicle entrapment efficiency relies on the stability of the vesicle which is highly dependent on the type and amount of surfactant forming the bilayers, the amount of both cholesterol and lecithin and the drug load. Although it is important, the effect of drug load is usually missed in many publications [37]. The effects of these different parameters were discussed.

3.1.1. Effect of surfactant type

Non-ionic surfactant is the main building block of niosomal vesicles. The stability – and consequently the entrapment efficiency – of the formed vesicle were highly affected by the intrinsic properties of the surfactant, such as, HLB, chemical structure and phase transition temperature [37]. The effect of using different traditional non-ionic surfactants as well as sugar esters on the entrapment efficiency of proniosomal formulations after hydration is shown in Table 1.

Interestingly, formulations containing surfactants of either low or high HLB exhibited relative high entrapment efficiency, with some approaching 100% (Span 20, Span 80, S-370, S-970, S-1670). The vesicle formation ability of hydrophobic non-ionic surfactants could be understood as the molecule geometry fulfilled a proper critical packing parameter where the highly lipophilic drug is expected to be housed almost completely within the vesicles bilayer [13,15]. However, hydrophilic surfactants were also able to form vesicles due to the presence of sufficient amount of cholesterol [12].

These results indicated that not only the HLB but also the chemical structure of the surfactant which is related to the length of the alkyl chain was also governing the entrapment efficiency. Surfactants of longer saturated alkyl chains showed higher entrapment efficiency [38]. Another important factor that commonly helps to explain the vesicles entrapment patterns is the gel to liquid phase transition temperature (T_c) of the surfactant forming the niosomal membrane which is directly proportional to the surfactant alkyl hydrocarbon chain length. Surfactants of higher T_c are more likely in the ordered gel form forming less leaky bilayers, thus having higher entrapment efficiency, while surfactants of lower T_c are more likely in the less ordered liquid form [12,39].

On the basis of HLB, alkyl chain length and phase transition temperature, results in Table 1 could be explained as follows:

- Both Span 20 and Span 80 showed high EE% (98.25, 98.34 respectively) since both are hydrophobic surfactants with low HLB.
- Tween 20 and Tween 80 showed relative lower EE% (81.75, 87.17 respectively) due to their high HLB, yet Tween 80 revealed higher results due to its longer alkyl chain.
- HLB seemed to be the least entrapment governing factor among SEs. All stearate SEs, S-370, S-970 and S-1670, revealed high entrapment efficiency regardless of their HLB values due to their long alkyl chain and high phase transition temperatures.

3.1.2. Effect of surfactant amount

Increasing the amount of surfactant, the main component responsible for vesicle formation significantly increased the EE% ($p < 0.05$). This might be attributed to the increase in the number of formed niosomes and consequently the volume of the hydrophobic bilayer domain, the available housing for entrapment vinpocetine hydrophobic drug [40,41].

3.1.3. Effect of cholesterol amount

Increasing the cholesterol content from 10 mg to 20 mg was accompanied by a significant increase in EE% ($p < 0.05$). However, further cholesterol increase resulted in EE% significant decrease ($p < 0.05$). This pattern was reported previously by Mokhtar et al. [25] and was suggested to take place due to the ability of cholesterol

to abolish gel to liquid phase transition of niosomal systems resulting in less leaky vesicles [37]. Moreover, cholesterol molecules accommodate itself as "vesicular cement" in the molecular cavities formed when surfactant monomers are assembled into bilayers to form niosomal membranes [37,42]. This space filling action was responsible for the increased rigidity, decreased permeability of cholesterol-containing membranes compared to cholesterol-free membranes and the improved entrapment efficiency. In contrast, increasing cholesterol beyond a certain concentration may compete with the drug for the space within the bilayers, hence excluding the drug and can disrupt the regular linear structure of vesicular membranes [25].

3.1.4. Effect of lecithin amount

Significant increase in EE% was observed with increasing lecithin content ($p < 0.05$) which is commonly added to increase the system stability [43]. This result was substantiated by the high transition temperature of hydrogenated lecithin used in this study (48 °C) [44] and its unique advantage over unhydrogenated one in enhancing the rigidifying effect of cholesterol and formation of less leaky membrane bilayers [12,45]. The presence of double bonds in the unhydrogenated phosphatidylcholine permit the chains to bend (undergo conformational rotations to give cis/trans conformations), causing the adjacent molecule not to be tightly close to the bent phosphatidylcholine molecule when they assembled to form the niosomal membrane; accordingly, the membrane become more permeable [41]. The opposite held true upon saturation of this double bond, and the inclusion of hydrogenated lecithin forces the bilayer molecules to get in order resulting in more rigid and less permeable vesicle membrane [45]. Similar results were previously reported [13,27].

3.1.5. Effect of drug load

Increasing the vinpocetine drug load from 3 to 7 mg led to significant decrease in EE% ($p < 0.05$). It should be pointed out that a fixed amount of vesicle components (surfactant, cholesterol and lecithin) produce a constant number of niosomes of definite entrapment efficiency [41] with precipitation of excess drug [25]. This was supported by microscopic investigation where crystals of precipitated drug were found after hydration of formula F18 of higher drug load (Fig. 1).

3.2. Vesicle size analysis

All formulae were of small vesicle size ranging from 0.63 to 2.52 μm with low polydispersity index and unimodal size distribution (Table 1), which favors transdermal delivery of the drug [46]. Direct proportionality did exist between the vesicle size and both chain length and degree of hydrophilicity of the surfactants forming the vesicle bilayer [47,48]. The effect of chain length was illustrated by Israelachvili who proposed an equation by which the minimum vesicular radius (R) could be calculated:

$$R = l_c/(1 - \upsilon/a_o l_c)$$

where R = minimum vesicular radius that a vesicle can attain, l_c = hydrophobic chain length, υ = hydrophobic chain volume, and a_o = hydrophilic head group area [48].

While for the other factor, it was found that as the hydrophilicity of the niosome bilayers increased, the water intake of the bilayers of these vesicles increased too resulting in larger vesicle size [47].

Using these two factors, the differences in the mean vesicle size could be explained: being more hydrophobic, Span-based niosomes showed smaller vesicle size than Tween-based niosomes which are more hydrophilic. Yet, within each class, the surfactant of higher chain length (Span 80 and Tween 80) produced a larger vesicle than those of smaller chain length (Span 20 and Tween 20). With regard to sugar esters proniosomes-derived niosomes, increasing the chain length among L-1965, M-1695, P-1670, S-1670 having similar hydrophilicity (all of HLB = 16), or increasing hydrophilicity among S-370, S-970, S-1670 having the same alkyl chain length (C18), larger vesicles were obtained consequently.

Upon variation of amounts of proniosomal ingredients, the following effects on vesicle size were observed. Increasing surfactant/lipid ratio led to an increase in vesicle size which was substantiated by the increase in the overall degree of hydrophilicity since SE L-1965 used in this study is hydrophilic in nature with HLB value of 16. The opposite held true with increasing the cholesterol amount which was associated with a decrease in the hydrophilicity of bilayers, thus limiting the water intake to the vesicles core and resulted in a subsequent decrease in mean vesicle size. Also, one can accept the increase in mean vesicle size by increasing lecithin content if we considered the long hydrocarbon chain of lecithin molecules (18 °C).

Finally, the increase in mean vesicle size with increasing drug load was attributed to the drug entrapped in the hydrophobic domain of the vesicle, causing the bilayer molecules to become apart from each other leading to an increase in vesicle size [49].

3.3. Scanning electron microscopy

Scanning electron micrographs revealed the formation of well-identified spherical niosomal vesicles with sharp boundaries after hydration of proniosomes (Fig. 2). The surface characteristics of proniosomes-derived niosomes of F5 and F14 containing 20 and 30 mg of cholesterol, respectively, have shown an increased vesicle wall rigidity with increasing cholesterol content (Fig. 2b).

3.4. Differential scanning calorimetry (DSC)

DSC thermograms of vinpocetine, sugar ester S370, empty and loaded niosomes are illustrated in Fig. 3. Vinpocetine, sugar ester S-370 showed endotherms at 149.3, 50.14 and 64.12 °C, respectively, corresponding to their melting temperatures. The dual melting points of SE S-370 confirmed the fact that it is a mixture of mono to pentaesters of stearic acid and sucrose, thus melting does not take place at a single temperature [50].

DSC thermogram of drug-free niosomes showed the appearance of a new sharp endothermic peak at 80.74 °C indicating the interaction between the molecules of sugar ester, cholesterol and lecithin and the formation of the double layer structure of the vesicle [49]. However, thermogram of vinpocetine loaded niosomes revealed a disappearance of the characteristic endothermic

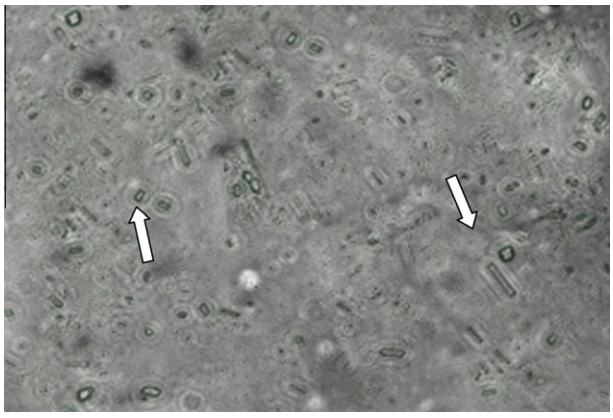

Fig. 1. Optical micrograph of niosomal dispersion of F18 showing insoluble drug crystals (40×).

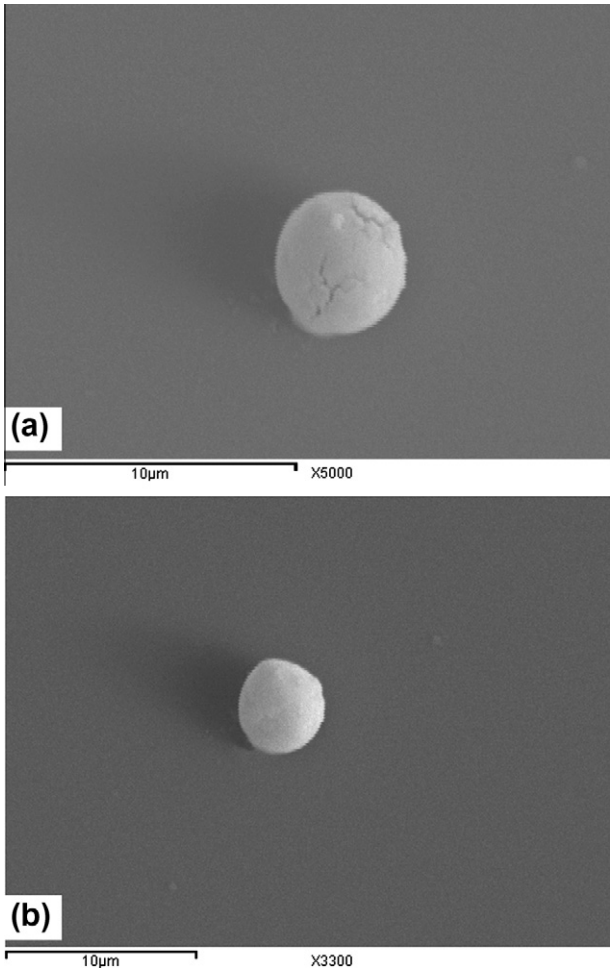

Fig. 2. Scanning electron micrographs of niosomes formed after hydration of (a) F5 containing 20 mg cholesterol and (b) F14 containing 30 mg cholesterol (Scale bar indicates 10 μm).

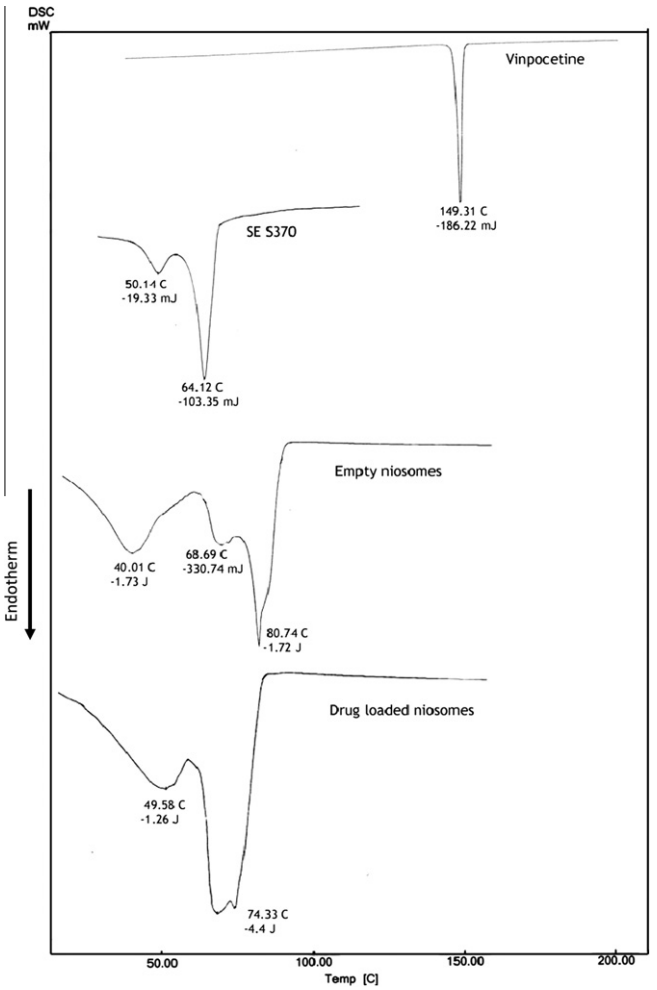

Fig. 3. DSC thermograms of vinpocetine, SE S-370 (F8), empty and drug loaded niosomes.

vinpocetine peak, and the endotherm of the niosomal bilayer was shifted from 80.74 to a very broad wide peak at 74.33 °C. These results suggest the dispersion and entrapment of vinpocetine into the bilayers of niosomal vesicles.

It should be pointed out that, despite the presence of cholesterol which was reported to abolish the gel to liquid phase transition [12], yet endothermic peaks are still present. This is acceptable if we consider low mol% of cholesterol used in this study (4–12%) which is much below the necessary concentration reported by Cable et al. [51] to abolish the gel to liquid phase transition.

3.5. In vitro release

Results of vinpocetine in vitro release from different formulae are illustrated in Fig. 4. It was apparent that the incorporation of vinpocetine in all proniosomal formulations led to significant slower release profiles ($p < 0.01$) compared to its alcoholic solution where 98% was released in 4 h. The kinetic analysis of all release profiles followed diffusion controlled mechanism with an initial relative fast release phase followed by a slower release one. The initial phase was due to the desorption of vinpocetine from the surface of niosomes while the drug release in the slower phase was regulated by diffusion through the swollen niosomal bilayers [52]. This profile could be advantageous if we considered the importance of epidermis saturation with initial fast drug released to achieve high concentration gradient required for successful drug delivery to the blood [17].

It is rather important to mention the inverse relation between the entrapment efficiency and the drug release. Entrapment efficiency is a measure of the vesicle ability to retain the drug; thus, the more the drug is retained in the vesicle, the slower the release profile will be [37]. Accordingly, factors that stabilize the vesicle membrane and increase the entrapment efficiency of a hydrophobic drug such as vinpocetine (namely, low HLB, long alkyl chain, high phase transition temperature) will slow down the release profile.

The general features of the release profile of the proniosome-derived niosomes prepared using conventional surfactants revealed significant increase ($p < 0.01$) in the percentage drug released with the increase in HLB since hydrophilic surfactants have higher solubilizing power on hydrophobic solutes in aqueous medium compared to hydrophobic surfactants [53]. (Tween 20 (F3) > Tween 80 (F4) > Span 20 (F1) > Span 80 (F2).)

It was interesting to note that sugar esters-based niosomes exhibited a fatty acid alkyl chain length dependent release, where sugar esters of short alkyl chain (F5: laurate, F6: Myristate, F7: Palmitate) increased the drug release more than stearate sugar esters of longer alkyl chain (F8, F9, F10). This pattern was also confirmed by insignificant difference ($p > 0.05$) in the percentages of drug released from stearate sugar esters having the same alkyl chain length and different HLB (S-370; HLB = 4, S-970; HLB = 9, S-1670; HLB = 16). A possible explanation of the obtained results is the ability of smaller fatty acid SEs to be built more completely into polymeric chains, thus creating open holes in the bilayers resulting in higher permeability and faster drug release [17]. Therefore,

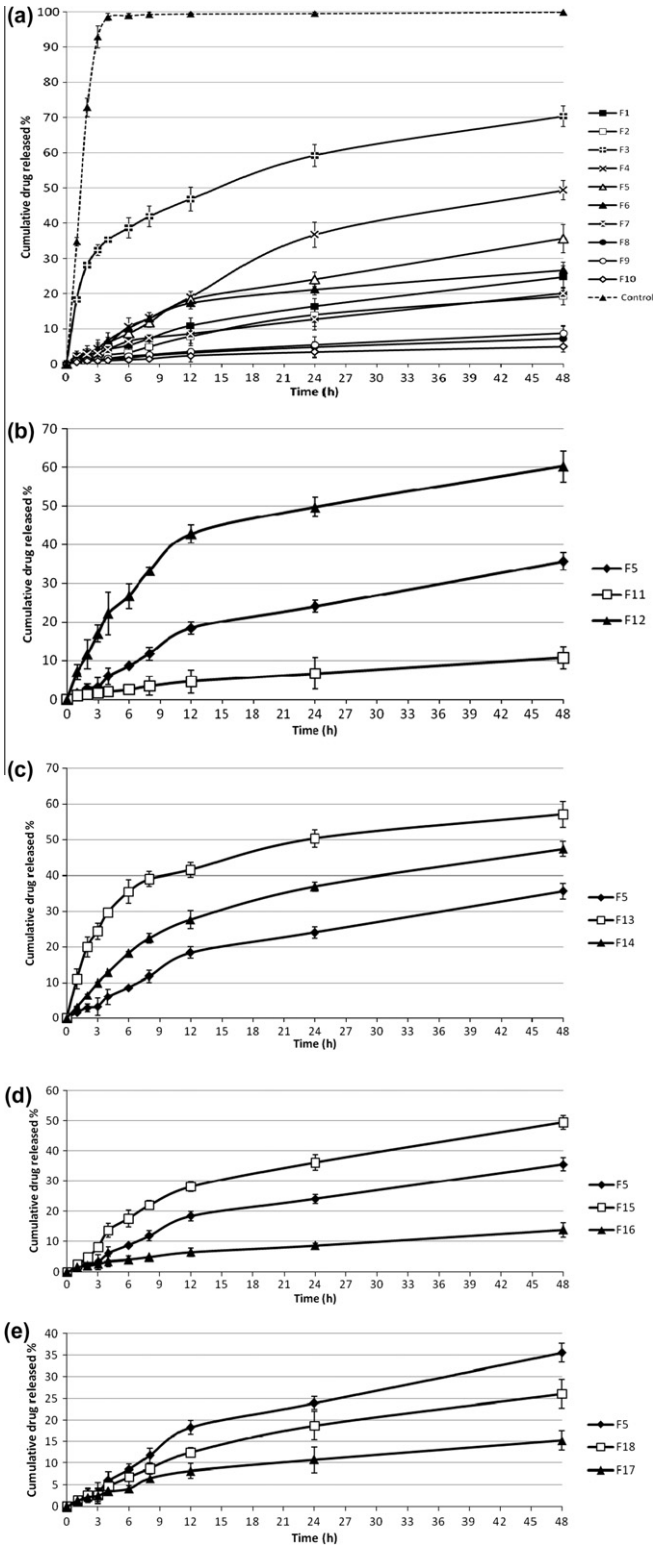

Fig. 4. (a) In vitro drug release from control and proniosomes prepared using different non-ionic surfactants. (b) In vitro drug release from proniosomes prepared using different amounts of SE L-1965. (c) In vitro drug release from proniosomes prepared using different cholesterol amounts. (d) In vitro drug release from proniosomes prepared using different lecithin amounts. (e) In vitro drug release from proniosomes prepared using different drug loads (All points represents mean ± SD, $n = 3$).

among sugar esters, F5 containing SE L-1965 with best release profile and satisfying EE% (85.37 ± 0.16) was chosen to progress to optimization studies.

Increasing the cholesterol amount from 10 to 20 mg and lecithin content from 90 to 270 mg resulted in a more intact lipid bilayer as a barrier for drug release which decreased its leakage and permeability, hindered the release of entrapped drug from the vesicles and led to a significant slow release profile ($p < 0.05$). [22,38]. However, further increase in cholesterol amount from 20 to 30 mg led to a significant increase in the released drug % after 48 h.

On the other hand, increasing the amount of the hydrophilic SE L-1965 from 180 to 270 mg was associated with higher drug release ($p < 0.05$) due to its high solubilizing power on the hydrophobic vinpocetine in aqueous medium [53]. In addition, the best release profile among different drug loads was observed upon using 5 mg vinpocetine.

Therefore, based on in vitro characterizations, two optimized formulae F19 and F20 based on Tween 20 as traditional non-ionic surfactant and SE L-1965, respectively, were further progressed to in vitro skin permeation (Table 2). The amount of surfactant was set to 270 mg to ensure the highest dissolution rate and the highest entrapment efficiency as well, low level of cholesterol and lecithin (10 mg and 90 mg respectively) were selected for optimal membrane stability and maximum release of entrapped drug. Five milligrams of vinpocetine was loaded which was optimally entrapped and released.

3.6. Ex vivo permeation

Results of ex vivo permeation studies upon using newly born rat skin are illustrated in Fig. 5 and Table 3. It is clear that although skin had some control on vinpocetine transdermal delivery and its release was slowed down by approximately 17–21% compared to its direct release to buffer solution in sink condition, yet the extent of drug permeation through the skin from both tested proniosomal formulations F19, F20 was still high with improved skin permeability. It should be noted that though newly born rat skin was not a precise model for human skin for percutaneous absorption studies, it could be used to gain insight about the general pattern and mechanisms. The absence of hair on the skin would avoid introduction of artifacts due to the methods used for hair removal in regular rats [30].

Interestingly, no lag phase could be detected and within one hour (minimum sampling time) vinpocetine could be detected in

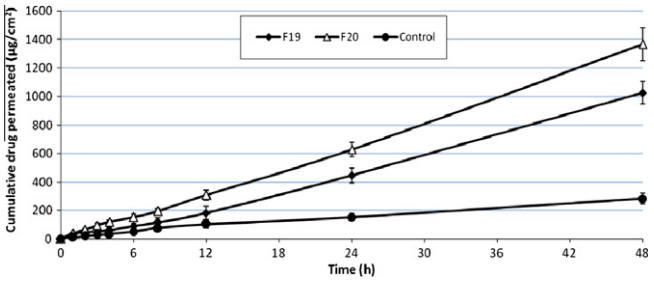

Fig. 5. Cumulative amount of vinpocetine permeated through rat skin (µg/cm^2).

Table 3
Permeation parameters of vinpocetine through mouse skin.

	J	P_{app}	ER
F19	22.67 ± 1.12	4.53 ± 1.26	4.32 ± 0.36
F20	29.02 ± 1.82	5.8 ± 1.33	5.52 ± 0.51
F20 (non-occlusive)	17.56 ± 1.92	3.51 ± 1.41	3.34 ± 0.86
Control	5.25 ± 0.74	1.05 ± 0.14	0

J: steady state flux (µg/cm^2/h); P_{app}: apparent permeability coefficients (cm/h × 10^{-3}); ER: enhancement ratio.

the receptor medium indicating that all processes (water permeation from the receptor compartment to the skin, proniosomes conversion to niosomes, vinpocetine release from the reconstituted niosomes and its permeation) took place very rapidly. Similar observations were previously reported by Vora et al. [14]. The proposed mechanism for improved permeability from F19 (Tween 20) and F20 (SE L-1965) based proniosomes may involve disruption of the densely packed lipids that fill the extra cellular spaces of the stratum corneum. This may be substantiated by the evidence that both Tween 20 and SE L-1965 contain a lauryl hydrocarbon chain of 12 carbon lengths which reported to be the most effective chain length to disrupt ceramide–cholesterol or cholesterol–cholesterol interactions, as this chain length corresponds to the chain length of the steroid nucleus of cholesterol [30,54].

Furthermore, SE L-1965 produced significant higher flux ($p < 0.05$) compared to Tween 20. In this sense, and based on prior report of ability of SE L-1965 to preferentially improve the skin permeation of ionized species [55], a postulation was made that overall flux of F20 was due to the permeation of both ionized and unionized species while F19 based on Tween 20 improved the permeation of the unionized species mainly. In order to verify this assumption, the permeability coefficients of unionized (P_{un}) and ionized (P_i) forms was calculated adopting the approach of Cazares-Delgadilloratio et al. [55]. The apparent permeation coefficient (P_{app}) was determined for F19 and F20 in three different pH, namely, 5, 7.4 and 9. P_{un} and P_i are, respectively, the slope and the intercept of the linear relationship between P_{app}/f_i versus f_{un}/f_i.

Mathematically, the fraction of unionized species for basic drugs is calculated as follows [55]:

$$f_{un} = 1/\left(1 + 10^{pKa-pH}\right)$$

The obtained $P_{app\text{-}F19}/f_i$ and $P_{app\text{-}F20}/f_i$ were plotted versus f_{un}/f_i. The resulting P_{un}/P_i ratio is illustrated in Fig. 6. Obviously, F19 containing Tween 20 improved the permeability of unionized species 4.6 times more than the ionized one. On the contrary, P_{un}/P_i of F20 containing SE L-1965 was less than one indicating higher permeation enhancement of ionized species over unionized one.

Applying the previous concept to our case, we found that about 55% of vinpocetine was in the unionized form while 45% was in the ionized form; thus, ionized part constitutes a large percentage of vinpocetine at the skin surface and improving its permeability would lead to a significant enhancement of the overall permeation.

The mechanism by which permeation of ionized species could be enhanced was related to the presence of aqueous intercellular micro-channels within the lipid matrix of the skin [55]. These micro-channels are formed by the hydrophilic groups carried on some types of lipids. Ayala-bravo et al. found that SE L-1965 in contact with human skin produces significant stratum corneum lipid extraction and fluidization [56] and therefore, it is expected to produce structural disorder of the intercellular lipids, increasing the volume and number of aqueous micro-channels resulting in a greater penetration of ions [55].

Nevertheless, F19 and F20 supported the permeation of the unionized part of vinpocetine by adsorption and fusion of the formed niosomes onto the surface of skin leading to a high thermodynamic activity gradient of drug at the interface, which is thought to be the main driving force for the permeation of lipophilic species [13]. This effect is signified by the presence of lecithin which is widely known to impart high affinity between the vesicle and skin surface layers [57].

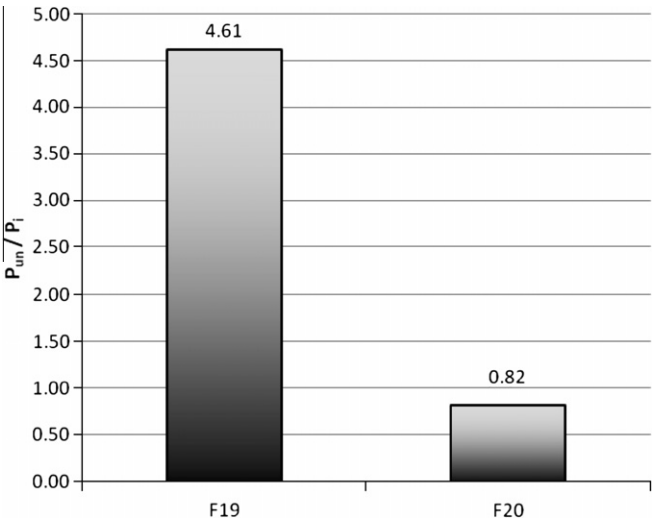

Fig. 6. The effect of using Tween 20 (F19) and SE L-1965 (F20) on the ratio between apparent permeability coeffecient of unionized vinpocetine to the apparent permeability coeffecient of ionized vinpocetine (Pun/Pi).

3.7. Effect of occlusion

Results shown in Table 3 illustrate the effect of occlusion on improving the conversion of proniosomes to niosomes with skin water and the consequent improvement in permeation profile. Enhancement by 66.7% in percutaneous permeation was imparted by occlusive compared to non-occlusive conditions. This result is expected if we consider the increase in the trapped endogenous water, normally diffusing through the stratum corneum and evaporating from the sweat glands (the increase is estimated by 45% in 1 day) under occlusion compared to 15% water contained in the stratum corneum under normal physiological state (non-occlusive). Improvement of percutaneous permeation rate of both hydrophilic and lipophilic compounds by increasing the degree of stratum corneum hydration even without the addition of external penetration enhancers was reported [58].

3.8. Stability studies

Results presented in Table 4 revealed that after storage for 90 days the mean vesicle size as well as drug entrapment efficiency percentage of F19 and F20 were not significantly different ($p > 0.05$) from the same formulae when freshly prepared. The ability of proniosomes-derived niosomes to retain their entrapment efficiency was previously reported by Abd-Elbary et al. [22].

These results suggest that proniosomes offered a more stable system that could minimize the problems reported about conventionally prepared niosomes like degradation by hydrolysis or oxidation, sedimentation, aggregation and fusion during storage.

3.9. Irritation studies

3.9.1. Measurement of skin fold thickening

It is widely known that epidermal injury caused by the irritating materials results in the recruitment of epidermal cells from the

Table 4
Change of the vesicle size and drug entrapment efficiency % (EE%) after storage for 90 days at 2–8 °C (Mean ± SD, $n = 3$).

Storage period	Vesicle size (μm)		EE (%)	
	F19	F20	F19	F20
Freshly prepared	1.62 ± 0.17	1.55 ± 0.26	98.74 ± 0.51	97.93 ± 0.82
90 days	1.81 ± 0.35	1.7 ± 0.32	95.24 ± 0.79	94.74 ± 1.14

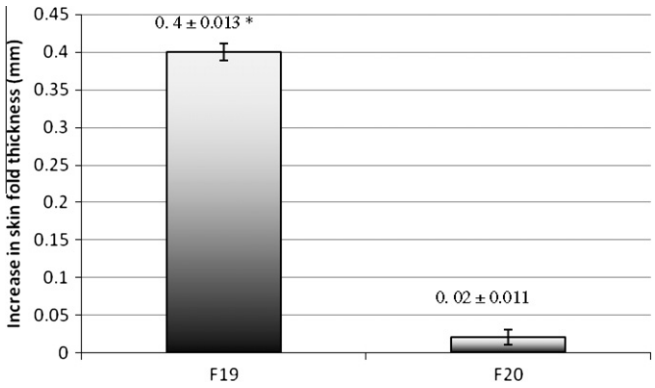

Fig. 7. The increase in skinfold thickness after 48 h of application of F19 and F20 (mean ± SD, $n = 3$). *Significant difference.

quiescent stage into the mitotic cycle by the activation of the phosphoinositol cycle, and the release of inflammatory mediators such as PGE2 will lead to increasing the epidermal proliferation which is manifested by increase in skinfold thickness [59]. As shown in Fig. 7, F20 led to a non-significant thickness increase ($p > 0.01$) compared to epidermis swelling caused by Tween in F19 after 48 h of application. Our results are in accordance with Lerk et al. [32] who reported the complete absence of sucrose laurate irritating effect.

SEs have drawn a worldwide interest as permeation enhancers of reduced irritation potential and due to absence of any type of toxicity, SEs are now approved by Food and Agriculture Organization (FAO), World Health Organization (WHO), in Japan, USA and Europe, as food additives owing to their high safety and excellent properties [60].

3.9.2. Histopathological evaluation

Photograph of normal rabbit skin (control) in Fig. 8a shows a thin epidermal layer and a thin, wavy stratum corneum with a distinct boundary between epidermis and dermis.

As shown in Fig. 8b, the application of SE L-1965-based proniosomes for 7 days led only to a mild hyperkeratosis (thickening of the horny layer) and minute epidermal erosion with no observed signs of inflammation observed. Evident signs of hydration were noticed in both epidermis and dermis with an increase in the water content manifested by moderate increase in the epidermis thickness together with widening of intercellular spaces and marked swelling of connective tissue fibers. Therefore, it was concluded that F20 caused no damage or harmful effects to the rabbit skin after 7 days of daily applications.

On the contrary, the application of F19 revealed certain degree of dermal damage (Fig. 8c) manifested by moderate hyperkeratosis with parakeratosis (immature keratinization that result in the appearance of nucleated cells in the horny layer), acanthosis (loss of intercellular connections resulting in loss of cohesion between keratinocytes) as well as increased neutrophiles and lymphocytes in the dermis (inflammatory cell infiltration).

In conclusion, the different irritation studies have pointed that F20 based on SE L-1965 proved to have a favorable safety profile and is not likely to cause irritation in clinical trials on humans. Thus, F20 was chosen to progress for the next in vivo absorption studies.

3.10. In vivo absorption study

The mean plasma concentration–time curves following the administration of Vinporal® oral tablet and SE proniosomal patch

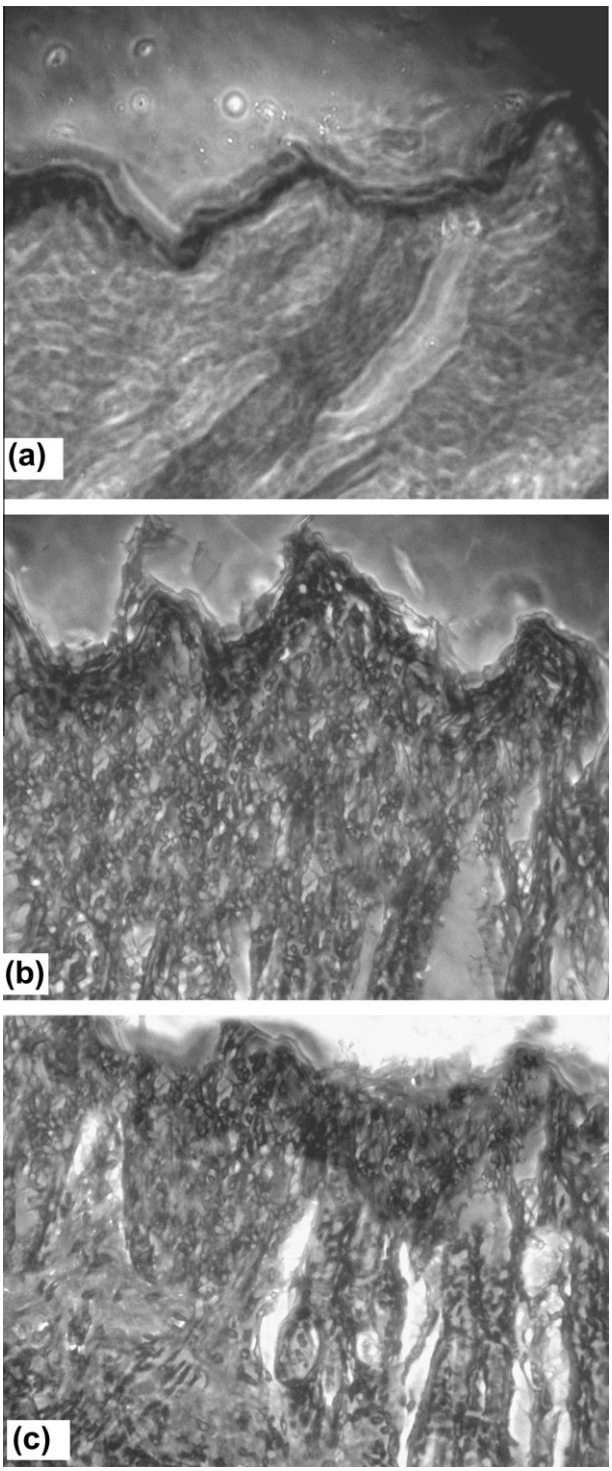

Fig. 8. Light microscopic photographs (100×) of rabbit skin (a) untreated control group (b) treated with F20 for 7 days (c) treated with F19 for 7 days.

both containing 5 mg are shown in Fig. 9. Mean pharmacokinetic parameters are presented in Table 5.

Remarkable differences in the shape of the concentration–time courses between the two treatments were found, expressed by rapid sharp peak of vinpocetine absorption from the oral tablet at 1.5 h followed by a fast decline in plasma drug levels. The value was in good agreement with that obtained by Abd-Elbary et al. [35]. The absorption of vinpocetine from proniosomal patch was much slower and extended over a longer period of time where the patch delivered large fraction of its drug content during the

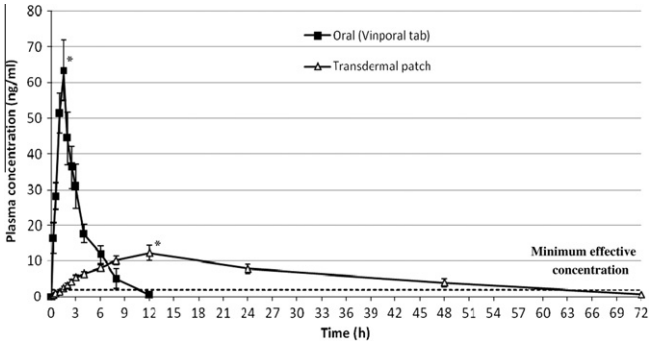

Fig. 9. Plasma concentration–time profile following administration of 5 mg vinpocetine from SE proniosomal patch (F20) and Vinporal® tablet (mean ± SD, $n = 12$). *Significant difference.

Table 5
Summary of mean pharmacokinetic parameters of vinpocetine after administration of 5 mg Vinporal® tablet and SE proniosomal patch to 12 volunteers.

Parameter	Vinporal® tab	SE proniosomal patch
T_{max} (h)	1.46 ± 0.14	11.67 ± 1.15
C_{max} (ng ml^{-1})	63.69 ± 8.32	12.44 ± 1.87
$AUC_{(0-t)}$ (ng h ml^{-1})	201.68 ± 14.39	405.70 ± 52.1
$AUC_{(0-\infty)}$ (ng h ml^{-1})	202.48 ± 14.48	417.70 ± 50.27
$t_{1/2}$ (h)	1.36 ± 0.27	13.94 ± 1.2
k (h^{-1})	0.53 ± 0.12	0.05 ± 0.004

first 12 h of the application period, followed by a slower release phase that extended the presence of vinpocetine in plasma for about 72 h. Moreover, the patch exhibited higher drug levels in the plasma from 6 to 12 h compared to Vinporal® tablet. The average C_{max} was significantly lower ($p < 0.05$) for the patch (12.44 ± 1.87 ng/ml) compared with the oral tablet (63.69 ± 8.32 ng/ml) while t_{max} was significantly higher ($p < 0.05$) in the case of the transdermal patch (≈12 h) compared with the oral tablet (≈1.5 h). The extent of absorption of vinpocetine from the patch expressed by AUC_{0-t} was determined to be about 101% larger and statistically significantly different when compared to the oral tablet. The relative bioavailability (F_{rel}) of vinpocetine SE proniosomal patch to the oral tablet was estimated to be on average 206%. The elimination half-lives of vinpocetine after oral and transdermal administration were 1.36 ± 0.27 h and 13.94 ± 1.2 h, respectively, and were statistically significant ($p < 0.01$).

Since these results were inconsistent with the pharmacokinetic theory in which absorption should not alter elimination, flip flop kinetics which refers to the situation in which the absorption half-life is much longer than the elimination half-life should be considered. The half-life of the decline of drug in the body from the patch now corresponds to the absorption half-life. Because the absorption was so slow such that much of the drug remained to be absorbed well beyond the peak time, the decay phase represented the drug being eliminated as fast as it was absorbed such that absorption is now the rate limiting step. Thus, the decay phase in this situation did not represent the elimination half-life of the drug but the rates of absorption and elimination. Therefore, the elimination half-life for an absorption rate limiting situation cannot be estimated from this situation.

Interestingly, as the minimum effective concentration (MEC) of Vin was reported to be 0.8 ng/ml [61], the SE proniosomal patch was able to maintain effective therapeutic concentration for about 48 h. Thus, one SE proniosomal patch can replace 6 tablets of vinpocetine using the same amount of drug in one tablet only (5 mg). The superior performance of SE proniosomal patch compared to oral tablets was attributed to the success of the patch to deliver vinpocetine transdermally to the general circulation avoiding the extensive first-pass effect that metabolizes 75% of orally ingested vinpocetine [62].

4. Conclusions

Taking into consideration the high efficiency in systemic delivery together with lack of irritancy and excellent safety profile, laurate SE proniosomes could be considered as very promising candidates as absorption and penetration enhancer for delivering Vin transdermally, where one patch containing the same drug load of one commercial tablet, was able to preserve a plasma concentration over the minimum effective concentration for 48 h and would be able to replace 6 commercial tablets improving patient compliance and ensuring better clinical outcomes.

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