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Terbinafine hydrochloride nanovesicular gel: *In vitro* characterization, *ex vivo* permeation and clinical investigation

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ABSTRACT

In this work, nanovesicular chitosan gels were prepared for dermal delivery of terbinafine hydrochloride (TBN HCl). Ethosomes and vesicles containing different types of penetration enhancers (PEs) *viz.* Terpenes (cineole and limonene), labrasol and transcutol were developed. The prepared vesicles were evaluated for physical characteristics as well as skin interaction. The selected vesicles were incorporated into chitosan gel. An *in vivo* animal study was done on rat induced superficial *Candida* infection model. Moreover, randomized double blind clinical study was done on patients to compare the effect of the selected nanovesicular gel against the market product. Results showed the formation of nearly spherical, mostly deformable vesicular systems with size range of 95.5–530 nm, zeta potential range of −0.1 to 15 mV and entrapment efficiency range of 20–96.7%. Penetration enhancer vesicles (PEVs) prepared with 4% limonene (ELI4) showed the highest percent of drug deposition in the skin (53%) and the highest local accumulation efficiency value (35.3). *In vivo* animal study showed that the lowest fungal burden produced with ELI4 chitosan gel. Clinical studies showed cure rate of 86% within 7 days treatment in case of limonene nanovesicular gel compared to 20% for market product (Lamisil® cream).

1. Introduction

Skin fungal infections have increased rapidly. Nowadays about 40 million people are affected with dermal fungal infections around the world. Many types of fungi are the causative agents of such infections like *Candida albicans* species, Dermatophytes and Tinea species. A wide variety of antifungal agents are available for either topical or systemic use. However, their use is associated with prolonged duration of treatment, low therapeutic value, local and systemic side effects. The main two obstacles in the treatment of skin fungal infections are the skin barrier nature against drug molecules and the low penetration power deeper into skin layers (Akhtar et al., 2015).

TBN HCl is an antifungal drug with a broad activity against wide spectrum of fungal types and species like Dermatophytes, *Aspergillus* species, *Candida* species and *Pityrosporum* yeasts. It is slightly soluble or very slightly soluble in water (Pharmacopoeia, 2009). The base, TBN, has octanol/water partition coefficient (log *p* value) of 3.3 and as a consequence, log D (which is logarithm of distribution coefficient of the salt) is dependent on the pH of the medium. The pKa value of TBN base is 7.1 while the molecular weight of TBN HCl is 327.90 Da (Sachdeva et al., 2010a). It is highly soluble in organic solvents as methanol and slightly soluble in acetone (Pharmacopoeia, 2009). It can be used either in the form of topical or oral formulations. Systemic treatment is always required for heavy cutaneous infection or nail infection with long period of treatment. In order to keep the drug above the minimum inhibitory concentrations during the entire treatment period, oral administration of large doses for prolonged periods, 2–6 weeks for skin infections, is necessary. In addition, oral administration has been shown to be associated with drug-drug interactions, hepatotoxicity, gastrointestinal and systemic side effects, lactose intolerance and other adverse effects. Hence an improved topical drug delivery approach could overcome these limitations as it provides immediate access to the site of infection and reduces unwanted systemic drug exposure. However; a major limitation of topical delivery for skin infections is the poor skin bioavailability.

Lipid based vesicles were studied in order to maximize the skin penetration ability of active agents. They are considered safe and non irritant with good patient compliance. However, early generations of topically applied vesicles were of poor skin penetration ability. This necessities the development of newer generations with higher skin penetration ability (Romero and Morilla, 2013). In this work, ethosomes and vesicles containing different types of PEs *viz.* Terpenes (cineole and limonene), labrasol and transcutol were developed in order to enhance dermal delivery of TBN HCl. The prepared vesicles were evaluated for physical characteristics as well as skin interaction. Nanovesicular chitosan gel was developed and *in vivo* animal study was done on rat induced superficial *Candida* infection model. Moreover, randomized double

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blind clinical study was done to compare the effect of the nanovesicular gel against the market product, Lamisil® cream.

2. Materials and methods

2.1. Materials

TBN HCl was a kind gift from Novartis, Cairo, Egypt. Soya phosphatidylcholine 99% (Epikuron 200) was supplied by Degussa Texturant Systems, Deutschland, Hamburg, Germany. Transcutol and labrasol were kind gifts from Gattefosse, Nanterre, France. Di-potassium hydrogen phosphate and Sabouraud dextrose agar were purchased from El-Nasr Pharmaceutical Co. For Chemicals, Cairo, Egypt. Limonene, cineole, Tween 80®, chloroform HPLC, ethanol HPLC, and ortho-phosphoric acid were obtained from Sigma chemical Co., St. Louis, USA. Acetonitrile HPLC and methanol HPLC were purchased from Fisher Scientific, Massachusetts, USA. Chitosan (low molecular weight, deacylation above 85%) was purchased from Euromedex, soufflé weyershiem, France. Lamisil® cream was purchased from Novartis, Cairo, Egypt.

2.2. Preparation of vesicles

Ethosomes (ETH) were prepared by classic mechanical dispersion method. Briefly, phospholipid (Epikuron 200, 200 mg) and TBN HCL (37.5 mg) were dissolved in 10 ml chloroform and subjected to complete evaporation of the solvent by rotary evaporator using deep vacuum (IKA Laboratories, Staufen, Germany). The thin lipid film was then hydrated by water-ethanol mixture solution (30% v/v) and vortexed to get ETH. The formulation (5 ml) was then homogenized at 15,000 min^{-1} for 5 min using high shear homogenizer (Ultra Turrax T25, IKA, Deutschland, Germany) to get uniform vesicles (López-Pinto et al., 2005). Liposomes (LIPO) were also prepared without addition of ethanol as a control. Moreover, PEVs containing labrasol, transcutol, limonene or cineole were prepared using the same method of preparation of ETH by adding PEs at low and high levels (4 and 10% w/v, respectively (0.2 and 0.5 g/5 ml, respectively)). PEs were added either to chloroform or hydro-alcoholic solution according to their solubility, so labrasol and transcutol were added to the hydro-alcoholic solution while limonene and cineole were added to the organic solvent. The formulation (5 ml) was then homogenized at 15,000 min^{-1} for 5 min using high shear homogenizer to get uniform vesicles.

2.3. HPLC determination of the drug

Drug detection was performed using (Agilent HPLC, 1200 series, California, USA) system with auto sampler. A C18 column (reversed-phase analytical HPLC columns, 5 μm PS, silica, length of 250 mm; an internal diameter of 2.1 mm, Thermo Scientific, Massachusetts, USA) was used as a stationary phase. The mobile phase consisted of acetonitrile: methanol:0.01 M di-potassium hydrogen phosphate (50:30:20 v/v) with pH adjusted to 7.4 by few drops ortho-phosphoric acid and the flow rate was adjusted to 1 ml/min. Column temperature was 35 °C and detection wavelength was 224 nm. This method was validated according to ICH guidelines for linearity, specificity accuracy, robustness and precision. The limit of detection (LOD) and limit of quantification (LOQ) were also calculated. The retention time was about 15 min. Calibration curve was constructed in a range of 0.1–40 μg/ml with correlation coefficient $r^2 = 0.9999$. The LOD and LOQ were 0.07 μg/ml and 0.2 μg/ml, respectively. Accuracy was ensured with relative standard deviation (RSD%) less than 2%.

2.4. Characterization of the prepared vesicles

2.4.1. Determination of particle size and polydispersity index

The particle size (PS) and polydispersity index (PDI) of the prepared ethosomes and PEVs were determined through dynamic light scattering (DLS) technique using Zetasizer Nano ZS (Malvern instruments, UK). The DLS yielded the mean diameter of the main population and the PDI. Samples were measured at 25 °C using disposable polystyrene cells in triplicates.

2.4.2. Measurement of zeta potential

The surface charge of vesicles was determined by the measurement of the zeta potential (ZP) calculated from their electrophoretic mobility by Malvern Zetasizer using disposable plain folded capillary zeta cells. All measurements were performed in triplicate of different batches.

2.4.3. Determination of entrapment efficiency percent

Entrapment efficiency percent (EE%) was determined by centrifugation method (Bendas and Tadros, 2007) with slight modification. 100 μl of each formula were diluted with 5 ml hydro-alcoholic solution and then centrifuged at 21,380 × *g* for 30 min at 4 °C using cooling centrifuge (Model Z216 MK, Hermle, Gosheim, Germany). The EE% was calculated according to the following equation:

$$EE\% = \left(\frac{Q_t - Q_s}{Q_t}\right) * 100$$

where Q_t is the theoretical amount of TBN HCl added, and Q_s is the amount of TBN HCl detected in the supernatant. In spite of limitations of the indirect method of determination of EE% (Xu et al., 2006), it was the feasible way to determine the amount of the entrapped drug.

2.4.4. Deformability index measurement

Measurement of deformability of vesicles was carried out by extrusion measurement through locally fabricated stainless steel pressure filter holder (El Zaafarany et al., 2010). Polycarbonate filters of definite pore size (Nucleopore®, 50 nm) were used and vesicles were extruded through them at constant pressure of 0.17 MPa. Deformability index (DI) was calculated according to the following equation (Salama et al., 2012):

$$DI = j/t\,(rv/rp)^2$$

where DI is the deformability index (ml/s), j is the amount of suspension extruded (ml), t is the extrusion time (s), rv is the vesicles size after extrusion (nm), rp is the filter membrane pore size (nm). The higher the DI value the greater the elasticity of the vesicles.

2.4.5. Differential scanning calorimetry studies

The thermal properties of pure TBN HCl, ETH, LIPO and representative PEVs (ET4) were investigated using differential scanning calorimetry (DSC). Samples (3–5 mg) were heated at a rate of 10 °C/min. to a temperature of 350 °C, using dry nitrogen as carrier gas with a flow rate of 25 ml/min.

2.5. Ex vivo studies

2.5.1. Skin preparation

The protocol for the *ex vivo* studies was reviewed and approved by the Ethical Committee, Faculty of Pharmacy, Ain Shams University. Albino rats (6–8 weeks old, 100–125 g) were supplied by Nile Pharmaceuticals and Chemical Industries, El Sawah Sq., Al Ameria, Cairo, Egypt. Albino rats were kept under standard laboratory conditions in 12 h light/dark cycle at 25 ± 2 °C. Rats were euthanized and the abdominal skin of each rat was excised using scissors and forceps. Hair on the skin of animals was removed using electrical clippers and then the subcutaneous tissues were surgically removed. Isopropyl alcohol was used to remove residual adhering fat of the dermis then the skin was cut into appropriate size. The skin was washed with de-ionized water then wrapped in aluminum foil and stored in a deep freezer at −20 °C until use within 2 weeks (Ahad et al., 2012).