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Research Article

Biopolymer-Based Transdermal Films of Donepezil as an Alternative Delivery Approach in Alzheimer's Disease Treatment

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Abstract. Matrix type transdermal films of donepezil (DNP) as an alternative delivery approach was designed to improve patient compliance to Alzheimer disease treatment. Sodium alginate, a natural polysaccharide, was used as matrix-forming agent in the optimization of transdermal films. Propylene glycol and *dl*-limonene was added into films as a plasticizer and permeation enhancer, respectively. As well as mechanical strength and bioadhesiveness of optimized transdermal films of DNP, the impact of *dl*-limonene concentration in films on DNP *in vitro* permeation across pig skin was assessed. Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) measurements were carried out to examine the effects of enhancer on *in vitro* conformational order of the *stratum corneum* intercellular lipids following permeation study. Results showed that transdermal formulations of DNP were suitable due to both mechanical and bioadhesive features of the films. *In vitro* skin permeation study indicated that *dl*-limonene at a concentration of 3% was optimum with high drug flux. ATR-FTIR results confirmed a more fluidized *stratum corneum* lipid state in the presence of *dl*-limonene, indicating its permeation enhancement effect. Regarding to achieve therapeutic levels of DNP, it seems to be feasible deliver DNP with transdermal films for the management of Alzheimer disease.

KEY WORDS: Alzheimer disease; donepezil; limonene; permeation enhancement; transdermal film.

INTRODUCTION

Alzheimer's disease has become an important health problem as a result of increased elderly population worldwide. This disease is characterized by the deficits in the cholinergic system and deposition of beta amyloid in the form of neurofibrillary tangles and amyloid plaques (1). Thus, cholinesterase inhibitors which are the first-class medicines used in the treatment of Alzheimer's disease have been attracted attention (2). Donepezil (DNP) is one of the reversible *acetylcholinesterase* inhibitors (3). Currently, it has been widely used *via* oral route in Alzheimer's disease. However, the oral administration of DNP has several limitations such as large fluctuations in plasma concentration levels associated with its high incidence of gastrointestinal side effects. In addition, Alzheimer patients have problems in continuing medication effectively and reliably due to suffering from dementia. Elderly patients have also difficulties to swallow dosage forms in oral treatment (4,5). Therefore, it is a challenge to develop novel dosage forms of drugs used in Alzheimer's disease to increase patient compliance.

Transdermal delivery offers a variety of superiorities over oral conventional dosage forms, including sustained drug release directly to the blood stream over a long period of time, bypassing of the gastrointestinal and hepatic elimination pathways, improved patient compliance and reduced gastrointestinal side effects (6). The success of a transdermal drug delivery system depends on the achievement of the drug to penetrate the skin in sufficient quantities to maintain therapeutic levels (7). However, *stratum corneum*, the outermost layer of skin, is an effective barrier for delivering of drugs. Mostly, penetration enhancers those reversibly altering its barrier function are included into transdermal formulations to overcome that limitation and to facilitate delivery of drugs across skin (8).

Terpenes are reported to be very safe and effective penetration enhancers for both lipophilic and hydrophilic drugs permeating human skin. The United States Food and Drug Administration (FDA) has classified these compounds as generally regarded as safe (GRAS) (8,9). The effects of terpenes as penetration enhancers are primarily related to their chemical structure as well as the physicochemical properties (10). The mechanism of permeation enhancing activity of terpenes have been attributed to increment of the drug partition into the *stratum corneum* and creating perturbation on the intercellular packing of the *stratum corneum* lipids (10,11). Limonene is a lipophilic hydrocarbon terpene (logP: 4.58), obtained from the lemon peel of *Citrus lemon* (12,13).

The choice of polymers used in transdermal films has also great impact on drug release and permeability as well as the elasticity and wearing properties of the formulations. Biopolymers obtained from natural sources, such as sodium alginate,

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chitosan, and pectin have been widely used in the optimization of matrix type of transdermal films (14). Sodium alginate is one of the biopolymers which plays a fundamental role in controlling the mechanism and rate of drug release from a dosage form. It is a natural polysaccharide, consisting chiefly of the sodium salt of alginic acid. Sodium alginate is generally regarded as a nontoxic and nonirritant material and is GRAS listed (15). Previously, the transdermal films in which sodium alginate was used as matrix polymer have shown good characteristics in terms of mechanical strength, flexibility, and bioadhesiveness of the films (16,17).

The aim of the present study was to design transdermal films of DNP composed of sodium alginate and *dl*-limonene, as polymer and permeation enhancer, respectively. In this perspective, the physicochemical, mechanical and bioadhesive properties of the developed film formulations were examined and the impact of *dl*-limonene on *in vitro* skin permeation of DNP was assessed. Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopic analysis on pig skin following administration of transdermal films during permeation studies were also performed in order to evaluate the effect of *dl*-limonene on skin barrier properties, *in vitro*.

MATERIALS AND METHODS

Materials

DNP was kindly provided from Santa Farma Drug Company (Turkey). Acetonitrile, *dl*-limonene, and Tween® 80 were supplied from Merck (Germany). Sodium alginate (medium viscosity, from brown algae) was purchased from Sigma (USA). All other reagents were used without additional purification.

Methods

Formulation of Sodium Alginate-Based DNP Transdermal Films

The codes and composition of transdermal films of DNP are given in Table I. The concentrations of the biopolymer as matrix agent (sodium alginate), plasticizer (propylene glycol), and the penetration enhancer (*dl*-limonene) were optimized based on data obtained from preformulation studies (data is not given).

Table I. The Codes and Composition of Transdermal Films of DNP (*w*/*w*, % on wet basis)

Compositions	TF-LM1	TF-LM3	TF-LM5	TF-CONT
Sodium alginate	4.5	4.5	4.5	4.5
Propylene glycol	7.5	7.5	7.5	7.5
Limonene	1.0	3.0	5.0	-
Tween® 80	1.0	1.0	1.0	1.0
Distilled water	86	84	82	87
DNP				
% *w*/*w*[a]	1.0	1.0	1.0	1.0
mg/cm^2[b]	3.3	3.3	3.3	3.3

TF-LM1, *TF-LM3*, *TF-LM5* transdermal formulations containing limonene as enhancer, *TF-CONT* control formulation
[a] On wet basis, amount of gel prepared=25 g per batch
[b] As a base finished product

In order to prepare transdermal films, firstly, sodium alginate was dispersed in water for 24 h. The mixture of Tween® 80 (1%) and *dl*-limonene (1, 3, or 5%) was homogenized in water at 9000 rpm for 5 min. Aqueous solution of DNP was added into the former mixture to form an emulsion. This emulsion was added to the sodium alginate gel base. As the last step, propylene glycol was added into the mixture as a plasticizer agent and stirred continuously until a homogeneous gel was obtained. The transdermal films of DNP were prepared by casting the gel dispersions on Petri dishes (θ, 9.8 cm) followed by drying at 40±2°C for 20 h. All transdermal formulations (TF-LM1, TF-LM3, and TF-LM5) were prepared freshly before *in vitro* permeation studies (max. 24 h). Dried films were stored in a desiccator wrapped in aluminum foil. A control formulation (TF-CONT) was also prepared following the same procedure and components without adding *dl*-limonene in order to assess the efficiency of the penetration enhancer.

Physicochemical and Mechanical Characterization of DNP Transdermal Films

Organoleptic Examination. Transdermal films of DNP were evaluated by visual inspection in terms of color, transparency, smoothness, flexibility, and homogeneity.

Uniformity of Weight. The uniformity of weight for each formulation was calculated in six pieces of 1.77 cm^2 film by calculating their average weight, and the deviation from average weight was determined.

Thickness. Film thickness of DNP transdermal films was measured with a handheld micrometer (QLR digit, IP4, PRC), and six replicates were taken on each transdermal film in different places. Mean values and standard deviations were calculated.

Uniformity of Drug Content. A known weight of film was dissolved and subsequently diluted with 0.9% (*w*/*w*) saline solution. Samples were filtered through membrane filters (0.45 μM, Millex LH) prior to HPLC analysis. Each film formulation was tested in triplicate and the results were expressed as the mean and standard deviation.

Elasticity. Elongation at break (%) indicates the flexibility of films and it has been defined as the ratio between the extension of the film at the point of rupture and the initial length of the sample (18). Elongation testing of sodium alginate-based transdermal films of DNP (TF-CONT, TF-LM1, TF-LM3, and TF-LM5) was performed using texture profile analyzer (TA-XT Plus, Stable Micro Systems, Surrey, UK) equipped with a 5-kg load cell. Rectangular film samples (10×80 mm) were clamped between the tensile grips of the texture analyzer and then the grips moved apart at a speed of 0.5 mm/s. The force and displacement were recorded. Studies were

performed in triplicate. The elongation at break was calculated using the following (Eq. 1):

$$\%\text{Elongation at break} = \left[\text{Increase in length at breaking point(mm)} \Big/ \text{Initial length(mm)}\right] \times 100 \quad (1)$$

In Vitro Bioadhesion Character. The bioadhesive strength of sodium alginate-based transdermal films of DNP (TF-CONT, TF-LM1, TF-LM3 and TF-LM5) was evaluated using the previously improved method (19). The measurement was performed using texture profile analyzer (TA-XT Plus, Stable Micro Systems, Surrey, UK) equipped with a 5-kg load cell and a bioadhesion test rig. Pig skin was utilized as the *in vitro* model membrane (thickness, 0.750 μm). The skin was allowed to hydrate with 50 μL 0.9% saline solution at 37±2°C prior to the experiment and then fitted on the bioadhesion test rig. A circular film sample (θ=1 cm) attached to the probe (Lucite International Ltd., Queens Gate, UK) with a double side adhesive tape (Tesa Verlegeband 05696–00010, Beiersdorf AG D-Hamburg). The probe was lowered onto the surface of the skin with a constant speed of 1 mm/s and the contact force of 2.5 N was applied. After keeping in contact for 60 s, the probe was moved upwards vertically at a constant speed of 1 mm/s. The work of adhesion (mJ/cm^2) was calculated from the area under the force-distance plot using Texture Exponent software package of the instrument (Eq. 2). Each experiment was carried out three times.

$$\text{Work of bioadhesion } \left(\text{mJ/cm}^2\right) = \text{AUC}_{1-2/}\pi r^2 \quad (2)$$

where πr^2=surface area of the membrane surface that is in contact with transdermal film formulations and AUC_{1-2}=area under the force-distance plot

In Vitro Skin Permeation of DNP from Transdermal Films

Skin Preparation. Full thickness skin was removed from the dorsal side of the freshly excised pig and used immediately. On the same day of the experiment, skin was freed from visible hair with scissors and dermatomed to a thickness of 750 μm (Zimmer Dermatome, Swindon, UK).

Permeation Experiments. In vitro skin permeation of DNP from transdermal films (TF-CONT, TF-LM1, TF-LM3, and TF-LM5) was investigated using Franz type diffusion cells (Permegear, Hellertown, USA) with an available diffusion area of 1.77 cm^2 and receptor volume of 12 mL. Phosphate buffered saline solution (pH 6.5) was used as receptor medium to maintain sink conditions in the receiver compartment. Sodium azide was added into receptor to prevent microbiological contamination. The receptor fluid was magnetically stirred at 500 rpm and thermostated at 37±1°C throughout the experiments. Skin was equilibrated in 0.9% saline solution for 30 min. and then mounted on diffusion cells. Transdermal film formulations were wetted with 200 μL of deionized water and mounted onto the *stratum corneum* side of the skin. The cells were occluded with Parafilm^{TM} (USA). One milliliter of aliquots was collected from the receptor at designed time intervals over 72 h and replaced by the same volume fresh medium to maintain a constant volume. The amount of DNP transported was determined by HPLC. Permeation experiments were conducted at least three times.

The flux, J (μg/cm^2 h^{-1}) of DNP was calculated from the slope of the plot of the cumulative corrected amount of DNP permeated per square centimeter of skin at steady state against time using linear regression analysis. The lag time (h) was estimated by extrapolating the linear part of the profile of cumulative drug amount *versus* time plot.

HPLC Analysis. The quantitative determination of DNP in this study was performed using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with a SPD-M20A UV/Vis detector, a LC 20A pump and a CN column (125 mm× 4.0 mm; 5 μm, Waters Symmetry). The mobile phase was a mixture of acetonitrile:triethylamine buffer solution (20 mM, pH 2.5) (25:75 *v/v*) filtered through membrane filters (0.45 μM, Millex LH) and eluted at a flow rate of 1 mL/min. Analyses were performed using a UV detection wavelength of 270 nm. The method was validated for selectivity, linearity, accuracy, and precision. It was found to be linear over the concentration range 0.2–10 μg/mL with a high correlation coefficient (r^2>0.999) and precise (intra- and interday variation, RSD <2%) and accurate (recovery >99%). There were no interfering peaks with DNP confirming the selectivity of the method. Stability studies showed that DNP was stable during 48 h in the mobile phase.

ATR-FTIR Spectroscopy Analysis

Following skin permeation experiments, the excess of formulation was gently blotted away from the skin surface, the skin was vacuum dried and an infrared spectrum on the treated skin site was recorded using an ATR-FTIR spectrometer (PerkinElmer Spectrum 100 FT-IR Spectrometer, Waltham, MA) in the frequency range 4000–650 cm^{-1} with a spectral resolution of 4 cm^{-1}. The peak positions were assigned using PerkinElmer Spectrum Version 6.0.2 software. Attention was focused on characterizing the occurrence of peaks near 2920 and 2850 cm^{-1} which were due to the asymmetric and symmetric C–H stretching vibrations, respectively. To obtain meaningful and accurate results, a normalization procedure was performed based on the literature (20). In order to minimize intersample variation,

the same piece of skin before treatment was used for normalization.

Statistical Analysis

The significance of the differences between values was assessed using GraphPad Prism Version 5.01 software. One-way ANOVA, followed by the Newman-Keuls multiple comparison tests were performed.

RESULTS AND DISCUSSION

Transdermal films of DNP was fabricated based on sodium alginate as matrix forming agent (4.5%) with adding propylene glycol (7.5%) to improve flexibility of films and *dl*-limonene (1, 3, or 5%) as enhancer to overcome the impervious nature of skin barrier and to enhance skin permeation rate of DNP.

The films TF-CONT, TF-LM1, TF-LM3, and TF-LM5 prepared in this study were thin, flexible, and transparent. The thickness, weight, and drug content of the film formulations are represented in Table II.

The thickness of the films was about 0.5 mm, and the standard deviation of weight of films was less than 2%. Both data indicate homogenously spread ability of sodium alginate gels on the plate surface while preparing transdermal films. The drug content uniformity of all formulations satisfied pharmacopeia requirements for transdermal drug delivery systems evidenced by the low standard deviation values, confirming the reproducibility of the manufacturing process (21).

The mechanical properties of transdermal films are strongly dependent on the type of the polymer and plasticizer as well as their concentrations in formulation. Thus, the mechanical characteristics of the films were evaluated firstly. The percentage of elongation values of the sodium alginate based transdermal films of DNP are given in Table III, and the graphs illustrating the distance at elongation point are shown in Fig. 1.

Elongation can be defined as the deformation in shape of the films putting under tensile stress (22). The point at which the sample piece breaks after a sufficient increase in length is referred as percent elongation break. It expresses the capability of a material to resist changes of shape without crack formation. Films intended for transdermal drug delivery must be flexible enough to follow the movements of the skin (18,22). It has been reported that an ideal transdermal film should be soft and tough that can be characterized by a high elongation value (23,24). According to our presented results, the control transdermal film (TF-CONT) exhibited a significantly lower flexibility compared to other transdermal films containing of *dl*-limonene ($p<0.05$). It was clear that increasing amount of the terpene tend to increase the mean value of elongation at break (%) with the maximum effect at 5% concentration of *dl*-limonene (%36.88±5.13). The increase in elasticity of the transdermal film formulations in the presence of *dl*-limonene could be due to its lipophilic nature that softens the bonds within the polymer sodium alginate, which is more hydrophilic in nature.

Table II. Thickness, Weight, and Drug Content Uniformity of the DNP Transdermal Films (Mean±SD, $n=6$)

Transdermal Film	Thickness (mm)	Weight (mg cm^{-2})	DNP content (%)
TF-CONT	0.53±0.05	47.46±1.80	97.21±0.57
TF-LM1	0.58±0.04	48.89±0.70	97.98±0.32
TF-LM3	0.54±0.06	53.82±1.82	97.17±0.47
TF-LM5	0.56±0.04	40.75±1.24	97.94±0.01

DNP donepezil, *TF-LM1*, *TF-LM3*, *TF-LM5* transdermal formulations containing limonene as enhancer, *TF-CONT* control formulation

Table III. Percent Elongation Values of Sodium Alginate-Based Transdermal Films of DNP (Mean±SD, $n=3$)

CODE	*dl*-Limonene (%)	Elongation (%)
TF-CONT	0	12.75±3.14
TF-LM1	1	21.05±4.42
TF-LM3	3	30.30±6.32
TF-LM5	5	36.88±5.13

DNP donepezil, *TF-LM1*, *TF-LM3*, *TF-LM5* transdermal formulations containing limonene as enhancer, *TF-CONT* control formulation

The adhesion of transdermal films to the skin surface is the other critical functional feature for the product safety, efficiency, and quality. Bioadhesion represents the degree of binding of the polymer to a biological membrane and assumes a key role in the effective delivery of drug molecules. Thus, the determination of bioadhesive properties of transdermal films is an important part of the formulation optimization studies (22,25). The measurement of bioadhesion strength is commonly performed by the use of a texture analyzer which measures the detachment force in tensile mode. This technique has been widely employed in measuring the *in vitro* bioadhesive strength of polymer-based films (16,17,19,22). We used dorsal skin of pig *in vitro* as the substrate for testing adhesive properties of the formulated films and a quantitative evaluation was made by measuring the work of adhesion. The results of the *in vitro* bioadhesion study are depicted in Figs. 2 and 3.

Transdermal films prepared with sodium alginate have shown good bioadhesive character with high bioadhesion work values, indicating that the carboxyl groups (–COONa) of sodium alginate are negatively charged strong hydrogen bonding groups which form hydrogen bonds with negatively charged skin components, that increase the bioadhesiveness of films (17). The data obtained also indicated that the incorporation of the penetration enhancer (*dl*-limonene) to the film formulation at 5% concentration was led to decrease in the work of adhesion dramatically (TF-LM5=0.69±0.05 mJ/cm^2).

In transdermal drug delivery, the most widely used approach to overcome the barrier characteristics of the *stratum corneum* is to add chemical permeation enhancers into transdermal formulations. Terpenes which are derived from plant essential oils have been widely used to enhance the skin permeation of both lipophilic and hydrophilic drugs (26). It has been recognized that hydrocarbon terpenes, such as limonene, provide better enhancement for lipophilic drugs than the hydrophilic ones. As well as the type of enhancer, optimization of enhancer concentration in formulation also has an impact on its efficacy (9).

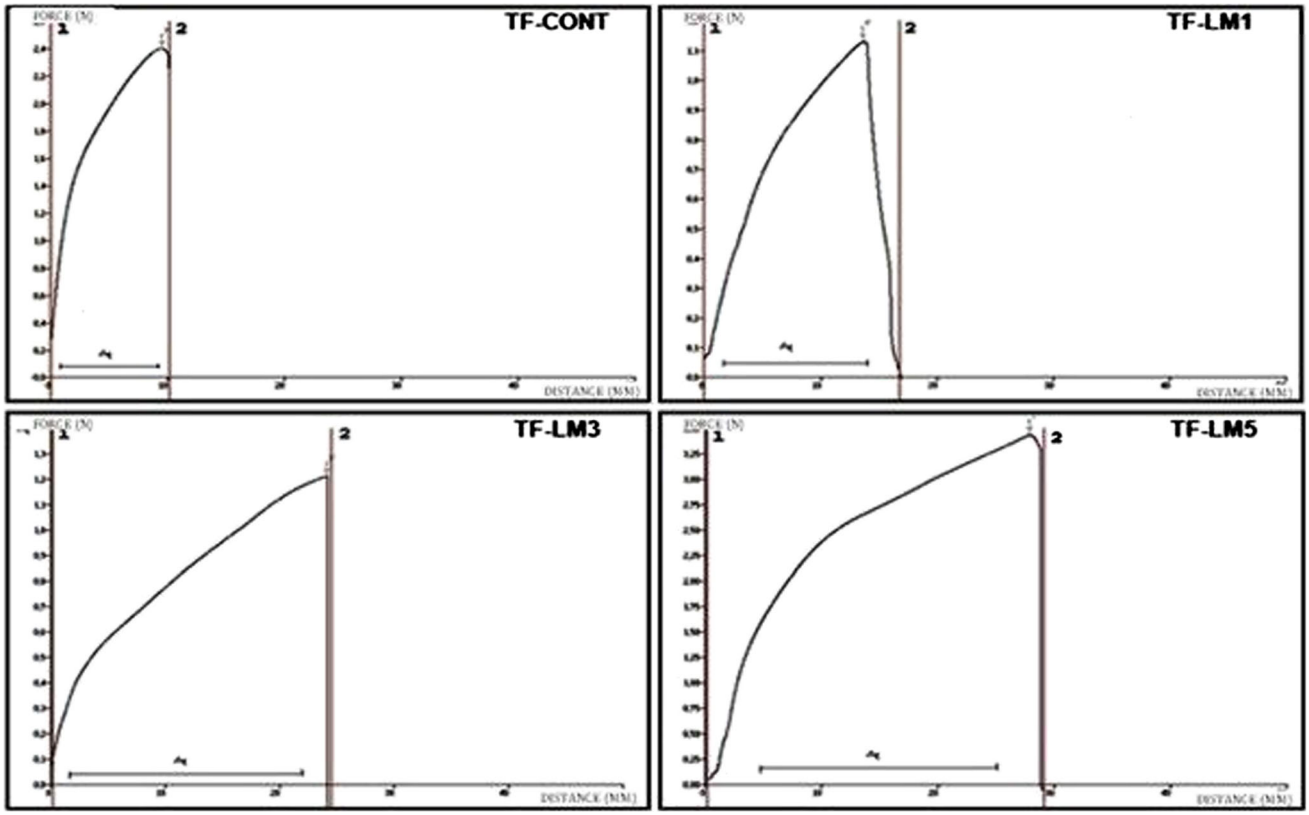

Fig. 1. Elongation graphs of sodium alginate based transdermal films of DNP, A_t=distance at the elongation point

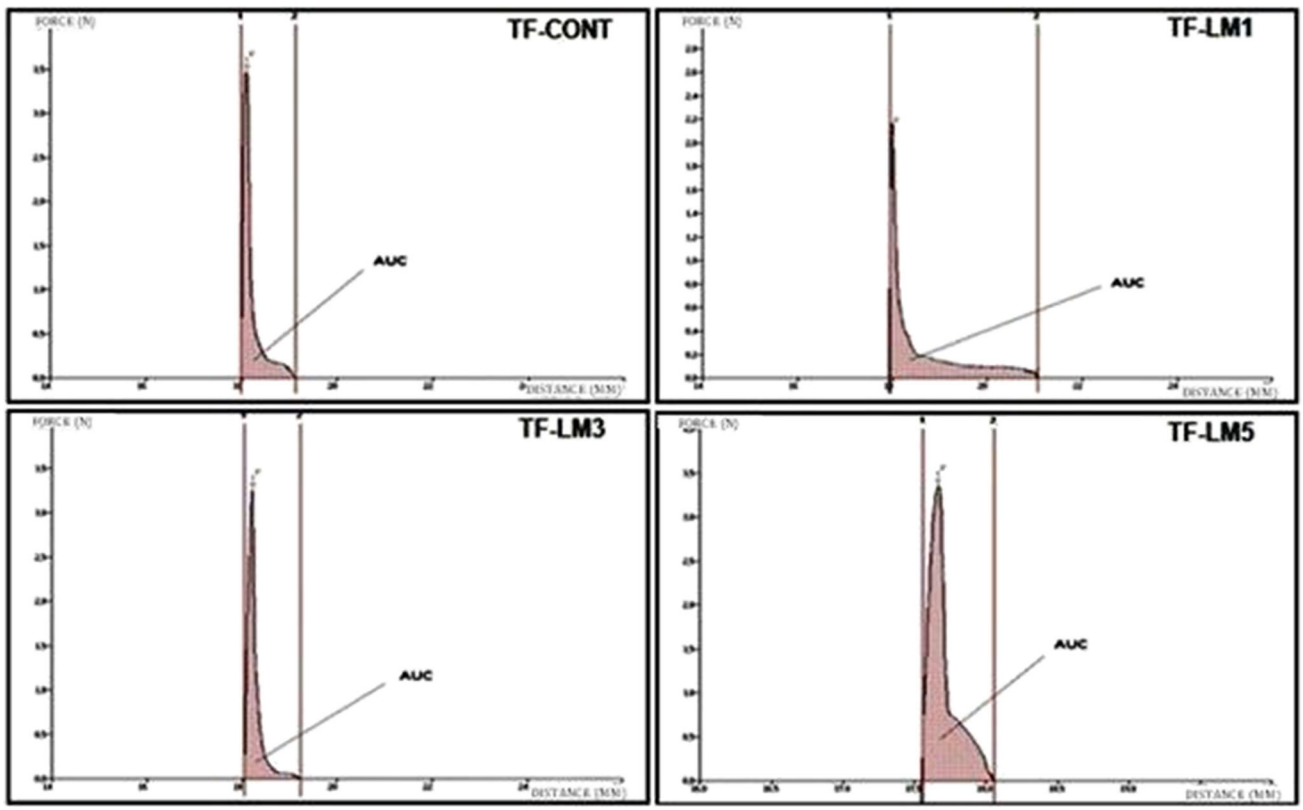

Fig. 2. *In vitro* bioadhesion force-time plots of sodium alginate based transdermal films of DNP

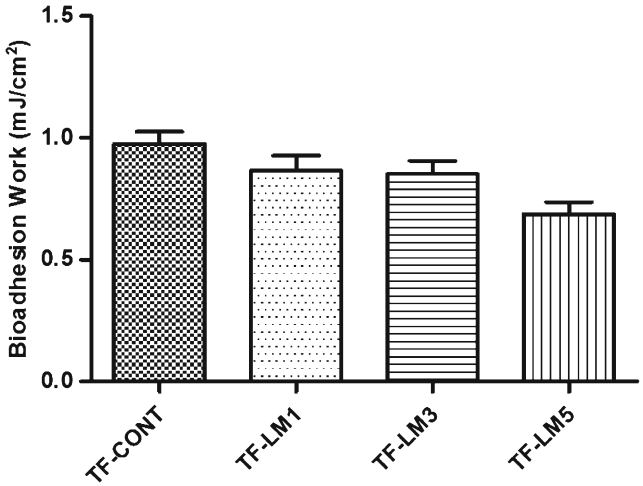

Fig. 3. *In vitro* bioadhesion work of sodium alginate based transdermal films of DNP

The effect of the enhancer added into transdermal films of DNP was assessed following *in vitro* permeation studies. Dermatomed pig skin was used as membrane model due to its comparability, with similarities existing for epidermal thickness, pelage density, lipid content, and general morphology to human skin. The permeability characteristics of pig skin are also shown to be close to that of human skin (27). Figure 4 shows the skin permeation profiles of DNP from transdermal films in the absence and presence of *dl*-limonene at three different concentrations (1, 3, and 5%). Steady state fluxes (J_{ss}), cumulative permeated DNP after 72 h (Q_{72}), lag times, and enhancement ratios for flux (ER_{flux}) for each formulation are presented in Table IV.

Following 72 h *in vitro* permeation, the maximum amount (Q_{72}) of DNP permeated from transdermal films not containing enhancer (TF-CONT) was 267.83±48.30 μg cm^{-2}, and the corresponding DNP flux was calculated to be 4.41±0.16 μg cm^{-2} h^{-1} (Table IV). A significant increase in transdermal permeation of DNP was observed with TF-LM3 containing 3% *dl*-limonene (818.47±92.45 μg cm^{-2}, $p<0.05$). The corresponding flux was 12.45±0.40 μg cm^{-2} h^{-1}, and the increase in flux was significant when compared to control formulation (TF-CONT) and other transdermal films having limonene at different concentrations (TF-LM1 and TF-LM3) ($p<0.05$). There was about a 2.82-fold increase in the drug flux

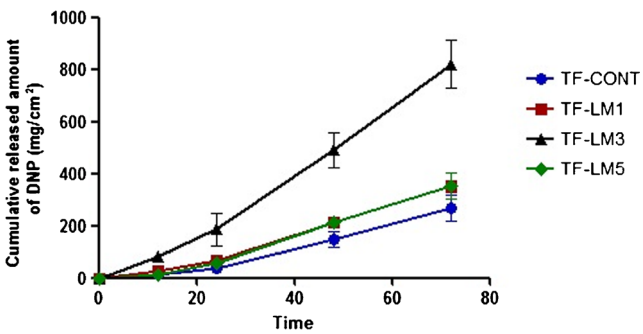

Fig. 4. The cumulative amount of DNP permeated across the dermatomed pig dorsal skin from sodium alginate based transdermal films in presence and absence of *dl*-limonene (±SD, $n=6$)

with TF-LM3 when compared with that obtained with TF-CONT ($p<0.05$). But, the obtained increase in Q_{72} and flux of DNP with formulations TF-LM1 and TF-LM5 was insignificant when compared with that of control (TF-CONT).

Data indicated when *dl*-limonene was added into films at 5% concentration, DNP flux was significantly lower than that of films consisting of (3%) *dl*-limonene ($p<0.05$). The increase in limonene concentration (above 3%) in transdermal films was resulted in decrease in flux of DNP. A similar, negative effect of enhancer concentration on permeation profile of valsartan was also reported previously by Rizwan *et al.* (26). Krishnaiah *et al.* also studied the transdermal permeation of ondansetron in the presence and absence of different terpenes (13). They also indicated that there was no significant difference on the penetration enhancing effect of limonene depending on increasing its concentration, due to plateau effect on the drug permeation across skin above 3%. Similarly, we also observed a plateau effect beyond 3% of *dl*-limonene in films, and there was no significant difference on the permeation enhancing effect of 1% *dl*-limonene when compared with that of 5% *dl*-limonene. It was reported that at high concentrations of limonene even a reduction of the enhancement activity can be seen (28). Our results indicated that 3% *dl*-limonene produced optimum enhancement in DNP permeation through skin. This might be due to the thermodynamic activity of DNP in transdermal films. Additionally, the bioadhesiveness of the formulation TF-LM5 was lower than those of other formulations. That would be an effect on the efficiency of transdermal films.

The lag time of DNP shortened significantly in the presence of *dl*-limonene in the transdermal film formulations compare to that observed with TF-CONT (Table IV). It has been reported that terpenes usually reduce the lag time for permeation which indicates an increase in drug diffusivity based on the ability of terpenes to modify the intercellular lipid arrangement in the *stratum corneum*. Thus, it can be considered that limonene modified the diffusivity of DNP through the pig skin. This result is in accordance with the literature (8,29).

Plasticizers are essential components to overcome the brittleness of the polymeric films. They soften the rigidity of the film structure by increasing the mobility of the polymer chains and improve the mechanical characteristics of the resulted films (23,24). Also, it has been found that the effect of the plasticizer on drug transport is related to the physicochemical properties of the permeant, in particular to its solubility in the plasticizer (30). In our study, propylene glycol (7.5%) was used as plasticizer and additionally, propylene glycol behaves as a co-solvent to increase the solubility of DNP in the formulations. Moreover, the combination of limonene with propylene glycol is reported as an effective enhancer system for several drugs (8,9). Synergistic effects between chemical penetration enhancers and more polar co-solvents like propylene glycol have been reported suggesting that the latter facilitate the solubilization of the former within the *stratum corneum* thus amplifying the lipid-modulating effect (8,31,32). Limonene in combination with propylene glycol has been found more effective than oxygenated linalool and cineole for improving the permeability of the lipophilic drug haloperidol across human skin (33). Studies suggest that limonene acts as

Table IV. Effect of *dl*-Limonene on Percutaneous Permeation of DNP from Sodium Alginate-Based Transdermal Film Formulations

Formulation	Flux (J_{ss}) (μg/cm^2/h^{-1})	Q_{24} (μg/cm^2)	Q_{48} (μg/cm^2)	Q_{72} (μg/cm^2)	Lag time (h)	ER_{flux}
TF-CONT	4.41±0.16	38.64±12.98	146.72±30.64	267.83±48.30	12.81±0.55	1
TF-LM1	5.51±0.08*	66.44±13.83	212.31±20.21	351.60±21.80	9.02±0.28*	1.24
TF-LM3	12.45±0.40**	187.25±61.84*	491.29±68.30*	818.47±92.45**	7.31±0.45**	2.97
TF-LM5	5.84±0.23*	55.93±13.90	214.44±14.74	353.48±52.21	11.72±0.55*	1.26

Each data point is the mean±SD of six determinations
TF-LM1, *TF-LM3*, *TF-LM5* transdermal formulations containing limonene as enhancer, *TF-CONT* control formulation, ER_{flux} enhancement ratios for flux
*$p<0.05$, significant when compared with TF-CONT; **$p<0.05$, significant when compared with TF-CONT, TF-LM1 and TF-LM5

a barrier-altering agent and causes a reversible change in the skin barrier. In combination with propylene glycol, limonene can increase the diffusion coefficient or the partitioning of the permeant into the skin (31,34). This enhancement mechanism is consistent with the findings obtained in our experiments.

Chemical enhancers have been postulated to cause a dynamic structural disorder in the *stratum corneum* intercellular lipids resulting in transdermal permeation enhancement (35). The activity of terpenes as transdermal penetration enhancers is based on a reversible disturbance of the *stratum corneum* intercellular lipid organization (36). It was reported that the hydrocarbon terpene limonene increases the skin permeation of drugs by disrupting the highly ordered structure of intercellular lipids and improving the partitioning of drugs in the *stratum corneum* (37). Small angle X-ray diffraction (SAXD) experiments showed that limonene in propylene glycol decrease the intensity of lipid-based reflections, suggesting disruption of lipid packing within the bilayers (38,39).

ATR-FTIR spectroscopy has been extensively used to study the phase behavior of the *stratum corneum* lipids since it was shown that the degree of the alkyl chain disorder results in a shift of the C–H stretching absorbances to higher wavenumber (40). Besides, ATR-FTIR analysis ensures complementary data about the interaction of penetration enhancers with *stratum corneum* (11). To investigate the conformational order of *stratum corneum* lipids after the *in vitro* permeation study, ATR-FTIR measurements were conducted on the skin sites treated with DNP transdermal films TF-CONT, TF-LM1, TF-LM3, or TF-LM5 following the *in vitro* permeation studies. The absorbance frequency shift of C–H asymmetric and symmetric absorbance peaks originating from the hydrophobic alkyl chain of *stratum corneum* intercellular lipids was analyzed and the results are given in Fig. 5a, b. The ATR-FTIR spectra of C–H asymmetric and symmetric absorbances are shown in Fig. 6.

The C–H symmetric and asymmetric band frequencies of untreated control skin were observed at 2851.1±0.16 and 2918.1±0.18, respectively (Fig. 5a, b). These frequencies support the presence of *stratum corneum* intercellular lipid organization in a mostly ordered state (35). It has been proven that C–H stretching bands at low frequencies are generally characteristic of a high content of *trans* conformers while bands at higher frequencies are associated with the presence of gauche conformers (40,41). Our ATR-FTIR results showed a more fluidized *stratum corneum* lipid state in the presence of *dl*-limonene as enhancer ($p>0.05$). ATR-FTIR spectra of the skin treated with transdermal film formulations exhibited a peak shift to higher wavenumber. The presence of 3% *dl*-limonene in the transdermal film formulation (TF-LM3) caused the greatest shift of both asymmetric and symmetric stretching vibrations to higher wavenumber (2921.1 cm^{-1} for C–H asymmetric and 2851.1 cm^{-1} for C–H symmetric stretching absorbances). This data is in accordance with the finding that terpenes have been shown to increase skin permeability by fluidizing the intercellular lipid structure of *stratum corneum* (31,42).

In order to optimize a feasible transdermal therapeutic system of DNP as an alternative delivery approach for the effective treatment of Alzheimer disease, the required steady-

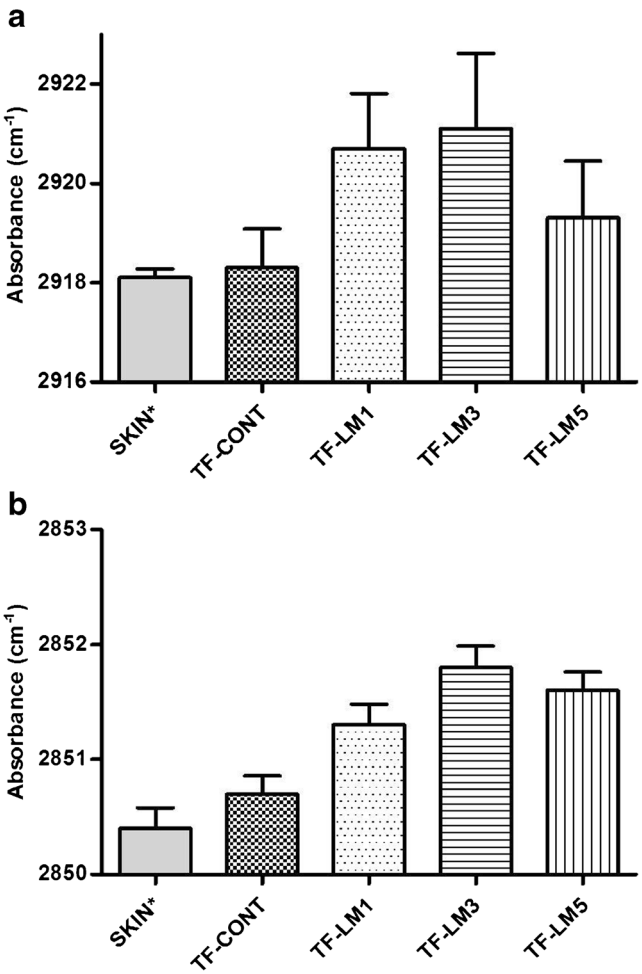

Fig. 5. a Peak positions of skin lipids C–H asymmetric stretching absorbances after application of sodium alginate-based transdermal films of DNP. **b**. Peak positions of skin lipids C–H symmetric stretching absorbances after application of sodium alginate-based transdermal films of DNP. *Asterisk* untreated control pig skin

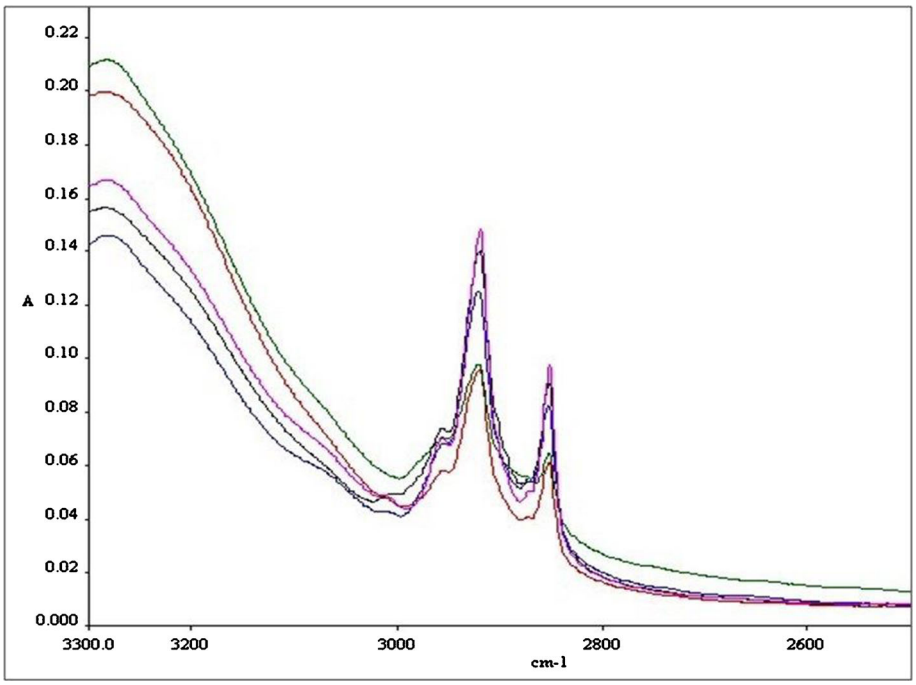

Fig. 6. ATR-FTIR spectra of skin lipids C–H asymmetric and symmetric absorbances after application of sodium alginate-based transdermal films of DNP, *pink*: untreated control pig skin, *black*: TF-CONT, *blue*: TF-LM1, *green*: TF-LM3, and *red*: TF-LM5

state plasma levels of DNP should be provided. In transdermal drug delivery, the permeation rate of drugs must equal the rate of elimination. Thus, considering pharmacokinetic parameters (body clearance and steady-state plasma level) of DNP, the required permeation rate to maintain therapeutic plasma dug levels should be calculated (43). Previously, the targeted transdermal permeation rate of DNP across skin has been estimated to be 16.5 μg cm^{-2} h^{-1} in order to provide its therapeutic response with applying a patch on skin surface with size of 20 cm^2 (44). Regarding to the data obtained in this study, the permeation rate of DNP across excised pig skin from transdermal films (TF-LM3) containing 3% of limonene as enhancer (12.45±0.40 μg cm^{-2} h^{-1}) was very close to the targeted permeation rate of DNP. Therefore, it could be said that therapeutic concentration of DNP might be achieved with optimized transdermal films of DNP patch with a size of about 25 cm^2, which is also a reasonable size in practice.

CONCLUSIONS

Based on the results obtained in the present work, it can be concluded that sodium alginate-based matrix type of transdermal films of DNP can be considered as suitable delivery systems due to good mechanical properties, high bioadhesiveness, and permeability characteristics of films. The efficacy of limonene used as permeation enhancer in transdermal films of DNP was shown with *in vitro* skin permeation data and its effect was also confirmed by ATR-FTIR spectroscopy analysis. Based on that *in vitro* data, it can be concluded that optimized transdermal films of DNP would deliver feasible required amount of DNP with a reasonable patch size. But, to test the validity of that *in vitro* data and to determine the *therapeutic* level of the drug, thus allowing an appropriate formulation, results should be supported by *in vivo* performance of transdermal films.

Conflicts of Interest The authors declare no conflict of interest.

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