International Journal of Pharmaceutics 416 (2011) 136–144

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Evaluation of skin permeation and anti-inflammatory and analgesic effects of new naproxen microemulsion formulations

Neslihan Üstündağ Okur[a], Şebnem Apaydın[b], N. Ülkü Karabay Yavaşoğlu[c], Altuğ Yavaşoğlu[d], H. Yeşim Karasulu[a,*]

[a] Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Ege, 35100 Bornova, Izmir, Turkey
[b] Ege University, Center for Drug R&D and Pharmacokinetic Applications, 35100 Bornova, Izmir, Turkey
[c] Department of Biology, Faculty of Science, Ege University, 35100 Bornova, Izmir, Turkey
[d] Department of Histology and Embryology, Faculty of Medicine, University of Ege, 35100 Bornova, Izmir, Turkey

ARTICLE INFO

Article history:
Received 22 February 2011
Received in revised form 12 June 2011
Accepted 16 June 2011
Available online 23 June 2011

Keywords:
Naproxen
Microemulsion
Anti-inflammatory
Analgesic effect
Histopathology

ABSTRACT

The aim of this study was to evaluate the potential application of microemulsions as a transdermal drug delivery for naproxen (Np). The pseudo-ternary phase diagrams were developed for microemulsions composed of isopropyl myristate, Span 80, Labrafil M, Labrasol, and Cremophor EL, ethanol and isopropyl alcohol and 0.5 N sodium hydroxide. The final concentration of Np in microemulsion systems was 10% (w/w). The microemulsions were characterised by conductivity, droplet size, viscosity and pH. Moreover, in vitro permeability studies were performed using diffusion cells from rat skin. The permeation rates of Np from microemulsions ($M1_{Np}$ and $M2_{Np}$) were higher than the commercial (C) gel formulation. The paw oedema test was performed in rats to evaluate the anti-inflammatory activity of Np. The volume increase in paw oedema after 6 hr was $0.71 \pm 0.46\%$ with $M2_{Np}$, whereas $M1_{Np}$ and C exhibited $6.48 \pm 2.71\%$ and $14.97 \pm 3.15\%$ increases in oedema, respectively. Additionally, a significant analgesic effect was detected in the hot plate and tail-flick tests for all test microemulsion and C formulations when compared with the control. Histopathological examination of the treated skin was performed to investigate changes in skin morphology. In conclusion, the microemulsion formulations, especially the $M2_{Np}$ formulation, may be used as an effective alternative for the transdermal delivery of Np.

1. Introduction

Naproxen (Np), (s)-6-methoxy-α-methyl-2-naphthalene-nacetic acid (Fig. 1), is a non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic effects that is used for the treatment of rheumatoid arthritis, osteoarthritis and traumatic contusions. NSAIDs are the most commonly used drugs to reduce pain and inflammation (Baboota et al., 2007; Escribano et al., 2003). Oral therapy of NSAIDs is very effective, but clinical use is often limited because of adverse side effects, such as irritation and ulceration of the gastro-intestinal mucosa. Topical administration of these agents can prevent these side effects associated with oral use and may help to maintain consistent plasma levels for long-term therapy from a single dose (Beetge et al., 2000; Jantharaprapap and Stagni, 2007).

Microemulsion is defined as a dispersion consisting of oil, surfactant, co-surfactant and aqueous phase, which is a single optically isotropic and thermodynamically stable liquid solution. Microemulsions are homogenous dispersions of water in oil (w/o) or oil in water (o/w) droplets stabilised by surfactants. Microemulsions have several advantages, such as enhanced drug solubility, good thermodynamic stability, and enhanced transdermal effect of conventional formulations (Attwood, 1994; Bolzinger et al., 2008; Chen et al., 2006; Polizelli et al., 2006; Yue et al., 2007).

In principle, microemulsions can be used to deliver drugs to patients via several routes, but the topical application of microemulsions has gained increasing interest (Peltola et al., 2003). Three main mechanisms have been proposed to explain the advantages of microemulsion for the transdermal delivery of drugs. First, the high solubility potential for both lipophilic and hydrophilic drugs in microemulsion systems might increase the thermodynamic activity towards the skin. Second, ingredients in the microemulsion that act as permeation enhancers might destroy the structure of the stratum corneum and increase the permeation of drug through the skin. Third, the permeation rate of the drug from microemulsion may be increased, since the affinity of a drug to the internal phase in microemulsion can be easily modified to favour partitioning into stratum corneum, changing its portion in microemulsion (Rhee et al., 2001; Zhao et al., 2006).

* Corresponding author. Tel.: +90 232 3436600/4067; fax: +90 232 3885258.
E-mail address: yesim.karasulu@ege.edu.tr (H.Y. Karasulu).

0378-5173/$ – see front matter.
doi:10.1016/j.ijpharm.2011.06.026

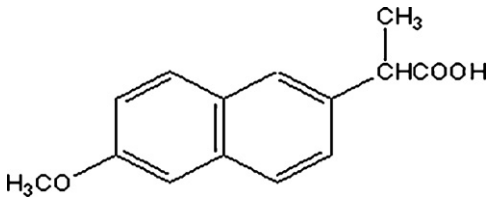

Fig. 1. Chemical structure of naproxen.

The aim of this study was to develop new Np microemulsion formulations ($M1_{Np}$ and $M2_{Np}$) to be used as transdermal drug carriers and to compare the in vitro permeation rate and anti-inflammatory and analgesic effects of Np in these formulations with the commercial topical gel (1 g gel contained 100 mg Np) formulation (C) as the first naproxen preparation that is administered by dermal route in Turkish Drug Industry. To validate the transdermal use of microemulsion formulations, we histopathologically examined the abdominal skin of microemulsion treated rats and compared it to that of control rats treated with either the C formulation or serum physiologic (SP).

2. Materials

Carrageenan, isopropyl myristate (IPM), acetonitrile and Cremophor-EL were purchased from Sigma, Germany. Span 80, methanol, isopropyl alcohol (IPA) and ethanol were obtained from Merck, Germany. Labrafil-M and Labrasol were gifts from Gattefosse, France. Np was a gift from Deva Holding (Turkey). All of the other chemicals and reagents used were of HPLC or analytical grade samples. Water was double distilled.

3. Methods

3.1. Preparation of microemulsions

Pseudo-ternary phase diagrams were constructed to obtain the concentration range of the components for the existing range of microemulsions. For each phase diagram, mixtures of IPM and surfactant/co-surfactant mixtures were prepared at weight ratios of 0.5:9.5, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1. These mixtures were titrated, drop-by-drop, with a sodium hydroxide solution (0.5 N) while being gently stirred at 25 ± 2 °C until the mixture became clear. After being equilibrated, the mixtures were assessed by visual characterisation.

After the microemulsion regions in the phase diagram were identified, typical microemulsion vehicles were selected and prepared at different component ratios. Composition of the microemulsion formulations (M1, M2) is given in Table 1. Naproxen was slowly incorporated into the microemulsion under stirring. After naproxen was entirely dissolved in the miroemulsion, the clear microemulsion-based formulation was obtained. The final concentration of Np in microemulsion systems ($M1_{Np}$, $M2_{Np}$) was 10% (w/w) (100 mg/g) (Table 1).

3.2. Determination of the solubility of naproxen

The solubility of Np was determined in double distilled water, IPM, ethanol, IPA, and surfactants (Labrafil-M, Labrasol, Cremophore EL, Span 80), in addition to M1 and M2 microemulsion formulations at 25 ± 2 °C. An excess amount of Np was dispersed into 2 mL solvents in different glass vials. Each vial was shaken for 72 h in a water bath at 25 ± 2 °C, and then the resulting mixture was centrifuged for 10 min at 12,000 rpm. The supernatant was filtrated through a membrane filter (0.2 μm Nylon, Millipore Millex-GN) before the drugs were assayed by HPLC. Each experiment was performed in triplicate.

Table 1
Percentage weight and batch composition of each microemulsion formulation in the presence or absence of Np and HLB values of formulations.

Formulations	M1 (w/o) formulation components		M2 (o/w) formulation components	
	5.916[a]		10.75[a]	
	M1 (%) (w/w)	$M1_{Np}$ (g)	M2 (%) (w/w)	$M2_{Np}$ (g)
IPM	26.22	2.36	8.53	0.77
Labrafil-M	–	–	3.97	0.36
Labrasol	1.39	0.13	–	–
Span 80	6.91	0.62	–	–
Cremophor EL	–	–	11.89	1.07
Ethanol	58.10	5.23	–	–
IPA	–	–	47.57	4.28
0.5 N NaOH solution	7.38	0.66	28.04	2.52
Naproxen	–	1	–	1

[a] HLB value.

3.3. Determination of n-octanol/water partition coefficient

To determine the n-octanol/water partition coefficient of Np, n-octanol phases were saturated with double distilled water for 24 h. A solution of Np (10^{-4} M) was prepared with double distilled water. Two millilitres of this solution were transferred to 10 mL assay tubes containing 2 mL of the organic phase. The tubes were stoppered and shaken for 24 h in a thermostated bath at 25 ± 2 °C. After being centrifuged for 15 min at 3500 U/min, the concentration of Np in the water phase was analysed by HPLC. The drug in n-octanol layer was diluted into the mobile phase before HPLC analysis. The partition coefficient was calculated according to Eq. (1) as described below (Fang et al., 2003).

$$\text{Partition coefficient} = \frac{\text{Np concentration in octanol phase}}{\text{Np concentration in aqueous phase}} \quad (1)$$

3.4. Characterisation of the microemulsions

The microemulsions were analysed for various physicochemical attributes. The average droplet size and polydispersity index of microemulsions in the presence or absence of Np were measured by photon correlation spectroscopy (Nano ZS, Malvern Instruments, UK). The dynamic light scattering (DLS) experiments were performed on a Zetasizer Nano ZS (Malvern Instrument Laboratory, Malvern, UK) instrument employing a He–Ne laser operated at 4 mW (λ_o = 633 nm), and a digital correlator. The viscosities of microemulsions were measured at 25 ± 2 °C using a viscosimeter (ULA spindle, equipped with a model ULA-40Y water jacket, Brookfield, USA). The pH of the microemulsions was detected at 25 ± 2 °C using a digital pH-meter (HI 221 – Mauritius). The refractive indexes of microemulsions were evaluated at 25 ± 2 °C using a refractometer (Atago RX-7000 CX – Japan).

Electrical conductivity of the microemulsions were studied at 25 ± 2 °C using a conductometer and conductometer probe (Jenway 4071 – UK). Experiments were performed in triplicate for each sample, and the results are presented as mean ± SD. The phase inversion temperature (PIT) of the microemulsions in the presence or absence of Np was measured using a conductometer. Twenty millilitres of all microemulsions were placed in a beaker. An electrode was completely immersed and fixed in the microemulsion, and the beaker was heated in a water bath whose temperature was increased 1 °C/min. The microemulsion was agitated with a stirrer, and the change in the conductivity was recorded (Bayrak and Iscan, 2004; Yue et al., 2007). Experiments were performed

in triplicate for each sample, and the results are presented as mean ± SD.

3.5. Stability of microemulsions

$M1_{Np}$ and $M2_{Np}$ formulations were stored at 5 ± 1 °C, 25 ± 2 °C and 40 ± 2 °C in the dark for 12 months. The physical stability of the microemulsions containing Np was studied via clarity and phase separation observation, droplet size, pH, refractive index , viscosity and electrical conductivity determination. Also, centrifuge tests were performed to assess the physical stability of microemulsions. The microemulsions were centrifuged for 30 min at 13,000 rpm for the centrifuge tests (Djordjevic et al., 2005; Yue et al., 2007). In the chemical stability tests, concentration of Np in the formulations was also investigated by HPLC analysis at 5 ± 1 °C, 25 ± 2 °C and 40 ± 2 °C for up to 12 months.

3.6. In vitro drug release studies

3.6.1. Animals and preparation of skin

The experimental protocol was approved by the Local Animal Ethical Committee of Ege University, Faculty of Pharmacy (Approval No. 2008/3-1). Male Wistar albino rats weighing 250 ± 20 g were purchased from the Experimental Animal Center of Ege University (Izmir, Turkey) for the in vitro, in vivo and histopathology studies. Rats were housed in a room maintained at 22 ± 1 °C with an alternating 12 h light–dark cycle. Animals had free access to pellet diet and water ad libitum. The rats were transported to a quiet laboratory at least 1 h before the experiment began. For the in vitro studies, all experiments were performed between 09:00 and 12:00 h in normal room light and temperature (22 ± 1 °C). The rats were sacrificed using carbon dioxide gas. Skin from the abdominal region was excised after the hair was removed with a depilatory, and then the subcutaneous fat and connective tissue were removed. The excised skins were washed, examined for integrity, stored at −20 ± 2 °C overnight and then used for the permeation experiments (Jain et al., 2007; Yuan et al., 2006).

3.6.2. In vitro permeation studies

Diffusion cells were used for the permeability studies of Np. Rat abdominal skin samples were mounted immediately on glass diffusion cells. These cells provided a diffusional area of 1.326 cm^2. Phosphate buffer pH 7.4 (10 mL, 600 rpm, 37 °C) was used in the receptor compartment. The donor compartment contained 500 mg microemulsion ($M1_{Np}$ or $M2_{Np}$) (containing 50 mg Np) or C (containing 50 mg Np) formulation. Approximately 10 mL of the receptor medium was withdrawn at predetermined intervals (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12 and 24 h) and replaced immediately with an equal volume of receptor solution to maintain a constant volume. All samples were filtered through a membrane filter (0.2 μm Nylon, Millipore Millex-GN) and immediately injected into an HPLC system that consisted of a UV spectrometric detector and C18 column (270 nm). Three replicates of each experiment were performed. All experiments were performed at 25 ± 2 °C. Sink conditions were maintained in the receptor compartment during in vitro permeation studies.

3.6.3. Analysis of the permeation data

Average cumulative amount of drug permeated per unit surface area of the skin was plotted versus time. The slope of the linear portion of the plot was calculated as flux (*Jss*) (Gupta et al., 2005), and the permeability coefficient was calculated using Eq. (2).

$$Kp = \frac{Jss}{Cv} \quad (2)$$

where *Kp* was the permeability coefficient, and *Cv* was the total amount of the drug.

The enhancement of drug penetration due to microemulsion-based formulations was noted as enhancement ratio (ER) (Huang et al., 2008), which was calculated using the following Eq. (3).

$$\mathrm{ER} = \frac{\text{Flux from microemulsion formulation}}{\text{Flux from commercial formulation}} \quad (3)$$

3.7. HPLC analysis of naproxen

The samples were analysed using the HPLC (HP Angilent 1100 series) system that included a separations module, a UV spectrometric pump and detector. The column was a ZORBAX Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 μm). The mobile phase contained methanol/acetonitrile/purified water (20/28/52, v/v/v) and 0.4 mL triethylamine (adjusted to pH 3.2 using orthophosphoric acid). The flow rate was adjusted to 1.5 mL/min, and the injection volume was 20 μL. The UV detection wavelength was 270 nm (Zakeri-Milani et al., 2005) and the retention time was 7.7 min. No interface of the other formulation components was observed. All samples were filtrated through an aqueous 0.2 μm pore size membrane filter (0.2 μm Nylon, Millipore Millex-GN) before injection.

The peak area correlated linearly with Np concentration in the range of 2.5–100.0 μg/mL with the lowest detection limit at 0.158 μg/mL, and the average correlation coefficient was 0.9986.

3.8. Assessment of anti-inflammatory activity

Anti-inflammatory activity was evaluated by inducing paw oedema using carrageenan in rats. Rats were deprived of food overnight and treated topically on the dorsal part of the hind paw with the 500 mg (containing 50 mg Np) of microemulsion formulations (M1, M2, $M1_{Np}$, $M2_{Np}$), C formulation (containing 50 mg Np) (positive control) or SP (negative control) 60 min before 0.1 mL 1% carrageenan in isotonic saline was injected subplantar into the left hind paw (Winter et al., 1962). The contralateral paw was injected with 0.1 mL saline and used as a control. The volume difference between the carrageenan and saline injected paws was used to evaluate the inflammatory response. Paw volume (*V*) was measured by water plethysmometer (Lettica, LE 7500, Barcelona, Spain) immediately before and 1, 2, 3, 4, 5 and 6 h after the injection of carrageenan into the plantar region of the left hind paw ($n = 7$ for each group). The percent inhibition of oedema induced by carrageenan was calculated for each group using Eq. (4).

$$\text{Inhibition of oedema } (\%) = \frac{V_{\text{control}} - V_{\text{treated}}}{V_{\text{control}}} \quad (4)$$

3.9. Assessment of analgesic activity

3.9.1. Tail flick test

The tail flick test was performed by focusing radiant heat on the dorsal surface of the tail. Latency, or the time it took the rats to withdraw their tails from a noxious thermal stimulus, was measured using a tail flick meter (MAY-TF 0703, Ankara, Turkey). To minimise tissue damage, a maximum latency of 30 s was imposed. For each set of experiments, 500 mg of microemulsion formulations (containing 50 mg Np) (M1, M2, $M1_{Np}$, $M2_{Np}$), C formulation (containing 50 mg Np) (positive control) or SP (negative control) were applied to an area of approximately 4 cm^2 on the dorsal skin. The nichrome wire was about 1/8 below the tail. Each rat was then tested before and 30, 45, 60, 75, 90, 105, 120 and 180 min after the topical administration of each formulation ($n = 7$ for each group). The analgesic potency was calculated by the following Eq. (5).

$$\text{ME (maximum effect) } (\%) = \frac{\mathrm{TL} - \mathrm{BL}}{\mathrm{CL} - \mathrm{BL}} \times 100 \quad (5)$$

where TL was test latency, BL was baseline latency and CL was cut-off latency.

3.9.2. Hot plate test

The hot plate test has been described previously by Baker et al. (2002). Rats were placed on an aluminium hot plate (MAY-AHP 0603, Turkey) kept at a temperature of 62 ± 0.5 °C for a maximum time of 30 s. The temperature of the plate was monitored at all times. For each set of experiments, 500 mg of microemulsion formulation (containing 50 mg Np) (M1, M2, $M1_{Np}$, $M2_{Np}$), C formulation (containing 50 mg Np) (positive control) or SP (negative control) were applied to an area of approximately 4 cm^2 on the dorsal skin. The reaction time was determined when animals licked their fore and hind paws and jumped before and 15, 30, 45, 60, 90, 120 and 180 min after the topical application of each formulation (n = 7 for each group). After each measurement, the plate was wiped with a damp cloth to remove traces of urine and faeces.

3.10. Histopathology studies

For histopathological examination, the dorsal aspect of the rat skin was carefully shaved using an electric shaver. Five hundred milligrams of microemulsion formulation (containing drug 50 mg) ($M1_{NP}$ or $M2_{NP}$), SP (negative control) or C formulation (containing drug 50 mg) (positive control) were topically applied for 24 h on male Wistar albino rat dorsal skin. After 24 h, rats were sacrificed using carbon dioxide gas. The transdermal formulations were removed, and the dorsal side of the rat skin was dissected and further processed for light microscopy. Each specimen was fixed in a 10% formaline solution for approximately 24 h, washed with tap water, dehydrated through an increasing ethanol series, immersed in xylene and, finally, embedded in paraffin wax at 56 °C. Paraffin blocks were cut serially at 5 μm using a rotary microtome (RM 2145, Leica Co., Nussloch Germany). Sections were stained with hematoxylene and eosin and examined by light microscope (Olympus BX-51, Japan). Skin that was not treated with any formulation served as a control.

3.11. Statistical data analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) to test the difference between the means of microemulsion formulation and C formulation permeation experiments in in vitro studies. The mean and standard deviation (SD) of n = 3 were calculated. Data were considered statistically significant at $P < 0.05$. For the in vivo studies, differences between drug treated and control groups were also evaluated using Dunnett's t-test. The mean and SD of n = 7 were calculated. A probability level of $P < 0.05$ was considered statistically significant.

4. Results and discussion

4.1. Preparation of microemulsions

The construction of pseudo-ternary phase diagrams was used to determine the composition of the aqueous phase, oil phase, surfactant, and co-surfactant from which the transparent, one-phase microemulsions were formed. The pseudo-ternary phase diagrams with various weight ratios of IPM, Labrafil M, Labrasol, Span 80, Cremophor EL, ethanol, IPA and 0.5 N NaOH solutions are described in Fig. 2. A stable microemulsion was formed when the content of surfactant and co-surfactant was more than 60%, as shown by the pseudo-ternary phase diagrams (Fig. 2a and b). Microemulsions contained 6.91% Span 80, 1.39% Labrasol, 58.10% ethanol and 26.22% IPM (Fig. 2a). This phase diagram was obtained by maintaining the surfactant/co-surfactant ratio at 1:7. IPM was employed as the oil phase to construct the microemulsion diagram, with Span 80-Labrasol and ethanol as the surfactant and co-surfactant, respectively. As shown in Fig. 2b, the composition of microemulsions was fixed at a constant 11.89% Cremophor EL, 3.97% Labrafil M, 47.57% IPA and 8.53% IPM. This phase diagram was obtained by holding the surfactant/co-surfactant ratio constant at 1:3. As described above, IPM was used as the oil phase to construct the microemulsion diagram with Cremophor EL-Labrafil M and IPA as the surfactant and co-surfactant, respectively. The other regions represent for the non-microemulsion region with an opaque appearance.

Table 2
The solubility of Np in microemulsions and microemulsion components at 25 ± 2 °C.

Component	Solubility (mg/mL)
IPM	4.798 ± 0.509
Labrafil-M	0.396 ± 0.041
Labrasol	0.550 ± 0.003
Span 80	0.178 ± 0.209
Cremophor EL	0.547 ± 0.021
Ethanol	123.427 ± 0.288
IPA	78.139 ± 0.159
Distilled water	1.141 ± 0.631
0.5 N NaOH solution	117.296 ± 2.565
M1	110.150 ± 2.149
M2	111.059 ± 1.153

4.2. Determination of naproxen solubility

It was necessary to find an appropriate solvent or oil-surfactant to dissolve Np in because only the dissolved drug can permeate to skin. Table 2 shows the solubility of Np in M1-M2 formulations and formulation components. The maximal drug solubility was measured in ethanol, 0.5 N NaOH solution, M2 and M1, and was found to be 123.427 ± 0.288 mg/mL, 117.296 ± 2.565 mg/mL, 111.059 ± 1.153 mg/mL and 110.150 ± 2.149 mg/mL, respectively. Therefore, we determined that the microemulsion structure contributed to the solubility. The large increase in drug solubility was most likely related to the formation of an interfacial surfactant film between the water and oil phase, which might lead to additional solubilisation sites for the drugs when compared to the molecular organisation of bulk surfactants in Table 2. Furthermore, studies have demonstrated that the unique structural organisation of the phases in microemulsions might contribute to additional solubility regions, which increases the load capacity of microemulsions when compared to unstructured solutions containing the same fraction of constituents (Kogan and Garti, 2006). This is consistent with Malcolmson et al. (1998), who have suggested that the major solubilisation advantage of microemulsions could be ascribed to the surfactant interfacial film of micellar structure. Micellar solutions might further increase the solubility of lipophilic drugs like NP.

4.3. Determination of n-octanol/water partition coefficient

Aqueous solubility and lipophilicity have been shown to influence membrane flux, therapeutic activity, and pharmacokinetic profiles of drugs. Lipophilicity is essential for transdermal penetration because the stratum corneum, the major barrier to drug permeation, is lipophilic and, in general, favours permeation by lipophilic drugs. Determination of the log P of n-octanol/water can be used to evaluate the lipophilicity of the drug. The log P value for Np was 3.18. This value indicates that Np possesses an appropriate lipophilicity for skin permeation (Zhao et al., 2008). This value was significantly non-polar; therefore, the permeation rate is controlled primarily by the aqueous strata of the Np layer in skin that

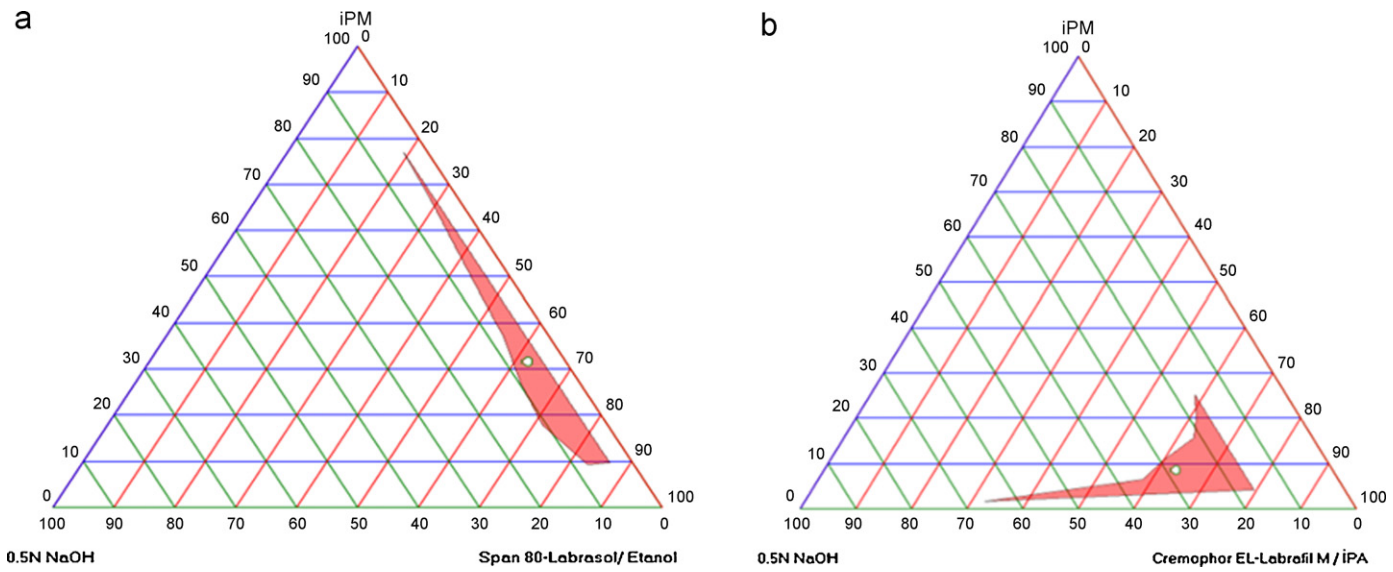

Fig. 2. The pseudo-ternary phase diagrams of the microemulsion. (a) The pseudo-ternary phase diagram of the microemulsion composed of IPM, Span 80, Labrasol, ethanol and water (0.5 N NaOH solution) (w/o). (b) The pseudo-ternary phase diagram of the microemulsion composed of IPM, Cremophor EL, Labrafil M, IPA and water (0.5 N NaOH solution) (o/w).

has a disrupted stratum corneum (Beetge et al., 2000; Fang et al., 2003).

4.4. Characterisation of the microemulsions

The characterisation parameters of the microemulsions are listed in Table 3. In the absence of Np, the average droplet size of M1 and M2 was 1.405 ± 0.040 nm and 5.617 ± 0.199 nm, respectively. However, in the presence of Np, the average droplet sizes of $M1_{Np}$ and $M2_{Np}$ were 1.007 ± 0.011 nm and 4.583 ± 0.560 nm, respectively (Table 3). In addition, the incorporation of Np into M1 and M2 resulted in a 0.4–0.9 nm decrease in the average droplet size (Table 3). These findings support a recent study that found the mean droplet size was significantly decreased after loading the drug (Cui et al., 2009). The polydispersity value described the homogeneity of the droplet size. All polydispersity values were smaller than 0.5. Therefore, these results indicate that the droplet size had high homogeneity. Incorporating the co-surfactant into the microemulsion resulted in a significant reduction in the viscosity of the formulations, with the flow changing to a simple Newtonian flow. Therefore, the viscosities of $M1_{NP}$ and $M2_{NP}$ were found to be 15.33 ± 0.577 cP and 10.33 ± 0.577 cP, respectively. The average pH of all microemulsions ranged from 6.39 ± 0.025 to 8.3 ± 0.025 (Table 3). The average refractive indexes of microemulsions ranged from 1.405 ± 0.002 to 1.461 ± 0.002 (Table 3). The incorporation of Np into M1 and M2 increased the pH and refractive index values.

There was a strong correlation between the specific structure of the microemulsion systems and their electrical conductive behaviour (Yue et al., 2007). The phase systems (o/w or w/o) of the microemulsions were determined by measuring the conductivity (Jenway 4071 – UK) of the microemulsions (Peltola et al., 2003). According to the conductivity measurements, the investigated microemulsion could be divided into w/o and o/w. The M1 (141.5 ± 0.1 μs/cm) formulation was detected as a w/o phase system, whereas the M2 (0.51 ± 0.01 ms/cm) formulation was determined to be an o/w phase system (Table 3).

The microemulsion, which was prepared with a nonionic surfactant, tended to form a w/o type emulsion at higher temperatures and an o/w type at lower temperatures (Bayrak and Iscan, 2004). The microemulsions of non-ionic surfactants of polyoxyetylene type became cloudy at elevated temperatures. The temperature at which the o/w microemulsion changed into w/o microemulsion was referred as the PIT. The PIT was defined as the temperature at which conductivity decreased sharply, which corresponded to a phase inversion from o/w to w/o. Because the conductivity of the system changed at this temperature, the PIT could be determined by changing the point of conductivity. Experimental evidence showed that, as the temperature increased, electrical conductivity of the microemulsions changed (Warisnoicharoen et al., 2000). Fig. 3 shows that increasing the temperature caused an increase in conductivity up to the PIT. However, sudden decreases were also detected after reaching the PIT. Moreover, electrical conductivity and PIT were slightly decreased in the microemulsion formulations loaded with Np. Warisnoicharoen et al. (2000) showed that the presence of electrolytes or drugs that were lipophilic like Np could lower the PIT, which illustrates the importance of determining microemulsion phase behaviour in the presence of drug (Warisnoicharoen et al., 2000). The cloudy dispersion became a transparent, volatile microemulsion upon reaching the PIT. Fig. 3a shows the changes in electrical conductivity of the w/o microemul-

Table 3
Characterisation of microemulsion formulations.

Formulation/Characterisation	M1	M2	$M1_{Np}$	$M2_{Np}$
pH	6.39 ± 0.025	6.62 ± 0.020	7.12 ± 0.01	8.3 ± 0.025
Viscosity (cP)	15.33 ± 0.577	10.33 ± 0.577	15.33 ± 0.577	10.33 ± 0.577
Droplet size (nm)	1.405 ± 0.040	5.617 ± 0.199	1.007 ± 0.011	4.583 ± 0.560
Conductivity	141.5 ± 0.1 (μs/cm)	0.51 ± 0.01 (ms/cm)	149.5 ± 0.1 (μs/cm)	0.59 ± 0.02 (ms/cm)
Polydispersity ± SD	0.399 ± 0.043	0.471 ± 0.067	0.225 ± 0.025	0.181 ± 0.001
Refractive index	1.405 ± 0.002	1.413 ± 0.001	1.452 ± 0.002	1.461 ± 0.002
PIT (°C)	50 ± 0.003	65 ± 0.010	41 ± 0.006	61 ± 0.020

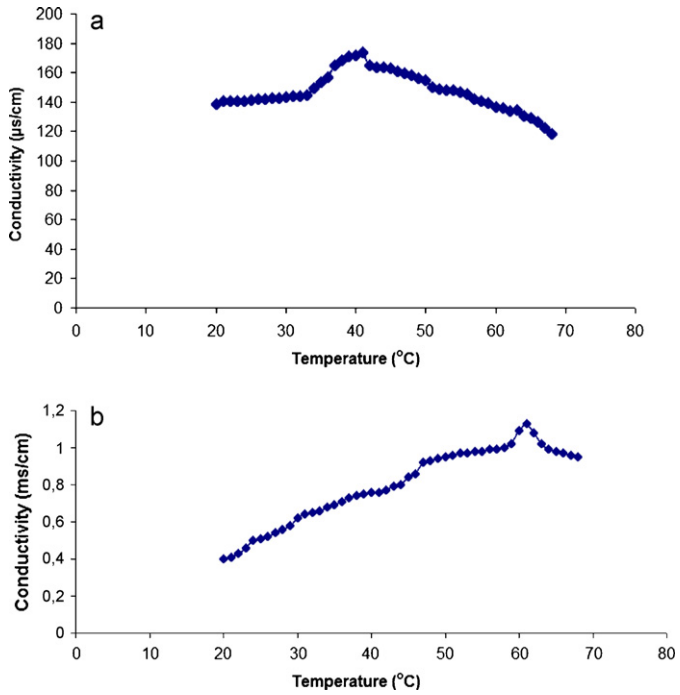

Fig. 3. Changes in microemulsion conductivity in the presence of Np with varying temperatures. (a) Change in the conductivity of $M1_{Np}$. (b) Change in the conductivity of $M2_{Np}$.

sion ($M1_{Np}$) as temperature increased. Fig. 3b shows the changes in electrical conductivity of the o/w microemulsion ($M2_{Np}$) as temperature increased. The line graphs of Fig. 3a and b show an increase in conductivity with temperature; however, sudden decreases were also detected after the PIT (Table 3).

4.5. Stability of the microemulsions

For the stability test, all microemulsion formulations were examined at 5 ± 1 °C, 25 ± 2 °C and 40 ± 2 °C in the presence or absence of Np. No change of particle size, refractive index, viscosity, pH or phase separation of Np was observed during 12 months of storage temperature at 5 ± 1 °C, 25 ± 2 °C and 40 ± 2 °C. The concentration of Np in microemulsions remained constant and no degradation was observed during storage for 12 months (Table 4). The microemulsion vehicles were isotropic, transparent dispersions, and, after centrifugation, no phase separation was observed. This demonstrated the physical stability of the tested microemulsions.

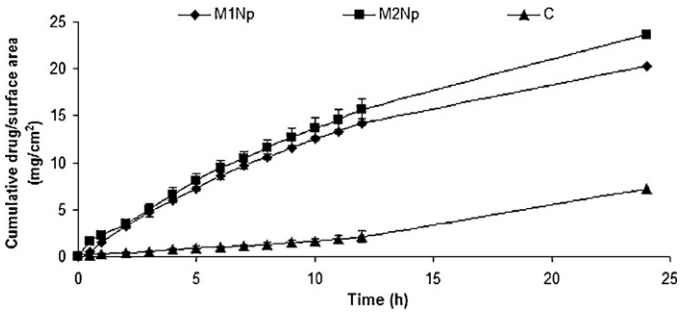

Fig. 4. In vitro permeation profiles of Np through excised rat skin from $M1_{Np}$, $M2_{Np}$ and C. Values are means of three experiments ± SD.

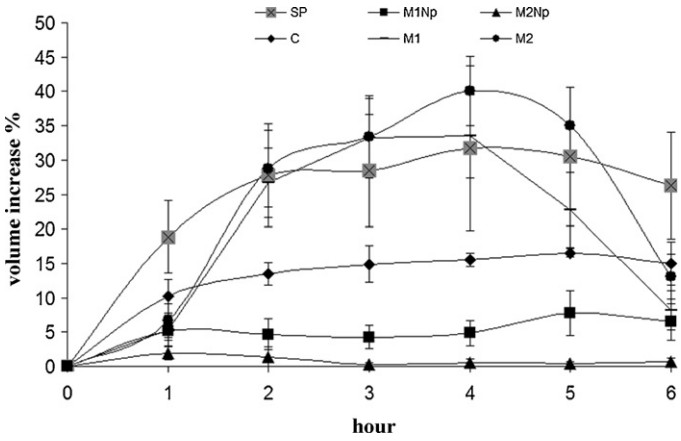

Fig. 5. Volume increase percentages over 6 h for $M1_{Np}$, $M2_{Np}$, C. 1, 2, and 3 h after injection. $P < 0.05$ when compared to SP. For $M1_{Np}$ and $M2_{Np}$, 4, 5, and 6 h after the injection. $P < 0.05$ compared to SP. Each point represents the mean of seven measurements ± SD.

4.6. In vitro permeation studies

The permeation parameters (permeability coefficient, lag time and flux) of $M1_{Np}$, $M2_{Np}$ and C are presented in Table 5. The permeation profiles of Np through rat skin are shown in Fig. 4. A steady increase of Np in the receptor chambers was observed over time. The percutaneous permeation parameters of the tested formulations were calculated. As shown in Table 5, the permeation rates of Np from $M1_{Np}$, $M2_{Np}$ and C were 1.238 ± 0.007, 1.308 ± 0.017 and 0.155 ± 0.006 mg/cm²/h, respectively. The permeation of $M1_{Np}$ and $M2_{Np}$ were significantly higher than C ($P < 0.05$). These results were consistent with previous studies demonstrating that microemulsions had transdermal transport behaviour and favourable solubilisation. This consistency was a result of the hydrophilic and lipophilic compositions of the

Table 4
Chemical stability of Np in the microemulsion formulations after 12 months of storage. Values are means of three experiments ± SD.

	$M1_{Np}$			$M2_{Np}$		
Time	5 °C (mg/mL) ± SD	25 °C (mg/mL) ± SD	40 °C (mg/mL) ± SD	5 °C (mg/mL) ± SD	25 °C (mg/mL) ± SD	40 °C (mg/mL) ± SD
$T_{initial}$	5.076 ± 0.050	5.102 ± 0.049	5.185 ± 0.050	5.187 ± 0.050	5.112 ± 0.049	5.035 ± 0.050
$T_{12\ months}$	5.025 ± 0.050	4.921 ± 0.051	5.010 ± 0.051	5.055 ± 0.051	4.994 ± 0.051	5.011 ± 0.050

Table 5
The permeation parameters and enhancement ratio of the $M1_{Np}$, $M2_{Np}$ and C.

Formulation	Permeation rate *Jss* (mg/cm²/h)	Lag time (h)	r^2	ER	*Kp* (×10⁻³ cm/h)
$M1_{Np}$	1.238 ± 0.007	0.551 ± 0.047	0.988 ± 0.063	7.987	2.4 ± 0.018
$M2_{Np}$	1.308 ± 0.017	0.957 ± 0.009	0.991 ± 0.877	8.438	2.6 ± 0.038
C	0.155 ± 0.006	0.062 ± 0.013	0.996 ± 0.001	–	0.3 ± 0.016

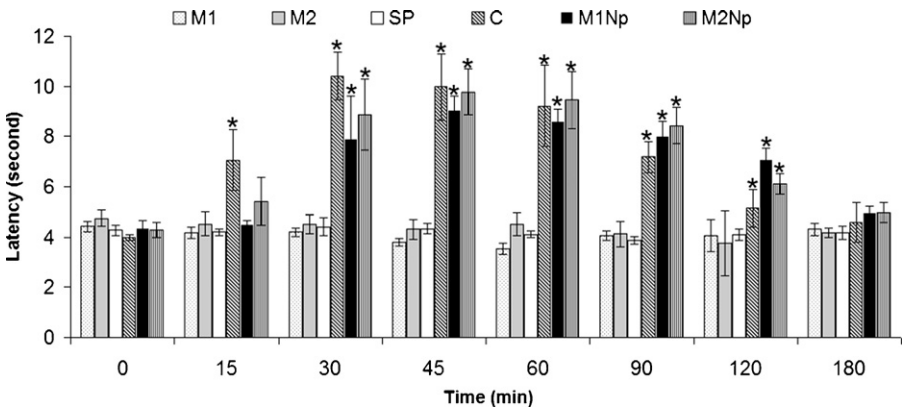

Fig. 6. Tail flick test results. *$P < 0.05$ compared to SP. Each point shows the means of seven determinations ± SD.

microemulsions that enhanced the activity in the system (Chen et al., 2004; Zhao et al., 2006). The microemulsions were able to reduce the interface tension between vehicle and skin because of their contact to the skin lipids, which resulted in faster permeation (Teichmann et al., 2007). Moreover, IPM has been shown to be an effective permeation enhancer (Goldberg-Cettina et al., 1995). Ethanol was shown to extract stratum corneum lipids and to disrupt barrier function, which improved the permeation of more hydrophilic drugs through the skin (Peltola et al., 2003). For this reason, these formulations ($M1_{Np}$, $M2_{Np}$) exerted a higher permeation rate for the transdermal release of Np than C formulation because of their compositions. Moreover, $M2_{Np}$ showed a higher mean transdermal penetration (*Kp* $2.6 \pm 0.038 \times 10^{-3}$ cm/h) value than the other formulations.

The lag time (the first time point that Np was detected) of formulations ranged from 0.551 ± 0.047 to 0.957 ± 0.009 h, indicating the drug permeation rates through rat skin were significantly affected by the composition of microemulsion formulation (Table 5).

The enhancement ratio (ER) was significantly increased in the $M1_{Np}$ (7.987 times) and $M2_{Np}$ (8.438 times) formulations as compared with C ($P < 0.05$) because these microemulsions contained permeation enhancers like Labrafil M, Tween 80, IPM and ethanol. The permeability parameters of the $M1_{Np}$, $M2_{Np}$ and C formulations are shown in Table 5. Shakeel et al. (2007) showed that, by using permeation enhancers like Labrafil, Triacetin, Tween 80, and Transcutol P, the ER was increased in nanoemulsions.

4.7. Assessment of anti-inflammatory activity

The carrageenan-induced oedema test was used to examine the in vivo effects of the developed microemulsion formulations. Intraplantar injection of carrageenan caused a time-dependent paw oedema in the rat, whereas the saline injection did not cause swelling. Transdermal application of the formulations ($M1_{Np}$, $M2_{Np}$, C) inhibited paw swelling. However, the M1, M2 and SP formulations did not inhibit paw swelling or exert any statistically significant anti-inflammatory effects ($P > 0.05$) (Fig. 5). The maximum inhibition for $M2_{Np}$ was observed after 3 h and lasted up to 6 h.

Previously, Mei et al. (2003) have reported that the formulations that had a small particle size showed the strongest acute anti-inflammatory activity. Similarly, the results of this study demonstrated that the $M1_{Np}$ and $M2_{Np}$ containing the smallest particle size (1.007 ± 0.011, 4.583 ± 0.560, respectively) possessed the strongest anti-inflammatory activity.

4.8. Assessment of analgesic activity

4.8.1. Tail flick test and hot plate test

C, $M1_{Np}$ and $M2_{Np}$ administration increased tail flick and hot plate latency when compared with control groups (SP, M1 and M2 administration in rats) (Fig. 6 and Table 6). The latent period did not change in the tail flick and hot plate tests in the control group animals. These results showed the analgesic activity of $M1_{Np,}$ $M2_{Np}$ and C (Table 6). In the tail flick test, the maximum analgesic response of $M2_{Np}$ was observed at 60 and 90 min. Moreover, the $M1_{Np}$ analgesic response peaked at 120 min. In the hot plate test, the maximum analgesic response of $M2_{Np}$ was determined to be 9.55 ± 0.45 at 45 min.

4.9. Histopathology studies

To determine the safety of the transdermal delivery system, histopathology studies were performed. We investigated the influence of formulations on skin irritation by topically applying each formulation. Microscopic images of rat dorsal skin that was treated with microemulsion ($M1_{Np}$ and $M2_{Np}$), SP or C formulations are

Table 6
The latency time of rats subjected to the hot plate test. Presented values are means of seven determinations ± SD.

Group (second)	Minute							
	0	15	30	45	60	90	120	180
SP	4.85 ± 0.15	4.75 ± 0.22	4.90 ± 0.24	4.85 ± 0.18	4.70 ± 0.22	4.40 ± 0.15	4.57 ± 0.14	4.34 ± 0.14
$M1_{Np}$	4.71 ± 0.28	5.08 ± 0.23	8.41 ± 0.72 *,#	9.28 ± 0.44 *,#	8.87 ± 0.43 *,#	7.41 ± 0.35 *,#	6.52 ± 0.28 *,#	5.34 ± 0.22*
$M2_{Np}$	4.55 ± 0.31	5.02 ± 0.29	8.31 ± 0.70 *,θ	9.55 ± 0.45*,θ	9.54 ± 0.42 *,θ	8.28 ± 0.37 *,θ	6.91 ± 0.32 *,θ	5.81 ± 0.39 *,θ
C	4.41 ± 0.24	8.35 ± 0.85*	9.38 ± 0.38*	8.88 ± 0.70*	9.02 ± 0.59*	8.54 ± 0.73*	5.62 ± 0.25*	4.87 ± 0.19
M1	4.85 ± 0.42	4.71 ± 0.32	4.68 ± 0.40	4.75 ± 0.37	4.51 ± 0.37	4.51 ± 0.41	4.48 ± 0.39	4.52 ± 0.42
M2	4.54 ± 0.25	4.34 ± 0.21	4.28 ± 0.29	4.34 ± 0.17	4.35 ± 0.22	3.94 ± 0.22	3.94 ± 0.24	3.92 ± 0.20

* SP; $P < 0.05$.
\# $M1_{Np}$; $P < 0.05$.
θ $M2_{Np}$; $P < 0.05$.

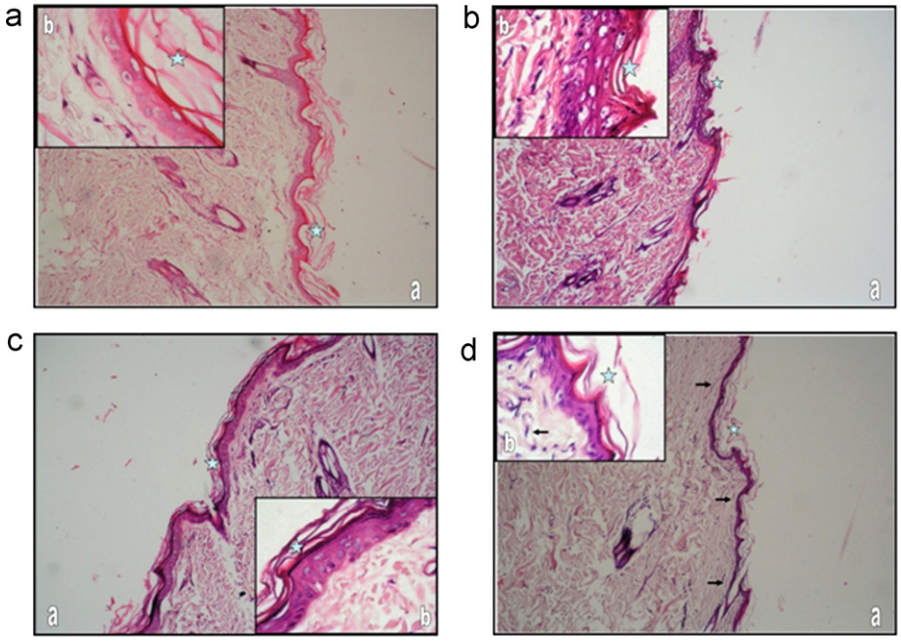

Fig. 7. Microscopic images of treated rat skin with the (a) $M1_{Np}$ microemulsion, (b) $M2_{Np}$ microemulsion, (c) SP and (d) C formulations. Compared to the negative control, the microemulsion formulations developed ($M1_{Np}$ and $M2_{Np}$) did not show any statistically significant histological changes on the skin ($P > 0.05$). (a) 10× (magnification value), (b) 100×, H&E (hematoxylene and eosin). Values are means of seven experiments ± SD; (☆) stratum corneum of epidermis in the rat skin; (→) cells increased in dermis of the skin.

shown in Fig. 7. In Fig. 7a and b, the stratum corneum layer became thinner; however, there was no apparent change in skin morphology (other skin layers and dermis) after the application of $M1_{Np}$ and $M2_{Np}$.

The stratum corneum, dermis and epidermis layers were normal following SP application (Fig. 7c). The stratum corneum layer became thinner and leaved subjacent layers after the application of C formulation. Furthermore, it was observed that cells were increased following dermis irritation (Fig. 7d). Compared to the negative control, the developed microemulsion formulations ($M1_{Np}$ and $M2_{Np}$) did not cause any statistically significant histopathological changes on the skin ($P > 0.05$). The irritation studies did not show visible irritation after application of M1 and M2 after 24 h on the dorsal skin of rats (data not shown). Thus, transdermal drug delivery of the developed microemulsions were considered to be safe.

5. Conclusions

Recently, many studies have focused on using microemulsions for various therapeutic delivery applications. The microemulsion is a promising transdermal drug delivery vehicle. In this study, new w/o and o/w microemulsions for the transdermal delivery of Np have been developed, fully characterised and shown to achieve percutaneous absorption rates of the drug that are consistent with an effective treatment. According to these results of the characterisation and in vitro permeation studies, $M2_{Np}$ was found to be the most effective formulation. The best formulation of $M2_{Np}$ consisted of 8.53% IPM, 3.97% Labrafil M, 11.89% Cremophor EL, 47.57% IPA and 28.04% NaOH solution. Moreover, the $M2_{Np}$ permeation rate was significantly higher when compared to $M1_{Np}$ and C formulations. Similarly, the anti-inflammatory activity of $M2_{NP}$ was stronger than that of C and $M1_{NP}$ in carrageenan-induced rat paw oedema. This study demonstrated that microemulsion formulations containing Np could be promising as an alternative anti-inflammatory and analgesic therapy. Furthermore, irritation and histopathological studies illustrated that the $M2_{NP}$ microemulsion formulation did not cause any skin irritation. Finally, the microemulsion formulation may be a promising vehicle for the delivery of Np transdermally.

Acknowledgements

This study was supported by University of Ege, Faculty of Pharmacy, and Department of Pharmaceutical Technology (08/ECZ/006).

References

Attwood, D., 1994. Microemulsions. In: Kreuter, J. (Ed.), Colloidal Drug Delivery Systems. Marcel Dekker, New York, pp. 31–71.
Baboota, S., Shakeel, F., Ahuja, A., Alı, J., Shaafiq, S., 2007. Desing, development and evaluation of novel nanoemulsion formulations for transdermal potential of celecoxib. Acta Pharmaceut. 57, 315–332.
Baker, A.K, Hoffmann, V.L.H., Meert, T.F., 2002. Dextromehorphan and ketamine potentiate the antinociceptive effects of μ- but nor delta- or kappa-opioid agonists in a mouse model of acute pain. Pharmacol. Biochem. Behav. 74, 73–86.
Bayrak, Y., Iscan, M., 2004. Phase inversion temperatures of triton X-100/1-butanol/hydrocarbon/water systems. J. Surfactants Deterg. 7 (4), 363–366.
Beetge, E., Plessis, J., Müler, D.G., Goosen, C., Rensburg, F.J., 2000. The influence of the physicochemical characteristics and pharmacokinetic properties of selected NSAIDs on their transdermal absorption. Int. J. Pharm. 193, 261–264.
Bolzinger, M.A., Briançon, S., Pelletier, J., Fessi, H., Chevalier, Y., 2008. Percutaneous release of caffeine from microemulsion, emulsion and gel dosage forms. Eur. J. Pharmacokinet. Biopharm. 68, 446–451.
Chen, H., Chang, X., Du, D., Li, J., Xu, H., Yang, X., 2006. Microemulsion-based hydrogel formulation of ibuprofen for topical delivery. Int. J. Pharm. 315, 52–58.
Chen, H., Chang, X., Weng, T., Zhao, X., Gao, Z., Yang, Y., Xu, H., Yang, X., 2004. A study of microemulsion systems for transdermal delivery of triptolide. J. Control. Release 98, 427–436.
Cui, J., Yu, B., Zhao, Y., Zhu, W., Li, H., Lou, H., Zhai, G., 2009. Enhancement of oral absorption of curcumin by self-microemulsifying drug delivery systems. Int. J. Pharm. 371, 148–155.
Djordjevic, L., Primorac, M., Stupar, M., 2005. In vitro release of diclofenac diethylamine from caprylocaproyl macrogolglycerides based microemulsions. Int. J. Pharm. 296, 73–79.
Escribano, E., Calpena, A.C., Queralt, J., Obach, R., Domenech, J., 2003. Assessment of diclofenac permeation with different formulations: anti-inflammatory study of a selected formula. Eur. J. Pharm. Sci. 19, 203–210.

Fang, L., Numajiri, S., Kobayashi, D., Morimoto, Y., 2003. The use of complexation with alkanolamines to facilitate skin permeation of mefenamic acid. Int. J. Pharm. 262, 13–22.
Goldberg-Cettina, M., Liu, P., Nightingale, L., Kurihara-Bergstrom, T., 1995. Enhanced transdermal delivery of estradiol in vitro using binary vehicles of isopropyl myristate and short-chain alkanols. Int. J. Pharm. 114, 237–245.
Gupta, R.R., Jain, S.K., Varshney, M., 2005. AOT water-in-oil microemulsios as a penetration enhancer in transdermal drug delivery of 5-fluorouracil. Colloids Surf. B 41, 25–32.
Huang, Y.B., Lin, Y.H., Lu, T.M., Wang, R.J., Tsai, Wu, P.C., 2008. Transdermal delivery of capsaicin derivative-sodium nonivamide acatate using microemulsions as vehicles. Int. J. Pharm. 349, 206–211.
Jain, S.K., Gupta, Y., Jain, A., Bhola, M., 2007. Multivesicular liposomes bearing celecoxib–β-cyclodextrin complex for transdermal delivery. Drug Deliv. 14, 327–335.
Jantharaprapap, R., Stagni, G., 2007. Effects of penetration enhancers on in vitro permeability of Meloxicam Gels. Int. J. Pharm. 343, 26–33.
Kogan, A., Garti, N., 2006. Microemulsions as transdermal drug delivery vehicles. Adv. Colloid Interface Sci. 123–126, 369–385.
Malcolmson, C., Satra, C., Kantaria, S., Sidhu, A., Lawrence, M.J., 1998. Effect of the oil on the level of solubilization of testosterone propionate into nonionic oil-in-water microemulsions. J. Pharm. Sci. 87 (1), 109–116.
Mei, Z., Chen, H., Weng, T., Yang, Y., Yang, X., 2003. Solid lipid nanoparticle and microemulsion for topical delivery of triptolide. Eur. J. Pharm. Biopharm. 56, 189–196.
Peltola, S., Saarinen-Savolainen, P., Kiesvaara, J., Suhonen, T.M., Urtti, A., 2003. Microemlsions for topical delivery of estradiol. Int. J. Pharm. 254, 99–107.
Polizelli, M.A., Telis, V.R.N., Amaral, L.Q., Feitosa, E., 2006. Formation and characterization of soy bean oil/surfactant/water microemulsions. Colloids Surf. A 281, 230–236.
Rhee, Y.S., Choi, J.G., Park, E.S., Chi, S.C., 2001. Transdermal delivery of ketoprofen using microemulsions. Int. J. Pharm. 228, 161–170.
Shakeel, F., Baboota, S., Ahuja, A., Ali, J., Aqil, M., Shafiq, S., 2007. Nanoemulsions as vehicles for transdermal delivery of aceclofenac. AAPS Pharm. Sci. Technol. 104, 1–9.
Teichmann, A., Heuschkel, S., Jacobi, U., Presse, G., Neubert, R.H., Sterry, W., Lademann, J., 2007. Comparison of stratum corneum penetration and localization of a lipophilic model drug applied in an o/w microemulsion and an amphiphilic cream. Eur. J. Pharm. Biopharm. 67, 699–706.
Warisnoicharoen, W., Lansley, A.B., Lawrence, M.J., 2000. Nonionic oil-in-water microemulsions: the effect of oil type on phase behaviour. Int. J. Pharm. 198, 7–27.
Winter, C.A., Risley, E.A., Nuss, C.W., 1962. Carrageenan-induced oedema in hind paw of the rat as an assay for anti-inflammatory drugs. Proc. Soc. Exp. Biol. Med. 111, 544–547.
Yuan, Y., Li, S., Mo, F., Zhong, D., 2006. Investigation of microemulsion system for transdermal delivery of melexicam. Int. J. Pharm. 321, 117–123.
Yue, Y., San-ming, L., Pan, D., Da-fang, Z., 2007. Physicochemical properties and evaluation of microemulsion systems for transdermal delivery of meloxicam. Chem. Res. Chin Univ. 23 (1), 81–86.
Zakeri-Milani, P., Barzegar-Jalali, M., Tajerzadeh, H., Azarmi, Y., Valizadeh, H., 2005. Simultaneous determination of naproxen, ketoprofen and phenol red in samples from rat intestinal permeability studies: HPLC method development and validation. J. Pharmaceut. Biomed. 39, 624–630.
Zhao, L., Fang, L., Xu, Y., Zhao, Y., He, Z., 2008. Effect of O-acylmenthol on transdermal delivery of drugs with different lipophilicity. Int. J. Pharm. 352, 92–103.
Zhao, X., Liu, J.P., Zhang, X., Li, Y., 2006. Enhancement of transdermal delivery of theophylline using microemulsion vehicle. Int. J. Pharm. 327, 58–64.