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Transdermal drug delivery using microemulsion and aqueous systems: Influence of skin storage conditions on the in vitro permeability of diclofenac from aqueous vehicle systems

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Abstract

The objective of this study was to evaluate the transdermal delivery potential of diclofenac-containing microemulsion system in vivo and in vitro. It was found that the transdermal administration of the microemulsion to rats resulted in 8-fold higher drug plasma levels than those obtained after application of Voltaren Emulgel. After s.c. administration (3.5 mg/kg), the plasma levels of diclofenac reached a peak of 0.94 μg/ml at $t = 1$ h and decreased rapidly to 0.19 μg/ml at $t = 6$ h, while transdermal administration of the drug in microemulsion maintained constant levels of 0.7–0.9 μg/ml for at least 8 h. The transdermal fluxes of diclofenac were measured in vitro using skin excised from different animal species. In three rodent species, penetration fluxes of 53.35 ± 8.19 (furry mouse), 31.70 ± 3.83 (hairless mouse), 31.66 ± 4.45 (rat), and 22.89 ± 6.23 μg/cm^2/h (hairless guinea pig) were obtained following the application of the microemulsion. These fluxes were significantly higher than those obtained by application of the drug in aqueous solution. In contrast to these results, a 'flip-flop' phenomenon was observed when frozen porcine skin (but not fresh skin) was significantly more permeable to diclofenac-in-water than to the drug-in-microemulsion. In fact, the drug penetration from the microemulsion was not affected by the skin storage conditions, but it was increased when an aqueous solution was applied. However, this unusual phenomenon observed in non-freshly used porcine skin places a question mark on its relevancy for in vitro penetration studies involving aqueous vehicle systems.

Keywords: Microemulsion; Transdermal drug delivery; Percutaneous penetration; Porcine skin model; Diclofenac

1. Introduction

Due to their unique physicochemical properties, microemulsions offer advantages over traditional topical and transdermal drug delivery formulations. Some of the advantages are spontaneous formation, thermodynamic stability, and a high solubilizing capacity for various drugs. The high solubilizing capacity of microemulsions enables them to increase the solubility of compounds and, in particular, to solubilize poorly water-soluble drugs. Both, increase in solute concentration and the tendency of the drug to favor partitioning into the stratum corneum make the microemulsion a useful vehicle to enhance transdermal drug permeability. Thus far, it has been shown in many studies that microemulsion formulations possessed improved transdermal and dermal delivery properties, mostly in vitro (Osborne et al., 1991; Trotta et al., 1996; Delgado-Charro et al., 1997; Parra et al., 1997; Dreher et al., 1997; Schmalfuss et al., 1997; Kreilgaard et al., 2000; Alvarez-Figueroa and Blanco-Mendez, 2001; Rhee et al., 2001; Lee et al., 2003), and several in vivo (Kemken et al., 1992; Kreilgaard, 2001; Kreilgaard et al., 2001; Sintov and Shapiro, 2004).

To investigate the possibility for transdermal delivery of drug-in-microemulsion systems, diclofenac was employed as a model compound. High-loaded ibuprofen sodium in the microemulsion was also tested for the purpose of comparison and for use as a supporting evidence of an unusual phenomenon found during the studies (see below). Diclofenac is a commonly used, highly effective non-steroidal anti-inflammatory agent (NSAID) in the management of acute conditions of inflammation and pain, musculoskeletal disorders, arthritis, and dysmenorhea. It is a non-selective cyclooxygenase inhibitor but possesses a slightly preferential cyclooxygenase-2 inhibition activity (Giuliano and Warner, 1999). Thus, although diclofenac is a relatively safe and tolerable NSAID, serious gastrointestinal adverse effects occa-

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sionally appear after oral administration. Owing to its adverse effects, its high portion of hepatic first-pass metabolism (∼50%) as well as its short biological half-life, the topical application of diclofenac provides, therefore, a preferred alternative to the oral dosage forms. The diethylammoniun salt of diclofenac in a topical medication, known as Voltaren Emulgel, has been reported to be particularly suitable for musculoskeletal pain and localized forms of non-articular rheumatism and inflammations of well-defined areas near the body surface (Kriwet and Muller-Goymann, 1993). Diclofenac salt is soluble in aqueous solutions as ionized salts and its penetration into the skin is dependent upon partitioning of the unionized form into the lipophilic phase of the topical emulsion. There are many published works attempting to increase the percutaneous penetration of diclofenac (Kriwet and Mueller-Goymann, 1995; Parra et al., 1997; Rhee et al., 2001; Bonina et al., 2001; Takahashi et al., 2001; Fini et al., 1999; Arellano et al., 1998; Boinpally et al., 2003; Escribano et al., 2003; Djordjevic et al., 2005). Several microemulsion formulations have also been used for transdermal diclofenac delivery (Kweon et al., 2004; Kantarci et al., 2005). In our laboratory, we have challenged this limitation and developed a water-in-oil microemulsion vehicle containing diclofenac, which is capable of carrying the drug quantitatively through the lipophilic layers of the skin.

The tested microemulsion is based on commonly used non-ionic surfactants, PEG-40-stearate and glyceryl oleate, and a non-irritant co-surfactant, tetraglycol. It has previously been shown that tetraglycol, which is generally used as a solvent in parenteral pharmaceutical products, possesses solvent properties for acetaminophen in a transdermal delivery system, increasing its load capacity and contributing to its percutaneous absorption (Sintov et al., 2003). In a more recent study, it was demonstrated that lidocaine can be delivered into the dermis at increased concentrations and through full-thickness skin at enhanced penetration rates when microemulsion vehicles containing polyoxyl stearate, glyceryl oleate, and tetraglycol were used (Sintov and Shapiro, 2004). In the present paper, we have studied the transdermal delivery potential of diclofenac from this type of microemulsions in vivo and in vitro. We have also studied the in vitro drug permeability using skin specimens excised from different animal species. For each animal model, the drug penetration flux obtained with microemulsion was compared to a plain aqueous solution. In addition, we have reported on a phenomenon that we came upon during the studies, placing a question mark on the validity of the commonly used frozen porcine skin for analyzing drug permeability from aqueous vehicle systems.

2. Materials and methods

2.1. Materials

Diclofenac and ibuprofen as sodium salts were obtained from Sigma, Rehovot, Israel. PEG-40 stearate and glyceryl oleate were obtained from Uniqema, Bromborough Pool, The Wirral, UK. Isopropyl myristate and tetraglycol were purchased from Sigma, Rehovot, Israel. A commercial formulation, Voltaren Emulgel® (Novartis) was obtained from a local pharmacy. High-performance liquid chromatography (HPLC) grade solvents were obtained from J.T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ).

2.2. Preparation of microemulsions

The formulation was prepared according to Sintov and Shapiro (2004). Shortly, water-in-oil (W/O) liquid microemulsions were prepared by dissolving PEG-40 stearate (surfactant) in tetraglycol (co-surfactant). Glyceryl oleate (surfactant) and isopropyl myristate (oil) were then dissolved followed by gentle mixing with distilled water. The co-surfactant/surfactants (CoS/S) weight ratio was 1.8, while the surfactants' ratio was 2:3 PEG-40 stearate/glyceryl oleate (Fig. 1). The microemulsion formed spontaneously at room temperature as a clear monophasic liquid. The final concentration of diclofenac sodium in the microemulsions was 1.0 wt%. The final concentration of ibuprofen sodium in the microemulsions was 16 wt%. As a convenient method to represent an increase of water content while decreasing CoS-S levels, 'water dilution lines' were drawn from the CoS-S apex (100% CoS-S) to the opposite oil side of the phase diagram (Fig. 1). Formulations along dilution lines 25:75 (at the margin of the microemulsion region of existence) and 40:60 (at the center of the microemulsion region) were prepared and analyzed for droplet sizes by light scattering.

2.3. Light scattering

The mean droplet size of the microemulsions was determined at 18.3 °C by dynamic laser light scattering (ALV Instruments Type CGS-3, ALV/LSE-5003 digital correlator, ALV GmbH, Langen, Germany). The scattering intensity measurements were performed at angles of 60°, 90°, 120°, and 150°. The droplet size was calculated using the Stokes–Einstein relationship.

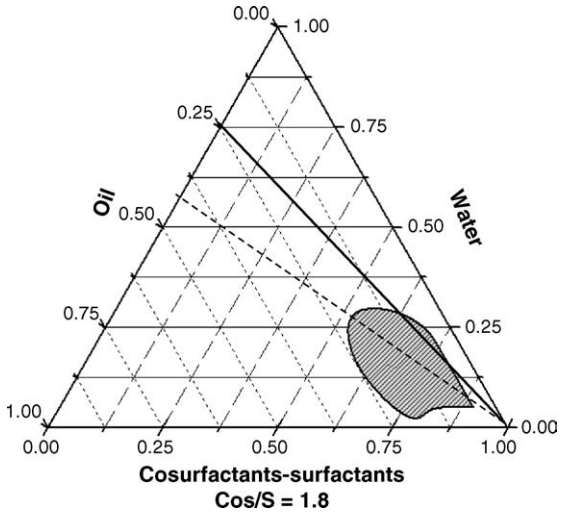

Fig. 1. Pseudo-ternary phase diagram of diclofenac-containing microemulsions. The co-surfactant/surfactants (CoS/S) ratio was 1.8, and the surfactants' ratio was 2:3 PEG-40 stearate/glyceryl oleate. The final concentration of diclofenac sodium in the formulations was 1.0 wt%. Note that the dilution line 25:75 (solid line) and 40:60 (dashed line) are drawn.

2.4. In vitro skin penetration study

The permeability of diclofenac through animal skin was determined in vitro with a Franz diffusion cell system (Crown Bioscientific, Inc., Clinton, NJ, USA). The diffusion area was 1.767 cm^2 (15 mm diameter orifice), and the receptor compartment volumes varied from 11 to 12 ml. The solutions in the water-jacketed cells were thermostated at 37 °C and stirred by externally driven, Teflon-coated magnetic bars. Each set of experiments was performed with at least four diffusion cells ($n \geq 4$), each containing skin from a different animal. Sprague–Dawley rats (males, 200–300 g), CD-1 hairless and ICR mice (males, 25–30 g, Harlan Laboratories Ltd., Jerusalem, Israel), or hairless guinea pigs (males, 350–400 g, Charles River Laboratories, Inc., USA) were sacrificed by aspiration of ethyl ether. The abdominal hair was clipped carefully and sections of full-thickness skin were excised from the fresh carcasses of animals. Full-thickness porcine skin was excised from fresh ears or rear abdomen of slaughtered white pigs (breeding of Landres and Large White, locally grown in the Institute of Animal Research, Kibbutz Lahav, Israel). After subcutaneous fat was removed with a scalpel, the transepidermal water loss (TEWL) was measured before the skin sections were mounted in the diffusion cells or stored at lower temperatures until used. TEWL examinations were performed on skin pieces using Dermalab® Cortex Technology Instrument (Hadsund, Denemark) and only those pieces that the TEWL levels were less than 15 g/m^2/h were introduced for testing. The skin pieces were either kept in the refrigerator (4 °C), frozen at −20 °C, or used as a fresh tissue within 1.5 h (rodents) or 7 h on ice (pigs) after animal death. Unused skin sections which were stored for more than 2 weeks were discarded. Due to unexpected data obtained with frozen porcine skin, TEWL was also measured at 1, 2, and 3 h after thawing the skin pieces (see Section 3). The skin was placed on the receiver chambers with the stratum corneum facing upwards, and the donor chambers were then clamped in place. The excess skin was trimmed off, and the receiver chamber, defined as the side facing the dermis, was filled with phosphate buffered saline (PBS, pH 7.4). After 15 min of skin washing at 37 °C, the buffer was removed from the cells. Diclofenac (or ibuprofen) containing formulations were applied on the skin, and the receiver chambers were refilled with a fresh phosphate buffered saline (PBS, pH 7.4). Samples (2 ml) were withdrawn from the receiver solution at predetermined time intervals, and the cells were replenished up to their marked volumes with fresh buffer solution each time. Addition of PBS to the receiver compartment was performed with great care to avoid trapping air beneath the dermis. The samples were taken into 1.5-ml amber vials and kept at −20 °C until analyzed by HPLC.

2.5. Pharmacokinetic study of transdermal diclofenac in rats

The animal protocol was reviewed and approved by the institutional Animal Care & Use Committee, which complies with the Israeli Law of Human Care and Use of Laboratory Animals. Each male Sprague–Dawley rats (330–400 g, Harlan Laboratories Ltd.) was anesthetized by i.p. injection of a combination of ketamine hydrochloride (75 mg/kg) and xylazine (5 mg/kg). Anesthesia was maintained with 0.1 ml ketamine (100 mg/ml) throughout the experiment.The hair on abdominal skin was trimmed off, and the TEWL was determined to verify skin integrity. The abdomen was then washed gently with distilled water. Volumes of 200 μl microemulsion containing 1% (w/w) diclofenac sodium were applied to the skin surface over an area of 1.7 cm^2 via open containers glued to the skin on one end with a silicon rubber. Blood samples were taken under anesthesia from the tail vein into heparanized tubes. After centrifugation, plasma samples were stored at −20 °C until analyzed for drug concentration. Before analyzing, 100 μl of plasma were taken with 200 μl of methanol (HPLC grade), vortexed and centrifuged for 15 min at 10,000 rpm. The supernatant was taken to a separate vial and injected to the HPLC as described below.

2.6. HPLC analysis of plasma and samples from receiver solutions

2.6.1. Diclofenac

Aliquots of 20 μl from each sample were injected into a HPLC system, equipped with a prepacked column (Betasil C_{18}, 5 μm, 250 mm × 4.6 mm, Thermo Electron Corporation, UK). The HPLC system (Shimadzu VP series) consisted of an autosampler and a diode array detector. The quantification of diclofenac was carried out at 280 nm. The samples were chromatographed using an isocratic mobile phase consisting of acetonitrile–sodium acetate buffer, pH 6.3 (40:60) at a flow rate of 1 ml/min.

2.6.2. Ibuprofen in the receiver solutions

Aliquots of 20 μl from each sample were injected into the same HPLC system, equipped with a prepacked C_{18} column (LiCrosphere® 60 RP-select B, 5 μm, 250 mm × 4 mm, Merck, Darmstadt, Germany). The quantification of ibuprofen was carried out at 225 nm. The samples were chromatographed using an isocratic mobile phase consisting of acetonitrile–sodium phosphate buffer, pH 3.0 (60:40) at a flow rate of 1.3 ml/min.

2.6.3. Calculation of the in vitro data

A calibration curve (peak area versus drug concentration) was constructed by running standard drug solutions in PBS for each series of chromatographed samples. In the in vitro testing, as a result of the sampling of large volumes from the receiver solution (and the replacement of these amounts with equal volumes of buffer), the receiver solution was constantly being diluted. Taking this process into account, the cumulative drug permeation (Q_t) was calculated from the following equation:

$$Q_t = V_r C_t + \sum_{i=0}^{t-1} V_s C_i$$

where C_t is the drug concentration of the receiver solution at each sampling time, C_i the drug concentration of the ith sample, and V_r and V_s are the volumes of the receiver solution and the sample, respectively. Data were expressed as the cumulative drug

permeation per unit of skin surface area, Q_t/S ($S = 1.767\ cm^2$). The steady-state fluxes (J_{ss}) were calculated by linear regression interpolation of the experimental data at a steady state:

$$J_{ss} = \frac{\Delta Q_t}{\Delta t \times S}$$

Apparent permeability coefficients (K_p) were calculated according to the equation:

$$K_p = \frac{J_{ss}}{C_d}$$

where C_d is the drug concentration in the donor compartment (1.0 wt% or 1.0×10^4 μg/ml), and it assumed that under sink conditions the drug concentration in the receiver compartment is negligible compared to that in the donor compartment.

3. Results and discussion

3.1. Microemulsion system

Microemulsions were prepared as those created previously (Sintov and Shapiro, 2004), being composed of a lipophilic phase, non-ionic surfactants, a co-surfactant, and water (Fig. 1). The spontaneously formed formulations were isotropic, transparent, and slightly viscous. All microemulsions formed along the dilution line 40:60 possessed a low electrical conductivity (50 μS/cm at the highest water content), implying that this water-in-oil microemulsion did not inverse to oil-in-water or to a bi-continuous form. The microemulsions, which were constructed along the dilution lines of 25:75 and 40:60, were obtained with a mean droplet size of approximately 15 nm diameter as measured by dynamic light scattering. The uniform droplet size may explain the similarity in the skin penetration rates obtained after application of microemulsions lying on the same dilution line with 20 and 30% water content (unpublished data). In the last decades, many extensive studies have been performed on microemulsions using short-chain or medium-chain alcohols as co-surfactants. The inclusion of these alcohols to microemulsions may limit their potential use due to their skin irritancy. Alcohol-free microemulsions containing no chemical enhancers, may improve drug penetration without causing skin irritancy (Delgado-Charro et al., 1997; Kreilgaard et al., 2000; Alvarez-Figueroa and Blanco-Mendez, 2001; Kemken et al., 1992; Sintov and Shapiro, 2004; Sintov et al., 2003; Schwarz et al., 1996; Shukla et al., 2003; Baroli et al., 2000; Lehmann et al., 2001). Polysorbates (Tween 80 and Tween 85) with 1,2-octanediol, poloxamers or propylene glycol as co-surfactants are commonly useful for the preparation of alcohol-free microemulsions (Alvarez-Figueroa and Blanco-Mendez, 2001; Kemken et al., 1992; Shukla et al., 2003; Baroli et al., 2000), however, PEG-8 caprylate/caprate (Labrasol) and polyglyceryl isostearate (Plurol Isostearique) combinations have also been used in several studies (Delgado-Charro et al., 1997; Kreilgaard et al., 2000; Alvarez-Figueroa and Blanco-Mendez, 2001). In our studies, we have used a different combination consisting of PEG-40 stearate/glyceryl oleate as surfactants and tetraglycol as the co-surfactant. This composition, which had already shown to enhance the skin permeability for acetaminophen (Sintov et al., 2003) and lidocaine (Sintov and Shapiro, 2004), increased the penetration rate of diclofenac as well. It has been demonstrated in the present report that incorporating diclofenac as sodium into the tested microemulsion enhanced drug penetration through the skin in vitro and in vivo.

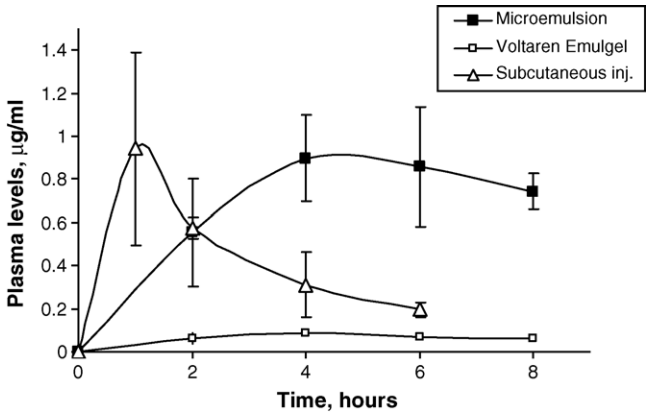

Fig. 2. Pharmacokinetic profiles of diclofenac after transdermal administrations of Voltaren Emulgel (200 mg, 1.7 cm^2, $n = 4$) and drug-containing microemulsion (200 μl, 1.7 cm^2, $n = 8$), compared to subcutaneous administration of 1.2 mg (3.5 mg/kg) ($n = 3$) drug to rats.

3.2. In vivo studies in rats

Fig. 2 shows the pharmacokinetic profiles of diclofenac after topical application of 1% (w/w) drug (as sodium salt) in microemulsion and in macroemulsion (Emulgel) compared to 3.5 mg/kg s.c. bolus injection of the drug. It should be noted that the introduction of the commercial Emulgel and the subcutaneous diclofenac dosage into this study was for demonstrative purposes only without any consideration as for the clinical implications of the therapeutic use of this drug. The transdermal administration of the microemulsion to rats resulted in 8-fold higher drug plasma levels than those obtained after application of the commercial macroemulsion. Our data relating the commercial product fall within the same range as determined in different animal species by previously published reports (Riess et al., 1986; Cevc and Blume, 2001). Drug plasma levels obtained during 4–8 h following microemulsion application were comparable to the peak plasma levels obtained following subcutaneous administration of a relatively high drug dosage (1.2 mg, 120 μl of 1% solution, dose = 3.5 mg/kg). After s.c. administration, the plasma levels of diclofenac reached a peak of 0.94 μg/ml at $t = 1$ h and decreased rapidly to 0.19 μg/ml at $t = 6$ h, while transdermal administration of the drug in microemulsion maintained constant levels of 0.7–0.9 μg/ml for at least 8 h. No immediate skin damage or irritation was observed in all the animals used.

The in vivo data, which have demonstrated significant drug absorption by the transdermal route (compared to an O/W emulsion), could be explained by the nano-sized aqueous droplets dispersed in the continuous lipophilic phase of the microemulsion. While diclofenac molecules are entrapped in the inner phase surrounded by an amphiphilic membrane, the skin naturally interacts with the continuous lipophilic phase. This situ-

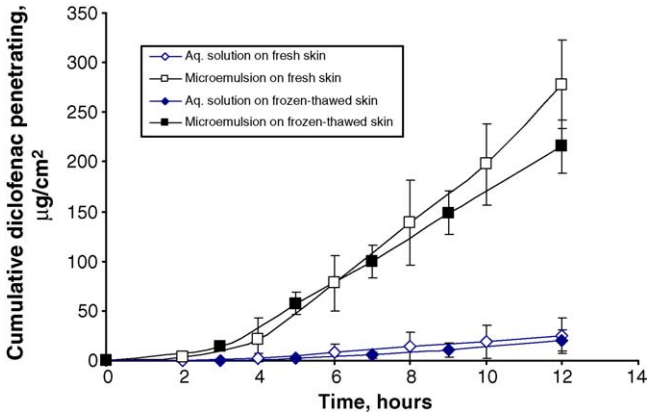

Fig. 3. In vitro percutaneous penetration of diclofenac through rat skin after application of microemulsion and plain aqueous solution. A comparison was also made between fresh skin (open symbols) and frozen skin (close symbols) ($n = 6$).

ation enables the aqueous droplets to move easily through the stratum corneum and carry the drug to the blood circulation.

3.3. In vitro studies using several sources of animal skin

The transdermal fluxes of diclofenac were measured in vitro using skin excised from different animal species. The percutaneous penetration of diclofenac from the microemulsion through Sprague–Dawley rat skin is illustrated in Fig. 3, while the penetration fluxes/permeability coefficients of the drug through rat, guinea pig and mouse skin are summarized in Table 1. In all three rodent species, the penetration fluxes obtained following the application of the microemulsion were significantly higher than those obtained by application of the drug in a aqueous solution ($p < 0.05$), which was used as a control. It can be seen from Fig. 3 and from the corresponding fluxes in Table 1 that (a) the microemulsion enhanced the penetration of diclofenac through rat skin by about 10 folds compared to the aqueous solution, and (b) the drug penetration rates from the aqueous solution through rat and hairless guinea pig skin were quite low compared to the values obtained when using mouse skin. It is also interesting to note that frozen rat skin, which had been thawed just before the penetration testing was somewhat less permeable to diclofenac from microemulsion with a flux value of $22.73 \pm 2.26\ \mu g\ cm^{-2}\ h^{-1}$ versus $31.66 \pm 4.45\ \mu g\ cm^{-2}\ h^{-1}$ obtained with fresh rat skin ($p < 0.05$, Student's t-test). This difference was not significantly observed in hairless guinea pig skin after microemulsion application. In rat skin, diclofenac flux obtained from the aqueous vehicle applied on either fresh or frozen skin was not significantly different. Unlike rat skin, the flux obtained from the aqueous vehicle applied on frozen hairless guinea pig skin was significantly higher than the flux obtained after application to fresh skin ($p < 0.05$). It can be seen from Table 1 that the highest permeable skin belongs to the mouse while pig skin exhibited the lowest permeability to diclofenac. In summary, the different species can be ordered according to their permeability as follows: mouse > hairless mouse > rat ≈ hairless guinea pig > pig (for drug in aqueous solution), and mouse > hairless mouse ≈ rat > hairless guinea pig > pig (for diclofenac in microemulsion).

3.4. The relatively high permeability of frozen porcine skin to aqueous vehicles

In contrast to the results obtained from rodents' skin, frozen pig's ear skin as well as the abdominal skin surprisingly showed a different pattern, in which higher quantities of diclofenac in the aqueous solution penetrated the skin than those penetrated from the microemulsion (Fig. 4 and Table 1). This 'flip-flop' phenomenon was not specific to the microemulsion system alone as can be seen in Fig. 5. Fig. 5 presents the percutaneous penetration of Voltaren Emulgel (macroemulsion containing high water concentration) in comparison to the aqueous drug solution, demonstrating that the aqueous solution is also superior to the oil-in-water macroemulsion. Thus, although the Emulgel is highly hydrophilic in nature its lipophilic ingredients

Table 1
Summary of diclofenac penetration fluxes (±S.D.) and permeability coefficients through skin of various animal species/sites at different storage conditions

Skin source (condition)	J ($\mu g\ cm^{-2}\ h^{-1}$)		$K_p \times 10^3$ ($cm\ h^{-1}$)	
	Microemulsion	Aqueous solution	Microemulsion	Aqueous solution
Pig's ear (frozen[a])	2.10 ± 1.51* ($n = 20$)	5.80 ± 2.38 ($n = 20$)	0.21 ± 0.15	0.58 ± 0.24
Pig's ear (refrigerated[b])	2.63 ± 1.00 ($n = 5$)	2.05 ± 0.96 ($n = 5$)	0.26 ± 0.10	0.20 ± 0.09
Pig's ear (fresh[c])	2.06 ± 0.68 ($n = 4$)	1.12 ± 0.45 ($n = 4$)	0.20 ± 0.07	0.11 ± 0.04
Pig's abdomen (frozen[a])	0.05 ± 0.03* ($n = 6$)	0.63 ± 0.47 ($n = 6$)	0.005 ± 0.003	0.063 ± 0.047
Hairless guinea pig's abdomen (fresh)	22.89 ± 6.23* ($n = 5$)	2.96 ± 1.14 ($n = 5$)	2.29 ± 0.62	0.29 ± 0.11
Hairless guinea pig's abdomen (frozen[a])	18.82 ± 7.77* ($n = 6$)	4.45 ± 0.62 ($n = 6$)	1.88 ± 0.77	0.44 ± 0.06
Rat's abdomen (fresh)	31.66 ± 4.45* ($n = 6$)	2.73 ± 1.81 ($n = 6$)	3.17 ± 0.44	0.27 ± 0.18
Rat's abdomen (frozen[a])	22.73 ± 2.26* ($n = 6$)	2.66 ± 1.49 ($n = 6$)	2.27 ± 0.22	0.27 ± 0.15
Mouse's abdomen (fresh)	53.35 ± 8.19* ($n = 6$)	22.02 ± 3.70 ($n = 6$)	5.33 ± 0.82	2.20 ± 0.37
Hairless mouse's abdomen (fresh)	31.70 ± 3.83* ($n = 6$)	12.47 ± 3.04 ($n = 6$)	3.17 ± 0.38	1.25 ± 0.30

[a] Between 2 and 14 days at −20 °C.
[b] 12 h at 4 °C.
[c] 7 h after slaughtering (kept on ice).
* Statistically significant difference ($p < 0.05$, Student's t-test) from the data obtained after drug application by an aqueous vehicle.

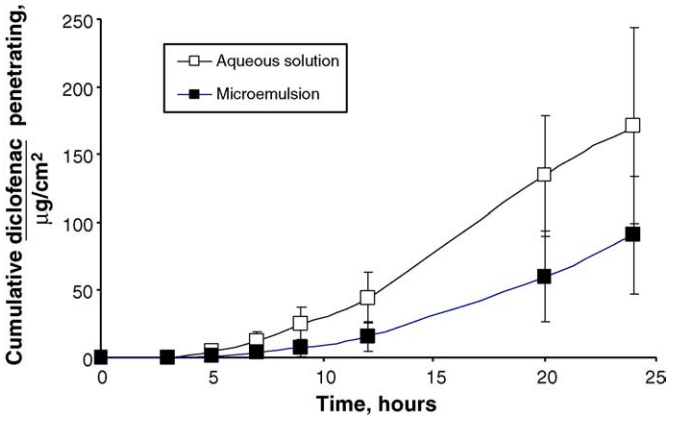

Fig. 4. In vitro percutaneous penetration of diclofenac through ear's porcine skin (frozen) after application of microemulsion and plain aqueous solution ($n = 20$).

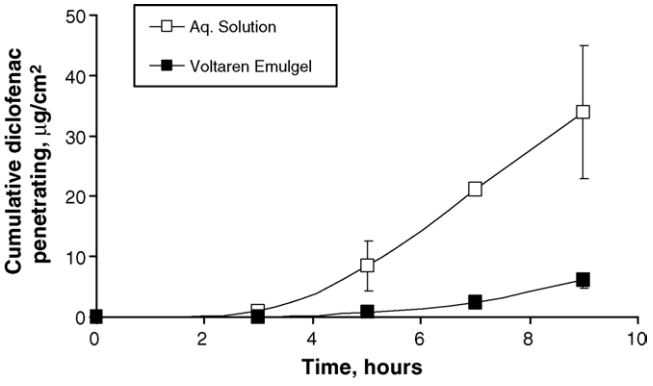

Fig. 5. In vitro percutaneous penetration of diclofenac through ear's porcine skin (frozen) after application of Voltaren Emulgel (Novartis) and plain aqueous solution ($n = 3$).

probably inhibited drug penetration through the frozen skin as compared with the penetration determined for diclofenac using plain aqueous solution. In another comparative study, it has been shown that ibuprofen sodium, which was dissolved in the same microemulsion vehicle as used for diclofenac (16%, w/w), penetrated at a lower rate than the rate measured after its application as a 16% aqueous solution (Fig. 6). The com-

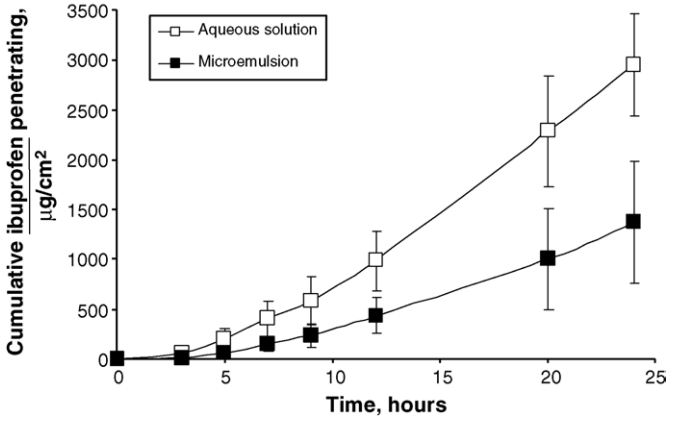

Fig. 6. In vitro percutaneous penetration of ibuprofen through ear's porcine skin (frozen) after application of microemulsion and plain aqueous solution ($n = 10$).

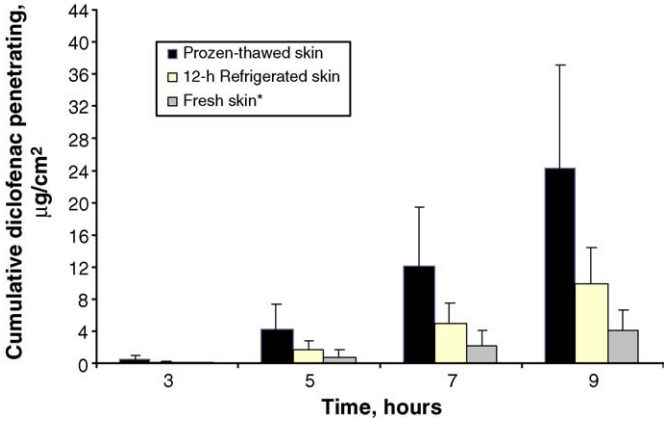

Fig. 7. In vitro percutaneous penetration of diclofenac through ear's porcine skin after application of plain aqueous solution. A comparison was made between fresh skin taken 7 h after pig's slaughtering (gray bars), 12-hr refrigerated skin (open bars) and frozen skin (black bars).

mon attribute of these results is that diclofenac and ibuprofen molecules in water can penetrate more easily and freely through the frozen porcine skin in vitro than drug in lipophilic vehicles. Since ibuprofen has demonstrated a similar phenomenon as obtained by diclofenac, it seems that the mechanism by which hydrophilic drug penetrates the skin does not principally depend on the drug, but is mainly based on skin–vehicle interactions. A possible argument that the shunt pathway may play a prominent role in this case, i.e., the microemulsion might have penetrated specifically through the hair follicles in hairy skin (rat and mouse), has been utterly negated by the comparison made between hairless and furry mice (Table 1). In an attempt to explain this abnormal phenomenon, "fresh" (7 h on ice after animal death) and 12-h refrigerated (4 °C) skin excised from pig's ears were tested for diclofenac penetration and compared to the frozen skin. As can be seen in Fig. 7, the cumulative permeating quantities of diclofenac increased significantly with the decrease in the storage temperature of the skin. Penetration fluxes of diclofenac increased from 1.12 ± 0.45 to 2.05 ± 0.96 and to 5.80 ± 2.38 μg/cm^2/h with application of aqueous drug solutions on fresh, refrigerated, and frozen porcine skin, respectively (Table 1). Interestingly, application of the microemulsion vehicle resulted in similar drug penetration fluxes regardless of skin conditions, i.e., no matter if the skin was fresh or had been stored at low temperatures. This may imply that after the freezing and thawing procedure, the lipid pathways in the skin do not practically change while the aqueous pathways alone in the lipid bilayers are modified and become more diffusible to water influx. This may explain why only the drug in aqueous solution (rather than drug in lipophilic vehicles) penetrated more rapidly through frozen skin than through fresh skin. Measurements of the transepidermal water loss after thawing of frozen skin samples showed an increase by 1.84 ± 0.48, 1.60 ± 0.51 and 1.69 ± 0.46 folds after 1, 2 and 3 h at conditioned room temperature, respectively, compared to the same samples before freezing ($n = 18$, $p < 0.05$). This significant increase in the water evaporation rate indicates that frozen skin became more permeable to water. Since the barrier to water transport is one of

the most essential properties of the stratum corneum, numerous studies have dealt with its composition and structure. After evidence has been provided, it is now well-established that the intercellular bilayered structure is responsible for this barrier (Elias, 1983; Friberg and Kayali, 1989; Friberg et al., 1990; Kayali et al., 1991). There are several possible mechanisms that may explain changes in the barrier properties of the skin: (a) perturbation of the lamellar lipid bilayers resulting in enhancement of hydrophilic and lipophilic drug penetration, (b) formation of macroscopic fractures and cracks that expose the polar groups in the bilayer, thus decrease the resistance of the skin towards water transport (Friberg and Kayali, 1989), and (c) loose cohesiveness of corneocytes due to modification/degradation of structural proteins in the interstices (Elias, 2004). The latter mechanism is based on Menon and Elias (1997), who suggested that an aqueous, expansible "pore" penetration pathway is created and bypasses the skin common barrier. We have found that although the permeability to aqueous solutions was modified in frozen skin, the permeability to hydrophobic vehicles (e.g., microemulsions) did not significantly change (Table 1). It may be assumed therefore that the frozen skin loses its resistance to water penetration by either mechanisms (b) or (c) or even both; however, the intercellular layered structure and the stratum corneum lipids remain intact. Mechanism (a) or perturbation of the lipid lamellar arrangement, however, is not related to the skin storage condition but rather connected to a possible disruption of the liquid crystalline structure of the bilayers (Friberg, 1990). The apparent difference between species, in which frozen rat and mouse skin did not demonstrate a significantly decreased resistance toward water penetration as found in frozen pig skin and frozen hairless guinea pig skin, still remained to be explained.

Besides the practical reasons, porcine ear skin has frequently been used as in vitro model that simulates penetration to and through human skin (Simon and Maibach, 2000; Schmook et al., 2001), However, a more recent study by Jacobi et al. (2005), who compared the penetration of flufenamic acid in porcine and human skin in vitro, has shown that the drug concentrations detected in human skin layers were smaller than in porcine tissue. In addition to the various differences in skin structure of rodents, pig, and human, which could make differences in the permeability of the skin to drugs, our data further indicate that differences may also be created at various storage conditions of the skin. Further work should be performed with human skin to compare and understand the processes occurring to the skin during storage.

4. Conclusion

In conclusion, based upon results for diclofenac transdermal penetration, it was found that topical delivery from the microemulsion vehicle studies was highly effective in vivo and in vitro. The transdermal administration of the microemulsion to rats resulted in 8-fold higher drug plasma levels than those obtained after application of Voltaren Emulgel. Drug plasma levels obtained during 4–8 h following topical microemulsion application were comparable to the peak plasma level obtained 1 h after subcutaneous administration of 3.5 mg/kg drug. The transdermal fluxes of diclofenac were also measured in vitro using skin excised from different animal species. The penetration fluxes obtained following the application of the microemulsion to fresh skin excised from different animal species were significantly higher than those obtained by application of the drug in aqueous solution. Finally, this report is one additional work that joins to a series of studies substantiating the advantage of the microemulsion system in enhancement of cutaneous drug permeability.

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