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Effects of penetration enhancers on *in vitro* permeability of meloxicam gels

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Abstract

Meloxicam (MLX) was formulated as a 0.3% hydroxypropylcellulose (Klucel®) gel. The effect of four different combinations of co-solvents (ethanol, glycol-PEG-400, propylene glycol, and water) on MLX permeability was determined *in vitro* throughout isopropyl myristate (IPM)-saturated cellulose membranes. The gel consisting of 2.5% Klucel, propylene glycol, ethanol, and water (1:1:1) showed superior permeability properties and it was selected as the base-gel to investigate the effect of three levels of the penetration enhancers: dimethylsulfoxide (1, 5, and 10% DMSO), tween20 (1, 2, and 5% TW20), oleic acid (0.4, 1, and 5% OA), and menthol (1, 2.5, and 5% MT). *In vitro* permeability was determined throughout IPM-saturated cellulose membranes and human cadaver skin. DMSO and TW20 did not improve permeability of MLX compared to the control gel at any of the levels tested. Menthol produced a statistically significant ($P < 0.001$), dose proportional increase in MLX flux with a peak at 5% (2.43 ± 0.47 μg/cm^2/h). Conversely, addition of OA peaked at 1% but decreased at the higher level (5%). There was no significant difference between the MLX amount recovered in stratum corneum and dermis across the different formulations tested. These findings show that the 0.3% MLX gel formulation containing 5% menthol can possibly deliver therapeutically relevant doses of MLX.

Keywords: Meloxicam; Skin permeability; Human cadaver skin; HPLC; Menthol

1. Introduction

Meloxicam (MLX) is a nonselective, nonsteroidal, anti-inflammatory drug (NSAID) with preferential inhibition of cyclo-oxygenase-2 (COX-2) over COX-1. MLX does not have documented cardiovascular toxicity at doses of less or equal to 15 mg/day which are recommended for the treatment of rheumatoid arthritis and osteoarthritis (Noble and Balfour, 1996). However, when orally administered nonselective NSAIDs may adversely affect the gastrointestinal tract and can even reduce the life expectancy of patients with rheumatoid arthritis (Whitehouse and Roberts, 1998). Transdermal delivery of MLX would avoid major gastrointestinal side effects and provide steady plasma levels from a single dose (Beetge et al., 2000). In addition, it has been demonstrated that NSAIDs promote local analgesia when administered locally through the skin (O'Hanlon et al., 1996).

The key barrier to transdermal drug delivery is the outermost layer of the skin, the stratum corneum (SC) (Ranade, 1991). The primary approach to overcome skin resistance to drug-penetration is the skillful selection of vehicles and penetration enhancers, substances that facilitate penetration by reversibly altering the structure of the skin.

In this study, MLX was formulated as a gel because of the favorable properties of this topical type of formulation. The effect of four different combinations of co-solvent (ethanol, PEG 400, propylene glycol, and water) was tested in order to select the one with the most promising properties and use it as the base for the testing of penetration enhancers. Four types of penetration enhancers were tested: the lipid disrupting agent oleic acid (OA) that increases the fluidity of SC lipids; the aprotic solvent dimethyl sulphoxide (DMSO) that denature proteins, change the intercellular keratin conformation, and interact with the intercellular lipid to distort their geometry; the non-ionic surfactant tween20 that can solubilise lipids within the SC; the terpene

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menthol that modify the solvent nature of the SC and usually decrease the lag time for permeation (Addicks et al., 1990).

2. Materials and methods

2.1. Chemicals

Meloxicam (MLX) was a gift from Boehringer Ingelheim, Biberach, Germany. Dulbecco's phosphate buffered saline, disodium ethylenediaminetetraacetic acid (Na_2EDTA), dimethyl sulfoxide (DMSO), sodium hydroxide (pellet), and oleic acid were from Sigma–Aldrich, St. Louis, MO. HPLC grade methanol, phosphoric acid, tween20, and alcohol-anhydrous, reagents were from J.T. Baker, Philipsburg, NJ. HPLC grade water was from EM Science, Gibbstown, NJ. Propylene glycol, isopropyl myristate, and L-menthol (3-P-methanol) crystal USP were from Spectrum Chemical MFG. CORP., New Brunswick NJ. Klucel® was from Hercules Inc., Wilmington, DE. Polyethylene glycol 400 was from Union Carbide Chemical, Bound Brook, NJ. Spectra/Por® 7 regenerated cellulose membranes were from Spectrum laboratories, Inc., Laguna Hills, CA.

2.2. Quantification of meloxicam

MLX was determined by High Performance Liquid Chromatography (HPLC) using a modified method from Choi (2005). The HPLC system consisted of a Water 717 plus Auto Sampler, Hitachi L-4250 UV–VIS Detector, Hitachi L-6200A Intelligent Pump, Perkin Elmer Nelson 900 series Interface, and the Perkin Elmer Software TotalChrom® navigator (PE Nelson-Version 6.2.1) data handling system.

Separation was achieved on a C18 column (Ascentis™, 10 cm × 4.6 mm, 3 μm particle size) eluted with a mobile phase consisting of methanol, water and phosphoric acid (69.9:30:0.1) delivered at a flow rate of 1 mL/min. Detection wavelength was 370 nm. Auto-sampler's temperature was kept at 12 °C. The injection volume was 20 μL. Typical retention time was 2.8–3.1 min. Peak area was used to determine MLX concentration. Calibration curves were linear in the range 0.1–100 μg/mL ($r^2 > 0.99$). The CV (%) for inter-day assays at 0.100 μg/mL (lowest limit of quantification LLOQ) and 100 μg/mL were 2.8 and 0.59, respectively.

2.3. Gel formulations

2.3.1. Selection of co-solvents

Klucel® was dispersed in a mixture of ethanol and Na_2EDTA under continuous magnetic stirring until it was homogeneous. MLX (300 mg) was then added to 30 g polyethylene glycol 400 (formulation #1), or to 30 g propylene glycol (formulation #2), or to 47 g of propylene glycol (formulation #3), or to 30 g of propylene glycol (formulation #4). Then 0.342 mL of a 10% NaOH solution were added to the dispersion and stirred until a clear solution was obtained. This MLX solution was then incorporated into the Klucel® gel base by stirring continuously until a homogeneous gel was obtained. USP Ethanol was added to adjust the total weight to 100 g for formulations #1 and #2, whereas a solution of ethanol/water was used to adjust the weight to 100 g for formulations #3 and #4. Table 1 reports the exact composition of the four gels. Formulation #4 gave the best results and it was selected as the control gel for the permeation enhancer studies. The following levels of permeation enhancers were tested: oleic acid (0.4, 1, and 5%), tween20 (1, 2, and 5%), menthol (1, 2.5, and 5%) and DMSO (1, 5, and 10%). The amount of distilled water added was varied to make a sufficient quantity to 100 g.

Table 1
Compositions of base-gel formulations (%, w/w)

	F #1	*F* #2	*F* #3	*F* #4
Meloxicam	0.3	0.3	0.3	0.3
Klucel®	2.5	2.5	2.5	2.5
10% (w/v) NaOH[a]	0.342	0.342	0.342	0.342
0.3% (w/v) Na_2EDTA[a]	5.0	5.0	5.0	5.0
Polyethylene glycol 400	30	–	–	–
Propylene glycol	–	30	47	30
Ethanol	62	62	30	30
Distilled water	–	–	15	31

[a] Considering density = 1 g/mL.

2.4. In vitro permeability studies through cellulose membrane saturated with isopropyl myristate (IPM)

In vitro diffusion studies were carried out using Modified Franz Diffusion Cell Apparatus with a diameter of 15 mm and a diffusional area of 1.76 cm^2. A Spectra/Por®7 (Laguna Hills, CA) regenerated cellulose membrane (thickness of 60–65 μm and a molecular weight cut-off of 1000) saturated with isopropyl myristate (IPM) was sandwiched between the lower cell reservoir and the glass cell-top containing the sample and secured in place with a pinch clamp. The receiving compartment (volume 13 mL) was filled with Dulbecco's phosphate buffered saline (DPBS) pH 7.4 (9.6 g/L). The system was maintained at 37 ± 0.5 °C by a water bath circulator and a jacket surrounding the cell, resulting in a membrane-surface temperature of 32 °C (McVean and Liebler, 1997; Suwanpidokkul et al., 2004). A Teflon™ coated magnetic bar continuously stirred the receiving medium to avoid diffusion layer effects. A 0.5 g sample was placed evenly on the surface of the membrane in the donor compartment that was sealed with aluminum foil and Parafilm® to prevent evaporation. Three hundred and fifty microlitres of receptor fluid were withdrawn from the receiving compartment at 15, 30, 45 min and 1, 1.5, 2, 3, 4, 6 and 8 h and replaced with 350 μL of fresh solution. Samples were added with 150 μL of methanol and 20 μL injected onto the HPLC.

2.5. In vitro permeability studies through human cadaver skin

The same apparatus and experimental procedure described in the previous section were used to perform human cadaver skin permeability studies. Full-thickness dermatomed human cadaver skin taken from the back region of Caucasian subjects was from the Ohio valley Tissue and Skin Center (Cincinnati,

Ohio). This skin was frozen in a 10% glycerol solution by the supplier and stored at −70 °C until the experiments. Cadaver skin was used immediately after thawing and prepared by hydrating it in Dulbecco's phosphate buffered saline pH 7.4 for 30 min at room temperature before being cut into a piece with a surface area of approximately 3 cm × 4 cm. Integrity of the skin was checked by visual examination of specimens and inspection of the cumulative amount versus time profiles to ensure that no holes or other imperfections were present. The thickness of the skin was measured by using a Starrett® 25 mm outside micrometer (K436 MRL-25) with 0.01 mm metric graduation. The skin was mounted between the donor and receiving compartments of the diffusion cell using a metal clamp. The skin was set in place with the stratum corneum facing the donor compartment and the lower skin side facing the receptor compartment. The receiving compartment was filled with DPBS pH 7.4 mixed with 0.1% gentamycin as an antimicrobial agent. The sampling times were 6, 8, 10, 12, 24, 30, 36 and 48 h.

2.6. Quantification of MLX in stratum corneum and dermis

At the end of the experiment (48 h), the gel remaining on the skin surface was removed by gentle washing with DPBS pH 7.4 to prepare the SC for the tape-stripping procedure. Adhesive tapes (Scotch 3 M Gloss Finish Transparent Tape) were applied with uniform pressure on the skin surface then removed for 35 times. MLX was extracted from the adhesive tapes using a solution of 70% DPBS pH 7.4 and 30% methanol and then sonicated for 15 min. This solution was transferred to a 5-mL volumetric flask and adjusted to volume with a solution of 70% DPBS pH 7.4 and 30% methanol. Before transferring to the HPLC sampling vials, the solution was filtered through VWR™ sterile syringe filters with 0.2 μm cellulose acetate membranes. The remaining skin was cut into small pieces and ground with mortar and pestle. A solution of 70% DPBS pH 7.4 and 30% methanol was added to extract MLX. The solution was transferred to a 25-mL volumetric flask, sonicated for 20 min and its MLX content determined by HPLC.

2.7. Data analysis

Data obtained by HPLC analysis were corrected for sampling effects. Cumulative quantity of MLX collected in the receiver (μg/cm^2) was plotted as a function of time. The flux value (J_{ss}, μg/cm^2/h) for each experiment was obtained from the slope (steady-state portion) of the linear portion of the data fitted by regression analysis (Komatsu and Suzuki, 1979). Lag time (L) was determined from the X-intercept of the regression line. The apparent permeability coefficient (P, cm/h) was obtained by dividing J_{ss} by the donor concentration (C_d) according to Fick's First Law of diffusion. For release data analysis, cumulative permeation (μg/cm^2) was plotted as a function of square root of time, where linearity is indicative of first order release (Higuchi, 1962). Release rate was estimated as the slope of such plots (μg/cm^2/$t^{0.5}$). Penetration-enhancing activities were expressed as enhancement ratios (ER), i.e., the ratio of the flux value with enhancer to that obtained without enhancer. Means, standard deviation (S.D.), coefficient of variation (%CV), and linear regression analyses were calculated using Microsoft Excel 2000. Analysis of variance (ANOVA), LSD post hoc test, and Box-plots were performed with SPSS 10.1 for Windows 2000.

3. Results and discussion

The present investigation was carried out to explore the possibility to deliver through the skin therapeutical effective amounts of a gel formulation of MLX, a potent nonselective NSAID prescribed for the chronic treatment of rheumatoid arthritis and osteoarthritis. The formulation of a gel requires a significant portion of liquid phase. MLX is poorly soluble in water (0.012 mg/mL) and has a good solubility in lipophilic solvents (Seedher and Bhatia, 2003) as suggested by the high value of the octanol–water partition coefficient (log P = 1.9). Consequently, it was necessary to dissolve MLX in a mixture of co-solvents and to add an equimolar amount of NaOH to achieve the desired concentration of 0.3% (w/w) in the final formulation. Commercially available topical preparations of piroxicam, a drug with a molecular structure and a pharmacological activity similar to MLX, have strength of 0.5–1.5%. Since MLX is two to three times more potent than piroxicam (Engelhardt et al., 1995) we selected a 0.3% strength, assuming we could obtain similar permeability rates.

Several different combinations of the co-solvents ethanol, water, propylene glycol, PEG 400 were investigated and the four most representative were selected on the bases of appearance, consistency, and ability to maintain MLX in solution. Formulations #1 and #2 were almost anhydrous and contained a high percentage of ethanol (62%) and 30% of PEG 400 or propylene glycol, respectively. Formulations #3 and #4 contained only 30% of ethanol, increasing percentages of water (15 and 31%), and different percentages of propylene glycols (47 and 30%) (Table 1). The permeability properties of these four combinations of co-solvents were then investigated *in vitro* in order to select the one with the most promising properties and use it as the base-gel for testing of penetration enhancers.

Steady-state fluxes (μg/cm^2/h) were (mean ± S.D.): 39.4 ± 0.19 for F #1, 52.8 ± 1.92 for F #2, 72.2 ± 0.28 for F #3, and 94.0 ± 1.93 for F #4. The permeability profiles of the four formulations are shown in Fig. 1. Solubility of MLX in its lipophilic form (free-base) at 25 °C is 0.012, 0.354, 0.307 and 3.76 mg/mL in water, ethanol, propylene glycol and PEG 400, respectively (Seedher and Bhatia, 2003). The results of this study show that as the solubility of MLX free-base decreased in the co-solvent mixture, flux through the IPM-saturated membrane increased. A high solubility in the vehicle lowers the chemical potential of a permeant and decreases the membrane/vehicle partition coefficient. Therefore, flux increases accordingly with the increasing chemical potential of MLX in the vehicles. Viscosity also decreased from F #1 to F #4 and might improve diffusivity of MLX within the gel and facilitate flux. Having the largest flux, gel formulation #4 was selected as the control gel for the following set of studies in which the effect of penetration enhancers was evaluated.

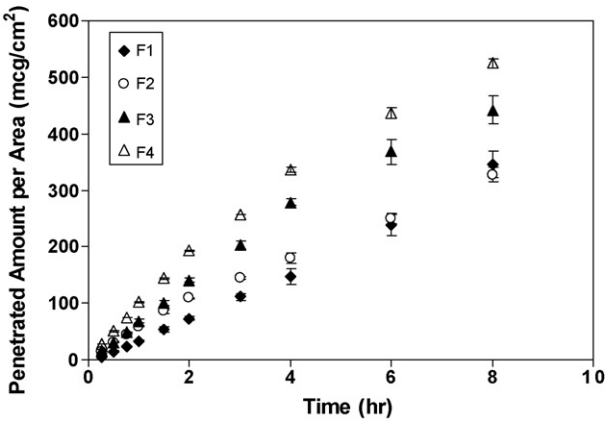

Fig. 1. Effects of co-solvents on the permeation profiles of MLX from gel formulation #1, 2, 3, and 4 through cellulose membrane saturated with IPM ($n = 3$; mean ± S.D.).

The addition of permeation-enhancing compounds to transdermal delivery systems may improve the penetration of drugs by modifying the thermodynamic activity of penetrants (e.g., changes in partitioning tendencies) or by altering the skin barrier properties (e.g., changes in fluidity of extracellular lipids). We studied the effect of four penetration enhancers (DMSO, tween20, oleic acid, and menthol) at three different concentrations each on the flux and diffusion characteristics of *F* #4 selected from the previous studies. All these penetration enhancers alter the stratum corneum at different levels. DMSO may possibly cause elution of DMSO-soluble components from the SC, denature proteins, change the intercellular keratin conformation, tween20 may emulsify lipids in SC and also promote the formation of micelles in the formulation, oleic acid may disrupt SC lipids and increase the formation of water channels, and finally menthol, a terpene, may cause a reversible disruption of the lipid domain and promote the formation of new polar channels. The presence of penetration enhancers may also change the thermodynamic activity of the drug (MLX) in the vehicle and consequently alter its permeability. To sort out these two different effects, the formulations containing penetration enhancers were first evaluated on cellulose membrane saturated with isopropyl myristate and then on human cadaver skin. In this way, we could detect possible negative interaction between the base-gel and the penetration enhancers (Penzes et al., 2005). Fig. 2 shows the permeation profiles. Table 2 reports flux, permeability coefficients, lag times, release rate (drug released per unit membrane area versus square root of time) and diffusion coefficient from these studies. The addition of tween20 and oleic acid significantly decreased the flux of MLX compared to the control gel ($P < 0.05$) whereas DMSO and menthol did not show a statistically significant effect ($P > 0.05$). Oleic acid at the highest concentration (5%, w/w) showed the maximum decrease in permeation followed by the highest concentration of tween20 (5%, w/w). The release profiles of the gels followed matrix diffusion kinetics (Higuchi, 1962) confirming that MLX was fully dissolved or suspended in the gel and, thus, the membrane used has no significant effect on the release of the drug.

Finally, the effects of penetration enhancers were tested on human cadaver skin as a barrier to assess which interaction with the SC is most favorable to MLX penetration.

The permeability profiles obtained are shown in Fig. 3. Table 3 reports the calculated steady-state flux (J_{ss}), permeability coefficient (k_p), lag time, and diffusion coefficients (D). Table 4 reports the enhancement ratio calculated for each level of penetration enhancer. The thickness of the human cadaver

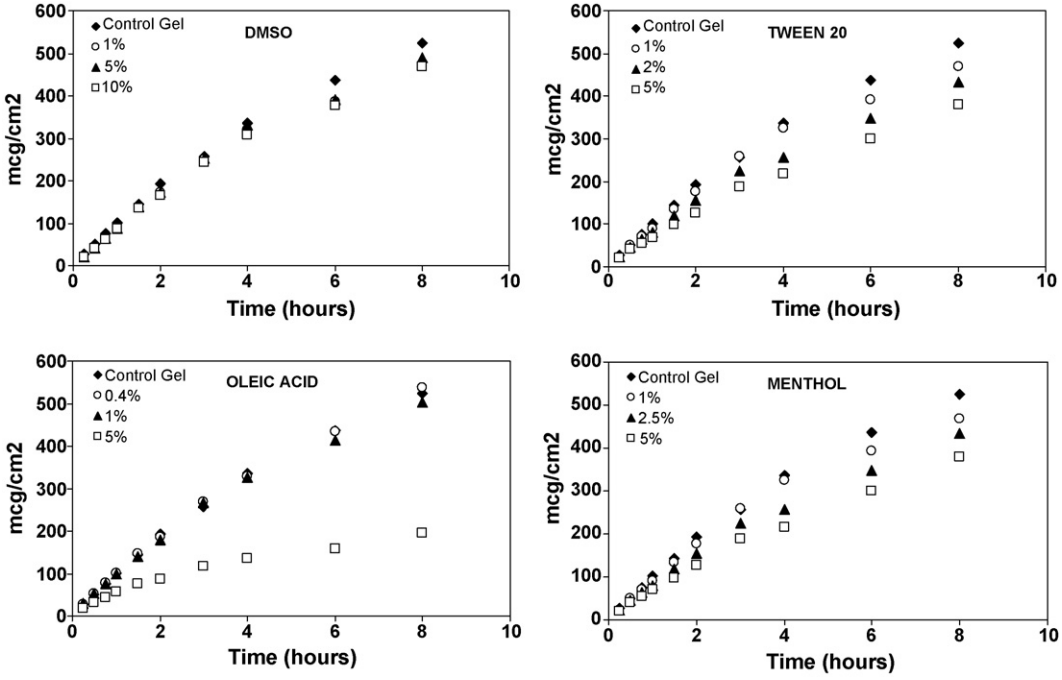

Fig. 2. Permeation profiles of meloxicam from gel formulations through IPM-saturated cellulose membranes at 32 °C. Data are presented as the average of two replicates.

Table 2
Flux (J_{ss}), permeability coefficient (k_p), lag time, release rate (RR), and diffusion coefficient (D) of MLX-gels through cellulose membrane saturated with IPM

		J_{ss} (μg/cm^2/h)	k_p (cm/h) × 10^{-2}	Lag time (h)	RR (μg/cm^2 h$^{1/2}$)	D (cm^2/h) × 10^{-3}
Gel control ($n=3$)		85.0 ± 1.57	2.83 ± 0.06	−0.14 ± 0.001	221 ± 10	4.26 ± 0.40
DMSO	1%	86.1; 82.0	2.87; 2.73	0.00; −0.07	210; 194	3.85; 3.27
	5%	85.9; 84.1	2.86; 2.80	−0.02; −0.02	206; 211	3.71; 3.87
	10%	82.0; 81.4	2.73; 2.71	−0.07; −0.01	203; 189	3.60; 3.12
TW20	1%	87.7; 81.9	2.92; 2.73	−0.03; −0.11	205; 197	3.66; 3.37
	2%	73.9; 72.1	2.46; 2.40	−0.08; −0.12	180; 177	2.82; 2.74
	5%	61.1; 58.5	2.04; 1.95	−0.13; −0.15	157; 152	2.15; 2.03
OA	0.4%	91.2; 82.7	3.04; 2.76	−0.10; −0.16	217; 229	4.11; 4.59
	1%	87.4; 84.4	2.91; 2.81	−0.12; −0.11	211; 210	3.87; 3.86
	5%	36.5; 33.8	1.22; 1.13	−0.46; −0.48	79.2; 72.3	0.55; 0.46
MT	1%	83.8; 81.1	2.79; 2.70	−0.12; −0.15	220; 213	4.24; 3.98
	2.5%	82.0; 78.0	2.73; 2.60	−0.16; −0.23	206; 190	3.71; 3.13
	5%	83.0; 75.9	2.77; 2.53	−0.19; −0.22	201; 187	3.52; 3.04

For experiments with two replicates both of them are presented separated by a semicolon.

skin ranged from 0.5 to 1.2 mm (mean ± S.D.: 0.87 ± 0.16 mm) and no correlation was found between permeability parameters and skin thickness. Presence of menthol produced a dose proportional ($r^2 = 0.99$) increase on MLX flux and permeability coefficient. The highest content of menthol (5%, w/w) also gave the highest enhancement ratio (27.5) followed by the 2.5% (10.3). The addition of oleic acid did not affect permeability in a dose dependent manner. The presence of 1% OA produced a six-fold increase in permeability. Conversely, at the 5% (w/w) level, enhancement ratio decreased to 5. This confirms the finding from IPM-saturated cellulose membrane that a high concentration of OA reduces release from the gel. Gels containing DMSO and tween20 showed no significant difference between the flux and permeability coefficient of MLX compared to the control gel.

DMSO is a dipolar aprotic solvent which is miscible with both water and organic solvents. It has the ability to accelerate the skin permeation of a wide variety of compounds including steroids, salicylates, and antimycotics (Walters, 1989). Although these improvements are often obtained at high strengths of DMSO (>60%) (Franz et al., 1995) we preferred to test low concentrations of DMSO because we were concerned with the toxicity of DMSO at high concentrations. Low concentrations of DMSO were reported to improve penetration for the drug piroxicam (Babar et al., 1990), prazosin (Reddy and Ghosh, 2001), and methyl nicotinate (Remane et al., 2006). However, we did not

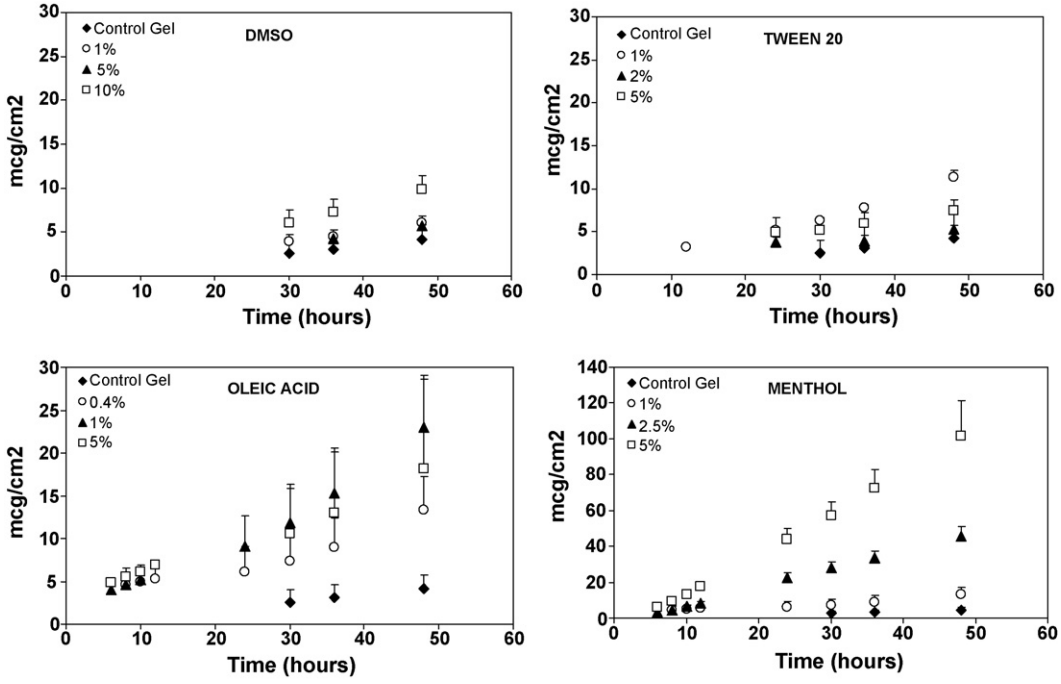

Fig. 3. Permeation profiles of meloxicam from gel formulations through human cadaver skin at 32 °C. Data represent mean ± S.D. ($n=3$).

Table 3
Flux (J_{ss}), permeability coefficient (k_p), lag time, and diffusion coefficient (D) of MLX-gels through human cadaver skin

		J_{ss} (μg/cm^2/h)	k_p (cm/h) × 10^{-5}	Lag time (h)	D (cm^2/h) × 10^{-6}	Stratum corneum %[a]	Skin residue %
Gel control		0.09 ± 0.02	3.03 ± 0.74	1.64 ± 16.2	0.12 ± 0.05	0.80 ± 0.32	4.75 ± 1.34
DMSO	1%	0.12 ± 0.00	3.89 ± 0.10	−3.33 ± 7.24	0.18 ± 0.01	0.63 ± 0.49	4.85 ± 1.20
	5%	0.11 ± 0.03	4.53 ± 1.41	−3.15 ± 8.58	0.18 ± 0.10	0.35 ± 0.14	4.56 ± 1.70
	10%	0.20 ± 0.02	7.07 ± 0.65	−0.99 ± 8.15	0.50 ± 0.10	0.33 ± 0.09	3.17 ± 0.36
TW20	1%	0.26 ± 0.02	8.61 ± 0.67	5.20 ± 0.58	0.83 ± 0.12	0.72 ± 0.30	6.70 ± 3.46
	2%	0.10 ± 0.03	3.08 ± 1.22	−2.36 ± 7.84	0.13 ± 0.08	0.46 ± 0.09	4.30 ± 2.99
	5%	0.13 ± 0.02	3.90 ± 0.06	−12.0 ± 17.0	0.21 ± 0.06	0.37 ± 0.17	4.21 ± 3.58
OA	0.4%	0.31 ± 0.04	11.1 ± 1.65	5.82 ± 9.18	1.17 ± 0.37	0.35 ± 0.10	3.52 ± 1.54
	1%	0.59 ± 0.07	18.4 ± 2.91	9.68 ± 4.21	4.28 ± 1.22	0.51 ± 0.25	5.33 ± 3.43
	5%	0.43 ± 0.04	14.9 ± 1.65	−5.45 ± 4.42	2.32 ± 0.54	0.26 ± 0.10	2.67 ± 1.51
MT	1%	0.34 ± 0.05	12.4 ± 2.10	9.83 ± 5.80	1.42 ± 0.49	0.69 ± 0.39	3.98 ± 1.40
	2.5%	0.98 ± 0.14	31.2 ± 5.89	1.03 ± 4.92	12.10 ± 4.01	0.61 ± 0.18	2.89 ± 2.34
	5%	2.43 ± 0.47	83.3 ± 19.2	5.79 ± 2.21	66.6 ± 41.8	0.51 ± 0.42	4.61 ± 1.17

The % remaining at $t = 48$ h in the stratum corneum and skin residue are also presented. Data are reported as mean ± S.D. ($n = 3$).
[a] Percentage of total recovery.

see any enhancing effect of DMSO on permeability of MLX ($P > 0.05$).

The addition of tween20 at any concentrations did not have a significant effect on flux, permeability coefficient, release rate, and diffusion coefficient compared to the control gel. Low concentrations of surfactants may emulsify stratum corneum lipids and improve permeability, however higher concentrations promote the formation of micelles in the vehicle that trap the permeant and decreases permeability. Results obtained from our studies confirm this hypothesis. In fact, permeability rate of MLX was reduced through IPM-saturated cellulose membrane as the percentage of tween20 increased. Conversely, there was no significantly different effect of tween20 on the permeability rate of MLX through human cadaver skin because the emulsification of stratum corneum lipids probably balanced the entrapment of MLX into micelles resulting in no changes of the overall permeability properties.

Oleic acid is an unsaturated fatty acid (18: $1^{\Delta 9}$). It can interfere with the SC permeability barrier either (a) by forming pools of fluid within the stratum corneum (Aungst, 1995) or (b) by disrupting the molecular packing of the lipid matrix (Francoeur et al., 1990; Naik et al., 1995). Results from this study indicate that the addition of 1 and 5% (w/w) oleic acid significantly increased the flux, permeability coefficient, and release rate of MLX through human cadaver skin compared to the control gel ($P < 0.05$). The enhancement ratios were found to be 6.09 and 4.92 for 1 and 5% (w/w) oleic acid, respectively. At the lowest concentration of OA (0.4%, w/w), there was an increase in flux and permeability coefficient (enhancement ratio: 3.65) but they were not statistically different from the control gel ($P > 0.05$). Apparently, permeability of MLX increases with concentration of OA up to 1% then decreases (Table 3) and then decrease. A large amount of oleic acid may hinder the partitioning of MLX out of the base-gel to the stratum corneum. This explanation is supported by the decreased permeability of MLX through IPM-saturated cellulose membrane at 5% (w/w) OA (enhancement ratio: 0.41). The results obtained are consistent with the study of Mortazavi and Aboofazeli (2003) who reported that the presence of 1% (w/w) oleic acid had the greatest effect on the flux of piroxicam. When the amount of oleic acid was increased from 1 to 5% (w/w), a decrease in flux was observed. In addition, Santoyo et al. (1995) found that the flux of piroxicam increased with increasing oleic acid concentration, reached a maximum at 5% (w/w), and then decreased at 10% OA (w/w) level.

Table 4
Enhancement ratio (ER) of various amounts of penetration enhancers incorporated in the 0.3% (w/w) MLX gel through cellulose membrane saturated with IPM and human cadaver skin

		IPM-cellulose membrane	Human cadaver skin
DMSO	1%	0.99	1.28
	5%	1.00	1.50
	10%	0.96	2.33
TW20	1%	1.00	2.84
	2%	0.86	1.02
	5%	0.70	1.29
OA	0.4%	1.02	3.65
	1%	1.01	6.09
	5%	0.41	4.92
MT	1%	0.97	4.08
	2.5%	0.94	10.3
	5%	0.93	27.5

L-Menthol is a terpene compound containing alcohols that has been widely used as skin penetration enhancers for a variety of compounds. Menthol was selected for our studies because it is also a refrigerant agent that induces a strong cooling sensation when applied to the skin and numbs the sensation of pain (Hashida and Yamashita, 1995), for this reason, it may provide an advantage for analgesic topical formulations. Out of all enhancers used in our studies, menthol at the concentration of 5% (w/w) has shown maximum permeation enhancement effect ($P < 0.001$) with an enhancement ratio of 27.5 followed by menthol at the concentration of 2.5% (w/w) ($P < 0.001$) with an enhancement ratio of 10.3. The addition of 1% (w/w) menthol

also increased the flux of MLX by 4.1-fold; however, there was no statistical difference compared to the control gel ($P > 0.05$). The mechanism of action of terpenes has been intensively studied. Kunta et al. (1997) reported that permeation enhancement of menthol could involve its distribution into the intercellular space of stratum corneum and the possible reversible disruption of the intercellular lipid domain. This would increase drug diffusivity. It is unclear whether the terpenes affect drug partitioning or not. From the permeability studies through IPM-saturated cellulose it is clear that L-menthol did not affect release from the gel. It should be noted that the gel contained 30% ethanol and that several studies demonstrated a synergistic action of ethanol with terpenes (Kobayashi et al., 1994).

Amounts of MLX retained in the stratum corneum (tape-stripping experiments) and in dermis were similar across all penetration enhancers and the control gel experiments (Table 3). They accounted for 4–6% of the applied dose showing that the different formulations did not affect MLX retention in the skin. Total recoveries from the diffusion experiments were determined by adding the amount of MLX from skin-surface wash (gel remaining on the skin surface), stratum corneum (tape stripping), dermis (skin residue), and receptor fluid at 24 h and were expressed as percentage of the applied dose as shown in Table 3. Total recoveries were similar across formulations (ANOVA) and accounted for 83% of total dose. Recoveries from gel formulations and solutions were always about 100%. However, recovery efficiency of the method used to extract MLX from dermis and SC was not determined therefore non-recoverable MLX may be the fraction trapped in the skin.

The average plasma concentration $C_{ave,ss}$ of MLX can be calculated from literature data as (Shargel et al., 2005):

$$C_{ave,ss} = \frac{AUC}{\tau} = \frac{F \times Dose}{CL} \times \frac{1}{\tau} = 0.533\ \mu g/mL$$

where the bioavailability factor (F) is 0.9, the dose is 7.5 mg, the dose interval (τ) is 24 h and the total body clearance (CL) is 528 mL/h (Ritschel and Kearns, 2004). $C_{ave,ss}$ represent a target average drug concentration that would maintain a desired pharmacological effect. Assuming that at steady state the elimination rate equals the input rate, the input-rate required to produce such a concentration can be calculated from the equation (Parikh and Ghosh, 2005):

$$\text{Input rate} = C_{ave,ss} \times CL = 281\ \mu g/h$$

In our studies, the highest flux (input rate) obtained from the addition of 5% (w/w) menthol was $2.43 \pm 0.47\ \mu g/cm^2/h$. Assuming that this flux could be reproduced *in vivo*, it can be estimated that a topical application area should be 116 cm^2 (10.8 cm × 10.8 cm). This is a reasonable size for a gel application.

In conclusion, this study shows that a MLX gel consisting of 2.5% Klucel® gel, 0.3% MLX, 5% menthol, and propylene glycol, ethanol, and water (1:1:1) can potentially deliver therapeutically relevant amounts of MLX through human skin.

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