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Effect of DC/mDC iontophoresis and terpenes on transdermal permeation of methotrexate: *In vitro* study

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Abstract

The systemic toxicity caused by methotrexate limits its use and transdermal delivery would be a possible alternative. Transdermal permeation of methotrexate loaded into polyacrylamide-based hydrogel patch, across mice skin was studied *in vitro* after pretreatment with terpenes and ethanol, alone or in combination with iontophoresis (DC/mDC). Polyacrylamide patches gave the maximum flux as compared to the copolymers of acrylamide and acrylic acid. Of the terpenes used, pure menthol showed maximum enhancement (38%), whereas pure limonene elicited a minimum of 9.9% enhancement. Binary combination of menthol and ethanol increased the permeation to 54.9%, which was further enhanced to 93.69% and 117% when used in combination with DC and square wave (mDC) iontophoresis, respectively. ATR-FTIR of the stratum corneum treated with terpenes showed a split in the asymmetric C–H stretching vibrations along with decrease in peak heights and areas of asymmetric, symmetric C–H stretching, C=O stretching and amide bands. A split in amide II band was observed with iontophoresis. ATR-FTIR studies suggest conformational changes in the lipid–protein domains thereby increasing permeation. Histopathological studies on treated skin samples, gave an insight about the anatomical changes brought by the application of various enhancers. Binary mixture of menthol and ethanol in combination with square wave gave best results.

Keywords: Terpenes; Methotrexate; DC/mDC iontophoresis; ATR-FTIR; Skin histopathology

1. Introduction

Methotrexate (MTX) is a folic acid antagonist with antineoplastic activity. The action of MTX in psoriasis is through local inhibition of DNA synthesis leading to mitotic suppression in the psoriatic hyperplastic epidermis when administered systemically (Weinstein et al., 1971) and by intradermal injection (New Berger et al., 1978). Methotrexate is administered orally or by intravenous/intramuscular injections. Weekly single oral dose of 10–25 mg/week or divided oral dose of 2.5 mg at 12 h interval for three doses each week is given. In case of injections, 10–25 mg by single IV or IM injection, once a week, is given until adequate response is achieved[1]. After optimal response has been achieved, dosage should be adjusted and reduced to the lowest possible dose. However, the systemic use of this drug causes numerous side effects like hepatic toxicity, bone marrow depression, leucopenia, thrombocytopenia, anaemia, ulcerative stomatitis, nausea, abdominal distress, etc. (Bookbinder et al., 1984; VanDooren-Greebe et al., 1994). So, it is desirable to deliver MTX by the transdermal route. Various topical forms (creams, ointments) for psoriasis have been tried (Van Scott and Reinertson, 1959; Nurse, 1963; Comaish and Juhlin, 1969; Stewart et al., 1972; Bjerring et al., 1986), but were ineffective for desired clinical use. MTX is hydrosoluble and is in dissociated form at physiological pH, so its capacity for passive diffusion is limited. To overcome the well-known barrier property of stratum corneum (SC), various chemical and physical enhancing techniques alone or in combination have been explored to enhance the transdermal permeation of MTX. Among the chemical enhancers used are, *viz*. dimethylsulphoxide, dimethylacetamide, azone, polyethylene glycol, propylene glycol, isopropyl ethanol and pure ethanol (Singh and Singh, 1995; Chatterjee et al., 1997), but some of these agents have been reported to cause adverse tissue reactions (Kligman, 1965).

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[1] http://www.rxlist.com/cgi/generic/mtx_ids.html.

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The physical enhancing technique like iontophoresis (DC) has shown to improve the transdermal delivery of MTX either in solution form or through a hydrogel patch (Alvarez-Figueroa et al., 2001; Stagni and Shukla, 2003; Tiwari et al., 2003; Alvarez-Figueroa and Blanco-Mendez, 2001). It has been reported that the iontophoretic delivery from hydrogels (Alvarez-Figueroa and Blanco-Mendez, 2001) was more effective than passive delivery from aqueous solution (Alvarez-Figueroa et al., 2001) of the drug. Syed et al. (2001), has reported that MTX 0.25% in hydrophilic gel was more effective than placebo and has reported that proper vehicle selection can improve the cutaneous penetration of MTX. Furthermore, it has been found that the drug release rates from a hydrogel can be controlled by changing the characteristics of the hydrogel during synthesis (Banga and Chien, 1993).

However, for delivery of proteins, genes and electrochemotherapy, the researchers are focusing on the different pulse waveforms like exponentially decaying pulses (Prausnitz et al., 1993; Pliquett and Weaver, 1996), square wave pulses (Denet and Préat, 2003; Heller et al., 1999) and these have shown better enhancement in drug permeation as compared to DC iontophoresis. The aim of the present research was to have a comprehensive study on the effect of different waveforms (mDC iontophoresis), *viz*. square, sine, triangle and exponentially decaying pulses and DC iontophoresis alone or in combination with terpenes/ethanol on transdermal permeation of MTX loaded in poylacrylamide hydrogel patch and also to study the anatomical changes brought up by their application onto mice skin morphology. In the present study GRAS category and FDA approved chemical enhancers, *viz*. terpenes (limonene, cineole and menthol) and ethanol either pure or in combination has been employed. Although pig skin is considered to be closest to human but due to ethical committee constraints we have chosen mice skin as a model.

2. Materials and methods

2.1. Reagents

Methotrexate(MTX) was a gift sample from Dabur (New Delhi, India), Acrylamide was obtained from Spectrochem (Mumbai, India), Acrylic acid from G.S. Chemicals (Mumbai, India), *N*,*N*-methylene bis acrylamide, potassium chloride and potassium dihydrogen phosphate from Sisco (Mumbai, India), sodium chloride from E. Merck (Mumbai, India), di-hydrogen-*o*-phosphate anhydrous from Qualikems fine chemicals (Mumbai, India), sodium hydroxide from Excelar (Mumbai, India), ammonium persulphate from Thomas Baker (Mumbai, India) and sodium metabisulphite from S.D. fine chemicals (Mumbai, India), Ethanol from Merck (Germany), cineole and limonene from Acros organics (New Jersey, USA) and menthol from Sigma chemicals (USA). All other chemicals used were of analytical grade. Deionised water having a resistivity of 18 MΩ or greater was used to prepare all solutions and buffers.

2.2. MTX hydrogels

The hydrogel patches were synthesized using acrylamide/acrylic acid monomer by solution polymerization method using redox initiators (0.2 mol% of the total weight of the monomer) and cross-linking agent *N*,*N*-methylene bis acrylamide (0.4 mol% of the total weight of monomer). The solution was bubbled with nitrogen gas for 5 min and poured into glass mould and allowed to polymerize at 40 °C. Various copolymers of acrylamide (Am) and acrylic acid (Ac) were synthesized. The monomers were taken in different proportions (Am:Ac; 1:1, 1:2, 1:3, 1:4, 2:1, 3:1, 4:1, 5:1, 6:1) with increasing and decreasing acrylic acid content. Homopolymers of polyacrylamide of varying cross-linking concentration (0.3–1.0 mol%) were synthesized similarly and repeatedly washed with double distilled water to remove the residual monomers. MTX (3 mg) was dissolved in 0.1N sodium hydroxide solution. The hydrogel patches, circular in shape with diameter of 2.3 cm were dipped in drug solution for 24 h for drug loading. Care was taken that no excess drug remained on the surface of the hydrogel patch by dipping them for a second in distilled water (30 °C) to remove the excess drug, if any on the surface of the hydrogel patch. It was observed that approximately 1 mg of the drug remains in the patch after 24 h permeation of MTX.

2.3. Skin preparation

All experiments were conducted according to the protocol approved by the Institutional Animal Ethics Committee (IAEC) of All India Institute of Medical Sciences (AIIMS) New Delhi, India. White albino mice ($n = 5$ in each group) were procured from AIIMS and sacrificed. The hair was removed from the dorsal portion using an animal hair clipper and the full-thickness skin was excised from the abdominal region. Fat adhering to the dermis side was cleaned by using a blunt scalpel and isopropyl alcohol, taking care not to damage the skin. Finally, the skin was washed in tap water and observed physically for any gross damage (Pillai and Panchagnula, 2004). The fresh skin was used, for *in vitro*, ATR-FTIR and histopathological studies.

2.4. In vitro permeation studies from MTX hydrogels

The mice skin was clamped between the two half-cells of the modified vertical Franz diffusion cell with epidermis facing the donor chamber and the area available for permeation was 5.72 cm^2. The skin was equilibrated for 1 h with phosphate buffer saline (pH 7.4) in the receptor chamber and was magnetically stirred throughout the experiment at thermostatically maintained temperature (37 ± 2 °C).

To study the effect of chemical enhancers, 500 μl of pure or ethanolic solution of terpenes (Table 1) were applied to the skin for 1 h. The excess enhancer was removed and the MTX loaded hydrogel patch was placed on the skin. For iontophoretic experiments (DC/mDC, with frequency of 1 kHz), current of 0.2 mA/cm^2 density was applied to the drug loaded hydrogel patch placed over the skin through silver–silver chloride electrode for 1 h. Current was also applied to the chemical enhancer pretreated skin, to see the combinational effect of chemical enhancer and iontophoresis. Control experiments were done using a placebo and the samples were analysed for any other products leaching from the skin/hydrogel.

Table 1
Different groups of enhancers used on the mice skin for permeation, ATR-FTIR and histological study

Group I (chemical enhancer)	Group II (physical enhancer)	Group III (combination of chemical and physical enhancer)
A. Ethanol	A. DC iontophoresis	A. Chemical enhancer + DC iontophoresis ($0.2\,mA/cm^2$ current density)
(i) 50% ethanol	(i) $0.2\,mA/cm^2$ current density	(i) 50% ethanol
(ii) 75% ethanol		(ii) 75% ethanol
(iii) Pure ethanol		(iii) pure ethanol
		(iv) Limonene
		(v) Cineole
		(vi) Menthol
		(vii) Limonene in ethanol
		(viii) Cineole in ethanol
		(ix) Menthol in ethanol
B. Terpenes	B. mDC iontophoresis ($0.2\,mA/cm^2$ current density)	B. Chemical enhancer + square wave ($0.2\,mA/cm^2$ current density)
(i) Limonene	(i) Square wave	(i) Menthol
(ii) Cineole	(ii) Sine wave[a]	(ii) Menthol in ethanol
(iii) Menthol	(iii) Triangle wave[a]	
	(iv) Exponential wave[a]	
C. 5% terpene in 75% ethanol (v/v)		
(i) Limonene in ethanol		
(ii) Cineole in ethanol		
(iii) Menthol in ethanol		

[a] These waveforms were not tried in combination with chemical enhancers as their flux was lower to square wave.

2.5. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) studies

After the treatment of the skin samples with different enhancers mentioned in Table 1 for 1 h, the skin was dabbed on a tissue paper. The samples were then subjected to ATR-FTIR using (Bio-RAD, FTS 135, FT-IR spectrophotometer). The spectra were recorded in the region 4000–400 cm^{-1} with an average of 32 scans and 8 cm^{-1} resolution. The peak height and areas of C–H stretching, C=O stretching and amide peak absorbance were measured for each sample.

2.6. Histological examination

Albino mice skin was subjected to different enhancers as described in Table 1 for 1 h. The treated skin was fixed in 10% formalin and then subjected to processing for histological examination. The skin samples were dehydrated by a series of graded ethanol then treated with xylene and finally embedded in paraffin blocks. Skin sections of 5 μm thickness were cut and stained with haematoxylin–eosin (H&E). The mounting of the stained sections was done in DPX and observed under light microscope using a scoring system (Table 2) adapted from Ingram and Grasso, 1975; Lashmar et al., 1989 with slight modifications. The total histological score (THS) was found to be 62 by taking an average from five animals.

2.7. Quantification of MTX

For MTX quantification in receptor solution, 0.5 ml samples were withdrawn at specified intervals from the receiver compartment and analyzed for the amount of drug by UV–vis

Table 2
Histological assessment method for *in vitro* scoring

(A) Epidermal changes	
1 Thinning of epidermis	
1/2 thinning	5
Less than 1/2 thinning	10
2 Destruction of epidermis	
Less than 1/4 of sectioned area	15
1/4 of sectioned area	18
1/2 of sectioned area	20
3/4 of sectioned area	25
Whole of sectioned area	30
3 Spongiosis	
Slight	1
Extensive	2
Microvesicle formation	3
Bullae formation	4
(B) Dermal changes	
4 Fractured collagen	
Focal upper dermis (focal)	1
Diffuse upper dermis (mild)	2
Focal deep dermis (moderate)	3
Diffuse deep dermis (severe)	4
5 Dermal edema	
Focal upper dermis (focal)	2
Diffuse upper dermis (mild)	4
Focal deep dermis (moderate)	6
Diffuse deep dermis (severe)	8
6 Appendageal changes	
Mild damage	2
Focal marked damage	4
Diffuse marked damage or loss	6

spectrophotometer (CARY 100 model) at 302 nm. The samples were also analyzed by HPLC using Waters 1525 binary pump attached to UV detector (Alvarez-Figueroa et al., 2001).

2.8. Data treatment, statistical analysis and mathematical modeling

The *in vitro* skin permeation data obtained were plotted as the cumulative amount of drug penetrated into the receptor compartment as a function of time. The percent enhancement in flux was calculated as follows:

$$\%\ \text{enhancement in flux} = \frac{\text{flux with enhancer} - \text{passive flux}}{\text{passive flux}} \times 100$$

All experiments were repeated five times and the values are expressed as mean ± S.D. Statistical analysis were made using Wilcoxon–ranksum test and the significance level was set at $p < 0.05$.

For curve fitting, a polynomial expression, $Y = At^3 + Bt^2 + Ct + D$, where Y = total amount and t = time, was utilized for a best curve fit to compare the rate of permeation of the drug, methotrexate with different physical and chemical enhancers. The *R*-squared value was found for each curve.

3. Results and discussion

3.1. Effect of hydrogels

When the concentration of acrylamide (Am) was increased in the copolymer (0.4 mol% cross-linking concentration) from 1:1 to 6:1 (Am:Ac), the iontophoretic flux of MTX increased from (12.01 ± 0.6) to (18.76 ± 0.78) μg/(cm^2 h) serially, whereas homopolymer of Am (PAm) depicted highest flux of (20.56 ± 1.02) μg/(cm^2 h) (Fig. 1). Changing the cross-linking concentration of PAm from 0.3 mol% to 1.0 mol%, the flux increased from (17.2 ± 0.9) to (28.28 ± 1.4) μg/(cm^2 h). This behavior is expected due to the accumulation of the drug on the surface of the hydrogel patches having higher cross-linking concentration. The modulus of the hydrogel increases as the cross-linking concentration is increased, hence the capacity of the drug to penetrate the hydrogel and distribute uniformly decreases, and as a result more amount of drug accumulates on the surface. The flux obtained from 0.4 mol% was higher than 0.3 mol% and did not show any necrosis or edema when applied on the skin, thus suggesting compatibility. The decrease in iontophoretic flux with increasing Ac content could be due to the interactions of the amino group of MTX with the acid functionality of the copolymer. It was found that, hydrogel patch having higher concentration of Ac was sticky and difficult to handle. Based on the above results, PAm patch of 0.4 mol% cross-linking concentration was selected, for all experiments for drug release. The control experiments showed absence of any absorbance at 302 nm thus confirming no leaching products from the hydrogel or skin at 302 nm.

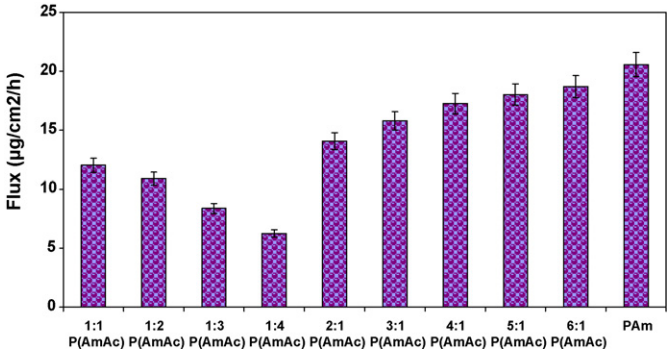

Fig. 1. Effect of copolymer composition on the iontophoretic permeation of methotrexate across mice skin.

3.2. Effect of chemical enhancers

Fig. 2a demonstrates the percent enhancement in flux of MTX with chemical enhancers (terpenes and ethanol) as pure or in combination. Out of the pure terpenes used, limonene gave

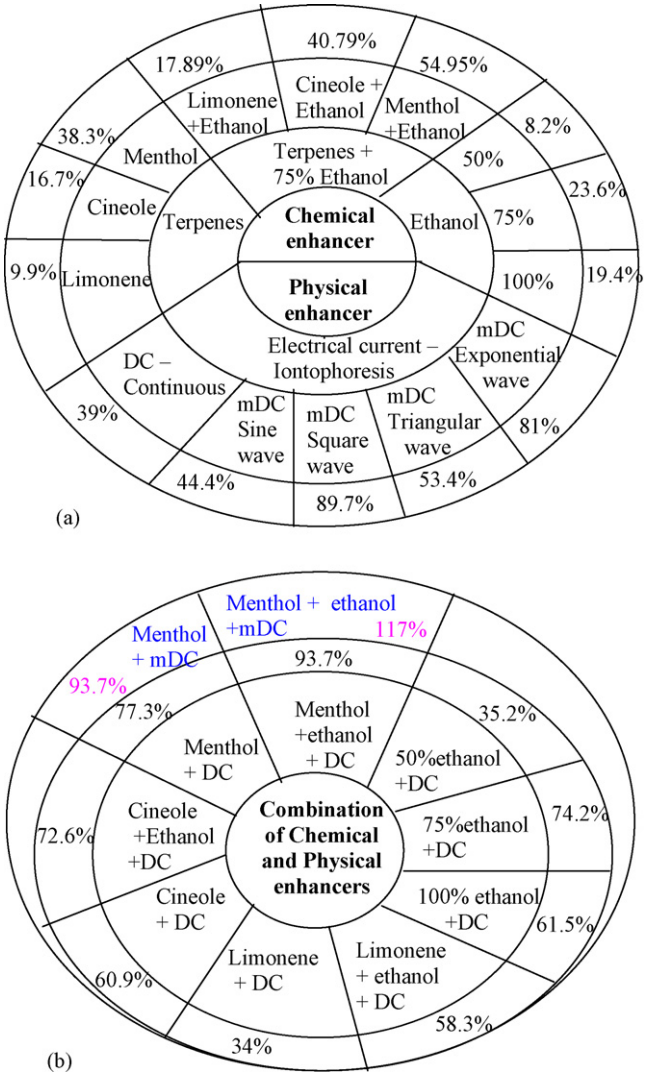

Fig. 2. (a) Different chemical and physical enhancers used along with % enhancement in flux and (b) different combination of chemical and physical enhancers used along with % enhancement in flux.

Table 3
Flux values obtained with different enhancers

Enhancer	Flux (μg/(cm^2 h))
Passive (control)	14.79 ± 0.72
50% ethanol	16.01 ± 0.80
75% ethanol	18.28 ± 0.92
100% ethanol	17.65 ± 0.85
Limonene	16.26 ± 0.83
Cineole	17.27 ± 0.89
Menthol	20.46 ± 0.98
Limonene + 75% ethanol	17.44 ± 0.90
Cineole + 75% ethanol	20.82 ± 1.10
Menthol + 75% ethanol	22.92 ± 1.17
DC iontophoresis	20.56 ± 1.12
mDC-sine wave	21.36 ± 1.16
mDC-square wave	28.06 ± 1.30
mDC-triangle wave	22.69 ± 1.13
mDC-exponential wave	26.79 ± 1.30
50% ethanol + DC	19.99 ± 0.99
75% ethanol + DC	25.77 ± 1.33
100% ethanol + DC	23.89 ± 1.27
Limonene + DC	19.86 ± 1.02
Cineole + DC	23.81 ± 1.20
Menthol + DC	26.22 ± 1.27
Limonene + 75% ethanol + DC	23.42 ± 1.24
Cineole + 75% ethanol + DC	25.53 ± 1.30
Menthol + 75% ethanol + DC	28.65 ± 1.44
Menthol + mDC (square wave)	28.65 ± 1.38
Menthol + 75% ethanol + mDC (square wave)	32.1 ± 1.45

minimum enhancement of 9%, and menthol gave maximum enhancement of 38% with respect to passive ($p < 0.05$). Ethanol was used in different proportions ranging from 50% to 100%. Although pure ethanol has been used by other research schools (Pillai et al., 2004; Pillai and Panchagnula, 2003), but in our studies, 75% ethanol demonstrated higher permeation. These findings support other studies conducted with ethanol (Megrab et al., 1995; Thomas and Panchagnula, 2003). The binary combination of terpene and ethanol showed higher flux as compared to their pure compositions. Ethanolic solution of menthol demonstrated maximum flux of (22.92 ± 1.17) μg/(cm^2 h) (Table 3) with 55% enhancement. The flux obtained was significantly different from the passive with $p < 0.05$.

The above behaviour can be explained as the terpenes act on the hydrogen bond network between the ceramides of the lipid bilayer of the skin. –OH group of menthol can accept or donate hydrogen bond, whereas in cineole, only hydrogen accepting moieties are present. Hence extent of disruption of hydrogen bond network is less and therefore permeation is less than menthol. Limonene shows least permeation as it has double bond and acts well with hydrophobic drugs (Jain et al., 2002).

Synergism in enhancement using terpene/ethanol combination has been reported earlier in case of LHRH (Bhatia and Singh, 1998). Weinstein et al., 1989 found that passive diffusion of 2% MTX solution across human skin was about 5 μg/(cm^2 h), whereas Aungst et al., 1990, reported a flux of 7.0 μg/(cm^2 h) from pure PG across human cadaver skin. Chatterjee et al., 1997, have reported a flux of (71.4 ± 18) μg/(cm^2 h) across hairless mice skin with 10% azone in PG system, which is much higher as compared to our results as azone causes complete delipidification of the SC barrier thus enhancing permeation. Whereas, with 100% ethanol, a flux of (1.59 ± 0.49) μg/(cm^2 h) has been reported. However, in our studies a flux (17.65 ± 0.85) μg/(cm^2 h) with 100% ethanol was achieved. The discrepancies in results among the similar chemical enhancers are due to the different permeability parameters, depending on the type and age of the mouse used.

ATR-FTIR spectrum of control and chemical enhancer treated mice skin are shown in Fig. 3a. The control sample demonstrated major absorption bands at 2852 cm^{-1} and 2920 cm^{-1} due to symmetric and asymmetric C–H stretching, respectively, ester band at 1744 cm^{-1}, a strong band at 1642 cm^{-1} due to C=O stretching vibrations of amide 1 band, and at 1545 cm^{-1} due to N–H bending vibrations of amide II. The ATR-FTIR spectrum of pure menthol and cineole pretreated SC depicted, a well-defined split in the asymmetric C–H stretching vibration into 2963 cm^{-1} and 2922 cm^{-1}, whereas no split was observed with limonene pretreatment. The difference in the results could be due to the hydrophilic, hydrophobic nature

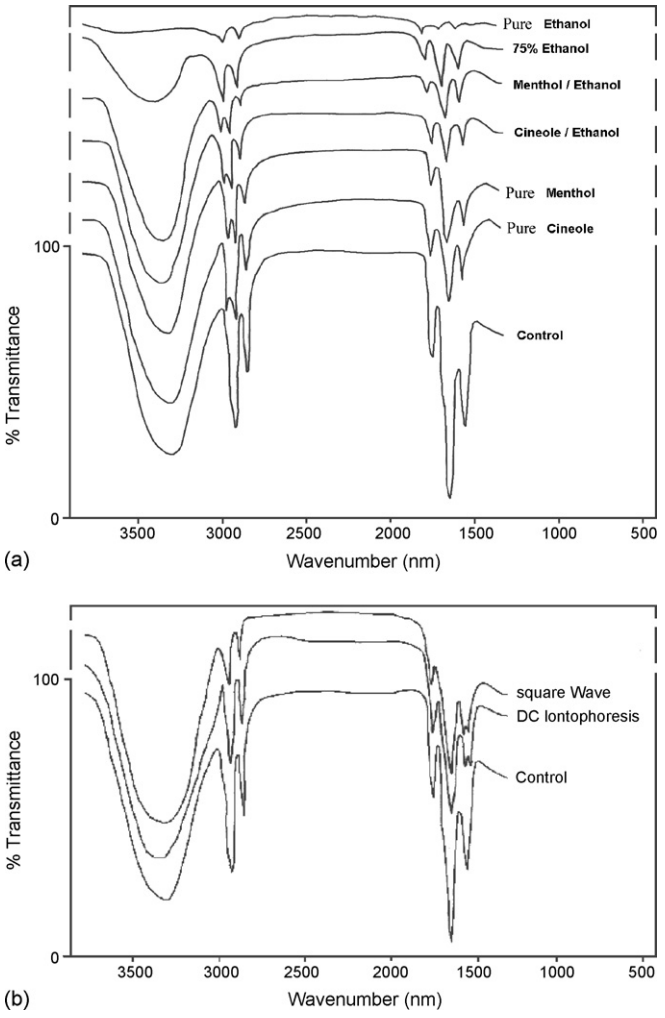

Fig. 3. ATR-FTIR spectra of mice skin: (a) effect of chemical enhancers, the spectra from top to bottom; pure ethanol, 75% ethanol, menthol in ethanol, cineole in ethanol, pure menthol, pure cineole, control and (b) effect of physical enhancers, the spectra from top to bottom are square wave (mDC), DC iontophoresis, control.

Table 4
Histological scoring based on the damages caused to the skin by different chemical enhancers

Chemical enhancer	Epidermal changes		Dermal changes			Total histological score (THS)
	Thinning of epidermis	Destruction of epidermis	Fractured collagen	Dermal edema	Appendageal changes	
Limonene	5	–	1	2	–	8
Cineole	5	–	1	2	–	8
Menthol	5	–	3	4	–	12
100% ethanol	5	20	3	6	2	36
75% ethanol	5	–	3	4	–	12
Cineole in ethanol	5	–	3	4	–	12
Menthol in ethanol	5	–	3	4–6	–	12–14

between menthol/cineole and limonene used but it is difficult to explain it on the basis of FTIR spectra alone. However, one can not exclude the possibility of change of conformation from eclipsed to gauge conformation giving rise to the two absorptional bands instead of one, thus decreasing the skin resistance and enhancing drug permeation. The binary combination of menthol/or cineole in ethanol, demonstrated vibrational bands of lower intensity as compared to their pure compositions (Fig. 3a).

Treatment with pure ethanol showed absence of O–H vibrations, shoulder of amide bands and the other peaks were weak in intensity. A sharp peak of ethanol that has partitioned into the SC was observed at 1053 cm^{-1} whereas with 75% ethanol, amide band, hydroxyl stretching and other bands were observed but have reduced intensity as compared to the control. The presence of water does not allow dehydration and complete removal of lipids. Furthermore, it has been reported (Vaddi et al., 2002) that ethanol causes removal of amide I and II bands when a pretreatment time of 12 h was given, however in our studies, a shoulder of amide bands was noticed with treatment time of 1 h. A lengthy pretreatment time with any chemical enhancer does not simulate the clinical situation and hence a lesser treatment time was adopted.

The extent of injury caused to the skin is dependent on the functionality of the enhancers and it was observed that the transdermal permeation increases with increase in histological score except with pure ethanol. The total histological score (THS) based on histological assessment of the various enhancers employed is given in Table 4 and Fig. 4 depicts, histological photomicrographs showing changes due to chemical enhancers alone or in combination with iontophoresis. The control sample showed well-defined stratum corneum, epidermis, appendages and only slight edema. Histopathological studies revealed that terpenes form pools in the skin (Fig. 4a), proving their uptake. The histological changes were less when cineole

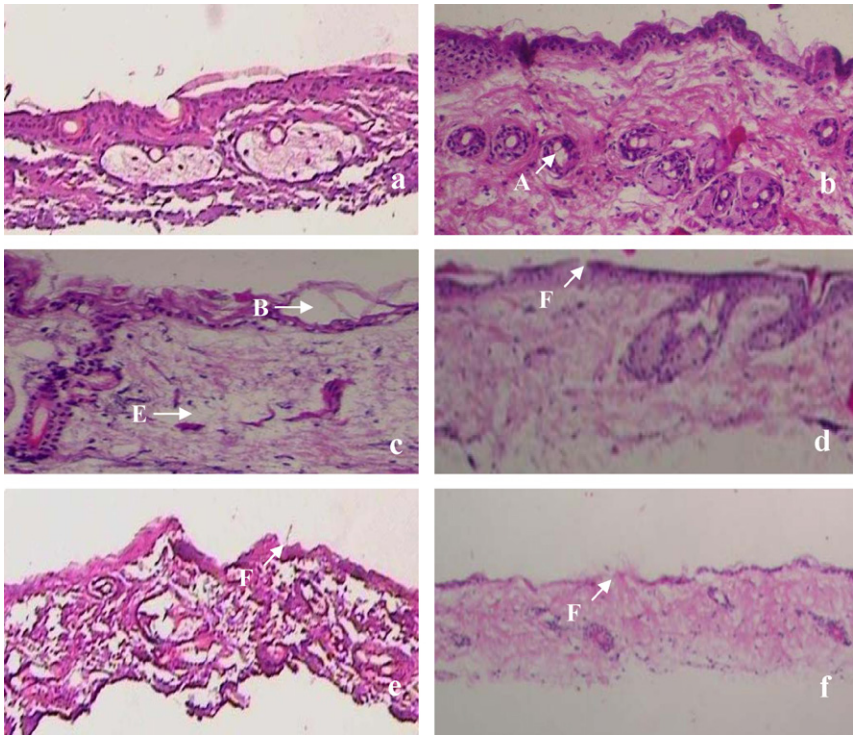

Fig. 4. Histological pictures of mice skin treated with various enhancers: (a) menthol; (b) ethanol 75%; (c) DC iontophoresis; (d) mDC(square wave); (e) menthol + DC iontophoresis; (f) menthol + square wave; where A, appendageal dilatation; E, edema; F, focal disruption of epidermis; B, bullae formation (H&E 200×).

(THS 8) was used as compared to menthol (THS 12). This can be attributed to the non-polar nature of cineole as compared to menthol. When pure ethanol (100%) was used as a penetration enhancer, there was significant destruction of the epidermis with partial loss and thinning, dilatation of appendages and fractured collagen (THS 36), whereas destruction of epidermal layer was absent with 75% ethanol (THS 12); however, epidermal thinning (Fig. 4b) was observed which can be correlated to changes in the conformations of the lipid bilayer, that is responsible for epidermal thinning. It has been seen in the permeation studies that the flux obtained with pure ethanol was lesser as compared to 75% ethanol, which could be attributed due to the dehydration caused by the pure ethanol as compared to 75% ethanol. These findings support other studies conducted with ethanol to enhance the permeation of estradiol and zidovudine (Megrab et al., 1995; Thomas and Panchagnula, 2003). The changes caused were in decreasing order, pure ethanol > menthol in ethanol > menthol = ethanol 75% = cineole in ethanol > cineole = limonene.

3.3. Effect of electrical current

The effect of iontophoresis (DC/mDC) on the *in vitro* permeation of MTX is shown in Fig. 2a. mDC iontophoresis using square wave pulses gave maximum enhancement of 89.72% with respect to passive, with flux of $(28.06 \pm 1.3)\ \mu g/(cm^2\ h)$ (Table 3), followed by exponential, triangle, sine and DC, respectively ($p < 0.05$). Alvarez-Figueroa and Blanco-Mendez (2001), have reported a cumulative delivery of $(1.69 \pm 0.42)\ \mu g/(cm^2\ h)$ in 10 h with acrylic acid hydrogel and $1.39\ \mu g/(cm^2\ h)$ with copolymer (1:1) of acrylic acid and acrylamide, using pig skin. The variation obtained, could be due to the permeability differences between the skin of animals used and also the nature of hydrogels employed. To best of our knowledge the effect of various wave forms has not been studied for the delivery of MTX. Based on the above observations, in all the experiments square wave was employed for mDC iontophoresis.

The ATR-FTIR spectra of mDC/DC iontophoresis showed a phenomenal split in amide II band into $1553\ cm^{-1}$ and $1541\ cm^{-1}$ (Fig. 3b). The split could be due to the disruption in hydrogen bonding associated with the head of ceramides, resulting in loosening of lipid–protein domains thus allowing higher flux as compared to the passive treatment. A higher decrease in peak height and area was noticed with all other vibrational bands in mDC iontophoresis as compared to DC. Additionally, mDC iontophoresis avoids polarization of the skin, thus increasing permeation (Odia et al., 1996; Johnson et al., 1998).

Histopathological studies with DC iontophoresis, revealed, focal disruptions of epidermis (less than 1/4 of sectioned area), 1/2 thinning of the epidermis and spongiosis with bullae formation (Fig. 4c). In the dermis, severe fractured collagen with moderate edema and mild appendageal damage was noticed (THS 36). The mDC current modes, *viz*. square (Fig. 4d), exponential, sine and triangle wave demonstrated comparable damage except that spongiosis showed microvesicle formation and therefore, marginally reduced histological scores (THS 35) as compared to the DC mode. Although, the damage caused by mDC were less as compared to DC, the flux obtained by mDC was found greater. This reverse behavior could be due to two reasons, firstly, mDC iontophoresis reduces skin resistance more effectively than DC and secondly, the current is being given in pulses, which depolarizes the skin. It has been reported (Denet et al., 2004; Dujardin et al., 2002), that square wave pulses induce a mild impairment of the barrier function of the skin but do not alter the viability of the skin. The decreased skin resistance is reflected in the increased permeability of the skin and therefore increased flux. Results of ATR-FTIR and histopathology further supports higher permeation with square wave than DC.

3.4. Effect of combination of chemical enhancer and current

A combination of iontophoresis and chemical enhancers allows greater amounts of drug to be delivered than either technique employed alone. It has also been reported that, the combinations of enhancers are often more effective compared to each of them used alone (Mitragotri, 2000, 2004). Since, sufficient permeation of MTX was not obtained when limonene was used, either alone or in combination with iontophoresis, the studies were focused on the effect of pure and ethanolic solution of menthol and cineole in combination with DC/or mDC iontophoresis using square wave.

Maximum enhancement of 117% with a flux of $(32.1 \pm 1.45)\ \mu g/(cm^2\ h)$ (Table 3) was obtained with ethanolic solution of menthol in combination to mDC (square wave), whereas 93.7% enhancement was obtained with pure menthol in combination with square wave (Fig. 2b). Flux was significantly different from passive ($p < 0.05$). With DC iontophoresis, the enhancement reduced to 77%, 61% and 74% with pure menthol, cineole and 75% ethanol, respectively, whereas, 93.7% and 72.6% enhancement was obtained with ethanolic solution of menthol and cineole, respectively (Fig. 2b).

The ATR-FTIR spectrum of ethanolic solution of menthol in combination with DC iontophoresis depicted split in the asymmetric C–H stretching vibrations and amide II band as well as reduce in the intensity of all other vibrational bands. However, with mDC iontophoresis the intensity of the peaks was further reduced. In case of cineole, the intensity of the other peaks was higher than those in the spectra of ethanolic solution of menthol. Histopathological studies as shown in Fig. 4e, revealed more destruction of epidermis with DC iontophoresis in comparison to mDC using square wave (Fig. 4f). However, flux shows the reverse relationship and is more with mDC. The reason for the above relationship has been described previously.

3.5. Mathematical modelling

A third order polynomial was chosen because data could not be presented by a quadratic. A higher order polynomial did not yield significant improvement in the least square error and was therefore avoided in the present analysis. The results are presented in Fig. 5a and b. Fig. 5a shows the effect of different chemical enhancers on the net permeation of methotrexate at a given time calculated by third order polynomial curve fitting.

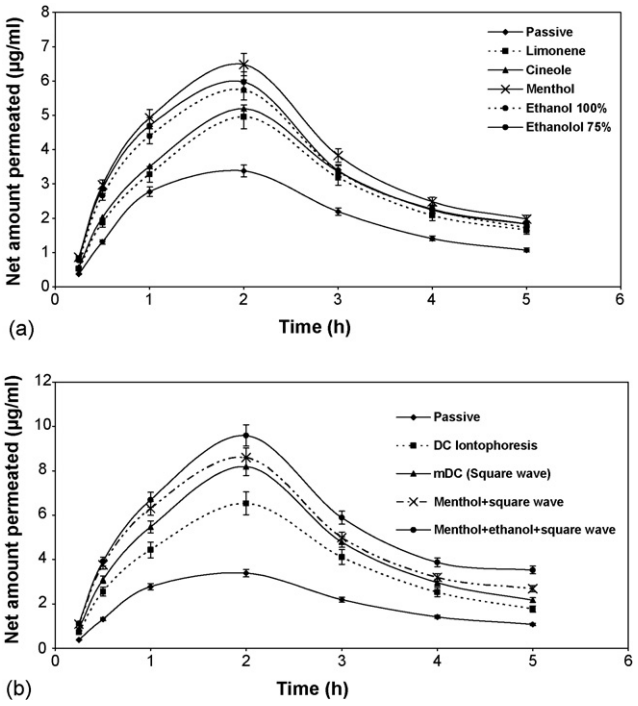

Fig. 5. Polynomial curve fitting: (a) effect of chemical enhancers and (b) effect of physical enhancer and combinational effect of chemical and physical enhancer.

The *R*-squared value for each curve was >0.99, thus indicating a good correlation between experimental and polynomial simulation. From the curve it can be clearly seen that with the passive experiments, that is, only with drug loaded hydrogel patch, we get an increase in permeation till 2 h and then there is decline and steady state is obtained. Here pore channel diffusion mechanism is taking place and drug diffuses via the least resistant pathway. After sometime, saturation level us reached and hence there is fall in permeation. Moreover, the swelling pattern of the hydrogel is Fickian in nature, which further supports that diffusion is the main mechanism of drug release. The effect of simple geometry on drug release is described by power–law model presented as follows:

$$M_t = M_\infty t^n$$

where M_t and M_∞ are the respective mass of drug release at time t, infinity and n is the diffusion exponent. Information about the release mechanism can be gained by fitting the drug release data and comparing the value of n to the semi-empirical value of various geometrys (Peppas and Ritger, 1987). The diffusion exponent for the drug release curve in water at pH 7.4 was found to be 0.47 ($n < 0.5$) thereby indicating that Fickian diffusion plays an important role in drug release from these hydrogels.

However, the hydrogel itself is not sufficient to achieve the desired drug levels (Chatterjee et al., 1997) hence permeation enhancers have been employed. It can be seen that there is sharp rise in permeation with the enhancers and then steady level is reached. These enhancers, terpenes and ethanol act mainly on the lipids and fluidise them thus reducing paracellular as well as intercellular pathways resistance. The rise is more with combination of physical and chemical enhancers used. This is in accordance to the lipid extraction caused by these enhancers. The ATR-FTIR and histopathological studies also support this.

4. Conclusion

From the results obtained, it can be concluded that square wave in combination with ethanolic solution of menthol gives the maximum enhancement of 117% with flux of (32.1 ± 1.45) µg/(cm^2 h) as compared to all other combinations. The use of combinational strategy allows maximum % enhancement, which could be due to the changes brought up in the conformation as well as hydrogen bonding in the lipid–protein domains of SC, as is evident from a split at C–H asymmetric stretching and amide II band in the ATR-FTIR spectra. The histopathological studies, showed maximum skin damage with ethanolic solution of menthol in combination with iontophoresis thus facilitating greater permeation. The permeation results obtained are encouraging and the work is further being extended on human cadaver skin.

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