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Studies on in vitro and in vivo transdermal flux enhancement of methotrexate by a combinational approach in comparison to oral delivery

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Abstract

Background: Methotrexate (MTX) causes systemic toxicity thereby limiting its use; hence, transdermal delivery would be a possible alternative. *Method*: A comparative in vitro/in vivo study was done to see the effect of the two-tier system of chemical and physical enhancers. MTX was loaded into polyacrylamide-based hydrogel patch to see the effect of enhancers. *Result*: Flux enhancement (161%) of MTX was achieved when ternary mixture of ethyl acetate:menthol:ethanol (1:1:1) was used in combination with square-wave iontophoresis for 1hour. Lower flux enhancement of 71%, 83%, and 93.5% was obtained in vitro with neat ethyl acetate, its binary composition with ethanol, and its ternary composition with ethanol and menthol, respectively, as compared to passive. However, with square-wave iontophoresis, it increased to 126%, 140%, and 161%, respectively. The mechanism of flux enhancement was supported by biophysical tools such as attenuated total reflectance–Fourier transform infrared spectroscopy (ATR–FTIR), scanning electron microscopy (SEM), and histopathology. ATR–FTIR studies demonstrated split in the asymmetric C–H vibration and amide II band with terpenes and iontophoresis, respectively. Additionally binary and ternary mixture of ethyl acetate demonstrated absence of ester peak accounting for lipid extraction. SEM of the skin samples treated with chemical enhancers in combination with square-wave iontophoresis showed both swelling and increased pore size of hair follicles, thus supporting higher permeation. Histopathological studies on treated skin samples of albino mice demonstrated epidermal thinning and focal disruptions, spongiosis, dermal edema, and appendageal dilatations. In vivo studies on mice demonstrated plasma concentration of 18.79μg/mL with ternary mixture of ethyl acetate in combination with square wave, which is twofold higher to oral delivery. The reversibility studies conducted in vivo on mice demonstrated that the histological changes induced by the above-mentioned enhancers were transient and reversible in 48 hours. *Conclusion*: The above results indicate that the above-mentioned enhancers are safe and well tolerated by the skin.

Key words: *ATR–FTIR; chemical enhancers; mDC iontophoresis; methotrexate; SEM; skin histopathology; transdermal delivery*

Introduction

Transdermal delivery of drugs, a noninvasive route of drug administration through the skin is one of the important areas of current research as it offers a number of advantages as compared to oral or parenteral route[1]. For the past two decades, the skin has gained importance as a means for the topical, regional, and systemic application of drugs. However, human skin is a remarkably efficient barrier, thus, presenting difficulties for the transdermal delivery of therapeutic agents[2]. Very few drugs having small-molecular weight such as scopolamine, nitroglycerin, nicotine, clonidine, fentanyl, estradiol, testosterone, lidocaine, and oxybutinin have been successfully delivered through transdermal patches[3,4]. However, it is difficult to transport ionized and high-molecular-weight therapeutic

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agents due to the impermeability of stratum corneum (SC), without any physical or chemical techniques of enhancement. Hence, various chemical[5,6], physical enhancement methods like iontophoresis[7–9], electroporation[10], ultrasound[11,12], and microneedles[13] have been investigated to overcome the barrier property of SC and thus enhance transdermal drug absorption.

Direct current (DC) iontophoresis with a constant current approach is the most common form of transdermal iontophoretic drug delivery[14,15]. However, it has been suggested that mDC iontophoresis can eliminate potential electrochemical burns and reduce skin irritation and sensation that would otherwise occur during long iontophoresis application[16,17]. The advantage of using mDC iontophoresis over DC is that it depolarizes the skin and reduces skin impedance more effectively, thus increasing permeation.

Methotrexate (MTX) is an antineoplastic agent that inhibits enzyme dihydrofolate reductase, inhibiting DNA synthesis and therefore has been used as a candidate for the treatment of psoriasis and rheumatoid arthritis at low doses and in high dose for the treatment of cancer[18]. Major side effects like hepatic toxicity, thrombocytopenia, fatigue, and anemia are caused by the systemic use of this drug[19]. Therefore, the transdermal route would be an effective alternative to deliver MTX and to reduce the side effects caused by oral delivery, but the passive diffusion of this drug is very limited. Therefore in this study, a combinational approach of flux enhancement has been carried out.

Hydrogels have attracted increasing attention because of their three-dimensional stability as well as their intrinsic network characteristics, which form a basis for swelling, adhesiveness, biocompatibility, and sustained/controlled drug release[20,21]. MTX formulation as a hydrogel patch facilitates sustained drug release[22] in contrast to solution or gel[23]. Various researchers have reported the delivery of MTX by ointments, creams, gels, microemulsions, hydrogels, chemical enhancers, and DC iontophoresis[18,22–30]. Our earlier publication[31] demonstrated the advantages of mDC iontophoresis on transdermal delivery. This study describes an extension of the work using a combination approach and its comparative study. Therefore, the aim of this study was to investigate the permeation enhancement in vitro as well as in vivo using ternary compositions of ethyl acetate/ethanol/terpene in combination with square wave on permeation of MTX across mice skin both in vitro and in vivo. A comparison between oral and transdermal delivery of MTX was done based on in vivo studies to assess the feasibility of its transdermal delivery. Additionally, a detailed study on the histopathological effects of different enhancers on skin was carried out by light microscopy. The recovery of the skin changes was assessed after 24 and 48 hours of the application of enhancers. A lot of work on transdermal delivery of drugs and the use of chemical and physical enhancers for permeation enhancement has been reported. However, enough attention has not been paid on the histocompatibility of these various physical and chemical enhancers. To the best of our knowledge, no comprehensive and comparative study on the usage of various combinations of the above-mentioned physical and chemical enhancers, with respect to the injury pattern of the skin, has yet been reported. This study summarizes the epidermal and dermal changes caused by the treatment of the different enhancers by histopathological assessment as well as reversibility of these skin changes with in vivo model.

Materials and methods

Reagents

MTX was a gift sample from Dabur India Ltd. (Kaushambi, Ghaziabad, India). Acrylamide was obtained from Spectrochem (Scientific Trading Company, Shakurpur, Delhi, India). *N*,*N*-Methylene-bis-acrylamide, potassium chloride, and potassium dihydrogen phosphate from Sisco Research Laboratory (Scientific Trading Company, Shakurpur, Delhi, India), sodium chloride from E. Merck (Scientific Trading Company, Shakurpur, Delhi, India), di-hydrogen-o-phosphate anhydrous from Qualikems (Scientific Trading Company, Shakurpur, Delhi, India), sodium hydroxide from Excelar (Scientific Trading Company, Shakurpur, Delhi, India), ammonium persulfate from Thomas Baker (Scientific Trading Company, Shakurpur, Delhi, India), and sodium metabisulfite from SD Fine Chemicals (Scientific Trading Company, Shakurpur, Delhi, India). Ethanol was obtained from Merck (Darmstadt, Germany), ethyl acetate from Excelar (St. Louis, MO, India), and menthol from Fluka, Sigma Chemicals (USA). All other chemicals used were of analytical grade. Deionized water having a resistivity of 18 MΩ or greater was used to prepare all solutions and buffers.

MTX hydrogels

The hydrogel patches were synthesized using acrylamide monomer by solution polymerization method as described earlier by Prasad et al.[31]

Characterization of hydrogels

Physical parameters—hardness test

The hydrogels after drying were subjected to hardness test using Shore D hardness testing machine.

Swelling

Weighed amount of hydrogel (PAm) was put in swelling medium (pH 7.4). At intervals, the swollen gels were taken out from the swelling medium, blotted dry, and weighed. The swelling studies were carried out for a maximum

period of 48 hours. Percent swelling was calculated using the following equation:

$$\text{Percent swelling} = \frac{W_s - W_d}{W_d} \times 100,$$

where W_s is the weight of swollen polymer and W_d is the initial weight of dry polymer.

The kinetics of swelling behavior was fitted into Peppas model ($M_t/M_\infty = kt^n$).

Fourier transform infrared spectroscopy

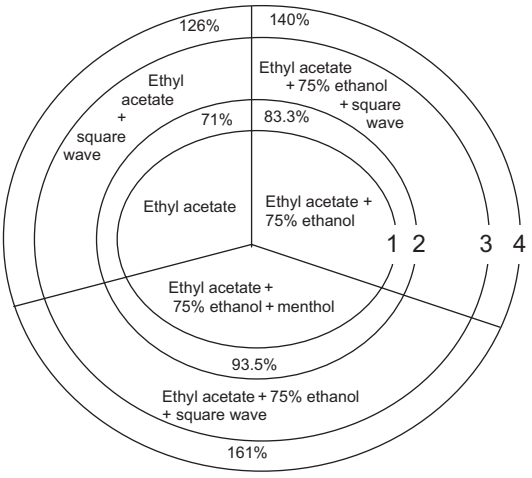
Fourier transform infrared spectrum of Am was recorded by KBr disk method and FTIR of PAm was recorded using thin film of hydrogels (Nicolet-007; Spectra Tech, Oak Ridge, TN, USA).

Skin preparation and in vitro permeation studies from MTX hydrogels

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 killed. The hair was removed from the abdominal region using an animal hair clipper and the full-thickness skin was excised. 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[32]. The fresh skin was used for in vitro, attenuated total reflectance–Fourier transform infrared spectroscopy (ATR–FTIR), and histopathological studies.

For permeation studies, 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 hour in phosphate buffer saline (pH 7.4) in the receptor chamber and was magnetically stirred throughout the experiment.

Enhancers employed were categorized into two groups (Figure 1). Group I: chemical enhancers [ethyl acetate; binary solution of ethyl acetate in 75% ethanol; ternary combination of ethyl acetate, 75% ethanol, and menthol (terpene)] and group II: mDC iontophoresis (square wave of 0.2 mA/cm^2 current density, 1 kHz frequency, and 50% duty cycle) in combination with the above-mentioned chemical enhancers. From group I, 500 µL of chemical enhancers alone or in various combinations was applied to the skin for a period of 1 hour. The excess chemical was removed by dabbing on Whatman filter paper no. 4 and the MTX-loaded hydrogel patch was placed on the skin for permeation studies. For group II, current was applied to the drug-loaded hydrogel patch placed over chemical enhancer pretreated skin through silver–silver chloride electrode for 1 hour. The experiments were done at thermostatically maintained temperature (37 ± 2°C).



Circle 1 Denotes the different combination of chemical enhancers (Group 1) used.
Circle 2 Gives the % enhancement in flux with the Group 1 chemical enhancers.
Circle 3 Denotes the combination of chemical and physical (mDC iontophoresis: square wave) enhancers used (Group 2).
Circle 4 Gives the % enhancement in flux with the Group 2 enhancers.

Figure 1. Different combination of chemical and physical enhancers used for the permeation of methotrexate across mice skin with % enhancement in flux with the respective enhancers.

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 spectrophotometer (CARY 100 model) at 302 nm. The samples were also analyzed by high-performance liquid chromatography (HPLC) using Waters 1525 binary pump attached to UV detector[23]. The samples were injected into C-18 column (Symmetry 5 µm, 4.6 × 250 mm^2, 100Å; Waters, Los Angeles, CA, USA) attached to C-18 guard column (Nova-pack; Waters). The flow rate of mobile phase was 1 mL/min and the mobile phase used was 0.1 M Na_3PO_4/methanol 75:25. The samples were monitored at 302 nm.

Data treatment, statistical analysis, and polynomial curve fitting

The cumulative amount of MTX permeated per unit skin surface area was plotted against time, and flux (J) was calculated as

$$J = \frac{dc}{dt}\frac{V}{A},$$

where V is the volume of solution in the receptor compartment of the diffusion cell, A is the area of the patch, and dc/dt is the change in concentration of drug in the receptor compartment solution of the diffusion cell with 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 ± SD Statistical comparisons were made using Student's t-test and the significance level was set at $P < 0.05$. For curve fitting, a third-order polynomial expression, $Y = At^3 + Bt^2 + Ct + D$, where Y is the total amount and t is the time, was utilized for a best curve fit to compare the rate of permeation of the drug, MTX, with different combinations of physical and chemical enhancers. The R^2 value was obtained for each curve.

Attenuated total reflectance–Fourier transform infrared spectroscopy study

The samples treated with different chemical and physical enhancers mentioned above were subjected to ATR–FTIR study using (Bio Rads Laboratories India Pvt. Ltd., Udyog Vihar, Gurgaon, India) FTS 135 FTIR spectrophotometer. The spectra were recorded in the region 4000–400 cm^{-1}. Each spectrum was an average of 32 scans with 8 cm^{-1} resolution. The peak height and areas of C–H stretching, C=O stretching, and amide peak absorbances were measured for each sample.

Morphological evaluation by scanning electron microscopy

The albino mice skin was treated with different physicochemical enhancers for 1 hour and fixed in electron microscopy (EM) fluid. After fixing the samples for 48 hours, they were washed with phosphate buffer saline and dehydrated using a graded series of ethanol solutions and finally dipped in acetone. The samples were air-dried and mounted on the base plate and then coated with silver using vapor deposition technique. The surface of the skin sample was investigated using Cambridge Stereoscan model S4–10 scanning electron microscope.

Histological examination by light microscopy

The enhancer-treated skin area was excised after 1 hour of in vitro experiment to study the effect of enhancers on skin and after 24 and 48 hours for in vivo experiment to see the reversal of injury after enhancer application. The excised skin was fixed in 10% formalin and then subjected to processing for histological examination by light microscope. 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 hematoxylin–eosin (H&E) stain. The mounting of the stained sections was done in Distyrene, Plasticizer and Xylene (DPX) and observed under light microscope using a modified score (Table 1 for in vitro and Table 2 for in vivo scoring)[33,34]. The final score reported was the average score from five animals.

In vivo studies

Albino mice (50–60 g, $n = 6$/experimental set) were used for in vivo studies. The hair from the abdominal region of the mice was removed 48 hours before the experiments using depilatory cream. Three mice were housed per cage, with cotton spread at the bottom. This was to avoid damage to the skin during the movement of animals. On the day of experiment, mice were anesthetized using ketamine hydrochloride injection (100 mg/kg) given intraperitonially. Two polyacrylamide patches, one containing

Table 1. Histological assessment method for in vitro scoring.

A	Epidermal changes	Score
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

Table 2. Histological assessment method for in vivo scoring.

A. Epidermal changes		
1	Epidermal thickening	
	2 × normal in places	1
	2 × normal generally	2
	2–3 × normal in places	3
	2–3 × normal generally	4
	More than 3 × normal	5
2	Increase in the cell layers of stratum granulosum	
	By 1 cell layer	1
	By 2 cell layers	2
	By 3 cell layers or more	3
3	Thickening of stratum corneum	
	2 × normal in places	1
	2 × normal generally	2
	2–3 × normal in places	3
	2–3 × normal generally	4
	More than 3 × normal	5
4	Hyperkeratosis	
	Mainly loose	1
	Half loose half compact	2
	Compact	3
	Compact severe	4
5	Parakeratosis	
	Focal	1
	Diffuse	2
6	Spongiosis	
	Slight	1
	Extensive	2
	Microvesicle formation	3
	Bullae formation	4
7	Destruction of the 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
B. Dermal changes		
1	Increase in the density and thickness of the collagen bundles	
	Focal upper dermis	1
	Diffuse upper dermis	2
	Focal deep dermis	3
	Diffuse deep dermis	4
	Diffuse deep dermis with displacement of appendages	5
2	Fractured collagen	
	Focal upper dermis	1
	Diffuse upper dermis	2
	Focal deep dermis	3
	Diffuse deep dermis	4
3	Infiltration of dermis	
	Focal	1
	Diffuse	2
4	Hyperaemia	
	Slight	5
	Moderate	10
	Extensive	15
5	Dermal edema	
	Focal upper dermis	2
	Diffuse upper dermis	4
	Focal deep dermis	6
	Diffuse deep dermis	8
6	Appendageal changes	
	Mild focal damage	2
	Focal marked damage	4
	Diffuse marked damage or loss	6

MTX and other swelled in saline, were affixed 1 cm apart on mice abdomen. Cathode was placed over the MTX patch and anode was placed over saline patch. For passive experiment, diffusion of MTX from polyacrylamide patches was conducted without any chemical or physical enhancer. Blood (0.2 mL) was drawn at predetermined time intervals and serum was separated by centrifuging the blood samples at 3300 × *g*. The serum was analyzed by HPLC as described earlier[23] using Waters 1525 binary pump attached to UV detector.

For chemical enhancement, binary combination of ethyl acetate and ethanol and ternary combination of ethyl acetate, ethanol, and menthol was applied slowly with micropipette drop by drop (500 μL) on different set of mice 30 minutes before placement of MTX-loaded patch on the mice to evaluate its effect on drug release. For physical enhancement, DC iontophoresis (0.2 mA/cm^2 current density) and mDC iontophoresis (square wave of 0.2 mA/cm^2 current density, 1 kHz frequency, and 50% duty cycle) were applied on the MTX hydrogel patch through the cathode on different set of animals for 1 hour to study its effect on drug release. Combinational effect of chemical enhancer (ternary combination of ethyl acetate, ethanol, and menthol) and square-wave iontophoresis was also studied. The enhancer was applied directly to the skin before the application of patch, followed by onset of square-wave iontophoresis for 1 hour. For blood withdrawal at regular intervals, the mice set was further divided into two subsets A and B. From subset A, the blood was drawn at 1, 2, 4, and 6 hours. From subset B, the blood was drawn at 1.5, 3, and 5 hours and analyzed as mentioned above.

For oral delivery, two sets of mice were considered for investigation (n = 6/set). One set served as control, and, to the other set, oral MTX (2 mg/mL) was administered with micropipette. The blood samples were drawn at regular intervals and analyzed as mentioned above for in vivo studies. The skin samples of the area of treatment were processed following in vivo experiment as mentioned earlier.

Results and discussion

Characterization of hydrogel

Polyacrylamide hydrogel patch having cross-linking concentration of 0.4 mol% selected for in vitro and in vivo studies has been discussed in our earlier publication[31]. Freshly prepared hydrogels were transparent and slippery to touch and the hardness index of 55 was obtained. The hydrogel patch was elastic and maintained its identity. The swelling studies at pH 7.4 suggested Fickian transport (diffusion exponent 'n' = 0.47). FTIR of polyacrylamide showed absence of C=C double bond thus confirming its formation (spectra not shown).

In vitro studies

Effect of chemical enhancers

The effect of chemical enhancers (group I—ethyl acetate; binary solution of ethyl acetate in 75% ethanol; ternary combination of ethyl acetate, 75% ethanol, and menthol) on the in vitro permeation of MTX is shown in Figure 1. All enhancers increased the transport of MTX in vitro. Maximum flux of 28.6 ± 1.41 μg/cm^2/h with 93.5% enhancement in permeation with respect to passive was obtained with the ternary combination of ethyl acetate, ethanol, and menthol, used in volume proportion of 1:1:1. Whereas the flux enhancement for neat ethyl acetate was 71% and for binary combination of ethyl acetate in ethanol was 83.3%. The results were much different from the passive with $P < 0.05$. The results obtained in this study are higher as compared to our earlier study[31], where we have reported 55% enhancement in flux with ethanolic solution of menthol. The above behavior can be explained as menthol acts on the hydrogen bond network between the ceramides of the lipid bilayer of the skin, thus causing disruption of hydrogen bond network, whereas ethyl acetate causes lipid extraction, thus accounting for higher permeation.

The effect of different chemical enhancers on the net permeation of MTX at a given time was calculated by third-order polynomial curve fitting. The R^2 value for each curve was >0.99, thus indicating a good correlation between experimental and simulated data. From the curve it is observed that, with the passive experiments (without physical/chemical enhancer), maximum permeation is at 2 hours, followed by a decline and a steady-state level. After saturation level is reached, a fall in permeation was observed (data not shown). The diffusion exponent for the drug release curve in water was found to be 0.47 ($n < 0.5$), thereby indicating the Fickian diffusion of drug release[35].

The drug release from the hydrogel patches passively was not found sufficient to achieve the desired clinical levels[29]. Chemical enhancers, ethyl acetate and ethanol, cause lipid extraction thus reducing paracellular as well as intercellular pathway resistance and increase drug permeation. The curve rise is more with ternary combination followed by binary mixture, followed by neat ethyl acetate. This is in accordance with the lipid extraction caused by these enhancers. The ATR–FTIR and histopathological studies also support this.

ATR–FTIR of normal mice skin demonstrated a broad absorbance at 3000–3600 cm^{-1} due to O–H stretching vibrations, major absorption bands of the lipids and proteins at 2920, 2852, 1743, 1642, and 1545 cm^{-1}, corresponding to asymmetric and symmetric C–H stretching vibrations, carbonyl stretching, amide I, and amide II, respectively (Table 3). The amount of the lipids and proteins in the SC has been found to be proportional to the amplitude and area of above-mentioned peaks. ATR–FTIR of neat ethyl acetate showed a shift of 6 cm^{-1} in asymmetric stretching vibration, lower intensity in both asymmetric and symmetric C–H stretching vibration and amide bands, and a shoulder due to ester peak (C=O) at 1743 cm^{-1}, signifying substantial lipid extraction. The spectra of binary and ternary combination showed almost identical spectra with shift and reduction in the intensity of all the absorption bands (Table 3). The elimination of ester peak at 1743 cm^{-1} with binary combination of ethyl acetate/ethanol and ternary combination suggests that these combinations not only bring changes in the conformation of lipid bilayer but also increase lipid extraction, thus enhancing permeation.

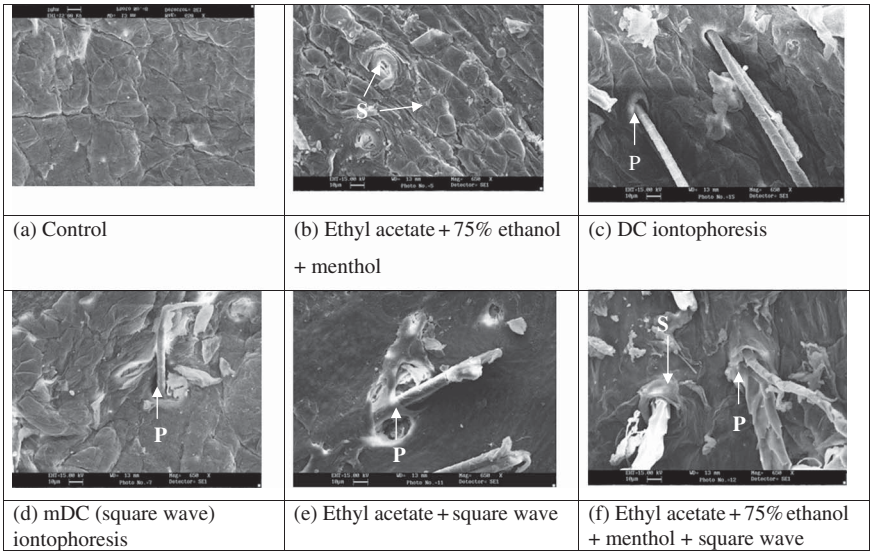
The scanning electron microscopy (SEM) of the skin samples treated with binary and ternary composition revealed extensive swelling as compared to the control sample as shown in Figure 2a and b. The extensive swelling could be due to the edema caused by the application of these chemical enhancers, which is further supported by light microscopy.

In case of light microscopy, the total histological score (THS) based on histological assessment of the various enhancers employed is depicted in Figure 3a for

Table 3. ATR–FTIR bands of stratum corneum lipids and protein, and percentage decrease in peak height after enhancer treatment for 1 hour.

	Asymmetric C–H (cm^{-1})	%↓↓	Symmetric C–H stretching (cm^{-1})	%↓↓	Ester C=O (cm^{-1})	%↓↓	Amide I (cm^{-1})	%↓↓	Amide II (cm^{-1})	%↓↓
A	2920	—	2852	—	1743	—	1642	—	1545	—
B	2926[a]	85.6	2852	82.8	1743	90.5	1647[a]	60	1545	80.3
C	2927[a]	92	2852	87	—	100	1648[a]	60	1545	82.7
D	2963[a], 2922	S	2865[a]	90.75	—	100	1648[a]	62	1545	82
E	2926[a]	90.2	2852	87.5	1743	94.5	1649[a]	63	1541, 1553	S
F	2927[a]	96.8	2852	91.7	—	100	1649[a]	63	1541, 1553[a]	S
G	2963[a], 2922	S	2865[a]	93.75	—	100	1649[a]	65	1541, 1553[a]	S

[a]Indicates shift of absorptional bands toward higher frequency and S indicates split in the absorptional band (due to unequal size of the split the % decrease has not been shown) A is control, B is ethyl acetate, C is binary mixture of ethyl acetate/75% ethanol, D is ternary mixture of ethyl acetate/75% ethanol/menthol, E is ethyl acetate + square wave, F is binary mixture of ethyl acetate/75% ethanol + square wave, and G is ternary mixture of ethyl acetate/75% ethanol/menthol + square wave.



(a) Control | (b) Ethyl acetate + 75% ethanol + menthol | (c) DC iontophoresis

(d) mDC (square wave) iontophoresis | (e) Ethyl acetate + square wave | (f) Ethyl acetate + 75% ethanol + menthol + square wave

Figure 2. Scanning electron micrographs of the mice skin treated with different enhancers depicting swelling in the cell surface and increase in pore size: (a) control, (b) ethyl acetate + 75% ethanol + menthol, (c) DC iontophoresis, (d) mDC (square wave) iontophoresis, (e) ethyl acetate + square wave, and (f) ethyl acetate + 75% ethanol + menthol + square wave. S, swelling; P, increase in pore size.

in vitro and Figure 3b for in vivo studies. The histological photomicrographs showed the variable changes due to different enhancers (figure not shown for in vitro studies). The control sample showed well-defined SC, epidermis, and appendages. The epidermal changes in ethyl acetate and binary combination of ethyl acetate/ethanol-treated skin were identical (score of six) with epidermal thinning and focal spongiosis. The dermal changes in case of binary mixture were higher and consisted of increased edema, fractured collagen with a THS of 19 (Figure 3a) in comparison to only ethyl acetate. The histological changes were highest with the ternary combination of ethyl acetate, ethanol, and menthol with THS of 24 (Figure 3a) with marked edema and separation of dermal collagen bundles in addition to epidermal thinning. The enhancement in flux when compared with passive permeation for ternary mixture having menthol was 93.5%, for binary composition of ethyl acetate/ethanol was 83%, and for neat ethyl acetate was 71%. The above results suggest that the flux enhancement is related to the anatomical changes caused by the enhancers. A preliminary study conducted earlier by Pillai and Panchagnula[36] also reported similar observation.

Effect of chemical enhancer and square wave

When ternary combination of ethyl acetate/ethanol/menthol and square wave was employed, a maximum flux of 38.6 ± 1.73 μg/cm^2/h with 161% (Figure 1) enhancement in permeation was obtained. With binary combination of ethyl acetate/ethanol and square wave, 140% enhancement was obtained, whereas with neat ethyl acetate and square wave 126% enhancement in flux with respect to passive was obtained ($P < 0.05$). ATR–FTIR, SEM, and light microscopy studies supported the different permeation results. It has been envisaged that the use of combinational therapy of chemical enhancers and square wave in this study causes 161% enhancement in permeation, which is higher as compared to our earlier published results[31] where 117% enhancement was achieved with ethanolic solution of menthol in combination with square wave. The difference in the results is possibly due to the use of ethyl acetate along with ethanolic solution of menthol, which causes higher lipid extraction. Moreover, the flux in this study is much higher as compared to the other research groups[22–29]. However, direct comparison between the fluxes obtained with different research groups cannot be compared due to the different animal species and membrane employed for the study.

The ATR–FTIR for all the above-mentioned enhancer combinations showed spectra identical to those of the chemical enhancers with lower intensity (Table 3), indicating importance of iontophoresis in enhancement. The ATR–FTIR of all the treated skin samples did not distinguish changes at the molecular and conformational level between the binary and ternary combinations used alone or with square wave; however, only reduction in the intensity of all absorption bands was noticed when binary to ternary combinations was employed.

The scanning electron micrographs of ternary mixture and square wave (Figure 2f) demonstrated effect due to square wave and chemical enhancers and elicited markedly swollen hair follicle with increase in pore size as well as the swollen skin surface, which suggest

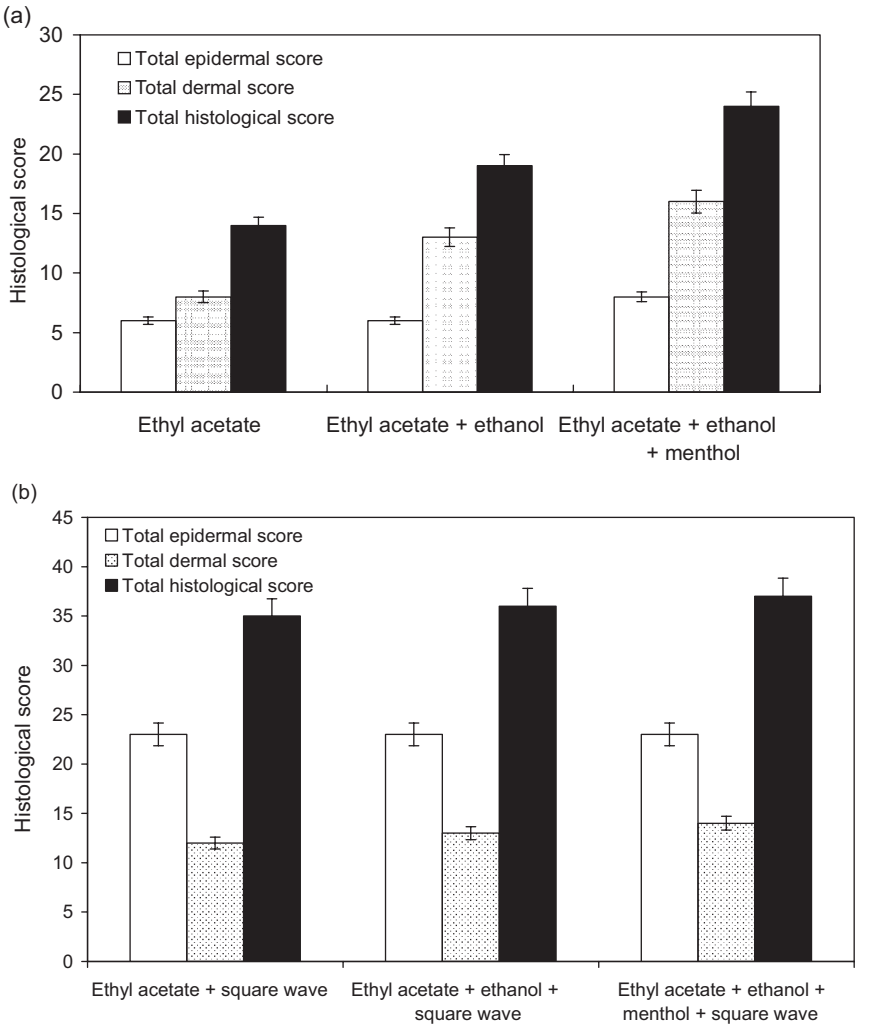


Figure 3. Histological score of mice skin, (a) after chemical enhancer treatment, (b) after a combination treatment of chemical enhancer and electrical current.

involvement of shunt pathways due to iontophoresis (Figure 2c and d) as well as edema formation[37]. SEM picture of neat ethyl acetate in combination with square wave (Figure 2e) showed hair follicle with increased pore size thus confirming their involvement in permeation.

The light microscopy studies showed that with binary combination of ethyl acetate/ethanol and square wave, there was epidermal thinning and focal disruption of the epidermis giving an epidermal score of 23, the dermis showed edema with moderate fractured collagen, and appendageal dilatation giving a dermal score of 13 (Figure 3b). With ternary combination of ethyl acetate/ethanol/menthol and square wave, the epidermal changes were identical; there was increase in appendageal dilatation, which raised the dermal score to 14 and maximum THS of 37 was obtained.

The results obtained from the different biophysical studies (reduced intensity in the FTIR bands, increase in pore size, and anatomical scoring data) supports the increase in flux.

In vivo studies

Pharmacokinetic study

The passive permeation of MTX from the hydrogel patch was very low (plasma concentration of 2.01 ± 0.34 μg/mL) and almost constant for a 6 hours period. As the amount of drug reaching the systemic blood circulation was almost negligible, enhancers (both physical and chemical) were used to enhance the drug permeation.

Pharmacokinetic study: effect of enhancers

With the enhancers, the plasma concentration of MTX showed initial rise and then declined with time. Among the chemical enhancer combination, plasma concentration of 9.11 ± 0.81 μg/mL (C_{max}) was obtained in 2 hours

Table 4. Pharmacokinetic parameters for transdermal delivery of MTX in vivo. Data represent mean ± SD ($n = 6$).

	Transdermal						
	Passive	EA + ethanol	EA + ethanol + menthol	DC	mDC (square wave)	EA + ethanol + menthol + mDC (square wave)	Oral
$C_{max\,(\mu g/mL)}$	2.01 ± 0.34	7.63 ± 0.68	9.11 ± 0.81	7.11 ± 0.28	11.1 ± 0.44	18.79 ± 0.75	8.89 ± 1.06
$t_{max\,(hours)}$	2	2	2	1.5	1.5	1.5	1
Skin conc. (μg/mL)	4.9	—	—	—	25.18	—	0.081

(t_{max}) with ternary combination of ethyl acetate/ethanol/menthol whereas with binary combination, C_{max} of 7.6 ± 0.68 μg/mL was obtained. With iontophoresis, higher C_{max} of 11.1 ± 0.44 μg/mL was obtained with mDC (square wave) as compared to DC iontophoresis (Table 4). When a combinational approach of chemical (ternary mixture) and physical enhancer (mDC) was employed, C_{max} increased to 18.79 ± 0.75 μg/mL (Table 4) and was achieved in 90 minutes ($P < 0.05$). The t_{max} obtained was different from chemical enhancer studies. It can be hypothesized that the permeation enhancement is due to the current acting both on the drug molecules as well as the skin. Hence it pushes the drug from the hydrogel patch into the skin and also acts on the lipid–protein domain of the skin creating pathways for drug permeation. When the external current is withdrawn after 1 hour, the amount of drug permeated into the skin slowly leaches into deeper tissues and reaches the blood circulation. Therefore, maximum concentration is obtained in 90 minutes. This combinational approach of enhancers helps in achieving higher blood concentration by making changes in the skin with the specific combination of chemical enhancers and mDC iontophoresis. The results obtained are higher than those obtained earlier[18]. They have reported a C_{max} of 0.5 μg/mL using current density of 0.2 mA/cm^2 on rabbit skin. The difference in results are possibly due to the different animal model used, drug reservoir employed, chemical enhancers, and the mode of iontophoresis used.

Histopathological studies by light microscopy

The light microscopy studies supported the above results. Skin injury observed with the ternary combination (ethyl acetate/ethanol/menthol) showed hyperkeratosis, focal spongiosis with epidermal score of 6. The dermal changes observed were moderate hyperemia and marked dermal edema (Figure 4a). The infiltration of dermis by inflammatory cells and fractured collagen were focal and a dermal score of 19 was noticed, thus THS obtained was 25. Binary combination of ethyl acetate and ethanol showed lesser skin injury.

DC iontophoresis showed focal disruptions of epidermis (Figure 4c) with bullae formation giving an epidermal score of 21. The dermis showed appendageal damage and edema with a score of 18. The edema formation is evident as the hydration effects of iontophoresis are responsible for enhanced permeation of the drug. The mDC (square wave) iontophoresis (Figure 4d), showed focal disruptions of the epidermal layer and separation from dermis, giving an epidermal score of 20. The dermis showed appendageal

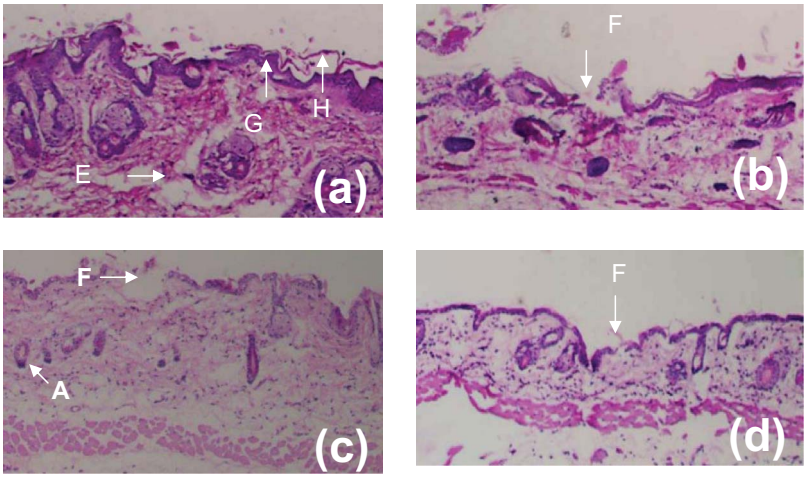


Figure 4. Photomicrographs of mice skin treated with various enhancers in vivo, (a) ethyl acetate + 75% ethanol + menthol, (b) ethyl acetate + 75% ethanol + menthol + square wave, (c) DC iontophoresis (0.2 mA/cm^2, current density), and (d) square wave. A, appendageal dilatation; E, edema; F, focal disruption of epidermis; G, granular cells; and H, hyperkeratosis (H&E), ×200.

damage and edema similar to DC current with less of fractured collagen, thus giving a dermal score of 17.

From the above results it appeared that the damage caused by mDC iontophoresis is less as compared to DC iontophoresis because in mDC iontophoresis the current is being given in pulses, with the result a continuous damage and repair process occurs. Earlier researchers have reported that the effects induced by square-wave pulses on the skin are mild and reversible[38,39], which is in accordance with our findings also.

The ternary combination of ethyl acetate, ethanol, and menthol with square wave showed maximum skin injury with epidermal score of 21, dermal score of 20, thus giving THS of 41. Therefore, higher concentration of MTX was found in the blood corresponding to higher skin injury, thus supporting the fact that higher the skin injury more is the decrease in skin resistance thereby resulting in higher permeation.

Reversibility

Reversibility studies conducted after 24 and 48 hours of the application of chemical enhancers alone or in combination with square wave showed that recovery process had started within 24 hours and is almost complete in 48 hours in most of the epidermal as well as dermal changes. The epidermis had become intact with no disruptions. Most of the enhancers and the combination showed hyperkeratosis after 48 hours, which might be a reactive response of the keratinocytes. The reversibility studies confirm that all the above-mentioned chemical and physical enhancers are well tolerated by the tissue and the injury caused is transient and reversible.

Oral versus transdermal delivery

The oral delivery of MTX showed blood concentration of 7.2 µg/mL in 2 hours and then there was sudden decline to 3.9 µg/mL in 3 hours that tapered to 1.6 µg/mL in 5 hours. Figure 5 demonstrates the effect of oral versus transdermal (passive and iontophoretic) delivery on MTX permeation. From the figure it is clear that the transdermal delivery (passive/iontophoretic) maintains a constant plasma level for a few hours before declining, whereas in case of oral, there is a sharp rise and sudden decline in plasma level. However, with passive transdermal delivery, permeation is very low. Hence, iontophoresis and chemical enhancers were used to increase the permeation. Figure 5 shows that with mDC (square-wave) iontophoresis, the plasma level is maintained for almost 5 hours before falling and the drug concentration in the blood is higher than that obtained with oral. When ternary mixture of ethyl acetate/ethanol/menthol was used in combination with mDC iontophoresis, the flux improved to significant level (Table 4) as against oral (C_{max} was two times oral), making it equivalent to systemic delivery. Moreover, the skin concentration was 300 times higher with mDC iontophoresis as compared to oral (Table 4). Thus, the transdermal route proves as an effective alternative to deliver MTX. However, more studies are needed to make it a clinically accepted method of administration.

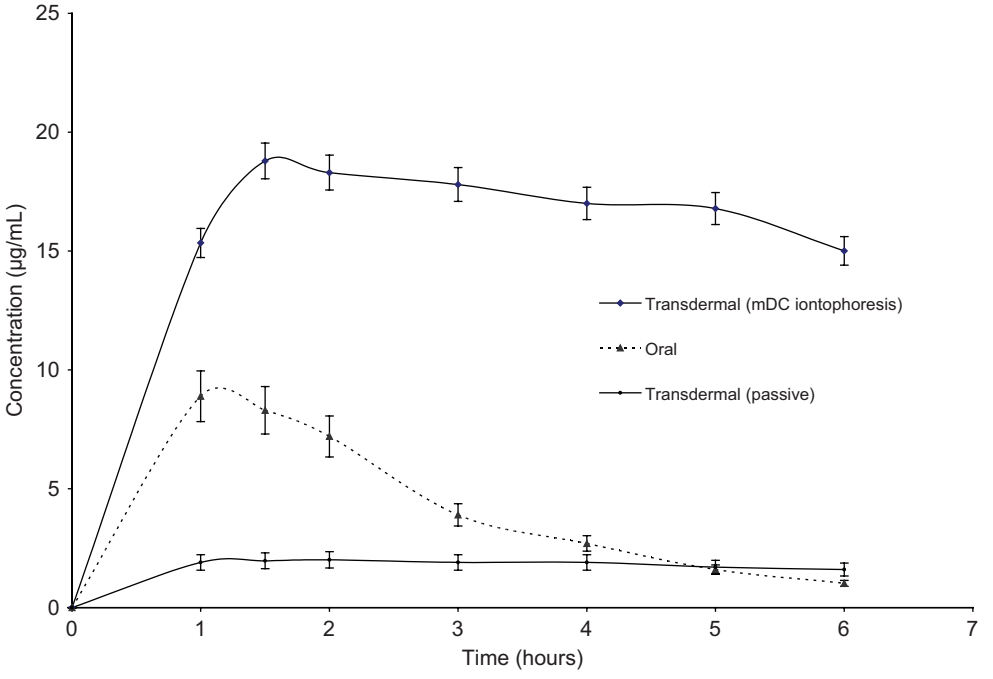


Figure 5. Effect of oral versus transdermal (passive and iontophoretic) delivery on MTX permeation. Data represent mean ± SD ($n = 6$).

Conclusion

From the above study, it is concluded that the ternary composition of ethyl acetate/ethanol/menthol in combination with square wave gave maximum enhancement in permeation of MTX both in vitro (161%) and in vivo (C_{max} 18.79 μg/mL) with respect to passive control as well as higher skin injury as compared to the other combinations used. Moreover, mDC iontophoresis was more effective than DC iontophoresis as it increases skin permeation while causing lesser skin injury. The biophysical assessment by ATR–FTIR, SEM, and light microscopy supports the enhancement in flux. Higher permeation by incorporation of square wave is due to its increased depolarizing effect on skin. SEM and light microscopy confirm that with combination of physical and chemical enhancers, both shunt as well as intercellular pathway resistance reduces, thereby increasing permeation. It has been seen that the incorporation of ethyl acetate into the system caused substantial amount of lipid extraction as is evident from ATR–FTIR.

Histopathologically, both in vitro and in vivo experiments showed a similar trend in the injury pattern but the in vivo skin changes were less as compared to the in vitro studies, as the protective response and the repair mechanisms are active in case of in vivo. From the above histopathological findings, it can be concluded that the injury caused to the skin by the different enhancers are transient and reversible with regeneration and organization of cells, both in epidermis as well as dermis within 48 hours. In designing any drug delivery system, there has to be a wise decision in choosing the enhancement modality. Balance between the type and degree as well as reversibility of injury has to be judged. To conclude, the reversibility studies confirm that all the above-mentioned chemical and physical enhancers are well tolerated by the tissue.

The transdermal delivery of MTX was more effective than oral route as it maintained higher plasma concentration and also higher concentration at the local skin site, which is clinically required for the treatment of skin disorders. With the above two-tier combination of enhancers, we have almost achieved a systemic delivery system and not withstanding the fact that no effect on histological and cellular structure was observed. However, further studies and clinical trials are needed to make it a commercially accepted method of administration.

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