

# Biophysical assessment of DC iontophoresis and current density on transdermal permeation of methotrexate

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## Abstract

**Introduction:** The effect of DC iontophoresis using low (0.2 mA/cm<sup>2</sup>) and high current density (0.5 mA/cm<sup>2</sup>) on transdermal permeation of methotrexate loaded into polyacrylamide hydrogel patch was investigated. **Results:** Flux of  $20.57 \pm 1.02 \mu\text{g}/\text{cm}^2/\text{h}$  and  $36.8 \pm 2.21 \mu\text{g}/\text{cm}^2/\text{h}$  was achieved with low and high current density DC iontophoresis, respectively. Attenuated total reflectance-Fourier Transform infrared (ATR-FTIR) spectra and microscopic studies of the treated skin samples supported the permeation results. A greater decrease in the peak height of asymmetric, symmetric C-H stretching vibration and ester peak was noticed with 0.5 mA/cm<sup>2</sup> current density as compared to 0.2 mA/cm<sup>2</sup> current density samples. Furthermore, an increase in the ratio of amide I and amide II bands from 2.6 to 11 with increase in current density was noticed, thus indicating that hydration levels are associated with iontophoresis and play an important role in increasing the drug permeation. Scanning electron microscopy revealed increase in pore size of the hair follicles. Light microscopy studies of the skin samples treated with low current density DC iontophoresis demonstrated epidermal thinning and focal disruptions, spongiosis and appendageal dilatations. With higher current density, disruption of epidermis in almost half of the sectioned area, loss of appendages and fractured collagen in the dermis was noticed. Moreover, the reversibility studies conducted *in vivo* on mice revealed that the recovery process had started within 24 h and is complete in 48 h for lower current density treated animals. However, the histological changes associated with 0.5 mA/cm<sup>2</sup> current density were not reversible in 48 h and edema, appendageal dilatations along with focal disruption of epidermis persisted. **Conclusion:** Hence our study suggests that high density current is not well-tolerated by the skin.

**Key words:** ATR-FTIR, current density, DC iontophoresis, methotrexate, skin histopathology

## INTRODUCTION

Transdermal drug delivery is a noninvasive route of drug administration into the body through the skin. It offers many advantages over other routes of conventional drug delivery.<sup>[1,2]</sup> However, only a few drug candidates have been successfully developed into suitable transdermal formulations because of the formidable skin barrier.<sup>[3]</sup> The highly lipophilic nature of the skin restricts the permeation of hydrophilic, high molecular weight and charged compounds through the stratum corneum into the systemic circulation. Therefore, different chemical<sup>[4,5]</sup>

and physical<sup>[6-8]</sup> methods for permeation enhancement have been used.

Transdermal iontophoresis is defined as application of an electrical potential that maintains a constant electric current across the skin and enhances the delivery of ionized as well as unionized molecules.<sup>[9]</sup> It uses an electrode of the same polarity as the charge on the drug to drive ionic drug molecules into the body.<sup>[10]</sup> The mechanisms of transdermal iontophoresis include electrorepulsion (a charged ion is repelled from an electrode with the same charge),<sup>[11]</sup> electro-osmosis (convective flow of solvent through a charged pore that occurs in response to the preferential passage of counter ions when the electric field is applied),<sup>[12]</sup> and current-induced skin permeability increment.<sup>[13]</sup> One of the major advantages of iontophoretic drug delivery is the ability to readily and precisely control the drug-delivery profile through modulating the current output.

Methotrexate (MTX) is an antineoplastic agent used for the treatment of cancer, psoriasis and rheumatoid arthritis. MTX inhibits the enzyme dihydrofolate reductase thus inhibiting DNA synthesis. At high doses it is used for the treatment of cancer and at low doses, it has immunosuppressive and anti-inflammatory properties and is used for the treatment of psoriasis

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and rheumatoid arthritis.<sup>[14]</sup> Various topical forms like ointments, creams and gels<sup>[15-18]</sup> have been tried as the systemic use of this drug causes many side effects mainly, hepatic toxicity and liver damage<sup>[19]</sup> but are still not available commercially.

The drug can be delivered either in solution formulation or through loading on to the hydrogel patches. Incorporation of the drug into hydrogels facilitates drug handling and release<sup>[20]</sup> and in case of iontophoretic delivery, allows the patient to remain ambulant.<sup>[21]</sup> It has been reported that the iontophoretic delivery of MTX from hydrogels<sup>[21]</sup> was more effective than passive delivery from aqueous solution.<sup>[22]</sup> It has been demonstrated that iontophoresis remarkably improved the transdermal delivery of MTX over passive diffusion.<sup>[14,23]</sup> More interestingly, a case of palmer psoriasis treated with iontophoresis of MTX has been reported<sup>[24]</sup> using current density for iontophoresis beyond the clinically acceptable limits. Although, the results with iontophoresis are promising, none of the papers give a detailed comparison between high and low current density used for iontophoresis.

The aim of the present study was to analyze the effects of current density on MTX permeation as well as to assess the skin injury caused by the above-mentioned physical enhancer by histological examination. Moreover, the reversibility of skin injury was studied *in vivo*.

## MATERIALS AND METHODS

### Reagents

MTX was a gift sample from Dabur (India), Acrylamide was obtained from Spectrochem Pvt. Ltd. (India), N, N-Methylene bis-acrylamide, potassium chloride and potassium dihydrogen phosphate from Sisco Research Laboratory (India), sodium chloride from E. Merck India Ltd. (India), dihydrogen-o-phosphate anhydrous from Qualikems fine chemicals Pvt. Ltd. (India), sodium hydroxide and ethyl acetate from Excelar Qualigens fine chemicals (India), ammonium persulphate from Thomas Baker chemicals Ltd. (India) and sodium metabisulphite from S.D. fine chemicals (India). Deionised water having a resistivity of 18 M $\Omega$  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 by Prasad *et al.*, 2007.<sup>[23]</sup>

### 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, New Delhi, India. White albino mice (n=5 in each group) were procured from AIIMS and sacrificed. 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.<sup>[25]</sup> The fresh skin was used, for *in vitro*, attenuated total reflectance-Fourier transform infrared (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<sup>2</sup>. The skin was equilibrated for 1h in phosphate buffer saline (pH 7.4) in the receptor chamber and was magnetically stirred throughout the experiment.

MTX-loaded patch was placed over the skin and DC iontophoresis using current density of 0.2 mA/cm<sup>2</sup> was applied for 1 h to the hydrogel patch through silver-silver chloride electrode and having same dimensions as the hydrogel patch to study the effect of low current density iontophoresis. Similarly the effect of high current density 0.5 mA/cm<sup>2</sup> was also studied. The experiments were done at thermostatically maintained temperature (37 $\pm$ 2 $^{\circ}$ C).

### Quantification of MTX, data treatment, statistical analysis and polynomial curve fitting

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 HPLC using Waters 1525 binary pump attached to UV detector.<sup>[22]</sup>

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

$J = (dc/dt) V/A$ , where V=volume of solution in the receptor compartment of the diffusion cell, A=area of the patch, and dc/dt=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:

$$\% \text{ enhancement in flux} = \left\{ \frac{\text{Flux with enhancer} - \text{passive flux}}{\text{passive flux}} \right\} * 100$$

All experiments were repeated five times and the values are expressed as mean  $\pm$  S.D. 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 = total amount and t = time, was utilized for a best curve fit to compare the rate of iontophoretic permeation of methotrexate with passive. The R-squared value was obtained for each curve.

### ATR-FTIR

The samples treated with iontophoresis mentioned above were subjected to ATR-FTIR spectroscopic study using Bio-RAD, FTS 135, FTIR spectrophotometer. The spectra were recorded in the region 4000-400 cm<sup>-1</sup>. Each spectrum was an average of 32 scans with 8-cm<sup>-1</sup> 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 iontophoresis for 1 h and fixed in EM fluid. After fixing the samples for 48 h, 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 iontophoresis-treated skin area (both *in vitro* and *in vivo*) was excised after 1 h to study the effect on skin and after 24 h and 48 h *in vivo* 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- $\mu\text{m}$  thickness were cut and stained with hematoxylin-eosin (HandE) stain. The mounting of the stained sections was done in DPX and observed under light microscope using a modified score [Table 1 for *in vitro* scoring].<sup>[26,27]</sup> The final score reported was the average score from five animals.<sup>[28]</sup>

## RESULTS AND DISCUSSION

### Effect of current density on permeation

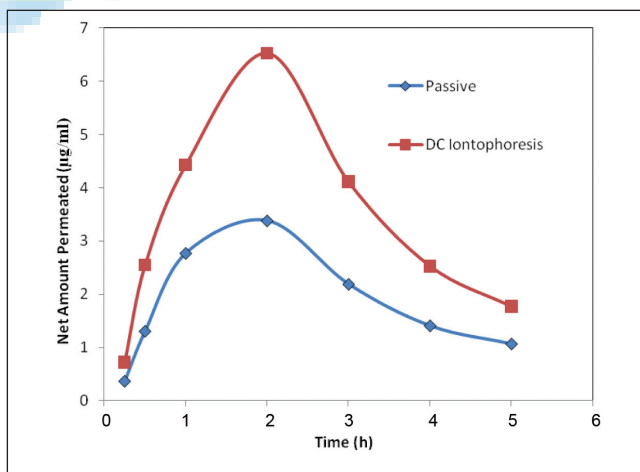
The flux obtained with DC iontophoresis using 0.2 and 0.5 mA/cm<sup>2</sup> current density was  $20.57 \pm 1.02 \mu\text{g}/\text{cm}^2/\text{h}$  and  $36.8 \pm 2.21 \mu\text{g}/\text{cm}^2/\text{h}$ , respectively ( $P < 0.05$ ). It was observed that MTX permeation increased with increasing current density. Similar results have been obtained by Cesares-Delgadillo, 2010,<sup>[29]</sup> although they have tried different current densities for a different drug.

### Polynomial curve fitting

A third order polynomial was chosen to model the experimental results as the data could not be presented by a quadratic. There was no significant improvement in the least square error by using a higher order polynomial, hence was not used for analysis. The results are presented in Figure 1 which shows the effect of DC iontophoresis on the net permeation of methotrexate at a given time calculated by third order polynomial curve fitting. A good correlation between experimental and polynomial simulation was obtained from the curve ( $R^2 > 0.9$ ). It can be observed from the curve that with the passive experiments (drug-loaded hydrogel patch), initially there is an increase in permeation, then there is decline and steady state is obtained. The drug diffusion is via the least resistant pathway, which reaches a saturation, so there is fall in permeation. Moreover, the swelling pattern of the hydrogel

**Table 1: Histological assessment method for *in vitro* scoring**

A Epidermal changes		
1	Thinning of epidermis	
	½ thinning	5
	Less than ½ 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



**Figure 1: Polynomial curve fitting: Effect of DC iontophoresis**

(0.4 mole%) is Fickian in nature (data not shown), which further supports that diffusion is the main mechanism of drug release.

Enhancement of permeation is required to achieve the desired drug levels.<sup>[18]</sup> From the curve, it can be seen that there is sharp rise in permeation with iontophoresis and then steady level is reached [Figure 1]. The ATR-FTIR and microscopic studies presented below also support this phenomenon.

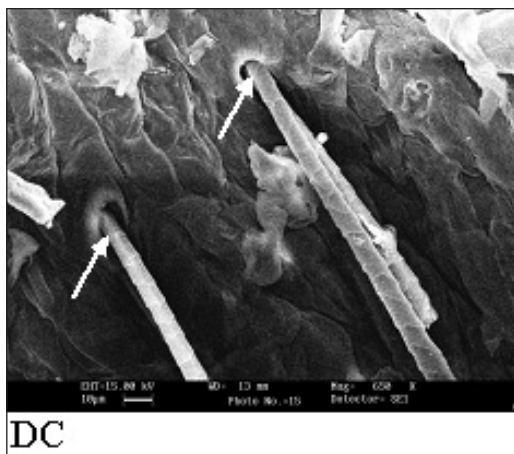
### ATR-FTIR studies

ATR-FTIR spectra of skin sample treated with current of 0.2 mA/cm<sup>2</sup> current density showed all the major peaks of the lipid and protein but with reduced intensity as compared to the control. A decrease of 28.12% and 31.25% in the peak height of asymmetric and symmetric C-H stretching vibration, respectively, as compared to the control, and 37.5% reduction in the ester peak was noticed [Table 2]. Furthermore, a decrease of 31.4% and 47.05% was noticed with amide I and amide II, respectively. With 0.5 mA/cm<sup>2</sup> current density iontophoretic samples, a greater reduction of 96.8%, 93.75% and 93.7% [Table 2] in asymmetric and symmetric C-H stretching and C=O stretching vibration, respectively, was obtained which was attributed to substantial amount of lipid extraction in the lipid protein domains. Moreover, an increase in the ratio of amide I and amide II bands from 2.6 to 11 with increase in current density from 0.2 to 0.5 mA/cm<sup>2</sup> was noticed, thus indicating that the hydration levels are associated with iontophoresis and play an important role in increasing the drug permeation.

The spectra also demonstrated a split in amide II band into 1553 cm<sup>-1</sup> and 1541 cm<sup>-1</sup>. The split could be due to the disruption in hydrogen bonding associated with the head of ceramides, breaking interlamellar hydrogen bonding of lipid bilayer and disrupting barrier property of SC, resulting in loosening of lipid-protein domains thus allowing higher flux as compared to the passive treatment.

### Scanning electron microscopy

The scanning electron micrographs of DC iontophoresis clearly showed increase in the pore size of the hair follicles [Figure 2]. This supports the involvement of shunt pathways for drug permeation



**Figure 2:** Scanning electron micrograph showing increase in hair follicles of the mice skin treated with DC iontophoresis

during iontophoresis.<sup>[30,31]</sup> Kajimoto *et al.*, 2011<sup>[32]</sup> have also reported the use of follicular pathways during iontophoresis.

### Histopathological studies

Figure 3 depicts a comparison between control, DC (0.2 mA/cm<sup>2</sup>) and DC (0.5 mA/cm<sup>2</sup>). From the histopathological studies it is clear that at higher current density, 0.5 mA/cm<sup>2</sup>, disruption of epidermis in almost half of the sectioned area was noticed [Figure 3b]. There was thinning of epidermis and spongiosis giving an epidermal score of 29. There was severe dermal edema with marked dilatation and loss of appendages and fractured collagen with a dermal score of 18. The dermal changes were higher as compared to iontophoresis with 0.2 mA/cm<sup>2</sup> current density.

Iontophoresis using lower current density of 0.2 mA/cm<sup>2</sup> showed focal disruptions of epidermis (less than ¼ of sectioned area) and bullae formation [Figure 3c] and an epidermal score of 24 was obtained. The dermis showed fractured collagen with moderated edema and mild appendageal damage, thus a dermal score of 12 was obtained. DC (0.5 mA/cm<sup>2</sup>) resulted in THS of 47 whereas at 0.2 mA/cm<sup>2</sup> DC, THS of 36 was observed indicating lesser skin injury.

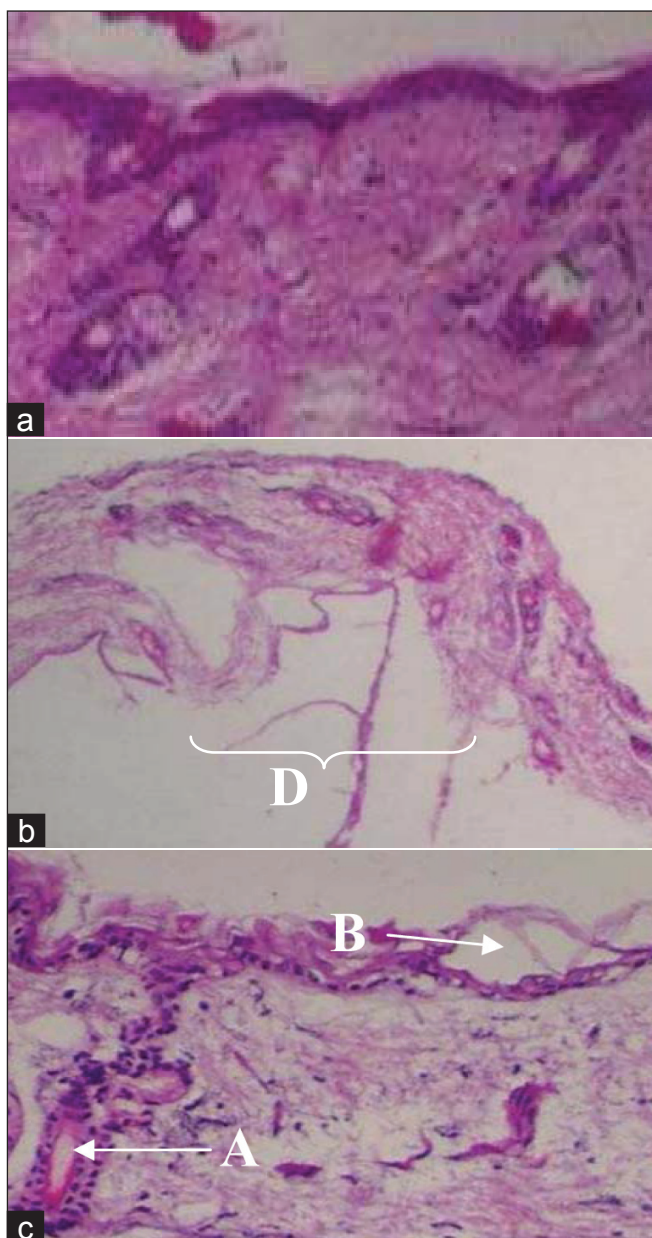
The severe edema formation is evident as the hydration effects of iontophoresis are responsible for enhanced permeation of the drug. This has been depicted by the ratio of amide I/II band in ATR-FTIR spectroscopy described previously. Application of current reduces the resistance of skin and the decreased resistance is reflected in the increased permeability of the skin and therefore increased flux. Current causes epidermal destruction as well as appendageal damage, as we could see in all the histological slides as well as from scanning electron microscopy, therefore shunt pathway for drug permeation become more operative on current application.<sup>[30-32]</sup> To summarize, with 0.5 mA/cm<sup>2</sup> current density, damage was higher as compared to 0.2 mA/cm<sup>2</sup>, which was in correlation with the flux observed. The higher current density provides more electromotive force that increases the flux. *In vivo* results in mice show that the damages associated with 0.5 mA/cm<sup>2</sup> current density were not reversible in 48 hours [Figure 4].

### Reversibility studies (*in vivo*)

Reversibility studies were conducted *in vivo* after 24 and 48 h of the application of iontophoresis. It was observed that recovery process had started in 24 h and almost total recovery of epidermal as well as dermal changes was found in 48 h with low current density DC iontophoresis, however with iontophoresis using 0.5 mA/cm<sup>2</sup> current density, edema along with focal disruption of the epidermis persisted [Figure 4]. The partial

**Table 2: Percentage decrease in peak height for lipids and protein absorption bands after current treatment for 1h**

	Asymmetric C-H	Shift	% ↓	Symmetric C-H	% ↓	Ester C=O	% ↓
Control	2920	-	-	2852	-	1743	-
0.2mA/cm <sup>2</sup>	2923	3	28.12	2852	31.25	1744	37.5
0.5mA/cm <sup>2</sup>	2928	8	96.8	2852	93.75	1744	93.7

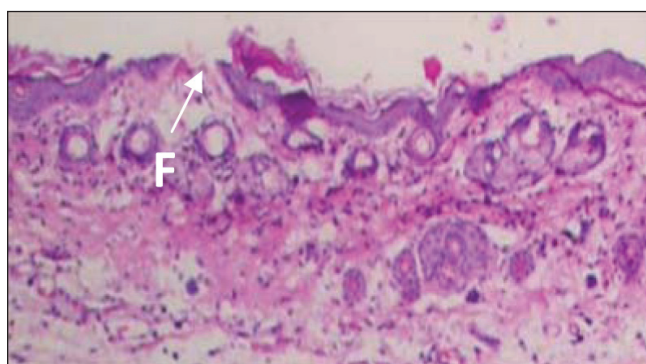


**Figure 3:** Photomicrographs of treated mice skin (*in vitro*) (a) Control, (b) 0.5mA/cm<sup>2</sup> current density (c) 0.2mA/c m<sup>2</sup> current density, (Where, A – Appendageal dilatation, B – Bullae formation, D – Destruction of epidermis) (H and E, ×200).

denudation of the epidermis with left over basal layer showed a rapid recovery as compared to areas with loss of basal layers as seen with 0.5 mA/cm<sup>2</sup> current density iontophoresis.

## CONCLUSIONS

It was observed that the transdermal permeation increased with increase in injury caused to the tissue. The higher current density (0.5 mA/cm<sup>2</sup>) increased the MTX flux tremendously, but the reversibility studies did not confirm its skin tolerance as the histological changes were not reversible in 48 h.



**Figure 4:** Photomicrograph of mice skin *in vivo* showing recovery after 48 h of application of iontophoresis (0.5 mA/cm<sup>2</sup> current density), where F – Focal disruption of epidermis. (H and E, ×200)

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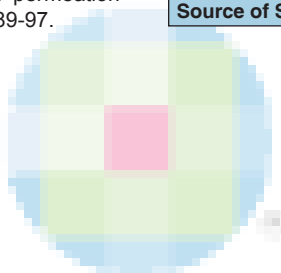
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