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Passive and iontophoretic transdermal penetration of methotrexate

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Abstract

The in vitro iontophoretic transdermal delivery of methotrexate (MTX) across pig skin was investigated. Cathodal iontophoresis considerably increased MTX skin permeation and accumulation as compared to the passive controls. The effect of NaCl and MTX concentrations in the vehicle were also studied. As expected, MTX iontophoretic transport decreased with NaCl content. On the other hand, MTX concentration did not modify its electrotransport in the range of concentrations considered (4.4–6.6 mM). The influence of the current density (0.25–0.5 mA/cm^2) was also investigated. The iontophoretic transport of MTX tends to increase with current density although this effect was not always statistically significant. Finally, the possibility of using anodal iontophoresis from an acid (pH 4.0–5.0) donor solution to deliver MTX was explored. This was limited due to the low solubility of MTX in acid pH. On the whole, this work that iontophoresis may be used to improve the topical application of MTX for the treatment of psoriasis.

Keywords: Iontophoresis; Methotrexate; Psoriasis

1. Introduction

Methotrexate (MTX, Fig. 1) is a folic acid antagonist with antineoplastic activity. It competitively inhibits the enzyme dihydrofolate reductase, leading to inhibition of DNA synthesis (Chládek et al., 1998). Since MTX inhibits mitotic activity, it is effective for the treatment of recalcitrant psoriasis (Vaidyanathan et al., 1985; Lu et al., 1997), a skin disorder which is characterized by hyperproliferation of epidermal cells, giving rise to regions of cutaneous thickening and erythematous lesions, typically on the back, the elbows, the knees, and the scalp (Ghadially et al., 1996; Tan and Ledwohl 1998).

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MTX is effective for the treatment of psoriasis when it is administered by the oral or parenteral route over long periods of time. However, the systemic use of this drug may provoke any of numerous side effects, including nausea, vomiting, fatigue, headache, dyspnea, leukopenia, thrombocytopenia, anemia, and hepatic toxicity (Bookbinder et al., 1984; Van Dooren-Greebe et al., 1994). To reduce such effects, it would clearly be preferable to administer MTX topically, and to this end there have been studies of its administration in ointments, creams, and gels (Vaidyanathan et al., 1985; Weintein et al., 1989; Hwang et al., 1995; Singh and Singh, 1995; Trotta et al., 1996; Chatterjee et al., 1997; Lu et al., 1997). However, no topical dosage forms of this drug are commercially available yet. A major problem is that MTX is hydrosoluble and has a high molecular weight, and is mostly in dissociated form at physiological pH; its capacity for passive diffusion is thus limited (Singh and Singh, 1995). Iontophoretic drug administration consists of the application of a weak electric field across the skin, in order to facilitate the penetration of charged molecules, or of neutral and polar molecules such as mannitol and glucose (Kim et al., 1993; Rao et al., 1993; Marconi et al., 1999). Iontophoretic techniques may therefore be useful for increasing transdermal penetration of MTX.

Iontophoretic transport is principally due to two phenomena: convective flow and electrorepulsion (Kim et al., 1993; Green, 1996). Since MTX is negatively charged at physiological pH (7.4), it must be delivered from the cathode, and will be driven from cathode to anode by electrorepulsion (Yoshida and Roberts, 1992). However, the convective flow which goes in the opposite direction at physiological conditions, will oppose the movement of MTX towards the anode.

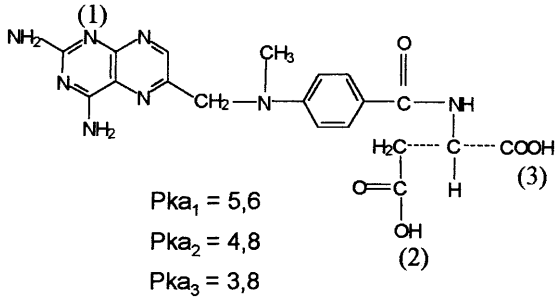

Fig. 1. Chemical structure of methotrexate (M.W. 454.56 g mol^{-1}, log *P* oct/water = −1.85).

Singh et al. (Singh and Singh, 1995) studied the combined effect of iontophoresis and enhancers such as DMSO (dimethylsulphoxide), DMF (dimethylformamide), DMA (dimethylacetamide) and Azone (1-dodecylazacycloheptan-2-one) on the transdermal penetration of MTX. They found that iontophoresis further increased the permeability coefficient of MTX as compared to that measured using the enhancers passively. However, these authors did not characterize the iontophoretic penetration of MTX in the absence of chemical enhancers.

The long-term aim is to develop an efficient topical dosage form for MTX. In this context, a study of factors possibly influencing electrotransport into and across pig skin, namely drug concentration, current density, ionic strength, and pH, is reported here.

2. Materials and methods

2.1. Reagents

Methotrexate hydrate (98%), Ag wire (99.99% pure, 1.0 mm diameter), AgCl (99%), Pt wire (99.99% pure, 0.5 mm diameter), and HEPES (99.5%) were all from Sigma-Aldrich Química S.A. (Spain). Methanol (HPLC quality) was from Merck S.A. All the other reagents (NaCl, ethanol, HCl, NaOH, Na_3PO_4) were analytical grade.

2.2. Skin

Skin was obtained from various regions (neck, hind leg, fore leg, back) of a 2–3-day-old pig that had been killed by cervical dislocation. The skin was frozen at −20°C until use. About 12 h before each experiment, the skin was thawed. Muscle and subcutaneous tissue remains were removed, and any hair was cut short. The skin was then cut into appropriately sized pieces which were placed in the diffusion cells with the epider-

mis facing the donor solution. In each series of assays skin from all of regions was used, to minimize inter-assay variability due to variability among the skin types.

2.3. Passive diffusion experiments

For these experiments vertical passive diffusion cells (area 4.15 cm^2) (Laboratory Glass Apparatus, USA), thermostatted at 37°C in a water bath (Selecta, Digiterm, Spain) and protected from light were used. The donor solution was 3 ml of 25 mM HEPES buffer pH 7.4 + / − 50 mM NaCl containing MTX at 2.0, 2.5, 2.8 or 3.0 mg/ml. The receptor solution was 4 ml of 25 mM HEPES buffer pH 7.4 + 154 mM NaCl. Both the donor and the receptor solutions were magnetically stirred (HP Variomag Multipoint Electronicrührer) throughout the experiment. The concentration of MTX in the receptor solution was determined after 6 and 24 h of passive diffusion, and the amount of MTX in the skin was determined at the end of the experiment. At each time the whole receptor solution was sampled and the receptor chamber refilled with fresh buffer.

2.4. Iontophoresis experiments

For these experiments, side-by-side diffusion cells were used (area = 0.78 cm^2; Crown-Bio Scientific, USA), again protected from light and magnetically stirred throughout the experiment. A constant direct current generated with a Kepco TES 2360LCR apparatus (Kepco Power Supply, USA) was applied. The voltage of the complete circuit and of each cell was measured hourly with a voltmeter (Freak, MY-63). Electrodes were Ag/AgCl, prepared by the method of Green et al. (Green et al., 1991). The donor solution (cathodic compartment) was 3 ml of 25 mM HEPES buffer pH 7.4 with NaCl at 0, 50 or 100 mM, and MTX at 2.0 or 3.0 mg/ml. The receptor solution was 4 ml of 25 mM HEPES buffer pH 7.4 + 154 mM NaCl. These experiments were performed at room temperature.

The effects of pH on the iontophoretic penetration of MTX were also investigated. To this end, experiments were performed in which the donor solution was 25 mM HEPES buffer pH 4.0 or 5.0 + 50 mM NaCl containing 0.034 or 0.072 mg/ml MTX, and the receptor solution was 25 mM HEPES buffer pH 7.4 + 154 mM NaCl. In these experiments the donor solution was placed in the anodic compartment, in view of the positive charge of MTX in this pH range.

In all of the above experiments, the concentration of MTX in the receptor solution was determined after 4, 7 and 10 h, and the amount of MTX in the skin was determined after 10 h. At each time the whole receptor solution was sampled and the receptor chamber refilled with fresh buffer.

2.5. MTX quantification

Quantification of MTX in receptor solutions was as follows. First, the receptor solution was filtered through a 0.45 μm polyvinyl difluoride filter (Millipore), then evaporated to dryness in a vacuum oven (Heraeus) at 50°C. The dry residue was then dissolved in 1.5 ml of the HPLC mobile phase (see below), and the solution was then centrifuged (Sigma 2-15, Laborzentrifugen, Germany) for 10 min at 3500 rpm. The supernatant was filtered, and MTX was quantified in the filtrate by HPLC, as below.

Quantification of MTX in skin was as follows. The skin was washed with ethanol (2 × 5 ml), then left in 10 ml of ethanol in the dark for about 48 h. The ethanol was then filtered through filter paper, and evaporated to dryness at 50°C under vacuum. The dried residue was then dissolved in 1.5 ml of HPLC mobile phase (see below) and centrifuged for 10 min at 3500 rpm. The supernatant was filtered through 0.45 μm polyvinyl difluoride filters, and MTX was quantified in the filtrate by HPLC, as below.

MTX was quantified by HPLC as per Lawson and Dixon (Lawson and Dixon, 1981), using a Merck Hitachi high-performance liquid chromatograph with an L-6200[a] pump, an L-4500 diode detector, an AS-4000[a] injector, and a D-6000 interface. The mobile phase was 0.1 M Na_3PO_4 (pH 6.7)/methanol 75:25 (degassed by vacuum filtration). Both the column and the precolumn were Lichrospher 100 RP-18 columns (5

Table 1
Cumulative transdermal delivery of methotrexate (MTX) (μg/cm^2 of skin) in each of the iontophoresis experiments (mean ± S.D, $n = 6$)

[MTX] (mg/ml)	[NaCl] (mM)	Current density (mA/cm^2)	[MTX] cumulative delivery		
			4 h (μg/cm^2)	7 h (μg/cm^2)	10 h (μg cm^{-2})
2	0	0.5	29.9 ± 11.8	100.4 ± 78.0	207.0 ± 154.6
2	50	0.5	18.4 ± 19.6	49.6 ± 44.4	70.3 ± 65.6
2	100	0.5	2.5 ± 3.1	9.0 ± 14.2	24.5 ± 28.7
3	0	0.5	40.0 ± 38.2	103.4 ± 89.8	184.6 ± 151.2
3	50	0.5	15.6 ± 15.4	32.5 ± 31.0	66.7 ± 54.1
2	0	0.25	17.1 ± 28.1	24.7 ± 35.0	57.8 ± 93.1
2	50	0.25	1.9 ± 2.6	6.4 ± 9.9	11.6 ± 17.8
2	100	0.25	6.5 ± 8.9	12.2 ± 15.9	21.9 ± 21.4
3	0	0.25	9.0 ± 18.0	19.2 ± 28.2	25.2 ± 31.6
3	50	0.25	3.6 ± 8.9	10.2 ± 21.9	19.3 ± 42.8

μm pore size) (Merck). Flow rate was 1 ml/min. System temperature was maintained constant at 35°C, and MTX was quantified on the basis of absorption at 305 nm. Sample volume was 100 μl. The detection limit was 2 μg/ml.

2.6. Data analysis

At least 4–6 replicates of each experiment were used. Results are presented in the text as means ± S.D.s. Data were analyzed by analysis of variance, and Dunn's tests or Student–Newman–Keuls tests for comparisons of multiple means. Statistical significance was fixed at $P < 0.05$.

3. Results and discussion

3.1. Passive diffusion experiments

These assays confirmed the scant ability of MTX to penetrate the skin by passive diffusion. MTX was only detectable in the receptor solution when the MTX concentration in the donor solution was ≥ 2.8 mg/ml: penetration after 24 h was 0.6 ± 0.4 and 2.9 ± 4.5 μg/cm^2 with 2.8 and 3.0 mg/ml of MTX in the donor solution respectively. These two means did not differ significantly.

Increasing MTX concentration in the donor solution led to significant increases in the amount of drug accumulated into the skin, which ranged from 2.38 ± 0.46 to 6.21 ± 0.50 μg/cm^2.

The results are in agreement with those of Weintein et al. (Weintein et al., 1989), who investigated the passive diffusion of MTX across human skin. These authors found that transdermal penetration of MTX from an aqueous donor solution containing 2% MTX was about 5 μg/cm^2 after 48 h.

It is clear then that transdermal delivery of MTX almost certainly requires some sort of enhancing technique, such as a chemical absorption-promoter (Chatterjee et al., 1997) or iontophoresis (Singh and Singh, 1995), since passive penetration into the skin may be sufficient for treatment of psoriasis, though this cannot be confirmed since the skin concentrations required for therapeutic effect are not know.

3.2. Iontophoresis of MTX

Iontophoresis has been proposed as a technique for enhancing skin penetration of antipsoriatic drugs such as khellin (Green, 1996) and MTX (Singh and Singh, 1995). In the present study, one aimed to identify the key factors controlling electrotransport of MTX.

Table 1 summarizes the results on iontophoretic transdermal penetration of MTX under the different conditions tested. As expected, the application of an electric current considerably increased penetration into the receptor solution: in all cases, the 10-h cumulative delivery of MTX was markedly

and significantly higher ($P < 0.05$) than in the corresponding passive-diffusion experiments.

Note that the influence of MTX concentration in the donor solution was studied only over the range 2.0–3.0 mg/ml (4.4–6.6 mM), because of the low solubility of MTX (Table 2). Furthermore, it was not possible to perform experiments with 3.0 mg/ml in medium with 100 mM NaCl, because of the even lower solubility of MTX at this ionic strength (Table 2).

The results of these experiments suggest that MTX concentration in the donor solution does not have a significant effect on iontophoretic trasdermal penetration. Results of this type have been predicted by Kasting (Kasting and Keister, 1989) in the absence of competing ions in the donor solution, and observed experimentally for hydromorphone (over the range 0.01–0.08 M) by Padmanabhan et al. (Padmanabhan et al., 1990). MTX concentration in the donor solution likewise had no significant effect in the presence of NaCl, but this may be attributable to the narrow concentration range considered and to the high inter-assay variability observed.

The effects of ionic strength on penetration were investigated by modifying NaCl concentration in the donor solution (0, 50 or 100 mM). Penetration declined with increasing ionic strength: however, the differences were statistically significant only when MTX concentration in the donor solution was 2.0 mg/ml and current density was 0.5 mA/cm^2. The apparent transport number of MTX was 6×10^{-4} in experiments with donor solution MTX concentration 2.0 mg/ml, current density 0.5 mA/cm^2, and NaCl concentration 100 mM, and increased only to 5×10^{-3} in experiments without NaCl. This low transport number indicates that the iontophoretic transport of MTX is limited by the presence of the highly mobile sodium ions at the anode and possibly by the comparably sized HEPES ions (M.W. 238 g/mol, 25 mM) at the cathode.

Table 2
Solubility of methotrexate (MTX) in the different donor solutions used in these experiments

Donor solution	Solubility of MTX (mg/ml)
HEPES 25 mM, pH 7.4	4.0
HEPES 25 mM + NaCl 50 mM, pH 7.4	3.1
HEPES 25 mM + NaCl 100 mM, pH 7.4	2.4
HEPES 25 mM + NaCl 50 mM, pH 5.0	0.09
HEPES 25 mM + NaCl 50 mM, pH 4.0	0.04

To investigate the influence of current density on MTX penetration, experiments were performed at 0.25 and 0.50 mA/cm^2. An increase in penetration with increasing current density is expected, given that the flow due to electrorepulsion (J_{ER}, mol/h per cm^2) of an ion i is given by:

$$J_{ER} = (t_N \cdot I)/z \cdot F$$

where t_N is the transport number of that ion, z its valency, F the Faraday constant, and I the current applied (Yoshida and Roberts, 1992; Burnette and Marrero, 1986). The amount of MTX delivered increased with increasing current density, but the observed differences were statistically significant only in the series of experiments in which MTX concentration was 3.0 mg/ml and NaCl concentration was 50 or 0 mM.

It is of interest to compare the results with those obtained using other penetration-enhancing techniques, such as microemulsions and chemical enhancers. In a study performed with hairless mouse skin using an MTX-saturated microemulsion of lecithin in water/PG (70:30), pH 5.0 (MTX solubility = 2.5 mg/g), the measured transdermal penetration rate was 5 ± 1 μg/cm^2 per h (Trotta et al., 1996). In another study (Weintein et al., 1989) performed with human skin, using a 2% MTX solution and the enhancers dodecylmethylsulfoxide and Azone, the cumulative delivery of MTX was 10–40 μg/cm^2 in 48 h (i.e. about 0.2–0.8 μg/cm^2/h), lower than in the present study.

In the present study, the concentration of MTX in the skin ranged from 1 to 10 μg/cm^2 in the different experiments (Table 3). Statistical analysis of these data suggests that the amount of MTX entering and remaining in the skin tends to increase with increasing current density, increasing MTX concentration, and declining NaCl concen-

Table 3
Methotrexate (MTX) recovered from the skin after the iontophoretic experimets (μg/cm^2, mean ± S.D, $n = 6$)

[MTX] (mg/ml)	[NaCl] (mM)	Current density (mA/cm^2)	[MTX] in skin (μg/cm^2)
2	0	0.5	6.9 ± 3.2
2	50	0.5	3.7 ± 2.6
2	100	0.5	7.1 ± 9.0
3	0	0.5	10.9 ± 6.1
3	50	0.5	4.7 ± 1.7
2	0	0.25	5.3 ± 2.5
2	50	0.25	5.4 ± 4.3
2	100	0.25	8.9 ± 10.4
3	0	0.25	1.4 ± 1.4
3	50	0.25	0.7 ± 1.7

tration in the donor solution. However, no clear pattern was observed. For example, a higher current density led to higher MTX accumulation only in the experiments performed with 3 mg/ml MTX. Similar values (13.5 ± 2.9 μg/cm^2) were obtained by Lu et al. (Lu et al., 1997) using rabbit ear skin and gels containing 0.5–1.0% of MTX and 4% *N*,*N*-diethyl-*n*-toluamide (DEET). The relatively high levels of MTX reached in the skin after iontophoresis suggest that this technique may be appropriate for the treatment of psoriasis; however, this cannot be confirmed until the concentration of MTX required for therapeutic effect is established.

Finally, the influence of pH on the iontophoretic penetration of MTX was investigated. At pH 4.0–5.0 the amine group of MTX is predominantly ionized ($\geq 80\%$), and MTX is therefore positively charged and zwitterionic, and must be delivered from the anode. In theory, anodic iontophoresis should offer the advantage of adding an electroosmotic component to the iontophoretic flux (Pikal, 1992). Furthermore, it has been proposed than the electroosmotic contribution becomes more relevant as the transport number of the drug considered decreases (Burnette, 1989; Pikal and Shah, 1990). However, MTX was not detected either in the receptor solution or in the skin after anodic iontophoresis at either pH. This is probably due to the low solubility of MTX in the donor solution (Table 2).

It therefore seems, that transdermal penetration of MTX can be effectively improved by iontophoresis. In same previous studies, the use of other absortion-promoting strategies such as microemulsions and chemical enhancers has resulted in similar values of transdermal penetration. However, iontophoresis offers some clear advantages, such as its versatility (as far as the control of drug delivery is concerned) and the fact that it is a physical method. In other words, the enhanced flux results from the interaction between the electric field and the ions present in the system. In view of this, a faster recovery of the skin barrier (soon after the current is turned off) and lower skin irritation are expected than when the skin barrier is chemically or structurally perturbed. In any case, the usefulness of iontophoresis for the treatment of psoriasis requires future sudies aimed at assessing: (a) the safety of iontophoretic delivery to psoriatic skin; (b) optimal MTX concentrations for therapeutic effect; and (c) measures of MTX iontophoretic transport and accumulation through a damage skin barrier such psoriatic skin.

4. Conclusions

Iontophoresis markedly improved transdermal penetration of MTX. Penetration was influenced by ionic strength (i.e. NaCl concentration) of the donor solution and current density. Specifically, penetration was highest in the absence of NaCl and at the highest current density tested (0.5 mA/cm^2). By contrast, penetration was not af-

fected by MTX concentration in the donor solution (range 4.4–6.6 mM). A variable amount of MTX was recovered from the skin, independently of the iontophoresis conditions. Attempts at delivery of MTX by anodic iontophoresis were not successful, probably because of the low solubility of this drug in acid medium.

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