The Effect of Chemical Enhancers on Tacrolimus Permeation through Rat Skin

**ABSTRACT**

**Purpose:** To achieve percutaneous delivery of tacrolimus to epidermis for the treatment of pigmentation disorders. **Methods:** Tacrolimus permeability parameters through rat skin were evaluated with and without chemical enhancers such as Eucalyptus oil, oleic acid, sodium lauryl sulfate. **Results:** The skin showed barrier for tacrolimus permeability through full skin and that diffusion into the skin was the rate-limiting step for drug flux. Transcutol and eucalyptus oil were the most effective enhancers as they increased flux 2 and 1.5-folds. Sodium lauryl sulfate disrupted the lipid-protein complex structure of the skin and thus increased diffusion coefficient 1.5-folds. Oleic acid demonstrated no enhancement effects on tacrolimus permeability. Two transition temperatures was found in hydrated skin thermograms around 40 and 140° C. The first transition belongs to transition from crystal to gel structure. It seems that structure responsible of this transition influenced by all chemical enhancers. The second transition occurred at 140° C and may reflects lipoprotein structures that influenced by all chemical enhancers except of oleic acid. **Conclusion:** Tacrolimus showed low permeability through rat skin because it has high molecular weight. Diffusion into skin was the rate-limiting step for permeability that successfully improved by Transcutol and eucalyptus oil.

**Key words:** Tacrolimus, percutaneous absorption, chemical enhancers, differential scanning calorimetry

**INTRODUCTION**

Permeation of drugs through the skin is the basis of transdermal delivery [1]. Transdermal drug delivery is associated with some advantages such as controlled drug delivery, continuous drug delivery (which is important for drugs with short biological half-life and low therapeutic indices), first-pass intestinal and hepatic bypass, avoidance of the gastrointestinal irritation (which is common with oral medications), and facilitation of drug localization at target site[1].

The two main steps in skin permeation are partitioning and diffusion through the stratum corneum and then viable epidermis, passage into the dermis and finally, systemic absorption or penetration into deeper tissues. The greatest barrier to drug penetration is the stratum corneum, the outermost layer of the skin [2]. The stratum corneum poses a formidable challenge to drug delivery systems. Several approaches have been used to improve entry of drugs into lower skin layer and deeper tissues. Chemical and physical permeation enhancers have been designed to facilitate delivery of high drug concentrations across the skin into systemic circulation or deeper tissues [3]. The classes of enhancers used and the mode of action of these agents vary [4]. Increased drug diffused in the skin, stratum corneum lipid fluidization, and increase in thermodynamic activity of drug in the skin and vehicles, as well as effect on drug partition coefficient, are the most common mode of action of chemical enhancers. Tacrolimus is a macrolid compound with immunosuppressive property that is applied in dermatological disease such as psoriasis [5] and vitiligo [6]. Tacrolimus is a phosphatase calcineurine inhibitor that is using for atopic dermatitis. This compound is a topical noncorticosteroidal immunosuppressive agent with lower skin permeability and without any atrophy adverse reaction in compare with corticosteroids [7; 8]. Percutaneous absorption of Tacrolimus, pimorcholimus, betamethasone and clobetasol through human and rat skin was studied [9]. Percutaneous delivery of tacrolimus to viable epidermis is the goal in the treatment of skin pigmentation disorders such as vitiligo [10]. Effectiveness of Tacrolimus for psoriasis treatment in comparison with calcipotrierol [11] and cyclosporine was studied. Topical Tacrolimus and cyclosporine A are potents immunosuppressants that are used systemically to treat several inflammatory skin conditions. Tacrolimus is 10–100 times more potent than cyclosporine A. They have been used topically in various clinical studies. Topical cyclosporine A is largely ineffective whereas topical tacrolimus is effective in treating atopic dermatitis [12]. For effective treatment, tacrolimus has to penetrate into the epidermis.

The aim of this study, therefore, is to develop a topical tacrolimus delivery system into epidermis and evaluate the effect of chemical enhancers on its permeation characteristics through rat skin.

**MATERIALS AND METHODS**

**Materials**

Tacrolimus was gift from Behvazan pharmaceutical company (Tehran, Iran). Eucalyptus oil, containing 70 % 1,8 cineole, was obtained from Barij Esrance Iranian Company in Kashan (Iran) while oleic acid, sodium lauryl sulfate and methanol supplied by Merck. Ethoxydiglycol (Transcutol CG) was kindly donated by Gattefosse (Faratin Company, Tehran, Iran), while potassium phosphate monobasic was purchased from Sigma. Water was deionized and filtered in-house was used. All other chemicals and reagents used were of analytical grade.

**Animal experiments**

Male Wistar rats weighing 200-250 g were used for the in vitro permeation study. After sacrificing under ether anesthesia, the abdominal skin hair was carefully removed with an electric clipper and razor without damaging. The skin was excised and any extraneous subcutaneous fat was removed from the dermal surface. Whole skin thickness was measured using a digital micrometer (AACO Company, France). The animals were treated according to the principles for the care and use of laboratory animals and approval for the studies was given by the Ethical Committee of the Ahvaz Jundishapour.
University of Medical Sciences. The guidelines followed were those laid down by the National Academy of Sciences and published by the National Institutes of Health (U.S. Department of Health & human services, office of laboratory animal welfare)

Solubility determination

The solubility of tacrolimus in water and buffer solutions was studied by equilibrating the suspension of excess amount of drug in 5 ml of medium and shaken gently for 24 h at 32 °C. It was then centrifuged for 10 min at 3000 rpm, filtered, diluted and analyzed by UV spectrophotometric (Cecil, England).

In vitro permeation study

Diffusion cells fabricated in-house and with an effective area of approximately 2.49 cm², were used for the permeation studies. Whole skin and epidermis samples were placed between donor and receptor chambers of the cells with the epidermal side facing the donor compartment. Skin samples were hydrated prior to their being used. The donor phase was filled with 3ml saturated aqueous solution of drug while the receptor compartment was filled with phosphate buffer (pH 7). Tacrolimus is soluble in this medium which also provided perfect sink conditions. Temperature was maintained at 37 ± 0.5 °C and the receptor chamber was stirred at 300 rpm. At predetermined time intervals, 0.5 ml of the receptor medium was withdrawn and immediately replaced with an equal volume of fresh buffer. The permeated amount of tacrolimus was determined by HPLC with UV spectrometry at 210 nm.

Effect of chemical enhancers on drug permeation across rat skin

Fully hydrated samples were used as controls. To minimize experimental errors arising from biological variability, each piece of skin was used as its own control. For pretreatment of skin samples, fully hydrated samples were pre-treated with putting 1 ml of a chemical enhancer on the surface of skin in the donor phase. The donor and receptor chambers were then washed with water and filled with aqueous saturated solution of trolamine salicylate and phosphate buffer (pH 7), respectively. The effect of chemical enhancers was evaluated for tacrolimus permeation through full skin samples. Transcutol, olive oil (containing 70 % 1,8-cineole) and eucalyptus oil were used as received without dilution while sodium lauryl sulfate was used as 1 % aqueous solution.

Differential scanning calorimeter (DSC)

DSC studies were carried out with a Mettler DSC facility (model: CH 8603). The complete hydrated skin samples were first immersed in a chemical enhancer on the surface of skin in the donor phase. The donor and receptor chambers were then washed with water and filled with aqueous saturated solution of trolamine salicylate and phosphate buffer (pH 7), respectively. The effect of chemical enhancers was evaluated for tacrolimus permeation through full skin samples. Transcutol, olive oil (containing 70 % 1,8-cineole) and eucalyptus oil were used as received without dilution while sodium lauryl sulfate was used as 1 % aqueous solution.

Data analysis

The cumulative amount of tacrolimus permeated through unit area of the diffusion surface into the receptor was calculated and plotted as a function of time. Flux (J) was calculated from the slope of the linear portion of the penetration curves and expressed as the mass of drug passing across 1 cm² of skin time. Steady state drug diffusion from a saturated solution through a skin membrane is represented as in Eq 2.

\[ J = P \cdot S = (K \cdot D \cdot h) \cdot S \] (Equation 2)

Where J is the flux of the drug and P is the permeability coefficient comprising of the membrane/vehicle partition coefficient (K), diffusivity coefficient (D) and skin thickness (h). P was calculated by dividing J by the drug’s saturated solubility (S) in the donor phase [14]. Enhancement ratios were calculated from permeation parameters after enhancer treatment divided by the same parameters for controls (Equation 3).

\[ E_r = \frac{\text{flux after enhancer treatment}}{\text{flux before enhancer treatment}} \] (Equation 3)

The statistical significance of the difference between various treatments was determined using one-way ANOVA. Differences were considered to be statistically significant at p < 0.05. Correlation analyses were performed by least square linear regression method. Correlation coefficients were examined for significance by Student’s t - test. All statistical analyses were conducted using SPSS software (SPSS 13.0 for Windows, SPSS Inc, Chicago, IL, USA)

RESULTS AND DISCUSSION

Effect of chemical enhancers on trolamine salicylate permeability

Permeability parameters after skin pretreatment with chemical enhancers in compare with control presents in table 1. The effect of chemical enhancers on tacrolimus permeability in compare with control is presented in table 2 as ER₃₀₀ (ratio of drug flux after and before skin pretreatment with enhancer) and ER₇₂₅ (drug diffusion coefficient after and before skin pretreatment with enhancer). Hydrated skin with no enhancer pretreatment and aqueous saturated solution of tacrolimus as donor phase served as control. The results indicate that eucalyptus oil, transcutol and sodium lauryl sulfate increased tacrolimus flux and diffusion coefficient significantly (p < 0.05) but oleic acid doesn’t have any enhancement effect. Transcutol provided the best enhancement of tacrolimus flux, increasing it approximately up to 2-fold relative to control, followed by eucalyptus oil (1. 55-fold) and sodium lauryl sulfate (1. 25-fold). All the chemical enhancers except oleic acid exerted significant effects on diffusion coefficient (p < 0.05), with transcutol showing the greatest enhancement effect on diffusion coefficient (up to 2 – fold). Transcutol and eucalyptus oil, with higher drug solubility in skin, increased flux 2-fold approximately. The effect of Transcutol (Ethoxydiglycol) and eucalyptus oil on flux was same as diffusion coefficient. This observation indicates that the main mechanism of action of Transcutol and eucalyptus enhancement activity is facilitation of drug partitioning and diffusion into skin. Transcutol is a powerful solubilizing agent and is miscible with polar and non-polar solvent [15]. Eucalyptus oil consists of 75 % 1,8-cineole. Cineole is a cyclic terpene that acts by creating liquid pools in stratum corneum and disrupting the lipid structure of the stratum corneum, thereby increasing the diffusion coefficient of polar and non-polar drugs in the membrane [16]. Therefore, it seems that cineole, by disrupting the lipid structure of the stratum corneum, increased the diffusion coefficient of tacrolimus but the effect of cineole on flux was not limited to diffusion coefficient. Comparison between the effect of sodium lauryl sulfate on flux and diffusion coefficient suggests that sodium lauryl sulfate increased flux by improvement the diffusion coefficient and the effect on partitioning phenomena was not important. The effect of sodium lauryl sulfate on skin permeability of hydrophilic compounds with log P < 3 has previously been reported [17].

It seems that the main barrier against tacrolimus penetration through rat skin is the diffusion through epidermis. This finding is in tandem with the physicochemical properties of tacrolimus which is a compound with high molecular weight. Permeation through membrane consists of two processes, partitioning and diffusion. Tacrolimus with lipophilic properties demonstrates good partitioning from aqueous solution into skin and so partitioning into stratum corneum isn’t rate limiting step. Therefore low permeation is due to diffusion through skin layers.

Earlier studies on skin absorption of tacrolimus disclosed a rate of percutaneous penetration for a 0.03-0.3% topical tacrolimus preparation of 3.1-6.8 ng/cm².hr [18]. Results obtained in present study indicate tacrolimus lower flux through rat skin. For achieving perfect therapeutic effects, high tacrolimus concentration should be obtained by applying chemical and physical enhancement methods. This goal was provided by topical application of liposomal formulation of tacrolimus that achieved nine times the concentration of tacrolimus at a target site than did systemic administration of tacrolimus [19].
Table 1: permeability parameters after pretreatment with chemical enhancers in compare with control (mean ± standard deviation, n = 5)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Flux (mg·cm⁻²·h⁻¹)</th>
<th>D (cm²·h⁻¹)</th>
<th>Tₘₕ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.026± 0.003</td>
<td>0.0007 ±0.0009</td>
<td>5.38 ±0.41</td>
</tr>
<tr>
<td>SLS</td>
<td>0.035 ±0.003</td>
<td>0.0012 ±0.0006</td>
<td>6.15±0.47</td>
</tr>
<tr>
<td>Eucalyptus oil</td>
<td>0.04 ±0.005</td>
<td>0.0014 ±0.0005</td>
<td>5.72 ±0.49</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>0.033± 0.004</td>
<td>0.0008 ±0.0001</td>
<td>4.59 ±0.4</td>
</tr>
<tr>
<td>Transcutol</td>
<td>0.055± 0.0053</td>
<td>0.0014 ±0.0002</td>
<td>4.83±0.28</td>
</tr>
</tbody>
</table>

Table 2: Effect of chemical enhancers on derived permeation parameters for tacrolimus through whole skin (mean ± standard deviation, n = 5)

<table>
<thead>
<tr>
<th>Chemical enhancer</th>
<th>ERₘₕ</th>
<th>ERₛ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic acid</td>
<td>1.2 ± 0.066</td>
<td>1.1 ± 0.171</td>
</tr>
<tr>
<td>Sodium lauryl sulfate</td>
<td>1.3 ± 0.11</td>
<td>1.72 ± 0.190</td>
</tr>
<tr>
<td>Eucalyptus oil</td>
<td>1.55 ± 0.37</td>
<td>1.58 ± 0.121</td>
</tr>
<tr>
<td>Transcutol CG</td>
<td>2.12 ± 0.12</td>
<td>2. ± 0.138</td>
</tr>
</tbody>
</table>

ERₘₕ = ratio of flux after and before treatment with enhancer; ERₛ = ratio of diffusion coefficient after and before treatment with enhancer

DSC
The thermogram of hydrated whole rat skin is shown in Fig 1. It shows two endothermic transition at around 40.7 ± 1.6 °C with an enthalpy of 2.33 ± 0.15 (mean ± SD, n = 3) and 145 ± 2.14 °C with an enthalpy of 56.5 ±3.22. The effect of chemical enhancers on transition temperature and enthalpy is shown in table 3.

Figure 1: Thermogram of hydrated whole rat skin

Table 3: Effect of chemical enhancer on the thermal properties of hydrated rat skin (mean ± SD, n = 3)

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Transition temperature T(°C)</th>
<th>Transition enthalpy ΔH (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.6±10.7</td>
<td>0.15±2.33</td>
</tr>
<tr>
<td>Sodium lauryl sulfate</td>
<td>2.1±14.5</td>
<td>3.22±56.5</td>
</tr>
<tr>
<td>0.9±100</td>
<td>1.2±140</td>
<td>1.87±25.31</td>
</tr>
<tr>
<td>Eucalyptus oil</td>
<td>0.6±40</td>
<td>0.5±1.08</td>
</tr>
<tr>
<td>Transcutol</td>
<td>1.5±150</td>
<td>3.2±84.01</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>1.8±130</td>
<td>3.3±35</td>
</tr>
<tr>
<td>0.9±50</td>
<td>1.5±155</td>
<td>51.14±3.83</td>
</tr>
</tbody>
</table>

In the literature, the main transition phase reported for hydrated rat skin occurred at 81 °C [13], 70.3 °C [20] that indicating lipid structure and 40°C as against the in our study that has been reported as changing from orthorhombic to hexagonal [21]. Another transition in rat skin occurred at 100°C [13] that related to lipidprotein structure. In present study it seams that transition that was happen at 140 °C according to lipidprotein structure. Following pretreatment of whole skin, all enhancers reduced enthalpy of Tₘₕ. Transcutol and eucalyptus oil provided the best and lowest decreasing of Tₘₕ enthalpy respectively. This finding indicates that all chemical enhancers induced structural changing and increased lipid fluidity in intercellular region. In the other hand all enhancers reduced enthalpy of Tₘₕ with highest and lowest effect according to sodium lauryl sulfate and oleic acid respectively. Denaturizing effect of sodium lauryl sulfate has been reported [17]. The thermograms of skin pretreated with sodium lauryl sulfate seem to confirm the effect of the enhancer on the diffusivity of the skin in that sodium lauryl sulfate increased diffusion coefficient more than flux. The effect of sodium lauryl sulfate on Tₘₕ was more than other enhancers. This finding indicates that structure responsible for Tₘₕ is the main barrier against drug diffusion through skin. However, the effect of transcutol on skin flux and the transition phase at Tₘₕ were more pronounced than that of sodium lauryl sulfate and eucalyptus oil. Two deductions can be made from this. First, the lipid structure related to Tₘₕ wasn’t the main barrier against tacrolimus diffusion. Second, diffusion through this lipid structure was not rate-limiting for flux.

CONCLUSION
The main barrier in tacrolimus permeability is diffusion into skin and therefore, to achieve a good percutaneous formulation with satisfactory dermal penetration, incorporation of Transcutol and eucalyptus oil would be of great advantage. Therefore, since tacrolimus is a non-irritating with perfect therapeutic property, but with low permeable character it could become a suitable candidate for the treatment of inflammatory skin conditions and vitiligo if it can be made to penetrate deeply to provide effective tissue concentration with the aid of enhancers such as Transcutol and eucalyptus oil.

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