ENHANCEMENT OF TRANSDERMAL DELIVERY BY SUPERFLUOUS THERMODYNAMIC POTENTIAL. I. THERMODYNAMIC ANALYSIS OF NIFEDIPINE TRANSPORT ACROSS THE LIPOIDAL BARRIER

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In vitro techniques were used to test certain concepts regarding the enhancement of the transdermal delivery of nifedipine from topical vehicles. Tested vehicles include the volatile solvent acetone, nonvolatile solvents such as propylene glycol and isopropyl myristate, volatile/nonvolatile mixtures, and those mixtures with a polymer additive. An ethylene-vinyl acetate (EVA) copolymer membrane was used as the lipoidal barrier for the diffusing drug. Not merely the rate of transport per unit area, but the activity coefficient and the diffusion coefficient of the penetrating agent in the barrier were obtained. Despite the 10000-fold difference in the vehicle concentration, water and several hydrophilic vehicles containing finely ground suspensions of the drug produced nearly the same rate of penetration. Some lipophilic solvents affected the barrier function of the EVA membrane to promote penetration of the drug. It has been found that the activity coefficient in the EVA membrane is very susceptible to wide variations by imbibition of such solvents. From volatile/nonvolatile mixtures, a transient enhancement in the transport of nifedipine across the membrane was observed. The increase in the flux was accounted for by the increase in the thermodynamic activity of the drug in the nonvolatile vehicle caused by the evaporation of the volatile component. The eventual decrease in penetration was the result of the drug precipitation from the supersaturated solution. The precipitation was inhibited and/or retarded during the entire time course of the experiments when a polymer additive was present. The steady-state fluxes from mixtures with a polymer additive were higher by about 3 to 5 times than that of the control experiment.

Keywords — transdermal delivery; nifedipine; penetration enhancement; thermodynamic potential; activity coefficient; diffusion coefficient; supersaturation; volatile component; polymer additive; diffusion model

Maximizing the rate of transdermal penetration of beneficial drugs is of great interest to pharmaceutical scientists. There are many agents capable of increasing the permeability of the skin. These include caustics, alkalis, strong acids, certain detergents, and organic solvents. All of these agents appear to have the ability to damage or alter the nature of the stratum corneum, thus reducing the diffusional resistance and increasing the permeability.

An alternative approach for enhancing the passive diffusion of a solute from a vehicle into the skin arise from controlling the escaping tendency or thermodynamics of the drug substance in the formulation. Higuchi\(^{1}\) pointed out that the maximum rate of delivery through the skin might be limited to that resulting from the thermodynamic activity of the pure drug substance. Therefore, effective delivery of drugs with poor dermal bioavailability may be accomplished by modifying the physical characteristics of the drug, e.g., by forming appropriate prodrug modifications. Without molecular modifications, any higher activity represents supersaturation with respect to the form. The superfluous thermodynamic potential, that is, the exceeding free energy of the penetrating agent in the metastable supersaturated solution, may increase the rate of transdermal delivery. Crystalline modifications (polymorphic forms) may exist at room temperature with different free energies and thermodynamic activities. In such instances the selection of the most energetic species will result in the fastest penetration. However, the more energetic the polymorph is, the lower is its stability. Those metastable systems may show a gradual change in properties.

Enhancement of percutaneous absorption by the use of volatile/nonvolatile solvent systems was reported.\(^{2,3}\) This can be accomplished so
that the large bulk of the volatile solvent is lost by evaporation immediately after application, concentrating the drug in the remaining vehicle. A supersaturated system can be attained having an unusually high thermodynamic activity of the drug in the nonvolatile vehicle. The accelerated percutaneous absorption at the early phase will be followed by the eventual decrease in penetration as the result of drug precipitation from the supersaturated solution.

Nifedipine is a calcium channel antagonist which undergoes extensive first pass metabolism in man and the use of this drug in the chronic treatment is limited by its rapid bioinactivation. It has been found that percutaneous nifedipine appears to be a promising administration route from the viewpoint of sustained and controlled drug absorption. However, an extensive application area may be needed for a therapeutic effect, and this may prevent good patient acceptance and compliance. Therefore attempts to increase percutaneous nifedipine absorption may be worthwhile.

It has been suggested that some polymer additives may influence the crystallization of drug from the solution. Therefore, one might expect that volatile/nonvolatile solvent systems in combination with polymer additives may prolong the metastable condition and the enhanced penetration may sustain over a sufficient period of the transdermal therapy. As the purpose of our work was to investigate the thermodynamic aspects of nifedipine transport across the lipoidal barrier, using a diffusion cell technique, and in particular how polymers modify them with the vehicle mixture of volatile/nonvolatile solvent system.

MATERIALS AND METHODS

All procedures were carried out under subdued lighting conditions in consideration of the high sensitivity of nifedipine to light.

Materials — Nifedipine JP XI grade was used. An internal standard (I.S.), diethyl 2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate, was synthesized according to the procedure reported previously. Methacrylic acid copolymer (Eudragit RS 100 L, Rohm Pharma GmbH, W. Germany), ethylcellulose (Ethocel STD 10PR/45PR, Dow Chemical Co., Midland, Michigan, U.S.A.), and hydroxypropylmethylcellulose phthalate 200731 (HP-55, JP XI grade, Shinetsu Chemical Ind., Co., Ltd., Tokyo, Japan) were used as received. All other chemicals were commercial products of reagent grade or JP XI grade. An ethylene–vinyl acetate copolymer (EVA) membrane (composition, ethylene–vinyl acetate, 90:10; thickness, 40 μm) obtained from Tamapoly Co., Ltd., Tokyo, Japan was used.

Diffusion Cell Studies — A model system for drug transport across the lipoidal barrier has been studied by a diffusion cell technique employing an EVA membrane. Since the skin barrier is generally considered to be lipid in nature, the EVA membrane method is claimed to be of value in the investigation of drug transport through the skin barrier. The diffusion cell (Kersco Engineering Consultants, Palo Alto, California, U.S.A.) was the same as that used by Sloan et al. and consisted of a plexiglass chamber (45 ml) with a side arm to allow sampling of the receptor phase, a polytetrafluoroethylene gasket. A polyethylene-coated star-head magnet provided efficient mixing. The opening in the lid left exposed an 8.0 cm² area on the EVA membrane through which penetration was measured. The receptor was filled with 0.9% NaCl solution or saline solution incorporated with 25% polyethylene glycol (PEG) 400 to maintain a sink condition. PEG 400 acts as a solubilizer to enhance the aqueous solubility of the relative water-insoluble nifedipine. All air bubbles were removed carefully from the lower surface of the membrane by tipping the cell. Test formulations were applied on the membrane surface and the volatile solvent allowed to escape. In some experiments, silicone grease was smeared around the central well and the top of each cell was sealed with a glass cover slip to prevent evaporation of the solvent. Each cell was placed on a magnetic stirrer in a thermostated chamber maintained at 37 °C. Samples of 100 μl were removed from the receptor phase via the side arm. The concentration of applied drug in the receptor fluid was measured by using high performance liquid chromatography (HPLC). The results reported for each experiment were the average values from three replicate diffusion cells.

The HPLC analysis was performed using a mixture of methanol and distilled water (6:4, v/v) as the mobile phase and a flow rate of 1 ml/min on a reversed-phase column (NOVA PAC C₁₈, Waters) with ultraviolet detection at
254 nm. An internal standard that is structurally similar to nifedipine was used to construct calibrations.

**Partitioning Studies** — The EVA membrane was cut into discs, weighed and placed in nifedipine solutions at 37 °C for 2 weeks. The partition coefficient was calculated from the change in nifedipine concentration of the solutions before and after the partition. The concentration of the drug was measured by using HPLC.

**Solubility Studies** — The solubility of nifedipine in the liquid vehicles was determined at 37 °C by rotating excess amounts in the appropriate vehicle for 24 h. The suspension was then centrifuged. The supernatant fluid was filtered, diluted with methanol and its concentration was determined by means of a spectrophotometer at 350 nm.

**Data Treatment** — The total amount of diffusing substance, \( M \), which has passed per unit area through the membrane in time \( t \) from the essentially constant donor penetrant concentration to the receptor phase at “sink” condition is given by:

\[
M = \frac{DC_0 t}{h} \left( \frac{hC_0}{6} - \frac{2hC_0}{\pi^2} \sum_{n=1}^{\infty} (-1)^n \frac{1}{n^2} \exp \left( -\frac{Dn^2\pi^2t}{h^2} \right) \right)
\]

where \( D \) is the diffusion coefficient; \( C_0 \) is the solute concentration at the membrane surface; and \( h \) is the thickness of the membrane.\(^{10}\) To determine \( D \) and \( C_0 \), each set for all experimental readings was fitted to Eq. (1) by an iterative least-squares computer program.\(^{11}\) Initial values for \( D \) and \( C_0 \) were estimated by the following manner.

As \( t \) in Eq. (1) approaches infinity, this expression approaches a straight line

\[
M = \frac{DC_0}{h} \left( t - \frac{h^2}{6D} \right)
\]

If we differentiate Eq. (2), we then obtain the steady-state flux \( dM/dt \), which is the slope of the straight line

\[
\frac{dM}{dt} = \frac{DC_0}{h}
\]

If a steady state plot is extrapolated to the time axis, the intercept so obtained at \( M = 0 \) is the lag time \( L \):

\[
L = \frac{h^2}{6D}
\]

From Eq. (4), \( D \) is estimated, since the membrane thickness \( h \) is known. Thus \( L \), together with the slope of the straight line, provide estimates of the solute concentration at the membrane surface \( C_\Phi \).

In our consideration, we have assumed that the membrane thickness is constant. In special instances this is a poor assumption and \( D \) can be underestimated. This is attributed to imbibition of the applied solvent by the barrier membrane and marked alteration in the membrane thickness.

Regression analyses of the experimental readings against time, within the steady-state time interval, yielded steady-state fluxes. The interpolation of the experimental readings was done by a cubic-spline function. The maximum flux was estimated as the maximum value in the derivative of the spline function.

**RESULTS AND DISCUSSION**

The approximate thermodynamic activity of pure nifedipine was estimated as \( 4.6 \times 10^{-6} \) mol l\(^{-1} \) at 37 °C with infinitely dilute solution in hexane or heptane having an activity coefficient of unity, as defined by Higuchi.\(^{12}\) Provided that all other factors are equal, the Higuchi theorem predicts that the maximal expected rate of percutaneous delivery should correlate with the values of the activities given. On this basis we would expect that nifedipine would be delivered about ten times faster than topical steroids such as hydrocortisone and hydrocortisone acetate (the activities of pure state (\( a_i \)) for these compounds were reported to be less than \( 2 \times 10^{-6} \) mol l\(^{-1} \))\(^{12}\) but it should be delivered much more slowly than molecules such as methyl testosterone (\( a_i = 1.23 \times 10^{-3} \) mol l\(^{-1} \)) and norethindrone acetate (\( a_i = 2.75 \times 10^{-3} \) mol l\(^{-1} \)).\(^{12}\)

After the diffusing molecules penetrate the barrier layer (the stratum corneum), they encounter an aqueous phase (the viable epidermis). Thus, any molecule which has extremely low water solubility and a high affinity for the barrier layer would not be transported further. For such
molecules, the rate-determining step is the clearance rate from the barrier rather than penetration of the barrier.

If we employ the same standard state as defined above, we can define activity coefficient as

$$\gamma_{ik} = \frac{a_i}{C_{ik}}$$  \hspace{1cm} (5)

where \(a_i\) is the activity of \(i\) in the system expressed in terms of the unit molar of hypothetically infinitely dilute reference system; \(\gamma_{ik}\) is the activity coefficient of \(i\) in a solvent, \(k\), in equilibrium with the reference system; and \(C_{ik}\) is the concentration of \(i\) in the solvent expressed in molar units. Activity coefficients so defined for most lipophilic drugs in water are much greater than 1. The estimated activity coefficient of nifedipine in dilute aqueous solution defined in this fashion is 1.3. It appears that clearance from the barrier as the rate determining process is not significantly important for nifedipine.

Figure 1 shows the average nifedipine transport across an artificial EVA membrane from each nifedipine suspension calculated from three individual experiments.

Equation (3) is equivalent to

$$\frac{dM}{dt} = \frac{aD}{\gamma h}$$  \hspace{1cm} (6)

where \(a\) is the thermodynamic activity of the drug in its vehicle and \(\gamma\) is the effective activity coefficient of the agent in the barrier phase. If it is assumed that the vehicle containing the penetrating chemical does not appreciably affect the barrier, the rate of penetration measured for different vehicles would be approximately constant provided that the thermodynamic activity of the drug in the vehicles was constant. Thus, despite the 10000-fold difference in the vehicle concentration, water and some hydrophilic vehicles, as well as a lipophilic vehicle such as castor oil containing finely ground suspensions of the drug (thermodynamic activity equal to that of pure nifedipine), produced nearly the same rate of penetration (Fig. 1-A).
TABLE I. Parameters for Nifedipine Penetrating Through EVA Membrane from Vehicles Containing Finely Ground Suspensions

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>$C_v$ (mg/ml)</th>
<th>$C_0$ (mg/ml)</th>
<th>$D$ (cm$^2$/s)</th>
<th>$\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water a)</td>
<td>0.012</td>
<td>0.373</td>
<td>$8.28 \times 10^{-10}$</td>
<td>0.0429</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.14</td>
<td>0.483</td>
<td>$5.95 \times 10^{-10}$</td>
<td>0.0331</td>
</tr>
<tr>
<td>PG</td>
<td>10</td>
<td>0.418</td>
<td>$8.13 \times 10^{-10}$</td>
<td>0.0383</td>
</tr>
<tr>
<td>PEG 400</td>
<td>99</td>
<td>0.444</td>
<td>$1.26 \times 10^{-9}$</td>
<td>0.0360</td>
</tr>
<tr>
<td>Acetone a)</td>
<td>181</td>
<td>2.243</td>
<td>$2.56 \times 10^{-9}$</td>
<td>0.0071</td>
</tr>
<tr>
<td>Castor oil</td>
<td>6.7</td>
<td>0.474</td>
<td>$8.28 \times 10^{-10}$</td>
<td>0.0338</td>
</tr>
<tr>
<td>IPM</td>
<td>2.0</td>
<td>2.643</td>
<td>$2.39 \times 10^{-9}$</td>
<td>0.0061</td>
</tr>
</tbody>
</table>

a) Donor phase was occluded to prevent evaporation of the solvent.

Not only lipophilic solvents, such as isopropyl myristate (IPM), diethyl sebacate and disopropyl adipate but also acetone affected the barrier function of the EVA membrane so as to promote penetration of the drug (Fig. 1-B). Whether this is due to the effect of such solvents on the activity coefficient ($\gamma$) or the diffusion coefficient ($D$) of the penetrating agent in the barrier or both has been investigated. Parameters for nifedipine penetrating through EVA membrane from vehicles containing finely ground suspensions are summarized in Table I.

$D$ and $C_0$ were estimated by a full curve analysis using all experimental readings and the fitting of data to Eq. (1). A significant danger in this method arises from possible inaccuracies in the transient flow data, forcing the equation to give the best fit to all the data will give equal weight to those points of questionable precision and may degrade the accuracy of the diffusional parameters deduced. Since $C_0$ is the product of the partition coefficient ($K = C_v / C_0$) and the vehicle concentration ($C_v$), the $C_0$ value can be estimated by an alternative approach. The partition coefficient for the aqueous solution was calculated as 27.3 from the partitioning study. The $C_v$ value estimated in this manner was 0.328 mg/ml in close agreement with the value computed by the full curve analysis.

Since the thermodynamic activity of the drug in the saturated solution is equal to that of the pure drug substance, the effective activity coefficient of nifedipine in the barrier phase ($\gamma$) can be calculated as

$$\gamma = \frac{a}{C_0} = \frac{4.6 \times 10^{-6}}{C_0}$$

where $C_0$ is expressed in molar units. Following the application of nifedipine suspensions in propylene glycol (PG), PEG 400, glycerol, and castor oil, the $\gamma$ and $D$ values varied to a lesser extent from those for the aqueous suspension (Table I). Significant changes both in $\gamma$ and $D$ were observed following the application of the vehicles such as IPM and acetone containing nifedipine suspensions, compared with those values for the aqueous suspension. Since the lag

![FIG. 2. Effect of the Volatile Cosolvent on the Transport of Nifedipine across an Artificial EVA Membrane from the Drug Solutions](image-url)

(1), 8 ml of nifedipine-IPM-acetone (3 : 12.5 : 12.5 : 72, weight ratio); (2), 8 ml of nifedipine-IPM-acetone (3 : 25 : 72); (3), 2 ml of nifedipine-IPM (2 : 98, suspension); (4), 8 ml of nifedipine-PG-acetone (3 : 25 : 72); and (5), 2 ml of nifedipine-PG (2 : 98, suspension). Bars represent the standard deviations.
TABLE II. Effect of the Volatile/Nonvolatile Solvent System on the Transport of Nifedipine across an Artificial EVA Membrane

<table>
<thead>
<tr>
<th>Vehicle mixture</th>
<th>Weight ratio</th>
<th>Steady-state flux (µg/h/cm²)</th>
<th>Maximum flux (µg/h/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP-PG</td>
<td>2:98</td>
<td>0.318</td>
<td>-</td>
</tr>
<tr>
<td>NP-PG-AT</td>
<td>3:25:72</td>
<td>0.928</td>
<td>2.282</td>
</tr>
<tr>
<td>NP-IPM</td>
<td>2:98</td>
<td>4.287</td>
<td>-</td>
</tr>
<tr>
<td>NP-IPM-AT</td>
<td>3:25:72</td>
<td>4.575</td>
<td>6.003</td>
</tr>
<tr>
<td>NP-IPM-PG-AT</td>
<td>3:12.5:12.5:72</td>
<td>4.495</td>
<td>17.825</td>
</tr>
</tbody>
</table>

a) NP, nifedipine; AT, aceton. b) Suspensions.

time $L$, $\gamma$ and $D$ for the IPM suspension were substantially unaltered by pretreatment of the EVA membrane with pure IPM for 1 h, rapid imbibition of the solvent by the barrier phase was indicated. $D$, the diffusion coefficient, is inversely proportional to the microscopic viscosity of the barrier phase and may be varied in this manner. The affinity of the drug for the barrier phase, that is, its activity coefficient in the EVA membrane, is also very susceptible to wide variations by imbibition of such solvents.

Figure 2 shows the nifedipine transport from vehicle mixtures of volatile and nonvolatile solvents. On application of the IPM-acetone mixture to the membrane, the acetone rapidly evaporated causing simultaneous precipitation of nifedipine. In this case, the penetration from the volatile/nonvolatile solvent system remained at a similar level to that of the simple nonvolatile solvent. From the PG-acetone mixture, a transient enhancement in the transport of nifedipine across the membrane was observed. Possibly, in this way, evaporation of the 72% (w/w) volatile solvent leaving the drug to concentrate into the nonvolatile component would cause temporal supersaturation and so enhance the penetration. The penetration curve of nifedipine from the IPM-PG-acetone mixture followed a similar pattern to that of the PG-acetone mixture.

The influence of the volatile/nonvolatile solvent system on the nifedipine transport is also shown in Table II. The maximum flux from the PG-acetone mixture was about 2.5 times the steady-state flux and 7 times that from the simple PG suspension. Steady-state fluxes from the IPM-acetone mixture, the IPM-PG-acetone mixture and from the simple IPM suspension were approximately identical. The maximum flux from the IPM-PG-acetone mixture was about 4 times the steady-state flux. If it is assumed that $\gamma$ and $D$ can be considered as constants during the entire time course of diffusion, the increase in the flux is accounted for by the increase in the thermodynamic activity of the drug in the nonvolatile vehicle. The eventual decrease in penetration was the result of the drug precipitation from the supersaturated solution.

FIG. 3. Effect of Polymer Additives on the Transport of Nifedipine across an Artificial EVA Membrane from the Volatile/Nonvolatile Solvent System

(C), 4 ml of nifedipine-IPM-PG-acetone (3:12.5:12.5:72, weight ratio); (1), 4 ml of nifedipine-Ethocel-IPM-PG-acetone (3:9:12.5:12.5:63); (2), 4 ml of nifedipine-HP-55-IPM-PG-acetone (3:9:12.5:12.5:63); and (3), 4 ml of nifedipine-Eudragit-IPM-PG-acetone (3:15:12.5:12.5:57). The receptor was filled with the saline solution incorporated with 25% polyethylene glycol 400 to maintain a sink condition. Bars represent the standard deviations.
TABLE III. Effect of the Volatile/Nonvolatile Solvent System in Combination with the Polymer Additives on the Transport of Nifedipine across an Artificial EVA Membrane

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Steady-state flux (μg/h/cm²)</th>
<th>$D$ (cm²/s)</th>
<th>$C_0$ (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.04 (20.02) a)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethocel</td>
<td>40.77</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HP-55</td>
<td>32.53</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eudragit</td>
<td>22.90</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eudragit $b)$</td>
<td>23.24</td>
<td>$1.97 \times 10^{-9}$</td>
<td>13.89</td>
</tr>
<tr>
<td>Eudragit $c)$</td>
<td>3.67</td>
<td>$1.95 \times 10^{-9}$</td>
<td>2.28</td>
</tr>
</tbody>
</table>

a) The value in parentheses is the maximum flux. b) The vehicle mixture was applied after evaporation of the volatile solvent under a gentle stream of nitrogen at 60 °C for 1.5 h. c) The vehicle mixture was applied under occlusive condition.

Figure 3 depicts the influence of the volatile-nonvolatile solvent system in combination with the polymer additives on the transport of nifedipine across the EVA membrane. The steady-state fluxes from mixtures with a polymer additive were higher by about 3 to 5 times than that of a control experiment (Table III). Those values were higher than even the maximum flux from the control mixture. In the absence of polymer additives, the precipitation proceeded with evaporation of the volatile solvent. In contrast, the precipitation was inhibited and/or retarded during the entire time course of the experiments when a polymer additive was present. The inhibitory effect of polymer additives on the crystallization of several drugs has been reported to clarify the mechanism of drug coprecipitation with the additives. 7

It is apparent that the enhancement of the transport seen in these experiments is due to the increase in the solute concentration caused by the evaporation of the volatile component. These penetrative effects cannot be due to the acetone exerting an affection or carrier mechanism in the barrier as the penetration is very low from the completely volatile solvent and from the occluded systems where evaporation was minimized and the solution remained on and in the membrane surface throughout the experiment (Fig. 4). When a vehicle mixture was applied after evaporation of the volatile component, the steady-state flux was unaltered from that of the intact mixture (Fig. 4 and Table III).

The diffusion coefficient for the evaporated mixture and that for the occluded mixture were estimated to be closely identical (Table III). On the other hand, a 6-fold difference in $C_0$ was detected between the two experiments, exactly corresponding with the difference in the steady-state flux. It is concluded that the superfusing thermodynamic potential, that is, the exceeding
free energy of the penetrating agent in the metastable supersaturated solution, may increase the rate of transport across the lipoidal barrier.

Diffusion studies with artificial membranes have long been used as one criterion for assessing the availability from topical formulations. There are obvious limitations of such methods and conclusions must be drawn with great caution. Certainly the partition coefficient for a drug between vehicle and membrane will not duplicate that between vehicle and human skin. But, regardless of the actual value of the partition coefficient for a penetrant present on the surface of the skin, it must be subject to its thermodynamic activity. Since the rate of permeation of the skin barrier is directly related to the thermodynamic activity of the drug achieved immediately above the barrier, the alteration in that activity should affect the passage of penetrant into an adjacent phase, whether that phase is polymer membrane or skin tissue. The degree to which this is actually true and its clinical implications must ultimately be established by correlation to in vivo studies using the same dermatological formulations. Details will be reported in the near future.\textsuperscript{13,14}

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REFERENCES