Effects of Penetration Enhancers on *In Vitro* Permeability of Zidovudine Gels

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**ABSTRACT**

Acquired immune deficiency syndrome (AIDS), which threatens to cause a great plague in present generation, was first identified in California in 1981. UNAIDS 2006 report showed 38.6 million children & adults to be infected with HIV/AIDS Worldwide. Most of the existing molecules have several drawbacks. Like in case of Zidovudine (Zido) drug which has low t\(_{1/2}\), first pass metabolism in liver, dose dependent hematological toxicity, poor bioavailability and also frequent oral dosing. The objective of this study was to prepare a transdermal delivery system for Zidovudine using different type of penetration enhancers incorporated in chitosan gel and to evaluate *in vitro* as well as *ex-vivo* permeation across rat skin. *In-vitro* drug diffusion and *ex vivo* permeation studies through rat skin showed a high diffusion and permeation with formulation Zido-J, Zido-G containing penetration enhancers as compared to Zido-F (Blank) and other formulations, so achieving therapeutically effective plasma concentrations would be possible with these formulations. No lag-time was observed in release of zidovudine from gels. Among all gel formulations 2%w/w chitosan gel had desirable viscosity and exhibit follow pseudoplastic flow.

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INTRODUCTION

Human Immunodeficiency Virus is a retrovirus that causes irreversible destruction of the immune system, leading to the occurrence of opportunistic infection and malignancies [1]. Many viruses eventually kill their host cells, resulting in disease and provoking an assault by the immune response of the host [2]. The worldwide viral diseases are Acquired immunodeficiency syndrome (AIDS), Dengue, Encephalitis, Hepatitis, Yellow fever [3]. Approximately million people are suffering from AIDS worldwide. This highlights the importance of treating AIDS [4]. It is characterized by gradual destruction of cell-mediated (T-cell) immunity; it also affects humoral immunity and autoimmunity because of the central role of the CD4+ T lymphocyte in immune reactions [5].

Chitosan is a biodegradable and non-toxic hydrophilic polysaccharide with excellent mucoadhesive and permeation enhancing properties [6]. The natural biopolymer chitosan is one of the most abundant polysaccharides in nature, second only to cellulose. Its sugar back bone consists of β-1,4-linked glucosamine with a high degree of N-acetylation, a structure very similar to that of cellulose, the only difference being the replacement of the hydroxyl moieties by amino groups. chitosan exhibits favourable biological properties such as nontoxicity, biocompatibility and biodegradability; it has attracted great attention in the pharmaceutical and biomedical fields. Because of the mucoadhesive, gel and film forming properties of chitosan find many applications in various fields [7].

It is crucial for the success of AIDS therapy to maintain the systemic drug concentration consistently above its targeted concentration throughout the course of the treatment [8-9]. Zidovudine (AZT) is clinically used for treatment of AIDS either alone or in combination with other antiviral agents. However, the main limitation to therapeutic effectiveness of AZT is its dose-dependent hematological toxicity, low therapeutic index, short biological half-life and poor bioavailability [10]. After oral administration, it is rapidly absorbed from the gastrointestinal tract exhibiting a peak plasma concentration of 1.2μg/ml at 0.8 h and also undergoes first pass metabolism [11]. In the systemic circulation, it is first converted to AZT triphosphate, which is pharmacologically active and prevents the replication of the HIV virus. The biological half life of AZT-triphosphate is 4 hours, so frequent administrations (3 to 4 times a day) of AZT are required to maintain constant therapeutic drug levels [12]. These limitations are usually associated with excessive plasma level of AZT immediately after intravenous or oral administration. Therefore it is proposed that a non-invasive zero order delivery is desirable to maintain the expected anti-HIV effect and to avoid strong side effects which may be attributed to exceeded plasma levels of these drugs immediately after intravenous or oral administration. Because of the high first pass metabolism, oral sustained delivery may not be a good option [13]. On the other hand, transdermal drug delivery may be helpful in maintaining suitable plasma concentration and could be useful in improving the bioavailability, avoiding side effect and frequent dosing [14].

The purpose of this study was to evaluate both in vitro and ex vivo transdermal enhancement potential of the permeation enhancers like menthol, oleic acid and propylene glycol using zidovudine as a model drug. The formulation containing oleic acid and propylene glycol in 5% combination was showed high permeability and steady state flux so achieving of therapeutic plasma concentration was possible with this formulation.
MATERIALS AND METHODS

Materials

Zidovudine was gift sample from Matrix Laboratories Limited, Hyderabad, India. Chitosan was purchased from Marine Chemicals, Cochin, India. All other reagents were analytical grade and used as such.

Saturation solubility studies

The saturation solubility of AZT was determined in water, ethanol, propylene glycol and the binary combinations of ethanol in water, propylene glycol in water and ethanol in propylene glycol. Excess AZT was added to known volumes of the solvent systems, vortexed for 2 min followed by sonication for 10 min to dissolve AZT and then equilibrated at 32 ±0.5°C for more than 48 h. The solution was centrifuged at 10000 rpm for 15 min. The supernatant was analyzed by UV spectrophotometer at 266 nm [15].

Development of Gel Formulation

Preparation of chitosan gel was done by adding 0.5%, 1%, 2% chitosan powder in 2% lactic acid solution; drug was dissolved in 66.6% ethanol solution and incorporated in to 2% polymer concentration. 5% w/w Menthol, propylene glycol, Oleic acid and mixture of oleic acid and propylene glycol were added to the above concentration (Table-1). These formulations were used for in-vitro experiments after 48 hrs.

Table 1: Preparation of Chitosan gel with different penetration enhancer

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Zidovudine (% w/w)</th>
<th>Chitosan (% w/w)</th>
<th>Penetration Enhancers (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Menthol</td>
</tr>
<tr>
<td>Blank gel-A</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Blank gel-B</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Blank gel-C</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Gel- Zido-D</td>
<td>5</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Gel- Zido-E</td>
<td>5</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Gel- Zido-F</td>
<td>5</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Gel- Zido-G</td>
<td>5</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Gel- Zido-H</td>
<td>5</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Gel- Zido-I</td>
<td>5</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Gel- Zido-J</td>
<td>5</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>
Evaluation of gel

The appearance of gels was inspected visually for clarity, colour and presence of any foreign particles. pH of the gels was determined by dissolving 1 gm of gel in distilled water under stirring to form a uniform suspension and then was measured using calibrated pH meter.

Viscosity

The Viscosity of all samples were determined to identify the minimum concentration of Chitosan required for the formation of gel with good viscoelastic properties by using a stress control Viscotech Rheometer (Reologica Instruments, Sweden). The test was carried out in the stress range of 0.05-100 Pa and the viscosity was measured at 25 °C which was maintained using cryostatic bath.

Fourier Transform Infrared Spectroscopy (FTIR) measurements

FTIR spectra of AZT loaded gels were recorded on (Jasco V-5300, Japan) in the range of 400 to 4000 cm\(^{-1}\) in KBr pellets prepared on KBr press (Spectra lab, India). For comparison, FTIR spectrum of pure AZT and chitosan was also recorded.

Animals

The animal skin was prepared using a protocol reported earlier (16). Male Wistar rats (250±10 g) were sacrificed by ether inhalation. The dorsal skin of animal was shaved and subcutaneous tissue was removed surgically and dermis side was wiped with isopropyl alcohol to remove adhered tissue. The skin was washed properly with phosphate buffer and used immediately.

In-vitro and ex-vivo drug diffusion study

In vitro release was studied using a cellulose membrane and rat skin. All formulations were subjected to \textit{in vitro} diffusion through cellulose membrane by using Franz diffusion cell with diffusional surface area of 3.14 cm\(^2\) and a receptor volume of 18 ml. Cellulose membrane was used as barrier between donor and receptor compartment. The membrane was mounted on a Franz diffusion cell. The receptor compartment was filled with phosphate buffer pH 7.4. It was jacketed to maintain the temperature 37 ±0.5 °C and was kept under constant stirring. Prior to application, the membrane was allowed to equilibrate at this condition for 60 min. Ex-vivo permeation studies across rat skin were carried out using a permeation set up described above except that instead of cellulose membrane, rat skin was used. The skin pieces were mounted over diffusion cells with the dermal side in contact with the receptor phase, equilibrated for 2 h. Subsequently, donor compartments were filled with 300 mg of gel formulation and then covered with aluminum foil to prevent evaporation of vehicle. Samples were withdrawn at definite time intervals from the receptor compartment followed by replacement with fresh volume of phosphate buffer solution and concentration of AZT in receptor samples was analyzed by UV spectrophotometric method. For each skin specimen, AZT permeated per unit area was calculated and plotted against time. The steady-state flux (J\(_{ss}\)) and lag time (L) were calculated from the slope and \(x\)-intercept of the linear portion fitted through the regression analysis (17).

RESULTS AND DISCUSSION

For developing gel formulation of zidovudine, the first step was to find the solubility system for AZT, so as to prevent precipitation of AZT. The saturation solubility of AZT was determined by equilibrating excess AZT in different solvent systems like water, ethanol, propylene glycol and their combinations like ethanol in
water, propylene glycol in water, and ethanol in propylene glycol at various concentrations. Saturation solubility of AZT varied among various binary combinations of ethanol, propylene glycol and water and was significantly higher than solubility of AZT in water alone. Among various solvent systems used ethanol in water (66.6% v/v) appeared to be a good vehicle with saturation solubility of 261.23 ± 2.05 mg/ml followed by polyethylene glycol in ethanol and polyethylene glycol in water with a saturation solubility of 170.43 ± 1.10 mg/ml and 135.90 ± 3.82 mg/ml respectively. Thomas et al, reported that 66.6% v/v ethanol in water binary mixture gave maximum flux and shorter lag time (18).

Prepared gels containing 5% w/w AZT were evaluated for appearance, pH, viscosity study, drug content uniformity, FTIR, in-vitro diffusion through cellulose membrane and rat skin. Chitosan gels (all batches) were found to be clear with slightly yellow in colour. The pH of all gel batches was between 5.98 ± 0.09 to 6.3 ± 0.30 indicating suitability for skin application.

Viscosity

It was seen from the flow curves (Fig. 1) that chitosan gels exhibit pseudoplastic flow with increasing chitosan concentration. Incorporation of zidovudine into the gels did not change the viscosity. Chitosan at all concentrations showed Non-Newtonian flow against applied stress. Gel containing 2% Chitosan is having desirable consistency.

![Viscometry of Chitosan at different concentrations.](image)

**Drug Content Uniformity**

Zidovudine was extracted from an accurately weighed amount of gel. The samples were appropriately diluted with phosphate buffer pH 7.4, sonicated for 10 min and AZT content was determined by UV spectrophotometer at 266 nm. The percent drug content of all formulations was more than 97.8 ± 0.41 indicating good loading efficacy and suitability of method adopted for preparation of gel.
Fourier Transform Infrared Spectroscopy Measurements

Transmission infrared spectra of chitosan powder, zidovudine and zidovudine gel were acquired to draw information on the molecular state of chitosan and zidovudine. Chitosan is an amino glucose characterized by a small proportion of amide groups via as a amide linkage with acetic acid. In the infrared spectrum, powder chitosan exhibited a broad peak at 3431 cm$^{-1}$, which is assigned to the N-H and Hydrogen boned O-H stretch vibrational frequencies while a sharp peak at 3610 cm$^{-1}$ is that of free O-H bond of glucopyranose units. Further, in the C-H stretch region of FTIR spectrum, the higher intensity peak at 2923 cm$^{-1}$ is assigned to the asymmetric and the lower intensity peak at 2857 cm$^{-1}$ is assigned to the symmetric modes of CH$_3$. The peaks at 1550 and 1599 cm$^{-1}$ were assigned to strong N-H bending vibrations of secondary amide, which usually occur in the range of 1640 to 1550 cm$^{-1}$ as strong band.

The spectra of pure AZT, pure Chitosan, AZT loaded chitosan gel are shown in figure (2). Pure AZT spectra indicated the presence of stretching bands in the 3500 – 3200 cm$^{-1}$ wave number region assigned to O-H stretching and O-H bending mode at 1630-1600 cm$^{-1}$, a band at 2102 cm$^{-1}$ assigned to C=N=N=N azide group, a band at 1694 cm$^{-1}$ assigned to C=O, a band at 1385 cm$^{-1}$ assigned to CH$_2$ and 1281 cm$^{-1}$ assigned to C-O-C and C-OH (20). Spectra of pure chitosan showed a strong band at 1710 cm$^{-1}$ which is characteristic peak of non-ionized carboxylic group and the spectra of AZT loaded chitosan gel showed the characteristic peak of carbonyl group at 1694 cm$^{-1}$ and azide group at 2102 cm$^{-1}$ indicated that the stable nature of AZT in gel formulation with no chemical interaction between chitosan and AZT.

![FTIR spectra](image)

**Fig.2.** FTIR spectra of (a) Chitosan, (B) Blank chitosan gel, (c) Drug (D) Drug loaded gel

**In-vitro drug diffusion studies**

Accumulated amounts of zidovudine in the receptor compartment as a function of time for all the conditions assayed are plotted in Fig. 3. All the enhancers formulated at 5% (w/w) in chitosan gel increase the penetration of AZT through the skin.

All the molecules tested increase AZT flux relative to control Zido-F (blank). All enhancers studied do not increase AZT flux to the same extent. The vehicle significantly increases AZT flux. Our results confirm the
capacity of ethanol to permeate through the stratum corneum [21] and alter the organization of its intercellular lipids, therefore, increasing skin permeability [22]. Oleic acid and propylene glycol significantly increases the flux of AZT across the skin respective to the control. Oleic acid has been found to increase the epidermal permeability through a mechanism involving the stratum corneum lipid membranes. Oleic acid is incorporated into skin lipid, disrupts molecular packaging and alters the level of hydration, thus allowing drug penetration [23]. It has also been described that at high concentrations oleic acid can exist as a separate phase within the lipid bilayers [24-25]. Our results confirm that it can work synergistically with ethanol and propylene glycol, as proposed by other authors [26]. Propylene glycol was selected as a more hydrophilic compound capable of increasing transdermal flux [27]. It has shown a moderate enhancing activity on the transdermal flux of AZT, probably due to its moderate capacity for disrupting the stratum corneum lipid packing. It was reported that oxygen containing terpenes, such as menthol, cineole etc form new aqueous pore pathways in the skin thus increasing the permeation of hydrophilic drugs like AZT (28). According to the lipid protein and partitioning theory, penetration enhancers may act by one or more of the three main mechanisms: disruption of the highly ordered lipid structure, interaction with intracellular protein to promote permeation through the corneocyte and increased partitioning (29). As terpenes are highly lipophillic, it is less likely that they interact with keratin, which has been proved by calorimetric studies where they did not alter protein endotherm (29-30). On the other hand, it was found that terpenes alter neither thermodynamic activity nor partitioning of AZT into stratum corneum from vehicle (31). Hence, the only other possible mechanism was disruption/alteration in the barrier property of stratum corneum. So oxygen containing terpenes, such as menthol, cineole predominantly act as, first by disrupting the existing lateral/transverse hydrogen bond network of the stratum corneum lipids bilayer probably by preferential hydrogen bonding of terpenes with ceramide head groups and secondly by facilitating the excessive hydration of ceramide head groups and thereby increase the breath of existing polar pathways or form new polar pathways. Based on the pharmacokinetics of AZT, a target flux between 0.2 and 0.6 mg/cm²/h is required across human skin from a 50 cm² patch in order to reach therapeutically effective plasma concentrations. However as rat skin is 3-5 fold more permeable than human skin (29-30-31). The minimum target flux would be approximately 1 mg/cm²/h. As the steady state flux of AZT across rat skin from our formulations Zido-J was above 1.1 mg/cm²/h, achieving therapeutically effective plasma concentrations would be possible with this formulation.

Maximum drug diffusion was observed from Zido-J containing 5% oleic acid and propylene glycol and Zido-G containing 5% menthol were added as penetration enhancers while minimum drug diffusion was observed from Zido-F (Control) are illustrated figure 3A. It was observed that only 2.12 mg/cm² of drug diffused from control preparation (Zido-F) in 14 h which is not sufficient to achieve and maintain therapeutic concentration of Zidovudine. So diffusion of Zidovudine can be significantly enhanced by penetration enhancer like mixture of 5% oleic acid and propylene glycol and 5% menthol. At 5% w/w enhancer concentration, diffusion of AZT increased from 2.12 mg/cm² to 12.88 mg/cm² in Zido-J containing 5% oleic acid and propylene glycol and 10.77 mg/cm² in Zido-G containing 5% menthol attained in 14 h which is sufficient to achieve and maintain therapeutic concentration of Zidovudine from chitosan gel formulation. In case of ex-vivo diffusion through rat skin, permeation profiles of Zidovudine across rat skin with and without 5% w/w enhancers in gel formulation are illustrated in figure 3B. Flux values of AZT from different formulations in decreasing order are as follows Zido-J > Zido-G > Zido-H > Zido-I > Zido-F (Control). Flux of Zidovudine in terpenes was significantly different from control and other formulations. However among all the enhancers, flux of Zidovudine with of 5% oleic acid and propylene glycol and 5% menthol were maximum and not significantly different from each other in their flux enhancement activities, although 5% oleic acid and propylene glycol showed higher flux values. These flux values were above the theoretical Zidovudine flux values (0.2 and 0.6
mg/cm$^2$/h) (15). The steady state flux of Zidovudine from gel formulations calculated from the linear portions of the cumulative amount permeated against time plots was 1.2 mg/cm$^2$/h from Zido-J respectively and the flux values were very high in comparison with passive Zidovudine flux across rat skin in the absence of ethanol and penetration enhancers (20). The lag time with the gel formulations were 4 h and flux during this period were only 0.788 mg/cm$^2$/h from Zido-J. Ethanol is known to be a skin penetration enhancer and alters the barrier property of the skin which is partly responsible for the high flux values. In addition, it was reported that oxygen containing terpenes, such as menthol form new aqueous pore pathways in the skin thus increasing the permeation of hydrophilic drugs like Zidovudine (15). According to the lipid protein and partitioning theory, penetration enhancers may act by one or more of the three main mechanisms: disruption of the highly ordered lipid structure, interaction with intracellular protein to promote permeation through the corneocyte and increased partitioning (28). So oxygen containing terpenes, such as menthol, predominantly act as, first by disrupting the existing lateral/transverse hydrogen bond network of the stratum corneum lipids bilayer probably by preferential hydrogen bonding of terpenes with ceramide head groups and secondly by facilitating the excessive hydration of ceramide head groups and thereby increase the breath of existing polar pathways or form new polar pathways. Based on the pharmacokinetics of AZT, a target flux between 0.2 and 0.6 mg/cm$^2$/h is required across human skin from a 50 cm$^2$ patch in order to reach therapeutically effective plasma concentrations. However as rat skin is 3-5 fold more permeable than human skin (30-31). The minimum target flux would be approximately 1 mg/cm$^2$/h. As the steady state flux of AZT across rat skin from our formulation Zido-J was above 1.2 mg/cm$^2$/h, achieving therapeutically effective plasma concentrations would be possible with these formulations.

![Fig.3](A) In-vitro diffusion of Zidovudine from different formulations through cellulose membrane, (B) Ex-vivo diffusion study from different formulations across rat skin.

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