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Selective complexing agents designed after adenosine receptor as percutaneous permeation enhancers of drugs

Venkata Ramana S. Uppoor[1], Jaymin C. Shah*

Department of Pharmaceutical Sciences, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425, USA

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Abstract

The objective was to evaluate selective complexing agents, naphthalene diamide diimide (I) and naphthalene diester diimide (II), as transport facilitators of adenosine across skin. Molecular modeling was used to show favored binding, energetically and conformationally, between adenosine and its selective complexing agents. The energy-minimized conformational structure of the adenosine–diamide diimide (I) complex indicated favored binding conformationally and energetically by potential energy saving of 8.26 kcal/mol. Permeation studies of adenosine alone (control) and with the complexing agent were conducted across polydimethyl siloxane (PDMS), whole thickness human skin and isolated human stratum corneum using vertical franz diffusion cells and infinite dose technique. Adenosine flux across PDMS was increased 10-fold by diester diimide (II) (0.28 μg/min per cm^2 versus 0.027 μg/min per cm^2 for the control). During permeation across whole thickness human skin, adenosine was primarily metabolized to inosine, which further metabolized to hypoxanthine, and adenine was formed in small amounts by a minor pathway. The V_{max} and K_m values for adenosine metabolism were 6.7×10^{-4} mM/min and 6.4×10^{-3} mM, respectively. Thus, extensive metabolism within the skin concurrent with diffusion prevented the estimation of mass transfer rates of adenosine alone or in the presence of the complexing agent. Adenosine permeation across isolated stratum corneum was not enhanced by diester diimide (II); however, increased amounts of hypoxanthine and inosine were found in the receptor phase, indicating enhanced uptake of adenosine. These results also suggested residual metabolic activity in the stratum corneum. A novel mechanism was demonstrated for permeation enhancement of drugs across lipophilic solid-phase and biological membranes, using lipophilic selective complexing agents as transport facilitators. However, the inherent metabolic activity of biological membranes profoundly influences drug transport and synthetic lipophilic membranes may therefore have limitations as models of biological barriers. Copyright © 1996 Elsevier Science B.V.

Keywords: Adenosine; Permeation enhancement; Selective complexing agents; Skin; Synthetic receptors; Transport facilitator

* Corresponding author. Tel.: + 1 803 7925366; fax: + 1 803 7920759.

[1] Present address: Division of Biopharmaceutics, Food and Drug Administration, Rockville, MD, USA.

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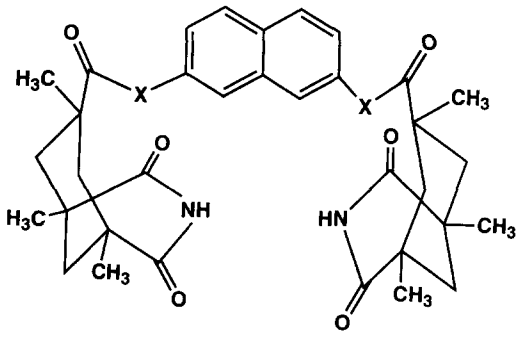
1. Introduction

Skin is an effective physical barrier for the entry of drugs and chemicals into the body. Methods to enhance permeation of drugs across skin have been reviewed recently (Gosh and Banga, 1993a,b,c). Permeation enhancers have been shown to increase drug permeation across skin by a variety of mechanisms, such as extraction of skin lipids by solvent action, disruption of semicrystalline organization of skin lipids resulting in partial liquefaction of the lipid matrix, and increasing skin uptake of drugs (Gosh and Banga, 1993b,c). Our objective was to develop a novel method of permeation enhancement of drugs, using selective complexing agents designed after receptors of biological molecules, which form tight complexes and, being lipophilic, may facilitate the transport of bound substrate across a biological macroscopic lipoidal membrane such as skin.

Molecules have been designed with structures that feature a cleft with functional groups attached to the interior which converge on a specific substrate by ionic, hydrogen-bond and aromatic-stacking interactions (Rebek, 1987[2]). These agents selectively recognize amino acids, nucleotides and metal ions, and bind to their specific substrates with high affinity (Rebek, 1987). Several agents (Fig. 1) have been designed with structures featuring a cleft with complementary hydrogen bonds for purine nucleotides, and they thus recognize purine nucleus (Benzing et al., 1988). These agents selectively bind adenosine and deoxyadenosine derivatives with high affinity (K_a for binding 3000–11 000 M^{-1}). These compounds are also extremely lipophilic and were shown to enhance transport flux of adenosine derivatives 3–10-fold across organic liquid membrane without enhancing the transport of cytidine or guanosine. This indicates that they act as selective transport enhancers of adenosine derivatives across the membrane. Lehn (1983) designed molecules that selectively recognize and bind the substrate, and which were lipophilic enough to be retained in the membrane but carry the substrate across, resulting in facilitated transport of the substrate across the membrane (Lehn, 1983). Similarly, selective permeability enhancement for the drug across a biological barrier may be achieved by their selective complexing agents if they are lipophilic. Facilitated percutaneous absorption using a non-selective ion-pairing agent was proposed earlier but significant enhancement was not observed (Hadgraft et al., 1986).



X = NH, Diamide diimide (I)
X = O, Diester diimide (II)

Fig. 1. Selective complexing agents of adenosine: naphthalene diamide diimide (I) and naphthalene diester diimide (II).

The objective of this study was to investigate if the selective complexing agents (Fig. 1) for adenosine can act as its permeation enhancers across skin. Since stratum corneum is the lipophilic rate-limiting barrier in transport of drugs across skin, these lipophilic, selective complexing agents could theoretically act as transport facilitators of adenosine across skin. We selected adenosine as the model substrate and studied its permeation across whole thickness human skin in the presence of selective complexing agents to achieve permeation enhancement.

2. Materials and methods

2.1. Materials

Adenosine, adenine, hypoxanthine and inosine were obtained from Sigma (St. Louis, MO). Selective complexing agents, naphthalene diamide diimide (I) and naphthalene diester diimide (II)

[2] Rebek has referred to these compounds as 'synthetic receptors'.

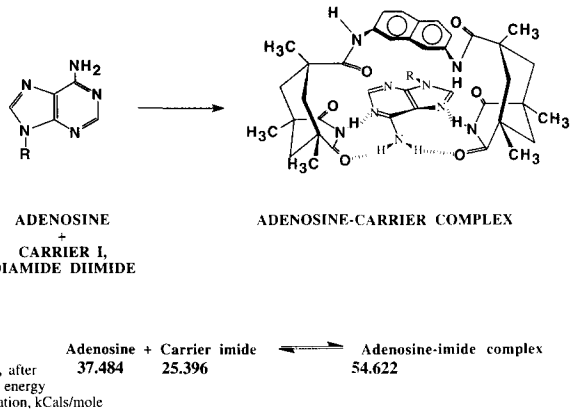


Fig. 2. The energy-minimized conformational structure, obtained using MAXIMIN2 in SYBYL, of the adenosine–diamide diimide (I) complex, indicating favored complex formation energetically and conformationally.

(referred to as diamide diimide (I) and diester diimide (II), respectively) were generous gifts from Dr Julius Rebek of Massachusetts Institute of Technology, Cambridge, MA. Acetonitrile, dimethyl sulfoxide (DMSO) and chloroform were obtained from Fisher Scientific, Norcross, GA. Lyophilized trypsin was obtained from GIBCO Laboratories (Gaithersburg, MD). Isolated human skin was obtained from local area plastic surgeons and defatted to prepare whole thickness human skin which was kept frozen at −70°C till the experiment. The thickness of the human skin was on average approximately 400 μm. Polydimethyl siloxane (PDMS) membrane (0.4 mm thickness) was a gift from Dow-Corning, Midland, MI.

2.2. Molecular modeling

Structures of adenosine, naphthalene diamide diimide (I) and the complex were first drawn, and the conformation with the lowest potential energy was obtained using energy minimization algorithm, MAXIMIN2 in SYBYL, a molecular modeling software. Energy calculations were done to determine if the complex formation is thermodynamically favorable (Fig. 2). These energy calculations were done in gas phase (free space); however, the complex formation in solution phase has already been demonstrated (Rebek, 1987; Benzing et al., 1988).

2.3. Assay method

A sensitive and specific isocratic high-performance liquid chromatography (HPLC) method for analyzing adenosine and its putative metabolites, hypoxanthine, inosine and adenine, was adapted from the literature (Hartwick et al., 1978). The chromatography was reversed phase using Waters C_{18} μBondapak column and mobile phase consisting of 14% methanol and 86% 0.01 M monobasic potassium phosphate buffer. The compounds of interest were detected and quantified with an ultraviolet spectrophotometer set at a wavelength of 254 nm.

2.4. Permeation study across PDMS membrane

This permeation study was conducted to verify if the selective complexing agents have the ability to enhance transport of adenosine through a macroscopic solid-phase lipophilic membrane, PDMS, by the mechanism proposed earlier. The permeation studies were conducted with a vertical franz diffusion cell with 0.9 cm diameter and 4.5 ml receptor volume at 37°C. Adenosine and the diester diimide II at a concentration of 0.025 M each were dissolved in chloroform due to the complexing agent's poor aqueous solubility, and 0.5 ml of this solution was applied to the membrane in the donor compartment. The donor chamber was covered to prevent evaporation of

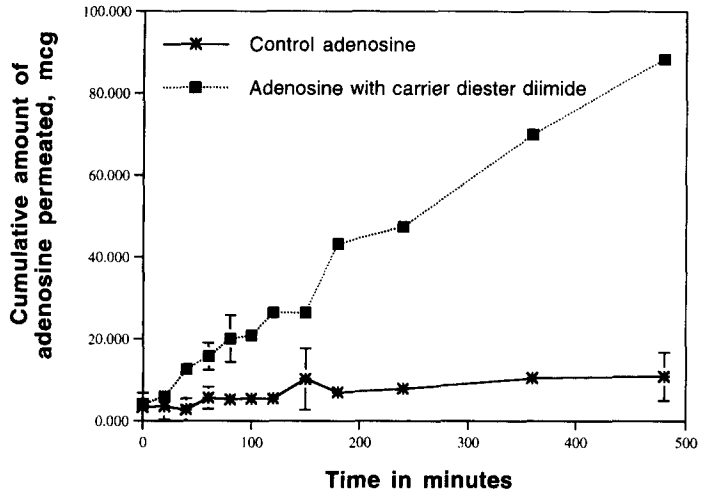


Fig. 3. Permeation profile of adenosine across PDMS (0.4 mm) membrane showing the permeation enhancing effect of diester diimide (II) ($n = 3$).

chloroform throughout the duration of the experiment. The PDMS membrane (0.4 mm thick) had been pre-soaked and washed with phosphate-buffered saline (PBS) to remove any impurities. Receptor fluid, 0.1 M PBS, was stirred at 900 rpm to maintain sink condition, and 0.3-ml samples were taken at various time intervals and analyzed for adenosine. Controls were also run without the complexing agent in the donor solution, in triplicate. The permeation profiles (Fig. 3) were constructed and the steady-state flux calculated from the initial linear region of the profile.

2.5. Permeation study across whole thickness human skin

This permeation study was conducted to determine if the complexing agents enhance permeation of adenosine through whole thickness human skin. The permeation studies were conducted using a vertical franz diffusion cell with 0.9 cm diameter and 4.5 ml receptor volume at 37°C. The whole thickness human skin was obtained after defatting excised human skin obtained from local area plastic surgeons. The skin was kept frozen at −70°C until use and thawed overnight at room temperature prior to the experiment. The thickness of the human skin was on an average approximately 400 μm. Due to the poor aqueous solubility of the complexing agents, DMSO was selected as the solvent for both adenosine and selective complexing agents. Adenosine and the complexing agent were applied at 0.075 M concentrations to the skin, and the donor chamber was covered during the entire experiment to prevent evaporation of the solvent. Receptor fluid, 0.1 M PBS, was stirred at 900 rpm to maintain sink condition, and 0.3-ml samples were taken at various time intervals and analyzed for adenosine. Controls were run without the carrier compounds in the donor solution, in triplicate, using skin from the same subject. Samples were drawn from both donor and receptor compartments and stored at 4°C until analyzed.

Since no adenosine was detected in the receptor phase and other peaks appeared in the chromatograms of the samples, either chemical degradation or extensive metabolism of adenosine during permeation through the skin was suspected. Therefore, the following experiment was conducted to verify the potential metabolism of adenosine by skin and also to eliminate the possibility of its chemical degradation.

Table 1
The experimental design used to invesigate if adenosine was undergoing degradation or metabolism

Group	Donor	Receptor	Skin	Reason or comment
I	Adenosine in DMSO	Phosphate buffer	Yes	To determine permeation of adenosine, if any
II	Adenosine in buffer	Adenosine in buffer	Yes	To determine metabolism on both dermal and SC surface of skin
III	Adenosine in buffer	Adenosine in buffer	No	Control: to determine chemical degradation in absence of skin

SC, stratum corneum.

2.6. Verification of adenosine metabolism by whole thickness human skin

Authentic standards were obtained for the putative adenosine metabolites, hypoxanthine, inosine and adenine, and the HPLC assay was validated to enable quantitation of adenosine and all its putative metabolites. The experimental design used for this study is described in Table 1. Studies were conducted with adenosine in contact with skin and appearance of metabolites was followed in both donor and receptor compartments. Group I consisted of adenosine applied as a solution in DMSO to whole thickness human skin while the receptor phase was PBS, to see if any adenosine permeated across skin and if any metabolites were detected in the receptor compartments. Group II consisted of adenosine in buffer (PBS) in contact with the stratum corneum surface of the human skin, along with adenosine in buffer in contact with the dermis surface of the skin in the receptor compartment at 37°C. Group III consisted of adenosine in buffer at 37°C in the diffusion cell without the skin, to see if it underwent chemical degradation. Samples were taken from both the donor and receptor compartments and analyzed for adenosine and its putative metabolites immediately. This experiment was conducted in triplicate and results depicted in Figs. 4–6.

2.7. Permeation study across isolated stratum corneum of human skin

Since extensive metabolism of adenosine was observed to occur in the dermis of the skin in the above experiment, adenosine permeation is confounded by its metabolism. Therefore, it was not possible to evaluate if the selective complexing agents enhance adenosine permeation across whole thickness skin, unequivocally. Stratum corneum is the rate-limiting barrier to permeation of the majority of chemicals and drugs and has been generally believed to be deficient in metabolic enzymes (Noonan and Wester, 1983; Liu et al., 1990; Tojo et al., 1994). Therefore, adenosine permeation across isolated stratum corneum may be expected to be free of metabolism. This will enable us to test our hypothesis: if the selective complexing agents enhance adenosine permeation across the lipophilic stratum corneum, the rate-limiting barrier of the skin.

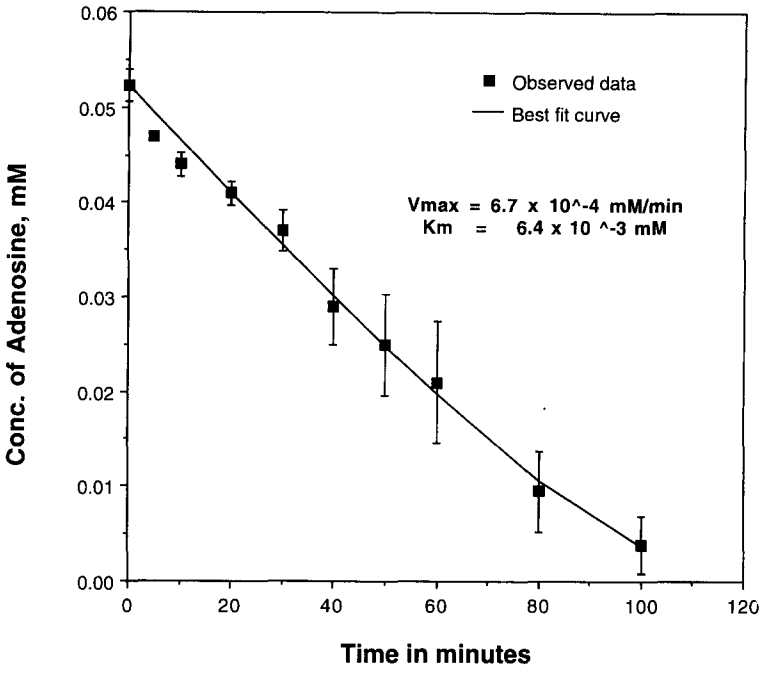
2.8. Isolation of stratum corneum

Stratum corneum was isolated from the whole thickness human skin by incubation with 0.25% (w/v) trypsin solution overnight at 37°C (Raykar et al., 1988). The stratum corneum was isolated as a thin sheet, and after washing with PBS, it was mounted on the franz diffusion cell and the permeation study conducted as described earlier for the whole thickness human skin. The results of this study are depicted in Fig. 7.

3. Results and discussion

3.1. Molecular modeling

Energy minimization on adenosine, diamide diimide (I) and the complex with the carrier showed that the formation of cleft in I, and the complex formation, are favored both conformationally and



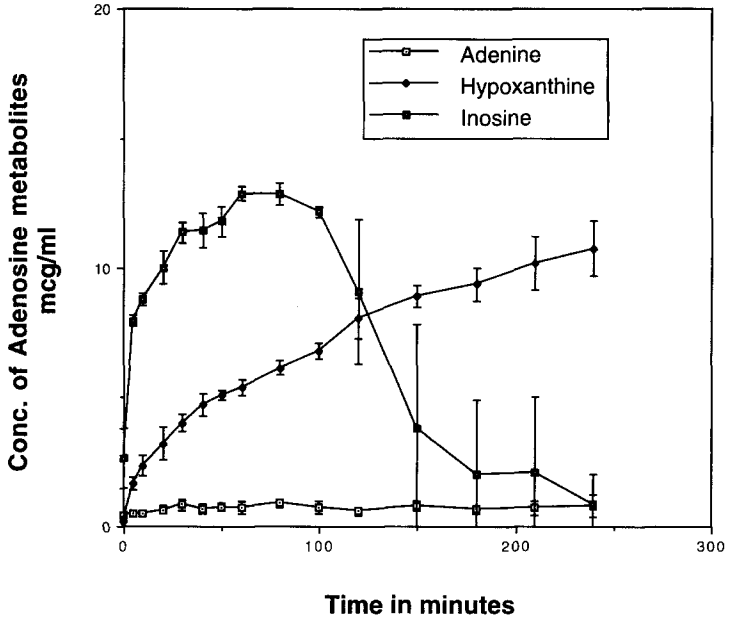


Fig. 4. (a) Declining concentration of adenosine in the receptor compartment (Group II, contact with the dermis of the skin) due to metabolism: data fit to the best curve described by Michaelis–Menten kinetics ($V_{max} = 6.7 \times 10^{-4}$ mM/min, $K_m = 6.4 \times 10^{-3}$ mM, $R^2 = 0.998$, $n = 3$). (b) Profiles of hypoxanthine, inosine and adenine formed due to adenosine metabolism during contact with the dermis surface of the whole thickness human skin (Group II, $n = 3$).

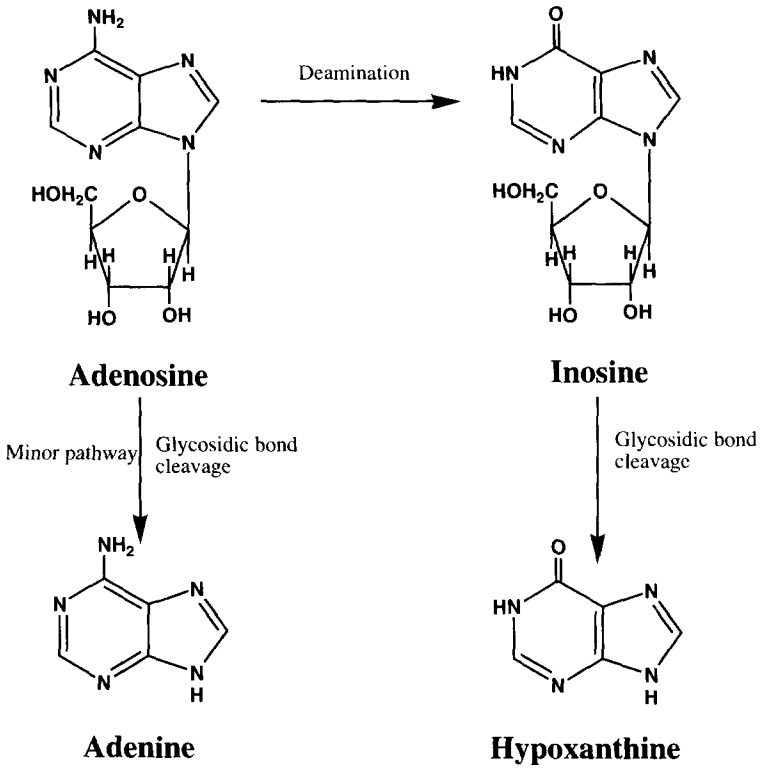


Fig. 5. The scheme for metabolism of adenosine in skin.

thermodynamically (Figs. 1 and 2). Energy minimization indicated favorable complex formation as a result of hydrogen bonding and aromatic ring stacking interactions, as shown in Fig. 2. Based on the potential energy of the adenosine–diamide diimide I complex, there is a potential energy saving of 8.26 kcal/mol upon complex formation. This is not surprising since these compounds were designed to bind selectively with adenine-based structures with high binding affinity (Benzing et al., 1988). Molecular modeling also showed that complex formation between the complexing agents I and II with cytidine and guanosine was not feasible conformationally as well as thermodynamically. Although the energy minimization for the conformations was conducted in gas phase (no solvent effects), the presence of solvent would not influence the binding significantly. Additionally, the selective binding of adenosine to these complexing agents in solvent has been demonstrated experimentally (Benzing et al., 1988).

3.2. Permeation studies across silicone (PDMS) membrane

Permeation profiles of adenosine alone and in the presence of diester diimide (II) across 0.4 mm PDMS silicone membrane as a model lipophilic barrier are compared in Fig. 3. Adenosine permeates through the PDMS membrane very poorly due to its very hydrophilic character; however, the diester diimide increases its permeation significantly. Adenosine flux (0.28 μg/min per cm^2) was increased 10-fold, with equimolar concentration of diester diimide (II) compared with the control (0.027 μg/min per cm^2). The increased transport (3–10-fold) of adenosine derivatives across chloroform liquid membrane by these complexing agents has already been reported (Benzing et al., 1988). The above results with PDMS membrane suggest that the same mechanism is still functional in a solid-phase lipophilic membrane, in enhancing transport of adenosine to the same extent.

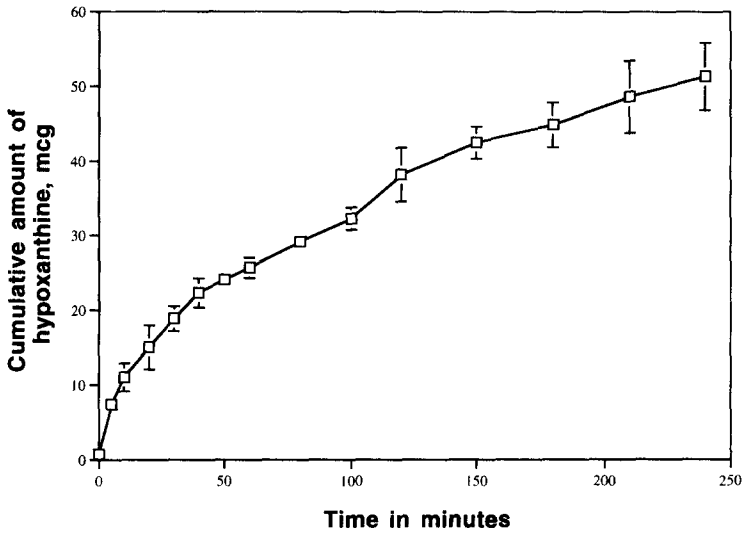


Fig. 6. The formation and the profile of hypoxanthine as detected in receptor compartment during adenosine permeation from DMSO solution across whole thickness human skin (Group I, $n = 3$).

3.3. Permeation studies of adenosine across whole thickness human skin

No adenosine was detected in the receptor phase even after long periods of permeation when adenosine was applied as saturated solutions in either PBS or in DMSO to the skin. However, other peaks were observed and their peak areas appeared to increase with time as the experiment progressed. This suggested that these unknown peaks were adenosine-related.

The increasing amounts of these unknown compounds in the receptor phase suggested adenosine metabolism and/or degradation, either during or after permeation through the skin. Although our objective was to study the effectiveness of complexing agents as permeation enhancers, it became necessary to investigate the observed percutaneous metabolism and/or degradation of adenosine further with the design described briefly in Table 1.

The possibility of chemical degradation of adenosine was eliminated since its concentration in buffer alone without the skin (Group III) was maintained constant for the entire duration of the experiment. When adenosine was in contact with both the dermis and the stratum corneum surfaces of the skin simultaneously (Group II), its concentrations in contact with the dermis surface of the skin decreased continuously in the receptor compartment (Fig. 4a). In addition to adenosine, three major peaks were identified and quantified as hypoxanthine, inosine and adenine, using standards of each and running the samples on an HPLC with a diode array ultraviolet spectrophotometer to monitor peak ultraviolet spectra and peak purity. This simultaneous increase in concentration of adenosine metabolites along with a decrease in adenosine concentration suggested its extensive metabolism when in contact with the dermis (Fig. 4a and b). In contrast, there was no decrease in adenosine concentration in contact with the stratum corneum surface of the skin. These results suggested metabolism of adenosine by the enzymes present in the epidermis and/or the dermis of the skin and not in the stratum corneum. This is not surprising, since the dermis is highly active metabolically and leaching of enzymes from the dermis into the receptor solution can also occur (Noonan and Wester, 1983; Liu et al., 1990; Tojo et al., 1994).

The adenosine data in Fig. 4a were fitted to the following implicit equation for the Michaelis–Menten kinetics to obtain the parameters, V_{max} and K_m for adenosine, using MINSQ® (Micromath, UT), a nonlinear regression software:

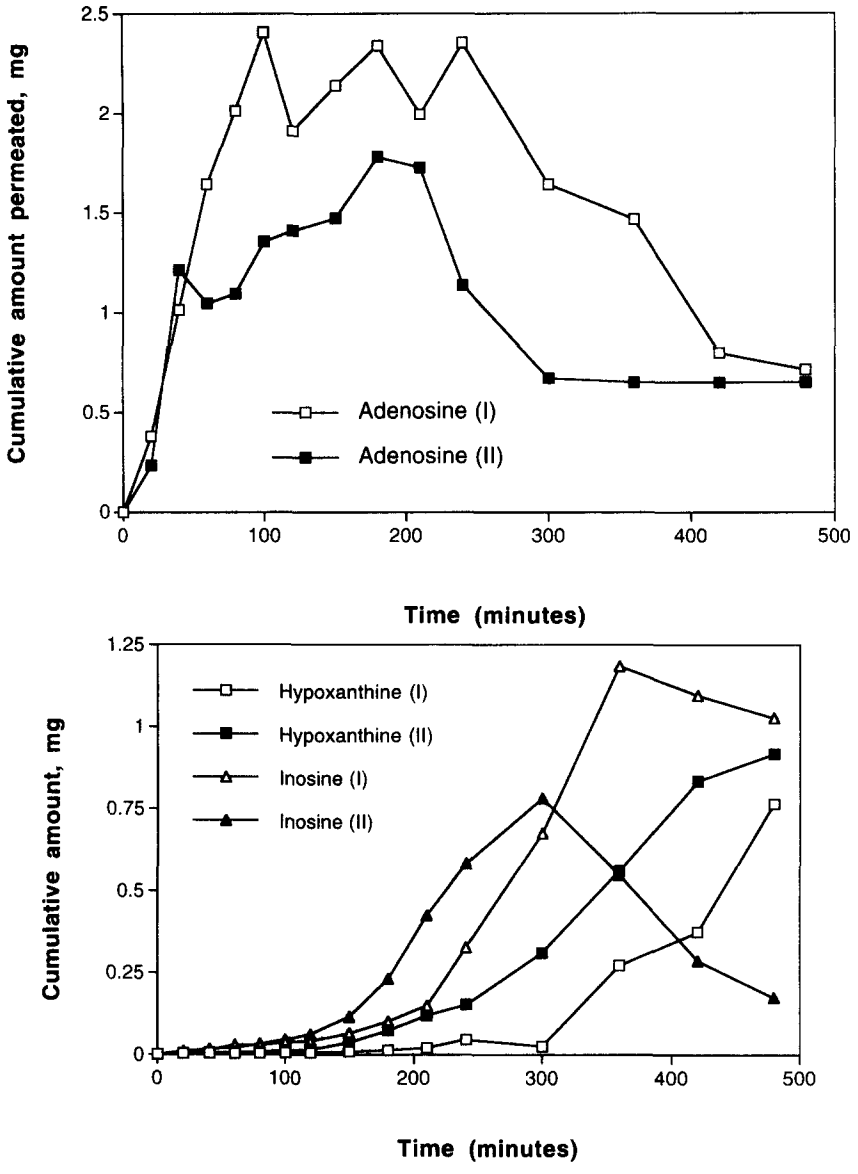


Fig. 7. The profiles of (a) adenosine and (b) its metabolites in the receptor compartment during permeation of adenosine across isolated stratum corneum without (I) and with (II) equimolar concentrations of diester diimide (II) ($n = 3$).

$$T = \frac{K_m \ln\left(\frac{C_0}{C}\right) + (C_0 - C)}{V_{max}}$$

where V_{max} is the maximum rate of metabolism in mM/min; K_m is the concentration of the substrate (adenosine) in mM, at half the maximum velocity, i.e. C at $V = V_{max}/2$; C_0 is the initial concentration in mM of the substrate and C is the concentration of adenosine at time T in min. There was very good fit between the observed data and the calculated curve based on Michaelis–Menten kinetics as seen in Fig. 4a, with a high model selection criteria (MSC) and R^2 of 0.996 or higher for all the three cells. The parameters were optimized independently using nonlinear regression. The

maximum rate (V_{max}) was 0.00067 ± 0.0001 mM/min, while K_m was 0.0064 ± 0.0004 mM.

The three metabolites detected and quantified were hypoxanthine, inosine and adenine. The formation and further metabolism of each of the metabolites as a function of time was obtained, as shown in Fig. 4b. Based on the above metabolite profiles, the scheme for adenosine metabolism in skin was presented, as shown in Fig. 5. Inosine is rapidly formed from adenosine by deamination followed by glycosidic bond cleavage to form hypoxanthine. Most of the adenosine is thus eventually converted to hypoxanthine. Adenine is formed in very small amounts by direct glycosidic bond cleavage of adenosine and is a relatively minor pathway. The kinetic scheme presented for adenosine metabolism in skin is consistent with the established metabolic pathway for adenosine in the human body (Hartwick et al., 1978). Adenosine is known to undergo deamination in the body by adenosine deaminase to inosine and then to hypoxanthine (Hartwick et al., 1978). Adenosine deaminase was reported to be present in skin by Ando et al. (1977). Later, Yu et al. showed metabolism of Ara-A (vidarabine), an adenosine-based drug, in skin by adenosine deaminase, resulting in the formation of inosine and then hypoxanthine (Yu et al., 1980). The V_{max} and K_m values of adenosine observed in our study are lower but consistent with those reported by Higuchi and Yu for Ara-A (Higuchi and Yu, 1987). However, in a recent study of adenosine permeation across human skin, no metabolism was reported (Kadir et al., 1988). The results of our study and those of previous studies clearly show extensive metabolism of adenosine-based compounds in human skin by adenosine deaminase.

When adenosine alone was applied as a saturated solution in DMSO to skin (Group I), no detectable adenosine levels were found in the receptor compartment, indicating complete metabolism of adenosine during its permeation through skin. However, the hypoxanthine concentrations increased in the receptor phase, as shown in Fig. 6. This suggested that adenosine was metabolized to inosine and then to hypoxanthine during permeation through the skin based on the proposed metabolic scheme (Fig. 5). Adenine may be formed in very small amounts.

3.4. Permeation studies of adenosine across isolated stratum corneum

The high level of adenosine metabolism within the whole thickness human skin concurrent with diffusion profoundly influenced its permeation and prohibited any control mass transfer rates to be obtained. The focus was therefore shifted to isolated stratum corneum, to study permeation enhancement by selective complexing agents. Since stratum corneum is the lipophilic barrier, limiting permeation of hydrophilic compounds such as adenosine, the lipophilic complexing agents will be expected to increase adenosine permeability primarily in the stratum corneum. The stratum corneum is composed of layers of dead corneocytes and is therefore believed to be devoid of enzymes present in the dermis. Hence, adenosine may not be metabolized during its permeation through stratum corneum. Permeation studies across isolated stratum corneum would thus be able to verify if the selective complexing agents act as permeation enhancers of adenosine.

Adenosine permeation profiles across stratum corneum when applied alone (control) and with the diester diimide (II) are compared in Fig. 7a. Adenosine permeates very rapidly through stratum corneum; however, there was very high variability. No significant difference was found between the permeation profiles of adenosine control and of adenosine when co-applied with equimolar amounts of diester diimide (II) (Fig. 7a). Hypoxanthine, inosine and very low levels of adenine were found in the receptor phase but after a small lag time (200 min), indicating that the stratum corneum had some metabolic activity (Fig. 7b). Increased amounts of hypoxanthine and inosine were found in the receptor phase after co-application of adenosine with diester diimide (II) (Fig. 7b). Although adenosine permeation was not significantly enhanced by diester diimide (II), the increased formation of hypoxanthine and inosine indicates that a greater amount of adenosine was metabolized, suggesting increased uptake of adenosine by the skin in the presence of

diester diimide (II). Since adenosine metabolism occurred concurrently with diffusion, steady state flux, permeability and diffusion coefficient could not be easily obtained. Permeation enhancement of adenosine by the selective complexing agent may have been limited by its metabolism, unlike the significant permeability enhancement obtained across the PDMS membrane (10-fold increase).

PDMS, being a silicone, is significantly more lipophilic than stratum corneum and, hence, it is significantly less permeable to adenosine than stratum corneum. The complexing agents, being very lipophilic, would be more efficient transport facilitators of bound substrates in lipophilic membranes such as PDMS and chloroform liquid membrane, in contrast to the stratum corneum. Secondly, the inherently high permeability of adenosine across stratum corneum may have limited the extent of permeation enhancement achievable with the complexing agents. Adenosine permeability across stratum corneum was already enhanced significantly by DMSO, the solvent for adenosine and the complexing agent. DMSO has been known to increase permeability of a variety of drugs and chemicals through skin (Gosh and Banga, 1993b,c), and has also been shown to enhance permeation of vidarabine (Ara-A), an adenosine derivative (Bergstrom et al., 1987). This already increased permeability of adenosine across skin may have further limited the extent of permeation enhancement achievable with the selective complexing agents. However, the insolubility of complexing agents in any polar and semipolar solvents limited choice of solvents to either chloroform or DMSO. Since chloroform is known to extract lipids from the skin and denature proteins, DMSO was selected as the solvent for co-application of adenosine and the complexing agents to skin.

The observed metabolism of adenosine in the stratum corneum raises a question regarding the assumption that the stratum corneum is devoid of metabolic enzymes since it is composed of layers of dead corneocytes. The observed metabolic activity of stratum corneum could be due to the incomplete separation of the viable epidermis, with residual layers of the viable epidermis still attached to the stratum corneum and/or back diffusion of some metabolic enzymes into the stratum corneum layer. The lag time in the appearance of hypoxanthine and inosine suggest that the enzymes may be leaching from the stratum corneum into the receptor compartment, resulting in metabolism of permeated adenosine. Enzyme distribution, primarily in the lower epidermis and dermis, has been better able to describe the results of metabolism of estradiol (Liu et al., 1990) and prednisolone derivatives (Tojo et al., 1994), than just assuming uniform distribution of enzymes in the entire skin, which is in contrast with the finding of adenosine metabolism in stratum corneum.

The 10-fold permeation enhancement across PDMS and the encouraging permeation results with isolated stratum corneum suggest that selective complexing agents can act as transport facilitators of substrates with which they bind with high affinity and specificity. Also, this mechanism would be specific for the substrate, and selective permeation enhancement could therefore occur without significant alteration of the membrane. Use of selective complexing agents such as permeation enhancers of drugs across lipoidal biological barriers for drug delivery is a novel approach and should be explored.

4. Conclusions

A novel mechanism for permeation enhancement of drugs across lipophilic solid-phase and biological membranes using lipophilic selective complexing agents as transport facilitators was demonstrated. Adenosine flux across PDMS was increased 10-fold by diester diimide II (0.28 μg/min per cm^2 versus 0.027 μg/min per cm^2 for the control). Adenosine was extensively metabolized during permeation across whole thickness human skin, thus profoundly influencing its percutaneous mass transfer. Isolated human stratum corneum was found to have metabolic activity. The inherent metabolic activity of biological membranes profoundly influences drug transport and, hence, synthetic lipophilic membranes have limitations as models of biological barriers.

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