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Effect of nerodilol, carvone and anethole on the *in vitro* transdermal delivery of selegiline hydrochloride

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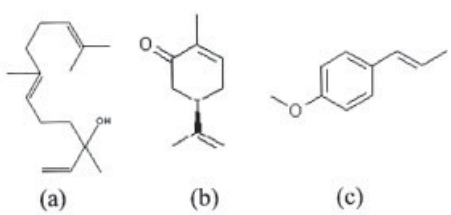
The aim of the study was to investigate the effect of terpene enhancers (nerodilol, carvone or anethole) on the *in vitro* transdermal delivery of selegiline hydrochloride with a broad objective of developing a membrane-moderated transdermal therapeutic system (TTS). The *in vitro* permeation studies were carried across the rat epidermis from hydroxypropyl methylcellulose (HPMC) gel drug reservoir containing selected concentrations of nerodilol, carvone or anethole and selegiline hydrochloride. The amount of selegiline hydrochloride permeated during the 24 h of the study (Q_{24}) from HPMC gel drug reservoir without terpene enhancer was 2169 ± 50 μg/cm^2 and the corresponding flux of the drug was 92 ± 1 μg/cm^2 · h. The amount of drug permeated and its flux increased with an increase in terpne concentration in HPMC gel drug reservoir. Nerodilol provided an approximately 3.2-fold increase in the flux of selegiline hydrochloride followed by carvone with a 2.8-fold increase, and anethole with a 2.6-fold increase. It is concluded that the terpene nerodilol, carvone and anethole produced a marked penetration enhancing effect on the *in vitro* transdermal delivery of selegiline hydrochloride that could possibly be used in the formulation of membrane-moderated TTS.

1. Introduction

Terpenes are of low cutaneous irritancy, generally regarded as safe, provide excellent enhancement ability, and appear to be promising candidates for transdermal formulations (Gao and Singh 1998). A variety of terpenes has been shown to increase the percutaneous absorption of both hydrophilic (Zhao and Singh 1999) and lipophilic drugs (Gao and Singh 1998), and thus could be used as penetration enhancers for increasing the transdermal permeation of hydrophilic selegiline hydrochloride.

Selegiline hydrochloride is a selective monoamine oxidase B (MAO B) inhibitor indicated for mood elevation in depression patients and parkinsonism (Baldessarini 1996). Following oral administration, selegiline hydrochloride is well absorbed and undergoes extensive first-pass metabolism. The absolute oral bioavailability averages 10%. A single 10 mg dose, administered in tablet form, produces a peak plasma concentration of about 2 to 2.1 ng/mL 0.5 to 2 h after administration. The recommended oral dosing regimen of selegiline hydrochloride for treating Parkinson's disease is 5 mg two times a day wherein the patient compliance may be low. Sustained release formulations may enable the selegiline dosing-frequency to be reduced, and therefore increase patient compliance. The transdermal administration of selegiline hydrochloride bypasses the first-pass effect, minimizes inter- and intra-patient variation and provides steady-state plasma concentration of the drug and long-term therapy from a single dose. Hence, the development of TTS for selegiline hydrochloride would be beneficial in providing an effective and safe therapy to patients suffering from parkinsonism. Preclinical and clinical studies are being carried out on TTS of selegiline hydrochloride (Bodkin and Amsterdam 2002; Mawhinney et al. 2003; Amsterdam 2003). However, the details on the formulation of such TTS are not known. Hence, the broad objective of the present study was to develop a membrane-moderated TTS of selegiline hydrochloride that provides a predetermined plasma concentration of the drug for predetermined period.

In the present investigation, a detailed study was undertaken so as to formulate a drug reservoir system necessary for designing a membrane-moderated TTS. As a first step, studies were carried out to select an optimal solvent system that could provide an optimal transdermal delivery. The study was extended to formulate a drug reservoir system with HPMC gel containing selected concentrations of a terpene enhancer such as nerodilol, carvone or anethole. The influence of the terpene enhancer (nerodilol, carvone



Chemical structure of (a) nerodilol, (b) carvone and (c) anethole

or anethole) on the *in vitro* transdermal permeation of selegiline was investigated in an attempt to overcome the barrier function of skin, and to provide the desired flux of selegiline hydrochloride across the skin. The findings of this study are expected to be useful in fabricating a membrane-moderated TTS of selegiline hydrochloride.

2. Investigations and results

In the determination of transdermal permeability of selegiline hydrochloride, the rat epidermis was used as a skin model. Although human cadaver skin may be the logical choice as a skin model for a product finally to be used in humans, it is not easily available for most of the investigators. It is more appropriate to use the skin of hairless mouse, hairless rat or pig as approximate substitute for human skin. In the present study, the excised rat skin was used as a skin model. Only the skin of male rats was used because it was difficult to obtain the required full-length skin from female rats due to the presence of mammary glands. The *in vitro* permeation studies using the excised rat skin would provide information to manipulate the design of TTS patch for achieving the desired permeation of the drug across human skin. This would be based on the extent of relationship between rat skin permeability when compared to human skin. The permeability of rat skin was reported to be about 3 times higher than that of human skin (Diez et al. 1991).

2.1. In vitro permeation of selegiline hydrochloride from ethanol-water solvent systems

The cumulative amount of selegiline hydrochloride permeated across the rat epidermis from the solvents like water, ethanol and co-solvents containing various ratios of ethanol and water is shown in Fig. 1. There was a lag period of about 1 to 2 h for obtaining steady-state permeability flux of selegiline hydrochloride through rat epidermis from all solvent systems used in the present study. This may be because of the time required for the skin to get saturated with the drug. The flux of selegiline hydrochloride across the rat epidermis from ethanol alone was found to be high when compared to that obtained from water and ethanol-water co-solvent systems. There was an increase in the amount of drug permeated from rat epidermis as the ethanol concentration increased in the ethanol-water co-solvent system. The flux of selegiline hydrochloride from ethanol alone was found to be higher than the all proportions of ethanol-water co-solvent systems. Thus, the cumulative amount permeated in 24 h (Q_{24}) from ethanol alone was 3554 ± 41 μg/cm^2. However, from water alone, the amount permeated was only 393 ± 15 μg/cm^2 indicating that ethanol is absorbed into the skin membrane where it acts as penetration enhancer (Berner et al. 1989). It is believed that alcohol interacts with the stratum corneum at sufficiently high concentration, and increases the permeation of drugs.

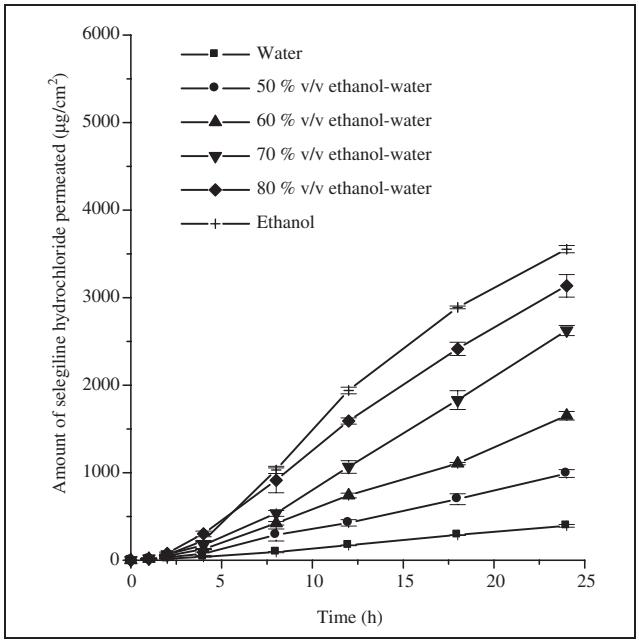


Fig. 1: Mean (±S.D.) amount of selegiline hydrochloride permeated across rat abdominal skin (n = 3) from ethanol, water and ethanol-water solvent systems.

The flux (J), permeability coefficient (k_p), enhancement ratio (ER) and percent of selegiline hydrochloride permeated at the end 24 h of study are given in Table 1. Skin permeation rate of selegiline hydrochloride from ethanol was higher than that from water. On adding ethanol to water, the flux of selegiline hydrochloride increased linearly up to a level of 60% v/v of ethanol in water and then increased drastically by further addition of ethanol.

2.2. Formulation of HPMC gel drug reservoir system

The observed flux of selegiline hydrochloride with 70% v/v ethanol-water solvent system was 111 ± 0.3 μg/cm$^2 \cdot$ h, which was 3.6 times more than that obtained with water. Because of high fluidity (may result in leakage from membrane-moderated TTS) and less stability, a solution of selegiline hydrochloride in 70% v/v ethanol-water needs to be formulated as a gel system by incorporating a suitable polymer such as HPMC at an appropriate concentration in the chosen 70% v/v ethanol-water solvent system. The HPMC was added to 70% v/v ethanol-water to prevent the crystallization of selegiline hydrochloride and thereby to improve the stability of the drug reservoir (Raghavan et al. 2000). Thus, a 2% w/w HPMC gel system containing 2% w/w of selegiline hydrochloride was pre-

Table 1: Mean[#] (±S.D.) flux (J), permeability coefficient (k_p), enhancement ratio (ER) and percent of selegiline hydrochloride permeated from various ethanol-water solvent systems across rat abdominal epidermis

Solvent system	J (μg/cm$^2 \cdot$ h)	k_p (cm/h $\cdot 10^{-3}$)	ER	% drug permeated
Water	16.68 ± 0.51	0.83 ± 0.03	1	5.29 ± 0.09
50% v/v Ethanol-water	41.93 ± 2.25*	2.11 ± 0.113*	2.56 ± 0.09*	13.63 ± 0.62*
60% v/v Ethanol-water	69.19 ± 1.82*	3.46 ± 0.091*	4.22 ± 0.18*	22.70 ± 0.66*
70% v/v Ethanol-water	111.25 ± 0.54*	5.56 ± 0.027*	6.78 ± 0.08*	36.08 ± 0.79*
80% v/v Ethanol-water	137.88 ± 7.47*	6.89 ± 0.37*	8.41 ± 0.59*	43.11 ± 3.14*
Ethanol	161.12 ± 1.54*	8.06 ± 0.08*	9.78 ± 0.22*	48.86 ± 5.63*

[#] Mean of three experiments (n = 3)
* Significant at $P < 0.001$ when compared to water

pared and evaluated for their drug content and stability. The HPMC gel system was found to contain about 99.1% of the drug indicating the uniform distribution of the drug in the reservoir. The HPLC chromatograms showed no additional peaks without a change in the retention time of selegiline hydrochloride indicating that the drug was stable in the reservoir system. Because of its high viscosity, 2% w/w HPMC gel may provide the required consistency so as to form a reservoir of the drug without any leakage.

The HPMC gel (2% w/w) containing selected concentrations of nerodilol (0%, 3%, 4%, 5% or 6% w/w), carvone (0%, 6%, 8%, 10% or 12% w/w) or anethole (0%, 1%, 2% or 3% w/w) and 2% w/w selegiline hydrochloride were prepared and evaluated for drug content, stability of the drug and *in vitro* transdermal permeation. The HPMC gel formulations were found to contain 99.1 to 99.8% of selegiline hydrochloride showing the uniformity of the drug content in the gel formulation. The stability of selegiline hydrochloride in HPMC gel containing varying concentrations of nerodilol, carvone or anethole was assessed by HPLC. The HPLC showed no additional peaks without a change in the retention time of selegiline hydrochloride indicating the stability of the drug in HPMC gel systems containing the chosen terpenes.

2.3. Effect of nerodilol

The cumulative amount of drug permeated across rat epidermis from HPMC gel drug reservoir containing selected concentrations of nerodilol was shown in Fig. 2. The percutaneous permeation parameters of selegiline hydrochloride from HPMC gel formulations with and without (control) nerodilol as penetration enhancer were given in Table 2. A synergistic effect on selegiline hydrochloride permeation was observed when nerodilol was incorporated in varying quantities in HPMC gel containing 70 : 30 v/v ethanol-water as a solvent system. When the data were analyzed, the amount of drug permeated fitted to zero order kinetics right from 2 to 24 h with a lag period of 1–2 h. The total drug used in study was accounted (mean total recovery 94.9%) when the drug content in the skin, donor compartment and receptor compartment was summed up. This indicates that there was a mass balance of the drug used in the study.

A marked effect of nerodilol on selegiline hydrochloride permeation was observed when incorporated in drug reservoir in varying quantity (Table 2). It may be observed from the results (Fig. 2) that there was a constant increase in the flux of the drug up to 5% w/w of nerodilol in HPMC gel, and such an increase in the flux and permeability coefficient (Table 2) was found to be significant ($P < 0.001$) when compared to that obtained with control (without nerodilol). But beyond 5% w/w of nerodilol, the increase in flux and permeability coefficient was insignificant ($P > 0.05$) when compared to that obtained with

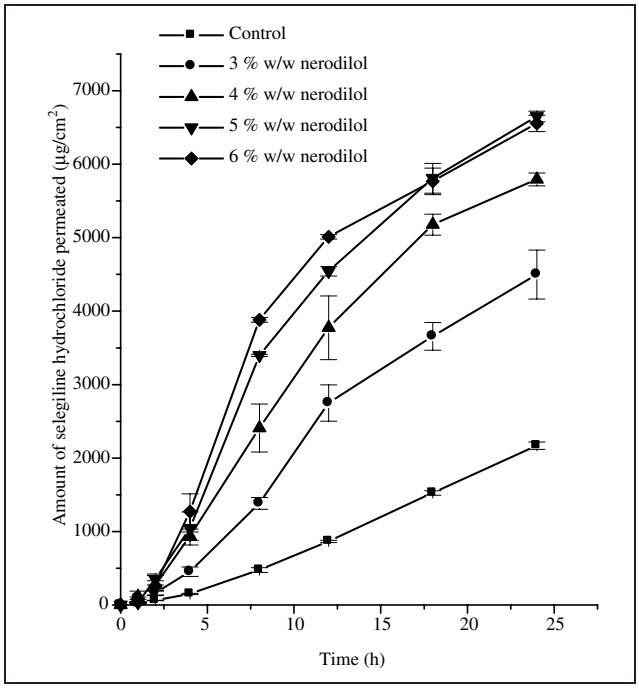


Fig. 2: Mean (±S.D.) amount of selegiline hydrochloride permeated across the rat abdominal skin (n = 3) from 2% w/w HPMC gel containing selected concentrations of nerodilol as a penetration enhancer

6% w/w of nerodilol. A plateau effect was observed beyond 5% w/w of nerodilol in the drug reservoir. There was about 3.2-fold increase in the permeation of the drug from the HPMC gel containing 5% w/w of nerodilol when compared to that obtained with control (without nerodilol).

2.4. Effect of carvone

The cumulative amount of drug permeated across rat epidermis from HPMC gel drug reservoir containing selected concentrations of carvone is shown in Fig. 3. The permeation parameters of selegiline hydrochloride across rat epidermis from HPMC gel drug reservoir system with and without carvone as penetration enhancer are given in Table 3. A synergistic effect on selegiline hydrochloride percutaneous permeation was observed when carvone was incorporated in varying quantities in HPMC gel containing 70 : 30 v/v ethanol-water as a solvent system.

A marked effect of carvone on selegiline hydrochloride permeation was observed when incorporated in drug reservoir in varying quantity (Table 3). However, there was a lag period of 1–2 h in the permeation of drug across the rat stratum corneum. It may be observed from the results (Fig. 3) that there was a constant increase in the flux of the drug upto 10% w/w of carvone in HPMC gel, and such an increase in the flux and permeability coefficient (Table 3) was found to be significant ($P < 0.001$) when compared to

Table 2: Effect of nerodilol on the permeation of selegiline hydrochloride from HPMC gel reservoir system across rat epidermis

Concentration of nerodilol (% w/w)	Q_{24} (µg/cm²)[a]	% Drug permeated[a]	DRS (µg/g)[a]	J (µg/cm² · h)[a]	k_p (cm/h · 10^{-3})[a]	ER
0 (control)	2169.64 ± 50.28	29.83 ± 0.69	977.26 ± 16.96	91.90 ± 1.11	4.59 ± 0.06	1
3	4498.62 ± 70.98*	54.35* ± 6.03	1402.36 ± 125.87*	202.25 ± 10.48*	10.11 ± 0.52*	2.20*
4	5792.76 ± 87.46*	79.66 ± 1.21*	1763.83 ± 153.01*	265.68 ± 3.42*	13.28 ± 0.17*	2.89*
5	6649.90 ± 72.8*	88.68 ± 1.01*	2147.94 ± 147.72*	295.48 ± 2.39*	14.77 ± 0.12*	3.22*
6	6556.19 ± 110.18*	90.15 ± 1.51*	2409.79 ± 72.83*	302.38 ± 3.52*	15.12 ± 0.18*	3.29*

[a] Mean ± S.D. (n = 3); DRS: Drug retained in skin after 24 h; Q_{24} cumulative amount of selegiline hydrochloride after 24 h; ER: Enhancement ratio
* Significant at $P < 0.001$ when compared to control

Table 3: Effect of carvone on the permeation of selegiline hydrochloride from HPMC gel reservoir system across rat epidermis

Concentration of carvone (% w/w)	Q_{24} (μg/cm^2)[a]	% Drug permeated[a]	DRS (μg/g)[a]	J (μg/cm^2 · h)[a]	k_p (cm/h · 10^{-3})[a]	ER
0 (control)	2169.6 ± 50.3	29.8 ± 0.7	977.36 ± 16.96	91.90 ± 1.11	4.6 ± 0.1	1.00
6	2968.5 ± 56.6*	40.8* ± 1.1	4312.36 ± 393.4*	122.9 ± 3.5*	6.1 ± 0.6*	1.34*
8	4827.6 ± 138.2*	66.4 ± 0.5*	4621.6 ± 349.6*	222.7 ± 9.2*	11.1 ± 0.5*	2.42*
10	5660.1 ± 86.9*	77.8 ± 0.6*	5124.0 ± 83.6*	257.8 ± 8.1*	12.9 ± 0.3*	2.81*
12	5560.1 ± 54.5*,#	76.5 ± 1.9*,#	5101.7 ± 83.6*	255.1 ± 10.1*,#	12.8 ± 0.5*,#	2.80*,#

[a] Mean ± S.D. (n = 3); DRS: Drug retained in skin after 24 h; Q_{24} cumulative amount of selegiline hydrochloride after 24 h; ER: Enhancement ratio
* Significant at $P < 0.001$ when compared to control
\# Not significant when compared to 10% w/w carvone

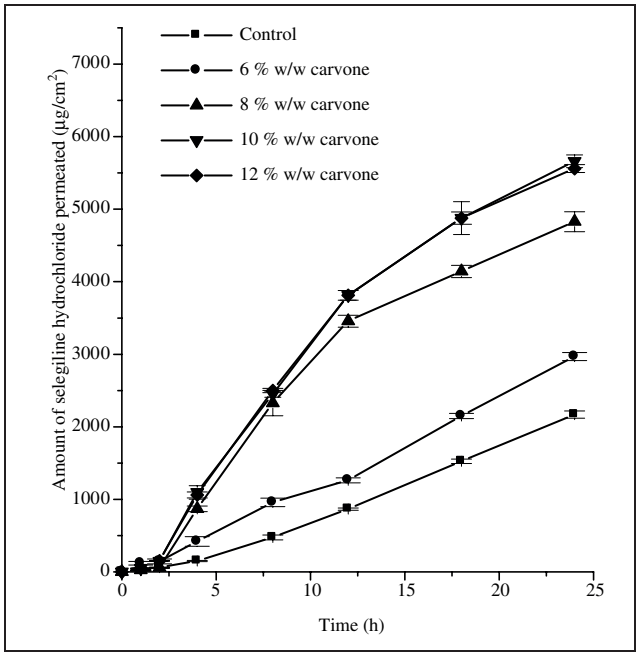


Fig. 3: Mean (±S.D.) amount of selegiline hydrochloride permeated across the rat abdominal skin (n = 3) from 2% w/w HPMC gel containing selected concentrations of carvone as a penetration enhancer

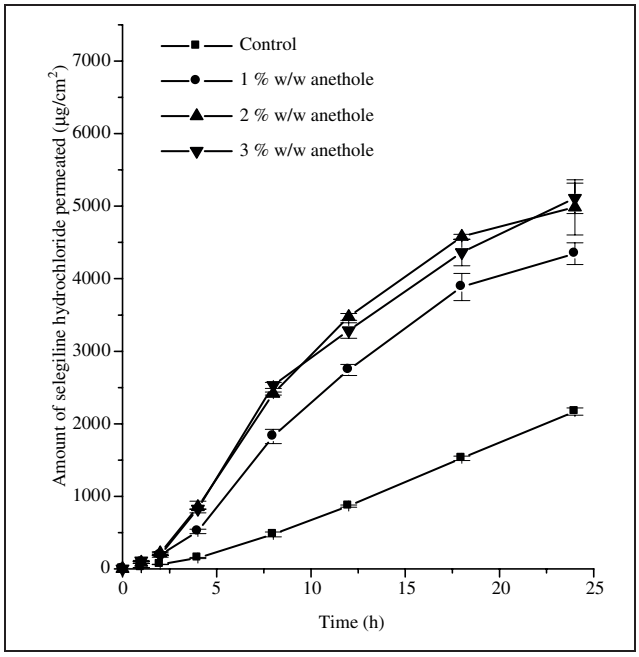


Fig. 4: Mean (±S.D.) amount of selegiline hydrochloride permeated across the rat abdominal skin (n = 3) from 2% w/w HPMC gel containing selected concentrations of anethole as a penetration enhancer

that obtained with control (without carvone). But beyond 10% w/w of carvone, i.e., with 12% w/w carvone the increase in flux and permeability coefficient was insignificant ($P > 0.05$) when compared to that obtained with 10% w/w of carvone. Thus, a plateau effect was observed beyond 10% w/w of carvone in the drug reservoir. There was an about 2.8-fold increase in the permeation of the drug from the HPMC gel containing 10% w/w of carvone when compared to that obtained with control (without carvone).

2.5. Effect of anethole

The cumulative amount of drug permeated across rat epidermis from HPMC gel drug reservoir containing varying amounts of anethole is shown in Fig. 4. The permeation parameters of selegiline hydrochloride from HPMC gel formulations with anethole as penetration enhancer are given in Table 4. The permeation of selegiline hydrochloride was enhanced when anethole was incorporated in varying quantities in HPMC gel containing 70 : 30v/v ethanol-water as a solvent system.

A marked effect of anethole on selegiline hydrochloride permeation was observed when incorporated in drug reservoir in varying quantity (Table 4). However, there was a lag period of 1–2 h in the permeation of drug across the rat stratum corneum. It may be observed from the results (Fig. 4) that there was a constant increase in the flux of the drug upto 2% w/w of anethole in HPMC gel, and such an increase in the flux and permeability coefficient (Table 4) was found to be significant ($P < 0.001$) when compared to that obtained with control (without anethole). But beyond 2% w/w of anethole i.e., with 3% w/w anethole in

Table 4: Effect of anethole on the permeation of selegiline hydrochloride from HPMC gel reservoir system across rat epidermis

Concentration of anethole (% w/w)	Q_{24} (μg/cm^2)[a]	% Drug permeated[a]	DRS (μg/g)[a]	J (μg/cm^2 · h)[a]	k_p (cm/h · 10^{-3})[a]	ER
0 (control)	2169.6 ± 50.3	29.8 ± 0.7	977.2 ± 16.9	91.9 ± 1.1	4.6 ± 0.1	1
1	4345.1 ± 149.3*	59.7* ± 1.0	4537.3 ± 74.9*	206.5 ± 13.4*	10.4 ± 0.1*	2.25*
2	4984.4 ± 380.4*	68.5 ± 1.2*	4989.7 ± 71.0*	241.8 ± 20.4*	12.1 ± 0.6*	2.63*
3	5107.9 ± 209.4*	70.2 ± 0.8*	5172.9 ± 32.7*	240.4 ± 17.9*	12.0 ± 0.5*	2.62*

[a] Mean ± S.D. (n = 3); DRS: Drug retained in skin after 24 h; Q_{24} cumulative amount of selegiline hydrochloride after 24 h; ER: Enhancement ratio;
* Significant at $P < 0.001$ when compared to control

HPMC gel, the increase in flux and permeability coefficient was insignificant ($P > 0.05$) when compared to that obtained with 3% w/w of anethole. A plateau effect was observed beyond 2% w/w of anethole in HPMC gel drug reservoir. There was about an 2.6-fold increase in the permeability of the drug from the HPMC gel containing 2% w/w of anethole when compared to that obtained with control (without anethole).

3. Discussion

The transdermal delivery of selegiline hydrochloride depends on its permeation through the stratum corneum, which in turn depends on the development of an optimal solvent system. Several solvents either alone or in various proportions with other solvents (co-solvents) are being investigated as solvent systems to overcome the low permeation of drugs through the skin. These solvent systems may modify the skin structure and open channels in the skin barrier (Kim et al. 1996) thus improving the skin permeability of drugs. For example, ethanol and propylene glycol are widely used as solvent systems that also work as co-solvents to solubilise the drugs (Walker and Smith 1996). Ethanol, used as a part of co-solvent system with water, has been demonstrated to increase the penetration of a variety of drugs through the skin barrier (Berner et al. 1989; Ho et al. 1998). As a result, the use of co-solvents, as a solvent system, may exert a profound influence on the percutaneous delivery of drugs from transdermal therapeutic systems. By changing the proportion of the solvents in a co-solvent system, an optimal transdermal permeation of the drugs could be achieved. In the present study, the effect of solvents (ethanol and water) and co-solvents (mixtures of ethanol and water) on the *in vitro* skin permeability of selegiline hydrochloride was studied in order to select the solvent system as the first step to develop a membrane-moderated transdermal therapeutic system. The chosen solvents include ethanol, water and mixture of ethanol and water in various proportions (co-solvents).

The flux of selegiline hydrochloride from ethanol-water solvent system in the ratio of 50 : 50 v/v, 60 : 40 v/v, 70 : 30 v/v and 80 : 20 v/v increased from 41 to 138 μg/$cm^2 \cdot h$ respectively. But the flux of the drug from 'ethanol alone' was higher when compared to all ethanol-water co-solvent systems (Table 1). The amount of selegiline hydrochloride permeated across the rat abdominal skin from ethanol-water co-solvent systems increased with an increase in ethanol content and also it further increased with ethanol alone (Fig. 1). The skin permeation of selegiline hydrochloride from ethanol alone was found higher than that in other ethanol-water systems. The permeation of selegiline hydrochloride was enhanced by about 10 times from ethanol alone when compared to that from water (Table 1). This may be due to the varying influence of ethanol on the biophysical properties of the stratum corneum (Seki et al. 1989). When ethanol alone was used as a solvent system in the donor compartment, ethanol molecules might have diffused in to the receptor fluid (70 : 30% v/v ethanol-water). This in turn might have increased the thermodynamic activity of the drug in the donor compartment. It is a desired phenomenon that the thermodynamic activity is higher in the donor compartment for an ideal drug reservoir system of membrane-moderated TTS. However, subsequent studies were carried out with equal concentration of ethanol in donor and receiver cells wherein the ethanol molecules do not diffuse readily into either of the cells.

The results of the *in vi*tro permeation studies across rat epidermis showed that the permeation of selegiline from ethanol alone was higher than from ethanol-water co-solvent systems. However, such high concentration of ethanol leads to a serious damage to the skin if chosen for use in membrane-moderated TTS. Thus, in the present study 70 : 30 v/v ethanol-water solvent system was chosen as the solvent system to formulate the drug reservoir system needed for the design of membrane-moderated TTS of selegiline hydrochloride.

The role of ethanol in transdermal drug delivery was reviewed by Williams and Barry (2004). Ethanol is commonly used in many transdermal formulations and is often the solvent of choice for use in patches. It is also commonly employed as a cosolvent with water for ensuring sink conditions during *in vitro* permeation experiments. As with water, ethanol permeates rapidly through human skin with a steady state flux of approximately 1 mg $cm^2 \cdot h$ (Berner et al. 1989). However, when using an ethanol water co-solvent vehicle, the enhancement effect of ethanol appears to be concentration dependent. Salicylate ion diffusion across human epidermal membranes was promoted up to an ethanol: water composition of 0.63 whereas higher levels of the alcohol decreased permeation (Kurihara-Bergstrom et al. 1990). It is probable that at higher ethanol levels dehydration of the biological membrane reduced permeation across the tissue. However, in the present study, transdermal permeation of selegiline increased with an increase in ethanol concentration. Ethanol can exert its permeation enhancing activity by increasing the solubility of poorly soluble drug in the donor phase (Pershing et al. 1990). Further, permeation of ethanol into the stratum corneum can alter the solubility properties of the tissue with a consequent improvement for drug partitioning into the membrane (Megrab et al. 1995). A further potential mechanism of action arising as a consequence of rapid ethanol permeation across the skin has been reported; solvent 'drag' may carry permeant into the tissue as ethanol traverses, although such a mechanism has been discounted for morphine hydrochloride permeation from ethanol and methanol containing formulations (Morimoto et al. 2002). In addition, ethanol as a volatile solvent may extract some of the lipid fraction from within the stratum corneum when used at high concentration for prolonged times; though not an 'enhancing' effect, such a mechanism would clearly improve drug flux through skin.

Because of low viscosity, the ethanolic solution of selegiline hydrochloride may leak out of the membrane-moderated TTS when sandwiched between the rate controlling membrane and drug-impermeable backing membrane. This could be avoided by inclusion of a suitable polymer such as HPMC or HPC that imparts the required viscosity to the chosen solvent system (70 : 30 v/v ethanol-water) and minimizes the spreadability of the drug reservoir. It may be noted that addition of HPMC, as a gelling agent, to 70 : 30 v/v ethanol-water has decreased the flux of the drug from 111 to 91 μg/$cm^2 \cdot h$. Thus this HPMC gel drug reservoir system may not be able to provide the desired plasma concentration of the drug for the desired time period. This in turn demands for the inclusion of a suitable percutaneous enhancer in the above formulated HPMC gel drug reservoir system.

Terpenes were reported to be effective penetration enhancers for both hydrophilic and lipophilic drugs (Gao and Singh 1998; Zhao and Singh 1999). Hence, terpenes were added to HPMC drug reservoir at varying concentrations. The concentrations of the penetration enhancers were se-

lected based on an earlier study (Krishnaiah et al. 2004). Wherever necessary, either higher or lower concentrations were used as per the expected flux of the drug across the skin. The percutaneous absorption of selegiline hydrochloride was enhanced significantly by the addition of terpene enhancers to the HPMC gel formulation (Fig. 2, 3 and 4). The amount of selegiline permeated across rat skin was almost linear from 2–18 h of the study. But there was no true steady-state linearity beyond 18 h which may be due to the pronouncing effect of the terpene enhancers resulting in the depletion of drug from the donor compartment. Such a depletion of drug from the donor compartment might be responsible for the absence of true steady-state flux. This could be rectified by increasing the concentration of the drug in the reservoir after completing necessary studies. The flux of selegiline hydrochloride was calculated using the linear regression analysis of the amount of drug permeated from 2 to 24 h. In this study, it may be noted that the tested terpene enhancers provided significant enhancement in the permeation of selegiline hydrochloride across rat epidermis when compared to control. Nerodilol provided about 3.2-fold increase in selegiline hydrochloride flux followed by carvone with a 2.8-fold increase, and anethole with a 2.6-fold increase. The maximum amount of selegiline hydrochloride permeated across rat epidermis was 6650, 5660 or 4984 $\mu g/cm^2$ at 5% w/w level of nerodilol, 10% w/w of carvone or 2% w/w of anethole in HPMC gel formulations respectively. Beyond these concentrations there was a constant effect observed in permeation of selegiline hydrochloride. Nerodilol was found to be the most effective terpene enhancer in promoting the permeation of selegiline hydrochloride followed by carvone and anethole.

Based on the flux of selegiline hydrochloride obtained with the three terpene enhancers, it appears that nerodilol is the most effective one at a concentration of 5% w/w providing a flux of about 295 $\mu g/cm^2 \cdot h$. The flux of the drug with 2% w/w or 3% w/w anethole remained the same at about 240 $\mu g/cm^2 \cdot h$ (Table 4). This showed that even with 5% w/w anethole, the flux may not increase further. The flux of selegiline hydrochloride obtained with 12% w/w of carvone was only 255 $\mu g/cm^2 \cdot h$. This clearly showed that nerodilol is the most effective terpene enhancer in promoting the permeation of selegiline hydrochloride followed by carvone and anethole. The results of the study on the enhanced percutaneous permeation of selegiline hydrochloride with the tested terpenes are in accordance with the other reports. Cornwell and Barry (1994) evaluated the effect of terpene enhancers on the percutaneous permeation of 5-fluorouracil across the skin. It was reported that nerodilol is the most effective chemical penetration enhancer in promoting the permeation of 5-fluorouracil. Furthermore, the high percutaneous enhancement activity of nerodilol was reported by Arellano et al. (1996) wherein it was found that nerodilol is an effective enhancer for the permeation of diclofenac sodium across the rat skin. The effective promoting activity of nerodilol was attributed to its amphiphilic structure that is suitable for alignment within the lipid lamellae of the stratum corneum and disrupting its highly organized packing (Cornwell and Barry 1994).

Most studies suggest that hydrophilic terpenes (alcohol, ketone, and oxide terpenes) are more effective in enhancing the permeation of hydrophilic drugs, whereas hydrocarbon terpenes (limonene and cymene) are more active in promoting percutaneous permeation of lipophilic drugs (Moghimi et al. 1997). Furthermore, Hori et al. (1991) studied the effects of terpenes on the permeation of propranolol hydrochloride (hydrophilic drug) and diazepam (lipophilic drug) as a model drugs. The purely hydrocarbon terpenes promoted percutaneous permeation of both hydrophilic (propranolol hydrochloride) and lipophilic (diazepam) drugs. However, the terpenes with hydrogen bonding ability only enhanced the flux of hydrophilic drug, propranolol hydrochloride (Hori et al. 1991). In the present study also, the terpenes nerodilol, carvone and anethole, with their hydrogen bonding ability, provided the enhanced permeation of hydrophilic (Rohatagi et al. 1997) selegiline hydrochloride (Log $P = 0.5315$) across the rat epidermis. Thus the skin permeation of hydrophilic selegiline hydrochloride might have been prominently enhanced by the lipophilic terpene enhancers nerodilol, carvone and anethole (Williams and Barry 2004).

The penetration enhancing activity of all the three terpenes, observed in the present study, was normalized based on the flux of the drug obtained across rat epidermis. Carvone provided a flux of about 258 $\mu g/cm^2 \cdot h$ at 10% w/w level whereas anethole provided almost the same flux (242 $\mu g/cm^2 \cdot h$) at 2% w/w level itself indicating that anethole is 5-times more effective than carvone in enhancing the permeation of selegiline hydrochloride across rat abdominal skin. Nerodilol provided a flux of about 302 $\mu g/cm^2 \cdot h$ at 5% w/w level whereas carvone provided a flux of about 242 $\mu g/cm^2 \cdot h$ at a concentration of 2% w/w indicating that anethole is 2-times more effective than nerodilol in enhancing transdermal permeation of selegiline hydrochloride. Thus, the penetration enhancing activity of the three terpenes in enhancing the *in vitro* transdermal permeation of selegiline hydrochloride was in the following order: anethole > nerodilol > carvone. The lipophilicity of the permeant as well as the enhancer molecule is thought to play an important role in determining the enhancer's promoting activity on the permeation of the drug across the skin (Hori et al. 1991; Sung et al. 2000). The log P values for anethole, nerodilol and carvone are about 3.39, 5.36 and 2.23 respectively whereas the Log P value of the permeant, selegiline hydrochloride was 0.5315 (Rohatagi et al. 1997). It appears that the lipophilic terpene enhancers, nerodilol, carvone and anethole increased transdermal permeation of the hydrophilic selegiline hydrochloride.

Terpenes act as penetration enhancers due to their ability to modify the solvent nature of the stratum corneum and thereby improve drug partitioning into the tissue (Williams and Barry 2004). Many terpenes permeate human skin well (Cornwell and Barry 1994), and large amounts of terpenes (up to 1.5 $\mu g/cm^2$) were found in the epidermis after application from a matrix type patch (Cal et al. 2001). With loss of terpenes, which are generally good solvents, from a formulation there could be an alteration to the thermodynamic activity of the permeant in the formulation. Terpenes may also modify drug diffusivity through the membrane. This is evident from the decreased lag time (Fig. 2, 3 and 4) and enhanced flux of selegiline hydrochloride when terpenes were incorporated in HPMC gel drug reservoir system prepared with 70% v/v ethanol-water solvent system (Tables 2, 3 and 4). Small angle X-ray diffraction studies indicated that the terpenes D-limonene and 1,8-cineole disrupt stratum corneum bilayer lipids, whereas nerolidol reinforces the bilayers, possibly by orientating alongside the stratum corneum lipids (Cornwell and Barry 1996). Spectroscopic evidence suggested that terpenes could exist within separate domains in stratum corneum lipids (Williams and Barry 2004).

Terpene enhancers are non- or relatively less toxic, less irritant and designated as "generally recognized as safe (GRAS)" by the Food and Drug Administration (Afouna et al. 2003; Gao and Singh 1998). In the present study, carvone at a concentration of 10% w/w was found to be effective in promoting the *in vitro* transdermal permeation of selegiline hydrochloride. But, the terpene enhancers are generally incorporated in transdermal formulations at a concentration not exceeding 5% w/w due to their possible adverse effects on long term usage in humans. Our earlier study involving the *in vivo* evaluation of carvone-containing TTS in human volunteers showed that carvone at a concentration more than 5% w/w showed no signs of irritation or sensitization upto 24 h of study (Krishnaiah et al. 2003a, 2003b). This indicates that even the present transdermal formulation containing 10% w/w of carvone may not produce adverse effects upto 24 h of application. Still, it is essential to conduct safety studies on carvone-containing transdermal formulations of selegiline hydrochloride developed in the present study to study the possible adverse effects on long term usage in humans.

For topical formulations, drug retained in skin is considered an important parameter. In the present study selegiline hydrochloride retained in the rat skin was determined at 24 h (Michniak et al. 1994; Bhatia et al. 1997). The drug retained in rat skin at the end of 24 h of study with nerodilol, carvone and anethole as penetration enhancers in HPMC gel were given in Tables 2–4. Carvone and anethole showed the highest drug content retained in the skin wherein the values were 5102 and 5173 μg/g of skin tissue. The drug retained in the skin was only 2408 μg/g of skin tissue when nerodilol was present as penetration enhancer in HPMC gel. There was a correlation between the drug remains of the skin and the flux values. This may suggest that the tested terpene enhancers increased the flux of selegiline hydrochloride by localizing the drug in the stratum corneum. Thus, the results of the study showed that the terpenes nerodilol, carvone and anethole have the potential in providing an optimal transdermal permeation of selegiline hydrochloride at 5% w/w, 10% w/w and 2% w/w level respectively in HPMC gel drug reservoir system. This prompts for further studies to fabricate and evaluate terpene-based membrane-moderated TTS of selegiline hydrochloride for use in humans.

4. Experimental

4.1. Materials

Selegiline hydrochloride and ondansetron hydrochloride were gift samples from M/s. Sun Pharmaceutical Industries Ltd., Baroda, India and M/s. Natco Fine Pharmaceuticals Ltd. Hyderabad, India respectively. The terpenes d,l-nerodilol (purity 98%), l-carvone (purity 99%) and trans-anethole (99%) were obtained from M/s. Merck-Schuchardt, Hohenbrunn, Germany. HPMC was a gift sample from M/s. Dr. Reddy's Labs, Hyderabad, India. Methanol and water (HPLC grade) were obtained from M/s. Qualigens Fine Chemicals, Mumbai, India. Other materials used in the study such as ethanol and potassium dihydrogen orthophosphate were of analytical grade (Qualigens).

4.2. Preparation of ethanol-water solvent system and HPMC gel drug reservoir

Ethanol and water were mixed in different ratios so as to obtain co-solvent systems of 50 : 50 v/v, 60 : 40 v/v, 70 : 30 v/v or 80 : 20 v/v of ethanol in water. To prepare 2% w/w HPMC gel, the HPMC powder was added to 70% v/v ethanol-water while being stirred by means of a stirrer (M/s Remi Motors, Mumbai, India) at 2,500 rpm, and the resulting mixture was mixed continuously at 37 °C until the gel was formed (1 h). Then, selegiline hydrochloride (2% w/w) followed by the terpene enhancer such as nerodilol (3%, 4%, 5% or 6% w/w), carvone (6%, 8%, 10% or 12% w/w) or anethole (1%, 2% or 3% w/w) were added to HPMC gel and mixed well for complete dissolution/ dispersion. The gel formulations were left overnight at room temperature (25 to 28 °C).

4.3. HPLC estimation of selegiline hydrochloride

The quantitative determination of selegiline hydrochloride was performed by HPLC. A gradient HPLC (Shimadzu HPLC Class VP series) with two LC-10AT VP pumps, a variable wave length programmable UV/VIS Detector SPD-10A VP, a CTO-10AS VP Column oven (Shimadzu), an SCL-10A VP system controller (Shimadzu), a disposable guard column LC-18 ((Pelliguard^{TM}, LC-18, 2 cm, Supelco, Inc., Bellefonte, PA) and a RP C-18 column (250 mm · 4.6 mm I.D., particle size 5 μm; YMC, Inc., Wilmington, NC 28403, U.S.A) was used. The HPLC system was equipped with the software "Class-VP series version 5.03 (Shimadzu)".

The mobile phase used was a mixture of methanol and 0.02 M potassium dihydrogen orthophosphate. The mobile phase components were filtered through a 0.45-μm membrane filter and pumped in the ratio of 70 : 30 at a flow rate of 1 ml/min. The column temperature was maintained at 40 °C. A series of drug solutions with varying quantity of selegiline hydrochloride ranging from 0.2 to 20 μg/ml and fixed concentration (2 μg/ml) of internal standard (ondansetron hydrochloride) were prepared and injected into the HPLC column. The eluent was detected by an UV detector at 206 nm, and the data were acquired, stored and analyzed with the software Class-VP series version 5.03 (Shimadzu). A good linear relationship was observed between the peak area ratio of selegiline hydrochloride to that of internal standard and the concentration of selegiline hydrochloride with a high correlation coefficient ($r = 0.9999$). The method was found to be precise (intra- and inter-day variation was found to be less than 3%) and accurate (mean recovery 98.4%). The standard curve, constructed as described above, was used for estimating selegiline hydrochloride in the skin permeates, drug retained in the skin after 24 h of study or in HPMC gel formulations. Required studies were carried out to validate the HPLC method of estimating selegiline hydrochloride in skin permeates and skin homogenates. Varying amounts of selegiline hydrochloride (0.5, 5 or 15 μg) and fixed quantity of internal standard (2 μg) were added to skin permeates or skin homogenates containing known concentration (5 μg/ml) of drug, and subjected to HPLC method as described above. There was a high recovery of selegiline hydrochloride ranging from 98.2 to 99.4% indicating the HPLC method, used in the present study, was highly accurate in estimating the drug either in skin permeates or skin homogenates.

4.4. Estimation of selegiline hydrochloride in HPMC gel drug reservoir

One gram of the HPMC gel formulation was accurately weighed, placed in 100-mL volumetric flask containing 30 ml of mobile phase, added with fixed concentration of internal standard (2 μg/ml), stirred for 30 min and made up to volume. The resultant mixture was filtered through a 0.45-μm membrane filter and injected into the HPLC system. The amount of selegiline hydrochloride was estimated from the standard curve as described above.

4.5. Preparation of rat epidermis

In the present study, rat epidermis was used as a skin model. Male albino rats (150–200 g) were obtained from M/s Ghosh Enterprises, Kolkata, India. They were euthanized using carbon dioxide asphyxiation before the experiments. The dorsal hair was removed with a clipper, and full thickness skin was surgically removed from each rat. The epidermis was prepared by a heat separation technique (Zhao and Singh 1999). The entire epidermis was soaked in water at 60 °C for 45 s, followed by careful removal of the epidermis. The epidermis was washed with water and used in the *in vitro* permeation studies. Such a transient heat treatment is unlikely to affect either the integrity or viability of the skin. However, the assumption is that transdermal absorption is a passive process, and that skin viability is therefore not any relevance. The epidermal membranes were examined for physical damage by using magnifying lens. The epidermis that is free from physical damage was used for *in vitro* permeation studies.

4.6. In vitro transdermal permeation studies

Modified Keshary-Chien diffusion cells (Keshary and Chien 1984) were used in the *in vitro* permeation studies. The rat epidermis, prepared as described above, was mounted between the two compartments of the diffusion cell with stratum corneum facing the donor compartment. High vacuum silicone grease was applied onto donor and receptor compartments and excessive skin at the sides was trimmed off to minimize the lateral diffusion. The effective diffusional area was 5.6 cm^2 and the volume of the receiver compartment was 35 ml. Two milliliters of 2% drug solution or two grams of HPMC gel drug reservoir without or with terpene enhancer (HPMC gel containing selected concentrations of nerodilol, carvone or anethole) containing 2% w/w of selegiline hydrochloride, were placed in the donor cell and covered with parafilm and aluminium foil to minimize the evaporation of the solution. Ethanol-water (70 : 30 v/v) solvent system was added to the receiver cell. The cells were maintained at 37 ± 0.5 °C by

placing on a magnetic stirrer with heater (M/s Remi Motors, Mumbai, India). It may be noted that 70% v/v ethanol-water is also effective against microbes that prevents the possible contamination of the skin and maintains the skin integrity during 24 h. The contents in the receiver compartment was stirred with help of a magnetic bar rotating at 500 rpm. The permeate samples (0.5 ml) were withdrawn from the receiver compartments at predetermined time intervals upto 24 h, and an equivalent volume of drug-free vehicle (70% v/v ethanol-water) was added to the receiver compartment to maintain a constant volume. The samples were assayed for selegiline hydrochloride by HPLC method as described above.

4.7. Estimation of drug retained in rat epidermis

At the end of the study, the skin sample was removed from the cells and washed briefly in methanol (20 ml) for 15 s (Bhatia et al. 1997) to remove the adhering HPMC gel drug reservoir. Following drying at room temperature for 10 min, the skin was cut into pieces and then homogenized in 4 ml of methanol for 10 min and sonicated for 30 min to leach out the drug. The samples were centrifuged, the supernatant liquid filtered through a 0.45-μm membrane filter, added with fixed quantity of internal standard (2 μg/ml) and analyzed for the drug content by HPLC method, as described above.

4.8. In vitro permeation data analysis and statistical analysis

The *in vitro* permeation parameters such as flux, permeability coefficient and enhancement ratio (ER) were obtained as described earlier (Krishnaiah et al. 2004**).** The difference observed in the permeation parameters with varying concentrations of nerodilol, anethole or carvone in HPMC gel drug reservoir was tested by using analysis of variance (ANOVA) and Duncan's multiple range test with the help of STATISTICA program. A value of $P<0.05$ was considered statistically significant.

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