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Effect of Nerodilol and Carvone on in vitro Permeation of Nicorandil Across Rat Epidermal Membrane

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ABSTRACT The objective of the study was to investigate the effect of nerodilol and carvone on the in vitro transdermal delivery of nicorandil so as to fabricate a membrane-moderated transdermal therapeutic system. The in vitro permeation studies were carried across the rat epidermal membrane from the hydroxypropyl methylcellulose (HPMC) gels (prepared with 70:30 v/v ethanol–water) containing selected concentrations of a terpene such as nerodilol (0% w/w, 4% w/w, 8% w/w, 10% w/w, or 12% w/w) or carvone (0% w/w, 4% w/w, 8% w/w, 12% w/w, or 16% w/w). The amount of nicorandil permeated (Q_{24}) from HPMC gel drug reservoir without a terpene was 3424.6 ± 51.4 μg/cm^2, and the corresponding flux of the drug was 145.5 ± 2.2 μg/cm^2·h. The flux of nicorandil increased with an increase in terpene concentration in HPMC gel. It was increased ranging from 254.9 ± 3.1 to 375.7 ± 3.2 μg/cm^2·h or 207.6 ± 4.7 to 356.7 ± 15.3 μg/cm^2· h from HPMC gels containing nerodilol (4% w/w to 12% w/w) or carvone (4% w/w to 16% w/w), respectively. Nerodilol increased the flux of nicorandil by about 2.62-folds whereas carvone increased the flux of the drug by about 2.49-folds across the rat epidermal membrane. The results of the Fourier Transform Infrared (FT-IR) study indicated that the enhanced in vitro transdermal delivery of nicorandil might be due to the partial extraction of stratum corneum lipids by nerodilol or carvone. It was concluded that the terpenes, nerodilol and carvone, produced a marked penetration enhancing effect on the transdermal delivery of nicorandil that could be used in the fabrication of membrane-moderated transdermal therapeutic systems.

KEYWORDS Nicorandil, in vitro permeation, Nerodilol, Carvone, Rat epidermal membrane

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INTRODUCTION

Scientists from various disciplines are bringing exciting developments in the field of enhanced skin permeability in the last decade. In spite of this excellent

achievement, transdermal patches exist only for a few drugs such as scopolamine, nitroglycerin, nicotine, clonidine, fentanyl, estradiol, testosterone, and oxybutinin (Prausnitz et al., 2004). This reflects the inability to deliver sufficient quantities of therapeutic agents across the skin to maintain the desired plasma concentration. The stratum corneum is the barrier to transdermal permeation of drugs across the skin (Bouwstra et al., 1991). Overcoming this barrier safely and reversibly is a fundamental problem that persists in the field of transdermal delivery. The stratum corneum is composed of dead, flattened cells filled with keratin in the form of regular array of protein-rich cells embedded in an intercellular and multicellular lipid domain running parallel to the skin (Elias, 1996). Barrier properties of the stratum corneum may be manipulated by using several techniques. The most promising technique is the use of penetration enhancers that allow drug permeation through the skin at an appropriate rate for a suitable time. Willams & Barry (2004) have reviewed the use of penetration enhancers for use in transdermal drug delivery systems. The various chemical penetration enhancers that have been studied are azone and its analogues, pyrrolidones, polyunsaturated fatty acids, alkanols, polymeric enhancers, nonionic surfactants, and terpenes. The safety of chemical penetration enhancers is of primary consideration while selecting them for use in the development of membrane-moderated transdermal therapeutic systems (TTS). Terpenes are of low cutaneous irritancy, generally regarded as safe, provide excellent enhancement ability and, thus appear to be promising candidates for transdermal formulations (Gao & Singh, 1998). A variety of terpenes has been shown to increase the percutaneous absorption of both hydrophilic and lipophilic drugs, and hence could be used as penetration enhancers for increasing the permeation of a lipophilic drug such as nicorandil (Zhao & Singh, 1998, 1999; El-Kattan et al., 2001).

Nicorandil, a potassium channel activator characterized by its arterial vasodilator properties, is used in the treatment of angina pectoris (Frampton et al., 1992; Kerins et al., 2001). It is subjected to hepatic first-pass metabolism following oral administration with systemic bioavailability of about 75% (Frydman, 1992). Because of its short elimination half-life (1 h), the drug has to be given at 10 to 20 mg twice daily (Frampton et al., 1992). Thus, the conventional therapy may result in higher fluctuations in plasma concentration of the drug resulting in unwanted side effects. Hence, the development of a TTS for nicorandil that could provide the desired constant drug delivery for a predetermined time period is beneficial for an effective and safe therapy of angina pectoris.

The various approaches for achieving transdermal drug delivery are reservoir-type membrane-moderated systems, adhesive diffusion-controlled systems, matrix diffusion-controlled systems, and microreservoir systems. Of these, reservoir-type membrane-moderated TTS are considered advantageous in providing the desired plasma concentration of drug for the predetermined time with minimal fluctuations. Thus, the broad objective of the present study was to design a membrane-moderated TTS of nicorandil. In this context, it was reported that nicorandil is a potential drug candidate for formulation as TTS after predicting its permeation across human skin based on its in vitro permeation through animal skin model (Sato et al., 1989, 1991). In addition to these preliminary reports, only one report existed in the literature on the development of monolithic TTS for nicorandil (Tipre & Vavia, 2002). Earlier studies showed that HPMC gel drug reservoir system prepared with 70:30 v/v ethanol–water solvent system containing 6% w/w of limonene was effective in promoting the in vitro transdermal delivery of nicorandil (Al-Saidan et al., 2004). The flux of nicorandil was 370.9 ± 4.2 $\mu g/cm^2 \cdot h$ from the HPMC gel reservoir system with 6% w/w of limonene, which was about 2.6 times the required flux to be obtained across rat epidermal membrane for producing the desired plasma concentration for the predetermined period in humans. In light of this observation, the present study was planned to study the penetration enhancing effect of two more terpenes, nerodilol and carvone on the in vitro transdermal permeation of nicorandil from HPMC gel formulations. Such a comparative study, with two more terpene enhancers, is useful in choosing the right transdermal formulation among the three terpene-containing HPMC gel drug reservoirs with respect to their ability in providing controlled in vivo drug release in humans with maximum safety (with no or minimal skin irritation/sensitization). Nerodilol- or carvone-containing HPMC gel formulations are hypothesized to enhance the in vitro transdermal delivery of nicorandil such that they could be used in the design of membrane-moderated TTS of nicorandil for use in humans.

EXPERIMENTAL

Materials

Nicorandil was obtained from M/s. Aarti Drugs Ltd., Mumbai, India. The terpenes d,l-nerodilol (purity 98%) and l-carvone (purity 99%) were obtained from M/s. Merck-Schuchardt, Hohenbrunn, Germany. The HPMC (USP/NF) was a gift sample from M/s. Dr. Reddy's Labs, Hyderabad, India. Acetonitrile (HPLC grade), water (HPLC grade), ethanol (AR grade), and potassium dihydrogen orthophosphate (AR grade) were obtained from M/s. Qualigens Fine Chemicals, Mumbai, India.

Preparation of HPMC Gel Drug Reservoir with Nerodilol or Carvone

The Composition of HPMC gel drug reservoir formulations containing nicorandil and selected concentrations of nerodilol or carvone are given in Table 1. 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 formation of gel (1 h). Then, nicorandil (4% w/w) followed by nerodilol (0% w/w, 4% w/w, 8% w/w, 10% w/w, or 12% w/w) or carvone (0% w/w, 4% w/w, 8% w/w, 12% w/w, or 16% w/w) was added to the HPMC gel and mixed well for complete dissolution/dispersion. The gel formulations were left overnight at room temperature (25 to 28°C).

HPLC Analysis of Nicorandil

The quantitative determination of nicorandil was performed by high performance liquid chromatography (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) were used. The HPLC system was equipped with the software "Class-VP series version 5.03 (Shimadzu)."

The mobile phase consisted of acetonitrile and 0.02*M* phosphate buffer. The mobile phase components were filtered before use through a 0.45-μm membrane filter and pumped in the ratio of 38:62 (acetonitrile: 0.02*M* potassium dihydrogen orthophosphate) from the respective solvent reservoirs. The flow rate of the mobile phase was maintained at 0.8 mL/min, and the column temperature was maintained at 40°C. A series of drug solutions with varying concentrations of nicorandil ranging from 0.2–20 μg/mL were prepared and injected into the HPLC column. The column pressure varied from 85 to 90 kgf/cm^2. The eluent was detected by a UV detector at 254 nm, the data acquired, stored, and analyzed with the software Class-VP series version 5.03 (Shimadzu). A good linear relationship was observed between the peak area of nicorandil and its concentration with a high correlation coefficient ($r = 0.9999$). The method was found to be precise (intra- and inter-day variation was found

TABLE 1 **Composition of HPMC Gel Drug Reservoir Formulations Containing Nicorandil and Selected Concentrations of Nerodilol or Carvone**

	Quantity present in the HPMC drug reservoir formulation (% w/w)				
Formulation	Nicorandil	Nerodilol	Carvone	HPMC	Ethanol–water (70% v/v) q.s.
I	4	0	—	2	100
II	4	4	—	2	100
III	4	8	—	2	100
IV	4	10	—	2	100
V	4	12	—	2	100
VI	4	—	0	2	100
VII	4	—	4	2	100
VIII	4	—	8	2	100
IX	4	—	12	2	100
X	4	—	16	2	100

to be less than 2.5%) and accurate (mean recovery 99.97%). The standard curve, constructed as described above, was used for estimating nicorandil in the skin permeates, drug retained in the skin (DRS) after 24 h of study, or in HPMC gel formulations. Required studies were carried out to validate the HPLC method of estimating nicorandil in skin permeates and skin homogenates. Varying amounts of nicorandil (0.4, 4, or 10 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 nicorandil ranging from 98.5 to 99.1% indicating the HPLC method, used in the present study, was highly accurate in estimating the drug either in skin permeates or skin homogenates.

Assay of Nicorandil in HPMC Gel Formulations

One gram of the HPMC gel formulation was accurately weighed, placed in 100-mL volumetric flask containing 30 mL of mobile phase, 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 nicorandil was estimated from the standard curve as described above.

Preparation of Rat Epidermal Membrane

In the present study, rat epidermal membrane was used as a skin model after removing the hair. It is possible that the hair removal process might affect the skin permeability. The use of hairless rat epidermal membrane appears to be an alternative. 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. Although human cadaver skin may be the choice as a skin model for the final product to be used in humans, it is not easily available for most of the investigators. Yet, the in vitro permeation studies across the rat skin model (rat epidermal membrane) would provide information to manipulate the design of TTS patch for achieving the desired permeation of the drug across human skin. Diez et al. (1991) while studying the in vitro permeation of five calcium channel antagonists across the skin of hairless rats reported a 3-fold difference in the permeability of rat skin and human skin. This information was utilized to optimize the amount of terpene enhancer to be incorporated in the reservoir for achieving the required flux of nicorandil in the present study. Such an approach saves a lot of time in optimizing the formulation of drug reservoir for use in the preparation of TTS patch of nicorandil.

The animals used were male albino rats (150–200 g) and obtained from M/s Ghosh Enterprises, Kolkota, India. They had free access to food and water until used for the study. The care of the rats was in accordance with institutional guidelines and institutional animal ethics committee approved the protocol. The rats 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 rat epidermal membrane was prepared by a heat separation technique (Zhao & Singh, 1999). The entire abdominal skin was soaked in water at 60°C for 60 s followed by careful removal of the epidermis. The rat epidermal membrane, so prepared, was washed with water and examined for physical damage by using a magnifying lens. The rat epidermal membrane, free from physical damage, was used for in vitro permeation studies.

In vitro Permeation Studies

Modified Keshary-Chien diffusion cells were used in the in vitro permeation studies. The rat epidermal membrane, prepared as above, was mounted between the compartments of the diffusion cells with stratum corneum facing the donor compartment (Keshary & Chien, 1984). The effective diffusion area was 6.6 cm^2, and the volume of the receiver compartment was 35 mL. Two grams of the HPMC gel drug reservoir system containing 4% w/w of nicorandil and selected concentrations of nerodilol (0% w/w, 4% w/w, 8% w/w, 10% w/w, or 12% w/w) or carvone (0% w/w, 4% w/w, 8% w/w, 12% w/w, or 16% w/w) were placed in the donor cell. Ethanol–water (70:30 v/v) was added to the receiver cell. The cells were maintained at 37 ± 0.5°C by a magnetic stirrer with heater (Remi Equipments, Mumbai, India). The contents in the receiver compartment were stirred with the help of a magnetic bar at 500 rpm. At predetermined time periods (1, 2, 4, 8, 12, 18, and 24 h), 0.5 mL samples were withdrawn from the receiver compartment and replaced with an equivalent volume of drug-free solvent (70:30 v/v ethanol–water)

so as to maintain a constant volume. The skin permeate samples were assayed for nicorandil by the HPLC method as described previously. During the in vitro permeation study, ethanol–water solvent sytem (70:30 v/v) was used in donor compartment to avoid changes in thermodynamic activity of the drug, and in recepotr compartment, it provides sink conditions.

Preparation of Rat Stratum Corneum for FT-IR Study

The rat epidermal membrane, prepared as above, was incubated for 4 h in a 1% w/v trypsin solution in phosphate buffered saline (pH 7.4) at 37°C. The tissue was then smoothed out on a flat surface and the mushy epidermis was removed by rubbing with a moistened cotton-tipped applicator. The resultant transparent stratum corneum was briefly washed with water, blotted dry, and used for FT-IR studies (Bhatia et al., 1997).

FT-IR Spectroscopy

The rat stratum corneum samples (1 cm^2) were treated separately with either 70:30 v/v ethanol–water, a solution of 10% w/w nerodilol in 70% v/v ethanol–water, or a solution of 12% w/w carvone in 70:30 v/v ethanol–water for 24 h. The treated stratum corneum samples (n = 3) were washed with 70% v/v ethanol–water, vacuum-dried (650 mm of Hg) at 21 ± 1°C for 2 days, and stored in desiccator to remove traces of the solvent (Okamoto et al., 1988). The completely dried samples of stratum corneum (20 mg) were then subjected to FT-IR (Shimadzu, Japan) and spectra were recorded in the frequency range 400–4000 cm^{-1} with 2 cm^{-1} resolution. Each spectrum was an average of 60 scans. Attention was focused on characterizing the occurrence of peaks near 2851 cm^{-1} and 2920 cm^{-1} which were due to the symmetric and asymmetric C–H stretching absorbance, respectively, corresponding to stratum corneum lipids. The FT-IR study was also carried out with untreated rat stratum corneum (washed with water only) which served as a reference spectrum.

Permeation Data Analysis and Statistics

The permeation parameters such as flux, permeability coefficient, and enhancement ratio were calculated as described earlier (Al-Saidan et al., 2004). The flux ($\mu g/cm^2 \cdot h$) of nicorandil was calculated from the slope of the plot of the cumulative amount of drug permeated per cm^2 of rat epidermal membrane at steady state against the time using linear regression analysis. The steady state permeability coefficient (k_p) of the drug across rat epidermal membrane was calculated by using the following equation: $k_p = J/C$, where J is the flux and C is the concentration of nicorandil in donor compartment. The enhancement ratio (ER) was the ratio of flux of the drug with enhancer to the flux of the drug without enhancer.

The difference observed in the permeation parameters of nicorandil obtained without and with selected concentrations of nerodilol or carvone contained in HPMC gel drug reservoir was tested by using analysis of variance (ANOVA) and Duncan's multiple range test with the help of the STATISTICA program (Release 4.5, StatSoft Inc., 1993). The significance of the difference in peak height or percent decrease in peak height of asymmetric and symmetric C–H stretching absorbance of rat stratum corneum lipids before and after treatment with either 10% w/w of nerodilol or 12% w/w of carvone was tested in a similar way. A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Since the flux of nicorandil across rat epidermal membrane was low (145.5 ± 2.2 $\mu g/cm^2 h$) from HPMC gel prepared with 70:30 v/v ethanol–water as a solvent system[15], one can regulate the transdermal flux by changing the patch size and letting the skin function as rate-controlling membrane. But the ideal requirement of a transdermal product is that the transdermal patch should control the flux, not the skin. This is because of the possible adverse effect of drug accumulation in the skin if skin is let to act as rate-controlling membrane. Thus, membrane-moderated TTS are considered advantageous over monolithic (matrix-type) TTS in proving controlled transdermal delivery with no possibility of adverse affects on skin, especially on long term usage. Hence, HPMC gel drug reservoir systems were formulated using terpenes as penetration enhancers for improving the flux of nicorandil across the skin for use in the design of membrane-moderated TTS.

In the present study the penetration enhancing effect of terpenes, nerodilol and carvone, on the permeation of nicorandil across rat epidermal membrane was studied to optimize the formulation of HPMC gel drug reservoir system containing nerodilol or carvone as a penetration enhancer. Nerodilol, a monoterpene (log $P = 5.36$), is free from toxic effects and a widely used penetration enhancer in the transdermal drug delivery of several drugs (Cornwell & Barry, 1991; Arellano et al., 1996; El-Kattan et al., 2000). Carvone, a cyclic ketone terpene (log $P = 2.23 \pm 0.25$), is free from toxic effects and has been approved as a penetration enhancer in the transdermal delivery of several drugs (Gao & Singh, 1998; Leonard et al., 1989). The amount of nerodilol or carvone needed to provide the required flux of nicorandil across the rat epidermal membrane from the reservoir system would be utilized in developing a membrane-moderated TTS for nicorandil. The concentrations of both nerodilol and carvone were selected after conducting the in vitro permeation study on a trial basis. They were initially incorporated at 1% w/w level, and then the concentrations gradually increased to a higher level depending on the observed penetration enhancing effect. In the present study the data were shown only for selected concentrations of nerodilol and carvone.

Terpene-containing HPMC Gel Drug Reservoirs

The rationale for inclusion of 70:30 v/v ethanol–water in HPMC gel is its ability to provide an optimal transdermal permeation of nicorandil due to its synergistic penetration enhancing activity when combined with a terpene (limonene) in the gel formulation (Al-Saidan et al., 2004). The role of ethanol in transdermal drug delivery was reviewed by Williams & 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·h (Berner, 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 et al., 1990). It is probable that at higher ethanol levels dehydration of the biological membrane reduced permeation across the tissue. 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 menthol 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.

The HPMC gel formulations were found to contain 98.2 to 100.6% of nicorandil indicating the uniformity of drug content in HPMC gel drug reservoir systems. The stability of nicorandil in HPMC gel containing either nerodilol (4% w/w, 8% w/w, 10% w/w, or 12% w/w) or carvone (4% w/w, 8% w/w, 12% w/w, or 16% w/w) was assessed by HPLC method. The HPLC chromatograms showed no additional peaks without a change in the retention time of nicorandil indicating the stability of the drug in HPMC gel drug reservoir containing selected concentrations of nerodilol or carvone.

Effect of Nerodilol on in vitro Skin Permeation

The amount of nicorandil permeated across rat epidermal membrane from HPMC gel drug reservoir containing selected concentrations of nerodilol was shown in Fig. 1. The total drug used in study was accounted (mean total recovery 95.6%) when the drug retained in the skin (DRS), donor compartment, and receptor compartment was summed up. This indicated that there was a mass balance of the drug used in the study. The maximum amount of nicorandil

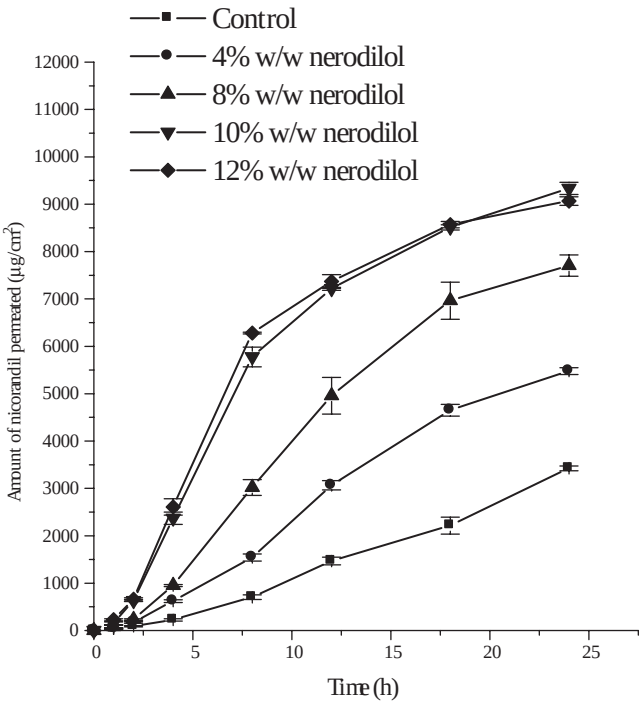

FIGURE 1 **Mean (±S.D.) Amount of Nicorandil Permeated Across the Rat Epidermal Membrane (*n* = 3) from 2% w/w HPMC Gel Containing Selected Concentrations of Nerodilol as a Penetration Enhancer.**

permeated during the 24 h of the study (Q_{24}) from the HPMC gel system without enhancer was 3424.6 ± 51.4 μg/cm^2 and the corresponding flux of the drug was 145.5 ± 2.2 μg/cm^2·h. The amount of drug permeated in 24 h increased with an increase in the concentration of nerodilol in the drug reservoir system up to a concentration of 10% w/w, but beyond this concentration there was no further increase in the amount of drug permeated, i.e., a plateau effect was observed beyond 10% w/w of nerodilol in the drug reservoir. The maximum flux of nicorandil observed with 10% w/w of nerodilol was 384.1 ± 4.6 μg/cm^2· h (Table 2).

Nerodilol showed significant effect on nicorandil permeation when it was incorporated in HPMC gel drug reservoir in varying quantities. The cumulative amount of nicorandil permeated over 24 h (Q_{24}) increased ranging from 5474.7 ± 72.0 to 9333.2 ± 129.4 μg/cm^2 from the HPMC gel drug reservoir containing 4% w/w to 12% w/w of nerodilol, and the corresponding flux of the drug was ranging from 254.9 ± 3.1 to 384.0 ± 4.6 μg/cm^2· h (Table 2). When the permeation data was analyzed, the amount of drug permeated fit for zero order kinetics right from 2 to 24 h with a mean lag period of about 1.5 h. It may be observed from the results (Fig. 1) that there was a constant increase in the flux of the drug up to 10% w/w of nerodilol in HPMC gel drug reservoir, and such an increase in the drug flux was significant when compared to control (without nerodilol). But with 12% w/w, the increase in the flux of nicorandil (375.7 ± 3.2 μg/cm^2· h) was insignificant ($P > 0.05$) when compared to that observed with 10% w/w of nerodilol (384.0 ± 4.6 μg/cm^2· h). As nerodilol concentration increased from 4% w/w to 10% w/w, the flux of nicorandil increased with an increase in permeability coefficient (Table 2). However, nerodilol, at a concentration more than 10% w/w in HPMC gel, showed a plateau effect on the permeation of nicorandil across the rat epidermal membrane. At the end of 24 h of the in vitro permeation study, nerodilol increased the skin content of nicorandil significantly ($P < 0.001$) in proportion to the concentration of nerodilol (Table 2) in HPMC gel drug reservoir. With increased concentration of nerodilol in HPMC gel, there had been an increase in the

TABLE 2 **Mean (±S.D.) Flux (J), Permeability Coefficient (k_p), Enhancement Ratio (ER), Amount of Drug Permeated in 24 h (Q_{24}), and Drug Retained in Rat Epidermal Membrane at the End of 24 h DRS in the in vitro Permeation Study Across Rat Epidermal Membrane (*n* = 3) from HPMC Gel Reservoir System of Nicorandil Containing Selected Concentrations of Nerodilol**

Concentration of nerodilol (% w/w)	*J* (μg/cm^2 h)	k_p (cm/h × 10^{-3})	ER	Q_{24} (μg/cm^2)	DRS (μg/g)
0 (control)	145.5 ± 2.2	3.64 ± 0.05	1.00 ± 0.0	3424.6 ± 51.4	1516.9 ± 98.6
4	254.9 ± 3.1*	6.37 ± 0.08*	1.73 ± 0.02*	5474.7 ± 72.0*	2069.5 ± 112.9*
8	358.6 ± 13.4*	8.99 ± 0.34*	2.51 ± 0.09*	7705.1 ± 224.0*	2691.4 ± 161.2*
10	384.0 ± 4.6*	9.60 ± 0.12*	2.69 ± 0.03*	9333.2 ± 129.4*	2801.6 ± 119.6*
12	375.7 ± 3.2*,#	9.25 ± 0.08*,#	2.62 ± 0.02*,#	9064.9 ± 92.0*,#	2798.7 ± 198.9*,#

*Significant at $P < 0.001$ when compared to control.
#Not significant at $P > 0.05$ when compared to 10% w/w nerodilol.

skin content of the drug that might have resulted in the increased flux of the drug.

Effect of Carvone on in vitro Skin Permeation

The cumulative amount of nicorandil permeated across rat epidermal membrane from HPMC gel drug reservoir containing selected concentrations of carvone was shown in Fig. 2. The total drug used in study

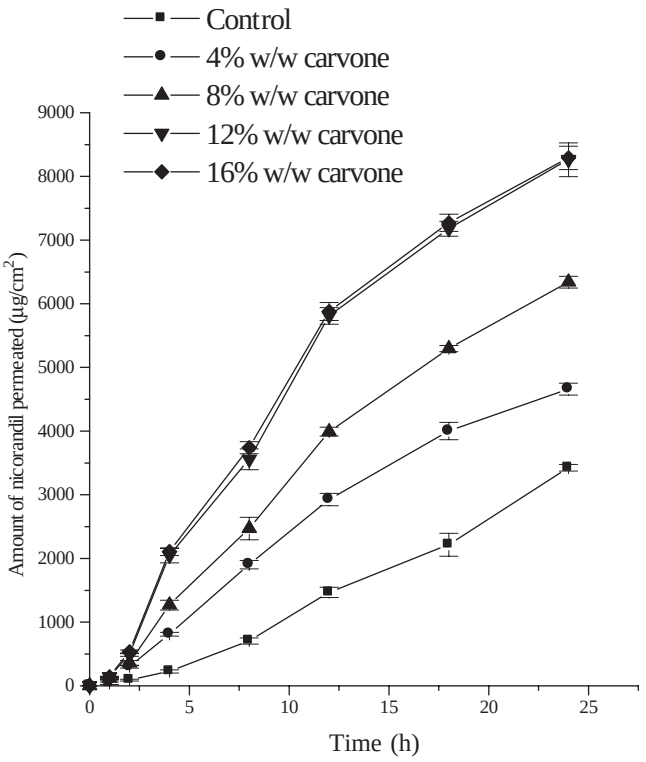

FIGURE 2 **Mean (±S.D.) Amount of Nicorandil Permeated Across the Rat Epidermal Membrane (*n* = 3) from 2% w/w HPMC Gel Containing Selected Concentrations of Carvone as a Penetration Enhancer.**

was accounted (mean total recovery 95.8%) when DRS, donor compartment, and receptor compartment was summed up. This indicates that there was a mass balance of the drug used in the study. When the data were analyzed, the amount of drug permeated fit to zero order kinetics right from 2 to 24 h (with a lag period of about 1 to 2 h). The maximum amount of nicorandil that permeated during the 24 h of the study (Q_{24}) from the HPMC gel system (without carvone) was 3224.6 ± 51.4 µg/cm^2 and the corresponding flux of nicorandil was 145.5 ± 2.2 µg/cm^2· h (Table 3). A marked effect of carvone on nicorandil permeation was observed when it was incorporated in HPMC gel in varying quantities. The cumulative amount (Q_{24}) of nicorandil permeated over 24 h increased ranging from 4659.6 ± 91.8 µg/cm^2 to 8291.6 ± 182.4 µg/cm^2 (Table 3) from HPMC gels containing 4% w/w to 12% w/w of carvone. The corresponding flux values were ranging from 207.6 ± 4.7 µg/cm^2·h to 356.7 ± 15.3 µg/cm^2· h (Table 3).

There was a constant increase in the permeability coefficient (k_p) of the drug as the concentration of carvone increased up to 12% w/w in HPMC gel prepared with 70% v/v ethanol–water (Table 3), and such an increase in the permeability coefficient (k_p) was significant ($P < 0.001$) when compared to control (without carvone). But with the incorporation of 16% w/w of carvone (8.9 ± 0.4 cm/h × 10^{-3}), there was a no further increase in the permeability coefficient (Table 3) of nicorandil when compared to that obtained with 12% w/w of carvone (9.2 ± 0.2 cm/h × 10^{-3}). However, the permeability coefficient decreased significantly ($P < 0.05$) with 16% w/w of carvone when compared to that obtained with 12% w/w of carvone in the reservoir. Thus, a steady effect was observed beyond 12% w/w of carvone in the HPMC gel. There was a 2.6-fold increase in the

TABLE 3 **Mean (±S.D.) Flux (J), Permeability Coefficient (k_p), Enhancement Ratio (ER), Amount of Drug Permeated in 24 h (Q_{24}), and Drug Retained in Rat Epidermal Membrane at the End of 24 h DRS in the in vitro Permeation Study Across Rat Epidermal Membrane (*n* = 3) from HPMC Gel Reservoir System of Nicorandil Containing Selected Concentrations of Carvone**

Concentration of carvone (% w/w)	J (µg/cm^2/h)	k_p (cm/h × 10^{-3})[a]	ER	Q_{24} (µg/cm^2)	DRS (µg/g)
0 (control)	145.5 ± 2.2	3.64 ± 0.05	1.00 ± 0.00	3424.6 ± 51.4	1516.9 ± 98.6
4	207.6 ± 4.7*	5.19 ± 0.12*	1.45 ± 0.04*	4659.6 ± 91.8*	1906.7 ± 121.9*
8	279.5 ± 2.9*	6.99 ± 0.07*	1.95 ± 0.02*	6339.6 ± 91.3*	2618.2 ± 88.7*
12	365.6 ± 8.7*	9.16 ± 0.23*	2.56 ± 0.07*	8262.6 ± 264.4*	3058.4 ± 136.8*
16	356.7 ± 15.3*,#	8.92 ± 0.38*,#	2.49 ± 0.11*,#	8291.2 ± 182.4*,#	3012.6 ± 192.6*,#

*Significant at $P < 0.001$ when compared to control.
#Significant at $P < 0.05$ when compared to 12% w/w carvone.

permeability of the nicorandil from the HPMC gel containing 12% w/w of carvone when compared with that obtained without carvone. The results of the study indicated that carvone at a concentration more than 12% w/w in HPMC gel showed steady effect in the permeation of nicorandil across the rat epidermal membrane. However, there was a lag period of about 1 to 2 h in the permeation of nicorandil across rat epidermal membrane with carvone. Also, carvone increased the skin content of nicorandil (DRS) with an increase in the concentration of carvone in HPMC gel at the end of 24 h of the in vitro permeation study (Table 3). Such an increase was statistically significant ($P < 0.001$) when compared to control. The increased drug content of rat skin membrane obtained with 12% w/w of carvone in the drug reservoir indicated that the drug might be occupying the lipid bilayers of the skin and increased the flux of nicorandil as suggested by other workers (Cornwell et al., 1994). Also, there was no further increase in the drug content in the rat skin membrane beyond 12% w/w of carvone in the HPMC gel drug reservoir.

It was reported that terpenes increase the drug percutaneous permeation mainly by disrupting the intercellular packing of the stratum corneum lipids (Zhao & Singh, 1998; Cornwell et al., 1994). Though a combination of FT-IR, Differential Scanning Calorimetry (DSC), and X-ray Diffraction (XRD) studies are needed to confirm this hypothesis, only FT-IR study was undertaken to find supporting evidence on the observed penetration enhancing effect of nerodilol and carvone on the permeation of nicorandil across rat epidermal membrane from HPMC gel.

FT-IR Studies

The FT-IR study provides an insight into the effect of nerodilol on the biophysical properties of the rat stratum corneum (Zhao & Singh, 1998, 1999; Takahashi et al., 2001). The extraction of stratum corneum lipids by chloroform–methanol solvent system led to dramatic decrease in the intensity (>95%) for the C–H stretching peaks, and increased the stratum corneum permeability by several orders of magnitude compared to that of untreated stratum corneum. There are several other reports on the use of FT-IR study to measure the changes in stratum corneum lipids with different solvent systems and penetration enhancers (Zhao & Singh, 1998, 1999; Takahashi et al., 2001). Figure 3 depicts FT-IR spectra from 3000–2700 cm^{-1} of rat stratum corneum pretreated with either water (water-washed), 70% v/v ethanol–water (control), 10% w/w nerodilol in 70% v/v ethanol–water, or 12% w/w carvone in 70% v/v ethanol–water. The lipid extraction resulting from the terpene treatment (10% w/w nerodilol or 12% w/w carvone) was evaluated by comparing the percent decrease in the peak intensities of asymmetric and symmetric C–H stretching absorbance with that of control (treated with 70:30 v/v ethanol–water). Also, the influence of solvent system (70:30 v/v ethanol–water) on the stratum corneum lipid extraction was assessed by comparing the percent decrease in the peak intensities of asymmetric and symmetric C–H stretching absorbance with that of stratum corneum alone (washed with water only).

The FT-IR study showed that the treatment of rat stratum corneum with 10% w/w nerodilol or 12% w/w carvone in ethanol–water (70:30 v/v) solvent system did not produce blue shift in the asymmetric and symmetric C–H stretching peak positions, i.e., treatment with ethanolic solution of nerodilol or carvone resulted in the occurrence of asymmetric and symmetric C–H stretching absorbance peaks at the same positions near 2851 cm^{-1} and 2920 cm^{-1} that correspond to the stratum corneum lipids. However, treatment with nerodilol or carvone showed a decrease in the heights of both asymmetric and symmetric C–H stretching absorbance peaks in comparison to those treated with ethanol–water (70:30 v/v) solvent system. Ethanol–water solvent system (70:30 v/v) decreased peak heights of asymmetric and symmetric C–H stretching absorbance by 21.82% and 26.92%, respectively, in comparison with those of water-washed (untreated) stratum corneum (Table 4). Treatment with ethanolic solution of nerodilol (10% w/w) decreased 65.62% and 73.10% in peak heights for asymmetric and symmetric C–H stretching absorbance, respectively, in comparison with the stratum corneum treated with a 70:30 v/v ethanol–water solvent system. Carvone, at a concentration of 12% w/w in 70:30 v/v ethanol–water, decreased 59.37% and 57.69% in peak heights for asymmetric and symmetric C–H stretching absorbance, respectively, in comparison with the stratum corneum treated with 70:30 v/v ethanol–water solvent system (control). The decrease in peak height indicated partial extraction of rat stratum corneum lipids, which in turn might have led to the enhanced flux of the drug across the skin (Goates & Knutson, 1994). Also, the partial extraction of stratum corneum lipids by

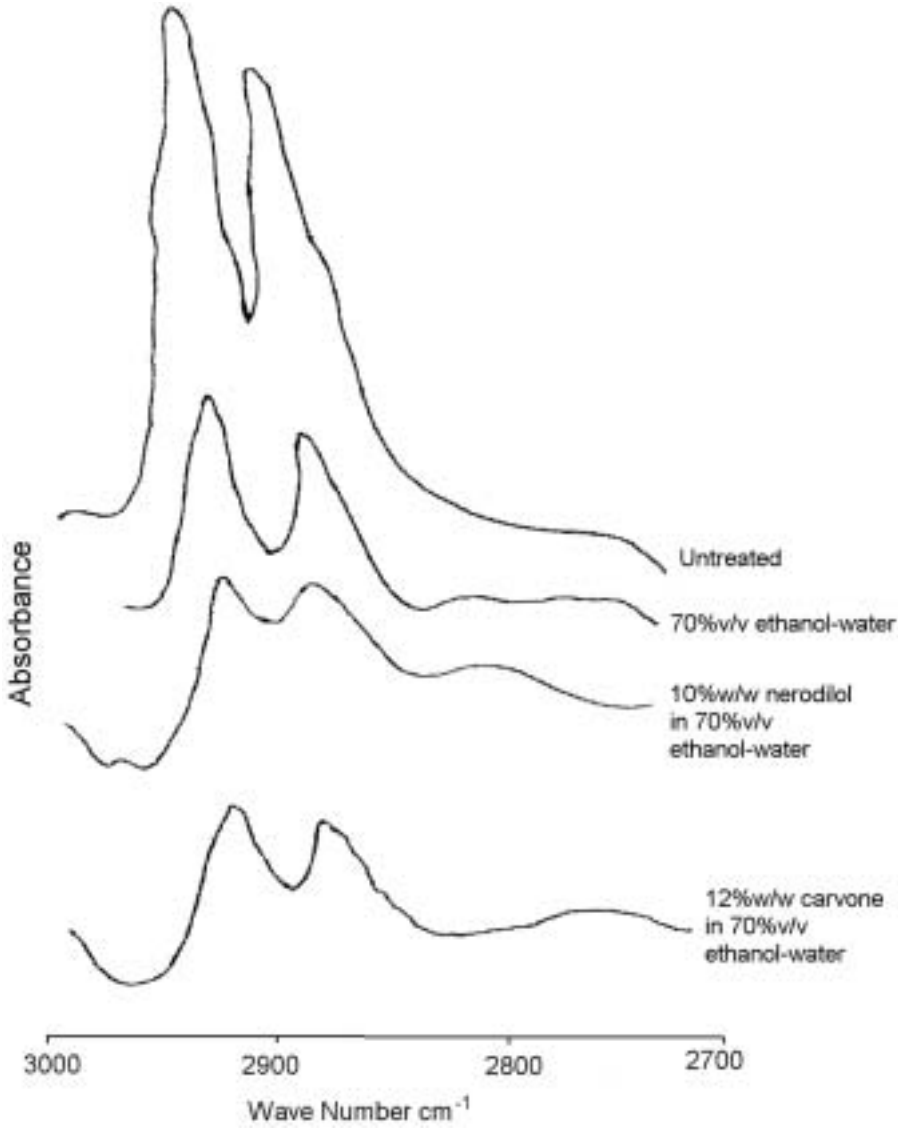

FIGURE 3 **FT-IR Spectra of Rat Stratum Corneum Showing Asymmetric and Symmetric Stretching Absorbance After Treatment with Either Water (Untreated), 70% v/v Ethanol–Water, 10% w/w Nerodilol in 70 v/v Ethanol–Water, or 12% w/w Carvone in 70% v/v Ethanol–Water.**

TABLE 4 **Mean (±S.D.) Peak Height of Asymmetric and Symmetric C–H Stretching Absorbance of Rat Stratum Corneum Lipids (n = 3)**

	Asymmetric C–H stretching absorbance		Symmetric C–H stretching absorbance	
Stratum corneum treated with	Peak height	% decrease in peak height[a]	Peak height	% decrease in peak height[a]
Untreated (washed with water only)	0.32 ± 0.01	—	0.26 ± 0.01	—
70:30 v/v ethanol–water (control)	0.25 ± 0.01[#]	21.82 ± 0.02	0.19 ± 0.05[#]	26.92 ± 0.03
10% nerodilol in 70:30 v/v ethanol–water	0.11 ± 0.01*	65.62 ± 0.03*	0.07 ± 0.01*	73.10 ± 0.05*
12% w/w carvone in 70:30 v/v ethanol–water	0.13 ± 0.01*	59.37 ± 0.02*	0.11 ± 0.01*	57.69 ± 0.05*

[a]% decrease in peak height = peak height from ethanol–water treated stratum corneum-Peak height from nerodilol (or carvone) treated stratum corneum)/Peak height from ethanol–water treated stratum corneum × 100.

*Significant at $P < 0.001$ when compared with 70:30 v/v ethanol–water treated rat stratum corneum (control).

[#]Significant at $P < 0.001$ when compared with untreated rat stratum corneum.

nerodilol or carvone might have increased the partitioning of the drug into the stratum corneum and thereby increased the DRS (Tables 2 and 3). Thus, the increase in transdermal permeation of nicorandil appears to be predominantly due to increased diffusion of the drug from the partially delipidized stratum corneum (Yum et al., 1994). However, such a hypothesis needs to be confirmed by carrying out DSC and X-RD studies.

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 & Barry, 2004). Mostly for this reason, the quantity of DRS at the end of in vitro permeation study increased with an increase in the concentration of nerodilol or carvone in the HPMC drug reservoir (Tables 2 and 3). Many terpenes permeate human skin well, 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 (Cornwell & Barry, 1994; 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 (Williams & Barry, 2004). This was evident from the decreased lag time (Figures 2 and 3) and enhanced flux of nicorandil when terpenes were incorporated in HPMC gel drug reservoir system prepared with 70% v/v ethanol–water solvent system (Tables 2 and 3). Small angle x-ray diffraction studies indicated that D-limonene disrupts stratum corneum bilayer lipids, whereas nerodilol reinforces the bilayers possibly by orientating alongside the stratum corneum lipids (Cornwell et al., 1996). Spectroscopic evidence suggested that terpenes could exist within separate domains in stratum corneum lipids (Williams & Barry, 2004).

Relative Penetration Enhancing Activity of Nerodilol and Carvone

The penetration enhancing activity of nerodilol and carvone (observed in the present study) and limonene, as reported in the earlier study (Al-Saidan et al., 2004), was normalized based on the flux of the drug obtained across rat epidermal membrane. All three terpenes provided an optimal drug flux ranging from 366 to 384 $\mu g/cm^2 \cdot h$, but with varying concentrations of the terpene in HPMC gel. The average flux of nicorandil with 6% w/w limonene (370.9 ± 4.2 $\mu g/cm^2 \cdot h$), 10% w/w nerodilol (384.0 ± 4.6 $\mu g/cm^2 \cdot h$), and 12% w/w carvone (365.6 ± 8.7 $\mu g/cm^2 \cdot h$) was about 374 $\mu g/cm^2 \cdot h$. This showed that there is a difference in the activity of the terpene enhancers on the transdermal permeation of nicorandil. The concentration of limonene, nerodilol, and carvone required producing a mean flux of about 374 $\mu g/cm^2 \cdot h$ was 6% w/w, 10% w/w, and 12% w/w, respectively. Thus, the penetration enhancing activity of the three terpenes in enhancing the in vitro transdermal permeation of nicorandil was in the following order: limonene > nerodilol > carvone.

The results of the study on the enhanced percutaneous permeation of nicorandil with the tested terpenes are in accordance with the other reports. Cornwell et al. (1994) evaluated the effect of terpene enhancers on the percutaneous permeation of 5-fluorouracil across the skin (Cornwell et al., 1994). It was reported that nerodilol was 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 was 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 et al., 1994).

Limonene is a hydrocarbon terpene (lipophilicity indicated by log P is 4.53 ± 0.23). Nerodilol is a saturated secondary alcohol (lipophilicity as denoted by log P is 5.36) and carvone is an unsaturated ketone, which is found particularly in the oils of caraway and dill. The lipophilicity of carvone as denoted by log *P* is 2.23 ± 0.25 (El-Kattan et al., 2000). Thus, the highly lipophilic nerodilol and limonene might have produced a higher penetration enhancing activity of weakly lipophilic nicorandil (Log $P = 0.43$) across the skin membrane when compared to a lesser lipophilic carvone (Moffat et al., 2004). A similar report existed in the literature that carvone was ineffective in promoting the percutaneous permeation of a lipophilic molecule, indomethacin, whereas D-limonene was highly effective (Okabe et al., 1989).

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, the effect of terpenes on the permeation of hydrophilic and lipophilic drugs was studied using propranolol hydrochloride (hydrophilic drug) and diazepam (lipophilic drug) as model drugs (Hori et al., 1991). 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 the hydrophilic drug propranolol hydrochloride (Hori et al., 1991). In the present study also, the terpenes nerodilol and carvone with their hydrogen bonding ability, provided the enhanced permeation of weakly lipophilic nicorandil (Log $P = 0.43$) across the rat epidermal membrane. Thus, the skin permeation of weakly lipophilic nicorandil might have been prominently enhanced by the lipophilic terpene enhancers, nerodilol and carvone (Williams & 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 (Gao & Singh, 1998; Afouna et al., 2005). In the present study, nerodilol and carvone were found to be effective in promoting the in vitro transdermal permeation of nicorandil at a concentration of 10% w/w and 12% w/w, respectively, 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 up to 24 h of study (Krishnaiah et al., 2003). This indicated that even the present transdermal formulation containing 12% w/w of carvone may not produce adverse effects up to 24 h of application. Still, it is essential to conduct safety studies on nerodilol- and carvone-containing transdermal formulations of nicorandil developed in the present study to investigate the possible adverse effects on long term usage in humans. The enhanced flux of nicorandil by either 10% w/w nerodilol or 12% w/w carvone across rat epidermal membrane, observed in this study, may give a useful selection of relatively safe penetration enhancer to aid transdermal drug delivery of nicorandil. The objective set to achieve nicorandil flux of about 400 $\mu g/cm^2 \cdot h$ across rat skin membrane was achieved with the incorporation of nerodilol or carvone in HPMC gel drug reservoir formulation. However, the permeation of nicorandil from HPMC gel containing either 10% w/w nerodilol or 12% w/w of carvone as penetration enhancer across adhesive-coated rate controlling membrane needs to be studied in the development of a membrane-moderated transdermal therapeutic system.

CONCLUSIONS

In vitro permeation study across rat epidermal membrane showed that both carvone and nerodilol enhanced the transdermal absorption of nicorandil from HPMC gel drug reservoir system. The FT-IR study on terpene-treated stratum corneum indicated that the enhanced in vitro transdermal delivery of nicorandil might be due to the partial extraction of stratum corneum lipids by nerodilol or carvone. Thus, the present study showed that nerodilol and carvone could be used as penetration enhancers in the fabrication of membranemoderated transdermal therapeutic systems of nicorandil.

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