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Effect of limonene on the *in vitro* permeation of nicardipine hydrochloride across the excised rat abdominal skin

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The aim of the present investigation was to study the effect of limonene on *in vitro* permeation of nicardipine hydrochloride across the excised rat abdominal skin from a 2% w/w hydroxypropyl cellulose (HPC) gel. The HPC gel formulations containing 1% w/w of nicardipine hydrochloride and selected concentrations of limonene (0% w/w to 12% w/w) were prepared, and subjected to *in vitro* permeation of the drug through excised rat abdominal epidermis. The drug content in the gels was found to be uniform and the drug was found to be stable in the HPC gel formulations. The permeation flux of nicardipine hydrochloride across rat epidermis was increased markedly by the addition of limonene to the HPC gels. A maximum flux was observed (246 ± 1 μg/cm^2/h) with an enhancement ratio of about 8 when limonene was incorporated at a concentration of 4% w/w. However, there was no further increase in the permeability of nicardipine hydrochloride beyond 4% w/w of limonene. The DSC and FT-IR data indicated that limonene increased the permeability of nicardipine hydrochloride across the rat epidermis by partial extraction of lipids in the stratum corneum. The results suggest that limonene may be useful for enhancing the skin permeability of nicardipine hydrochloride from transdermal therapeutic system containing HPC gel as a reservoir.

1. Introduction

Many studies showed that the lipoid domain, the integral component of the stratum corneum must be breached if a drug is to be delivered via the transdermal route at an appropriate rate [1–7]. Several enhancement techniques such as iontophoresis, electroporation [8], or the application of drug eutectic mixtures [9, 10] have been developed to overcome the impervious nature of the stratum corneum. However, the most widely employed technique is the use of chemical penetration enhancers, which reversibly perturb the permeability barrier of the stratum corneum. Various compounds being investigated as penetration enhancers are azone and its analogues [11–15], fatty acids [16], alcohols [17], pyrrolidones [18], sulfoxides [19], and terpenes [20–22]. However, the skin has a special role as a major barrier in protecting a living body from cutaneous exposure to toxic chemicals; hence the safety of the permeation enhancers is of primary consideration.

Terpenes are of low cutaneous irritancy and provide excellent enhancement ability, and appear to be promising candidates for improving transdermal drug delivery [7]. A variety of terpenes has been shown to increase the percutaneous absorption of both hydrophilic [23] and lipophilic drugs [7, 24–26] and thus could be used as penetration enhancers for increasing the permeability of nicardipine hydrochloride. Nicardipine hydrochloride, a potent calcium channel antagonist, is used in the treatment of angina pectoris and hypertension [27]. The terminal half-life of nicardipine hydrochloride after single oral administration in human subjects varies from 2 to 4 hours. After oral administration, nicardipine hydrochloride undergoes extensive first-pass elimination, and inter-and intra-subject variability of plasma concentration is observed [28]. Because of the first-pass elimination, oral bioavailability [28] of nicardipine hydrochloride in human subjects has been reported to be as low as 30–35%. Thus, the conventional oral administration may result in higher fluctuation in plasma concentration of the drug resulting in unwanted side effects. Hence, the development of a transdermal therapeutic system for nicardipine hydrochloride that could provide a predetermined constant drug delivery would be beneficial for an effective and safe therapy of hypertension. In a recent study, it was reported from our laboratory [29] that ethanol and water solvent system in the ratio of 70 : 30 v/v was a suitable vehicle for the transdermal delivery of nicardipine hydrochloride. However, it was necessary to improve the permeation rate of nicardipine hydrochloride by a suitable penetration enhancer.

In the present study, the penetration enhancing effect of limonene, a naturally occurring terpene, on the *in vitro* percutaneous absorption of nicardipine hydrochloride from the 2% w/w of hydroxy propyl cellulose (HPC) contained in 70 : 30 v/v of ethanol and water across the excised rat abdominal epidermis was investigated. Limonene is free from toxic effects and has been approved as a penetration enhancer ($\log P = 4.58 \pm 0.23$) in the transdermal delivery of several drugs [22, 24, 25, 30–37]. Thus the specific goal of the study was to investigate the usefulness of limonene as a penetration enhancer on the transdermal permeability of nicardipine hydrochloride such that the required flux of the drug could be provided from the hydroxy propyl cellulose (HPC) gel reservoir of the transdermal therapeutic system. The amount of limonene needed to provide the required flux of the drug through rat skin from HPC gel would be utilized in developing a membrane-moderated transdermal therapeutic system for nicardipine hydrochloride.

2. Investigations, results and discussion

The broad objective of the study was to develop membrane-moderated transdermal therapeutic systems for nicardipine hydrochloride. In this context, *in vitro* drug permeation studies carried out through the skin of hairless rats [38] indicated that nicardipine is the most potential drug candidate among the 5 calcium channel antagonists (nifedipine, nitrendipine, nicardipine, felodipine and nimodipine) that were investigated for the possibility formulating them as TTS. In general the free-base of a drug is used for transdermal delivery due to its lipophilic character [38]. There are reports on the possibility of transdermal delivery of nicardipine hydrochloride because of its optimal lipophilicity [15, 25, 26, 39, 40]. In view of this,

studies were carried out to select a solvent system for the transdermal transport of nicardipine hydrochloride using rat abdominal skin as a model. It was found that 70 : 30 v/v ethanol-water is an optimal solvent system for the transdermal transport of nicardipine hydrochloride [29]. In the present study, the penetration enhancing effect of limonene on the permeability of nicardipine hydrochloride across the excised rat epidermis from a 2% w/w hydroxypropyl cellulose (HPC) gel formulation containing 70 : 30 v/v of ethanol and water as a solvent system was investigated. The HPC gel containing selected concentrations of limonene (1% w/w, 2% w/w, 4% w/w, 8% w/w or 12% w/w) were prepared and subjected to uniformity of drug content, *in vitro* skin permeability studies and stability studies. The HPLC method used for the quantitative determination of nicardipine hydrochloride was found to be precise and accurate as indicated by less than 2.5% of CV (inter-and intra-day variation) and high recovery (99.98%). The HPC gel formulations were found to contain 100.20 to 102.02% of nicardipine hydrochloride indicating the uniformity of drug content in HPC gel. The stability of nicardipine hydrochloride either in ethanol-water (70 : 30 v/v) solvent system or HPC gel containing limonene (1% w/w, 2% w/w, 4% w/w, 8% w/w or 12% w/w) was assessed by HPLC. The HPLC chromatograms showed no additional peaks without a change in the retention time of nicardipine hydrochloride indicating the stability of the drug in both the solvent system (73 : 30 v/v of ethanol : water) and the HPC gel.

The cumulative amount of drug that permeated across the excised rat abdominal epidermis from the HPC gel containing selected concentrations of limonene is shown in the Fig. The maximum amount of nicardipine hydrochloride that permeated during the 24 h of the study (Q_{24}) from the HPC gel system (without enhancer) was 734 ± 24 μg/cm^2 and the corresponding flux of the drug was 30.9 ± 0.8 μg/cm^2/h. A marked effect of limonene on nicardipine hydrochloride permeation was observed when it was incorporated in the HPC gel in varying quantities. The cumulative amount of nicardipine hydrochloride that permeated over 24 h (Q_{24}) was found increased ranging from 2337 ± 75 to 5490 ± 22 μg/cm^2 from the HPC gel containing 1% w/w to 12% w/w of limonene, and the corresponding flux values were ranging from 103 ± 3 to 246 ± 1 μg/cm^2/h. However, there was a lag period of 3 to 4 h in the permeation of the drug through stratum corneum. It may be observed from the results that there was a constant increase in the flux of the drug up to a content of 4% w/w limonene in the HPC gel, and such an increase in the flux was found to be significant when compared to control (without limonene). But beyond 4% w/w of limonene (either 8% w/w or 12% w/w), the increase in the permeability flux of nicardipine hydrochloride (238 ± 5 μg/cm^2/h or 240 ± 6 μg/cm^2/h) was insignificant ($P > 0.05$) when compared to that observed with 4% w/w of limonene (246 ± 1 μg/cm^2/h). When the data were analysed, the amount of drug permeated fit for zero order kinetics right from 3 to 24 hours with a mean lag period of about 3.5 h. As limonene concentration increased from 0 to 4% w/w, the permeability flux of nicardipine hydrochloride was found increased as indicated by an increase in both the permeability coefficient and enhancement ratio (Table 1), but beyond 4% w/w of limonene, a plateau effect was observed. There was an about 8-fold increase in the permeability of the drug from the gel containing 4% w/w of limonene. The results of the study indicate that limonene, at a concentration more than 4% w/w in HPC gel, has a plateau effect on the permeability of nicardipine hydrochloride across the rat abdominal skin.

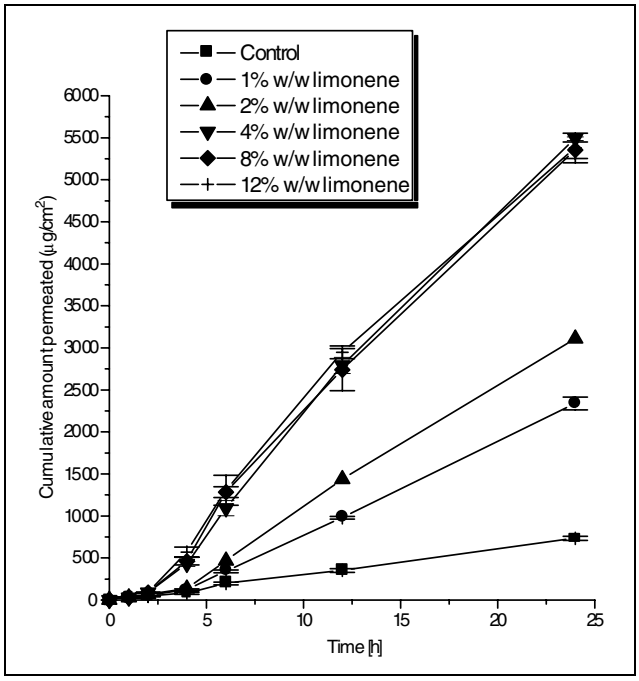

Fig.: Mean (± s.d.) amount of nicardipine hydrochloride permeated from HPC gel containing various concentration of limonene through excised rat abdominal epidermis

The total drug used in study was accounted when the drug content in the skin, donor compartment and receptor compartment was summed up. The mean total recovery of nicardipine hydrochloride in various studies was $99 \pm 1\%$ indicating a mass balance of the drug used in the study. It is interesting to note that limonene increased the skin content of nicardipine hydrochloride significantly ($P < 0.001$)

Table 1: Effect of limonene on the percutaneous permeability parameters of nicardipine hydrochloride from HPC gels

Concentration of limonene (% w/w)	Q_{24} (μg/cm^2)[a]	Drug released (%)	J (μg/cm^2/h)[a]	K_p (cm/h × 10^3)[a]	ER[a]	DCS (μg/g)[a]	Solubility at 37 °C (mg/ml)[a]
0	734 ± 24	8.6 ± 0.3	30.9 ± 0.8	3.1 ± 0.1	1	1200 ± 201	224 ± 1
1	2337 ± 75	27.3 ± 0.9	103 ± 3.2	10.0 ± 0.3	3.3 ± 0.2	1698 ± 123	225 ± 1
2	3109 ± 188	36.3 ± 2	139 ± 8	13.9 ± 0.8	4.5 ± 0.03*	2356 ± 200	224 ± 3.1
4	5490 ± 22	64 ± 0.3	246 ± 1	24.6 ± 0.1	7.9 ± 0.2*	3526 ± 356	224 ± 1.2
8	5352 ± 96	62 ± 1	238 ± 5.4	23.8 ± 0.5	7.7 ± 0.1*	3626 ± 546	221 ± 2
12	5378 ± 175	62 ± 2	240 ± 6.5	24.0 ± 0.7	7.7 ± 0.3*	3896 ± 698	220 ± 1.2

Q_{24} cumulative amount of nicardipine hydrochloride after 24 h; J = permeability flux; K_p = permeability coefficient; ER: enhancement ratio of nicardipine hydrochloride; DCS: drug content in skin after 24 h

[a]: mean ± s.d, n = 3

*: significant at $P < 0.001$ when compared to control (0% w/w)

in proportion to the concentration of limonene at the end of 24 h of the *in vitro* permeability study (Table 1). With increased concentrations of limonene in HPC gel, there has been an increase in the skin content of the drug that might have resulted in the increased permeability of the drug. The solubility studies (Table 1) showed that limonene had no influence on the solubility of nicardipine hydrochloride indicating that the penetration enhancer did not increase permeability by affecting the solubility of the drug.

It was reported that terpenes increase the drug percutaneous permeation mainly by disrupting the intercellular packing of the SC lipids [20, 24, 31]. D-Limonene enhanced the percutaneous absorption of ketoprofen from acrylic gel patches [41]. The action of D-limonene on stratum corneum was linked to a disruption of lipid packing within the bilayers and/or to a disturbance in the stacking of the bilayer [37]. Menthol and cineole enhanced the permeability of indomethacin through the skin [42]. Terpenes in combination with ethanol increased the permeability of a hydrophilic drug, 5-fluorouracil, through porcine epidermis [43]. Hence, DSC and FT-IR studies were carried out to confirm such a hypothesis on the observed penetration enhancing effect of limonene on the permeability of nicardipine hydrochloride through rat epidermis from HPC gel.

The FT-IR study provides an insight into the effect of limonene on the biophysical properties of the rat stratum corneum [23, 24, 32]. The extraction of SC lipids by chloroform-methanol solvent system led to a dramatic decrease in the intensity (>95%) for the C−H stretching peaks, and increased the SC permeability by several orders of magnitude compared to that of untreated SC. There are several other reports on the use of FT-IR study to measure the changes in SC lipids with different solvent systems and penetration enhancers [23, 24, 32]. The treated stratum corneum of the rat was vacuum dried, and was stored in desiccator for FT-IR study [33, 34, 44]. This resulted in the evaporation of the limonene and ethanol, and allowed studying the changes in the C−H stretching absorbance caused by limonene. This also allowed us to eliminate interference in C−H stretching absorbance caused by enhancers. Table 2 shows the peak heights under the asymmetric and symmetric C−H stretching. The lipid extraction resulting from the terpene treatment (1% w/w to 12% w/w limonene) was evaluated by comparing the intensities of the asymmetric and symmetric C−H stretching absorbances after treatment with penetration enhancer to those corresponding peaks with ethanol-water (70 : 30 v/v) treatment (control). Also, the influence of solvent system (70 : 30 v/v) on the SC lipid extraction was assessed by comparing the percent decrease in the peak intensities with that of stratum corneum alone (water treated).

The results of the FT-IR showed that the treatment of stratum corneum with different concentrations of limonene in ethanol-water (70 : 30 v/v) solvent system did not produce blue shift in the asymmetric and symmetric C−H stretching peak positions. However, they all showed a decrease in absorbance intensities for both asymmetric and symmetric C−H stretching absorbances in comparison to the stratum corneum treated with ethanol-water (70 : 30 v/v) solvent system. Ethanol-water solvent system (70 : 30 v/v) decreased peak heights for asymmetric and symmetric C−H stretching absorbances by 22.3% and 23.9% respectively, in comparison with water-treated stratum corneum (Table 2). Limonene at a concentration of 4% w/w produced a greater decrease in peak heights for C−H stretching absorbances in comparison with the stratum corneum treated with a concentration of 1% w/w or 2% w/w. The decrease in peak height may be because of the extraction of the stratum corneum lipids [45]. There was no significant effect on the stratum corneum on treatment with higher concentration of limonene (8% w/w or 12% w/w) when compared to 4% w/w of limonene. Extraction of the lipids of stratum corneum leads to the enhanced percutaneous absorption of drugs [46]. Our findings suggest that extraction of the stratum corneum lipids by limonene/ethanol-water led to an increase in the permeability of nicardipine hydrochloride. The increase in permeability may be predominantly due to increased solute diffusivity in the partially delipidized stratum corneum [47]. As expected the partially delipidized stratum corneum was highly permeable to the non-polar drug, nicardipine hydrochloride, used in this study.

In order to obtain more supporting information of lipid components of the stratum corneum treated with different concentrations of limonene (0% w/w−12% w/w), a DSC study was carried out. The DSC study is useful for characterizing the phase transition of the lipid bilayers [48]. Endothermic peaks corresponding to the phase transition of constituent lipids were observed at 58.6 °C, 70.6 °C and 86.2 °C in both 70% v/v ethanol-treated (vehicle) and water-treated stratum corneum. However, there was broadening of the peaks of the stratum corneum treated with 4% w/w, 8% w/w or 12% w/w limonene. The results of the study indicated that treatment with limonene (4% w/w to 12% w/w) showed a pronounced effect on the extrac-

Table 2: Mean (± s.d.) peak height of asymmetric and symmetric C−H stretching absorbances of rat stratum corneum lipids (n = 3)

Stratum corneum treated with[##]	Asymmetric C−H stretching		Symmetric C−H stretching	
	Peak height	Decrease in peak height[a] (%)	Peak height	Decrease in peak height[a] (%)
Water	0.32 ± 0.01	–	0.25 ± 0.01	–
70% v/v Ethanol[*]	0.25 ± 0.01	21.80 ± 0.008	0.19 ± 0.00	24.00 ± 0.008
1% Limonene/70% v/v ethanol[#]	0.20 ± 0.01	20.00 ± 0.009	0.17 ± 0.01	10.53 ± 0.008
2% Limonene/70% v/v ethanol[#]	0.12 ± 0.01	52.00 ± 0.006	0.14 ± 0.01	26.32 ± 0.005
4% Limonene/70% v/v ethanol[#]	0.07 ± 0.01	72.00 ± 0.008	0.06 ± 0.01	68.42 ± 0.006
8% Limonene/70% v/v ethanol[#]	0.07 ± 0.01	72.00 ± 0.006	0.06 ± 0.01	68.42 ± 0.009
12% Limonene/70% v/v ethanol[#]	0.07 ± 0.01	72.00 ± 0.008	0.06 ± 0.01	68.42 ± 0.008

[a]: % decrease in peak height = (peak height from ethanol-water treated stratum corneum − peak height from enhancer treated stratum corneum)/peak height from ethanol-water treated stratum corneum × 100

[*]: significant ($P < 0.001$) when compared with water treated stratum corneum

[#]: significant ($P < 0.001$) when compared with water treated stratum corneum

tion of lipids in the stratum corneum. Ethanol (70% v/v), the vehicle, also resulted in the extraction of lipids in the stratum corneum in comparison with the water-treated stratum corneum indicating an additional effect of limonene beyond ethanol (70% v/v) alone. The extraction of lipids out of the stratum corneum might have enhanced percutaneous absorption of nicardipine hydrochloride as was evidenced by increased flux (Table 2). The observed lag period in the permeation may be due to the delay in the action of solvent and/or limonene on the stratum corneum lipids.

In earlier studies, Krishnaiah et al. [49, 50] reported that the maximum flux of nicardipine hydrochloride was obtained with 8% w/w of menthol (227 ± 1 μg/cm^2/h) or 12% w/w of carvone (243 ± 1 μg/cm^2/h) that was incorporated in HPC gels as penetration enhancer. Menthol lipophilicity is denoted by log P 2.02 ± 0.15) and carvone lipophilicity [22] is denoted by log P 2.23 ± 0.25. But in the present study, the maximum flux of nicardipine hydrochloride (246 ± 1 μg/cm^2/h) was obtained with a lower quantity of the penetration enhancer (4% w/w of limonene). This indicates that limonene appears to be a better penetration enhancer than menthol or carvone, which were required at higher quantity. The results suggest that a hydrocarbon terpene such as limonene (lipophilicity indicated by log P is 4.53 ± 0.23) is more active towards lipophilic drugs (for example, nicardipine hydrochloride in the present study) where as hydrophilic terpenes such as menthol and carvone are more effective in enhancing the permeation of hydrophilic drugs [22]. This is in accordance with the report that terpenes with relatively high lipophilic index values provide significant penetration enhancing activity [51]. Limonene was not completely soluble at 4% w/w concentration in the gel as compared to hydrophilic terpenes such as carvone and menthol that were completely soluble even at 8% w/w concentration in 70% v/v of ethanol [49, 50]. The effect of enhancers on the permeation of a drug usually depends on the physicochemical characteristics of both the drug (permeant) and the enhancer [21].

The enhanced permeability flux of nicardipine hydrochloride by limonene at 4% w/w level through rat epidermis, observed in this study, may give a useful selection of relatively safe penetration enhancer to aid transdermal drug delivery.

3. Experimental

3.1. Materials

Nicardipine hydrochloride and limonene were obtained from M/s ICN Biomedicals, USA and Merck-Schuchardt, Germany, respectively. Hydroxy propyl cellulose (HPC) was a gift sample from M/s. Dow Chemical Company, USA and was of pharmacopoeial quality. Acetonitrile (HPLC grade) was obtained from M/s. Qualigens Fine Chemicals, Mumbai, India. Triple distilled (TD) water was used. Other materials used in the study include ethanol (analytical grade) and potassium dihydrogen phosphate (analytical grade) were obtained from M/s. Qualigens Fine Chemicals, Mumbai, India.

3.2. HPLC analysis

The quantitative determination of nicardipine hydrochloride was performed by HPLC. A gradient HPLC (Shimadzu HPLC Class VP series) with two LC-10AT VP pumps, variable wave length programmable UV/VIS Detector SPD-10A VP, CTO-10AS VP Column oven (Shimadzu), SCL-10A VP system controller (Shimadzu), a disposable guard column LC-18 (Pelliguard™, LC-18, 2 cm, Supelco, Inc., Bellefonte, PA.) and RP C-18 column (150 mm × 4.6 mm I.D., particle size 5 μm; Flexit Inc., Pune, India) 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 acetonitrile and 0.02M KH_2PO_4 in the ratio of 60 : 40. The mobile phase components were filtered (0.45 μm P.T.F.E. membrane) and pumped at a flow rate of 1 ml/min. The column temperature was maintained at 40 °C. The eluent was detected by UV detector at 239 nm and the data were acquired, stored and analyzed with the software "Class-VP series version 5.03 (Shimadzu)". A standard curve was constructed for nicardipine hydrochloride in the range of 0.01 to 2 μg/ml. A good linear relationship was observed between the concentration of nicardipine hydrochloride and the peak area of nicardipine hydrochloride with a high correlation coefficient ($r = 0.9999$). The required studies were carried out to estimate the precision and accuracy of this HPLC method of analysis of nicardipine hydrochloride. The standard curve constructed as described above was used for estimating nicardipine hydrochloride in the solution samples of solubility studies, skin permeates, drug content in the skin after 24 h of *in vitro* permeability study or in HPC gel formulations.

3.3. Solubility studies

Excess nicardipine hydrochloride was added to 10 ml of 70% v/v of ethanol: water solvent system containing selected concentrations of limonene (1% w/w, 2% w/w, 4% w/w, 8% w/w or 12% w/w) and vortexed. The test tubes containing the mixture was immersed in a water bath at 37 °C and allowed to equilibrate with intermittent shaking. The samples (0.5 ml) were obtained as function of time (12 h, 24 h and 36 h) and filtered through 0.4-μm membrane filter, the filtrate was suitably diluted and the concentration of nicardipine hydrochloride was estimated by HPLC method.

3.4. Preparation of HPC gels

The composition of the HPC gel formulation is shown in Table 3. To prepare 2% w/w hydroxy propyl cellulose gel, HPC powder was added to 70% v/v ethanol while being stirred by means of a stirrer (M/s. Remi Motors, India) at 2500 rpm, and the resultant mixture was mixed continuously at 37 °C for about 1 h until gel formation. Nicardipine hydrochloride (1% w/w) and limonene (1% w/w, 2% w/w, 4% w/w, 8% w/w or 12% w/w) were added to HPC gel, and mixed well for complete dissolution. The gel formulations were left overnight at ambient temperature.

3.5. Quantitative determination of nicardipine hydrochloride in HPC gel formulations

One gram of the drug reservoir (HPC 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 0.45-μm membrane filter and injected into the HPLC system. The amount of nicardipine hydrochloride was estimated using the standard curve as described above.

3.6. Preparation of rat abdominal skin

The animals used for the preparation of skin were male albino rats (150–200 g) 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 the institutional guidelines. The rats were euthanised 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 the heat separation technique [23], which involved soaking of the entire abdominal skin in water at 60 °C for 45 s, followed by careful removal of the epidermis. The epidermis was washed with water, and used for the *in vitro* permeability studies.

3.7. In vitro permeability studies

Modified Keshary-Chien diffusion cells [49] were used in the *in vitro* permeation studies. The rat epidermis, prepared as described above, was mounted between the compartments of the diffusion cell with stratum corneum facing the donor compartment. The effective diffusional area was 3.5 cm^2 and the volume of the receiver compartment was 24 ml. Three grams of HPC gel, without or with limonene (1% w/w to 12% w/w) containing 30 mg of nicardipine hydrochloride, was placed in the donor cell. Ethanol (70% v/v) was added to the receiver cell to maintain sink condi-

Table 3: Composition of HPC gel formulation containing nicardipine hydrochloride and selected concentrations of limonene

Ingredients	Quantity present in gel formulation (%w/w)					
	I	II	III	IV	V	VI
Nicardipine hydrochloride	1	1	1	1	1	1
Limonene	0	1	2	4	8	12
HPC	2	2	2	2	2	2
Ethanol 70% v/v q.s.	100	100	100	100	100	100

tions. The cells were maintained at 37 ± 0.5 °C by placing on a magnetic stirrer with heater (M/s. Remi Motors, India). The contents in the receiver compartment were stirred with the help of a magnetic bar rotating at 500 rpm. The permeate samples were withdrawn from the receiver compartment at different time intervals up to 24 h, and an equivalent volume of drug-free solvent (vehicle) was added to the receiver compartment to maintain a constant volume. The samples were assayed for nicardipine hydrochloride by HPLC method as described above. After 24 h of the study, the skin sample was removed from the cells, washed briefly in methanol (25 ml) for 15 s [13, 14] to remove the adhering HPC gel drug reservoir. Following drying at room temperature for 10 min, the skin was cut into pieces and then homogenized in 4 ml methanol. The samples were centrifuged, the supernatant layer was filtered through 0.2-μm membrane filter and analyzed for the drug content by HPLC method.
The stability of nicardipine hydrochloride in ethanol and water (70 : 30 v/v) solvent system, HPC gel formulations containing limonene (1% w/w, 2% w/w, 5% w/w, 8% w/w or 12% w/w) was assessed by HPLC. The chromatogram was observed for additional peaks, if any.

3.8. Preparation of rat stratum corneum

The rat epidermis was incubated for 4 h with 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 transparent stratum corneum obtained was floated briefly on water, blotted dry and used in DSC [52] and FT-IR studies [53].

3.9. Fourier transform infrared (FT-IR) spectroscopy

A typical FT-IR spectrum of rat stratum corneum shows separate lipid and protein peaks. The study of lipid biophysics by observing the peaks caused by C−H stretching vibrations would be helpful in identifying the influence of the penetration enhancer proposed in the study. The absorbances of stratum corneum lipids occur near 2851 and 2920 cm^{-1} for the symmetric and asymmetric C−H stretching vibration respectively. The change in the amount of stratum corneum lipids on treatment with penetration enhancer has been correlated with C−H stretching absorbance intensity [53].
The stratum corneum lipid extraction leads to a decrease in the C−H stretching absorbance intensity. The evidence for the assignment of the C−H stretching peaks comes from the study carried out by Casal and Mantsch [54]. In the present study, the rat stratum corneum obtained by procedure described above, was treated with selected concentrations of limonene (1% w/w−12% w/w) in 70%v/v ethanol for 24 h. The treated stratum corneum samples were vacuum-dried (650 mm of Hg) at 21 ± 1 °C for two days and stored in a dessiccator to remove the traces of the solvent [44]. The completely dried samples of the stratum corneum were then subjected to FT-IR (Shimadzu, Japan). The attention was focussed on characterizing the occurrence of peaks near 2851 cm^{-1} and 2920 cm^{-1} for symmetric and asymmetric C−H stretching absorbencies respectively. The FT-IR experiments were performed in triplicate.

3.10. Differential scanning calorimetric study of stratum corneum

The rat stratum corneum was treated with different concentrations of limonene (1% w/w to 12% w/w) in 70%v/v ethanol for 24 h. The treated stratum corneum samples were vacuum-dried (650 mm Hg) at 21 ± 1 °C for two days and stored in dessiccator to remove the traces of the solvent [44]. The change in the structure of rat stratum corneum was assessed by Differential Scanning Calorimetry (DSC 220C, Seiko Instruments, Inc. Japan) in terms of phase transition temperature of its lipid component [53]. The samples were scanned at 1 °C/min over the temperature range of 30–110 °C.

3.11. Permeation data analysis and statistics

The nicardipine hydrochloride concentration in the skin permeate samples was corrected for sampling effects according to the equation described by Hayton and Chen [55]:

$$C^1{}_n = C_n\,(V_T/V_T - V_S)\,(C^1{}_{n-1}/\,C_{n-1}) \quad (1)$$

where $C^1{}_n$ is the corrected concentration of the n^{th} sample, C_n is the measured concentration of nicardipine hydrochloride in the n^{th} sample, C_{n-1} is the measured concentration of the nicardipine hydrochloride in the $(n-1)^{th}$ sample, V_T is the total volume of the receiver fluid, and V_S is the volume of the sample drawn.
The flux (μg/cm^2/h) of nicardipine hydrochloride (J) was calculated from the slope of the plot of the cumulative amount of nicardipine hydrochloride permeated per cm^2 of skin at steady state against the time using linear regression analysis [55, 56]. The steady state permeability coefficient (K_p) of the drug through rat epidermis was calculated using the following equation [58]:

$$K_p = J/C \quad (2)$$

where 'J' is the flux and 'C' is the concentration of nicardipine hydrochloride in the gel. The penetration enhancing effect of limonene was calculated in terms of enhancement ratio (ER), and calculated by using the following equation [20]:

$$ER = K_p \text{ with penetration enhancer}/K_p \text{ without penetration enhancer} \quad (3)$$

Statistical comparisons were made using analysis of variance (ANOVA) and Duncan's multiple range test with the help of STATISTICA program (Release 4.5, statSoft Inc., 1993). A value of $P < 0.05$ was considered statistically significant.

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