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In vitro percutaneous permeability enhancement of nimodipine by limonene across the excised rat abdominal skin

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The purpose of the present study was to investigate the effect of limonene on the *in vitro* permeation of nimodipine across the excised rat abdominal skin from a 2% w/w hydroxypropyl methylcellulose (HPMC) gel drug reservoir system. The HPMC gel formulations containing 1.5% w/w of nimodipine and selected concentrations of limonene (0% w/w to 8% 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 HPMC gel formulations. The flux of nimodipine across rat epidermis was markedly increased by the addition of limonene to the HPMC gels. A maximum flux of nimodipine was observed (203 ± 0.6 μg/cm$^2 \cdot$ h) with an enhancement ratio of about 5.7 when limonene was incorporated in HPMC gel at a concentration of 4% w/w. However, there was no further increase in the permeability of nimodipine beyond 4% w/w of limonene in the HPMC gel. FT-IR data indicated that limonene increased the permeability of nimodipine across the rat epidermis by partial extraction of lipids in the stratum corneum. The results suggest that limonene is useful for enhancing the skin permeability of nimodipine from transdermal therapeutic systems containing HPMC gel as a reservoir.

1. Introduction

In the development of transdermal therapeutic systems the skin permeation is a key factor because of the barrier properties of the stratum corneum (Hadgraft et al. 1989). Many studies have shown that the lipid domain, the integral component of the skin barrier, must be breached if the drug is to be delivered transdermally at an appropriate rate (Gao and Singh 1998). Considerable research has been carried out on increasing the permeability of drugs through the stratum corneum. The most promising technique is the use of percutaneous penetration enhancers that allow drug permeation through the skin at an appropriate rate for a suitable time. Terpenes are receiving much attention as penetration enhancers. They were reported to have high percutaneous enhancement abilities and low cutaneous irritancy (Okabe et al. 1990; Obata et al. 1991, 1993). Moreover, varieties of terpenes have been shown to increase the percutaneous absorption of a number of drugs (Gao and Singh 1998; Krishnaiah et al. 2003, 2004; Hanif et al. 1998; Okabe et al. 1989), and thus could be used as penetration enhancers for increasing the permeability of nimodipine.

Because of its short elimination half-life (1–2 h), nimodipine has to be given frequently (30 mg three to four times daily). Thus, the conventional therapy with tablets, capsules or oral liquids may result in higher fluctuation in plasma concentration of the drug resulting in unwanted side effects. Hence, the development of a transdermal therapeutic system of nimodipine 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 that an ethanol-water solvent system, in the ratio of 60 : 40 v/v, was a suitable vehicle for the transdermal delivery of nimodipine (Krishnaiah et al. 2004). However, it was necessary to improve the permeation rate of nimodipine by a suitable chemical penetration enhancer to provide a predetermined constant transdermal delivery of the drug in humans. Hence, it was planned to investigate the percutaneous penetration enhancing effect of a terpene, limonene, on the *in vitro* percutaneous permeation of nimodipine from a 2% w/w of hydroxypropyl methylcellulose (HPMC) gel containing an ethanol-water solvent system (60 : 40 v/v) across the excised rat abdominal epidermis. Limonene, a cyclic terpene (log P = 4.58 ± 0.23), is free from toxic effects and a widely used penetration enhancer (Okabe et al. 1989; Zhao and Singh 1998). Thus, the specific aim of the study is to determine the penetration enhancing effect of limonene on the transdermal permeability of nimodipine in order to provide the required flux of the drug from the hydroxypropyl methylcellulose (HPMC) reservoir of the transdermal therapeutic system.

2. Investigations, results and discussion

Studies were carried out to design a HPMC gel drug reservoir system containing carvone as a penetration enhancer (Krishnaiah et al. 2003, 2004). Based on these studies it was planned to study the penetration enhancing effect of limonene. Hydroxypropyl methylcellulose (HPMC) was added to 60% v/v ethanol-water solvent system to impart desired rheological properties and to prevent the crystallization of nimodipine thereby to improve the stability of the drug reservoir (Raghavan et al. 2000). HPMC gels containing selected concentrations of limonene (1% w/w, 2% w/w, 4% w/w, 6% w/w or 8% w/w) were prepared (Table 1) and evaluated for drug content, stability of the drug and *in vitro* skin permeability. The HPLC method used in quantitative determination of nimodipine was found to be precise and accurate as indicated by less than 2.5% of CV (inter- and intra-day variation) and high recovery (98.9%) respectively. The stability of nimodipine either in ethanol-water (60 : 40 v/v) or in HPMC gel containing limonene was assessed by HPLC. The HPLC chromatograms showed no additional peaks without a change in the retention time of nimodipine indicating the stability of the drug.

Table 1: Composition of 2% HPMC gel formulation containing 1.5% nimodipine and selected concentrations of limonene

Ingredients	Quantity present in gel formulation (%w/w)					
	I	II	III	IV	V	VI
Limonene	0	1	2	4	6	8
Ethanol-water 60 : 40% v/v q.s.	100	100	100	100	100	100

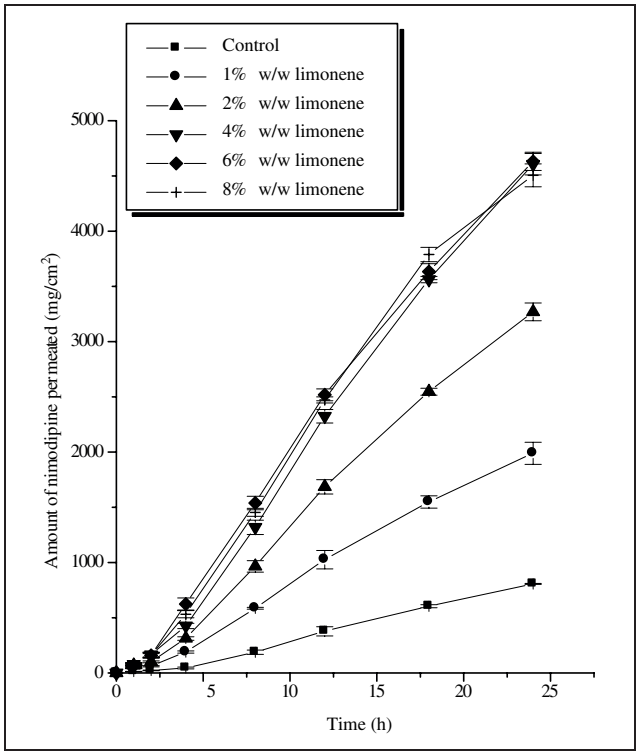

Fig. 1: Mean (± s.d.) amount of nimodipine permeated across the rat abdominal skin from 2%w/w of HPMC gel containing selected concentrations of limonene as a penetration enhancer; —■— Control; —●— 1% w/w limonene; —▲— 2% w/w limonene; —▼— 4% w/w limonene; —◆— 6% w/w limonene; –+– 8% w/w limonene

The cumulative amount of nimodipine permeated across the excised rat abdominal epidermis from HPMC gel containing selected concentrations of limonene is shown in Fig. 1. When the data were analyzed, the amount of nimodipine permeated fitted to zero order kinetics right from 2 to 24 h. The maximum amount of nimodipine permeated during the 24 h of study (Q_{24}) from the HPMC gel system without enhancer was 806 ± 4 μg/cm^2 and the corresponding flux of nimodipine was 35.5 ± 1.7 μg/cm$^2 \cdot$ h. The amount of drug permeated in 24 h study was found increased with an increase in the concentration of limonene in the drug reservoir system up to a concentration of 4% w/w. The cumulative amount (Q_{24}) of nimodipine permeated over 24 h was found increased ranging from 1998 ± 99 to 4513 ± 108 μg/cm^2 from HPMC gels containing 1% w/w to 8 % w/w of limonene. The corresponding flux values were ranging from 87 ± 2.7 to 202 ± 5 μg/cm$^2 \cdot$ h. However, there was a lag period of 2 h in the permeation of the drug through the stratum corneum. It may be observed from the results that there was a constant increase in the flux (J) of the drug upto 4% w/w of limonene in HPMC gel, and such an increase in the flux was found to be significant ($P < 0.001$) when compared to control (without limonene). But with the incorporation of either 6% w/w or 8% w/w of limonene, the increase in the flux of nimodipine was insignificant ($P > 0.05$) when compared to that obtained with 4% w/w of limonene (Table 2). As the limonene concentration increased from 1% w/w to 4% w/w, the flux of nimodipine was found increased (Figure 1) as indicated by an increase in both the permeability coefficient and enhancement ratio. The results of the study indicate that limonene, at a concentration more than 4% w/w, has a constant effect on the permeability of nimodipine across the rat abdominal skin.

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 nimodipine in various studies was $98 \pm 1\%$ indicating a mass balance of the drug. It is interesting to note that limonene increased the skin content of nimodipine significantly ($P < 0.001$) in proportion to the concentration of limonene in HPMC gel at the end of 24 h of the study. The increased drug content in rat's skin upto 4% w/w of limonene in the drug reservoir indicates that the drug might occupy the lipid bilayers of the skin thereby increasing the transdermal permeability of nimodipine (Williams and Barry 1991; Cornwell et al. 1994). Also there was no further increase in the drug content of the rat's skin beyond 4% w/w of limonene in the HPMC gel drug reservoir. The solubility studies showed that limonene did not increase the solubility of nimodipine.

It is possible that limonene might increase the drug permeation by partitioning the drug into the stratum corneum lipids. In this study limonene was completely soluble in the solvent system. The higher enhancement activity of 4% w/w limonene (hydrocarbon terpenes with a log P value of 4.52) can be attributed to their higher thermodynamic activity in the gel. Similar findings were reported by Obata et al. (1993) who demonstrated that D-limonene had higher enhancement activity for diclofenac at 1% concentration compared to L-menthol at the same concentration in 40% ethanol-buffer solution. The solubility of D-limonene in 40% ethanol-buffer was lower than that of L-menthol, resulting in a higher D-limonene thermodynamic activity. The high thermodynamic activity of limonene might have promoted the interfacial transfer of nimodipine

Table 2: Effect of limonene on the percutaneous permeation of nimodipine from HPMC gels

Concentration of limonene (% w/w)	Q_{24} ($\mu g/cm^2$)[a]	J ($\mu g/cm^2 \cdot h$)[a]	k_p ($cm/h \times 10^3$)[a]	ER[a]	DCS ($\mu g/g$)[a]	Solubility at 37 °C (mg/ml)[a]
0	806 ± 4	35.5 ± 1.7	2.4 ± 0.06	1	298.5 ± 2.8	8.2 ± 1.00
1	1998 ± 99	87.3 ± 2.7*	5.8 ± 0.09*	2.5 ± 0.03*	898.5 ± 2.8*	8.8 ± 1.6
2	3269 ± 11	143.5 ± 4.4*	9.6 ± 0.11*	4.1 ± 0.01*	1167.4 ± 65.4*	8.8 ± 1.3
4	4605 ± 97	201.4 ± 3.0*	13.4 ± 0.29*	5.7 ± 0.04*	2098.4 ± 51.7*	9.1 ± 1.4
6	4631 ± 82	203.0 ± 0.6*	13.5 ± 0.06*	5.7 ± 0.01*	2189.3 ± 45.3*	9.0 ± 1.5
8	4513 ± 108	202.7 ± 5.1*	13.5 ± 0.51*	5.7 ± 0.07*	2206.8 ± 65*	8.3 ± 1.9

Q_{24}: Cumulative amount of nimodipine permeated upto 24 h; DCS: Drug content in skin after 24 h; ER: Enhancement ratio of nimodipine
[a]: Mean ± s.d (n = 3)
*: Significant at $P < 0.001$ when compared to control

by increasing its solubility into stratum corneum and thus flux of the drug.

It was reported that terpenes increase the drug percutaneous permeation mainly by disrupting the intercellular packing of the SC lipids (Williams et al. 1991; Cornwell et al. 1994). Hence, FT-IR studies were carried out to confirm such a hypothesis on the observed penetration enhancing effect of limonene, a cyclic terpene, on the permeability of nimodipine through rat epidermis from HPMC gel.

The FT-IR studies provide an insight into the effect of limonene on the biophysical properties of the rat stratum

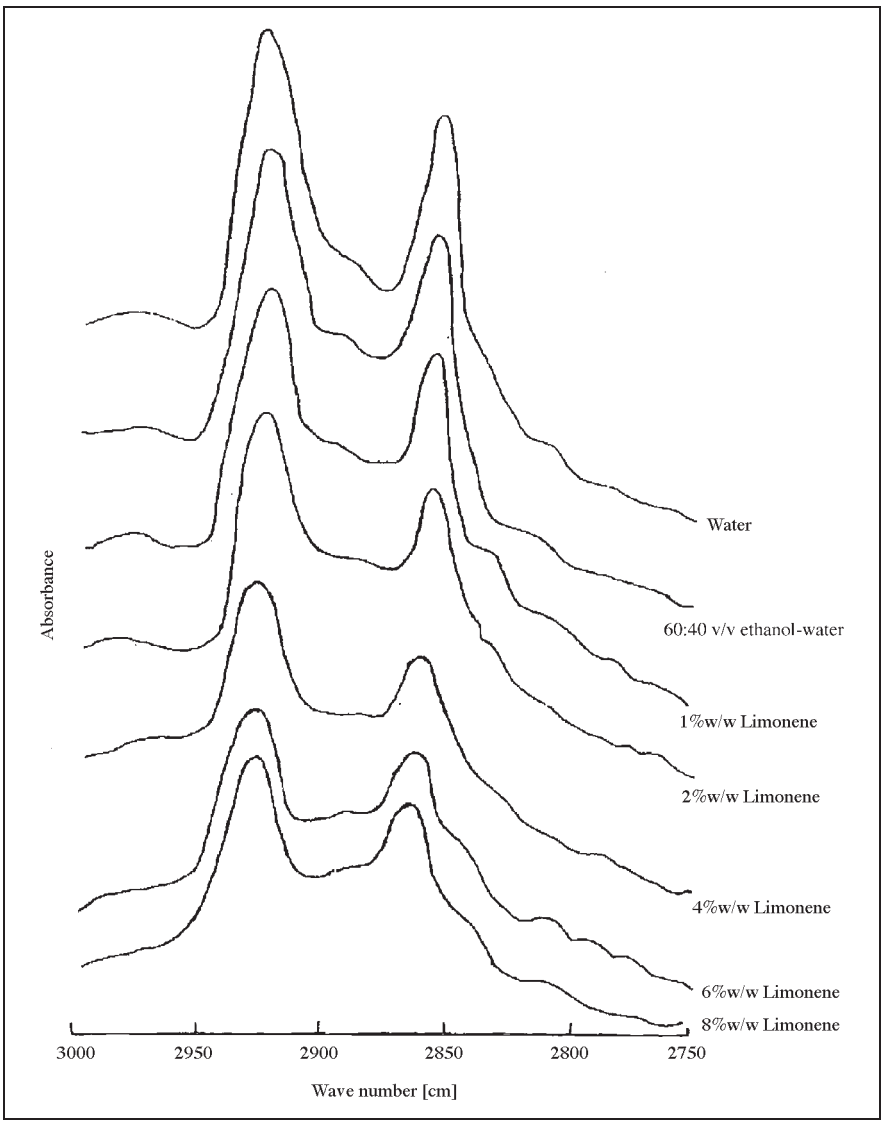

Fig. 2: FT-IR Spectra showing asymmetric and symmetric C–H stretching absorbances of rat's stratum corneum treated with various concentrations of limonene in 60 : 40 v/v ethanol-water, water and 60 : 40% v/v of ethanol

Table 3: Effect of limonene on 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	0.34 ± 0.01	–	0.24 ± 0.01	–
60%v/v ethanol	$0.26 \pm 0.01^{\#}$	$23.5 \pm 0.005^{\#}$	$0.19 \pm 0.01^{\#}$	$20.8 \pm 0.007^{\#}$
1% Limonene in 60%v/v ethanol	0.17 ± 0.01	34.6 ± 0.003*	0.14 ± 0.01	26.3 ± 0.005*
2% Limonene in 60%v/v ethanol	0.14 ± 0.01*	46.2 ± 0.004*	0.13 ± 0.01*	31.6 ± 0.005*
4% Limonene in 60%v/v ethanol	0.09 ± 0.01*	65.4 ± 0.005*	0.07 ± 0.01*	63.2 ± 0.006*
6% Limonene in 60%v/v ethanol	0.08 ± 0.01*	69.2 ± 0.005*	0.06 ± 0.01*	68.4 ± 0.006*
8% Limonene in 60%v/v ethanol	0.08 ± 0.01*	69.2 ± 0.004*	0.06 ± 0.01*	68.4 ± 0.005*

[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 X 100

#: Significant at $P < 0.001$ when compared with water-treated stratum corneum

*: Significant at $P < 0.001$ when compared with 60% v/v ethanol-treated stratum corneum

corneum (Okamoto et al. 1988; Goates et al. 1994). There are several other reports on the use of FT-IR to measure the changes in stratum corneum lipids with different solvent systems and penetration enhancers (Okamoto et al. 1988; Williams and Barry 1991). The treated stratum corneum of the rat was vacuum-dried, and stored in a desiccator for FT-IR study (Okamoto et al. 1988; Goates et al. 1994). This resulted in the evaporation of limonene and ethanol, and allowed studying the changes in the C–H stretching absorbance caused by limonene. Fig. 2 depicts IR spectra from 3000–2750 cm^{-1} of the stratum corneum pretreated with different concentrations of limonene (0% w/w to 8% w/w) in 60 : 40% v/v ethanol-water solvent system. Table 3 shows the peak heights under the asymmetric and symmetric C–H stretching absorbances of the rat stratum corneum pretreated with different concentrations of limonene in ethanol-water (60 : 40 v/v). The heights of these two peaks were proportional to the amount of lipids present the stratum corneum. The lipid extraction resulting from the terpene treatment (1% w/w to 8% w/w of limonene) was evaluated by comparing the intensities of the asymmetric and symmetric C–H stretching absorbances after treatment with limonene to those corresponding peaks with ethanol-water (60 : 40 v/v) treatment. Also, the influence of the ethanol-water solvent system (60 : 40 v/v) on stratum corneum lipid extraction was assessed by comparing the percent decrease in the peak height with that of stratum corneum alone (water treated).

The results of the FT-IR study show that treatment of the stratum corneum with selected concentrations of limonene in 60 : 40% v/v ethanol-water did not produce blue shift in the asymmetric and symmetric C–H stretching peak positions. However, they all showed significant decrease in absorbance intensities for both asymmetric and symmetric C–H stretching absorbances in comparison to that of the stratum corneum treated with a 60% v/v ethanol-water solvent system. Limonene, at a concentration of 4% w/w, produced a greater decrease in peak heights for C–H stretching absorbances. There was no significant effect on the stratum corneum with higher concentration of limonene (6% w/w or 8% w/w) when compared to that obtained with 4% w/w of limonene. The decrease in peak height may be due to extraction of the stratum corneum lipids (Goates et al. 1994). Extraction of the lipids of stratum corneum leads to an enhanced percutaneous absorption of drugs (Golden et al. 1986). Our findings in the FT-IR study suggest that the enhancement in the transdermal permeability of nimodipine by limonene in combination with 60 : 40% v/v ethanol-water may be due to extraction of the stratum corneum lipids by limonene. The increase in permeability may be predominantly due to increased solute diffusivity from the partially delipidized stratum corneum (Yum et al. 1994). As expected, the partially delipidized stratum corneum was highly permeable to the non-polar drug nimodipine. It also appears from the results of the present study that limonene increased drug permeation through improved drug partitioning into the barrier as hypothesized by Moghimi et al. (1996). This is evident from the increased drug content in the rat abdominal skin. Thus, the increased transdermal permeability of nimodipine might be due to partial extraction of stratum corneum lipids and improved portioning of the drug into the stratum corneum. It is also possible that limonene might interact with stratum corneum proteins enhancing the percutaneous permeability of nimodipine (Moghimi et al. 1996).

In an earlier study, Krishnaiah et al. (2004) reported that the maximum flux of nimodipine across the rat abdominal skin was obtained with incorporation of 10% w/w of carvone (165 ± 4.4 $\mu g/cm^2 \cdot h$) in HPMC gels as penetration enhancer. The lipophilicity of carvone (El-Kattan et al. 2000) as denoted by a log P of 2.2 ± 0.25. But in the present study, the maximum flux of nimodipine (203 $\mu g/cm^2 \cdot h$), was obtained with a lower concentration of penetration enhancer (limonene, 4% w/w). This indicates that limonene appears to be a better penetration enhancer than carvone, which was required at higher quantity. The results suggest that a hydrocarbon terpene such as limonene (lipophilicity indicated by log P of 4.5 ± 0.23) is more active towards lipophilic drugs whereas hydrophilic terpene such as carvone is more effective in enhancing the percutaneous permeation of hydrophilic drugs (El-Kattan et al. 2000). This is in accordance with the report that terpenes with relatively high lipophilic index values provide significant penetration enhancing activity (Takayama et al. 1993).

Limonene was not completely soluble at a concentration of 4% w/w in the gel as compared to carvone that was completely soluble even at 10% w/w concentration in 60% v/v of ethanol-water solvent system (Krishnaiah et al. 2004). Giannakou et al. (1998, 2002) studied the effect of a combination of cineole and caprylic acid on the transdermal permeation of nimodipine. Greater permeation rates were achieved with an increase in concentration of caprylic acid, in a drug reservoir containing cineole. It may be noted that limonene alone at a concentration of 4% w/w showed a

percutaneous penetration enhancing effect on nimodipine. The effect of chemical penetration enhancers on the percutaneous permeation of a drug usually depends upon the physicochemical characteristics of both the drug (permeant) and the enhancer (Cornwell and Barry 1994).

The enhanced flux of nimodipine by limonene at 4% w/w level through rat abdominal epidermis, observed in this study, may be useful in the selection of a relatively safe penetration enhancer to aid transdermal drug delivery. However, the permeability of nimodipine from HPMC gel containing 4% w/w of limonene as penetration enhancer through the skin/rate-controlling membrane composite needs to be studied in the development of a transdermal therapeutic system. Further studies are also required to find the influence of limonene on the permeability of nimodipine in human volunteers. Such studies were carried out and the results were promising.

3. Experimental

3.1. Materials

Nimodipine was a gift sample from M/s Micro Labs, Banglore, India. Limonene was obtained from Merck-Schuchardt, Honebourn, Germany. Hydroxypropyl methylcellulose (HPMC) was a gift sample from M/s Dr. Reddy's Laboratories Ltd., Hyderabad, India, and was of USP/NF quality. Acetonitrile and water (HPLC grade) were obtained from M/s Qualigens Fine Chemicals Ltd., Mumbai, India. Other materials used in the study such as ethanol were of analytical grade (supplied by M/s Qualigens Fine Chemicals Ltd., Mumbai, India).

3.2. Solubility studies

An excess of nimodipine was added to 10 mL of 60% v/v of ethanol-water containing selected concentrations of limonene (0% w/w, 1% w/w, 2% w/w, 4% w/w, 6% w/w or 8% w/w) and vortexed. The amber colored bottles containing the mixture were 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 a 0.45-μm membrane filter. The filtrate was suitably diluted, and subjected to HPLC analysis to estimate the solubility of nimodipine.

3.3. HPLC analysis of nimodipine

The quantitative determination of nimodipine 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 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 acetonitrile and water. The mobile phase components were filtered through a 0.45-μm membrane filter and pumped from the respective reservoirs in the ratio of 58 : 42 at a flow rate of 1 mL/min. The column temperature was maintained at 40 °C. The eluent was detected by an UV detector at 237 nm, and the data was acquired, stored and analyzed with the software Class-VP series version 5.03 (Shimadzu). A standard curve was constructed for nimodipine in the range of 0.01 to 40 μg/mL. A good linear relationship was observed between the concentration of nimodipine and the peak area of nimodipine with a high correlation coefficient ($r = 0.99999$). The studies were carried out to estimate the precision and accuracy of this HPLC method of analysis of nimodipine. The standard curve, constructed as described above, was used for estimating nimodipine in the skin permeates, HPMC gel formulations and to find drug content in the skin after 24 h of study.

3.4. Preparation of HPMC gels

The composition of HPMC gel formulations is given in Table 1. To prepare 2% w/w of hydroxypropyl methylcellulose gel, HPMC powder was added to 60% v/v of 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 for about 1 h until the formation of gel. Nimodipine (1.5% w/w) and limonene (1% w/w, 2% w/w, 4% w/w, 6% w/w or 8% w/w) were added to the HPMC gel, and mixed well for complete dissolution. The gel formulations were left overnight at ambient temperature.

3.5. Quantitative determination of nimodipine in HPMC gel formulation

One gram of the HPMC drug reservoir was accurately weighed, placed in a 100-mL volumetric flask containing 30 mL of mobile phase, stirred for 30 min and made upto volume. The resultant mixture was filtered through a 0.45-μm membrane filter and injected into the HPLC system. The amount of nimodipine 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 could have a 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 using the heat separation technique (Zhao and Singh 1999) that 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. The rat skin which was free from physical damage was used for in vitro permeation studies.

3.7. In vitro transdermal permeability studies

Modified Keshary-Chien diffusion cells (Keshary and Chien 1984) were used in the *in vitro* transdermal 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. High vacuum silicone grease was applied onto the 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 grams of HPMC gel, without or with limonene (1% w/w to 8% w/w) containing 30 mg of nimodipine was placed in the donor cell, which was covered with parafilm and aluminium foil to minimize the evaporation of the solution and degradation of the drug from light. Ethanol (60% v/v) was added to the receiver cell to maintain sink conditions. Generally as receptor solutions, aqueous phases are used for polar drugs and ethanolic phases for lipophilic drugs (e.g. nimodipine). The ethanol-water (60 : 40 v/v) co-solvent system provided solubility at a level of more than 10% of its aqueous solubility. Hence, the ethanol-water (60 : 40 v/v) co-solvent system was used in the receptor compartment to maintain sink conditions. It may be noted that 60% v/v ethanol-water is also effective against microbes preventing the possible contamination of the skin and maintains the skin integrity during 24 h. The cells were maintained at 37 ± 0.5 °C by placing on a magnetic stirrer with heater (M/s Remi Motors, Mumbai, India). The contents in the receiver compartment were stirred with the help of a magnetic bar rotating at 500 rpm. The permeate samples (0.5 mL) were withdrawn from the receiver compartments at different time intervals up to 24 h, and an equivalent volume of drug-free solvent (60%v/v ethanol-water) was added to the receiver compartment to maintain a constant volume. The samples were assayed for nimodipine by HPLC as described above. The stability of nimodipine in ethanol-water, or in HPMC gel containing limonene was assessed by HPLC. The chromatogram was observed for additional peaks, if any.

3.8. Estimation of drug content in skin samples

After 24 h of the study, the rat skin was removed from the cells and washed briefly in methanol (25 mL) for 15 s (Michniak et al. 1994, 1993) to remove the adhering HPMC gel drug reservoir. Following drying at room temperature for 10 min, the whole skin was cut into pieces and then homogenized in 10 mL of methanol. The samples were centrifuged, the supernatant layer filtered through a 0.45-μm membrane filter and analyzed for the drug content by HPLC.

3.9. 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, so obtained, was floated briefly on water, blotted dry and used in FT-IR studies (Bhatia et al. 1997).

3.10. Fourier Transform Infrared (FT-IR) spectroscopy

The absorbances of stratum corneum lipids occur near 2851 and 2920 cm^{-1} for the symmetric and asymmetric C–H stretching vibrations respectively. The change in the amount of stratum corneum lipids have been correlated with C–H stretching absorbance intensity (Yui et al. 1992). The evidence for the assignment of the C–H stretching peaks comes from the study carried out by Casal and Mantsch (1984). In the present study, the rat stratum corneum was treated with selected concentrations of limo-

nene in ethanol-water for 24 h. The treated stratum corneum samples were vacuum-dried (650 mm Hg) at $21 \pm 1\,^{\circ}C$ for two days and stored in a dessiccator to remove the traces of the solvent (Okamoto et al. 1988). The completely dried samples of the stratum corneum were then subjected to FT-IR (Shimadzu, Japan) spectroscopy. The attention was focused 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.

3.11. Permeation data analysis and statistics

The nimodipine concentration in the skin permeate samples was corrected for sampling effects according to the equation described by Hayton and Chen (1981)

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

where C_n^1 is the corrected concentration of the n^{th} sample, C_n is the measured concentration of nimodipine in the n^{th} sample, C_{n-1} is the measured concentration of the nimodipine 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 ($\mu g/cm^2 \cdot h$) of nimodipine was calculated from the slope of the plot of the cumulative amount of nimodipine permeated per cm^2 of skin at steady state against the time using linear regression analysis (Konsil et al. 1995; Ho et al. 1998). The steady state permeability coefficient (k_p) of the drug through rat epidermis was calculated using the following equation (Yamune et al. 1995):

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

where J is the flux and C is the concentration of nimodipine in the gel. The penetration enhancing effect of limonene was calculated in terms of enhancement ratio (ER), and was calculated using the following equation (Williams and Barry 1991):

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

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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