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Effects of some terpenes on the *in vitro* permeation of LHRH through newborn pig skin

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Received August 25, 2008, accepted September 5, 2008

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Pharmazie 64: 110–115 (2009) *doi: 10.1691/ph.2009.8243*

The objective of this work was to investigate the effect of oxygen containing terpenes (carvacrol, menthol and carvone) at 5%w/v in hydroalcoholic mixtures (40% ethanol) on the permeation of LHRH across newborn pig skin *in vitro*. In addition, the amount of LHRH retained in the skin after 24 h of diffusion was determined. It was found that the passive permeation of LHRH was very limited. Although percutaneous absorption of LHRH improved in the presence of the enhancers, a significant enhancement was observed only with carvacrol, an aromatic terpene. The rank order of enhancement ratio for skin permeation was found to be carvacrol > carvone > menthol. The enhancers also affected the retention of LHRH in the skin. The rank order of enhancement ratio for skin retention was carvone > carvacrol > menthol. The results of the *in vitro* skin metabolism study of LHRH using fresh newborn pig skin showed that the degradation products were detected and the amount of the degraded LHRH increased with increasing duration of incubation time.

1. Introduction

The stratum corneum, the outermost layer of the epidermis, is generally considered to be the rate-limiting barrier for the drug transportation across the skin (Barry 1983). In order to overcome this barrier property, various skin permeation techniques, utilizing both chemical and physical strategies, have been employed (Trommer and Neubert 2006). Amongst these approaches, the use of penetration enhancers which reversibly reduce the barrier of the stratum corneum is one of the most effective, popular and simple methods. A wide range of chemical substances including terpenes have been shown to promote the percutaneous absorption of permeants (Akimoto and Nagase 2003; Williams and Barry 2004; Herai et al. 2007)

Luteinizing hormone releasing hormone (LHRH), also known as gonadotropin releasing hormone (GnRH), is a potent decapeptide used for the treatment of several disorders including hypogonadism, endometriosis, female infertility and prostatic carcinoma. In mammals, the primary structure of LHRH is pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly.NH_2. It is synthesized in the cell bodies of the hypothalamic neurons and is transported by the hypothalamic-hypophyseal portal system to the anterior pituitary gland. It acts on the pituitary to selectively stimulate the gonadotrope cells to release luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH bind to receptors in both the testis and the ovary and stimulate gonadal production of sex steroids and gametogenesis, respectively (Schneider et al. 2006). LHRH is not therapeutically effective when taken orally because it undergoes proteolytic enzyme degradation in the gastro-intestinal tract, extensive first-pass hepatic metabolism, and has a short elimination half-life of 4.5 min. LHRH is hydrophilic, of large molecular weight (1182), and positively charged at physiological pH (Heit et al. 1994); thus passive transdermal delivery is very limited and enhancement strategies are essential. One strategy is the use of iontophoresis to improve the transport of LHRH across the skin. Since LHRH is positively charged at physiological pH, it can, in principle, be subjected to iontophoresis. Several studies have described details of the iontophoretic delivery of LHRH (Miller et al. 1990; Srinivasan et al. 1990; Heit et al. 1993; Raiman et al. 2004). Another approach is the use of chemical enhancers, alone or in combination with iontophoresis. For example, it has been found that pretreatment of pig epidermis by *N*-methyl 2-pyrrolidone and isopropyl myristate significantly promotes the permeability of LHRH compared with a control (Bhatia and Singh 1997). When a combination of enhancer pretreatment (oleic acid/propylene glycol) with iontophoresis is compared with iontophoresis alone or enhancer pretreatment alone the percutaneous absorption of LHRH through human epidermis is greatly enhanced (Smyth et al. 2002). In addition, the use of terpenes as penetration enhancers has been suggested. Terpenes have GRAS (generally recognized as safe) status granted by the U.S. Food and Drug Administration (FDA). In addition, terpenes appear to have high percutaneous enhancing abilities, with low skin irritancy and low systemic toxicity. As skin penetra-

tion enhancers, terpenes have been employed directly or in combination with propylene glycol or ethanol (Cornwell and Barry 1995; Moghimi et al. 1996; Vaddi et al. 2002a, b; Songkro et al. 2003). Several authors have reported that there is a synergistic effect on the transport of drugs (i.e. diclofenac sodium and tamoxifen) through the skin when terpenes are used with ethanol or propylene glycol (Obata et al. 1991; Takayama and Nagai 1994; Yamane et al. 1995; Zhao and Singh 2000). In addition, it has been reported that 5% linolenic acid or 5% limonene, a nonpolar hydrocarbon terpene, in combination with ethanol significantly enhances the passive flux of LHRH through human epidermis treated with enhancers (Bhatia and Singh 1999). A similar finding has been reported in porcine epidermis when limonene (5%) in combination with ethanol is used alone or in combination with iontophoresis (Bhatia and Singh 1998). Although several LHRH studies have focused on the use of hydrocarbon terpenes as penetration enhancers, the effect of other types of terpenes (i.e. oxygen containing polar terpenes) has not been well investigated.

In the current study, the oxygen containing polar terpenes carvacrol, menthol and carvone were selected to be used as skin penetration enhancers for LHRH transport. These enhancers were chosen because they have remarkable penetration enhancing activity for several drugs, in particular propranolol hydrochloride as shown in our previous study (Songkro et al. 2003b). Carvacrol (5% in 50% ethanol) has been shown to improve the percutaneous absorption of haloperidol across rat skin *in vitro* (Vaddi et al. 2002a). Moreover, of all the terpenes studied, carvacrol (5% in 40% ethanol) has exhibited the greatest penetration enhancement for the permeation of propranolol hydrochloride across newborn pig skin *in vitro* (Songkro et al. 2003b). Menthol has been found to accelerate the permeation of ketoprofen (Rhee et al. 2001) and ketotifen (Kimura et al. 2007). Carvone (5%) in combination with ethanol (50%) showed penetration enhancing activity for 5-fluorouracil (Gao and Singh 1997) and carvone in a mixture of ethanol : glycerin : phosphate buffer solution (60 : 10 : 30) enhanced the skin penetration of diclofenac sodium (Nokhodchi et al. 2007).

The effects of selected terpenes on the percutaneous absorption of LHRH were studied *in vitro* using a modified Franz diffusion cell. The concentration of these terpenes was set at 5% (w/v) as this concentration has been shown to give effective enhancement on the skin permeability of propranolol hydrochloride (Songkro et al. 2003b). Additionally, in an attempt to further accelerate the percutaneous absorption of LHRH, terpenes combined with ethanol (40% v/v) were used. Due to restrictions on the use of human skin, a validated skin membrane model was chosen in this study using excised skin of newborn pigs that had died of natural causes shortly after birth, as previously described (Songkro et al. 2003a).

In addition to acting as a diffusional barrier, skin is a metabolically active organ and the most metabolically active layer is found to be the viable epidermis below the stratum corneum (Tauber 1982). The metabolic processes that are catalyzed by the enzymes present in skin affects the bioavailability and the pharmacological and toxicological activity of drugs. In contrast, if there is no metabolism of the drug in the viable epidermis there will be free passage of the unchanged drug into the systemic circulation. When compared with other mucosal membranes, skin contains fewer proteolytic enzymes (Lee 1988). Therefore, first-pass metabolism of drugs absorbed through skin is reduced when compared to other membranes. Bi and Singh (1998), who studied the metabolism of LHRH in several intact pig tissues (i.e. buccal, liver, nasal and skin) and in tissue homogenates, have indicated that skin had minimal proteolytic activity towards LHRH, particularly when the skin is intact. Furthermore, the same authors (Bi and Singh 2000) have found that the degradation of LHRH in the skin can be further minimized by the use of suitable proteolytic enzyme-inhibitors such as bestatin.

In addition to overcoming the diffusion barrier, the stability of LHRH in skin needs to be considered. For this reason, the potential for metabolic deactivation of LHRH by the skin was also evaluated by exposing the LHRH solution to freshly excised newborn pig skin.

2. Investigations, results and discussion

The amount of LHRH that permeated through newborn pig skin was expressed as the cumulative amount permeated per unit area. The plots of cumulative permeation of LHRH versus time for each terpene are shown in Fig. 1. The permeation parameters of LHRH are summarized in the Table.

As seen from Fig. 1, passive transport of LHRH through newborn pig skin was extremely low when no enhancer was used (control). This is partly due to the large molecular weight of the compound (MW 1182). In the control, the amount of LHRH that penetrated through newborn pig skin in the first 10 h could not be detected by HPLC analysis. For the first 4 h the permeation of LHRH was slightly enhanced by the terpene enhancers when compared with the control. From 8–24 h, the cumulative amount of penetrated drug was greatest with carvacrol ($p < 0.5$). According to the permeation parameters shown in the Table, the three terpenes showed a tendency to increase the permeation of LHRH across the skin; however, only carvacrol significantly enhanced the penetration of LHRH compared to the control ($p < 0.05$). Menthol and carvone, which were effective for transdermal delivery of

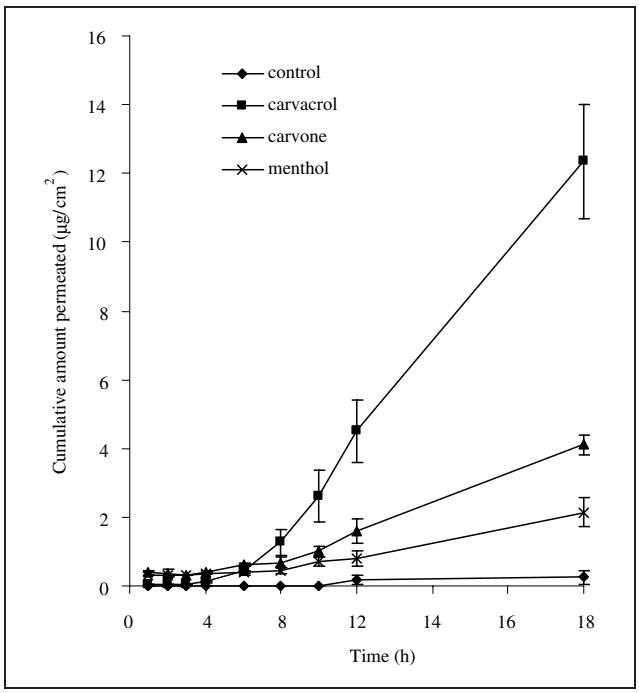

Fig. 1: Permeation profiles of LHRH through full-thickness newborn pig skin over 24 h. Each data point is the mean ± SEM ($3 \leq n \leq 4$)

Table: Effect of terpenes on the permeation parameters of LHRH through newborn pig skin

Enhancer	n	J_{SS} (μg/cm²/h) (mean ± SEM)	P ($\times 10^{-4}$) (cm/h) (mean ± SEM)	ER_P	Q_{24} (μg/cm²) (mean ± SEM)	SC_{24} (μg/cm²) (mean ± SEM)	ER_{SC}
None (control)	4	0.0072 ± 0.0082	0.14 ± 0.16	1.0	0.25 ± 0.15	5.37 ± 0.45	1.0
Menthol	3	0.0904 ± 0.0179	1.81 ± 0.36	12.9	2.14 ± 0.41	12.69 ±1.46	2.4
Carvone	3	0.2116 ± 0.0066	4.23 ± 0.13	30.2	4.12 ± 0.28	24.28 ± 4.03*	4.5
Carvacrol	4	7.8730 ± 0.6325*	15.75 ± 1.26	112.5	12.34 ± 1.68	15.14 ± 0.47*	2.8

5% terpene in 40% ethanol
n = number of determinations
Q_{24}, cumulative amount of LHRH permeated in receptor fluid at 24 h
SC_{24}, skin content of LHRH at 24 h
ER, enhancement ratio calculated as permeation parameter after enhancer treatment divided by the corresponding parameter from control (control = 1.0)
* There were significant differences in the skin parameters (J_{SS} or SC_{24}) ($p < 0.05$, ANOVA)

propranolol hydrochloride (Songkro et al. 2003b), did not significantly promote the permeation of the decapeptide. The ER_p of carvacrol was 112.5, whereas the ER_p of carvone and menthol were about 30 and 13, respectively. Based on the chemical structures of the terpenes, it is reasonable to assume that the remarkable enhancing activity of carvacrol can be attributed to its phenolic nature rather than to the presence of a hydroxyl group. The effects of a phenolic structure on the penetration enhancing ability of terpenes have been reported previously (Kunta et al. 1997; Songkro et al. 2003b).

The accumulation of LHRH in skin was significantly increased with carvone and carvacrol when compared to control ($p < 0.05$) (Table). Overall, carvone showed the greatest enhancing activity in skin retention of LHRH ($ER_{sc} = 4.5$).

Based on the LHRH data and the data from propranolol hydrochloride in our previous investigation (Songkro et al. 2003b), it was noted that carvone, the enhancer with moderate penetration enhancing activity, was likely to retain the permeants in the skin better than carvacrol, the enhancer with greatest penetration enhancing activity.

It has been suggested that the retention of permeants in the skin is related to the log K (octanol/water) values; the higher the log K values attain, the greater the skin retention of permeants (Potard et al. 2000). With respect to the log K values, it was surprising that the accumulation of LHRH in skin by terpenes was in the order of carvone > carvacrol > menthol, even though the calculated log K of terpenes was in the order of carvacrol (3.23) > menthol (3.02) > carvone (2.83). This was, at least in part, due to the fact that octanol cannot precisely mimic the heterogeneous structure of the stratum corneum. Owing to the polar nature of LHRH, hydrogen bonding between LHRH and each terpene or skin components (i.e. keratin, ceramides) is likely to occur. In this case, it is likely that the strongest hydrogen bonding occurs between carvone and the drug thus producing a much higher amount of drug retained within the skin.

In view of the low skin permeation of LHRH in the current investigation, further studies using these terpenes with physical enhancement methods such as iontophoresis are suggested.

The ratio of cumulative amount permeated into receptor fluid at 24 h was divided by the skin content of LHRH at 24 h and plotted as shown in Fig. 2. The highest ratio was obtained with carvacrol followed by carvone and menthol. This ratio may indicate whether an enhancer is suitable for transdermal drug delivery or local drug targeting in skin. In this case, the ratio between these two parameters was less than 1, suggesting that these terpenes enhanced accumulation of the drug within the skin rather than enhancing permeation of the drug through the skin into the receptor fluid. Nevertheless, it may be concluded that carvacrol was more effective in enhancing the permeation of LHRH through the skin than carvone or menthol.

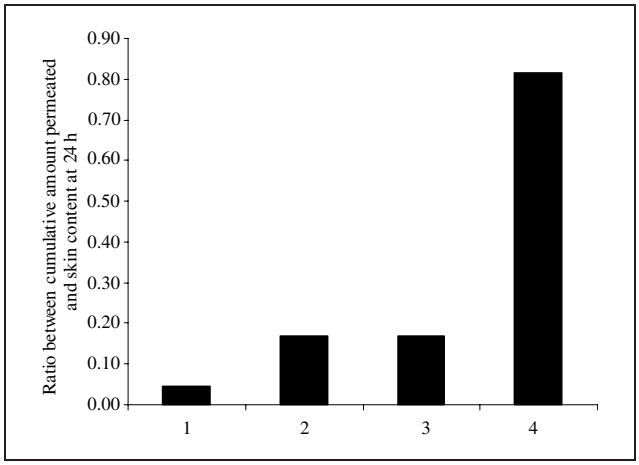

Fig. 2: Ratio between cumulative amount permeated in receptor fluid and skin content of LHRH at 24 h. Key: 1 control; 2 menthol; 3 carvone; 4 carvacrol

During the *in vitro* skin permeation experiments, degradation products of LHRH were detected after 12 h of permeation. The amount of degradation products found in the skin extracts and 50% methanol washes was very low (less than 5%). However, higher percentages of metabolites were observed in the receptor fluid, for example, 15% of degraded LHRH was detected at 24 h when carvacrol was used as enhancer.

The percutaneous parameters used to determine the correlation between skin penetration and skin accumulation of LHRH are shown in Figs. 3 and 4. As seen from Fig. 3, no correlation existed between the permeability coefficient and the skin accumulation of LHRH ($R^2 = 0.0909$). Similarly, there was no correlation between the amount of LHRH in the receptor fluid at 24 h and the skin accumulation of LHRH ($R^2 = 0.1272$) (Fig. 4). Lack of correlation between skin permeation and retention has been reported previously (Kumar et al. 1992; Songkro et al. 2003b).

As seen from Fig. 5, no degradation products were found in the control (fresh skin incubated in IPB at 32 °C). However, there were degradation products of LHRH after it was incubated with excised fresh skin and IPB. In the current experiment, two peaks of degradation products were found at the retention time of 4.9 and 8.2 min. The amount of degraded LHRH increased with the length of the incubation period whilst the amount of intact LHRH decreased with increasing incubation time. After 2 h, only 5% degradation products were detected. By 12 h, the amount of degraded LHRH had increased to 90%.

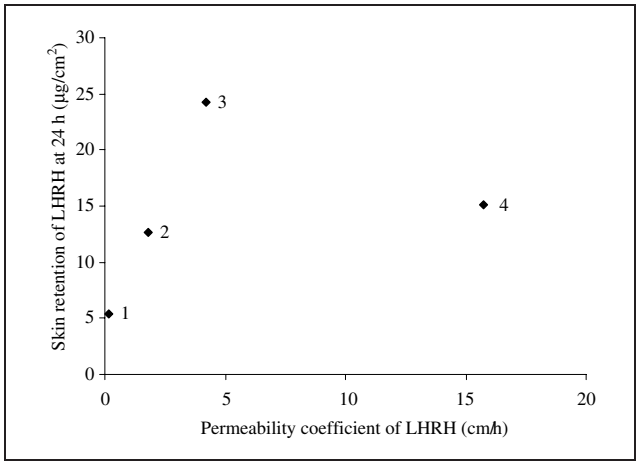

Fig. 3: Relationship between permeability coefficient of LHRH and its skin retention at 24 h. Key: 1 control; 2 menthol; 3 carvone; 4 carvacrol

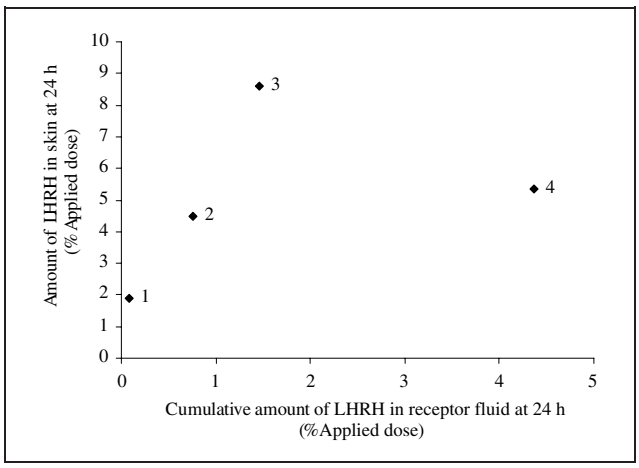

Fig. 4: Relationship between amount of LHRH in receptor fluid and in skin at 24 h. Key: 1 control; 2 menthol; 3 carvone; 4 carvacrol

Interestingly, the amount of degraded LHRH occurring in the skin metabolism study was much greater than that in the skin permeation experiments. This was possibly due to the fact that in the cutaneous metabolism study, the LHRH solution was in direct contact with viable layers of skin whilst in the permeation experiment, the large molecular weight of LHRH limited its transport across skin and therefore the majority of LHRH remained in contact with the stratum corneum (nonviable layer). In addition, the higher amount of degradation products of LHRH in receptor fluid, which was in direct contact with the dermal side, indicated that the degradation was in part due to the dermis. This is in accord with the study by Miller et al. (1990) who found that solutions of LHRH (Sörensen's phosphate buffer) contacting the stratum corneum of hairless mouse skin were stable over 40 h while solutions contacting the dermal side were unstable. The authors concluded that LHRH was metabolized when in contact with the dermal side of hairless mouse skin.

Since the skin is metabolically active and LHRH has a short *in vivo* half-life, it is not surprising that skin enzymes could cause the degradation of LHRH. Thus, for LHRH delivery via a transdermal route, the metabolic degradation by the enzymes present in the skin needs to be taken into account.

In conclusions, carvacrol was the most suitable penetration enhancer for percutaneous absorption of LHRH since the permeation of LHRH was significantly enhanced by carvacrol. In addition, degraded LHRH was observed during the skin permeation experiment. Carvone was able to retain LHRH in the skin more than carvacrol or menthol. In both permeation and retention studies, menthol showed the least effects for LHRH. No correlation could be found between skin permeation and skin retention of LHRH. The skin metabolism study revealed that enzymes present in the viable layers of skin could metabolize the decapeptide LHRH.

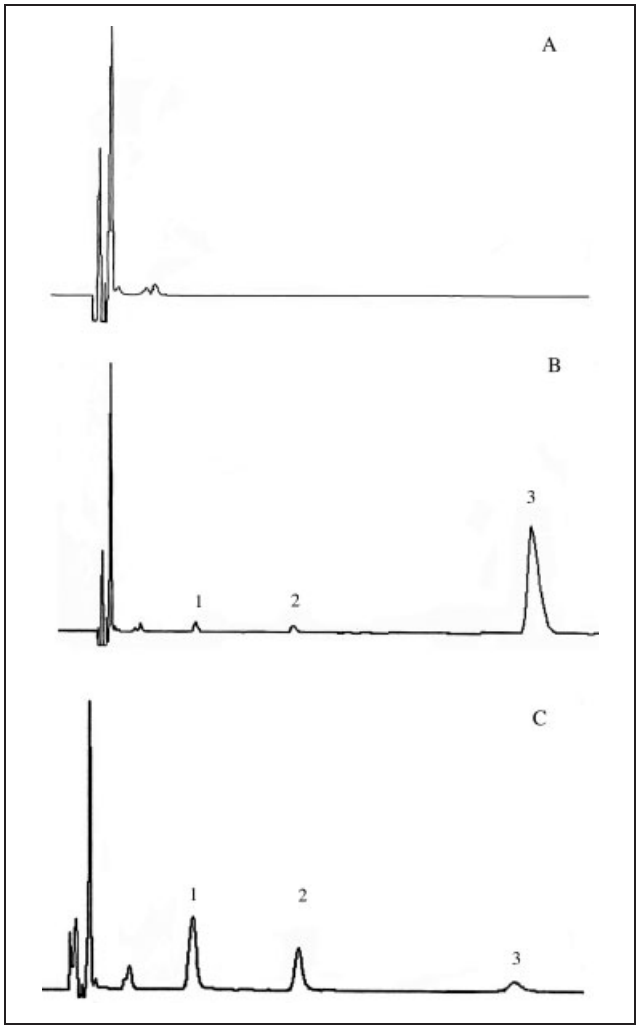

Fig. 5: HPLC chromatograms of LHRH incubated with fresh full thickness newborn pig skin at 32 °C. Key: A, control chromatogram of skin incubated in isotonic phosphate buffer pH 7.4; B, chromatogram of LHRH incubated with skin for 2 h; C, chromatogram of LHRH incubated with skin for 12 h. Key: 1 and 2, degradation products; 3, intact LHRH

3. Experimental

3.1. Chemical agents

LHRH was obtained from American Peptide Company (Sunnyvale, CA, USA). Carvacrol (98%), menthol (99%) and carvone (98%), were obtained from Aldrich Chemical Company, Inc. (WI, USA) and used as supplied. Sodium dihydrogen orthophosphate monohydrate, disodium hydrogen orthophosphate anhydrous, sodium chloride, acetonitrile (HPLC grade), trifluoroacetic acid (100%, HPLC grade), sodium hydroxide and absolute ethanol were supplied by BDH Laboratory Supplies (U.K.). All chemicals were at least reagent grade except where specified. Water was purified by a Milli-Q System (Millipore) which yielded a resistivity 18.2 MΩcm and was used to prepare all the solutions.

3.2. Skin preparation

Newborn pigs (*Sus scrofa*, Duroc male × Large White female) that had died of natural causes shortly after birth were obtained fresh from a local farm (Dunedin, New Zealand). The study was carried out with the ap-

proval of the Otago University Animal Ethics Committee, University of Otago, New Zealand. A tissue retrieval approval form was sufficient for the use of newborn pig skin.

The full thickness flank skins of newborn pigs weighing 1.4–1.8 kg were used. The epidermal hair at the flank area was clipped with electric hair clippers. The hair was carefully removed as close as possible to the skin without damaging the skin. The skin was then rinsed under running tap water in order to remove hair from the surface and then excised with a scalpel and a number 24 surgical blade. The subcutaneous fat and underlying tissues were carefully dissected from the dermal surface. The skin pieces were rinsed with isotonic phosphate buffer pH 7.4 (IPB), blotted dry with paper towels, wrapped in aluminium foil and stored at −20 °C for up to a month (Harrison et al. 1984). The skin samples were allowed to thaw, then were cut into 4.5×4.5 cm^2 pieces, placed in IPB and hydrated at room temperature overnight. The *in vitro* experiments were conducted on samples obtained from a minimum of 3 newborn pigs. Before the *in vitro* skin permeation experiments, the integrity of skin was determined by measurement of electrical resistance using silver chloride electrode system. Only skin pieces with electrical resistance greater than 20 kcm^2 were acceptable (Songkro et al. 2003a).

3.3. LHRH formulation

LHRH (0.05% w/v) and each terpene (5% w/v) were prepared in a hydroalcoholic mixture containing 40% v/v ethanol. A control formulation contained only LHRH in a hydroalcoholic mixture.

3.4. In vitro skin permeation and retention studies

The experiments were performed in a modified Franz's diffusion cell (Hanson Model 57–6M, Hanson Research Corporation, California, USA). The skin piece was mounted between the donor and the receptor compartments of the diffusion cell which was maintained at 37 °C. The skin sample was placed with the epidermal side facing towards the donor compartment and the dermal side bathed by the receptor fluid. The diffusion cell and skin were firmly clamped together providing a 1.77 cm^2 area exposed for diffusion. The donor (2.5 ml) and receptor compartments (6.5 ml) were filled with degassed IPB and the receptor medium was stirred at a speed of 200 rpm using a magnetic stirrer (Variomag® Telemodul 40 S, Germany). The electrodes were made from silver wire (1.00 mm diameter, 99.99% purity) obtained from a local company (Clyde Hill Glass, Dunedin, NZ). The electrode system was Ag/AgCl. The silver chloride electrode was prepared by electroplating Ag wire in 3 M potassium chloride to form a layer of AgCl. After equilibration of skin at 37 °C for 1 h, the Ag electrode was introduced into the donor chamber while the AgCl electrode was positioned below the skin, via the sampling port of the receptor compartment in order to complete a circuit across the skin membrane. Resistance measurements were made by applying a small electrical potential across the electrodes (0.3 V) which were connected in series with the power supply and with a highly sensitive multimeter (Fluke Corporation, Washington, USA). Utilising Ohm's law, the measurement of current was used to calculate the resistance. After the electrical resistance measurement, the IPB was removed from the donor compartment. One ml of each drug formulation was applied onto the skin surface and covered in order to prevent evaporation. At least three replications were performed for each formulation. At suitable time intervals (1, 2, 3, 4, 6, 8, 10, 12, and 24 h), 500 µl samples were removed from the receptor compartment and replenished immediately with an equal volume of fresh buffer (37 °C). Samples were assayed for LHRH by HPLC.

The retention of LHRH in the skin was determined following the method of Michniak et al. (1993) with slight modifications. At the end of a given time interval (24 h), the skin samples were removed from the diffusion cells and washed briefly in 50% methanol. The contact time of the skin with 50% methanol was minimized so that only surface LHRH was removed from the skin. The skin samples were cut with scissors and were then homogenized in 10 ml of 50% methanol using a DIAX 900 homogenizer (Heidolph, Germany) at a medium setting. The skin homogenate was centrifuged using a (KR-20000T centrifuge (Kubota Tokyo, Japan) at 15,000 rpm (3000 g) for 20 min. Supernatants were collected and frozen at −20 °C until HPLC analysis.

Recovery experiments were performed on six individual diffusion cells. The drug content in 50% methanol washes, representing the total donor concentration remaining after 24 h, the skin drug content measured in µg per diffusion area and the total corrected receptor concentration (µg/6.5 ml) at 24 h were added up. These results yielded greater than 75% recovery of the original concentration of LHRH. Loss of LHRH may have occurred through side diffusion in the skin, adsorption onto the glass surface of the diffusion cell, removal in the skin homogenate, or possible degradation of the model compound by cutaneous enzymes.

3.5. In vitro skin metabolism study

The method used in this study was modified from that reported in previous publications by Miller et al. (1990) and Bi and Singh (2000). Fresh skin samples were prepared identically to those used in the permeation studies. During the experiment, screw-cap glass vials containing 50 µg/ml of LHRH in IPB were maintained in a shaking water bath (32 °C). The total volume of solution in each vial was 5 ml. A 1.77 cm^2 piece of skin was added to each vial after 30-min temperature equilibration. Three vials contained LHRH solution and newborn pig skin. A fourth vial contained only skin and IPB and served as a control. At appropriate time intervals (from 0–24 h), 200 µl samples were withdrawn and analyzed using HPLC for the parent LHRH peak as well as additional peaks due to LHRH metabolism.

3.6. HPLC analysis of LHRH

LHRH and LHRH degradation products were measured by isocratic liquid chromatographic assay (Wen et al. 2002). A Jupiter C18 column (250×4.6 mm, 5 micron, Phenomenex, California, USA) was used to determine the amount of model compound. The HPLC system consisted of a Shimadzu pump (model LC-10 AT VP, Shimadzu, Japan) and a Shimadzu UV-Visible detector (model SPD-10 AVP, Shimadzu, Japan). The mobile phase consisted of 0.1% trifluoroacetic acid in 21% (v/v) acetonitrile in water adjusted to pH 2.5 with sodium hydroxide. The injection volume was 100 µl and the flow rate was 1 ml/min at room temperature. The absorbance was detected at 215 nm. The linearity range for this method was between 1–30 µg/ml. The retention time for the parent molecule was 15 minutes. Peak areas were analyzed and monitored for changes over the time course of the experiments. Validation of the method was performed to ensure that the coefficient of variation was less than 5%, both intra-day and inter-day. The detection limit of LHRH (signal-to noise ratio of 3 : 1) was 56 ng/ml.

3.7. Data analysis

The cumulative amount of the drug permeation (Q_t) was calculated from Eq. (1) (Zhang et al. 2007):

$$Q_t = V_r C_t + \sum_{i=0}^{t-1} V_s C_i \quad (1)$$

where C_t is the drug concentration of the receptor fluid at each sampling time, C_i is the drug concentration of the i^{th} sample, and V_r and V_s are the volumes of the receptor fluid and the sampling solution, respectively. Data were expressed as the cumulative drug permeation per unit of skin surface area, Q_t/S ($S = 1.77$ cm^2). The cumulative amount of drug in the receptor fluid per unit area of skin was plotted against time (T). The steady-state fluxes (J_{SS}) were calculated from the linear portion of the slope using linear regression as shown in Eq. (2):

$$J_{SS} = \Delta Q_t/(\Delta T.S) \quad (2)$$

The apparent permeability coefficient (P) was calculated from Eq. (3):

$$P = J_{SS}/\Delta C \quad (3)$$

where ΔC is the concentration gradient and is related to the donor and receptor concentrations. However, in the present study, the receptor concentration can be neglected as sink conditions apply. Therefore, ΔC can be assumed to be equal to the donor concentration (Roy and Manoukian 1995).

The enhancement ratio (ER) for the skin parameter was calculated from Eq. (4) (Goodman and Barry 1988).

$$ER = \frac{\text{skin parameter for enhancer treated skin}}{\text{skin parameter for control}} \quad (4)$$

Statistical analysis of the data was performed using one-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$. A Tukey's comparison test was used if the ANOVA indicated that there was a significant difference. Minitab release version 12.1 (Minitab Inc., State College, PA, USA) was used for statistical analysis.

Acknowledgement: The authors would like to thank the New Zealand Government for financial support for SS.

References

Akimoto T, Nagase Y (2003) Novel transdermal drug penetration enhancer: synthesis and enhancing effect of alkyldisiloxane compounds containing glucopyranosyl group. J Control Release 88: 243–252.

Banga AK, Bose S, Ghosh TK (1999) Iontophoresis and electroporation: comparisons and contrasts. Int J Pharm 179: 1–19.

Barry BW (1983) Skin transport. In: Barry BW (ed.) Dermatological formulations: percutaneous absorption, Marcel Dekker, New York, pp. 95–126

Bhatia KS, Singh J (1998) Mechanism of transport enhancement of LHRH through porcine epidermis by terpenes and iontophoresis: permeability and lipid extraction studies. Pharm Res 15: 1857–1862.

Bhatia KS, Singh J (1999) Effect of linolenic acid/ethanol or limonene/ethanol and iontophoresis on the *in vitro* percutaneous absorption of LHRH and ultrastructure of human epidermis. Int J Pharm 180: 235–250.

Bhatia KS, Singh J (1997) Percutaneous absorption of LHRH through porcine skin: Effect of *N*-methyl 2-pyrrolidone and isopropyl myristate. Drug Dev Ind Pharm 23: 1111–1114.

Bi M, Singh J (1998) Degradation of lutinizing hormone releasing hormone in buccal, liver, nasal and skin tissues. Int J Pharm 175: 269–273.

Bi M, Singh J (2000) Stability of lutenizing hormone releasing hormone; effects of pH, temperature, pig skin and enzyme inhibitors. Pharm Dev Technol 5: 417–422.

Cornwell PA, Barry BW (1995) Effects of penetration enhancer treatment on the statistical distribution of human skin permeabilities. Int J Pharm 117: 101–112.

Gao S, Singh J (1997) Mechanism of transdermal transport of 5-fluorouracil by terpenes-carvone, 1,8-cineole and thymol. Int J Pharm 154: 67–77.

Goodman M, Barry BW (1988) Action of penetration enhancers on human skin as assessed by the permeation of the permeation of model drugs 5-fluorouracil and estradiol. Part 1. Infinite dose technique. J Invest Dermatol 91: 323–327.

Harrison SM, Barry BW Dugard PH (1984) Effects of freezing on human skin permeability. J Pharm Pharmacol 36: 261–262.

Heit MC, McFarland A, Bock R, Riviere JE (1994) Isoelectric focusing and capillary zone electrophoretic studies using luteinizing hormone releasing hormone (LHRH) and its analogue. J Pharm Sci 83: 654–656.

Heit M, Williams P, Jayes F, Chang S, Riviere J (1993) Transdermal iontophoretic peptide delivery; *in vitro* and *in vivo* studies with luteinizing hormone releasing hormone. J Pharm Sci 82: 240–243.

Herai H, Gratieri T, Thomazine JA, Bentley MVLB, Lopez RFV (2007) Doxorubicin skin penetration from monoolein-containing propylene glycol formulations. Int J Pharm 329: 88–93.

Kimura C, Nakanishi T, Tojo K (2007) Skin permeation of ketotifen applied from stick-type formulation. Eur J Pharm Biopharm 67: 420–424.

Kumar S, Malick AW, Meltzer NM, Mouskountakis JD, Behl CR (1992) Studies of *in vitro* skin permeation and retention of a leukotriene antagonist from topical vehicles with a hairless guinea pig model. J Pharm Sci 81: 631–634.

Kunta JR, Goskonda VR, Brotherton HO, Khan MA, Reddy IK (1997) Effect of menthol and related terpenes on the percutaneous absorption of propranolol across excised hairless mouse skin. J Pharm Sci 86: 1369–1373.

Lee VH (1988) Enzymatic barriers to peptides and protein absorption. Crit Rev Ther Drug Carrier Syst 5 (2): 69–97.

Michniak BB, Player MR, Chapman JM, Sowell JW (1993) *In vitro* evaluation of a series of Azone analogs as dermal penetration enhancers. I. Int J Pharm 91: 85–93.

Miller LL, Kolaskie CJ, Smith GA, Rivier J (1990) Transdermal iontophoresis of gonadotrophin releasing hormone (LHRH) and two analogues. J Pharm Sci 79: 490–493.

Moghimi HR, Williams AC, Barry BW (1996) A lamellar matrix model for stratum corneum intercellular lipids IV. Effects of terpene penetration enhancers on the permeation of 5-fluorouracil and oestradiol through the matrix. Int J Pharm 145: 49–59.

Nokhodchi A, Sharabiani K, Rashidi MR, Ghafourian T (2007) The effect of terpene concentrations on the skin penetration of diclofenac sodium. Int J Pharm 335: 97–105.

Obata Y, Takayama K, Machida Y, Nagai T (1991) Combined effect of cyclic monoterpenes and ethanol on percutaneous absorption of diclofenac sodium. Drug Des Discov 8: 137–144.

Potard G, Laugel C, Schaefer H, Marty JP (2000) The stripping technique: *in vitro* absorption of five UV filters on excised fresh human skin. Skin Pharmacol Appl Skin Physiol 13: 336–344.

Raiman J, Koljonen M, Huikko K, Kostiainen R, Hirvonen J (2004) Delivery and stability of LHRH and Nafarelin in human skin: the effect of constant/pulsed iontophoresis. Eur J Pharm Sci 21: 371–377.

Rhee YS, Choi JS, Park ES (2001) Transdermal delivery of ketoprofen using microemulsions. Int J Pharm 228: 161–170.

Roy SD, Manoukian E (1995) Transdermal delivery of ketorolac tromethamine: Permeation enhancement, device design, and pharmacokinetics in healthy humans. J Pharm Sci 84: 1190–1196.

Schneider F, Tomek W, Gründker C (2006) Gonadotropin-releasing hormone (GnRH) and its natural analogues: A review. Theriogenology 66: 691–709.

Smyth HD, Becket G, Mehta S (2002) Effect of permeation enhancer pretreatment on the iontophoresis of luteinizing hormone releasing hormone (LHRH) through human epidermal membrane (HEM). J Pharm Sci 91: 1296–1307.

Songkro S, Purwo Y, Becket G, Rades T (2003a) Investigation of newborn pig skin as an *in vitro* animal model for transdermal drug delivery. S.T.P. Pharma Sci 13: 133–139.

Songkro S, Rades T, Becket G (2003b) The effects of *p*-menthane monoterpenes and related compounds on the percutaneous absorption of propranolol hydrochloride across newborn pig skin. I. *In vitro* skin permeation and retention studies. S.T.P. Pharma Sci 13: 349–357.

Srinivasan V, Su MH, Higuchi WI, Behl CR (1990) Iontophoresis of polypeptides: effect of ethanol pretreatment of human skin. J Pharm Sci 79: 588–591

Takayama K, Nagai T (1994) Limonene and related compounds as potential skin penetration promoters. Drug Dev Ind Pharm 20: 677–684.

Tauber U (1982) Metabolism of drugs on and in the skin. In: Brandau R, Lippold BH (eds) Dermal and transdermal absorption, Wissenschaftliche Verlagsgesellschaft MbH, Stuttgart, p. 132–149

Trommer H, Neubert RH (2006) Overcoming the stratum corneum: the modulation of skin penetration. A review. Skin Pharmacol Physiol 19: 106–121.

Vaddi HK, Ho PC, Chan SY (2002a) Terpenes in ethanol: haloperidol permeation and partition through human skin and stratum corneum changes. J Control Release 81: 121–133.

Vaddi HK, Ho PC, Chan SY (2002b) Terpenes in propylene glycol as skin-penetration enhancers: permeation and partition of haloperidol, Fourier transform infrared spectroscopy, and differential scanning calorimetry. J Pharm Sci 91: 1639–1651.

Wen J, Davies N, Ledger R, Butt G, McLeod B, Tucker IG. (2002) Isocratic liquid chromatographic assay for monitoring the degradation of luteinizing hormone releasing hormone by extracts from the gastrointestinal tract of possums. J Chromatogr B 779: 221–227.

Williams AC, Barry BW (2004) Penetration enhancers. Adv Drug Deliv Rev 56: 603–618.

Yamane MA, Williams AC, Barry BW (1995) Terpene penetration enhancers in PG/water co-solvent systems: effectiveness and mechanism of action. J Pharm Pharmacol 47: 978–989.

Zhang C, Yang Z, Luo J, Zhu Q, Zhao H (2007) Effects of cinnamene enhancers on transdermal delivery of ligustrazine hydrochloride. Eur J Pharm Biopharm 67: 413–419.

Zhao K, Singh J (2000) Mechanism(s) of *in vitro* percutaneous absorption enhancement of tamoxifen by enhancers. J Pharm Sci 89: 771–780.