Transdermal Penetration of Synthetic Peptides and Their Penetration Enhancement Caused by Some Terpene Compounds

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The work presented in this paper represents a study of the rate and extent of transdermal penetration of three synthetic hexapeptides consisting only of glycine (Gly) and phenylalanine (Phe) as the constituent amino acids and they include Phe-Gly-Gly-Gly-Gly-Gly (Pep-1), Phe-Phe-Gly-Gly-Gly-Gly (Pep-2), and Phe-Phe-Phe-Gly-Gly-Gly (Pep-3). The present study demonstrated the extent to which the peptides having a high metabolic stability were transdermally transported from the various vehicles. The results of this study appear to indicate that minor differences in the lipophilicity of the synthetic hexapeptides have a slight influence on the rate and extent of transport. In the presence of terpene permeation enhancers, together with ethanol (*i.e*., menthone/EtOH, carveol/EtOH or cineole/EtOH), the peptides were more rapidly penetrated through the skin and among the terpenes tested, cineole was the most effective for all three peptides. The maximum enhancement ratio of approximately 2 was achieved by cineole in 50% ethanol solution.

Key Words : Transdermal, Synthetic peptide, Permeation enhancer, Terpene

Introduction

A number of peptides and proteins have been investigated as therapeutic agents ever since the establishment of amino acid sequences of oxytocin, insulin and vasopressin in the early 1950s.[1] Moreover, recent advances in solid phase synthetic techniques for peptides have accelerated their availability. The FDA has increasingly approved many peptide and protein drugs for marketing since the early 1980s. Thus, peptide and protein drugs are expected to play an important role as therapeutic agents to treat, in particular, cancer, pain and diseases related to cardiovascular, metabolic and immune systems.

Transdermal route of delivery offers a number of advantages over oral and parenteral routes of administration such as increased patient compliance and provision of sustained or controlled delivery of the drug. The skin, however, has excellent barrier properties, which has resulted in only a small number of highly potent compounds being delivered by this route.[2]

The work presented in this paper represents a study of the rate and extent of transdermal penetration of three synthetic hexapeptides (Table 1) consisting only of glycine (Gly) and phenylalanine (Phe) as the constituent amino acids and they include Phe-Gly-Gly-Gly-Gly-Gly (Pep-1), Phe-Phe-Gly-Gly-Gly-Gly (Pep-2), and Phe-Phe-Phe-Gly-Gly-Gly (Pep-3). These peptides were chosen due to their proven stability against aminopeptidases and carboxypeptidases which are the main cause of the degradation of peptides while passing through the skin.[3,4] Based on the literature,[5] since peptides containing an unnatural D-amino acid on or near the amino acid terminal of the peptides showed much greater metabolic stability, the amino terminal of the synthetic hexapetides employed in this study was substituted with D-Phe. And this enhanced metabolic stability was demonstrated previously.[3] Additionally some terpenes including carveol, cineole and menthone which have used, along with ethanol, to enhance the transdermal permeation of small peptide, thyrotropin releasing hormone were tested for their ability to improve the synthetic peptides.[6]

Table 1. Molecular Weights and Log Distribution Coefficients of Synthetic Peptides Studied

Peptide Name	Amino Acid Sequence	Molecular Weight	Log D
Pep-1	D-Phe-Gly-Gly-Gly-Gly-Gly	450	−4.18
Pep-2	D-Phe-Phe-Gly-Gly-Gly-Gly	540	−2.65
Pep-3	D-Phe-Phe-Phe-Gly-Gly-Gly	630	−0.79

Experimental Section

Materials and reagents. The hexapeptides tested were synthesized by using repetitive base cleavage of α-amino protective groups in Solid Phase Peptide Synthesis as described elsewhere.[3] All peptides studied in this experiment were radiolabelled with C14-glycine unit and were synthesized in house. Phosphate-buffered saline pH 7.4 tablets were obtained from Sigma-Aldrich Company (St. Louis, MO). OptiPhase HiSafe 3 liquid scintillation cocktail were supplied by Fisher Chemicals (Loughborough, UK). All other chemicals and solvents were of HPLC or analytical grade.

HPLC analysis of peptides. The HPLC system consisted of a quaternary pump (Hitachi L-7100), an autosampler (Hitachi L-7200), a reverse-phase Capcell Pak ODS column (5 μm, 4.6 mm × 25 mm, Shiseido), a UV/Vis detector (Hitachi L-7400) and Berthold LB506-C-1 radiochemical detector.

The mobile phase consisted of acetonitrile/water mixture containing 0.1% trifluoroacetic acid (TFA). The percentage of acetonitrile in each mobile phase for the peptides were as follows; 7.5%, 17.5%, and 25% for Pep-1, Pep-2, and Pep-3, respectively. The flow rates were 1.5 mL/min (Pep-1 and Pep-2) and 1.4 mL (Pep-3). Detection was conducted both spectrometrically (distribution coefficient and radiochemically (penetration study).

Measurement of distribution coefficient of peptides. The *n*-octanol/PBS distribution coefficient of the peptides was measured prior to investigating the peptide permeation study across the skin. PBS and *n*-octanol were shaken to co-saturate in a 50 mL screw capped test tube for 24 h at 37 °C. The two phases were then separated by centrifugation at 2,000 rpm for 10 min. The aqueous phase was used to prepare the peptide solutions (500 μg/mL). The aqueous peptide solution (3 mL) was added to the organic phase (3 mL) and shaken in a shaker-bath at 37 °C. After equilibrium (24 h), the two phases were separated following centrifugation at 2,000 rpm for 10 min. Samples were taken from the aqueous phase and analyzed for each peptide by HPLC as described above. Log D values were calculated from distribution coefficients.

***In vitro* skin penetration study.** The skin was excised from the back of male Sprague Dawley rats (7-8 weeks old, 180-220 g). After removal of hair on the skin surface and any underlying muscle tissue and fat, the skin tissue was mounted in Franz diffusion cells with a diffusional surface area of 1.76 cm^2 ml and a volume of receptor compartment of 11 mL. The receptor compartment was filled with PBS pH 7.4, stirred with a magnetic bar for uniform mixing of the contents. Skin penetration of the peptides was examined using the following three basic compositions of the donor formulations: (i) 100% PBS, (ii) 50%(w/w) PBS and 50% (w/w) ethanol, and (iii) a homogenous enhancer solution of 3%(w/w) terpene (cineole, carveol, or menthone), 47% (w/w) ethanol and 50%(w/w) PBS. These formulations contained labeled peptide (5 μCi/mL) and unlabeled peptide (1 mg/mL) and would enable us to study level and rate of the penetration of the peptides used and the influence of terpenes on the peptide flux. The level of 3% of the enhancers was selected on the basis that it is the maximum concentration of terpenes allowing for homogeneous solutions with the same levels of PBS and ethanol for all three tested terpenes.

Data treatment. Skin flux can be experimentally determined as follows:

$$J_{ss} = (dQ/dt)_{ss} \cdot 1/A$$

where J_{ss} is the steady-state flux (%/cm^2 per hr), A is the area of skin tissue (cm^2) through which the permeation of the peptide takes place, $(dQ/dt)_{ss}$ is the percentile amount of peptide passing through the skin per unit time at steady-state (%/hr). The cumulative amount of peptide permeated through rat skins was plotted as a function of time. The permeation rate of peptides at steady-state (J_{ss}, %/cm^2 per h) through rat skin was calculated from the slope of linear portion of the cumulative amount permeated through the rat skins per unit area versus time plot.

Statistical analysis. Statistical analysis of data sets for each group of measurements was performed using one-way ANOVA and *P* values of 0.05 or less were considered statistically significant.

Results and Discussion

The molecular weights and measured log D values of the

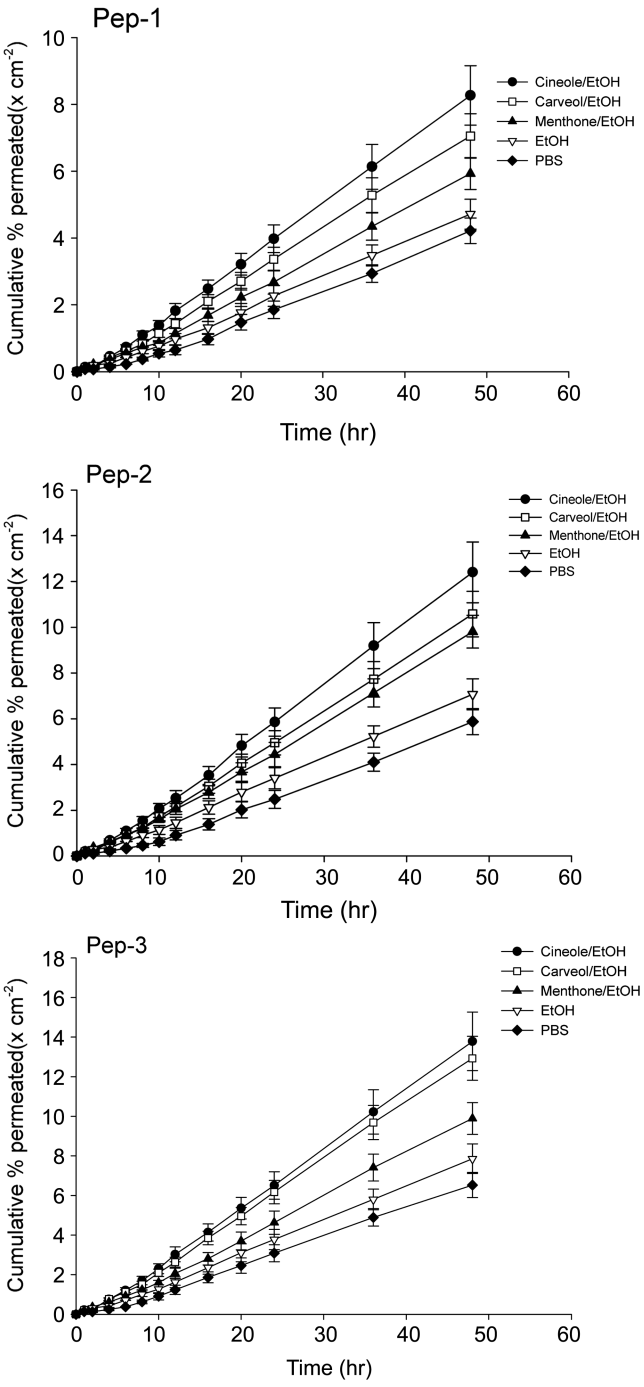

Figure 1. *In vitro* skin permeation profiles of Pep-1, Pep-2 and Pep-3 from various formulations. Mean ± SD, n = 5.

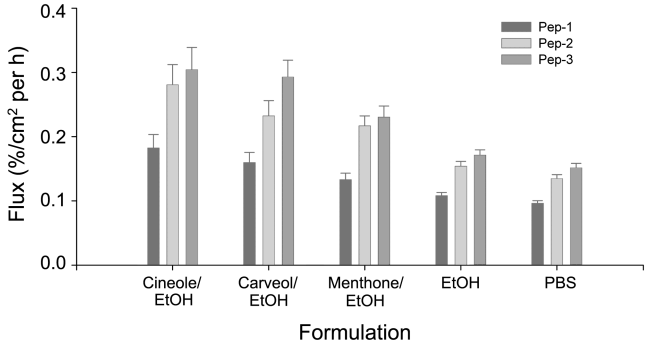

Figure 2. Steady-state fluxes of Pep-1, Pep-2 and Pep-3 from various formulations. Mean ± SD, n = 5.

Table 2. Influence of vehicles and terpene compounds on the permeability of Pep-1, Pep-2 and Pep-3. Mean ± SD, n = 5

Peptide	Formulation	Flux (%/cm^2 per h)	ER[#]
Pep-1	PBS	0.096 ± 0.004	–
	EtOH	0.108 ± 0.005	1.13
	Menthone/EtOH	0.133 ± 0.010[*]	1.39
	Carveol/EtOH	0.160 ± 0.015[*]	1.67
	Cineole/EtOH	0.182 ± 0.021[**]	1.90
Pep-2	PBS	0.135 ± 0.006	–
	EtOH	0.154 ± 0.007	1.14
	Menthone/EtOH	0.217 ± 0.016[*]	1.61
	Carveol/EtOH	0.232 ± 0.024[*]	1.72
	Cineole/EtOH	0.281 ± 0.031[**]	2.08
Pep-3	PBS	0.152 ± 0.007	–
	EtOH	0.171 ± 0.008	1.13
	Menthone/EtOH	0.231 ± 0.017[*]	1.52
	Carveol/EtOH	0.293 ± 0.026[**]	1.93
	Cineole/EtOH	0.304 ± 0.035[**]	2.00

[*]Significantly different from PBS group at $P < 0.05$. [**]Significantly different from PBS group at $P < 0.01$. [#]Enhancement ratio (ER) was obtained from flux value obtained with EtOH, Menthone/EtOH, Carveol/EtOH or Cineole/EtOH divided by flux obtained with PBS vehicle.

synthetic peptides tested are shown in Table 1. Also the permeabilities as expressed to be flux of the peptides are shown in Figure 1, 2 and Table 2. Of the peptides tested, Pep-1 is the most hydrophilic and Pep-3 is less hydrophilic than Pep-2 and Pep-3. This was expected because among the peptides Pep-1 contains least number of phenylalanine residue which is more lipophilc than glycine and, on the other hand, the number of phenylalanine in Pep-3 is the greatest.

The *in vitro* skin transport studies were carried out utilizing excised rat skins for 48 h. From the skin permeation studies (Fig. 1), the excised rat skin was permeable to a series of synthetic peptides consisted of phenylalanine and glycine having different hydrophilicity and it is well established that less hydrophilic peptide was penetrated through the skin to a greater extent at a faster flux than more hydrophilic peptide in the formulations employed. For instance, the flux value of Pep-1 in PBS the lowest (0.096 ± 0.004%/cm^2 per h) but Pep-3, the most lipophilic peptide, in PBS showed the fastest flux (0.152 ± 0.007%/cm^2 per h). This trend was demonstrated in other formulations (*i.e.*, EtOH, Menthone/EtOH, Carveol/EtOH or Cineole/EtOH).

Table 2 and Figure 2 show the rate of peptide transport changed in the presence of the permeation enhancers. The peptide permeation enhancers examined were shown, probably along with ethanol, to have a beneficial effect upon the skin transport of the peptides studied. In the presence of ethanol (50%), the flux values of three peptides increased but this increase was not significant. However, the addition of terpenes in combination with ethanol resulted in a considerable increase in the fluxes of three peptides. As shown in Table 2, among the terpenes studied the permeation enhancing effect in the presence of 3% cineole for all three peptides was statistically obvious at the confidence limit tested (*i.e.* $P < 0.01$) and it increased the flux of three peptides approximately two times, compared to when delivered in PBS alone (Table 2). In contrast, the enhancement ratio observed in the presence of ethanol and menthone or carveol was less than 2 for all three peptides. When menthone was used with ethanol, the enhancement ratio was 1.39 (Pep-1), 1.61 (Pep-2) and 1.52 (Pep-3), respectively and it was further increased to 1.67 (Pep-1), 1.72 (Pep-2) and 1.93 (Pep-3) by the addition of carveol in ethanol. In the presence of cineole in aqueous ethanol solution, between 8.27% and 13.78% of the tested peptides were transported after 48 h.

There has been little information available with regard to the direct influence of ethanol upon the skin permeability but several studies have demonstrated that alcohols may exert a topical action increasing transport of drugs into the underlying tissue probably by lipid extraction that occurs as a result of lipid solubilization.[7] It has also been reported that the mechanism by which terpenes act as a skin penetration enhancer may be through disturbing the lipid bilayer structure of the stratum corneum, thereby improving the diffusion coefficient of polar drugs in the membrane.[8] Even though the terpenes increased skin penetration rate of the peptides tested, the maximum enhancement ration of 2 was not greater than those seen in the literatures. The reason for this is probably because the intensity of the effect of terpene is greatly affected by the lipophilicity of the drug being transported and vehicle employed.[9] Thus, despite the fact that Pep-3 is the most lipophilic compound among the three peptides, since the log D value of Pep-3 is −0.79 indicating this peptide is more soluble in water the effect of the terpenes was not quite greater than the results reported previously.[10,11] The percentile amount penetrated in the present study does not represent the maximum value as the peptides and the terpenes were employed at low concentrations (*i.e.*, peptide amount of 1 mg/mL and 3% of the terpene, respectively) and no optimization studies were carried out to identify the best formulation in terms of the rate and extent of skin absorption. Further study may be performed to find out the transport pathway.

In conclusion, the present work demonstrated the extent to which the peptides having a high metabolic stability were

transdermally transported from the various vehicles. The results of this study appear to indicate that minor differences in the lipophilicity of the synthetic hexapeptides have a slight influence on the rate and extent of transport. In the presence of terpene permeation enhancers together with ethanol, the peptides were more rapidly penetrated through the skin and among the terpenes tested, cineole was the most effective for all three peptides. The maximum enhancement ratio of approximately 2 was achieved by cineole in 50% ethanol solution.

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