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Enhanced transdermal delivery of a dipeptide by Dermaportation

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Abstract

Poor skin permeability and stability limits the application of peptides to the skin. Enhanced skin permeation could offer new therapies for a range of dermatological and cosmetic applications. The aim of this study was to investigate the application of novel magnetic field enhancement technology to peptide delivery across the skin. Ala-Trp was used as a model dipeptide. Stability of the dipeptide in a range of conditions and with exposure to skin was determined. Dermaportation-magnetic field technology increased the *in vitro* permeability coefficient of Ala-Trp across human epidermis from 7.7 x 10^{-4} cm/h with passive diffusion to 1.94 x 10^{-2} cm/h with Dermaportation. Ala-Trp was unstable with exposure to human epidermis. Following permeation across the epidermis a degradation product was detected in the receptor solution with the amount increasing up to 6 h. Given the susceptibility of peptides to degradation in the skin it is essential that they are delivered rapidly across the skin in order to

maximise the opportunity for delivery of the native peptide. Dermaportation offers a potential new delivery method for skin delivery of peptides for a range of dermatological and cosmetic applications.

Key words:

Peptide delivery, transdermal, skin penetration enhancement, inductive energy, peptide stability

INTRODUCTION

Developments in biotechnology have led to the increased availability of therapeutic peptides and anti-cytokines. Currently the most common mode of administration is parenteral injection but alternatives are actively being sought. The transdermal route offers a potential alternative since it avoids first pass metabolism in the liver, permits controlled release and the skin exhibits less enzymatic activity than many other routes of administration. The site of action may be within the skin, such as new treatment options for skin conditions such as psoriasis and dermatitis or cosmeceutical outcomes,[1] or it may require systemic transport. The latter is much more challenging as larger doses are generally required and the skin provides an effective barrier to the delivery of peptides and proteins.

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A number of small peptides have been identified for their therapeutic or cosmetic potential in the skin. For example, carnosine (β-Ala-His) is implicated in biological activities such as wound healing and as an antioxidant.[2] The native peptide has very low affinity for the skin and does not permeate beyond the first layer of the stratum corneum, the outermost region of the epidermis.[2] However, when a palmitoyl chain is attached to the terminal $–NH_2$ group the lipopeptide has been shown to diffuse into the stratum corneum, epidermal and dermal skin layers and remain localised without any systemic activity.[2] Lys-Thr and Lys-Ser dipeptides have been used in personal care compositions to improve the cosmetic appearance of skin, hair and nails.[3] Lys-Thr and N-acyl derivatives and esters have also been used to treat or minimize signs of skin ageing such as wrinkles, skin lines and large pores.[4] A number of other small peptides are also formulated or under investigation for their anti-ageing potential.[5,6,7,8,9]

The lipophilic stratum corneum is a major barrier to the penetration of hydrophilic compounds into and through the skin.[10] Peptides are usually charged at physiological pH and contain both acidic and basic functional groups. Consequently their physicochemical characteristics do not favour permeation across the skin. It is therefore desirable to increase the transport of peptides across the stratum corneum in order to target a sufficient quantity to the site of action to achieve their therapeutic potential.[11,12] The most commonly used approaches include chemical modification (glycosylation, amino acid sequence modification, pegylation and cyclisation),[31] formulation innovations (vesicles and chemical enhancers), the use of physical energy to disrupt the lipoidal barrier (iontophoresis, sonophoresis and electroporation) or ‘minimally invasive’ methods (microneedles, stratum corneum ablation). Formulation and chemical enhancement techniques are well established with small molecules and

may be valuable to enhance the delivery of small peptides for dermatological and cosmetic applications. Chemical penetration enhancers may also be useful for enhancing delivery of small peptides but their clinical application is limited due to irritancy.[12] Transdermal iontophoresis has been extensively investigated and is now commercially available for the delivery of small molecules in a number of clinical applications such as patient controlled analgesia (Ionsys: Alza Corp/JNJ) and local anaesthesia (LidoSite: Vyteris Inc, NJ, USA). Iontophoresis applies a mild electric current to drive molecules across the skin[13] and has been shown to successfully enhance skin penetration of a number of peptides including LHRH,[14] TRH,[15] cyclosporin[16] and arginine vasopressin.

In addition to the limitation imposed by poor percutaneous penetration of peptides, they may also be subject to cutaneous metabolism. This is a particular problem if delivery is slow as the combination of poor penetration and cutaneous metabolism result in ineffective peptide concentrations at their target site. For example, Altenbach et al demonstrated that the model dipeptide tyrosine-phenylalanine (Tyr-Phe) was extensively metabolised by the skin with detection of elevated levels of the constituent amino acids and minimal amounts of the parent peptide in the receptor.[17] Enhanced skin delivery of peptides is therefore essential to provide effective amounts at their target site within the skin and reduce the impact of cutaneous metabolism.

In this study a novel physical skin penetration enhancement technology was investigated for peptide delivery. Dermaportation (OBJ Ltd.) is a Magnetic Field (MF) platform technology that applies electromagnetic pulses to enhance skin permeation and push target molecules away from the field. Biological and therapeutic effects of electromagnetic fields and inductive effects on biological tissues have been widely

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reported, for example, enhancement of healing of venous ulcers and bone fractures and effects on a range of cellular functions.[18,19] Dermaportation -MF uses a low voltage (3V) and does not require direct physical contact with the skin to produce diffusion enhancement. This offers potential advantages, particularly in wound management and drug delivery into the skin**.** In addition, the fabrication of small self contained Dermaportation patches is possible due to the low energy requirements. The standard Dermaportation system (DP1001) generates a specific pattern of energy pulses that have been shown to induce transdermal flux increases during field exposure of a number of small molecules, such as α-aminolevulinic acid,[20] diclofenac diethylammonium salt[21] and lidocaine hydrochloride.[22] It is proposed that Dermaportation energy influences both the molecular movement of drug molecules in the epidermis and the ordered structure of the stratum corneum lipid bilayers. The precise mechanism of enhancement is an area of continuing investigation.

The aim of the present study was to assess the effect of Dermaportation (DP1001) on the transdermal delivery of a model dipeptide (Ala-Trp) through human epidermis using an in vitro skin diffusion model. The stability of the dipeptide was also assessed at different temperatures and conditions.

RESULTS AND DISCUSSION

Chromatography

Ala-Trp was analysed by UV detection using HPLC. The dipeptide eluted without any interfering peaks at a retention time of 9.1 min. The linearity obtained by the HPLC method was 0.9999 over the range of the calibration curve (0.975-31.25 μg/mL) for Ala-Trp standard solutions. Fig. 2 is a typical HPLC chromatogram of Ala-Trp

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analysed 8 h after (a) passive and (b) Dermaportation administration to human epidermis.

A calibration curve was obtained by plotting the peak area versus concentration of standards injected. The CV for precision, determined from the relative standard deviation (n = 6), was 0.27% for 0.98 µg/mL, 0.15% for 7.80 µg/mL and 0.13% for 31.25 µg/mL Ala-Trp standard solutions in PBS. The intraday variation was 0.69% and 0.43% and the interday variation was 0.55% and 0.38% at 7.80 and 15.63 µg/mL Ala-Trp standard solutions in PBS, respectively. These are within the acceptable criteria for intra and interday repeatability of R.S.D. < 2%.

The level of detection (LOD), calculated as greater than three times the baseline noise level in the assay, was 49 ng/mL. The level of quantitation (LOQ), calculated as greater than 10 times the baseline noise level in the assay, was 165 ng/mL. The suitability of the assay with PBS which had been in contact with skin for 4 h to simulate a diffusion cell study was demonstrated, with no interfering peaks detected.

The HPLC method permitted the detection of 7.8 µg/mL of Ala-Trp sample with 92.11% accuracy and 15.63 µg/mL of Ala-Trp sample with 93.16% accuracy.

Stability of the dipeptide in solution

The degradation profile of Ala-Trp at different temperatures is shown in Fig 3. The stability data was presented as the percent remaining of the drug at varying temperatures and conditions. It was observed that when the dipeptide solution was placed at 37ºC in contact with the skin about 11% of the drug was lost in 6 h. This is in agreement with previous reports that lysine and tyrosine containing dipeptides are unstable when in contact with the skin.[13] In our experiment the skin was previously

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frozen therefore much of the enzymatic activity was lost, yet substantial degradation was observed. It is expected that the dipeptide would be extensively degraded where the cutaneous enzymes were preserved. The drug was most stable at refrigeration temperature of 4ºC with 3% degraded over the 6 h period. The dipeptide was relatively stable with only 4.6% being lost at 37ºC with no skin and at room temperature in dark conditions. A separate preliminary study was conducted to assess the stability during Dermaportation and passive diffusion and it was found that the dipeptide collected from the receptor was relatively stable during the time of the experiment. No significant amounts of degradation products, which were present in samples in contact with the skin, were detected. Fig. 2 is a typical HPLC chromatogram of Ala-Trp analysed 8 h after (a) passive and (b) Dermaportation suggesting that either Dermaportation does not alter the Ala-Trp structure or the degraded products may have a very strong affinity to the skin therefore may not diffuse through the skin. The possibility of the latter is under our investigation.

Dipeptides have previously been shown to decompose in the skin due to the biodegradability of peptides in the presence of cutaneous enzymes.[9] Skin proteases have been isolated and characterized however it is not known which peptide sequences or peptides are most susceptible to enzymatic degradation in human skin.[23,24] Previous studies have suggested that peptides can undergo extensive enzymatic metabolism in the viable skin and hence it is particularly desirable to increase the permeation of these drugs across the skin by overcoming the problems of stability and transport.[25,26]

Dermaportation of Ala-Trp through human epidermis

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In this study the influence of the novel penetration enhancement technology, Dermaportation on epidermal penetration of Ala-Trp in vitro was determined. The experimental protocol involved the standard in vitro model system of Franz cells stirred by magnetic stirrers. Preliminary experiments with a model compound were used to evaluate potential interference between the Dermaportation magnetic energy and the magnetic stirrers. There was no significant difference in skin penetration of the model compound with and without magnetic stirrers. Dermaportation appears to permit a substantial amount of native dipeptide to penetrate through the skin structure over the 8 h application period.

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The cumulative amount of Ala-Trp penetrating the human epidermis to the receptor solution over time was plotted for Dermaportation and passive application (Fig.4). The results indicated an increase in the amount and rate of Ala-Trp diffusion through human epidermis over 8 h in cells where Dermaportation was applied, as compared to cells where the dipeptide solution was applied without Dermaportation (Treat: $F_{1,16} = 23.97$, $p < 0.001$; Treat by Time interaction: $F_{13,208} = 10.33$, $p < 0.001$). An analysis per time point, demonstrated that the Dermaportation-treated cells had already a significantly higher amount of the dipeptide in the receptor compartment at 10min ($Treat_{t=10m}$: $F_{1,16} = 12.93$; $p < 0.005$). At t = 0, all groups were equal ($Treat_{t=0}$: $F_{1,16} = 0$, $p < 0.001$), demonstrating that no dipeptide was present in the receptor compartment before the experiment was started.

The estimated transdermal flux was calculated over the linear portion (0.33-2h) of the curve as 19.427 μg/cm^2.h for Dermaportation and 0.778 μg/cm^2.h for passive diffusion. The estimated permeability coefficient for Ala-Trp was 1.94×10^{-2} cm/h with Dermaportation and 7.7×10^{-4} cm/h with passive diffusion (Table 3). The transdermal permeability coefficient (kp) of the dipeptide in porcine skin when

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measured for passive diffusion has been reported to be 1.39×10^{-2} cm/s by Lin *et al,* in comparison with current study of 7.7×10^{-4} cm/h. However it is not valid to directly compare the two permeability coefficients since they were determined using different skin models. In the current study Dermaportation-MF was applied for the first 4 hours and a steep increase in cumulative amount permeating to the receptor was observed in 20 mins (38.23 and 1.02 µg/cm^2 for Dermaportation and passive application respectively). This difference may be due to an initial push into the skin applied by the magnetic field. There was a steady increase in the amount of dipeptide permeating over the entire period of the experiment (Fig 4). Over the first 20 mins, the first 2 h, the first 4 h, and the entire 8 h period, the Ala-Trp diffusion flux in Dermaportation diffusion cells was larger than for the passive cells ($Treat_{Flux20}$: $F_{1,16} = 12.93$, $p < 0.005$; $Treat_{Flux120}$: $F_{1,33} = 20.79$, $p < 0.005$; $Treat_{Flux4hr}$: $F_{1,16} = 13.50$, $p < 0.005$; $Treat_{Flux8hr}$: $F_{1,16} = 12.58$, $p < 0.005$). Dermaportation enhanced the permeability of Ala-Trp in the first 20 min by up to 37.3-fold over the passive diffusion rate. This finding is in agreement with previous reports in which Dermaportation was shown to increase the transdermal diffusion of a low molecular weight hydrophilic compound, 5-aminolevulinic acid and the local anaesthetics lidocaine and tetracaine hydrochloride in *in vitro* experiments.[20,21] Tables 1 and 2 list the flux and correlation coefficients for the stability study and the skin diffusion experiments. At present the mechanism of action of Dermaportation as a skin penetration enhancement technique has not been elucidated. It is known that magnetic fields can influence a number of cellular functions[18,19] but the effect on the skin has not been evaluated. We are currently conducting ATR-FTIR (attenuated total reflectance – fourier transform infra-red spectroscopy) studies to evaluate the effect of the Dermaportation magnetic field on stratum corneum lipids.

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Stability of Ala-Trp through human epidermis

In the stability study the rate of degradation of the dipeptide at 37°C without skin was about 9.4 times lower than the rate of degradation at 37°C when the dipeptide solution was left in contact with skin. At lower temperatures (i.e. at room temperature, room temperature and dark and at 4°C) the mechanism of degradation was different than at higher temperatures and it was assumed to follow a biphasic degradation pattern. The amount of the product that eluted at about 7.5 mins, which was suspected to be the degradation product, steadily increased till about 6 h. The total amount of the dipeptide and the percentage of degradation product were plotted against time and the rate was determined from the slope of the linear portion of the curve from 0.33 to 3 h. The rate of degradation which was determined in both studies showed a higher level in the stability study than that of the skin diffusion experiment with Dermaportation. This may be because the samples in the skin diffusion studies were collected from the receptor compartment which has a lower concentration of the dipeptide and is affected by the skin permeability of the dipeptide and degradation product. A lower percentage of degradation product with passive diffusion was detected since very low amounts of the dipeptide actually traversed the skin, which led to lower degradation in the receptor chamber. Previous studies on the characterization of aminopeptidases showed that Ala-Trp was susceptible to hydrolysis by aminopeptidase–I but no definite structures of the degradation products were reported.[27] Although tryptophan was suspected to be one of the likely degradation products, tryptophan residues in peptides or proteins can also undergo oxidation. Few studies have focussed on identification of compounds formed from these peptides. Simat et al attempted to identify tryptophan degradation compounds by subjecting the amino acid itself and

peptides containing tryptophan to an oxidising agent.[28] Comparing the transepidermal flux values in this study, it was seen that the permeation of the degradation product is lower than that of the native dipeptide. We have speculated that Ala-Trp may undergo hydrolysis or enzymatic degradation which results in the formation of a degradation product which could be an alanine or tryptophan related compound. This compound was detected after the diffusion of the native peptide through human epidermis in a level much lower than expected as the dipeptide's degradation product, suggesting that this degradation product has a tendency to remain in the skin. In addition, it was also observed that its permeation through the skin was enhanced by Dermaporation. Fast permeation of the dipeptide by Dermaportation could also mean less contact time for the peptide with the skin enzymes and more rapid delivery to the target site, both of which would greatly benefit the therapeutic outcome.

When iontophoresis was used as a penetration enhancement tool by Altenbach et al it was observed that there was no iontophoretic and passive transport of Tyr-Phe without the addition of an enzyme inhibitor.[17] This was due to the susceptibility of the peptide to hydrolysis. We cannot directly compare the transdermal flux values from this study to literature data since there has been very limited work done on Ala-Trp as a model dipeptide. This preliminary study indicates that the delivery rate of the peptide can be enhanced with Dermaportation when compared to passive diffusion. Dermaportation may provide an effective means of delivering molecules which are highly susceptible to degradation like dipeptides, in higher amounts and in a relatively short duration of time.

CONCLUSION

Dermaportation significantly enhanced the trans-epidermal delivery of Ala-Trp in vitro over an 8 h period when compared to passive diffusion. The dipeptide was shown to be unstable on exposure to human epidermis with an increasing amount of degradation product evident in the receptor phase over the 8 h period. Given the susceptibility of peptides to degradation in the skin it is essential that they are delivered rapidly across the skin in order to maximise the opportunity for delivery of the native peptide. Dermaportation offers a potential new delivery method for skin delivery of peptides for a range of dermatological and cosmetic applications.

MATERIALS AND METHODS

Chemicals

Alanine-Tryptophan (Ala-Trp), a model dipeptide with a molecular weight 275.30 Da was purchased from Sigma Aldrich (USA). Acetonitrile, HPLC solvent was supplied by JT Baker (USA) and phosphate buffered saline solution (PBS) was prepared according to the United States Pharmacopoeia.

HPLC instrumentation and conditions

The samples were analysed by reverse phase HPLC (Agilent 1100 system) which consisted of a quaternary pump (G1311A), autosampler (G1313A), degasser (G1312A) equipped with a photo diode array detector (G1321A). Separation was achieved on a Phenomenex Jupiter C_{18} 300A column (5 µm, 4.6mm×150mm). Integration was undertaken using Chemstation software.

The elution was performed at ambient temperature using a mobile phase gradient at a flow rate of 1 mL/min and the wavelength of detection was 210 nm.

Buffer A was 0.1% TFA and Buffer B was 0.1% TFA in acetonitrile. The dipeptide was eluted using a combination of isocratic and linear gradient protocol; Buffer B was held at 10% for 5 min followed by a linear gradient from 10 to 100% over 10 min and the dipeptide eluted at 9.1 min. All samples were analysed by HPLC using injection volumes of 20 µL.

HPLC Analysis

Calibration curves were obtained using 0.9, 1.9, 3.9, 7.81, 15.62 and 31.25 µg/mL of Ala-Trp standard solutions in PBS at pH 7.4. Linearity (quoted as R^2) was evaluated by linear regression analysis, which was calculated by the least square regression method. The precision of the assay was determined by injecting three standard concentrations (0.9, 7.8 and 31.25 µg/mL Ala-Trp) six times on the HPLC. The intraday repeatability was assessed by injecting 7.8 and 15.62 µg/mL Ala-Trp standards six times at different times in a day. The interday repeatability was determined by injecting 7.8 and 15.62 µg/mL Ala-Trp standards six times on 3 different days. The intra- and interday repeatabilities were quoted as the coefficient of variance. The minimum detectable and quantifiable limits (LOD and LOQ) were measured by diluting Ala-Trp in PBS to give a concentration range from 0.9 to 31.3 µg/mL and then injected on the HPLC. Accuracy of the analytical method was determined for in vitro skin diffusion studies as follows. Two separate samples of PBS which had been in contact with human epidermis for 24h at 37°C were spiked with Ala-Trp standards to give final concentrations of 7.8 and 15.6 µg/mL Ala-Trp, respectively. Each spiked sample and standard was injected six times on the HPLC and the percentage difference between each standard and the corresponding spiked sample was calculated.

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Stability Study

The stability of Ala-Trp was determined at different temperatures and with having the dipeptide in contact with skin to provide an estimate of the stability of the drug during the skin diffusion experiments. The following temperatures were used 37°C, room temperature, room temperature (dark) and at 4°C. Vials containing 3 mL of 1 mg/mL dipeptide solution were stored under these conditions. 100 µL samples were withdrawn at 0, 20, 45, 60 min, 2h, 3h, 4h, 5h and 6h. The samples were then diluted to give a final theoretical concentration of 20 µg/mL and were analysed by HPLC.

Human Skin Preparation

Full thickness human skin samples excised from female patients undergoing abdominoplasty at Perth hospitals were refrigerated immediately after surgery. Sampling was approved by the Human Research Ethics Committee of Curtin University (Approval number HR132/2001) and was conducted in compliance with the guidelines of the National Health and Medical Research Council of Australia. The following procedure was used to obtain epidermal sheets. The subcutaneous fat was removed by dissection, the full thickness skin then immersed in water at 60°C for 1 min, aloowing the epidermis to be teased off the dermis.[29,30] The epidermis was placed onto aluminium foil, air dried and stored at –20°C until required.

Skin permeation of Ala-Trp

In vitro permeation studies across human epidermis were performed in Pyrex glass Franz-type diffusion cells (enabling permeation across skin sections of cross sectional area 1.18 cm^2; receptor volume approximately 3.5 mL: Figure 1). Epidermal

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membrane was placed between the donor and receptor compartments and allowed to equilibrate for 30 min with the receptor solution (PBS pH 7.4) which was stirred continuously with a magnetic flea. The receptor compartment of the cell was immersed in a water bath at 37±0.5°C. PBS (1 mL) was placed in the donor compartment, allowed to equilibrate for 30 min and the conductivity across the epidermis was measured using a digital multimeter to determine membrane integrity. The PBS solutions were then removed from the donor and receptor compartments and the receptor refilled with fresh pre-warmed PBS. The donor solution, which consisted of 1mL of 1mg/mL Ala-Trp in PBS was added. Samples of the receptor phase were withdrawn and replaced with an equal volume of fresh pre-warmed (37°C) PBS over an 8 h period. The Ala-Trp content in the samples was determined using HPLC. Dermaportation coils were added to the exterior of the donor compartment and energy applied for 8 h (Dermaportation cells), whilst other cells had no external Dermaportation energy applied. 9 replicates were conducted for passive and Dermaportation experimental protocols. The cumulative amount of drug permeated through the epidermis (μg/cm^2) versus time (h) was plotted. The flux of Ala-Trp through the human epidermis for both passive and Dermaportation cells was determined from the slope of the plot of cumulative amount versus time and expressed as μg/cm^2.h. Permeability coefficients (cm/h) were calculated for Ala-Trp for both passive and Dermaportation using the following equation,

Permeability coefficient Kp (cm/h) = Flux (J) / Concentration (C)

Statistical Analysis

The cumulative amount of target drug in the receptor chamber was determined over the entire period of testing, and the differences between the diffusion by passive and

Dermportation were analysed using ANOVA with the Factors Treat and Time (GLM procedures, SAS Institute). Further, flux rates (µg/cm^{2}h) were calculated from the slope of the linear relationship between the cumulative amount and time per group. Two separate flux rates were calculated, (i) the rate of the active drug diffusion or transport in the first 20 min, and (ii) the average diffusion rate over the first 2 h . An ANOVA with Fisher LSD post hoc comparisons on the Factors Treat and Experiment was used for the flux rates. A result was considered significant with $p < 0.05$. Enhancement Ratios with respect to passive diffusion were calculated from statistically significant flux values only.

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Figure and Table legends:

Figure 1 Schematic of skin diffusion experimental cell with Dermaportation coil in place

Figure 2: Chromatograms for Ala-Trp analyzed after 8h (a) Passive and (b) Dermaportation

Figure 3: Ala-Trp degradation profile in solution at varying conditions

Figure 4: Cumulative penetration of dipeptide across human epidermis for passive (■) or Dermaportation (▲) applied from 0-8h (mean ± sem: n=9)

Table 1: Rate of degradation of Ala-Trp at different temperatures and conditions

Table 2: Rate of permeation of dipeptide and degradation product with Dermaportation and passive diffusion

Table 3: Skin permeation of Ala-Trp with Dermaportation and passive diffusion

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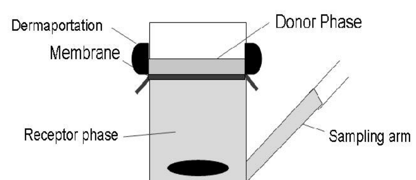

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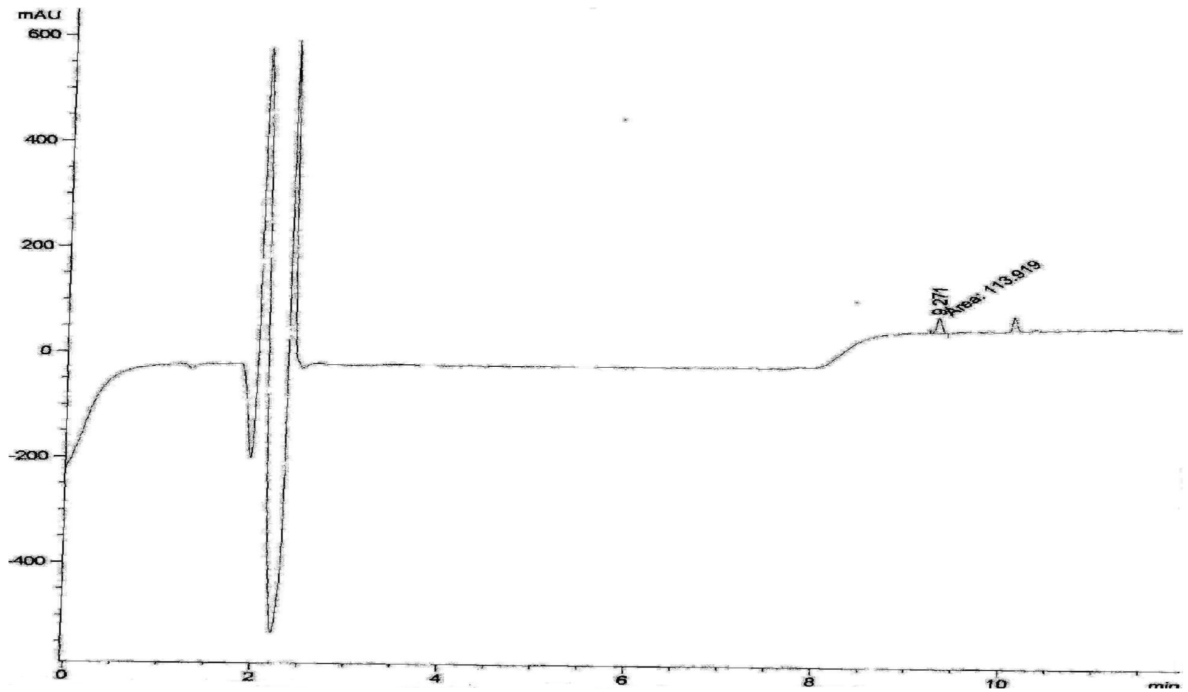

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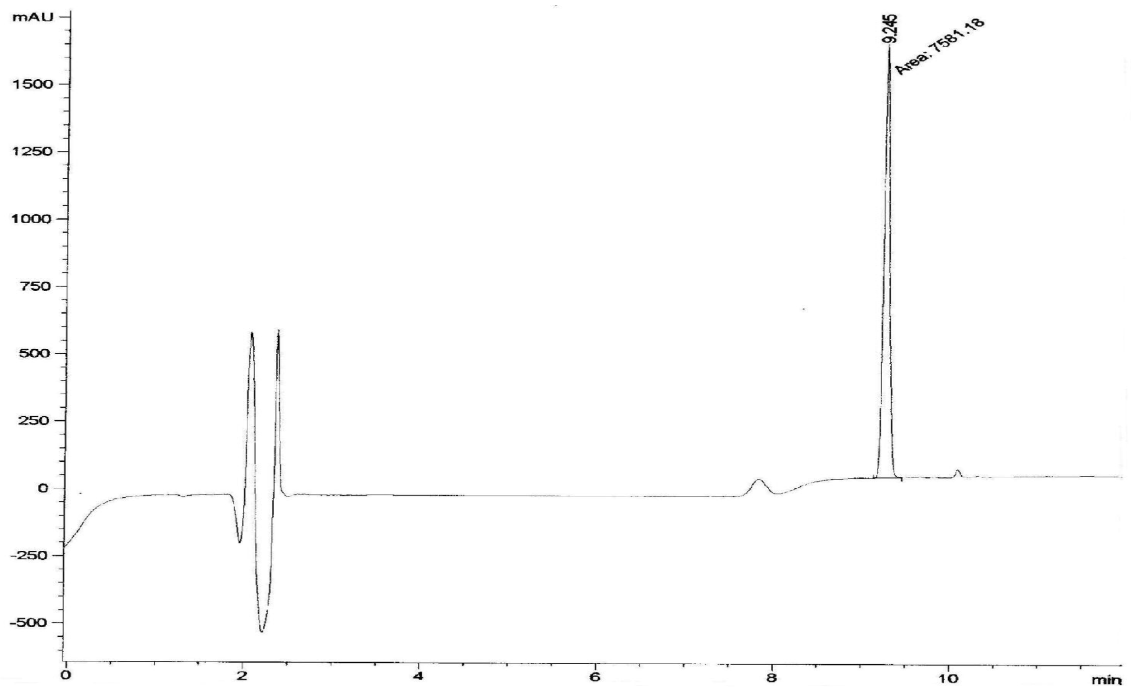

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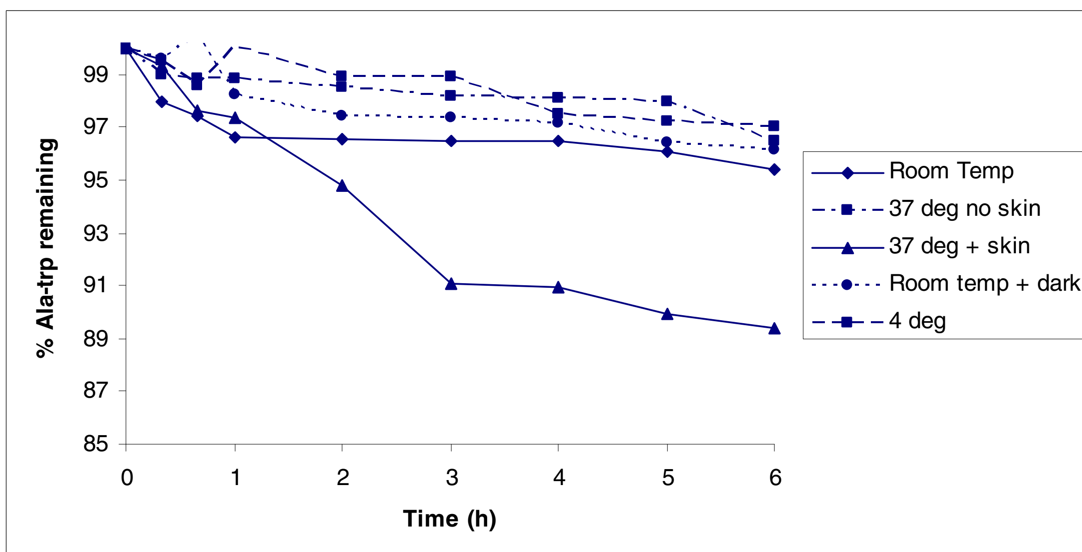

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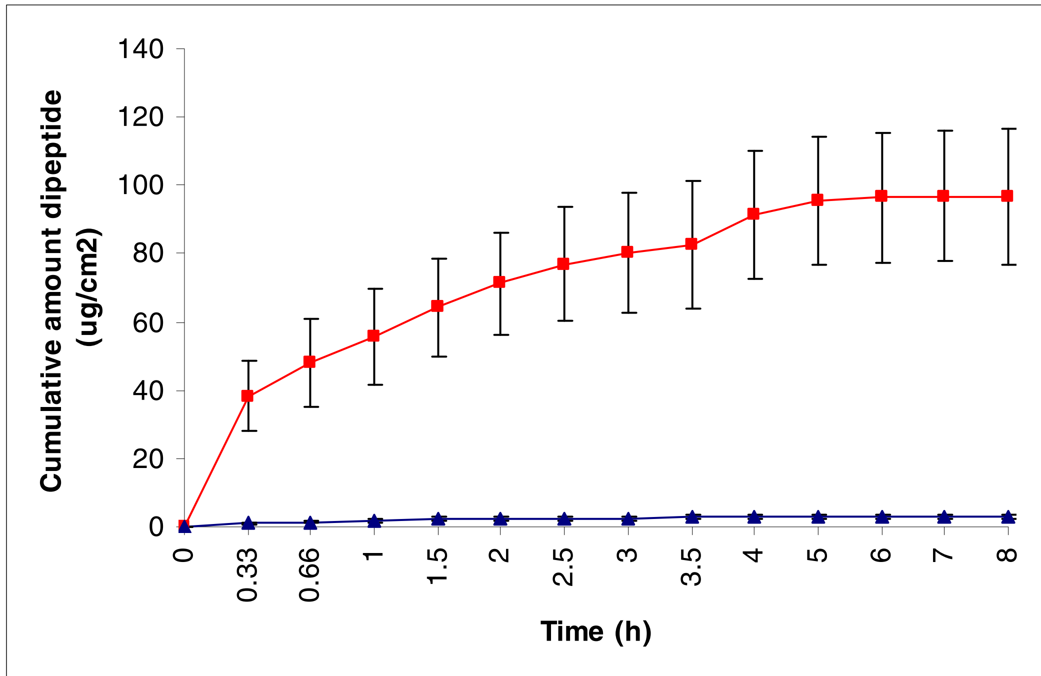

	Dipeptide					*Degradation product (amount)* 37°C with skin	*Degradation produ* 37°C with ski
	Room temperature	37°C without skin	37°C with skin	Room temperature and dark	4°C		
^{2}.h)	4.9	2.98	28.34	10.64	1.89	6.61	0.66
ation ent (R^2)	0.6434	0.981	0.9818	0.6792	0.1159	0.9828	0.9835

	Dipeptide		*Degradation product*		*% of degradation product*	
	Dermaportation	**Passive**	**Dermaportation**	**Passive**	**Dermaportation**	**Passive**
Flux (µg/cm^2.h)	19.427	0.7782	2.364	0.1393	2.392	0.021
Correlation coefficient (R^2)	0.9789	0.9404	0.9936	0.9311	0.9962	0.0011

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Treatment	**Cumulative amount at 8 h (μg/cm^2)**	**Transdermal flux (μg/cm^{2}h^{-1})**	**Permeability coefficient (k_p) (cm/h)**	**Enhancement ratio (0-2 h)**
Dermaportation	96.49	19.42	0.01942	34.67
Passive	2.77	0.77	0.00077	