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International Journal of Pharmaceutics 157 (1997) 113–121

international journal of pharmaceutics

Terpenes and ethanol enhance the transdermal permeation of the tripeptide thyrotropin releasing hormone in human epidermis

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Received 22 May 1997; received in revised form 15 July 1997; accepted 22 July 1997

Abstract

The effect of penetration enhancers on the percutaneous absorption of thyrotropin releasing hormone (TRH) across human epidermis was studied using flow-through diffusion cells with infinite dose technique. Steady-state penetration of TRH under control conditions, 0.27 ± 0.01 $\mu g/cm^2 \times h$, was achieved within 4 h and maintained for 40 h. The presence of 50% ethanol increased the permeation of TRH to 0.83 ± 0.02 $\mu g/cm^2 \times h$. The addition of individual terpenes as 3% cineole, carveol and menthone in combination with 47% ethanol increased the penetration of TRH to 0.92 ± 0.03, 1.07 ± 0.02 and 1.05 ± 0.03 $\mu g/cm^2 \times h$, respectively. The major effect of terpene compared to the addition of ethanol alone is not the increase in maximum flux, but a more rapid arrival at steady-state flux. This effect is most pronounced for cineole where maximum flux was registered 6 h after application. When calculated as total amount of penetrated TRH during the 40-h study period, the values measured in the presence of cineole, carveol and menthone with ethanol were 33.9 ± 0.8, 38.0 ± 1.6 and 33.3 ± 4.6 $\mu g/cm^2$, respectively. With a dermal patch area of 20 cm^2 this corresponds to a delivered dose in the order of 400 μg TRH per 24 h. This demonstrates the feasibility of achieving transdermal delivery of small peptides as TRH at physiologically relevant amounts by passive systems using penetration enhancers. © 1997 Elsevier Science B.V.

Keywords: Transdermal delivery; TRH; Peptides; Terpene; Ethanol; Diffusion chamber

1. Introduction

Transdermal delivery is an attractive route to administer peptide drugs. The advantages include

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PII S0378-5173(97)00235-4

more reliable uptake compared to gastrointestinal variable absorption and first-pass metabolism, continuous drug input which permits use of drugs with short elimination half-lives, rapid termination of drug delivery, and potentially improved patient compliance. The basic requirement of a transdermal drug delivery system is for the drug to penetrate the outermost layer of the skin, the stratum corneum, which is comprised of keratin-rich cells embedded in multiple lipid bilayers. Several studies demonstrate the capability of peptide drugs to penetrate across the skin (Meyer et al., 1988; Ruland et al., 1994). The transdermal fluxes, however, are reported to be low. Consequently, it is advantageous to facilitate the transdermal transport of peptides by the use of penetration enhancers (Hoogstraate et al., 1991; Hori et al., 1991). Natural volatile oils are commonly of low cutaneous irritancy and are therefore good candidates for useful skin penetration enhancement (Williams and Barry, 1992). Monoterpenes have been shown to be effective penetration enhancers for both hydrophilic drugs (Williams and Barry, 1991; Yamane et al., 1995) and lipophilic drugs (Okabe et al., 1989; Williams and Barry, 1991). These investigations test the enhancement effect on drug uptake by pre-treating the skin with neat terpenes followed by measurement of the absorption of the drug from saturated aqueous solutions.

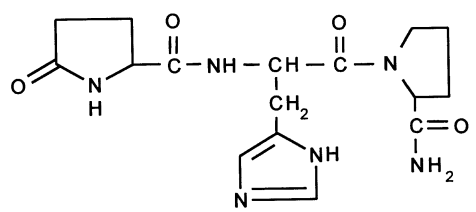
Thyrotropin-releasing hormone (TRH) is a peptide of potential clinical value for the treatment of various neurologic and neuropsychiatric disorders, including depression, schizophrenia, brain injury, acute spinal cord trauma, and Alzheimers disease (Kelly, 1995; Pitts et al., 1995). This tripeptide was the first hypothalamic hormone to be identified and chemically characterised as pGlu-His-Pro amide. Subsequently, TRH was shown to stimulate the release of thyroid-stimulating hormone (TSH) and prolactin from the pituitary, and to play a central role in regulating the pituitary-thyroid axis. Potent cardiovascular effects, including cerebral vasodilatation, are also elicited by the peptide (Koskinen and Bill, 1984; Koskinen, 1989).

In the present study, we examined: (i) the baseline level of TRH penetration through human epidermis from a buffered saline solution, (ii) the enhanced penetration of TRH by ethanol, and (iii) the enhanced penetration of TRH in a vehicle of ethanol and terpene (cineole, carveol, or menthone). To permit the study of the early phase of enhancer-induced changes in the permeability of the stratum corneum, the penetration enhancers were added to the donor compartment concurrently with the penetrant studied. The chemical structures of the tripeptide TRH and the individual enhancers are illustrated in Fig. 1.

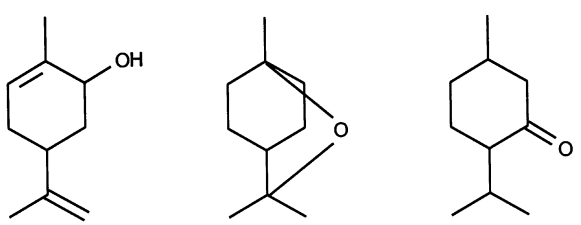
2. Materials and methods

2.1. Materials

The model permeant was TRH (L-pyroglutamyl-L-histidyl-L-prolineamide acetate), supplied by Sigma (lot. no. 33H5820). The terpenes used in their commercial form were cineole (99%), carveol (97%) and menthone (90%) (Sigma). The receptor



Thyrotropin releasing hormone
Mw: 362.4



Carveol
Mw: 152.2

Cineole
Mw: 154.3

Menthone
Mw: 154.3

Fig. 1. The structural formulae of the tripeptide TRH and the terpenes used as penetration enhancers.

fluid used was phosphate-buffered saline (PBS) solution, pH 7.4.

2.2. Preparation of epidermal membranes

Caucasian breast skin was obtained from plastic surgery. Excess fatty and connective tissues were removed, and the samples were stored at −70°C. Epidermal membranes, incorporating the anucleate stratum corneum and nucleate epidermal tissue, were prepared by the heat separation technique of Kligman and Christophers (1963). Specimens rich in appendages (≥ 6 appendages per piece) were discarded to reduce the risk of damage to the membrane during preparation and to avoid excessive transappendageal penetration. Epidermal membranes were collected on support grids and hydrated for 18 h in PBS before mounting in the diffusion cell.

2.3. Flow through diffusion cell

The penetration studies were performed in an automated system using miniature flow through diffusion cells. The design of the diffusion cells has been described previously by Magnusson et al. (1997). Sink conditions were maintained by a flow of degassed PBS solution through the receptor compartment. To maintain constant conditions of the permeant, a system for continuous exchange of the donor compartment fluid has been designed. This avoids the changes in donor fluid composition caused by penetration of test substance and by dilution due to water flux from the receptor compartment normally associated with finite dose experiments (Magnusson et al., 1997). An intermittently running peristaltic pump provides the donor compartment with fresh donor solution. The level in the donor compartment is maintained by a suction tube, fed by the same peristaltic pump, placed at the desired level in the compartment. The volume of the solution in the donor compartment can thus be maintained at 120–150 μl. By running the system continuously it can be used for washing the epithelial membranes between different stages in the experiment. The experiments were performed using two individual blocks with a total of eight diffusion cells per test. The cell has a nominal diffusion area of 0.50 cm^2. Thermostated blocks, each containing four receptor compartments and maintained at 32.0 ± 0.1°C, were placed directly above a Fractomin® autosampler. The donor compartments were covered by a Teflon plug to prevent evaporation. All parts in contact with the epidermal membrane, donor fluid and perfusion fluid are made of stainless steel, Teflon or polythene.

2.4. Penetration experiments

Prior to all experiments, the integrity of the barrier layer was verified for each epidermal membrane by the determination of water permeability; 100 μl of a [^{3}H]-H_2O solution (9.25 MBq/ml) was applied to the donor compartments. Perfusion media, 4.0 ml/h, was sampled at 15-min intervals for 1.5 h. Samples collected, or standards, were brought up to a volume of 2.0 ml by adding distilled water, to which 4.0 ml Ready Gel™ cocktail scintillation fluid was added. The radiolabelled penetrant was determined by liquid scintillation counting (Beckman LS 5000 CE scintillation counter). Membranes with a water permeability of < 5.0 $\mu l/cm^2 \times h$ were accepted for further experiments. Prior to the determination of TRH permeability, the [^{3}H]-H_2O solution was washed from the membrane with PBS solution for a minimum of 3 h, while continuously replacing the donor solution. This step reduces the residual [^{3}H]-activity to background levels.

All permeability experiments were performed using a 5.0 mg/ml concentration of TRH in the final solution. With a solubility of TRH in water of 10 mg/ml (data from supplier) this corresponds to a 50% saturated water solution. Penetration of TRH was determined using the following three basic compositions of the donor solutions: (1) PBS for the determination of control levels of TRH flux, (2) 50% (w/w) PBS and 50% (w/w) ethanol was used to study the effect of ethanol on the penetration of TRH, and (3) a homogenous enhancer solution of 3% (w/w) terpene (cineole, carveol, or menthone), 47% (w/w) ethanol and 50% (w/w) PBS was used to study the effect of terpenes on the TRH flux. The 3% level of enhancer was chosen on the basis that it is the

maximum concentration of terpenes allowing for homogenous solutions with the same levels of PBS and ethanol for all three tested terpenes. No data are available on the solubility of TRH in ethanol. The solubility of TRH in methanol is similar to that for water, 10 mg/ml, (data from supplier). The polar characteristics of TRH, partition coefficient (octanol/water, pH 7.4) of 0.0376 (Dowty et al., 1992) suggest that the polarity of the solvent is one of the main factors determining solubility. As the electric dipole moments for methanol and ethanol are close, 1.70 and 1.69 respectively (West, 1976), the solubility for TRH can be assumed to be similar in the two alcohols. The effect on solubility of the addition of 3% terpene is assumed to be negligible.

At the start of the experiment, 100 μl of the respective solution was applied to the donor compartment. The compositions of the solution in the donor compartments were maintained constant during the entire duration of the experiment by the continuous exchange of the test solutions at the rate of 50 μl/h. All the epidermal membranes were prepared from the same skin source. Perfusion media with a flux of 1.0 ml/h was sampled for 40 h and analysed by radio-immunoassay.

2.5. RIA analysis of TRH

Thyrotropin releasing hormone was analyzed by radio-immunoassay (Euro-Diagnostica, Apeldoorn, The Netherlands), that uses a polyclonal antibody raised in rabbit against TRH. The radio-immunoassay was performed according to the manufacturer's directions with the exception that no extraction with ethanol was performed before the sample was analyzed. The assay has a detection limit of 5 pg/ml and an interassay variation of less than 10%.

2.6. Statistics

The results were statistically analysed by Student's t-test (two-tailed) for unpaired observations and by regression analysis. Statistical comparisons were performed between the TRH values in the presence of ethanol and/or terpene vs. the control penetration of the peptide. A difference was considered statistically significant if $p \leq 0.05$. Values are reported as means $\pm$ S.E.M.

3. Results

Isolated human epidermis was found to be permeable to the thyrotropin releasing hormone. The measured control penetration of TRH was 0.27 ± 0.01 $\mu g/cm^2 \times h$ at steady-state (Fig. 2). Steady-state flux was obtained within 4 h of application with a significant increase between the samples taken at 2 and 4 h ($p \leq 0.05$, Student's t-test). In the presence of ethanol, the flux of TRH showed an increase up to the measurement at 14 h, in contrast to PBS alone, and the rate of increase during the period of 2–14 h was 0.07 $\mu g/cm^2 \times h^2$. The flux of TRH was significantly higher compared to control conditions from 12 h and later ($p \leq 0.05$, Student's t-test). The value measured at 14 h, 0.82 ± 0.06 $\mu g/cm^2 \times h$, is not significantly different from the steady-state conditions measured after 14 h. Expressed as permeability coefficients, the penetration of TRH under control conditions and TRH with ethanol, was 5.4 and 16.6×10^{-5} cm/h, respectively. The enhancement factor for TRH was 3.1 in the presence of ethanol, compared to the control flux of TRH.

The presence of 3% terpene (cineole, carveol or menthone) and 47% ethanol significantly increased the flux of TRH, compared to the control penetration of the peptide (Fig. 2). In the presence of cineole and ethanol, the flux of TRH showed a rapid increase with a maximum after 6 h. Statistically significant differences of the TRH flux in the presence of cineole and ethanol were observed at 6 h and all subsequent observations ($p \leq 0.05$, Student's t-test), compared to the control flux of TRH. From 10 h onwards, the flux of TRH is stable with a mean value of 0.92 ± 0.03 $\mu g/cm^2 \times h$ during the period of 10–40 h. This corresponds to a permeability coefficient of 18.4×10^{-5} cm/h. Expressed as an enhancement factor for TRH in the presence of cineole and ethanol compared to the control flux of TRH, the value was 3.4. Carveol and menthone in combination with ethanol increased the flux of TRH, not as rapidly as with cineole, but with an apparent gradual

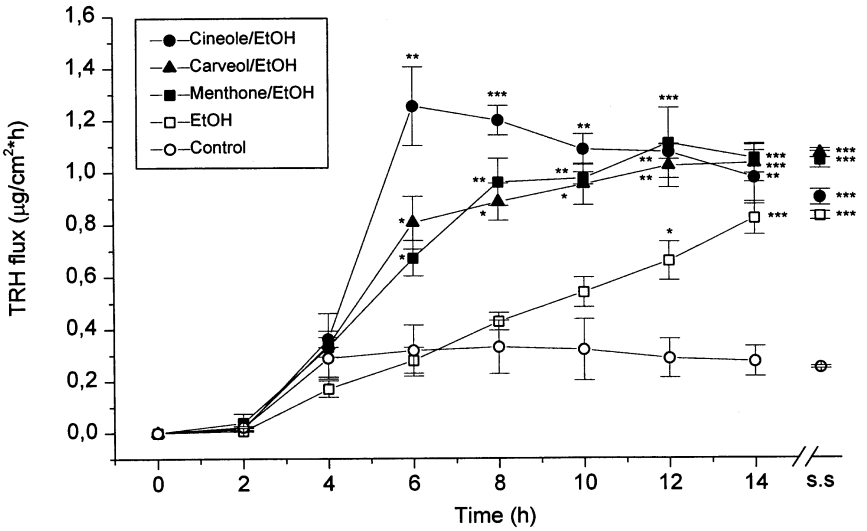


Fig. 2. Penetration of TRH through human epidermal membranes. Filled symbols represent the values for flux measured in the presence of 3% terpene (cineole, carveol or menthone) in combination with 47% ethanol and 50% PBS ($n = 3$). Open squares represent the values for the vehicle solution of 50% ethanol/PBS ($n = 5$) and open circles represent the control values for TRH ($n = 3$). Single symbols represent the steady-state (s.s) flux at the interval 14–40 h. Values are means ± S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ for presence of ethanol and/or terpene vs. control penetration of TRH, unpaired Student's t-test.

increase up to 12 h. At steady-state, 14–40 h, the measured fluxes of TRH are 1.07 ± 0.02 and 1.05 ± 0.03 $\mu g/cm^2 \times h$, respectively. Expressed as enhancement factors compared to the control flux of TRH, the values were 4.0 and 3.9, respectively. This corresponds to a permeability coefficient for TRH in the presence of carveol of 21.4×10^{-5} cm/h, and in the presence of menthone of 21.0×10^{-5} cm/h. Statistically significant differences in the flux of TRH between the solution containing ethanol and the solutions containing terpene/ethanol were observed at 6 h and all subsequent observations ($p \leq 0.05$, Student's t-test).

The total amount of penetrated TRH as a function of time is illustrated in the cumulative plots in Fig. 3. The amount of penetrated TRH under control conditions during 40 h was 10.0 ± 1.7 $\mu g/cm^2$. The presence of ethanol increased the amount of penetrated TRH to 26.5 ± 2.8 $\mu g/cm^2$. In the presence of terpene (cineole, carveol or menthone) and ethanol, the amounts of penetrated TRH after 40 h were 33.9 ± 0.8, 38.0 ± 1.6 and 33.3 ± 4.6 $\mu g/cm^2$, respectively (Fig. 3). Statistically significant increases of total penetrated TRH amounts were observed in the presence of terpene/ethanol or ethanol alone compared to control conditions ($p \leq 0.05$, Student's t-test). Significantly more TRH penetrated in the presence of terpene/ethanol compared to ethanol alone ($p \leq 0.05$, Student's t-test). This difference is mainly dependent on the reduced lag time observed for the solutions containing terpene.

4. Discussion

During the past decade, the feasibility of the dermal route for systemic drug delivery has been established. The model chosen for the studies presented here is one were penetrant and penetration enhancers are introduced concurrently. The set-up is relevant for the situation where a drug is transdermally administered via a patch containing, in addition to the drug, a penetration enhancer. The integrity of each epidermal membrane was controlled by measurement of water flux, and the values obtained are in agreement with previously reported data (Magnusson et al., 1997). The tripeptide studied, TRH, was adminis-

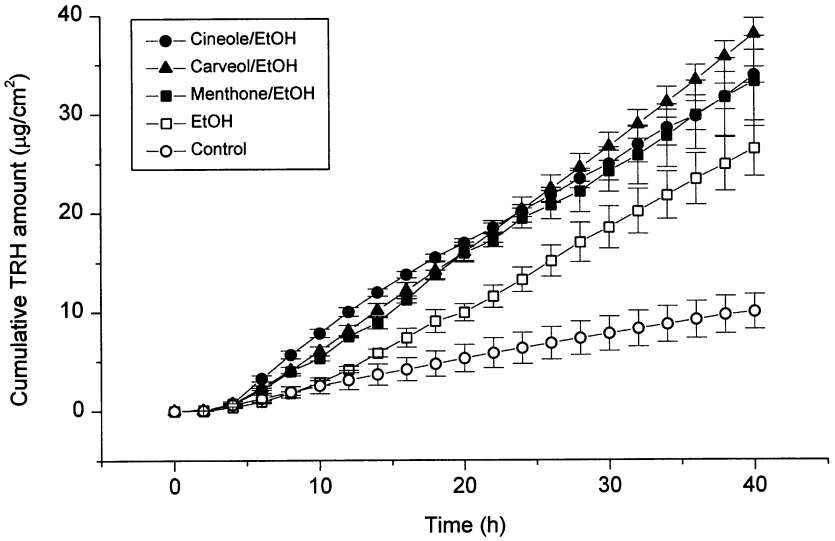


Fig. 3. Cumulative plot of TRH penetration as a function of time. Filled symbols represent the values for flux measured in the presence of 3% terpene (cineole, carveol or menthone) in combination with 47% ethanol and 50% PBS. Open squares represent the values for the vehicle solution of 50% ethanol/PBS and open circles represent the control values for TRH. Values are means ± S.E.M.

tered by infinite dose technique. This was to avoid the errors in finite dose design due to the changes in the donor fluid composition caused by loss of substance during penetration and by dilution through influx of receptor fluid. The design presented here has shown its utility through the ability to provide steady-state conditions during 40 h of application.

At a concentration in the donor compartment of 5.0 mg TRH/ml, the tripeptide was capable of passing the human epidermis at a flux rate of 745 pmol/h per cm^2. In the presence of 50% of the enhancer ethanol, the maximum flux of TRH increased approximately three times. The mechanisms by which ethanol induces microstructural changes and increased diffusivity are not fully understood. Lipid extraction and osmotic expansions may be the most plausible explanations (Krill et al., 1992; Williams and Barry, 1992). Fig. 2 illustrates a more rapid increase during the initial phase for the flux of TRH, in the presence of both terpene and ethanol compared to ethanol alone. In the presence of ethanol, TRH showed a steady and gradual increase in flux during the period of 2–14 h. In contrast, the addition of terpenes results in a situation where the increase in flux is most pronounced during the period from 2 up to 8 h (in the case of cineole up to 6 h). After 6 h of application, the fluxes of TRH under control conditions and in the presence of cineole and ethanol, were already at maximum levels (Fig. 2). At the same time only 30% of the maximum ethanol effect was reached and approximately 70% of the effects of carveol/ethanol and menthone/ethanol. This apparently slow effect of ethanol alone compared to the combinations of ethanol and terpene is also illustrated by the right shift of the ethanol curve in Fig. 3. An interesting observation is that neither ethanol alone nor the combination of terpene and ethanol has any significant effect on the flux of TRH during the period 2–4 h. The enhancers fail to induce an earlier onset of the TRH flux through the epidermal membrane. This suggests that the initial binding to, and the saturation of, the epidermal membrane by TRH is not affected by these enhancers. The enhancing effect by terpenes or ethanol is not observed until the initial stages of the penetration process are established. Alternatively, the time period for establishing the effect

on the lipid structures of the stratum corneum by the enhancers is such that no effect can be detected during the first 4 h of the preparation.

The presence of both terpene and ethanol in the donor solution increased the flux of TRH approximately four times, compared to the control conditions during steady-state. In a previous study we determined the permeability coefficient for ethanol in a solution of 50% ethanol/PBS to be $1.9 \pm 0.2 \times 10^{-3}$ cm/h (Magnusson et al., 1997). For the smaller and more polar water molecule, the permeability coefficient was $3.6 \pm 0.5 \times 10^{-3}$ cm/h. The penetration of ethanol and water increased in the presence of 3% carveol by a factor of 4.4 and 3.5, respectively. This coincides with the enhancing effect for carveol on TRH flux found in this study. This result supports the hypothesis proposed by Magnusson et al. (1997) that enhancer-induced changes in water permeability could be used as a predictive tool for the flux of other moderate sized polar substances.

According to Fick's first law, the driving force for diffusion is the activity gradient over the membrane. The calculation of enhancement factors thus needs to take this into account. According to the solubility data for TRH, the PBS solution of 5.0 mg TRH/ml can thus be considered as a 50% saturated solution. Based on the data for methanol the solubility of TRH is assumed to be similar to that for water. Further, the solubility in a water/ethanol mixture can be estimated to be in the same range. As a rough estimate, the PBS/ethanol and the PBS/ethanol/terpene would have the same activity of TRH as the PBS/TRH solution. A lower solubility of TRH, that is an increase in saturation, could partly explain the enhancement factors observed in the presence of ethanol and ethanol/terpene. However, compared to a 50% saturated solution under control conditions, the maximum increase would be in the range of two for a saturated solution. The observed enhancement factors in the range of four are thus interpreted as a true enhancement of flux in the presence of ethanol and ethanol/terpene. The strict application of Fick's first law requires ideal conditions. The stratum corneum is a complex multilamellar structure with possible differing concentration gradients of the different components of the donor solutions. In addition, interaction between ethanol and terpenes with e.g. the lipids of the stratum corneum could require a more complex model to fully explain the conditions for penetration of TRH observed under the different conditions used here.

The mechanism by which terpenes act as penetration enhancers is probably through disrupting the lipid structure of the stratum corneum, thereby increasing the diffusion coefficient of the polar drug in the membrane (Williams and Barry, 1992). Somewhat surprising is the result that ethanol is as effective an enhancer as the terpenes tested. This said with the reservation that only one combination of concentrations has been tested. The effects of ethanol and terpenes is also not additive. The net result for both the combination of terpene/ethanol and ethanol alone has an enhancement factor of approximately 3–4. The main difference is the slower onset of the effect with ethanol alone. This result suggests that ethanol and the terpenes exert their effect on the same point in the membrane but possibly by different mechanisms. If ethanol has its effect by slowly extracting lipids from the stratum corneum, the terpenes more rapidly modifies the bilayer structure of the same lipid components. The extraction of lipids by ethanol, when these have already been modified by terpenes, has little or no influence on the TRH flux. The low enhancing effect could be explained by the physicochemical properties of TRH which results in a slower diffusion through the stratum corneum compared to a more lipophilic compound. TRH is a weakly basic tripeptide with a partition coefficient (octanol/water, pH 7.4) of 0.0376 (Dowty et al., 1992). There is good evidence that the stratum corneum is much more permeable to neutral molecules than the salts of the weak acids or bases (Swarbrick et al., 1984). The relatively low enhancement factors obtained compared to other studies (Okabe et al., 1989; Williams and Barry, 1991; Yamane et al., 1995) could also be explained by the fact that the investigators pretreated the skin with neat terpene.

The success of transdermal systems depends on the ability of the drug to permeate the skin in sufficient quantities to elicit its desired therapeutic

effect. The effectiveness of transdermal delivery of TRH via the use of enhancers can be assessed by comparing the penetrated amount of the peptide under control conditions with the amount in the presence of the enhancers. If the area of the patch for transdermal delivery is 20 cm^2, it would be possible to deliver 130 μg of TRH over 24 h from a saline solution. In the presence of 50% ethanol, the delivered amount of TRH would increase two times to 260 μg over 24 h. By incorporating penetration enhancers, such as terpenes in the presence of ethanol, a penetrated amount of approximately 400 μg TRH over 24 h should be possible. The difference between 260 and 400 μg TRH is mainly due to the reduced lag time in the presence of terpene and ethanol compared to ethanol alone. The total amount of 400 μg TRH is almost in the same order (500 μg) as the amount of TRH given to humans by infusion or injection during 24 h (Mitsuma and Nogimori, 1984; Lampe et al., 1989). The amount delivered in this study does not represent the maximally obtainable value since the peptide and the terpene were applied in low concentrations (TRH amount of 5 mg/ml respectively 3% of the terpene), and no optimisation studies were done concerning the type of vehicle.

In conclusion, this study demonstrates that this approach, where the enhancer and co-solvent are concurrently added with the penetrant, may be useful for enhancing the delivery of peptides across human skin. The topical application of a peptide with a putative therapeutic application may provide a practical route for local delivery in the treatment of certain dermatopathies. Further studies should be done in order to show whether systemic effects can be elicited by the transdermally-delivered peptide.

Acknowledgements

This study was supported by the National Defence Research Establishment, Umeå, Sweden. The authors are grateful to Assistant Professor Bo Forslind for valuable discussions, Mrs Birgitta Nilsson for technical assistance, and to the personnel at the Department of Plastic Surgery, Umeå University Hospital, for the supply of excised skin.

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