Dermal Peptide Delivery Using Enhancer Molecules and Colloidal Carrier Systems – Part I: Carnosine

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Key Words

Peptide • Penetration enhancer • Microemulsion • Skin penetration • 1,2-pentylene glycol • Carnosine

Abstract

Due to the lipophilic properties of the uppermost skin layer of the stratum corneum (SC), it is highly challenging to attain therapeutic concentrations of active substances; hydrophilic drugs, in particular, penetrate poorly. The purpose of this study was the improvement of the topical bioavailability of the hydrophilic dipeptides L-carnosine and its related compound N-acetyl-L-carnosine. Different strategies were investigated. On the one hand, an enhancer molecule, 1,2-pentylene glycol (PG), was added to a standard preparation, and on the other hand, a microemulsion (ME-PG) system was developed. Both were compared to the standard formulation without an enhancer molecule. For all 3 preparations, the penetration of the peptides in ex vivo human skin was investigated. This allows statements to be made regarding dermal penetration, localization and distribution of the active substances in each skin layer as well as the influence of vehicle variations, in this case, the addition of PG or the incorporation of N-acetyl-L-carnosine in an ME-PG system. For L-carnosine and N-acetyl-L-carnosine, the use of the standard prepa-

ration with PG resulted in a significant increase of the substance within the SC. Approximately 6-fold and higher dipeptide concentrations in the SC and in the viable skin layers were detected at all experimental periods compared to the formulation without the enhancer molecule and the ME-PG. High concentrations of the compounds were found after a short period of time in the viable skin layers after applying the enhancer molecule, even in concentrations of 5%. The application of the colloidal carrier system did not lead to a higher penetration rate of N-acetyl-L-carnosine in comparison to both standard preparations, although it must be said that the microstructure of the investigated ME-PG might not have been optimal for the hydrophilic properties of the dipeptide.

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Introduction

Since the skin is the largest organ of the human body it is an important target site for the application of drugs. However, in order to reach therapeutic drug concentrations, the uppermost barrier, the stratum corneum (SC), has to be penetrated [1]. Due to the lipophilic properties of the SC, hydrophilic drugs, in particular, penetrate

poorly. Therefore, to attain topical bioavailability of hydrophilic active substances, like peptides or peptide-like substances, is highly challenging. The application of peptide drugs is a growing field in therapeutics because extensive proteolytic degradation and poor permeability through the intestinal mucosa in the gastrointestinal tract exclude oral administration [2]. The aim of this study was to attain bioavailability of skin-relevant dipeptides via cutaneous application. Carnosine, with its molecular weight of 226.23 Da and its partition coefficient (log P) of -2.972 ± 0.436 [3] and related compounds such as N-acetyl-L-carnosine (molecular weight 268.27 Da; log P -2.559 ± 0.561 [3] or carbinine are natural histidine-containing dipeptides. They are constituents of several tissues. Studies have demonstrated that carnosine has excellent potential to act as a natural antioxidant with hydroxyl-radical-, singlet oxygen-scavenging and lipid peroxidase activities [4, 5]. However, the level of carnosine in tissues is controlled by a number of enzymes transforming carnosine by decarboxylation, acetylation or methylation into its related substances such as N-acetyl-L-carnosine and carbinine or into the amino acids histidine and β -alanine [6]. Pegova et al. [7] have shown that, although N-acetyl-L-carnosine and carbinine are metabolically related to carnosine, they are not substrates for the enzyme carnosinase or other dipeptidases. Therefore, both are more suitable for therapeutic treatment as antioxidants because of their ability to prolong and potentiate physiological response [7–9].

The focus of this project was to investigate the penetration of L-carnosine and – because of the expected biodegradation of the peptide – of N-acetyl-L-carnosine into human skin from a standard preparation (hydrodispersion gel). For the preclinical assessment of the topical applicability of the test preparations, *ex vivo* penetration tests in human skin were carried out. This permits conclusions regarding the dermal penetration, localization and distribution of the active substances in each skin layer on a certain time-scale (concentration-time-profile). Furthermore, the influence of vehicle variations, in this case, the addition of 1,2-pentylene glycol (PG) or the incorporation of N-acetyl-L-carnosine in an o/w microemulsion (ME-PG) system, can be monitored.

PG is a short-chain molecule with hydrophilic and lipophilic moieties. Its amphiphilicity provides an excellent solubilizing capacity for cosmetic ingredients or poor water-soluble drugs and, hence, may improve their penetration behavior. The amphiphilicity of the molecule might be the reason for its ability to interact with biological membranes and oil/water interfaces in formulations.

Duracher et al. [10] and Heuschkel et al. [11] already showed that higher 1,2-alkanediols like PG are able to enhance the dermal availability of model drugs even in low concentrations. Furthermore, PG is an excellent moisturizer and antimicrobial agent. Its multifunctional properties make this colorless and odorless liquid suitable for many cosmetic formulations [12]. Aside from the addition of the enhancer molecule to standard preparations, the use of colloidal carrier systems such as MEs is also an appropriate strategy for the improved dermal delivery of drugs. MEs offer benefits like high solubilization capacity, noninvasive administration or easy preparation and application. Due to their penetration-enhancing properties, these systems are able to increase the therapeutic levels of drugs with unfavorable physicochemical properties. MEs are thermodynamically stable systems of oil and water stabilized by surfactants and cosurfactants. MEs usually appear in different microstructures. According to the major compound of the system, they can be categorized as water-in-oil (w/o) or oil-in-water (o/w) MEs with a droplet-like structure embedded in a monodisperse main phase. Bicontinuous structures can occur, if the system contains equal amounts of oil and water. However, the common feature of all appearing microstructures in MEs is that they are highly dynamic, undergoing continuous and spontaneous fluctuations. Although research of MEs as carriers for peptide drugs is at an early stage, it has proven to be of interest [1, 13]. Therefore, the penetration of the model substances L-carnosine and N-acetyl-L-carnosine was also investigated using the most interesting carrier system.

Materials and Methods

Materials

L-carnosine, N-acetyl-L-carnosine, PG (Hydrolite 5) and hydrodispersion gel (HDG) were obtained by courtesy of Symrise AG (Holzminden, Germany). Formic acid was supplied by Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). Plantacare 2000 UP and Cetiol B were kindly offered by Cognis GmbH (Düsseldorf, Germany). Phospholipid GmbH (Köln, Germany) donated Phospholipon 90 G. HPLC grade methanol was obtained from VWR International, (Darmstadt, Germany). Water was of bidistilled quality. Human breast skin was kindly offered by the Department of Dermatology and Venereology with the approval of the independent ethics committee of the Medical Faculty at the Martin Luther University Halle-Wittenberg.

Formulations

The HDG, a nontransparent and semisolid formulation, consisted of 1.0% PEG-9 tridecyl ether/PEG-5 octanoate, 3.0% cetyl octanoate, 2.0% stearic acid, 0.25% acrylates/C10–30 alkyl ac-

rylate crosspolymer, 3.0% liquid paraffin, 4.0% octyldodecanol, 0.5% dimeticone, 0.5% sodium hydroxide for pH adjustment (pH 7.0) and purified water up to 100.0%. For the penetration experiments, HDG was partially modified by adding 5% PG (HDG-PG) to replace 5% of the purified water.

The ME-PG was made of 8.33% Phospholipon 90 G, 16.67% Plantacare 2000 UP, 20% PG, 5% Cetiol B and purified water up to 100.0%. The active ingredient concentration in each test formulation was 2.0%. All given percentage values represent % (w/w). The ME-PG system has already been characterized and used for penetration in previous work [14].

Penetration Studies *ex vivo*

The investigations were carried out using full-thickness excised human skin from reduction mammoplasty. The tissue sections were postoperatively cleaned with mull pads and isotonic NaCl solution. The subcutaneous adipose tissue was mechanically dissected and discarded. Circular pieces of skin (20 mm in diameter) were punched, hermetically sealed in tinfoil, packed in an occlusive polyethylene bag and stored at -20°C for 2–3 weeks.

For the experiment, the skin pieces were completely defrosted at room temperature and the surface was dried using cotton pads. The investigations were performed in a glass Franz diffusion cell [15]. Phosphate-buffered saline pH 7.4 was used as acceptor phase. The specimens were stretched on the diffusion cell which was preheated to 32°C , enabling the underside of the skin (with the filter gauze) to directly hit the acceptor medium which was continuously stirred in order to reduce the thickness of the diffusion layer. Skin specimens of 3 donors were used. Three pieces of skin from each donor were used per test preparation, one for each of the respective application periods of 30, 300 and 1,000 min.

An amount of 20 mg of each formulation was applied directly onto the skin under nonoccluded conditions. To determine the amounts of penetrated active substance at the respective application period, the skin specimens were removed from the diffusion cell and the remaining preparation (residue) was carefully eliminated from the surface using a cotton pad. Three punch biopsies (0.2827 cm^2 in size) were taken (Kromayer punch, Stiefel-Laboratorium, Offenbach, Germany) and the individual skin layers were separated horizontally using a cryomicrotome (Jung, Heidelberg, Germany) according to the scheme presented in table 1.

The different skin layer sections were placed in 1.5-ml Eppendorf tubes. A mixture of methanol and water (70:30), acting as extracting agent, was added with a volume of between 300 and 500 μl depending on the expected amount of the active ingredients. The tubes were then mixed using a vortex mixer for about 1 h, kept refrigerated overnight and mixed again the next day for another hour before analysis. Both substances that were removed from the skin surface using swabs were extracted for 12 h with 5 ml of a mixture of methanol and water (70:30). Quantification of L-carnosine and N-acetyl-L-carnosine in all samples was carried out by HPLC.

Analytics

HPLC analysis of L-carnosine and N-acetyl-L-carnosine was carried out using Agilent 1100 LC System (Agilent, Waldbronn, Germany) equipped with a vacuum degasser, a binary pump, an autosampler and a diode array detector. The LC column used was 124/4 Nucleosil 300-7 Protein RP4 (Macherey-Nagel GmbH & Co. KG, Düren, Germany) maintained at a temperature of 40°C for

Table 1. Skin layer separation scheme for L-carnosine and N-acetyl-L-carnosine

Nonpenetrated remedy	swab
Stratum corneum	10 μm
Viable epidermis	4 sections of 20 μm = 80 μm
Dermis 1	5 sections of 40 μm = 200 μm
Dermis 2	5 sections of 40 μm = 200 μm
Dermis 3	5 sections of 40 μm = 200 μm
Dermis 4	5 sections of 40 μm = 200 μm
Dermis 5	5 sections of 40 μm = 200 μm
Permeated part	and remaining stump acceptor fluid

L-carnosine, and Lichrospher 60 RP select B 5- μm 125 $\times$ 4 mm (Merck Chemicals, Darmstadt, Germany) at a temperature of 25°C for N-acetyl-L-carnosine. The mobile phases consisted of (A) water adjusted to a pH value of 3.75 using formic acid and (B) methanol. The gradient program was 0–6 min 100% A, 6–7 min 100% A to 50% B, 7–14 min 50% B and 14–15 min 100% A, at a flow rate of 1.5 ml/min for L-carnosine and 1.0 ml/min for N-acetyl-L-carnosine. The injection volume of each sample was 10 μl . Detection of L-carnosine and N-acetyl-L-carnosine was carried out at wavelengths of 222 and 210 nm, respectively.

Statistics

Statistical significance was determined by 1-way ANOVA followed by Dunnett's Test as post hoc analysis. Differences were considered as significant at $p < 0.05$.

Results and Discussion

Skin penetration of L-carnosine and N-acetyl-L-carnosine was determined using Franz diffusion cells. Penetration profiles of both dipeptides were generated to evaluate the influence of the enhancer molecule PG and also the influence of the ME-PG on the availability of N-acetyl-L-carnosine in the skin.

In figure 1 the relative amounts of L-carnosine achieved at different points of time following the application of HDG and HDG-PG within the different skin compartments are plotted.

The dipeptide concentrations found in the SC were low for both formulations. With increased incubation time, the amounts of L-carnosine found in this compartment stayed on the same level, i.e. under 1.5%. The addition of PG had no influence on the penetrated amount of peptides in the uppermost layer. Since the SC is known for its high lipophilicity, the hydrophilic L-carnosine most likely did not accumulate, but rather penetrated into

Fig. 1. Comparison of the relative amounts of L-carnosine in the stratum corneum (SC), the viable epidermis (EP) and the dermis (DR) following application of an HDG and HDG-PG at different incubation times (data given as mean $\pm$ standard deviation, $n = 9$; # $p < 0.05$, * $p < 0.01$). No L-carnosine was detected in the acceptor compartment.

the more hydrophilic viable skin layers. Compared to SC, higher amounts of L-carnosine could be detected in the viable epidermis after only 30 min. Differences between both formulations could be observed after 100 min of incubation time. The application of HDG-PG resulted in significantly higher concentrations when compared to the preparation without the enhancer molecule. An approximately 6-fold increase could be detected. After 300 min, the same trend was evident but generally lower concentrations were detected. As found for the viable epidermis, the use of the enhancer molecule resulted in higher concentrations of L-carnosine within the dermis in comparison to the HDG alone. L-carnosine concentrations up to 17.66% of the applied dosage were found, whereas less than 5% could be detected when only the HDG was applied. Already 100 min later, a 5-fold higher L-carnosine content could be determined in the viable epidermis and dermis. In all experiments and at all experimental periods, no L-carnosine could be identified in the acceptor compartment. The results of the penetration studies for L-carnosine showed that the HDG-PG increased the penetration of the dipeptides significantly.

However, the total recovered L-carnosine content (i.e. the sum of L-carnosine in the swab, all skin layers and the acceptor phase) after 300 min of application time indicates the instability of L-carnosine in skin tissues. This

was already observed in preliminary experiments. The instability of the active ingredient could already be observed after incubation times of 30 and 100 min and could also be an explanation why no concentrations of the active substance were found in the acceptor compartment. Approximately 30% of the applied dose was rediscovered.

For this reason, penetration profiles of the biodegradable-stable dipeptide N-acetyl-L-carnosine were also determined. Although L-carnosine showed promising results and indicated that the enhancer molecule had a positive influence on the penetration of the active ingredient, the results have to be confirmed because of the low recovery rate in the skin. The penetration profiles of N-acetyl-L-carnosine achieved at different points of time after the application of HDG, HDG-PG and an o/w ME-PG system within the different skin compartments are displayed in figure 2.

The use of HDG-PG resulted in significantly i.e. with a trend towards higher dipeptide concentrations in the SC and the viable skin layers represented by the viable epidermis and the dermis. After 1,000 min of incubation, 57% of the applied N-acetyl-L-carnosine had penetrated into the viable skin layers and the acceptor compartment, but the standard preparation without PG only showed concentrations of 38%. Interestingly, the use of the col-

Fig. 2. Comparison of the relative amounts of N-acetyl-L-carnosine in the stratum corneum (SC), the viable epidermis (EP), the dermis (DR) and the acceptor compartment (AC) following application of an HDG and HDG-PG as well as a microemulsion (ME-PG) at different incubation times (data given as mean $\pm$ standard deviation, $n = 9$; # $p < 0.05$, * $p < 0.01$).

loidal carrier (ME-PG) system resulted in the lowest penetrated amount of 20% of the applied dose. The highest concentrations of dipeptide were found in the dermis; hereby confirming the trend observed for L-carnosine. The peptide did not accumulate in the lipophilic skin barrier, but penetrated into the more hydrophilic viable skin layers. Although the HDG without the added PG also offered appropriate liberation and penetration properties for N-acetyl-L-carnosine, the addition of the penetration modulator to the gel resulted in a faster and higher penetration into the skin because its hydrophilic prop-

erties limited the dipeptide partitioning and penetration into the lipophilic SC. The bioavailability of the active substance was improved and high concentrations could be achieved at the target sites. For N-acetyl-L-carnosine, the addition of PG revealed (similar to L-carnosine) better penetration results compared to the HDG and, interestingly, also when compared to the o/w ME-PG.

The incorporation of diols, particularly PG, is widely used in topical pharmaceuticals or cosmetic products. The enhancement properties of PG have been studied extensively. The precise role of the enhancer molecule is not

yet totally clear. A modification of the nonpolar route is discussed as well as a solvation of the alpha-keratin and an adsorption to hydrogen-binding sites [16, 17]. In addition, a solvent drag effect of the glycol was suggested [18]. Furthermore, the penetration of the diol molecules into the SC and thereby a modification of the drug solubility in the skin and its partition coefficient [19] and an increased driving force for diffusion can be considered [11, 20]. Although most studies were performed examining propylene glycol as an enhancer substance, they seem to be conferable to higher glycols [11].

However, only a few studies have dealt with the enhancement properties of PG used in this study. Duracher et al. [10] investigated the effect of propylene glycol and PG on the permeation of caffeine. They found that PG in small concentrations of 2.5 or 5% was superior to propylene glycol in binary water solutions and the control relating to the permeation enhancement of the hydrophilic drug. They also observed a dose-dependent effect. Evenbratt and Faergemann [21] studied the effect of 1,5-pentylene glycol in different concentrations on the penetration of terbinafine. The experiments revealed that the best drug concentrations in the skin were obtained with an enhancer concentration of 5%. They did not measure a dose-dependent effect like Duracher et al. [10] because the higher 1,5-pentylene glycol amount of 20% resulted in lower terbinafine in the skin and almost similar substance concentrations in the receptor fluid. The authors suggested that this was most likely caused by a better interaction of the drug with the enhancer molecules at lower amounts, and the fact that the SC is lipophilic so that a high amount of terbinafine was absorbed there.

Heuschkel et al. [11] investigated the modulation of the skin penetration of the lipophilic compound dihydroavananthramide D by 1,2-alkanediols. They assumed that the penetration enhancement observed when a mixture of 4% of PG and 1,2-butylene glycol was added to a hydrophilic cream could be caused by the favored partitioning into the skin due to an increased solubility of dihydroavananthramide D within the cream. They also compared 2 different vehicles, the hydrophilic cream and the HDG used in this study containing the 4% glycol mixture. Improved penetration results were obtained for the drug after applying the HDG. They assumed that this might be as a result of the collapse of the gel structure when the gel contacts with the electrolyte-containing skin surface causing the fast release of both the drug and the glycols. This could also explain the good penetration results of the dipeptides from the gel without an enhancer molecule discussed in this study.

Although MEs are known for enhancing the penetration properties of both hydrophilic and lipophilic drugs, the use of the o/w ME-PG as a carrier for N-acetyl-L-carnosine resulted in the lowest penetration rates compared to the HDGs. In particular, the amounts in the acceptor compartment differed significantly when compared to the application of both HDGs. One explanation for this could be that N-acetyl-L-carnosine was presumably not located in the colloidal phase of the ME-PG. Since the peptide was hydrophilic peptide it can be assumed that it had distributed into the monocontinuous phase which consisted partly of water and partly of PG. Thereby, a high drug mobility could not be realized and the diffusion was not improved. Another reason could be the excellent solubilization capacity of the investigated ME-PG. Due to the location in the main phase, the thermodynamic activity is probably much lower when compared to a location in the colloidal phase. In this case, the o/w ME-PG might not provide optimal conditions for the dermal delivery of the N-acetyl-L-carnosine with its specific physicochemical properties. In contrast to Heuschkel et al. [22] who detected higher dihydroavananthramide D concentrations in the acceptor compartment after applying an ME compared to a semisolid formulation with enhancer molecules, in this study, deeper skin layers could not be reached with a hydrophilic compound such as N-acetyl-L-carnosine.

Conclusions

It can be assumed that PG is a suitable enhancer molecule for the dermal penetration of hydrophilic peptides, even in concentrations as low as 5%. High concentrations of the peptides were found after a short period of time in the viable skin layers and no L-carnosine was detected in the acceptor compartment of the Franz diffusion cell. The application of a colloidal carrier system did not lead to a higher penetration rate of N-acetyl-L-carnosine, although it must be said that the microstructure of the ME-PG used might not have been optimal for the hydrophilic properties of this dipeptide.

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