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RESEARCH ARTICLE

Physicochemical characterization of native glycyl-L-histidyl-L-lysine tripeptide for wound healing and anti-aging: a preformulation study for dermal delivery

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Abstract

This study investigates the physicochemical properties of glycyl-histidyl-lysine-copper (GHK-Cu) to support the development of a formulation for effective topical delivery. The solubility and distribution coefficients (log D) were investigated using conventional methods and GHK concentrations were quantified with a validated stability-indicating reversed phase high performance liquid chromatography (RP-HPLC) method. In addition, the stability of GHK-Cu under stressed conditions and the compatibility with some potential formulation components were assessed. The peptide was susceptible to hydrolytic cleavage under basic and oxidative stressors and to a lesser extent acidic stress with first-order degradation profiles. Surprisingly, the peptide was stable in water and in pH (4.5–7.4) buffers for at least two weeks at 60 °C. The HPLC in conjunction with mass spectrometry identified three key degradation products, one of which was the constituent amino acid histidine. The distribution coefficients in octanol-phosphate buffered saline indicated the highly hydrophilic nature of GHK-Cu with log D values between −2.38 and −2.49 at pH range of 4.5–7.4. Furthermore, GHK-Cu was compatible with Span 60 based niosomes but less stable in the presence of the negatively charged lipid dicetyl phosphate. In summary, the preformulation studies provided information useful to deliver the GHK-Cu complex by carrier.

Keywords

Copper carrier peptide, glycyl-L-histidyl-L-lysine copper, mass spectrometry, skin delivery, stability-indicating HPLC

History

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Introduction

The tripeptide glycyl-histidyl-lysine (GHK) spontaneously forms a high affinity complex with copper (II) ions forming glycyl-histidyl-lysine-Cu (GHK-Cu, Figure 1)[1]. X-ray analysis identified the residues glycine and histidine as copper binding whereas the side chain of lysine may be involved in the recognition of receptors responsible for the uptake of copper into cells[2]. Therefore, GHK is also known as a carrier peptide, facilitating the transportation of the trace element copper in the body.

To date, GHK-Cu has been investigated as a wound healing and anti-aging cosmetic agent following topical applications. The importance of copper to cells was well described by Rucker et al.[3] Lysyl oxidase is a copper containing quinoprotein produced within the mitochondria of fibroblasts; the copper is involved in the electron-transfer of targeted peptidyl lysyl groups in tropocollagen and tropoelastin. This activity is responsible for the cross-linking in connective tissue, which is vital in providing resistance to elastolysis and collagenolysis by nonspecific proteinases. Without the export of lysyl oxidase from fibroblasts mechanical changes become evident in the skin[4]. A 12 week trial of topically administered GHK-Cu in 71 volunteers demonstrated improvements in fine lines, viscoelastic properties, thickness and density of the skin with no irritation reported[5]. Other trials report significant improvements in skin appearance[6], increased dermal keratinocyte proliferation and increased pro-collagen synthesis[7].

Maquart et al.[8] found dose related effects of GHK-Cu to include increases in dry weight, total protein, collagen and glycosaminoglycan content in rat skin. In addition, the GHK-Cu complex functions as an activator of tissue remodeling[9] by increasing secretion of matrix metalloproteinase-2 (MMP-2) which is involved in wound healing and necessary for tissue remodeling[10]. GHK-Cu also modulates the expression of tissue inhibitors of the metalloproteinases, TIMP-1 and TIMP-2, from fibroblasts which plays a vital role in the regulation of collagen degradation[10].

It is well known that topical delivery of hydrophilic peptides to the dermis, where fibroblasts reside, is a significant challenge due to the skin's natural barriers. Permeation into the skin depends on physicochemical factors such as molecular size, stability, binding affinity, partition coefficient and solubility[11]. It is ideal to have a molecular weight of less than 500 Da, moderate partition coefficient (log P) between 1 and 3 and no or few polar centres[12]. Despite the established biological efficacy of GHK-Cu in dermal tissues and its wide use in topical formulations in pharmaceutical and cosmetic industry, little physicochemical data is available for the tripeptide in literature. Conato et al.[13] investigated the stability of GHK-Cu complex showing that the complex is most stable at pH 4–6 with dissociation taking place when pH <4. Binding of copper takes place in a tridentate manner through the terminal amino, first amido and imidazole nitrogens as confirmed

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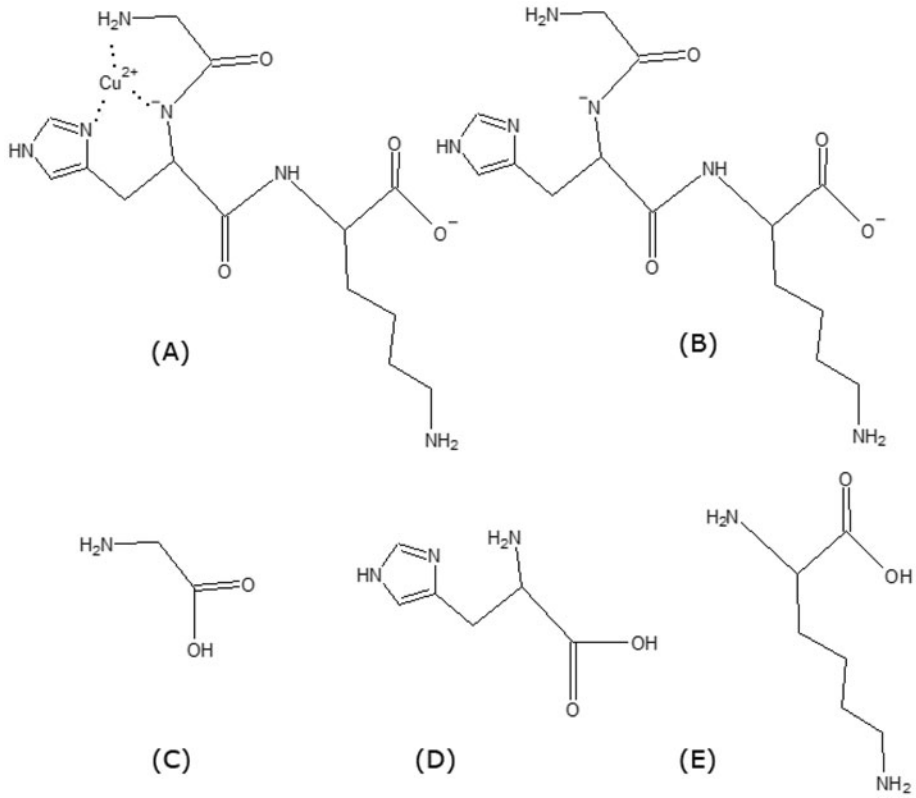

Figure 1. Chemical structures of GHK-Cu (A) and tripeptide GHK (B) and its constituent amino acids: glycine (C), histidine (D) and lysine (E).

by X-ray analysis[2]. When the pH increases (>6) the complex undergoes stoichiometric changes, reducing the binding to copper. Mazurowska et al.[1] investigated the structures of GHK-Cu in different pH solutions (pH range 1.8–7.4) using electrospray mass spectrum analysis showing this dissociation to be complete at pH 1.8.

In this study the physicochemical properties of GHK, in particular, the solubility, partition coefficient and chemical stability, are investigated. A rapid stability-indicating high-performance liquid chromatography (HPLC) method was developed according to International Conference on Harmonization (ICH) guidelines[14]. These guidelines require the forced degradation of the parent compound under a variety of conditions and the separation of the degradation products from the parent allowing the analysis of individual products. The major degradation products were identified using liquid chromatography-mass spectrometry (LC-MS) and degradation pathways proposed. pH-dependent n-octanol-PBS distribution coefficients (log D) of GHK were investigated to predict the permeability of the tripeptide complex into the skin. Stability of the peptide was also examined in the exposure to lipid based delivery system components, particularly niosomes. These self-assembled surfactant based vesicles have previously been used successfully to deliver the encapsulated hydrophilic actives into the dermis with no transdermal permeation[15]. This preformulation data will shed light on the development of topical formulations for the delivery of GHK and complex to the dermis.

Materials and methods

Materials

GHK-Cu (98%, the remainder being constituent amino acids) was obtained as a powder from Salkat (Auckland, New Zealand). HPLC-grade acetonitrile (ACN) methanol and trifluroacetic acid (TFA) were purchased from Ajax Finechem (Auckland, New Zealand). Hydrogen peroxide (30%), sodium hydroxide pellets, sodium dihydrogen phosphate, disodium hydrogen phosphate and hydrochloric acid (37%) were purchased from Scharlau (Barcelona, Spain). Span 60 (sorbitan monostearate) and cholesterol were purchased from Croda (Auckland, New Zealand). The charged lipid dicetyl phosphate was purchased from Sigma-Aldrich (Auckland, New Zealand). L-Histidine monohydrochloride monohydrate (MW 209.6) was procured from Sigma-Aldrich (Auckland, New Zealand). Milli-Q water used in the preparation of buffers was obtained by reverse osmosis (Millipore, Billerica, MA) of demineralized water.

Stability-indicating HPLC assay development and validation

A HPLC system (Agilent 1200, Agilent Corporation, Germany) comprising a quaternary pump, an autosampler and a photo diode-array (PDA) detector was used for the stability indicating method. A Hewlett Packard 1100 HPLC system coupled with an Agilent Technologies Quadrupole LC/MS 6150 (Agilent Corporation, Germany) was used for LC/MS studies. An Aeris peptide column (250×4.6 mm ID, 3.6 µm XB-C18) coupled with a C18 security guard column (4×3.0 mm, 5 µm) purchased from Phenomenex (Auckland, New Zealand) was used on both machines. Data acquisition was by ChemStation software at 229 nm (Agilent Corporation, Germany). The chromatographic separation was achieved using an isocratic method with a mobile phase of (1:99, v/v) acetonitrile:Milli-Q water (TFA 0.1% in both phases, pH 2) maintained at 25 °C. The flow rate of mobile phase was set at 1 mL min^{-1} and an injection volume of 10 µL was used throughout the study.

Serial dilutions were performed from a stock solution (20 mg mL^{-1} GHK-Cu in 0.1% TFA:Milli-Q) to yield working solutions of 5, 10, 25, 50 and 100 µg mL^{-1}. These were prepared in

triplicate in line with the ICH guidelines[16] and used to evaluate the linearity of the assay.

Repeatability was evaluated by assaying three replicates of three chosen concentrations from the calibration curve, whereas the reproducibility, also known as ruggedness, was similarly determined over a period of three days. Inter-day repeatability was based on data collected over a period of three days[17]. Precision was determined from reproducibility and repeatability of sample injections.

The accuracy of the HPLC method was calculated from three replicate injections of 15, 40 and 80 μg mL^{-1} GHK-Cu quality control (QC) solutions, prepared from a separate stock solution, in Milli-Q repeated over three days. This represents low, medium and high concentrations on the derived calibration curve. The limit of detection (LOD) was defined to be three times the baseline noise level whilst the limit of quantification (LOQ) was defined as ten times the baseline noise using a blank as comparison.

Forced degradation studies and kinetics

GHK-Cu in aqueous solutions was exposed to various stress conditions to produce degradation products to test the stability-indicating nature of the assay. Aliquots of 10 mg mL^{-1} GHK-Cu in water, 0.5 M hydrochloric acid (HCl, 60 °C), 0.5% hydrogen peroxide (22 °C) and 0.5 M sodium hydroxide (NaOH, 60 °C) were used to generate degradation[18,19] of 5–20%. These were kept in closed Eppendorf tubes with minimal headspace to prevent evaporation. At set time points samples were taken and neutralized with a quenching solution (a 1 M phosphate pH 7.4 buffer, in which GHK was found to be stable for at least 7 d in a preliminary study), filtered and immediately analyzed using the stability-indicating HPLC method in triplicate. Peak purity analysis was performed on all generated GHK peaks using photodiode array detector (PDA) to determine that a pure single peak eluted. The threshold was set[20] at 99.99%.

The observed first order degradation rate constant (K_{obs}) was calculated from the slope of a log C versus time (t) plot of Equation (1)[21].

$$\log(C/C_o) = -k_{obs}t/2.303 \quad (1)$$

where C is the concentration of GHK remaining at time (t); C_o is the concentration of GHK when t is equal to zero.

In addition, stability of GHK was also observed under pH 5.5 and 7.4 (in 0.1 M phosphate buffers) for a period of 2 weeks at 60 °C to assess the stability in physiological conditions in/on the skin, respectively.

Degradation product identification using liquid chromatography-mass spectrometry (LC/MS)

Mass spectrum analysis was conducted to identify the degradation products generated in the forced degradation samples. Samples were diluted and run through the HPLC system using the same mobile phase as described earlier with the MS running in a positive ionization mode (capillary voltage −4500 V). Data was acquired for 10 min over a 50 to 1000 *m/z* range. Confirmation of the final product was performed by HPLC using 0.1 mg mL^{-1} solution of pure histidine monohydrate.

Equilibrium aqueous solubility

Solubility was determined by added GHK-Cu powder into 500 μL Milli-Q water. The samples ($n = 3$) were mechanically shaken, in a Labnet Revolver Rotator (Labnet International, Edison, NJ), at 20 rpm for 3 d at 25 ℃ to reach equilibrium. Upon completion, the samples were left for 4 h at 25 ℃ before being centrifuged at 15 000 rpm for 10 min to remove the undissolved peptide. Samples were then taken from the supernatant and analyzed with HPLC in triplicate.

n-Octanol/ buffer distribution coefficients (log D)

The distribution coefficients of GHK between n-octanol and three different phosphate buffered solutions (0.1 M, pH 7.4, 5 and 4.5) were determined using a shake flask method[22]. The three pH values investigated were chosen to fall within the normal pH range found on the facial skin (pH 4–5.9)[23] and in the dermis (pH 7.4)[24].

Both *n*-octanol and aqueous buffers were mutually saturated for at least 12 h before use. A 20 mg mL^{-1} stock solution was diluted to give final solutions of 150 μg mL^{-1}. Aliquots of the GHK-Cu solutions were mixed with n-octanol in a volume ratio of 1:9 in screw-capped glass vials. Given the hydrophilic nature of GHK-Cu, a relatively large volume of n-octanol was used in this study to allow more accurate measurement of the distribution. These were vortex mixed for 2 min, placed into a rotating mixer and left for 72 h to equalize. After equilibration, the samples were left for 3 h to allow for phase separation. The aqueous portion was taken and centrifuged to remove any remaining n-octanol and diluted with mobile phase (1:10) before injection into the HPLC. The distribution coefficient (D) was calculated using Equation (2)[25]:

$$D = C_o/C_w = (C_{initial} - C_E)/C_E \quad (2)$$

where C_o is the concentration of GHK in *n*-octanol; C_w is the total concentration of GHK in buffer; $C_{initial}$ and C_E are the concentrations of GHK in buffer initially and at equilibrium, respectively.

Stability with common niosomal components

To assess the compatibility with a potential lipid based delivery system, niosomes, an aliquot (10 mL) of GHK-Cu solution was formulated into niosomes and placed into a stability chamber (Binder, BF 240, Germany) at 40 °C 75% RH for 4 weeks before analysis with the stability-indication HPLC assay.

Niosomes were formulated from cholesterol and Span 60 with and without the surface charge modifying surfactant dicetyl phosphate (DP) (molar ratios 6:10:0.5 respectively). Drug-loaded niosomes were prepared using a modified thin-film hydration method[26]. Briefly, niosome lipid components, with a total amount of 100 mg were dissolved in methanol (1 mL) and warmed to 60 °C. GHK-Cu water solution (75 mg/mL, 100 μL) was then added to the methanol solution and vigorously stirred for 5 min. This mixture was then placed into a round bottom flask in a rotary evaporator (Büchi, Germany) under reduced pressure to evaporate the methanol allowing formation of a thin film. The thin film was then rehydrated with 10 mL of Milli-Q water at 55 °C forming a niosomal suspension.

The above niosomes suspension was stored at 60 °C for 4 weeks to determine the stability. To allow maximum exposure to niosomes the untrapped GHK-Cu was left in the suspension. Samples were then taken, centrifuged for 1 h (Sorvall Wx Ultra 80, Fisher Scientific) at 176 000 G. The supernatant was diluted appropriately with the mobile phase and analyzed using HPLC to determine any degradation peaks.

Results and discussion

HPLC method development and validation

The retention time of GHK under the chromatographic conditions was 8.02 min with sharp and symmetrical peaks. It should be

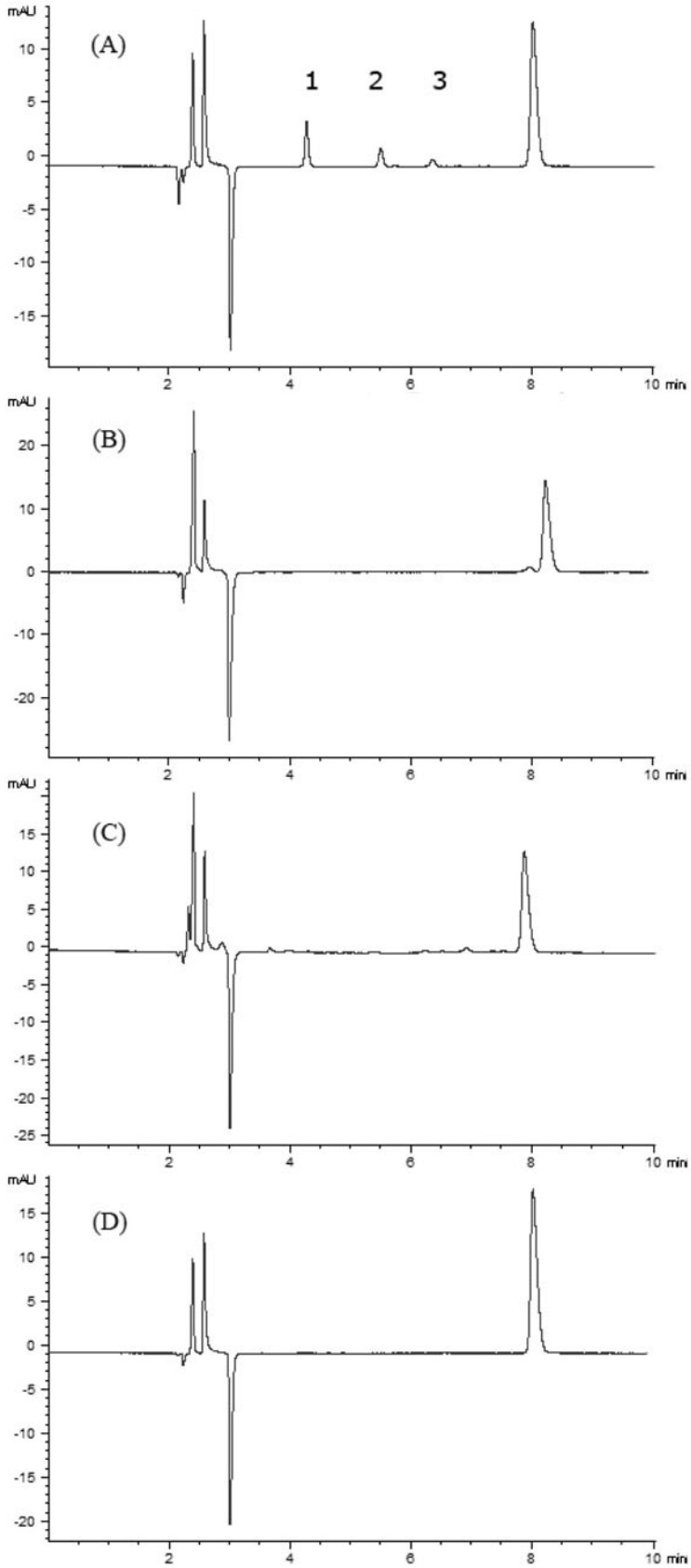

Figure 2. Chromatograms of the degradation products of GHK. GHK-Cu exposed to 0.5 M hydrochloric acid (A, 19.7% degraded) at 60 °C for 1 week, 0.5 M sodium hydroxide (B, 14.1% degraded) at 60 °C for 24 h, 0.5% hydrogen peroxide (C, 15.8% degraded) for 1 h at 22 °C and in Milli-Q water (D, no degradation seen) for 2 weeks at 60 °C. Further degradation would lead to no further clearly identifiable degradation peaks forming.

pointed out that at the low pH due to the addition of TFA in the mobile phase, GHK would no longer carry the copper ion due to the competition of the proton to the binding site[13]. Therefore, the chromatographic peak was the peptide alone, rather than its complex with copper (II).

Under these chromatographic conditions, the degradation peaks generated under the stressed conditions eluted separately to the parent compound peak (Figure 2), indicating the assay was stability indicating. The stability-indicating nature was further confirmed by the peak purity analysis with peak purity index for

Table 1. Nominal and measured concentrations, percentage recovery and linear regression parameters for the developed GHK assay.

	Nominal concentration (μg/mL)							
Set number	5	10	25	50	100	R^2	Slope	Intercept
1	4.7	10.0	24.7	50.3	98.0	0.999	1.29	0.28
2	5.0	9.5	24.2	49.1	99.9	0.999	1.35	−0.71
3	5.2	9.9	25.0	49.7	100.9	0.998	1.27	0.12
4	4.7	9.6	24.2	49.2	98.9	0.999	1.26	0.09
5	4.9	9.7	25.1	49.6	100.5	0.999	1.27	−0.32
RSD (%)	4.37	2.43	1.49	0.99	1.19			
Recovery (%)	99.30	98.09	98.89	99.29	99.69			

Table 2. Precision and accuracy data of the stability-indicating HPLC assay of GHK (results expressed as means ± SD, $n = 5$).

	Intra-day ($n = 3$)			Inter-day ($n = 5$)		
Theoretical concentration (μg/mL)	Determined concentration (μg/mL)	Accuracy (%)	RSD (%)	Concentration found (μg/mL)	Accuracy (%)	RSD (%)
15	14.80 ± 0.5	98.6 ± 2.9	2.60	15.06 ± 0.4	98.5 ± 1.7	0.85
40	39.55 ± 0.7	98.9 ± 1.3	1.66	39.42 ± 0.8	98.5 ± 1.6	1.37
80	80.62 ± 1.8	100.7 ± 1.8	1.16	79.69 ± 1.2	99.6 ± 1.3	1.30

all generated GHK peaks > 99.99%, in the presence of potential degradation products[27]. Linear regression analysis of the calibration curve indicated that the standard curve was linear ($R^2 > 0.9998$) in the range of 5–100 μg mL^{-1}. The recovery of GHK ranged from 98.09 ± 2.43% to 99.69 ± 1.19% (Table 1).

The LOD and LOQ were determined to be 0.55 and 1.8 μg mL^{-1}, respectively. The percentage recovery of the QC samples (Table 2) provided the precision and accuracy for the method. The inter-day and intra-day precision for three batches ($n = 5$) were found to be between 1.3 to 2.7%, and 1.1 to 2.6%, respectively, with recoveries between 98.6 and 100.8%. All the above values comply the ICH acceptance criteria[16].

Forced degradation

Figure 2 demonstrates that different degradation products were formed when exposed to various stressors. GHK-Cu exposed to 0.5 M hydrochloric acid (HCl) at 60 °C for 1 week showed 80.3% remaining. Three separate peaks were seen on the chromatogram (Figure 2A). In contrast, GHK-Cu exposed to 0.5 M sodium hydroxide (NaOH) at 60 °C required only 24 h to degrade to 85.9% (Figure 2B). The fastest degradation was seen in 0.5% hydrogen peroxide (H_2O_2), with 84.2% remaining after 1 h at 22 °C (Figure 2C). Surprisingly, under elevated temperature conditions (60 °C), the tripeptide GHK-Cu water solution, and those solutions at pH 5.5 and 7.4, equivalent to those in and on the skin[23,24], showed no detectable degradation over 14 d (Figure 2D).

Under strong alkaline conditions, blue precipitate was observed, most likely due to the formation of copper hydroxide. The addition of the ''quenching solution'' (pH 7.4), however, re-dissolved the hydroxide precipitate and allowed for the further analysis of GHK-Cu.

These results indicate that the GHK peptide is markedly affected by both high and low pH of the solution and possibly also to oxidative stress. These findings are commonly reported in peptides/proteins as there are numerous accounts of susceptibility to oxidation, temperature[28,29] and acidic/basic environments[30]. The results highlight that it is vital to maintain the pH for the conservation of peptide/protein stability *in vitro* and *in vivo* when developing dermal delivery systems[31].

Degradation product identification using LC-MS

MS assay of the major HPLC peak of GHK found the peptide-copper complex had dissociated as the molecular weight matched GHK alone.

Exposure to hydrogen peroxide, sodium hydroxide and hydrochloric acid caused the breakdown of GHK (Figure 2). Acid hydrolysis led to three major degradation products forming. Positive ion electrospray ionization mass spectra of Product 1, 2 and 3 yielded a *m/z* 284, 213 and 156, respectively. The structures of Products 1, 2 and 3 are proposed in Figure 3. The mass of Product 3, the final degradation product that could be detected, is consistent with histidine (MW = 155).

Degradation products for both basic and oxidative stress conditions could not be found or identified. This could, in part, be due to the half-life of the degradation products being shorter than their acidic counterparts. This was demonstrated in a paper by Wu et al. whereby the degradation rate, following a similar degradation pathway[25], increased with an increase in pH. The hypothesized degradation pathways of GHK, as a result of the hydrolytic cleavage of peptide bonds NH–C = O, in acidic condition are illustrated in Figure 4.

The carbon atom on the carbonyl group of glycine near the bond with histidine will have a significant positive character. The bond resonance dipole between the oxygen and carbon pulls the electrons away from the carbon. The amino group is also more electronegative and it too pulls electrons away contributing to the positive nature of the carbonyl carbon. This would leave the amide bond on the glycine vulnerable to nucleophilic cleavage.

Based on the above theory, a similar degradation process is considered to take place between lysine and histidine creating a number of successive products (Figure 4). Degradation begins with the predicted products, Product 1 and Product 2, corresponding to *m/z* 284 and 213. The loss of glycine in Product 1 impacts on the formation of copper complexes. Copper complexes are primarily driven by the glycine and histidine residues[13]. Cleavage between lysine and histidine, Product 2, leaves the copper binding site intact. The coordination path is typical of oligopeptides containing histidine as a second residue[13]. Further acidic hydrolysis leads to the formation of the major peak,

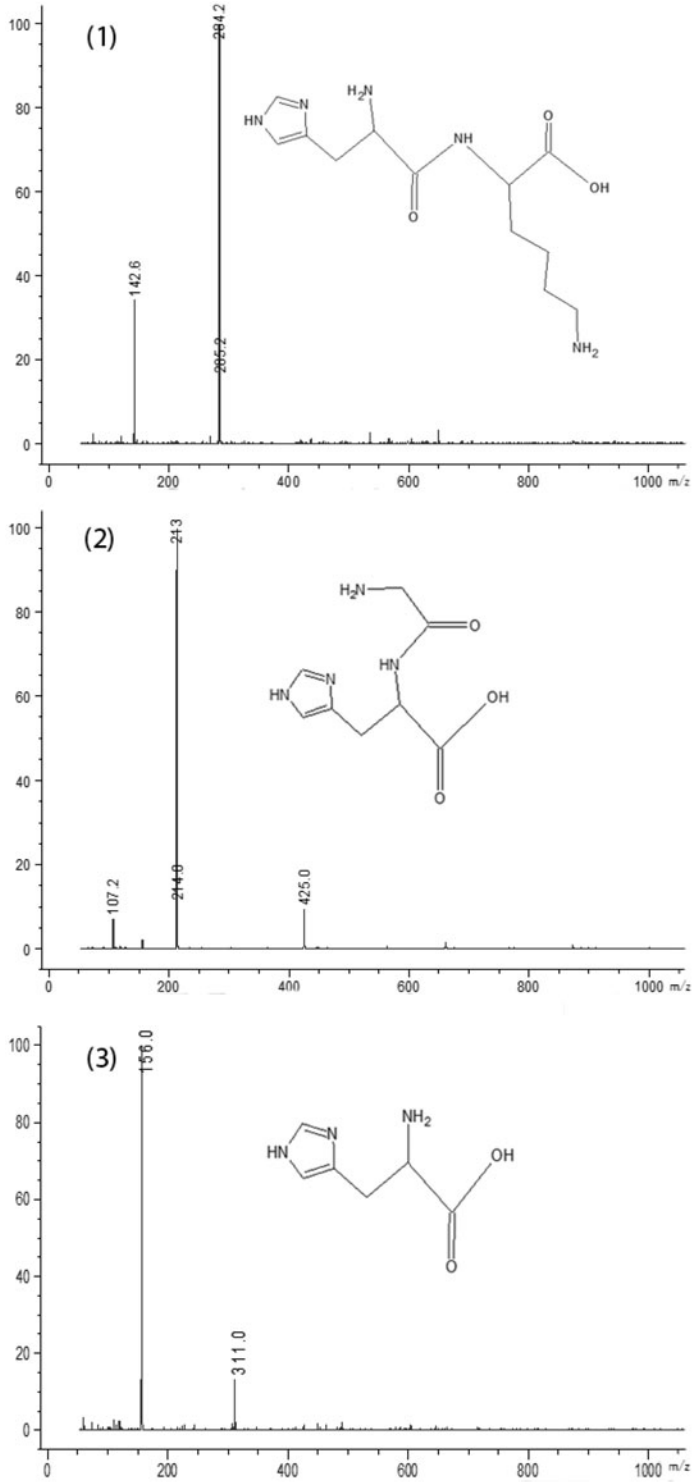

Figure 3. Positive ion electrospray ionization mass spectra for degradation Products 1, 2 and 3 found in acidic solutions of GHK-Cu.

Product 3, which coincidently is histidine. This was confirmed by the identical retention times of a standard histidine solution ($10\,\mu g\,mL^{-1}$).

The lysine residue is important for biological activity therefore the only degradation product that may be biologically active is Product 1.

Chemical kinetic studies

Figure 5 shows the degradation plots of GHK under various conditions over a 24-h period due to the relatively quick degradation observed. The plot of the logarithm of % GHK remaining over time had correlation coefficients (R^2) of between 0.977 and 0.993. This indicates first-order degradation kinetics. Degradation occurred with a relationship of K_{obs} being oxidation > basic > acidic. The K_{obs} values provided an estimated half-life ($t_{1/2}$) for each condition (Table 3).

Similar results may not be seen *in vivo* as the extreme conditions experienced during these forced degradation experiments are not replicated. There was no observed degradation seen in the samples kept in Milli-Q water, or the buffers at physiological pH of skin (pH 5.5 and 7.4) at 60 °C for two weeks. This infers better stability under normal physiological conditions. However Conato et al.[13] demonstrated that the half-life of GHK, when tested *in vitro* by incubating the peptide with human serum, is 16 min due to enzymatic degradation.

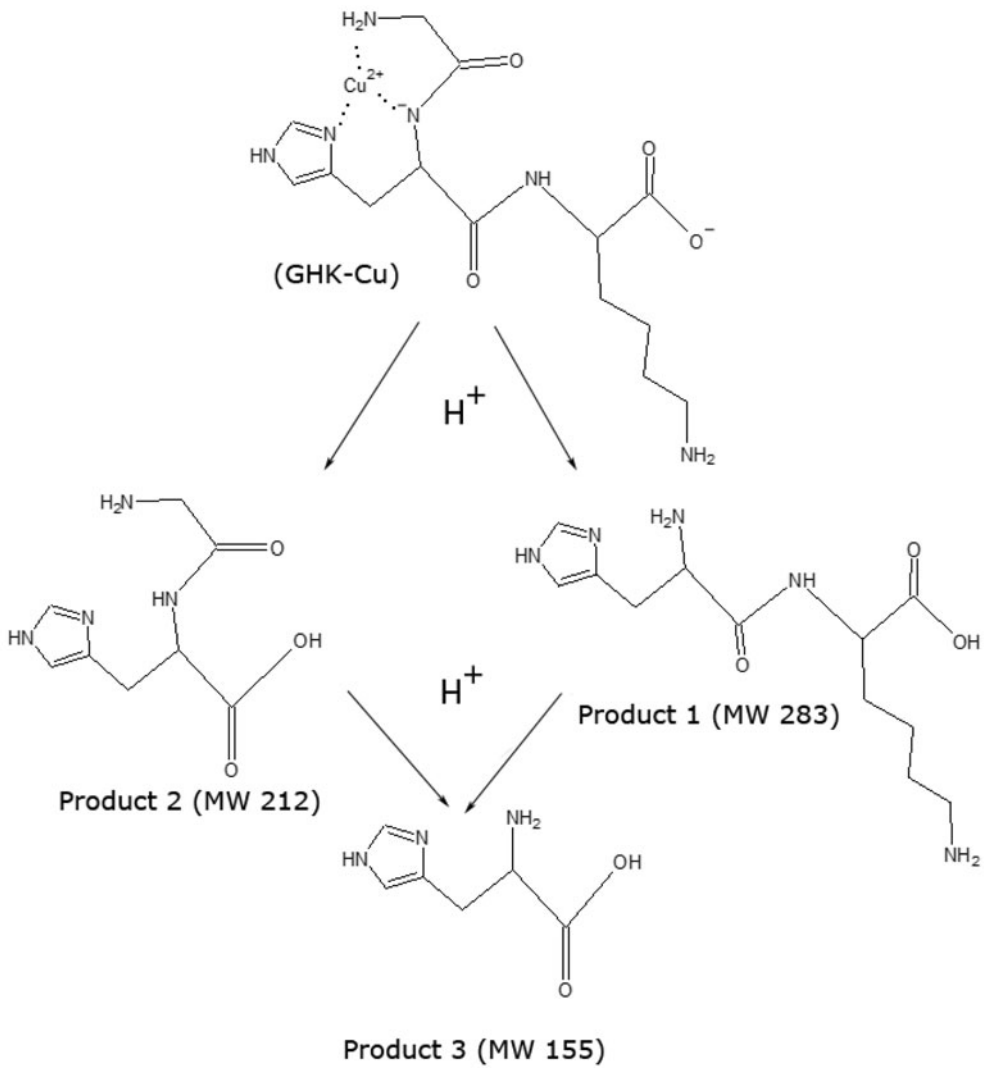

Figure 4. Proposed acidic degradation pathway of GHKCu in acidic solution.

Figure 5. The first order degradation profiles of GHK-Cu in various medium stored at 60 °C, with the exception of hydroxide at 22°C, over a 24-h period. Data were obtained by quantifying GHK ($n = 3$).

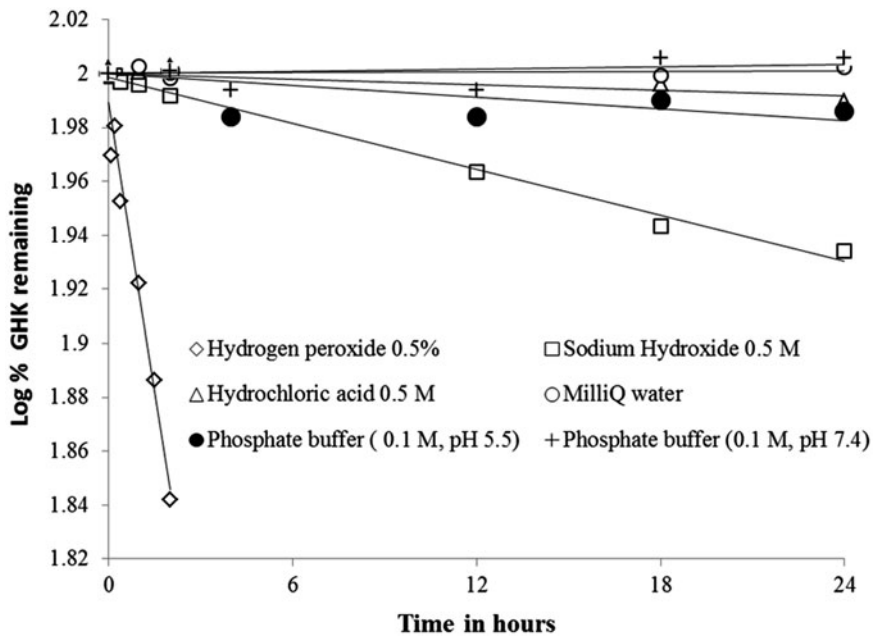

Table 3. First order kinetic parameters of GHK in aqueous solutions under various conditions.

Medium	K_{obs} (day^{-1})	$t_{1/2}$ (day)	Temperature
0.5% H_2O_2	0.182	0.16	$22 \pm 2\,^{\circ}C$
0.5 M NaOH	0.006	4.81	$60 \pm 2\,^{\circ}C$
0.5 M HCl	0.001	28.8	$60 \pm 2\,^{\circ}C$

Table 4. *n*-Octanol-buffer distribution coefficient of GHK-Cu at variable pH conditions at 25 °C (results expressed as mean values ± SD, $n = 3$).

pH	Aqueous concentration (μg/ml)	Percentage remaining in buffer	D (Equation (2))	log D
7.4	131.38 ± 0.25	99.68 ± 0.19	3.18×10^{-3}	-2.49 ± 0.35
5.5	131.25 ± 0.15	99.59 ± 0.12	4.13×10^{-3}	-2.38 ± 0.13
4.5	131.37 ± 0.27	99.68 ± 0.21	3.22×10^{-3}	-2.49 ± 0.33

Aqueous solubility

The aqueous solubility of GHK-Cu solubility at 25 °C was 325.09 ± 4.38 mg mL^{-1} ($n = 3$). This can be classified as ''freely soluble'' according to the Formulation in Pharmacy Practice[32].

Determination of *n*-octanol/buffer distribution coefficient of GHKCu

Lipophilicity is an important factor used to predict permeation into the skin[33] and in formulation selection[34]. Lipophilicity is indicated by the partition coefficient and more importantly and accurately by the distribution coefficient, which takes into account the ionized and un-ionized species in varying pH conditions. The log D values are reported in Table 4.

These results indicate that the GHK-Cu complex is highly hydrophilic, and is consistent with the high water solubility. The terminal amino group is less basic than the amino group on the lysine residue. This is in part due to the proximity of the former to the carbonyl group of the first peptide bond. This group is electron-withdrawing and according to the Lewis theory, will cause the terminal amino group to be less likely to be an electron pair donor. The side chain of the histidine residue, however, can act as a nucleophile. The interim imidazolium ion exists in either of the two ionic and tautomeric states. Either of the two nitrogens in the imidazole ring can release a proton to produce conjugate base forms of imidazole. The deprotonation of imidazole takes place at pH 14.58, physiological pH and under acidic conditions[35]. Protonation will take place three sites in the imidazole ring as well as the terminal amino group (lysine first and then glycine).

The negative log D values can thus be explained by the presence of the imidazole (planar 5-membered ring), which is highly polar as a result of the proton being located on either of the two nitrogen atoms (dipole of 3.61D). Furthermore, the functional groups of the amino residues are normally charged over the pH range 2–11 due to the corresponding logK values within the pH range previously reported[13]. LogK is the protonation constant measured through techniques of nuclear magnetic resonance spectroscopy[36] or potentiometry[13]. It has been reported that logK values for COO^- (alpha), N (im), NH_2 (alpha) and NH_2 (terminal) are 2.73, 6.45, 7.82 and 10.53 respectively. The results for the physiological pH conditions are consistent with the literature whereby it was found that GHK protonated at the lysine amino group[13].

Based on the results it is concluded that GHK is very hydrophilic at the physiological pH range tested. The log D values are much smaller than the optimal log P values, 1–3 for skin absorption[37]. Therefore it is expected that an appropriate formulation would enable GHK and complex to cross into the dermis more effectively. For better dermal delivery, lipid-based carrier systems may be useful to enhance the permeation of the peptide through the lipophilic epidermis layer of the skin[38]. Furthermore, the high solubility in water of GHK may favorably allow a high content of the peptide entrapped in the aqueous cores of the carriers to achieve effective delivery to the target, the fibroblasts in dermis.

Stability with some common niosome components

This work was originally performed to assess the suitability of lipid based systems to enhance the delivery of GHK and complex into the skin. The physicochemical parameters discussed highlight the need for such a system. The method examined the stability of GHK-Cu, which was exposed to all excipients during formulation and storage, reflecting the chemical compatibility of the lipids with the peptide entrapped in the vesicles before comprehensive optimization began. Samples kept at 40 °C with cholesterol and Span 60 showed no observed degradation for the 4 week period. Only samples formulated with dicetyl phosphate contained a degradation peak with retention time and UV spectrum corresponding to the degradation Product 1 – histidine (Figure 2). The results suggested that the breakdown of GHK formulated in the niosomes with a composition of 0.6:1:0.05 molar ratio of cholesterol:span 60:dicetyl phosphate in Milli-Q water had a half-life of 89.95 days.

This degradation might be explained in a similar manner to the forced degradation seen. Chemically dicetyl phosphate has a phosphoric acid moiety, which in more basic solutions may donate a hydrogen atom and ionize with a negative charge. This then leads to nucleophilic cleavage of the peptide as seen with the stronger acid hydrolysis samples.

Conclusion

The stability-indicating HPLC method for GHK allowed observation of stability of the peptide under various stressed conditions. The LC-MS assay verified the sequential hydrolytic cleavage of the peptide with three main degradation products. GHK-Cu is freely water soluble, highly hydrophilic and chemically stable throughout the pH range of normal skin conditions. This preformulation work incentivizes the consideration of lipid-based carrier systems, such as niosomes, for the dermal delivery of GHK-Cu with the active being incorporated in the aqueous cores. The lipid-based carrier should not contain dicetyl phosphate as this caused the breakdown of GHK in a similar manner to exposure to highly acidic conditions.

Declaration of interest

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