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*Research Article*

# Synthesis of Mono-PEGylated Growth Hormone Releasing Peptide-2 and Investigation of its Biological Activity

**Xiaoyu Hu,[1] Beihua Xu,[1,2] and Ziniu Zhou[1]**

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***Abstract.*** The purpose of this study was to investigate an efficient synthetic route to the mono-PEGylated growth hormone releasing peptide-2 (GHRP-2) and its biological activity *in vivo*. The commercially available key PEGylating reagent, mPEG-NHS ester, was successfully utilized to the synthesis of mono-PEGylated GHRP-2, during which the PEGylation profiles of GHRP-2 were monitored by high-performance liquid chromatography (HPLC). The product was purified by cation exchange chromatography, and its biological activity was conducted in rats. The desired mono-PEGylated GHRP-2 as the major product was readily obtained in anhydrous aprotic solvent, such as dimethyl formamide (DMF) and dimethylsulfoxide (DMSO), when the molar ratio of mPEG-NHS ester to GHRP-2 was fixed to be 0.8:1. The products were characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The evaluation of the biological activity for the products showed that the mono-PEGylated GHRP-2 gave a more stable activity than GHRP-2, suggesting that PEGylation led to the increase in the half-life of GHRP-2 in plasma without greatly impairing the biological activity. PEGylation of the GHRP-2 is a good choice for the development of the GHRP-2 applications.

**KEY WORDS:** growth hormone; growth hormone releasing peptide-2; mono-PEGylated GHRP-2; mPEG-NHS; PEGylation.

## INTRODUCTION

Growth hormone releasing peptide-2 (GHRP-2), a peptide with six amino acid units (D-Ala-D-beta-+Nal-Ala-Trp-D-Phe-Lys-$NH_2$), is one of the most potent members of the GHRP family (1,2). It can significantly promote the release of growth hormone (GH), improving the blood GH concentration in animals and humans (3–6). Unfortunately, GHRP-2 is readily degraded *in vivo*, with its biological half-life ($T_{1/2}$) for about 30 min only, causing the GH concentration to reach its peak value around 15 min after administration (3,6). The therapy should thus be administered periodically daily, which caused extra time, money, and inconvenience for patients. Therefore, it is desirable to develop long-acting analogues of GHRP-2, which became our focus in this study.

PEGylation has successfully been applied to many proteins to improve their biological properties (7). Indeed, the PEG modification of proteins and peptides has greatly improved their performance by increasing their aqueous solubility, enhancing the stability in the body, extending their half-life, and reducing the immunogenicity of protein drugs (8,9). However, there is limited reported work on the PEG modification of GHRP-2, and it is difficult to evaluate whether PEGylation is beneficial since the PEGylation of the proteins or peptides varied from substrate to substrate. In addition, the PEGylation efficiency is determined by many factors including the site of PEGylation and the physical properties of the PEG, etc. (10–12).

According to Grace and co-workers, mono-PEGylation at specific site on the protein has been found to provide a product with decent biological activity, whereas multiple PEGylation would impair the activity of the modified protein (13). It is also believed that growth hormone-releasing factor ($GRF_{1–29}$) modified by the single linear PEG chain is a desirable form, which maintain, *in vivo*, a biological activity no lower than that of the unmodified molecule (14). Therefore, mono-PEGylated GHRP-2 became a target of choice in our study.

As far as the synthesis of mono-PEGylated GHRP-2 is concerned, challenge came from PEGylation. Since GHRP-2 has two free amine groups ($\alpha$-$NH_2$ on the N-terminus of alanine at position A, $\varepsilon$-$NH_2$ on the lysine side chain at position B, Fig. 1.), thus leading to the competition for amine acylation, which would afford to a mixture of mono- and di-PEGylated products. Nevertheless, mono-PEGylation may be achieved to give the desired product with good quality and high biologically activity by optimizing reaction conditions.

In this study, we focused our efforts on the efficient synthetic route to mono-PEGylation. In order to obtain an optimum reaction condition, buffer solution, dimethylsulfoxide (DMSO), and dimethyl formamide (DMF) would be used as solvents for the modification. From a

[1] Department of Pharmacy, Zhejiang Pharmaceutical College, No. 888, Yinxian Avenue, Ningbo, 315100, People's Republic of China.
[2] To whom correspondence should be addressed. (e-mail: xvbeihua@163.com)

 

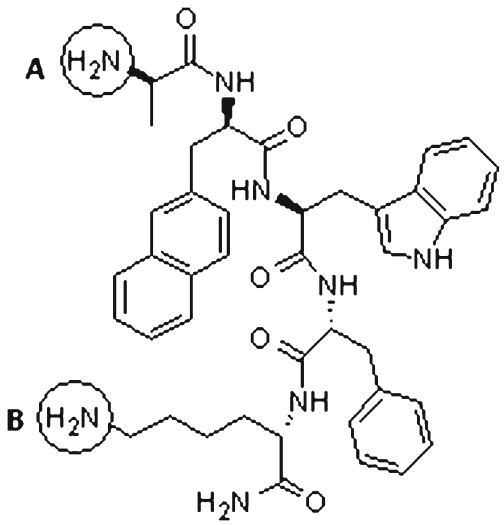


**Fig. 1.** The chemical structure of GHRP-2

chemistry perspective, the solubility and stability of the peptide in aqueous and organic solution were the critical parameters for the coupling process. While buffer solution is widely used as the solvent for the reaction of protein or peptide, the activated PEG (mPEG-NHS ester, which was used in this study) may be unstable in the aqueous buffers. To PEGylate proteins, a large excess of the PEG-NHS ester is often required to overcome the competitive hydrolysis of PEG-NHS ester itself in aqueous solution (15). To mitigate the competitive hydrolysis reaction, organic solvents such as anhydrous DMF and DMSO were used in this study. Another key factor, molar ratio of the mPEG-NHS ester to GHRP-2, would also be studied. An optimum molar ratio would then be identified based on the acylation performance of the commercially obtained PEGylating reagent.

Finally, the pharmacological behavior of mono-PEGylated GHRP-2 has been characterized by intravenous (i.v.) injection in rats. The induction of endogenous GH in rats by the mono-PEGylated GHRP-2 compared to parent GHRP-2 was examined.

## MATERIALS AND METHODS

### Materials

*O*-[(*N*-Succinimidyl)succinyl-aminoethyl])-*O*′-methylpolyethylene glycol [mPEG-NHS ester (MW, ~5000)] was purchased from Shanghai Jing Yu Biotech Co., Ltd., Shanghai China(batch number, PA20110627). The GHRP-2 was obtained from GL Biochem Co., Ltd., Shanghai China (batch number, P100519-SY055255), and the rat growth hormone (rGH) ELISA kit was purchased from Chemical Technology Co., Ltd., Shanghai Branch, Shanghai China. The trifluoroacetic acid (TFA) was obtained from Sigma (St Louis, MO, USA). High-performance liquid chromatography (HPLC) grade methanol and acetonitrile were obtained from J.T. Baker (Philipsburg, NJ, USA). Dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) were dried over calcium hydride before use. All the other chemicals were of analytical grade and purchased from China Sinopharm Shanghai Chemical reagent Company, Shanghai China.

### Preparation and Characterization of PEGylated GHRP-2

A 15-ml aliquot of GHRP-2 in anhydrous DMF (or anhydrous DMSO, borate buffer pH 7.4, borate buffer pH 8.4 as the solvent, 1 mg/ml solvent) was mixed with various molar amount of mPEG-NHS ester. The resulting reaction mixture was stirred at room temperature for 30 mins, and the reaction progress was monitored by reverse-phase HPLC. The yield (%) was calculated based on the ratio of the peak area for the amount produced over that for the initial GHRP-2 amount. After the reaction, the DMF or DMSO solutions were diluted and dialyzed against deionized water with a molecular weight cutoff of 1000 to remove the organic solvent and any unreacted GHRP-2 when DMF or DMSO was used as the solvent, or the reaction solutions were dialyzed to remove unreacted GHRP-2 with borate buffer of pH 7.4 or borate buffer of pH 8.4 as the solvent. The reaction mixture was then concentrated by evaporation at 40°C, and purified by an SP-Sepharose column, following the method described by Eun Ji Park previously (16). White powder product was obtained by concentration of the collected eluents in the lyophillizer. The molecular mass of the products was verified with a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry.

### HPLC Analysis

Chromatographic conditions were as follows: column, ODS C18 column (250 mm×4.6 mm, 5 μm); gradient elution, mobile phase A, 0.1% [vol/vol] TFA/water and mobile phase B, 0.1% [vol/vol] TFA/acetonitrile; a typical gradient started from A/B (80:20) and ended with A/B (32:68) over 0–12 min; flow rate was 1.0 ml/min; and detection wavelength was set at 215 nm with injection volume of 20 μl.

The procedure to obtain a standardization curve was as follows: for the preparation of 80 mg/l stock solution of GHRP-2, accurately weighed GHRP-2 (4.0 mg) was put in a 50-ml volumetric flask, to which was added acetonitrile to dissolve and dilute to the set volume. For the reparation of concentration of 4.0, 6.4, 8.0, 24.0, and 40.0 mg/l GHRP-2 series solution, 0.5, 0.8, 1.0, 3.0, and 5.0 ml of the stock solution were drawn and put in a 10-ml volumetric flask, diluted with acetonitrile to constant volume, followed by injection and chromatograms recording. The regression equation was $A$=1.479$C$−1.458 ($r$=0.999), indicating that the peak area of the GHRP-2 has a good linear relationship within the concentration of 4–80 mg/l region.

### Drug Release Study

The stability of mono-PEGylated GHRP-2 was studied using hydrolytic reaction in buffer at different pH of 7.4, 8.4, and 9.4, respectively. Borate buffer (0.1 M) solutions of mono-PEGylated GHRP-2 (1 mg/ml) were prepared and placed into 3-ml vials. The closed vials were then placed on the water bath and incubated at 37°C. The concentration of released GHRP-2 was determined by HPLC according to the method described previously at predetermined time points. The rate of drug release was determined by the concentration of GHRP-2 (released from mono-PEGylated GHRP-2) at certain time

points divided by its initial concentration of mono-PEGylated GHRP-2.

### Biological Activity Study

The *in vivo* biological activities of intact and PEGylated GHRP-2 were evaluated by monitoring the serum rat growth hormone (rGH) levels in female Sprague–Dawley rats (200± 20 g, 6 weeks old, obtained from Zhejiang Academy of Medical Sciences, Zhejiang, China). The rats were maintained under constant environmental conditions (22°C±1°C; 50%± 5% relative humidity). Twelve rats were fasted for one night prior to the experiment. These rats were divided randomly into three groups, one group for administration of mono-PEGylated GHRP-2 (physiological saline as the solvent, 0.18 μmol/ml of mono-PEGylated GHRP-2, at a dose of 0.04 μmol/ rat), another group for administration of GHRP-2 (physiological saline as the solvent, 0.18 μmol/ml, at a dose of 0.04 μmol/rat), and the last group for administration of the equivalent physiological saline. Anesthesia was induced by inhalation of ethyl ether. After the i.v. administration, the blood samples were collected at different time intervals over 6 h from tail veins and centrifuged at 5°C at 10,000 rpm for 5 min immediately. GH concentrations were determined using an rGH ELLISA kit. The assay was performed according to the manufacturer's instructions. Animal studies were performed according to the Principles of Laboratory Animal Care Guide by the National Institutes of Health (Bethesda, MD, USA).

## RESULTS AND DISCUSSION

### PEGylation Profiles of GHRP-2 Using mPEG-NHS

The PEGylation profiles of GHRP-2 using mPEG-NHS ester were monitored by HPLC as shown in Fig. 2, and the

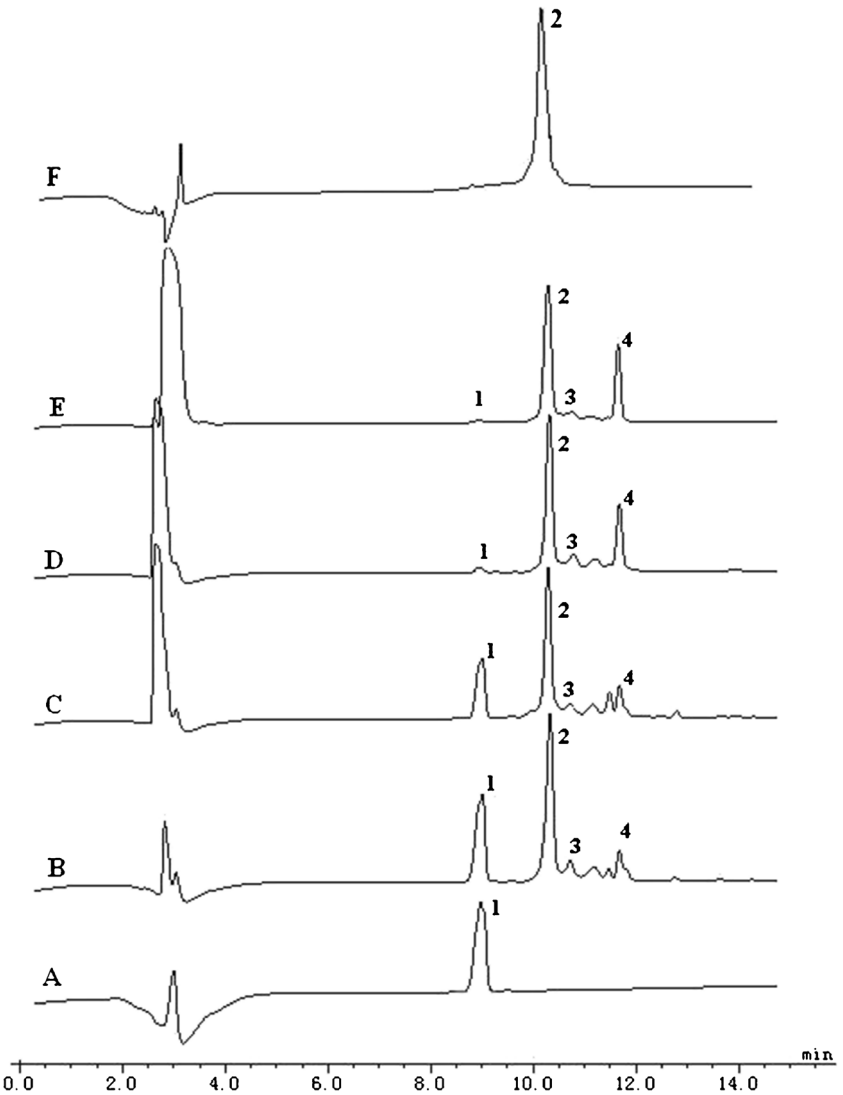


**Fig. 2.** HPLC analysis of PEGylated GHRP-2 in reaction solutions. The molar ratio of mPEG-NHS to GHRP-2 was fixed to be 0.8:1: *A* GHRP-2 in water (control), *B* reaction solution in borate buffer (pH 7.4), *C* reaction solution in borate buffer (pH 8.4), *D* reaction solution in DMSO, *E* reaction solution in DMF, and *F* isolated product **2** in water. *1* GHRP-2, *2* product **2**, *3* product **3**, and *4* product **4**

PEGylation reaction displayed different profiles with the variation of solvents. When the molar ratio of mPEG-NHS ester to GHRP-2 was set to be 0.8:1, product **2** and product **4** were found to be the major products in DMF and DMSO solution after 30 min of coupling. However, the buffer solutions appeared to be less effective reaction media, for comparatively large amount of unreacted GHRP-2 (1) remained, which was due to the instability of mPEG-NHS ester in the borate buffer (15).

In order to identify the major products **2** and **4** (which are corresponding to peaks 2 and 4, respectively) on HPLC of Fig. 2, the reaction solution was subjected to ion-exchange separation, subsequent NMR (Fig. 3) and MALDI-TOF-MS analysis (Fig. 4). Product **2** and **4** had the similar NMR spectroscopic characteristics, and the PEGylated structural unit was believed to be represented by the region from 3.1 to 3.7 ppm, corresponding to the repeating unit ($-CH_2CH_2-O$) as the characteristics of PEG (Fig. 3b). The observed mass of MALDI-TOF-MS analysis for product **2** (5870 Da) was consistent with the expected mass of mono-PEGylated GHRP-2 (about 5800 Da). The observed mass of product **4** (6687 Da), which was greater than that of product **2** by about 818 Da, was believed to be for the coupling product of two equivalents of GHRP-2 (MW, 818) with one equivalent of PEG-NHS molecule (1) (MW, 5000). The introduction of this side product may be caused by impurity NHS-PEG-NHS ester, which was commonly introduced during the production process of mPEG-NHS ester. Because polyethylene glycol monomethyl ether (mPEG/MeO-PEG-OH), as the synthetic starting material for mPEG-NHS, was often mixed with polyethylene glycol (HO-PEG-OH), which was related to the presence of water during the polymerization process. The content of HO-PEG-OH in MeO-PEG-OH could reach as high as 10% (17). As a result, HO-PEG-OH led to the production of NHS-PEG-NHS ester, which made the coupling of two molecules of GHRP-2 with one of PEG possible.

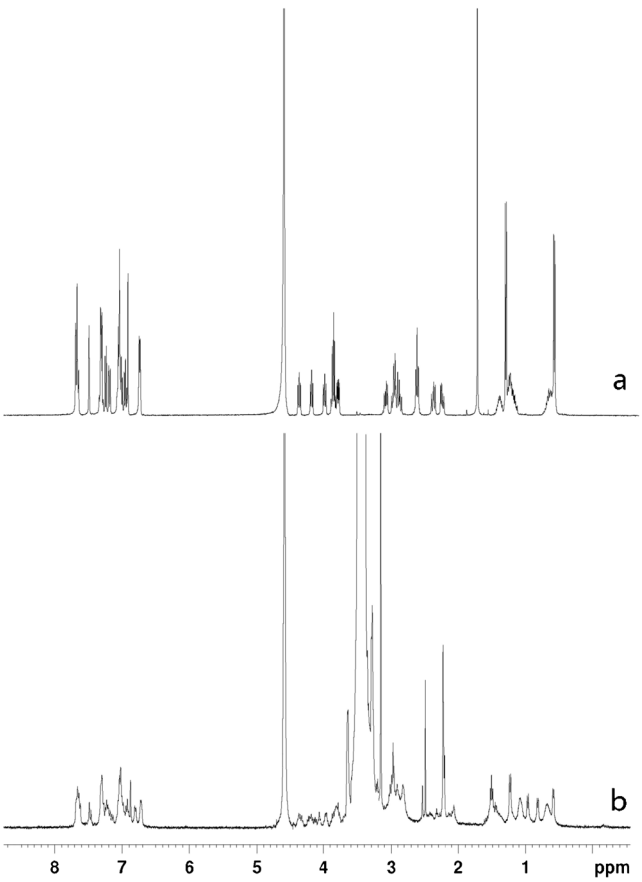


**Fig. 3.** $^1$H NMR spectra: **a** free GHRP-2 in $D_2O$ and **b** PEGylated GHRP-2 in $D_2O$

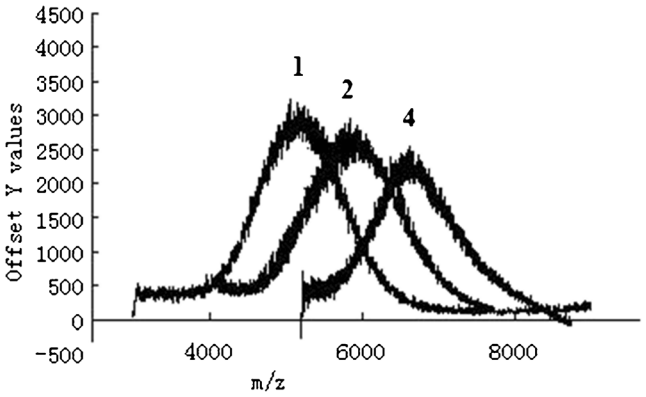


**Fig. 4.** MALDI-TOF MS of the PEGylated GHRP-2 [MATRIX:CHCA(H20/CAN=50/50)]. *1* PEG-NHS; *2* product **2**; *4* product **4**

The mono-PEGylated GHRP-2 was believed to be the mixture of two regio-isomers, for GHRP-2 can be modified by acylation with PEGylating reagent at two sites: one on the alanine terminus and the other on the lysine side chain. Therefore, acylation at one of the two sites gave the regio-isomers, and acylation at both two sites afforded the di-PEGylated GHRP-2. Since the separation of the isomers were difficult and may not be necesssary, they were thus subjected to the later biological activity analysis without separation.

Although the increased amount of mPEG-NHS ester made the reaction complete in buffer solution, more by-products were observed as a result (Fig. 5). As it was mentioned earlier, GHRP-2 had the tendency to be di-PEGylated due to the presence of two free $NH_2$ groups. The by-products were difficult to be isolated, while the starting material GHRP-2 was easy to be removed during dialysis against deionized water. In order to obtain the desired product with as little by-product or starting material as possible, while maintaining an optimum yield for the desired product, it is conceived that using DMF or DMSO as the solvent in the reaction would make it happen.

Meanwhile, the dependence of the mono-PEGylation of GHRP-2 on the molar amount of the PEGylating reagent was examined (Table I). The reaction was performed in the same aqueous or organic solvents as previously used by varying the molar ratio of mPEG-NHS ester to GHRP-2 from 0.4:1 to 2:1. Although increasing the amount of PEGylating reagent initially accelerated the production of mono-PEGylated GHRP-2, the amount of the desired product started dropping significantly after the molar ratio reached about 0.8:1 in DMF or DMSO, for excess mPEG-NHS ester led to more byproducts. The formation of mono-PEGylated GHRP-2 was determined by more factors in borate buffer (than in aprotic solvents), including the molar ratio of mPEG-NHS ester to GHRP-2, the

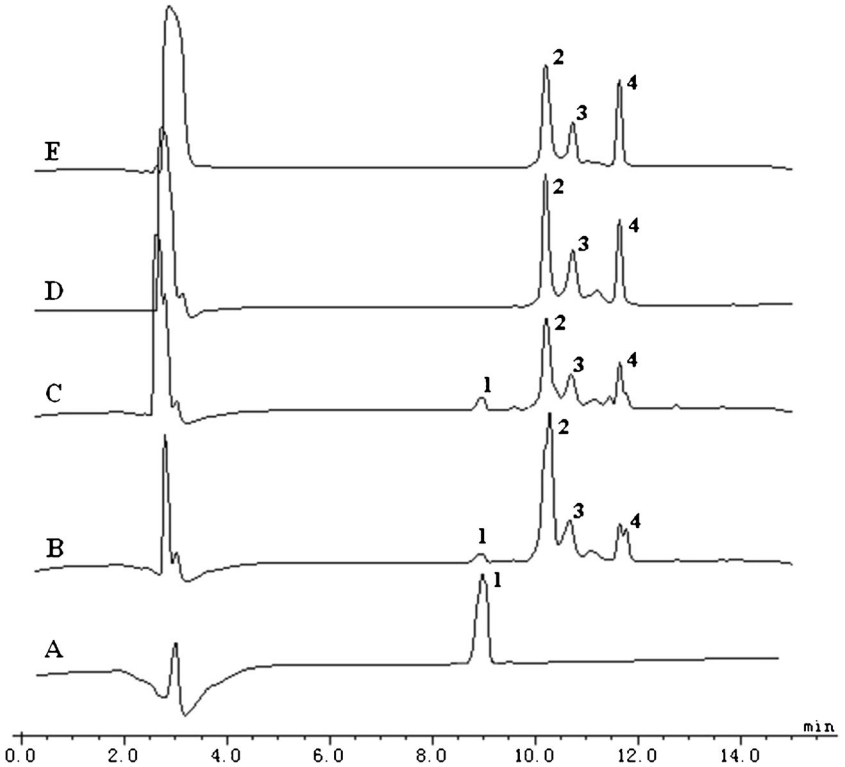


**Fig. 5.** HPLC analysis of PEGylated GHRP-2 in reaction solutions. The molar ratio of mPEG-NHS to GHRP-2 was fixed to be 1.2:1: *A* GHRP-2 in water (control), *B* reaction solution in borate buffer (pH 7.4), *C* reaction solution in borate buffer (pH 8.4), *D* reaction solutionin DMSO, and *E* reaction solution in DMF. *1* GHRP-2; *2* product **2**; *3* product **3**; *4* product **4**

lability of mPEG-NHS ester to hydrolysis, and the reactivity towards GHRP-2. Reactions advanced more quickly at pH 8.4 than at pH 7.4, while hydrolysis half-life of mPEG-NHS ester was shorter at pH 8.4 than at pH 7.4, which was demonstrated and explained by Nojma (15). As a result, more by-products and lower yields were observed when using borate buffer in our study (Figs. 2 and 5, Table I).

Other reaction parameters were also investigated. It was found that decreased amount of the reaction solvent would cause more by-products. The temperature, however, was not a key reaction factor, as the mono-PEGylated GHRP-2 production at 60°C did not display a much better profile than a reaction temperature at 25°C. In addition, a prolonged reaction time did not help raise the reaction yield if compared with a 30-min reaction.

**Table I.** The Yields of Product **2**(Mono-PEGylated GHRP-2) Under Different Reaction Conditions (%)

| | The reaction solvent | | | |
|---|---|---|---|---|
| The molar ratio of GHRP-2 to mPEG-NHS ester | DMF | DMSO | Borate buffer (pH 7.4) | Borate buffer (pH 8.4) |
| 0.4 | 44.5 | 40.5 | 33.6 | 35.5 |
| 0.8 | 64.4 | 62.9 | 49.8 | 52.1 |
| 1.2 | 61.1 | 58.7 | 55.5 | 56.1 |
| 2 | 25.8 | 24.7 | 20.3 | 23.3 |

## Drug Release Study

The drug release rate was determined in the buffer solutions with results shown in Fig. 6. Compounds were incubated in the presence of borate buffers with (a) pH 7.4, (b) pH 8.4, and (c) pH 9.4, and the concentration of GHRP-2 was

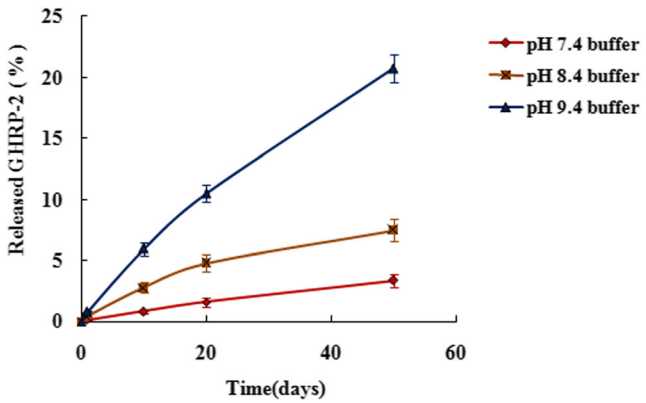


**Fig. 6.** Kinetics of hydrolysis of mono-PEGylated GHRP-2 in borate buffer at 37°C

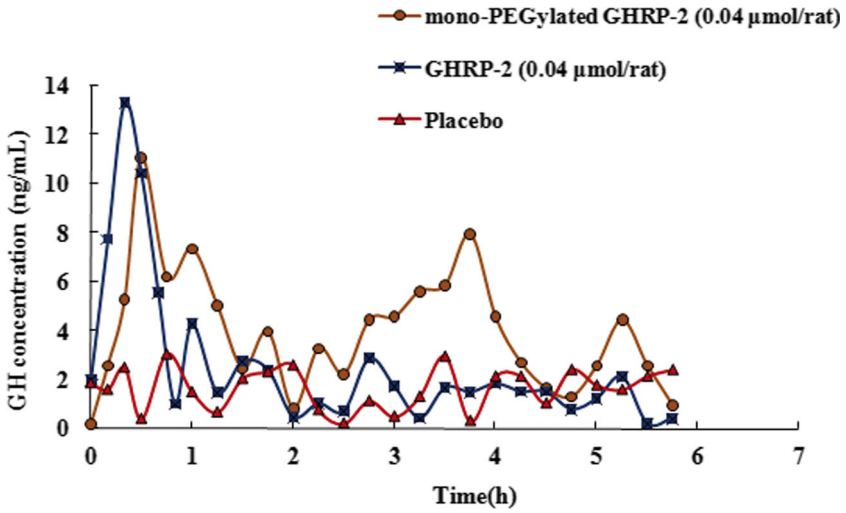


**Fig. 7.** Individual GH serum concentration time course following single intravenous administration of a 0.04 µmol/rat dose of the mono-PEGylated GHRP-2, GHRP-2, or placebo in rats

determined using HPLC at the time points indicated in Fig. 6. Fifty days after incubation, release rates of mono-PEGylated GHRP-2 in borate buffer were found to be 2.1% (pH 7.4), 7.2% (pH 8.4), and 20.5% (pH 9.4), respectively. Therefore, it can be concluded that mono-PEGylated GHRP-2 was stable in weakly alkaline conditions.

### Biological Activity

The profile of individual pharmacodynamic response to different treatments drew a clear comparison of the effects, as it is shown in Fig. 7. After intravenous administration of GHRP-2 or mono-PEGylated GHRP-2, a main peak of GH serum concentration was found to be at about 15–30 mins after dosing. More importantly, the rat groups treated with PEG-modified GHRP-2 showed repeated GH peaks after dosing within the observation time. The intensity of the peaks decreased with the time went by, but stronger than the basal endogenous GH profiles. The results indicated that the mono-PEGylation of GHRP-2 did not impair its biological activity; on the contrary, it prolonged its half-life in plasma.

## CONCLUSION

Due to the presence of two reactive amine groups, a mixture of mono- or multi-PEGylated GHRP-2 could be obtained theoretically. HPLC and MALDI-TOF MS showed that an anhydrous reaction condition was superior to the traditional buffer solution for the synthesis of mono-PEGylated GHRP-2, which mainly attributed to higher stability of mPEG-NHS reagent in anhydrous solvent. The PEGylated GHRP-2 showed good stability in weakly alkaline buffer. The bioactivity study also suggested that the mono-PEGylation of GHRP-2 increases the effectiveness of the GH response in rats. Overall, a strategy for the peptide stabilization such as PEGylation is successful to the applications of GHRP-2.

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