Preparation and Characterization of PEGylated Terlipressin

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ABSTRACT: Terlipressin was chemically modified by reaction with succinimidyl propionate- monomethoxy polyethylene glycol (mPEG-SPA). To determine the PEGylated degree, the position and the optimized condition for PEGylated terlipressin, the reactions were monitored in different pH value buffers at different molar ratios by reversed-phase high performance liquid chromatography (RP-HPLC). Tryptic digestion and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used. The results showed that the amount of mono-PEG-terlipressin was higher at lower pH value and lower content of PEG. Meanwhile, the amount of di-PEG-terlipressin was higher at higher pH value and higher content of PEG under the conditions investigated. The position of PEGylated terlipressin was confirmed by tryptic digestion. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 116: 3220–3224, 2010

Key words: terlipressin; PEGylated degree; molar ratio; pH value

INTRODUCTION

Terlipressin, as a single polypeptide consisting of 12 amino acids, is an analog of vasopressin used as a vasoactive drug in norepinephrine-resistant septic shock, hepatorenal syndrome and bleeding esophageal varices.[1,2] As a polypeptide, terlipressin was subjected to chemical and enzymatic degradation and has a short biological half-life in the body. To overcome the rapid clearance of polypeptide from the blood circulation and its *in vivo* instability, chemical modification of therapeutic polypeptides with polyethylene glycol (PEG) has been utilized as a novel approach.[3–5] It might be the most widely used polymer for drug conjugation to obtain functional bioconjugates possessing increased resistance to proteolytic degradation, increased solubility, decreased immunogenicity, and low toxicity.[6,7] The difficulty in the physicochemical characterization of PEGylated polypeptides was due to their heterogeneity with respect to the distribution of both the number and position of attached PEG molecules and inherent polydispersity of PEG itself.[3]

The pharmacological properties of the PEGylated polypeptides was influenced by the attached number of PEG molecules.[8,9] Despite the obvious importance of controlling the PEGylation, there had been relatively few published studies on controlling the degree of PEGylated terlipressin and the study of PEGylated terlipressin was not reported in literature. MALDI-TOF MS has recently been demonstrated as a convenient and powerful method to characterize PEGylated polypeptides and proteins.[8,10–12] In this contribution, the utility of RP-HPLC and MALDI-TOF MS for monitoring the formation of PEGylated terlipressin was investigated by varying the molar ratio of PEG molecules to peptide and the pH value of the reaction medium. The position of PEGylated terlipressin was confirmed by tryptic digestion.

EXPERIMENTAL

Materials

Succinimidyl propionate-monomethoxy PEG (mPEG-SPA) and terlipressin were purchased from Beijing Kaizheng and GL Biochem, respectively. α-cyano-4-hydroxycinnamic acid (α-CHCA) and trifluoroacetic acid (TFA, HPLC grade) were obtained from Sigma. Acetonitrile (HPLC grade) was supplied from Fisher Scientific. All other chemicals were of analytical grade and obtained commercially.

Preparation and separation of PEGylated terlipressin

mPEG-SPA, corresponding to molar excesses of 1–6, was added to a terlipressin solution (3 mg/mL) in

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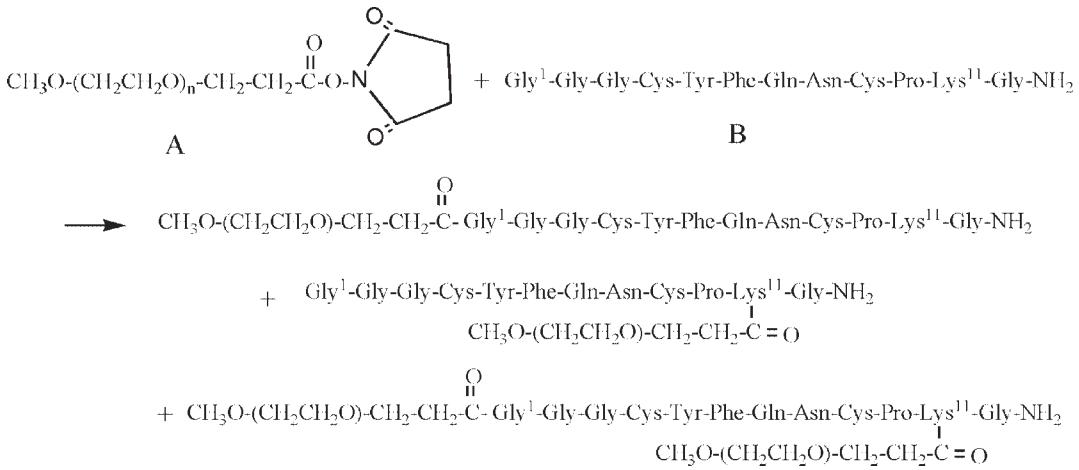

Figure 1 Reaction of mPEG-SPA (A) and terlipressin (B, a disulfide bond between the two Cys).

50 mmol L^{-1} phosphate buffers of different pH values (pH = 6–8). The reaction mixtures were shaken gently at 30°C for 60min. The PEGylation reactions were quenched by adjusting the pH to 3.5 with phosphoric acid. The PEG-terlipressin mixture was applied to a Diamosil C18(2) column (250 mm × 4.6 mm, 5 μm, Dikma) for RP-HPLC analysis with Waters 2695–2996 by UV detection at 210 nm at 30°C. Gradient elution was carried out at a flow-rate of 1.0 mL/min with solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile).The following gradient profile was chosen: 5–50% B over 35min. After an additional elution for 10 min with 100% B followed by 10 min with 5% B, the system was ready for the next injection. The HPLC fractions corresponding to respective peaks were collected by Waters 600E-2487 HPLC, separately, purged off with nitrogen, and stored at 4°C.

Identification of PEGylation number and position

A tryptic digestion was performed by adding 4 μL of trypsin (5 mg/mL in water) to 20 μL of 50 mmol L^{-1} phosphate buffer solution (pH = 7.0) containing the respective peaks of PEG- terlipressin (~ 10 μg/mL) at 40°C for 120 min. Unmodified terlipressin was also treated under the same conditions. The respective peaks and the respective tryptic digested samples were directly applied to MALDI-TOF-MS instrument and the MALDI- TOF-MS was carried out using an autoflex III smartbean (Bruker, Germany). Samples were prepared by mixing each peak fraction with the matrix solution, which was a saturated solution of α-CHCA in 50% of water in acetonitrile. The sample mixture was spotted into a well of the sample plate and dried by vacuum evaporation before mass spectrometry. Data for 2 ns pulses of the 337 nm nitrogen lasers were averaged for each spectrum in a linear mode, and positive ion TOF detection was performed using an accelerating voltage of 25 kV. The external calibration was performed using a mixture of peptide mass standards, the mixture of angiotensin I, ACTH (clip1 ~ 17), ACTH (clip 18 ~ 39), ACTH (clip 7–38), and bovine insulin.

RESULTS AND DISCUSSION

Reaction equation of mPEG-SPA and terlipressin was showed in Figure 1. Terlipressin contains two primary amino groups (the α-amino group at the N-terminus and the ε- amino groups of Lys^{11}), which are highly active to PEGylation reaction (Fig. 1). So two mono-PEG-terlipressins and one di-PEG-terlipressin would be produced. Figure 2 showed the MALDI-TOF MS of PEG. The number molecular weight of PEG was 4880. The PEG MS peak had a normal distribution, as expected for a polymer, with

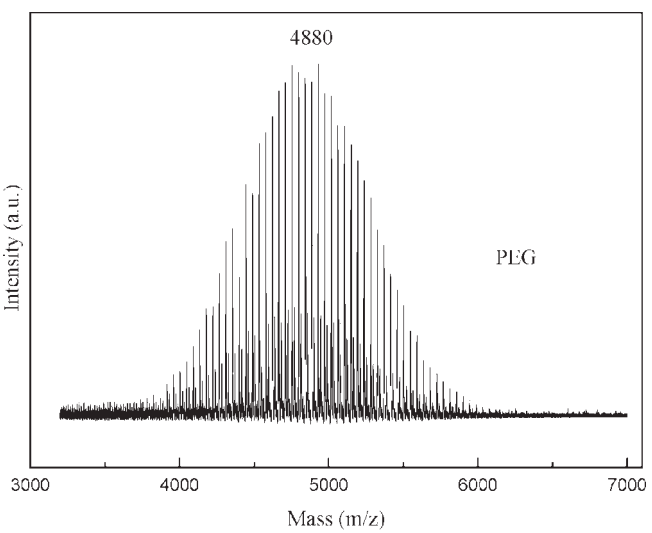

Figure 2 MALDI-TOF MS of PEG.

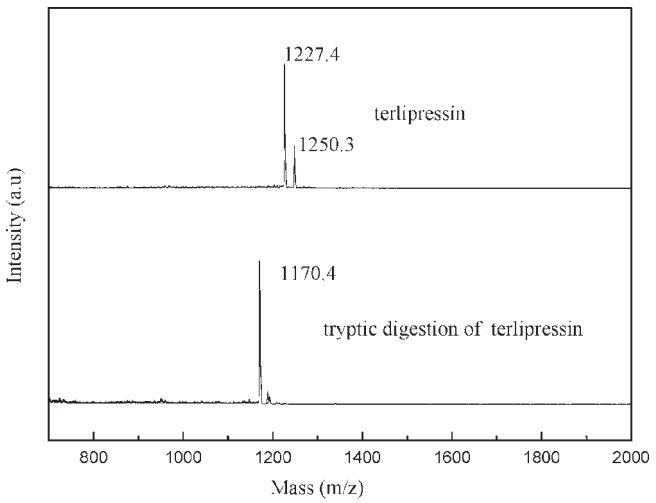

Figure 3 MALDI-TOF MS of terlipressin and tryptic digestion of terlipressin.

peak interval of 44 mass units (PEG monomer unit, —CH_2—CH_2—O—).

Figure 3 showed the MALDI-TOF MS of terlipressin and tryptic digestion of terlipressin. The molecular ion peak of terlipressin was observed at m/z 1227.4, and an unknown peak was observed at m/z 1250.3, which can be attributed to the Na^+ adduct of terlipressin. The endoproteinase trypsin generally cleaves only the carboxy-terminal side of the lysine and arginine residues.[12] Tryptic digestion of native terlipressin results in two fragments, Gly^1- Lys^{11}-OH, corresponded to the peak of 1170.4 m/z in Figure 3 and H-Gly^{12}-NH_2.

Figures 4 and 5 showed the RP-HPLC diagrams for the reactions at different molar ratios of the reactants and at different pH at molar ratio of 1 : 2 (terlipressin: PEG), respectively. The representative PEG-terlipressin was separated by RP-HPLC into three peaks (a, b, and c), (Fig. 4). The first peak (a), (retention time = 14.2 min) was identified to be unmodified terlipressin

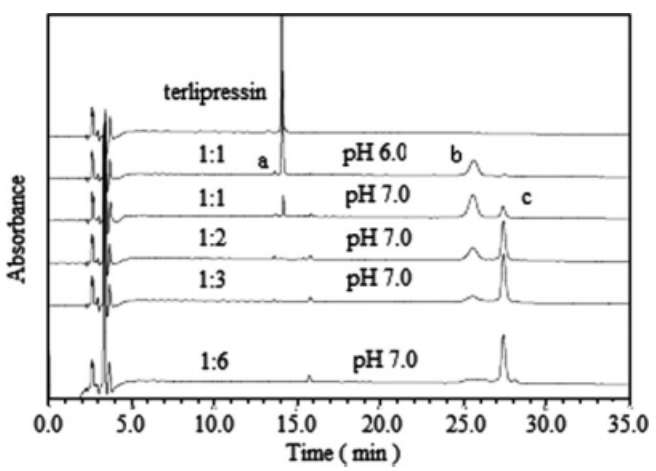

Figure 4 RP-HPLC diagram for the reactions at different molar ratios of the reactants (terlipressin: PEG).

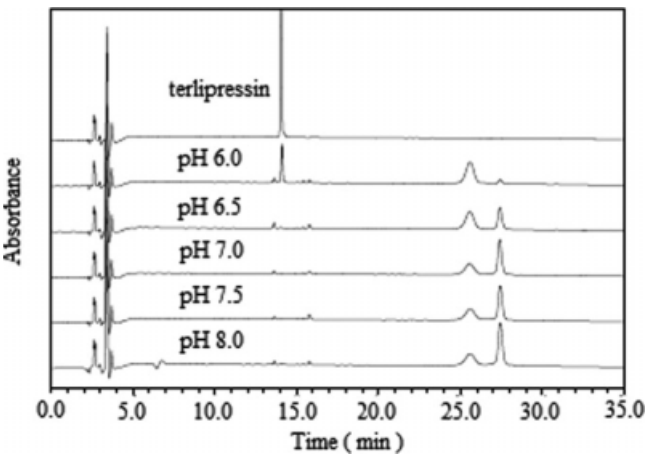

Figure 5 RP-HPLC diagram for the reactions at different pH of the reactants.

as shown in Figures 4 and 5. The other peaks were isolated, and their molecular masses were determined by MALDI-TOF MS. Peaks (b) and (c) had the distribution, which was due to the molecular weight distribution of the conjugated PEG.

Figure 6 showed the MALDI-TOF MS of the peaks (b), (c) and tryptic digestion of the peaks (b), (c). The molecular weight of the second peak (b), (retention time = 25.6 min) showed a main distribution at 6110 m/z indicating mono-PEG-terlipressin. The two different mono-PEG-terlipressins (PEG-Gly^1-terlipressin and PEG-Lys^{11}-terlipressin) could not be completely separated by using RP-HPLC, mainly due to the small physicochemical differences between them. The third peak (c), (retention time = 27.4 min) was found to be approximately at 10,990, which corresponded to the di-PEG-terlipressin.

The PEGylation reaction of peptides was controlled by varying the stoichiometry of the components and the pH values of the reaction. The effect of molar ratios of the reactants and the reaction pH on the PEGylation reaction of terlipressin was monitored by RP-HPLC (Figs. 4 and 5). The extent of the PEGylation reaction is inversely proportional to the amount of free peptide because of PEG molecules have no UV absorbance.[13] The yield of mono-PEG-terlipressin was the mole number of mono-PEG-terlipressin divided by that of total terlipressin. As expected, the yield of PEGylated terlipressin increased as the content of PEG increases (Fig. 4).

With increasing PEG content, a significant increase in the amount of di-PEG-terlipressin was observed (Fig. 4). The yield of di-PEG-terlipressin (74%) was highest at a molar ratio of 1 : 6. At a molar ratio of 1 : 1 and pH = 7.0, mono-PEG-terlipressin (70%) was more predominant than di-PEG- terlipressin (18%). At a molar ratio of 1 : 2, terlipressin was completely reacted.

The dependence of PEGylation of terlipressin on the pH values of the mixtures was also monitored at

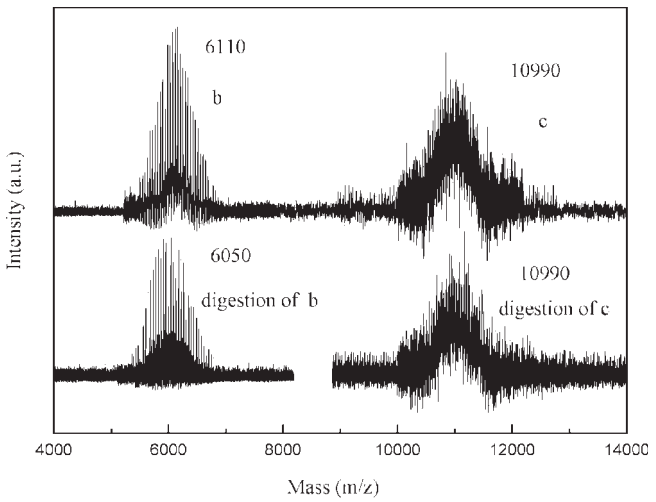

Figure 6 MALDI-TOF MS of the peaks (b and c) before and after tryptic digestion.

a molar ratio of 1 : 2, varying the pH values from 6.0 to 8.0 (Fig. 5). The production of PEGylated terlipressin increased as the pH increases. At pH 6.0 and a molar ratio of 1 : 2, the amount of di-PEG-terlipressin was little (7%), and increased with increasing pH value. So mono-PEG- terlipressin could be efficiently produced at pH 6.0. At pH = 7.0, the amount of intact terlipressin was hardly any. The yield of di-PEG-terlipressin (62%) was highest, while mono-PEG- terlipressin (38%) was lowest at pH = 8.0 and a molar ratio of 1 : 2. This effect is mainly related to the difference in reactivity between α-(N-terminus) and ε-amino (Lys residue) groups at different pH. The primary amine residues in protein have different p*K*a values: pKa 7.8 for N-terminal α-amino group and 10.1 for ε-amino group in lysine residues.[14] In addition, the reaction of mPEG- SPA to primary amine groups of terlipressin took place primarily by nucleophilic substitution reaction which was a kind of primitive reaction between carboxylic acid derivatives and amines: the attack of unprotonated amine group to the carbonyl groups of succinimidyl propionate.[15] It indicated that the two primary amines (one α and the other ε) were reactive to PEGylation at high pH, whereas, at low pH, selective unprotonation of the N-terminal α-amine group would be more reactive than the ε-amine groups in lysine residues. In addition, once PEG binds to Gly^1-terlipressin, the Lys^{11} position has a reduced chance to form a chemical bond with PEG due to the steric hindrances induced by the closed attached PEG molecule at the Gly^1 position. So the yield of mono-PEG-terlipressin was higher at lower pH value and lower content of PEG, such as pH = 6.0 and the molar ratio of 1 : 1 (as shown Fig. 4), but di-PEG-terlipressin (1%) was the lowest at the above condition. The amount of di-PEG-terlipressin was higher at higher pH value and higher content of PEG. In a word, mono-PEG-terlipressin and di-PEG-terlipressin could be adjusted by the molar ratio or pH. The molar ratio and pH combined would produce more efficient adjustment.

The amino acid residues conjugated with polymer PEG showed the resistance of the peptide to proteolytic digestion because of the steric hindrance of the PEG strands.[3,12,16] So the PEGylation sites were determined by measurement of the mass change based on the PEG resistance to the proteolytic cleavage during tryptic digestion. When the mono-PEG-terlipressin was treated with tryptic digestion, mass peaks surrounding 6050 m/z were produced (as shown Fig. 6). As expected, di-PEG-terlipressin showed no mass change (as shown Fig. 6). This confirmed the position of attached PEG molecules. As terlipressin has only one lysine residue and no arginine, PEGylation at this site would show no mass change when treated with tryptic digestion. While the tryptic digestion of the mono-PEG-terlipressin modified at the N-terminus would result in the reduced mass fragment.

The result also indicates that the most of mono-PEG-terlipressin consisted of PEG-Gly^1- terlipressin at pH = 6.0 and the molar ratio of 1 : 1 (terlipressin to mPEG-SPA). This is due to the N-terminal α-amine group being more reactive than the ε-amine groups in lysine residues, at lower pH value, such as pH = 6.0. Moreover, mPEG-SPA would prefer to react with N-terminal α-amine group of terlipressin under the environment of the amount of relative little mPEG-SPA.

CONCLUSIONS

In this study, preparation and identification of PEGylated terlipressin were investigated. The degree of PEGylation for terlipressin could easily be controlled through adjustment of the molar ratios and pH value. The amount of mono-PEG-terlipressin was higher at lower pH value and lower content of PEG. However, di-PEG-terlipressin was higher at high pH value and higher content of PEG under conditions investigated. The position of PEGylated terlipressin was confirmed by tryptic digestion.

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