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Synthesis and Chromatographic Separation of Monomethoxypolyethylene Glycol Modified Insulin

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Abstract: Insulin was modified with monomethoxypolyethylene glycol (MPEG)-succinimidyl succinate and succinimidyl ester of carboxymethyl MPEG. Effects of reaction solvents, initial molar ratio of MPEG derivative to insulin and reaction time on PEGylation of insulin were investigated by 2,4,6-trinitrobenzenesulfonic acid spectrophotometric assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sephadex G75 size exclusion chromatography (SEC), ion exchange chromatography (IEC) and reversed phase-high performance liquid chromatography (RP-HPLC) were applied to separate PEGylated insulin. IEC and RP-HPLC were proved to be efficient tools on separation of different PEGylated insulin species.

Keywords: Insulin, monomethoxypolyethylene glycol, PEGylation, size exclusion chromatography, ion exchange chromatography, reversed phase-high performance liquid chromatography

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INTRODUCTION

Being a primary therapy for insulin-dependent diabetes mellitus (IDDM; Type I diabetes), insulin faces many problems typical to protein pharmaceuticals, including poor physical and chemical stability, susceptibility to proteolysis, immunogenicity and antigenicity, and a relatively short plasma half-life. Therefore, the optimal blood glucose control and satisfactory patient compliance can hardly be achieved through conventional subcutaneous injection treatment.

Great deals of efforts have been made to overcome the shortcomings of insulin therapy (1–6). Among them, conjugation of insulin with monomethoxypolyethylene glycol (MPEG) is most advantageous. As compared with parental protein, conjugates of MPEG and insulin exhibit altered properties, including improved stability, enhanced resistance to proteolysis, prolonged circulating life-time in vivo, decreased immunogenicity and antigenicity with most biological activity retained (1, 6), which could help to enhance the therapeutic efficiency.

There are three free amino groups on insulin including GlyA1, PheB1, and LysB29. PEGylation of insulin via reaction with lysine and N-terminal amines generally produced heterogeneous conjugates depending on the number and position of the attached PEG molecules. Efficient methods of analysis and separation of PEGylated insulin mixture are needed because only one or a few species among conjugates' mixture substantially exhibit the optimal biological properties. It was reported that 2,4,6-trinitrobenzenesulfonic acid (TNBS) spectrophotometric assay (7) could provide information of the average modification extent of PEGylated proteins, while sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) could separate different PEGylated protein species with various MPEG chains attached (8). Therefore, simultaneous use of these two methods could be a good choice for analysis of PEGylated insulin mixture. In addition, separation and purification methods of PEGylated proteins were currently dominated by size exclusion and ion exchange chromatography (9), while RP-HPLC was rarely used. Actually, a successful separation of positional isomers of PEGylated growth hormone-releasing factor (1–29) by RP-HPLC has been reported by Youn (10), indicating RP-HPLC was a powerful analysis and separation method for PEGylated proteins.

In this study, insulin was modified with MPEG-succinimidyl succinate (MPEG-SS) and succinimidyl ester of carboxymethyl MPEG (MPEG-SCM). TNBS spectrophotometry and SDS-PAGE were used together to evaluate conjugation processes according to information of the average amino modification extent of PEGylated insulin mixture and preliminary distribution specifications of different PEGylated insulin species. Furthermore, SEC, IEC, and RP-HPLC were employed to separate the PEGylated insulin mixture, and an efficient separation of individual PEGylated insulin species was achieved.

EXPERIMENTAL

Materials

Crystalline porcine insulin (28 IU/mg, batch No. 000305) was purchased from Xuzhou Biochemical Co. (Xuzhou, China). Monomethoxypolyethylene glycol 5000 (MPEG5000) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were from Sigma Chemical Co. (USA). Acetonitrile of HPLC-grade was from Merck (Germany). All other reagents were of the highest grade available and were obtained commercially.

PEGylation of Insulin

Before reaction with insulin, the MPEG5000 was activated to produce MPEG5000-succinimidyl succinate (MPEG5000-SS) and the succinimidyl ester of carboxymethyl MPEG5000 (MPEG5000-SCM) according to the methods of Abuchowski (11) and Andreas (12) with some modifications, respectively. Then, a single-factor research was performed to investigate the effects of reaction solvents, initial molar ratio of MPEG derivative to insulin and reaction time on PEGylation of insulin using MPEG5000-SS. Firstly, insulin was dissolved in the following solvents: 0.1 M borate buffer pH 7.4, 8.0, 8.4, 9.0, DMF/water (10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, v/v), and DMF/0.1 M borate buffer pH 7.4, 8.0, 8.4, 9.0 (60:40, v/v) to produce 1 mg/mL solution of insulin. MPEG5000-SS reacted with insulin at initial molar ratio of 5:1 in various solvents for 4 h, respectively. Secondly, MPEG5000-SS reacted with insulin at initial molar ratio of 1:1, 2:1, 3:1, 5:1, 10:1 in DMF/0.1 M borate buffer pH 7.4 (60:40, v/v) for 4 h, respectively. Thirdly, MPEG5000-SS reacted with insulin at initial molar ratio of 5:1 in DMF/0.1 M borate buffer pH 7.4 (60:40, v/v) for 0.5, 1, 2, 3, 4, 6, 8 h, respectively. The reaction solutions were stirred and quenched by addition of glycine (2 times of MPEG derivatives by molar ratio), respectively. Then the reaction mixture was dialyzed against 0.01% (m/v) NH_4HCO_3 solution and lyophilized. PEGylation of insulin using MPEG5000-SCM will follow the processes based on the results of the above single-factor research. For convenience, the corresponding products of PEGylated insulin were marked as MPEGss-insulin and MPEGscm-insulin, respectively.

TNBS Assay and SDS-PAGE

The average modification extent of PEGylated insulin mixture was determined by TNBS spectrophotometry (7). Reaction mixtures after dialysis in section 2.2 were analyzed by SDS-PAGE (Mini-Protean® III, Bio-Rad, USA) under non-reducing conditions according to Laemmli (13), with 7.5–15% polyacrylamide gel.

Separation of PEGylated Insulin

Size Exclusion Chromatography (SEC)

The PEGylated insulin mixture was subject to SEC on a column (1.0 × 50 cm) packed with Sephadex G75 (Amersham Biosciences, Sweden) at room temperature. The column was equilibrated and eluted with 0.1 M acetate buffer pH 4.0, and 1 mL sample was loaded. The flow rate was 1 mL/min, and the elution profile was monitored at 280 nm. Intact insulin, *N*-hydroxysuccinimide (NHS), and MPEG5000-SS were analyzed as process controls.

Ion Exchange Chromatography (IEC)

The PEGylated insulin mixture was subject to IEC on a Protein-Pak^{TM} SP 8HR cation exchange column (1.0 × 8 cm, Waters, USA) connected to FPLC system (600E, Waters, USA) at room temperature. The column was equilibrated with 0.05 M acetate buffer pH 4.0, and 100 μL sample was loaded. The flow rate was 1.2 mL/min. The column was eluted by 0–0.5 M sodium chloride linear gradient in acetate buffer pH 4.0 for 40 min after 10 min's application of equilibration buffer, and the elution profile was monitored at 280 nm. The target fractions were pooled respectively, and subsequently applied to SDS-PAGE.

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

The PEGylated insulin mixture was subject to RP-HPLC on a column of Diamonsil C18 (4.6 × 150 mm, 5 μm, Dikma, USA) connected to HPLC system (LC 10AT, Shimadzu, Japan) at room temperature. Gradient elution was carried out at a flow rate of 1.0 ml/min^{-1} with 0.1% (v/v) trifluoroacetic acid (TFA) in water (eluent A) and 0.1% (v/v) TFA in acetonitrile (eluent B), and the elution profile was monitored at 220 nm. For MPEGss-insulin, a linear gradient from 30 to 51% eluent B for 35 min was performed. While for MPEGscm-insulin, the gradient program was set as follows: 34–38% B over 5 min, 38–43% B over 15 min, 43–45% B over 7 min.

RESULTS AND DISCUSSION

TNBS Assay and SDS-PAGE Analysis of the Conjugation Products

Effect of reaction solvents on PEGylation of insulin was shown in Fig. 1. Among the 17 kinds of solvents, 0.1 M borate buffer pH 8.0, DMF/water (90:10, v/v) and DMF/0.1 M borate buffer pH 7.4 (60:40, v/v) resulted in

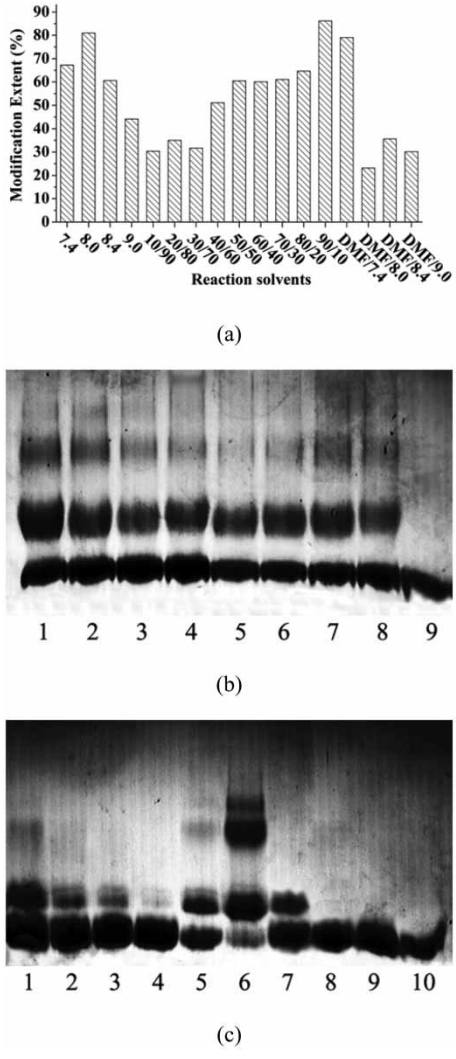

Figure 1. Effect of reaction solvents on synthesis of MPEGss-insulin. MPEG5000-SS reacted with insulin at initial molar ratio of 5:1 in the corresponding solvents for 4 h, respectively. (a) Effect of reaction solvents on modification extent of MPEGss-insulin mixture. The labels on horizontal axis from left to right respectively represent the following solvents: 0.1 M borate buffer pH 7.4, 8.0, 8.4, 9.0, DMF/water (10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, v/v), and DMF/0.1 M borate buffer pH 7.4, 8.0, 8.4, 9.0 (60:40, v/v); (b) and (c) SDS-PAGE of reaction mixtures after dialysis (MWCO 3500). Lane 1–9 in (b) were reaction mixtures in 0.1 M borate buffer pH 7.4, 8.0, 8.4, 9.0, DMF/water (50:50, 60:40, 70:30, 80:20, v/v), and intact insulin; lane 1-10 in (c) were reaction mixtures in DMF/water (40:60, 30:70, 20:80, 10:90, 90:10, v/v), DMF/0.1 M borate buffer pH 7.4, 8.0, 8.4, 9.0 (60:40, v/v), and intact insulin.

relatively higher modification extent. Besides, heavy-colored bands of insulin and light-colored bands of PEGylated insulin especially highly PEGylated insulin (di- or tri-PEGylated) in SDS-PAGE profiles indicated that the yield of PEGylated insulin in 0.1 M borate buffer pH 8.0 and DMF/water (90:10, v/v) was lower than that in DMF/0.1 M borate buffer pH 7.4 (60:40, v/v). It is probably ascribed to the synergistic action of reactivity of amino groups of insulin and the hydrolysis rate of MPEG-SS and MPEGss-insulin. Only the deprotonated amino groups on proteins could react with MPEG derivatives. Higher pH could lead to much more deprotonated amino groups and consequently bring about higher modification extent. However, too high a pH would increase the hydrolysis rate of the instable succinimidyl and succinyl ester linkage in MPEG-SS before it reacted with insulin and succinyl ester linkage in MPEGss-insulin. Thus, solvents with higher pH value such as borate buffer pH 9.0 and DMF/borate buffer pH 8.0, 8.4, 9.0 (60:40, v/v) brought much lower modification extent as well as light-colored highly PEGylated insulin bands or even absence of PEGylated insulin bands.

It was suggested from Fig. 2 that PEGylation of insulin is a reaction molar ratio dependent process. Modification extent of insulin increased along with the reaction molar ratio. Mono-PEGylated insulin was the main product when reaction molar ratio of MPEG-SS to insulin was below 3:1. Further increase of the reaction molar ratio would greatly increase the content of highly PEGylated insulin.

The effect of reaction time was shown in Fig. 3. Modification extent of insulin increased along with reaction time. It is obvious in Fig. 3a that the reaction reached a plateau phase when it went through for 4 h. This tendency was also indicated by SDS-PAGE profile (Fig. 3b). Besides, it was indicated by Fig. 3b that most mono-PEGylated insulin had been produced in the first 30 min of the conjugation reaction. After that, the content of the mono-PEGylated insulin decreased along with time, while the content of highly PEGylated insulin increased.

Separation of PEGylated Insulin

The SEC profile of the PEGylated insulin mixture was shown in Fig. 4. The first peak in Fig. 4d and the single peak in Fig. 4e corresponded to PEGylated insulin. The second peak in Fig. 4d could be considered as unreacted insulin and NHS. Despite that SEC was able to separate the PEGylated insulin from intact insulin and NHS, the chromatograms did not exhibit heterogeneity of MPEG-insulin conjugates, indicating the heterogeneous composition of PEGylated insulin mixture could not be resolved by SEC on Sephadex G75. It is probably attributed to the limited resolving power of Sephadex gel on molecules with minor differences in molecular weight and size.

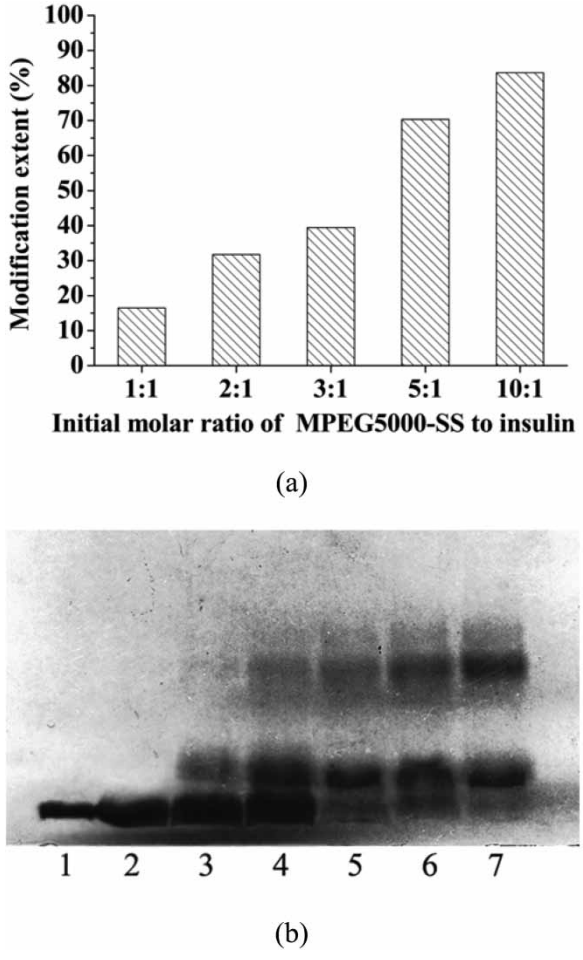

Figure 2. Effect of initial molar ratio of MPEG5000-SS to insulin on synthesis of MPEGss-insulin. MPEG5000-SS reacted with insulin at initial molar ratio of 1:1, 2:1, 3:1, 5:1, 10:1 in DMF/0.1 M borate buffer pH 7.4 (60:40, v/v) for 4 h, respectively. (a) Effect of initial molar ratio of MPEG5000-SS to insulin on modification extent of MPEGss-insulin mixture; (b) SDS-PAGE of reaction mixtures after dialysis (MWCO 3500). Lane 1–7 was intact insulin, insulin incubated 4 h with MPEG5000, and reaction mixtures at initial molar ratio of MPEG5000-SS to insulin of 1:1, 2:1, 3:1, 5:1, 10:1, respectively.

IEC using sodium chloride linear gradient elution was then employed to resolve the heterogeneous composition of the PEGylated insulin mixture and the elution profile was shown in Fig. 5. The four fractions in Fig. 5 were pooled and analyzed by SDS-PAGE, respectively. The SDS-PAGE profile (Fig. 6) indicated that fraction 1 may contain tri-PEGylated insulin besides

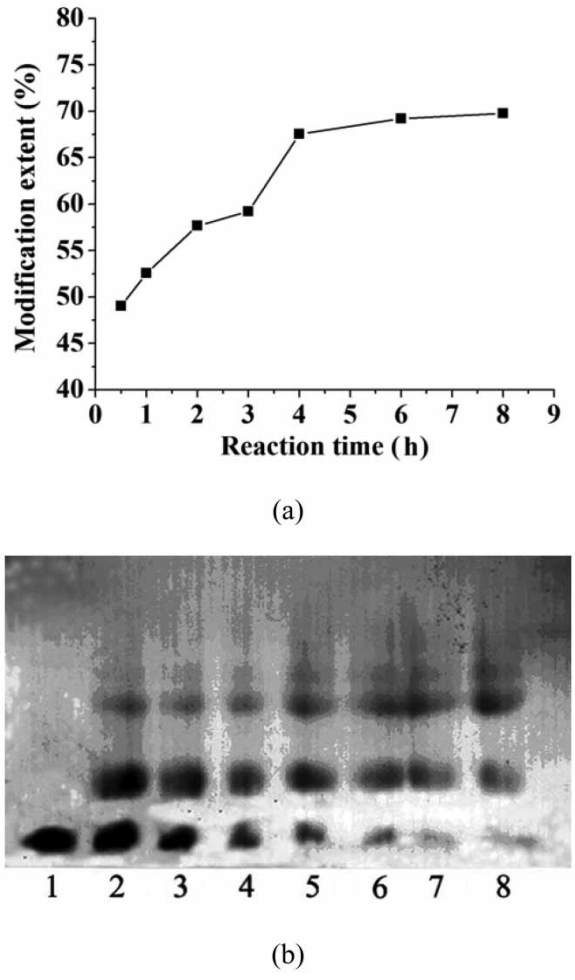

Figure 3. Effect of reaction time on synthesis of MPEGss-insulin. MPEG5000-SS reacted with insulin at initial molar ratio of 5:1 in DMF/0.1 M borate buffer pH 7.4 (60:40, v/v) for 0.5, 1, 2, 3, 4, 6, 8 h, respectively. (a) Effect of reaction time on modification extent of MPEGss-insulin mixture; (b) SDS-PAGE of reaction mixtures after dialysis (MWCO 3500). Lane 1–8 was intact insulin and reaction mixtures with reaction time of 0.5, 1, 2, 3, 4, 6, 8 h, respectively.

the excess inactive MPEG residues from hydrolysis of MPEG derivative, and fraction 2 to 4 corresponded to di-PEGylated insulin, mono-PEGylated insulin and unmodified insulin in turn. The elution sequence was found to be inversely corresponding to the degree of MPEG substitution on insulin. It was agreed well with the explanation that PEGylation via amines in amide bond linkage would neutralize the positive charge of insulin and simultaneously

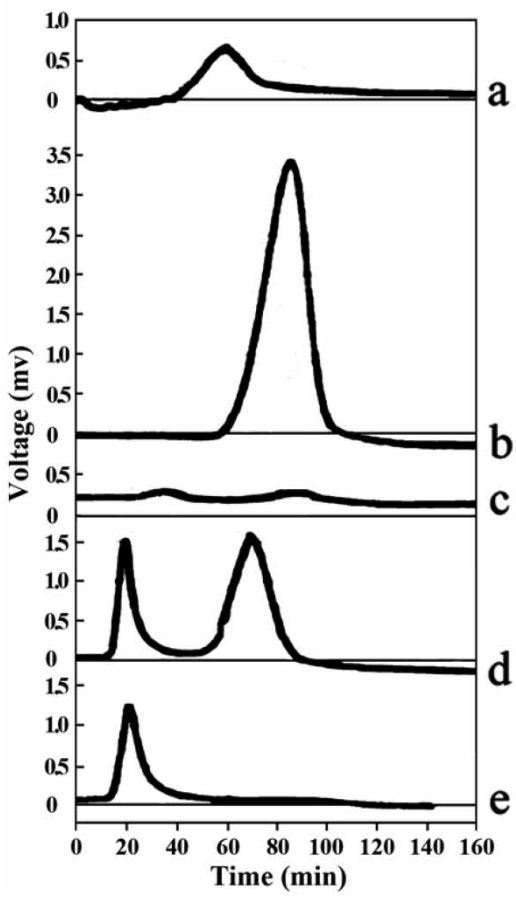

Figure 4. Chromatogram of size exclusion chromatography of MPEGss-insulin mixture. (a) intact insulin; (b) NHS; (c) MPEG5000-SS; (d) reaction mixture of MPEG5000-SS with insulin before dialysis; (e) reaction mixture after dialysis (MWCO 8000).

shield surface charge of it, and consequently weakened the interaction between PEGylated insulin and ion exchange media (14). Fraction 1, 2, and 3 (namely tri-, di-, and mono-PEGylated insulin) were analyzed by matrix assisted laser desorption and ionization-time of flight mass spectrometry (MALDI-TOF MS) later to acquire the molecular weight of three species of PEGylated insulin (spectra was not shown here). Tri-PEGylated insulin could not be detected due to relatively lower desorption/ionization/detection efficiency of insulin with large amounts of PEG on its surface. Molecular weight of di-PEGylated insulin and mono-PEGylated insulin was identified to be about 15.9 KDa and 10.8 KDa. Considering the molecular

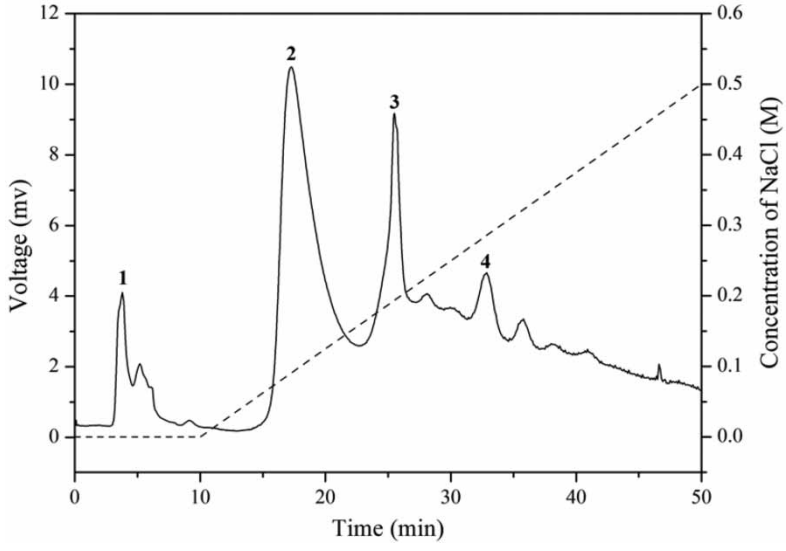

Figure 5. Elution profile of separation of MPEGss-insulin mixture by cation exchange chromatography.

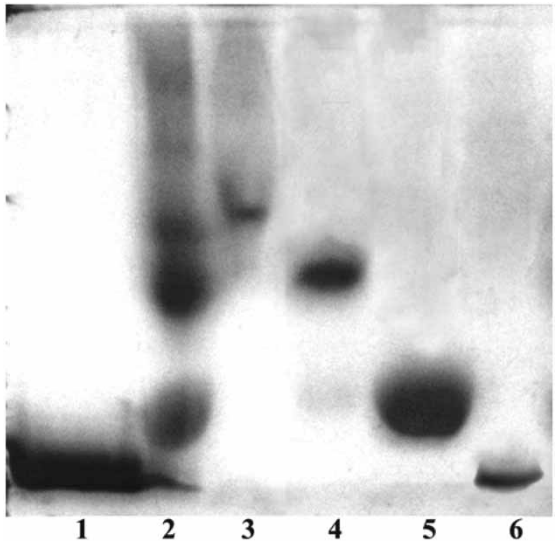

Figure 6. SDS-PAGE analysis of fractions eluted from cation exchange chromatography. Lane 1: insulin; lane 2: $MPEG_{SS}$-insulin mixture; lane 3: fraction 1 in Fig. 5; lane 4: fraction 2 in Fig. 5; lane 5: fraction 3 in Fig. 5; lane 6: fraction 4 in Fig. 5.

weight of insulin (5.8 KDa) and MPEG (5 KDa), the result of MALDI-TOF MS agreed well with the calculated molecular weight of di-PEGylated insulin (15.8 KDa) and mono-PEGylated insulin (10.8 KDa).

RP-HPLC using gradient elution was also conducted to separate individual PEGylated insulin species, and satisfying separation was achieved. There are grouped peaks in Fig. 7a during 10–15 min which may be assigned to insulin and insulin with succinate tag produced by hydrolysis of MPEGss-insulin conjugates. The later peaks after 20 min were considered as mono-, di-, and tri- MPEGss-insulin species in turn. As for MPEGscm-insulin conjugates, three main peaks in Fig. 7b between 9–25 min were considered as mono-, di-, and tri- MPEGscm-insulin species in turn. There are no grouped peaks like that of MPEGss-insulin, which proved that MPEGscm-insulin is more stable than MPEGss-insulin. The retention time of individual PEGylated insulin species increases with the increasing of PEGylation degree, somehow correlating with an increase of hydrophobicity of the conjugative molecule since the PEG chains are known as amphiphilic polymeric entities (15). Excess inactive MPEG residues from hydrolysis of MPEG

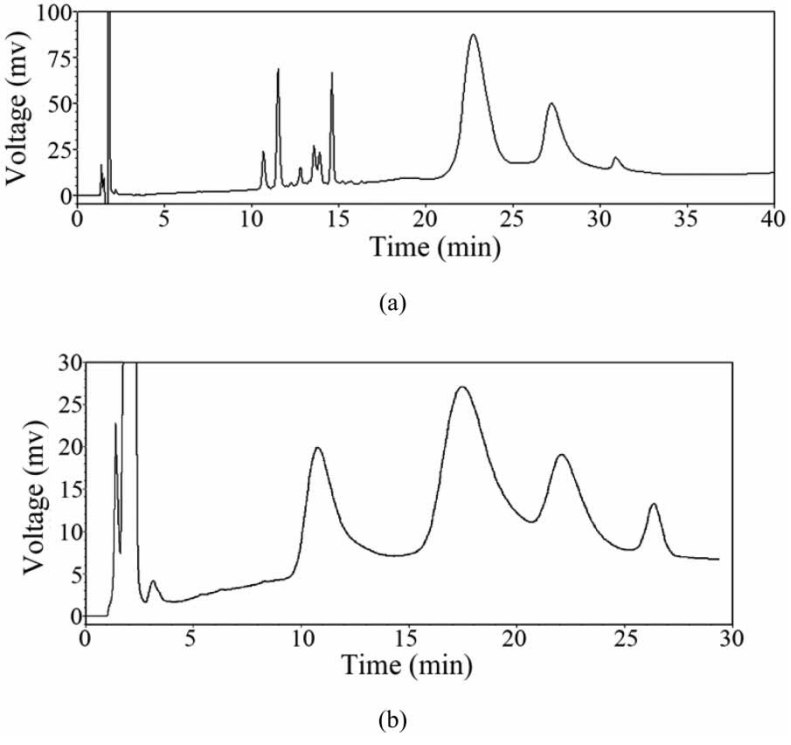

Figure 7. Chromatogram of separation of MPEGss-insulin and MPEGscm-insulin mixture by RP-HPLC. (a) MPEGss-insulin; (b) MPEGscm-insulin.

derivative in products of conjugation reaction would influence the chromatographic behavior of individual PEGylated insulin. It is supposed that removing excess free MPEG residues by IEC would help to improve the separation of PEGylated insulin species.

Compared with TNBS spectrophotometry and SDS-PAGE, RP-HPLC as an analytical method of PEGylated insulin could provide information about the modification extent and the heterogeneous distribution of PEGylated insulin species in a single run. Meanwhile, stability of the conjugates could also be exhibited based on the good separation of de-PEGylated products from the intact conjugates, which may be the apparent advantages of this method.

CONCLUSIONS

In this study, PEGylated insulin was prepared by the reaction of insulin and the succinimidyl active ester of MPEG. Although it appeared neither TNBS assay nor SDS-PAGE alone could provide sufficient information about PEG modification of insulin, simultaneous use of TNBS spectrophotometry and SDS-PAGE was proved to be efficient to evaluate conjugative processes of insulin with PEG. Besides, a strong cation exchange (sulfopropyl) chromatography using sodium chloride linear gradient elution and C18 RP-HPLC chromatography using acetonitrile containing trifluoroacetic acid gradient elution were developed for separation of different PEGylated insulin species with high-resolution. IEC and RP-HPLC are valuable tools for separation of different PEGylated proteins species, and is promising for the rational design of specific PEGylated peptides with optimal bioactivity and in vivo behavior in a future study.

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