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Utilization of a precolumn with size exclusion and reversed-phase modes for size-exclusion chromatographic analysis of polysorbate-containing protein aggregates

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ABSTRACT

Size-exclusion chromatography (SEC) is a useful method for quantification of protein aggregates because of its high throughput capacity and highly quantitative performance. One of the problems in this method concerns polysorbates, which are well-known additives for protein-containing products to prevent protein aggregation, but frequently interfere with the photometric detection of protein aggregates. We developed a new SEC method that can separate polysorbates from protein sample solutions in an on-line mode with a precolumn with size exclusion and reversed-phase mixed modes. The precolumn can effectively trap polysorbates in aqueous mobile phase, and the trapped polysorbates are easily eluted with acetonitrile-containing aqueous mobile phase to clean the precolumn. Small parts of protein aggregates may be also trapped on the precolumn depending on temperature and proteins. Setting appropriate column temperature can minimize such inconvenient trapping of aggregates.

1. Introduction

Nowadays, protein-based pharmaceutical products have rapidly been advanced. The situation increases demands for development of analytical technologies for protein aggregates, which are common sources of protein instability and are considered to concern their potential to elicit immune responses [1–5]. Various analytical methods that can evaluate protein aggregates have been developed such as analytical ultracentrifugation and asymmetric field flow fractionation [6–10]. However, these methods still have several weak points, for example, low throughput, need for professional skills, or difficulty in method development [10]. In contrast, size-exclusion high performance liquid chromatography (SEC-HPLC) with high throughput capacity and highly quantitative performance is frequently used and indispensable for quantification of protein aggregates.

In SEC-HPLC, proteins and their aggregates are separated based on the difference in the permeation property into pores of stationary phase (e.g., silica-based polymeric beads). Larger molecules (e.g., aggregates) or non-spherical (e.g., straight chain) molecules in a mixture are rapidly excluded and therefore eluted from the resin pores, while smaller molecules with greater access to the pores are eluted more slowly [11]. In many marketed biopharmaceuticals, nonionic surfactants such as polysorbate 80 (PS80) and polysorbate 20 (PS20) are included as stabilizers to protect the active protein against denaturation or aggregate formation [12–16]. The molecular mass of the polysorbates is usually lower than that of most of proteins and protein aggregates. However, the polysorbates present in some formulations are eluted at retention times close to those of protein aggregates and then interfere with aggregate quantitation in SEC-HPLC. This interference becomes a serious problem to perform sensitive and quantitative SEC-HPLC methods capable of quantitating protein aggregates in formulated products. Unfortunately, it is very difficult to selectively separate detergents from protein solutions. Some methods are reported to separate detergents from protein solutions [17–22], but these methods take time because they cannot be connected directly to an analytical column and the recovery of protein is not 100%. Gunturi et al. proposed to utilize potassium phosphate buffer containing isopropyl alcohol as a mobile phase in SEC-HPLC [23]. They found that the peaks of the polysorbates were completely disappeared when isopropyl alcohol content was increased up to 20–25%

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range in the mobile phase [23]. However, isopropyl alcohol at such high concentrations may cause some damage to proteins. Therefore, some separation methods without use of organic solvents are strongly desired for long time. In this study, we will propose a new SEC-HPLC method that can separate the polysorbates from protein samples in an on-line mode using a precolumn with mixed characteristics of size exclusion phase and reversed-phase. We will also show the significance of the temperature control in the on-line separation to perform sensitive and quantitative SEC-HPLC methods.

2. Experimental

2.1. Chemicals and reagents

Sodium monobasic phosphate, sodium dibasic phosphate and human serum albumin (HSA, lyophilized powder, fatty acid free, globulin free, ≥99%, product # A3782) were from Sigma–Aldrich (St. Louis, MO, USA). Sodium chloride (NaCl) was from Wako Pure Chemicals (Tokyo, Japan). Dulbecco's phosphate-buffered saline (PBS) was from Nissui Seiyaku (Tokyo, Japan). Polysorbate 80 (PS80) was from NOF Corp. (Tokyo, Japan), while polysorbate 20 (PS20) was from Croda (East Yorkshire, England). Synagis injection containing Palivizumab was purchased from Abbvie (IL, USA). The pure standard sample of recombinant human erythropoietin (r-HuEPO) was obtained from Kyowa Hakko Kirin (Tokyo, Japan).

2.2. Sample preparations

HSA and synagis injection were reconstituted by addition given amount of water and dialyzed with PBS for 24 h. The HSA was diluted with the PBS to 3 mg/mL, and the synagis injection was diluted with the PBS to 1 mg/mL. The commercially available r-HuEPO was also dialyzed with the PBS and diluted with the PBS to 1 mg/mL. Heat-induced r-HuEPO aggregate-containing samples were prepared by heating the diluted r-HuEPO sample for one day at 40 °C. PS80 and PS20 were diluted with the PBS to 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1 mg/mL. All the samples were stored at 2–8 °C until the HPLC characterization described in the next section.

2.3. SEC-HPLC methods

SEC-HPLC was performed with an Agilent 1100 chromatography system connected to a Tosoh TSKgel G3000SW$_{XL}$ (300 mm × 7.8 mm, 5 μm particle size). The chromatographic control, data acquisition and data analysis were performed using Chemstation (Agilent Technologies). The samples were set on an auto-sampler (as an accessory of the Agilent 1200 chromatography system) at 4 °C in a refrigerator. The UV detector was operated at a wavelength of 215 nm. The mobile phase for the SEC-HPLC method was the PBS. The mobile phase was filtered with a 0.45-μm Millipore filter and was degassed with an online degasser. The flow rate was 0.5 mL/min and the column was maintained at 25 °C unless otherwise noted. In order to prevent automatic stopping (due to condensation detection at lower temperatures), the leak sensor of the column oven was turned off. The method run time was set to 40 min.

A Shodex MSpak GF-4A (10 mm × 4.6 mm, 9 μm particle size) was used as a precolumn to separate the polysorbates. The column, when necessary, was connected to the front of the main column (Tosoh TSKgel G3000SW$_{XL}$) with a stainless steel capillary and fittings.

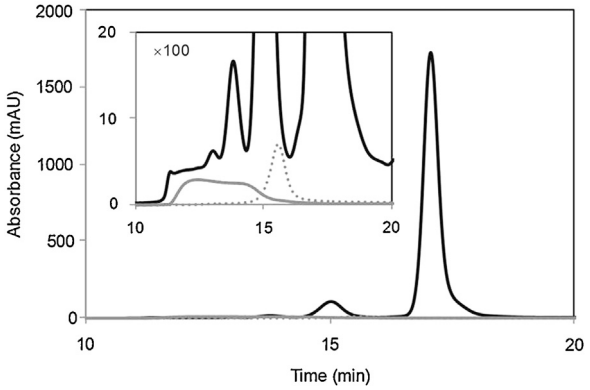

Fig. 1. Chromatograms of the HSA sample (black solid line; 3 mg/mL × 10 μL), diluted PS80 sample (gray solid line; 1 mg/mL × 40 μL) and the diluted PS20 sample (gray dash line; 1 mg/mL × 40 μL) on the SEC-HPLC system without the precolumn at 25 °C (flow rate: 0.5 mL/min, UV detection: 215 nm).

3. Results and discussion

3.1. SEC-HPLC characteristics of HSA aggregates and polysorbates

Fig. 1 shows chromatograms of the HSA sample and the diluted polysorbate samples on the SEC-HPLC system without the precolumn at 215 nm. Spectrophotometric detection of proteins is frequently performed at 280 nm based on the absorption of tryptophan and aromatic amino acid residues. However, the photometric detection at 215 nm due to amide bonds is required for highly sensitive detection of protein targets at extremely low concentrations such as protein aggregates in protein-based pharmaceutical products. The main peak at a retention time of 17 min corresponds to HSA monomer, while the peaks at 15, 14.5, 14 min correspond to dimer, trimer and tetramer of HSA, respectively. Larger aggregates were eluted in the region of retention times from 11 to 14 min. In order to characterize the aggregation, the peak analysis of this region is very important. Unfortunately, PS80 and PS20 were eluted at retention times very close to that of as the aggregates of HSA. Although the molecular mass of PS80 and PS20 are smaller than that of HSA, the polysorbates were eluted at retention times earlier than the time expected from the molecular mass. The reason may be that PS80 and PS20 can be formed in a micellar state in an aqueous solvent. This is a typical example of the interference from PS80 and PS20 in the SEC-HPLC quantification of aggregate contents as reported before [23].

We tried in this work to separate the polysorbate's peaks from the protein-derived ones by using a precolumn method because on-line analysis without any specific separation pretreatment of protein samples is convenient for chromatographic analysis of protein aggregates in protein-based pharmaceutical products. One of separation modes to be utilized for this purpose may be electrostatic interaction in anion or cation exchange. However, separation conditions for ion exchange chromatography have to be tuned for every protein, because the isoelectric points of proteins are different from each other. Another separation mode may be hydrophobic interaction in reversed-phase. One of weak points of this method is that the method requires organic solvents or ammonium sulfate in elution buffer. The situation may give some damages to proteins and may cause the dissociation of some non-covalent aggregates in reversed-phase chromatography and hydrophobic interaction chromatography. As a result, we have considered that some porosity silica gel with some hydrophobic characteristics may be effective to remove hydrophobic surfactants with relatively large molecular mass such as PS80 and PS20. In preliminary experiments we tried to use two kinds of anion exchange columns