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Quantitation of low concentrations of polysorbates in high protein concentration formulations by solid phase extraction and cobalt-thiocyanate derivatization

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HIGHLIGHTS

- Low levels of polysorbates can be extracted very efficiently by an Oasis HLB solid phase extraction cartridge.
- Guanidine HCl is an excellent reagent to release polysorbates from proteins, enhancing SPE extraction capability for polysorbates.
- Cobalt-thiocyanate reagent reacts specifically and uniformly with extracted polysorbates, and the derivative is measured spectrophotometrically at 620 nm.
- The developed method is able to accurately and precisely quantify 30–40 mg L^{-1} polysorbate with LOQ of 10 mg L^{-1} in up to 300 g L^{-1} protein formulations.

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ABSTRACT

A spectrophotometric method was developed to quantify low polysorbate (PS) levels in biopharmaceutical formulations containing high protein concentrations. In the method, Oasis HLB solid phase extraction (SPE) cartridge was used to extract PS from high protein concentration formulations. After loading a sample, the cartridge was washed with 4 M guanidine HCl and 10% (v/v) methanol, and the retained PS was eluted by acetonitrile. Following the evaporation of acetonitrile, aqueous cobalt-thiocyanate reagent was added to react with the polyoxyethylene oxide chain of polysorbates to form a blue colored PS–cobaltothiocyante complex. This colored complex was then extracted into methylene chloride and measured spectrophotometrically at 620 nm. The method performance was evaluated on three products containing 30–40 mg L^{-1} PS-20 and PS-80 in ≤70 g L^{-1} protein formulations. The method was specific (no matrix interference identified in three types of protein formulations), sensitive (quantitation limit of 10 mg L^{-1} PS) and robust with good precision (relative standard deviation ≤6.4%) and accuracy (spike recoveries from 95% to 101%). The linear range of the method for both PS-20 and PS-80 was 10 to 80 mg L^{-1} PS. By diluting samples with 6 M guanidine HCl and/or using different methylene chloride volumes to extract the colored complexes of standards and samples, the method could accurately and precisely quantify 40 mg L^{-1} PS in up to 300 g L^{-1} protein formulations.

1. Introduction

Polysorbate 20 (PS-20) and polysorbate 80 (PS-80) are widely used as excipients in protein biopharmaceutical formulations [1] because they have high surface activity [2,3], minimum binding to proteins [4], and low toxicity [5,6]. Polysorbates play a critical role in preventing protein surface adsorption at liquid–liquid, liquid–solid or liquid–air interfaces, which can lead to surface-induced denaturation and aggregation [7,8]. Many studies have demonstrated that polysorbates have a protective effect on protein stability during protein processing such as refolding [9], mechanical stress such as agitation, shaking or stirring [10–13], freeze thawing [14], freeze drying [15], and reconstitution of the lyophilized protein [16], as well as for formulations containing silicone oil droplets [12]. Because of its significance, it is necessary to monitor PS levels within a defined range during various stages of the protein formulation development. For this purpose, developing an accurate method is of importance.

Polysorbates consist of a core sorbitan group and four polyoxyethylene oxide side chains with $w+x+y+z=20$, one of which is esterified with a fatty acid, oleic acid for PS-80 or lauric acid for

Abbreviations: PS, polysorbate; SPE, solid phase extraction; UF/DF, ultrafiltration/diafiltration; DS, drug substance; SD, standard deviation; RSD, relative standard deviation; mAb, monoclonal antibody; Abs, absorbance.

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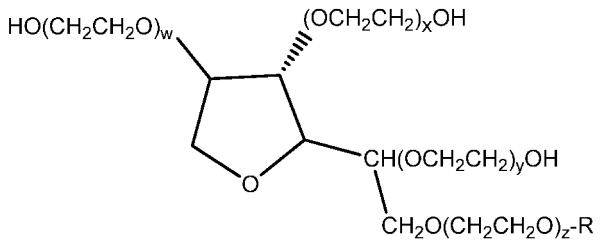

where w + x + y + z = 20 and R = oleic acid on PS-80 or R = lauric acid on PS-20.

Fig. 1. Chemical structures of PS-20 and PS-80.

PS-20 (Fig. 1). Because of the variable length of each side chain, the resulting product is a mixture of molecules with various sizes rather than a single uniform compound and the number of possible molecular structures could be greater than 1500 [17]. Furthermore, fatty acid itself is a diverse mixture of different fatty acid esters. For example, lauric acid in polysorbate 20 is only 40–60% of the total number of fatty acid species while oleic acid in polysorbate 80 is ≥58% of its total [18], which provides further diversity of the PS molecules.

Heterogeneous molecular structures of polysorbates with very weak ultraviolet absorption have caused many analytical challenges. In many monoclonal antibody (mAb) formulations, protein concentrations are higher than 150 g L^{-1}. The increasing protein concentrations in samples create an additional challenge to the method accuracy and precision as it is difficult to remove the proteins completely and residual proteins may interfere with PS quantitation. On the other hand, the concentration of polysorbate used is very low, and it is as low as 40 mg L^{-1} in some mAb formulations. These four factors (heterogeneous molecular structures, weak ultraviolet absorption, low PS levels and high protein concentrations) are compound in current mAb formulations.

Various chromatography techniques, including reversed phase, size exclusion, and ion exchange chromatography, have been reported for the determination of non-ionic surfactants [17,19–21]. These chromatographic methods cannot be directly used to determine polysorbates in high protein concentration formulations due to column fouling. More recently, Hewitt et al. developed a direct injection method for determining PS-20 in protein solutions using mixed-mode chromatography and evaporative light scattering detection [22]. This method is rapid and can accurately determine PS-20 from 100 to 300 mg L^{-1} in formulations containing 30–185 g L^{-1} proteins. An effort was made to adopt the method for quantifying <50 mg L^{-1} PS-20 in 70 g L^{-1} mAb formulations. However, it was not able to quantify low PS levels accurately because residual proteins were found to co-elute with PS-20, resulting in an unacceptably high PS-20 recovery (data not shown). The present paper described an accurate procedure for determination of polysorbates in high protein formulations using solid phase extraction, derivatization, and spectrophotometric measurement. The method is able to quantify both PS-20 and PS-80 concentrations with a quantitation limit of 10 mg L^{-1} in high protein concentration formulations.

2. Experimental

2.1. Materials

Sodium chloride and acetone were purchased from Fisher Scientific (Pittsburgh, PA); cobalt nitrate hexahydrate, ammonium thiocyanate and methanol from Sigma Aldrich (St. Louis, MO); methylene chloride, polysorbate 20 and polysorbate 80 from Avantor Performance Materials (Phillipsburg, NJ); guanidine HCl from MP Biomedicals (Solon, OH); and acetonitrile from Burdick & Jackson (Muskegon, MI). Solid phase extraction vac cartridges (Oasis MAX, Oasis WAX, and Oasis SPE HLB with 1 cc volume and 30 mg sorbent) were obtained from Waters (Milford, MA). All solutions were prepared in purified water with minimum 18 MΩ cm resistivity.

2.2. Reagents

A cobalt-thiocyanate solution was prepared by dissolving 50 g ammonium thiocyanate, 15 g cobalt nitrate hexahydrate and 25 g sodium chloride in 250 mL water. The solutions of 1–6 M Guanidine HCl were made by dissolving 9.55–57.30 g guanidine HCl in 100 mL water, respectively. Various concentrations (v/v) of methanol and acetonitrile were prepared by mixing appropriate volume of the solvent with water. A 1000 mg L^{-1} PS stock solution (w/v) was prepared by dissolving 0.1 g polysorbate 20 or 80 in 100 mL water. The PS working standards (10, 20, 40, 60 and 80 mg L^{-1}) were diluted from the 0.1% stock solution with water.

2.3. Assay procedure

Oasis SPE HLB cartridges were attached into the Visiprep SPE Vacuum Manifold (standard, 24-port model, Sigma–Aldrich) and conditioned with 1 mL methanol followed by 1 mL water. Drug substance (DS) samples were diluted with water so that the PS concentration in the samples was within the standard curve range of 10 and 80 mg L^{-1}. However, in order to cover 50–150% of a targeted PS concentration, the ideal PS concentration in the diluted sample should be between 30 and 50 mg L^{-1}. If the protein concentration in samples was >70 g L^{-1}, samples were also properly diluted with water so that the protein concentration was around 70 g L^{-1} or below. Therefore, this assay procedure can determine only 40 mg L^{-1} in a formulation containing up to 70 g L^{-1} proteins. For determination of 40 mg L^{-1} PS in a formulation with >70 g L^{-1} proteins, the detailed procedure is described in Section 3.3.

One mL of diluted DS sample was loaded onto the conditioned cartridge. The stopcocks were turned on slowly to achieve a vacuum pressure of −10 in Hg and allow the samples to flow through the cartridges slowly. The cartridges were then washed with 1 mL 4 M guanidine HCl and 2 × 1 mL 10% (v/v) methanol. At the load and wash steps, the stopcock was turned off before the solvent meniscus reached the top of the sorbent bed. After washing, the samples were eluted with 2 × 1 mL acetonitrile. The eluted samples were dried with nitrogen gas at 15–18 psi in a Turbo Vap LV evaporator (Caliper, Hopkinton, MA) at 60 °C for 20 min. The working standards (1 mL each) without the SPE treatment were also dried under the same conditions.

The dried PS samples and standards were derivatized using the modified procedure described by Brown and Hayes [23]. The detailed procedure is as follows: to the dried sample or standard, 1 mL of the cobalt-thiocyanate reagent was added to form a blue colored PS–cobaltothiocyanate complex. The blue colored complex was extracted into 1 mL methylene chloride. After the complete separation of two phases, the lower chlorinated solvent layer was transferred into a quartz cuvette with a 1-cm path length. The absorbance (Abs) was measured at 620 nm [23] on a Varian Cary 50 Spectrophotometer (Varian Inc., Palo Alto, CA). A standard curve ranging from 10 to 80 mg L^{-1} PS-20 or PS-80 was constructed and the concentration of PS in the unknown samples was determined accordingly.