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Use of ferric thiocyanate derivatization for quantification of polysorbate 80 in high concentration protein formulations

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ABSTRACT

Quantitation of polysorbate 80 in high protein formulation using solid-phase extraction (SPE) followed by derivatization with cobalt thiocyanate and UV measurement of the complex at 620 nm resulted in lower recovery of polysorbate 80. Dilution of protein samples with water improved the recovery of polysorbate, however, it compromised the sensitivity of the method when cobalt thiocyanate was used for derivatization. The presented work discusses an evaluation of alternative approaches for increasing the sensitivity of the quantitation method for polysorbate using ferric thiocyanate and molybdenum thiocyanate. Ferric thiocyanate complex of polysorbate 80 exhibited the highest sensitivity among the metals thiocyanate evaluated in the current work. The improvement in the sensitivity through derivatization with ferric thiocyanate allowed 10-fold dilution of a 140 mg mL^{-1} protein sample without affecting the recovery or compromising the sensitivity of polysorbate 80 quantitation, indicating that this methodology could be used as an alternate approach for the quantitation of polysorbate 80 in high concentration protein formulations.

Stability of ferric thiocynate and cobalt thiocyanate complex was also studied. When these complexes were allowed to equilibrate for 1 h between an organic layer containing polysorbate/thiocynate complex and an aqueous layer containing un-reacted metal thiocyanate, it resulted in the most reproducible UV absorbance measurements.

The SPE method for quantification of polysorbate 80 using ferric thiocyanate for derivatization provided accuracy (% spike recovery) within 107%, reproducibility (%relative standard deviation) less than 11.7%. The method is linear from 0.0001 to 0.008% polysorbate 80 concentrations in the formulations with protein formulations as high as140 mg mL^{-1}.

1. Introduction

Proteins in general are prone to physical and chemical degradation that often result into aggregation to form multimers and/or chemical modifications such as deamidation, oxidation, etc. Controlling the levels of protein aggregation in biotherapeutics is crucial for the product quality and safety of patients. To stabilize proteins, they are commonly formulated in excipients containing surfactants such as polysorbate 20 and polysorbate 80, commercialized as Tween 20™ and Tween 80™ [1]. Presence of polysorbate 20 in protein formulations was shown to reduce shear during manufacturing of monoclonal antibody products [2]. Controlling the levels of polysorbate in protein formulation within a target level during manufacturing of protein products is essential. Most protein formulations contain polysorbates from 0.006 to 0.1% (w/v). It becomes a challenge to measure polysorbate at such low levels owing to the fact that these surfactants do not have a strong UV absorbing chromophore, thus cannot be quantified using spectrophotometric methods. Numerous analytical methods employing various detection techniques have been reported for the accurate quantitation of polysorbates. These methods include reversed phase HPLC using condensation nucleation light scatting [3], evaporative light scattering [4], and charge aerosol detection [5]. Acid hydrolysis of polysorbate followed by HPLC determination of the free lauric acid was also used as an indirect measurement of polysorbates [6].

For the measurement of polysorbates in protein formulations, most methods require removal of the protein from the sample to eliminate interference from the protein. Some of the commonly used methods for trace quantitation of polysorbate in complex matrices uses extraction of polysorbate using liquid–liquid extraction (LLE) [7,8]. LLE methods are quite labor intensive and therefore, less preferable for routine testing [9]. Recently, the extraction of polysorbate 80 from therapeutic formulations using solid phase extraction (SPE), followed by evaporative light scattering detection was reported [10]. In our laboratory, polysorbate 20 and polysorbate 80 in the formulations

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containing high protein concentration are separated using SPE, followed by derivatization with cobalt–thiocyanate (CoSCN) and the quantitation of the polysorbate–thiocyanate complex using UV spectrophotometry [11]. UV measurement of derivatized polysorbate 80 was preferred due to general availability of UV spectrophotometers in most testing laboratories. For high protein concentration formulations, we encountered an issue of a decrease in the recovery of polysorbate when using the cobalt thiocyanate derivatization and UV spectrophotometry measurement. The loss of the polysorbate from the protein solution was attributed to the binding of polysorbate to protein [12,13]. A solution to improve the recovery was dilution of the sample with water, which likely reduced the protein–polysorbate interaction. Dilution of the samples, however, compromised the sensitivity of the cobalt thiocynate method. These observations led us to evaluate alternative approaches to increase the sensitivity for the quantitation of polysorbate 80 in the diluted protein samples.

Molybdenum(V) was reported to be selectively extracted in the presence of tungsten(III) using polysorbate 80 [14]. In this paper we report an investigation of polysorbate–thiocyanate complexes of other metals such as iron and molybdenum and their UV spectrophotometric measurement for the determination of the surfactant in protein containing samples. The objective of the method development was to improve the sensitivity of the existing cobalt thiocyanate method for trace quantitation of polysorbate 80. In addition, the goal of the current work was to develop an accurate method that provides consistent measurements of polysorbate in the diluted high protein formulations to facilitate specification testing for the release of protein product lots.

2. Material and methods

The procedure for extraction of polysorbate 80 from protein samples was adopted from that developed by Kim and Qiu [11]. Cobalt thiocyanate solution was prepared by dissolving 15 g of cobalt nitrate hexahydrate, 50 g of ammonium thiocyanate and 25 g of sodium chloride in 250 mL water. Similarly, ferric thiocyanate was prepared by dissolving 2.7 g of iron(III) chloride hexahydrate and 3.0 g of ammonium thiocyanate into 100 mL of water. Molybdenum thiocyanate was prepared by dissolving 2.7 g of molybdenum(V) chloride and 3.0 g of ammonium thiocyanate into 100 mL of water. Polysorbate used in these studies was purchased from Avantor Performance Materials (Phillipsburg, NJ, USA). Polysorbate 80 standards were prepared from 0.0001 to 0.01% (w/v) by serial dilution of a 0.1% (w/v) stock solution with water.

Solid phase extraction cartridges used in this procedure were Oasis HLB with 1 mL bed volume purchased from Waters (Milford, MA, USA). These cartridges were conditioned with methanol and water prior to loading the samples. The SPE cartridges were attached to a vacuum manifold to elute the solutions used in the extraction procedure. 1 mL of protein samples were loaded to the conditioned SPE cartridges. Protein from the SPE was eluted with 3 M guanidine hydrochloride solution, followed by a water wash to remove any residual guanidine hydrochloride. Polysorbate 80 retained by the SPE resin was eluted with 1 mL acetonitrile. The extract containing polysorbate in acetonitrile was dried under nitrogen. To the dried extraction containing polysorbate 80, 1 mL of aqueous metal thiocyanate was added to form a polysorbate/thiocyanate complex. Excess metal thiocyanate was then removed by liquid–liquid extraction with 1 mL of methylene chloride that contained derivatized polysorbate 80 and the unreacted ferric thiocyanate remained in the aqueous layer. Concentration of polysorbate/thiocyanate complex in the methylene chloride layer was measured using UV spectrophotometer, Cary 50 (Agilent, DE, USA) at the specified wavelengths. UV spectra were also collected from 200 to 800 nm for polysorbate/thiocyanate complexes of the metals.

Concentration of polysorbate 80 was determined by linear regression analysis of the calibration curve plotted for the standards. A monoclonal antibody product at >120 mg mL^{-1} protein concentration formulated with polysorbate 80 targeted at 0.01% (w/v) was used in the present studies. Pre-formulated protein samples without the presence of polysorbate 80 were used to determine spike recovery of the method.

3. Results and discussion

3.1. Loss of polysorbate 80 in high protein concentration formulation

In our laboratory, polysorbate 80 in monoclonal antibody products is routinely quantitated using SPE extraction followed by cobalt thiocyanate derivatization [11]. However, when this analytical method was employed to a monoclonal antibody product with a protein concentration greater than 120 mg mL^{-1}, recovery of the targeted polysorbate 80 was rather unsatisfactory (58%), as provided in Table 1. Upon dilution of the samples with water, spike recovery significantly improved with increasing dilution, as evident from the data provided in this table. While there was no significant difference in the observed spike recoveries between 4-fold versus 5-fold dilution, a dilution factor for 5-fold was chosen in order to ensure the robustness of the method. The range for the specification for polysorbate 80 in this drug product was 50 to 150% of the target PS80 concentration in the product. Therefore, the calibration curve needed to be adjusted to 25 to 200% of the target polysorbate 80 concentration so that it was wide enough to encompass the specification range (refer to Table 1). However, 5-fold dilution of the sample resulted in a lower UV absorbance of polysorbate/cobalt thiocyanate complex such that it was near the limit of quantitation of 0.0005% (w/v) of the method. In order to increase the sensitivity to allow the measurement of polysorbate 80 below 0.0005% (w/v), alternative approaches were evaluated for the situation when a dilution factor of 5-fold or greater became necessary.

3.2. Evaluation of other metal thiocyanate complexes

Polysorbate 80 forms a stable complex with molybdenum thiocyanate relative to its tungsten thiocyanate complex [14]. The stable molybdenum thiocyanate complex was, therefore,

Table 1
Improvement of polysorbate 80 recovery with sample dilution.

Dilution factor	Spike recovery (%)	Target[a] polysorbate 80 concentration, % (w/v)	Dilution factor[b] adjusted specification range, % (w/v)	Standard curve range[c]
No dilution	58	0.0100	0.0500–0.0150	0.00250–0.0200
2	79	0.00500	0.00250–0.00750	0.00125–0.0100
4	90	0.00250	0.00125–0.00375	0.000625–0.00500
5	90	0.002000	0.00100–0.00300	0.000500–0.00400

[a] Target concentration is the expected concentration of polysorbate 80 in the product after dilution factor.
[b] Dilution factor adjusted specification range is specification range of 50 to 150% of the target PS80 concentration divided by the dilution factor.
[c] Standard curve range is 25 to 200% of the target PS80 concentration.