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Alkaline hydrolysis to increase the selectivity of colorimetric determination of polysorbate

Andrea Engelmaier, Heinz Anderle, Alfred Weber*

Shire, Analytical Development, Vienna A-1220, Austria

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ABSTRACT

Here, we describe a straightforward sample pretreatment step for the colorimetric cobaltthiocyanate determination of polysorbate, which circumvents the assay's shortcomings due to interference of protein and does not require complex instrumentation. Protein-containing test samples are hydrolyzed with strong alkali at 100 °C, neutralized and clarified by filtration before applying the colorimetric assay. The modified method performs with appropriate accuracy and precision, allowing specific polysorbate measurement in the presence of Triton X-100 during virus inactivation, determination of residual amounts of polysorbate in the final products and measurement of polysorbate 80 in final formulated products. The alkaline hydrolysis step, primarily designed to provide the assay's reliability in the presence of protein, also enhances its selectivity towards interference by the non-ionic detergent Triton X-100 and increases its robustness against changes in the fatty acid moiety of polysorbate as it released the fatty acid essentially contributing to the known heterogeneity of polysorbates. These results demonstrate that with sample pretreatment the handy colorimetric assay, not requiring complex instrumentation, can be used to measure polysorbate 80 concentrations in intermediates and final products of therapeutic protein solutions.

1. Introduction

Viral safety is a prerequisite for therapeutic proteins, especially for plasma-derived medicinal products. Substantial progress has been made in virus safety by practically eliminating the risk of virus transmission [1,2] by designing, introducing and validating specific steps to remove or inactivate viruses during the manufacturing [3,4]. The EMA guideline on plasma-derived medicinal products [5] recommends adding “specific procedures to inactivate/remove viral contaminants” in the manufacturing strategy for all plasma products including “two distinct effective steps which complement each other in their mode of action”. Solvent/detergent (S/D) treatment [6], the incubation with tri-*n*-butylphosphate (TNBP) and a non-ionic detergent such as Triton X-100 or polysorbate 80 (PS 80), has been shown to inactivate lipid-enveloped viruses such as HIV, HBV, and HCV. This procedure specifically disrupts the viral lipid envelope, while minimally affecting the integrity of therapeutic proteins except lipoproteins.

Polyoxyethylene sorbitan monooleate (polysorbate 80, Tween 80; PS 80) [7] is the most commonly used virucidal detergent. To be compliant with specified and validated limits of detergent concentration during virus inactivation and to monitor the efficiency of the subsequent detergent removal procedure, the detergent concentration has to be measured in the presence of varying and occasionally high concentrations of plasma proteins. In addition, PS 80 is used as a common surfactant in biopharmaceutical formulations protecting proteins against stress caused by interfacial contacts and reducing surface adsorption to a minimum [8]. Measurement, however, is hampered by the chemical composition and the structural diversity of polysorbates [9–13] and the low reactivity of the compound missing a chromophore to be used for monitoring in separation techniques. In addition, the respective sample matrix containing sometimes high protein concentrations and the presence of other physico-chemically related detergents such as alkylphenol ethers with varying numbers of ethyleneoxide

Abbreviations: AOAC, American Association of Official Analytical Chemists; CAD, charged aerosol detector; ELSD, evaporative light scattering detector; EMA, European Medicines Agency; HPLC, high performance liquid chromatography; TNBP, tri-*n*-butylphosphate; S/D, solvent/detergent; OD, optical density; (a)PCC, (activated) prothrombin complex concentrate; PS 80, polysorbate 80; RSD, relative standard deviation; RT, room temperature (18–25 °C).

* Corresponding author. Shire, Analytical Development, Industriestraße 67, 1220 Vienna, Austria.
E-mail address: alfred.weber@shire.com (A. Weber).

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residues contribute to the analytical challenge. A colorimetric assay based on complexation of polyoxyethylene chains with cobalt-thiocyanate and extraction of the complex into chloroform [14] was originally developed to determine the concentration of polyethylene glycol fatty acid esters in aqueous solutions. This method cannot be used universally though, due to the presence of protein, which as in other assays strongly interferes with the measurement [15]. To overcome such matrix effects, several protein removal techniques have been described, which are time-consuming, have limited sample throughput, or require complex instrumentation. Thus, measurement with separation-based techniques require hydrolysis of the fatty acid followed by reversed phase high performance liquid or gas chromatography [16–18]. Without sample pretreatment the use of an evaporative light scattering detector (ELSD) [19,20], a charged aerosol detector (CAD) [21] or a condensation nucleation light scattering detector [22] have been developed to measure polysorbates after HPLC separation. Recently, a gas chromatographic method has been described based on the measurement of ethylene glycol diacetate formed after hydrolysis and acetylation of surfactants that contain ethylene glycol [23]. In contrast to the plethora of methods requiring complex instrumentation, simpler methods with the potential of automation are rare. Only a fluorescence-based method for the rapid determination of PS 80 content in therapeutic monoclonal antibody products has been described recently [24]. The use of this method, however, is restricted to the measurement of monoclonal antibodies.

Here, we present alkaline hydrolysis [25] as a universal protein removal method to eliminate its interference on the colorimetric cobalt-thiocyanate assay and enable determination of the actual detergent concentration during virus inactivation process and in final formulated biotherapeutic products. This sample pretreatment step also enhances the selectivity of the colorimetric assay towards PS when used in combination with Triton X-100 and eliminates the influence of the fatty acid moiety of PS 80 on the assay as alkaline hydrolysis results in release of the fatty acid moiety.

2. Material and methods

2.1. Materials

All chemicals used were of analytical grade unless otherwise stated. NaOH, acetic acid 96%, cobalt nitrate hexahydrate, methylene chloride (Lichrosolv®), Triton X-100, TNBP and ammonium thiocyanate were purchased from Merck. Two lots of PS 80 (Tween 80) from bovine and from vegetable source came from ICI. Aqueous solutions of the detergent with a content of 1% (w/v) were prepared and used for analysis. The following plasma protein solutions from Baxter BioScience (Vienna, Austria, now Shire) were used: human serum albumin, immunoglobulin G, antithrombin, the protein C concentrate CEPROTIN, activated prothrombin complex concentrate (aPCC) and prothrombin complex concentrate (PCC), the high-purity factor IX concentrate IMMUNINE, and orosomucoid (α_1-acid glycoprotein). The lyophilized human reference plasma preparation was obtained from Technoclone (Vienna, Austria). Recombinant protein preparations factor FIX (RIXUBIS) and von Willebrand factor (VONVENDI) were used (both from Shire, Vienna, Austria).

Alkaline hydrolysis was carried out in 100 × 15 mm polypropylene tubes with screw caps (Greiner, Austria), the subsequent color reaction in glass tubes with screw caps (Sovirel, Szabo, Vienna, Austria). Minisart cellulose acetate 0.2 μm-filters (Sartorius, Sigma, Vienna, Austria) were used. A LKB Ultrospec K4053 spectrophotometer using 1 cm Suprasil quartz cells with screw-caps (Hellma, Germany) was used for measurement. All steps except hydrolysis were performed at room temperature (RT; 18–25 °C).

2.2. Alkaline hydrolysis

The aqueous sample/standard/control (1 mL) with a PS 80 concentration of maximum 400 μg/mL was mixed with 0.5 mL 10 M NaOH and kept in a boiling water bath for 60 ± 10 min. After chilling to RT, the hydrolysate was neutralized by adding of 0.5 mL 10 M acetic acid. The neutralized hydrolysate was kept at RT for at least 30 min before filtration with a cellulose acetate Minisart filter. Test samples with a protein content of less than 1 mg/mL were mixed with an albumin solution before hydrolysis to prevent PS 80 losses due to surface adsorption. In particular, 0.1 mL of a 20% (w/v) human albumin solution (Baxter, Vienna, Austria) added to 1 mL of the respective sample was effective in preventing unwanted adsorption.

2.3. Color reaction and measurement

The color reaction was performed as described by Brown and Hayes [14], applying down-scaled volumes, extraction into methylene chloride instead of chloroform, and measurement at 320 nm to reach appropriate sample throughput and sensitivity. Briefly, 1 mL of the neutralized hydrolysate or the aqueous essentially protein-free PS solution was mixed with 3 mL cobalt-thiocyanate reagent (3% [w/v] cobalt nitrate hexahydrate, 20% [w/v] ammonium thiocyanate in distilled water, prepared freshly each day). Then, 2 mL methylene chloride was added. After vigorous shaking, the phases were allowed to separate at RT for at least 30 min. Finally, spectrophotometry was used to measure the methylene chloride phase in screw-capped cuvettes against a methylene chloride blank at 320 nm. The seven non-zero reference standards with PS 80 concentrations of 5, 10, 20, 50, 100, 200 and 400 μg/mL, prepared by spiking an aqueous PS 80 solution to a PCC sample, were analyzed in duplicates each time the assay was carried out. As alkaline hydrolysis compensated the detrimental influence of every protein solution tested so far, other protein solutions with a protein content of maximum 30 mg/mL should be suitable as protein matrix for the standard curve. Furthermore, due to the underlying assay principle other polysorbates than PS 80, differing in their fatty acid moieties, could be used for the calibration curve if required, although out study focused on PS 80.

2.4. Optimization of hydrolysis time

Appropriate hydrolysis time was determined with aPCC as protein matrix as this complex protein matrix interfered with the colorimetric assay, even after precipitating the protein and measuring the obtained supernatant (data on file). This matrix contains a broad spectrum of plasma proteins, such as inter-α-trypsin inhibitor, complement C4, vitronectin and prothrombin [26,27] and was spiked with 100 μg/mL PS. Alkaline hydrolysis was performed as described keeping samples in the boiling water bath for 15–120 min. Further processing was carried out as described (see 2.3).

2.5. Comparison of PS of different origin

Concerns over risks associated with bovine spongiform encephalopathy in the late 90s [28,29] led to the introduction of a vegetable-derived form of PS 80 manufactured from corn oil instead of bovine tallow. To evaluate whether the origin of PS 80 affects assay performance, PS 80 from vegetable and bovine source were analyzed in the presence of aPCC using alkaline hydrolysis. The color reaction was also carried out in the absence of protein without sample pretreatment.