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A highly sensitive high-performance liquid chromatography-charged aerosol detection method for the quantitative analysis of polysorbate 80 in protein solution

Authors
Zhen Long, Guobin Shen, Melanie Neubauer, Katherine Lovejoy, Lvye Liu, Xiaoda Liu, Yan Jin, Xingguo Liu

Thermo Fisher Scientific, Shanghai, People's Republic of China

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Biopharmaceutical, polysorbate 80, Tween 80, protein, charged aerosol detection

Goal

To describe the development of a highly sensitive charged aerosol detection (CAD) method for the determination of polysorbate 80, also known as Tween™ 80, in biopharmaceutical products.

Introduction

Polysorbates, such as polysorbate 20 and polysorbate 80, are non-ionic surfactants. They are commonly used in biotherapeutic formulations to prevent surface adsorption and stabilize proteins against aggregation induced by stress, such as agitation and shear.[1] For quality control purposes, it is important to determine the concentration of polysorbate in the final products. However, the quantitative analysis of polysorbate is challenging—polysorbate is a complex mixture of many different species, which lack natural UV chromophores, and is therefore difficult to analyze by UV detection. Also, chromatographic separation often leads to peaks or peak groupings that consist of many unresolved components and poor peak shapes, thus making accurate and sensitive quantitation problematic.

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Chinese Pharmacopia 2015 introduced a derivatization method for the quantitative analysis of polysorbate 80,[2] which is used by manufacturers of protein-based therapies. Such a preparation method can improve the sensitivity of the detection of Tween 80 (Figure 1). However, reagents that are commonly used for the derivatization of Tween 80, such as cobaltous thiocyanate and dichloromethane, are toxic. In addition, the derivatization method is time-consuming, because the pretreatment of the polysorbate takes more than three hours. Further improvements are also needed in terms of sensitivity, accuracy, repeatability, and selectivity versus non-Tween 80 substances in a formulation.

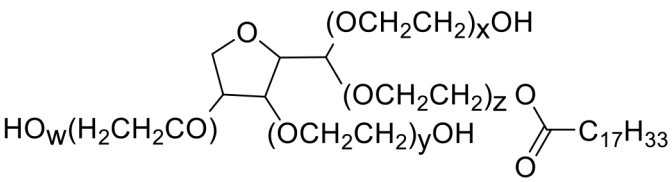

Figure 1. Structure of Tween 80 (x + y + z + w = 20).

CAD is a universal detection technique that can be used to detect non-volatile and some semi-volatile compounds with or without a strong UV chromophore.[3,4] Shi,[1] Fekete,[5] and Dixit[6] developed CAD methods for determining polysorbates in protein formulations. Compared to methods developed with evaporative light-scattering detection (ELSD),[7] CAD, as widely reported, is significantly more sensitive and its response its less dependent on analyte chemical structure.[8,9] The latter is particularly important to achieve accurate quantitation of Tween, since it consists of many different chemical species whose relative concentration can vary widely between manufacturer and lot. In this study, a previously reported method[10] was adapted for the quantitative analysis of Tween 80 using a new generation CAD, the Thermo Scientific™ Dionex™ Corona™ Veo™ detector. The quantitative parameters, including limit of detection (LOD), limit of quantification (LOQ), linearity, and precision, were systematically investigated, and then the method was used for the determination of Tween 80 in a protein formulation.

Experimental

Equipment and software

The Thermo Scientific™ UltiMate™ 3000 Dual Rapid Separation LC (RSLC) system was used, which includes:

- SRD-3600 Integrated Solvent and Degasser Rack (P/N 5035.9230)
- DGP-3600RS Dual Gradient Rapid Separation Pump (P/N 5040.0066)
- WPS-3000TRS Rapid Separation Wellplate Sampler, Thermostatted (P/N 5840.0020), equipped with a 100 µL sample loop and a 100 µL syringe
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.000)
- Thermo Scientific™ Dionex™ Corona™ Veo™ RS charged aerosol detector (P/N 5081.0020)
- Thermo Scientific™ Chromeleon™ Chromatography Data System software, version 7.2

Consumables

- Thermo Scientific™ Target2™ Polypropylene Syringe Filters (0.2 µm, 30 mm, P/N 03-376-219)
- Mixed-mode anion exchange column (2.1 × 20 mm, 30 µm)

Reagents and standards

- Deionized (DI) water, 18.2 MΩ·cm resistivity (generated from the Thermo Scientific™ Barnstead™ GenPure™ Pro UV-TOC Water Purification System, P/N 50131948)
- Isopropanol, HPLC Grade (Fisher Scientific™ P/N UN1219)
- Formic acid (FA), MS Grade (Fisher Scientific P/N TS-28905)
- Polysorbate 80, MP Biomedicals (Sigma®)

Preparation of standard solutions

Stock standard 1

Dissolve 10.0 mg of Tween 80 standard in 10 mL of DI water. The concentration of Tween 80 in stock standard 1 is 1000 mg/L.

Stock standard 2

Dissolve 1 mL of stock standard 1 in 10 mL of DI water. The concentration of Tween 80 in stock standard 2 is 100 mg/L.

Mixed standard solutions for calibration and sensitivity

For calibration, prepare 10, 20, 30, 40, and 50 mg/L of Tween 80 by diluting 100, 200, 300, 400, and 500 µL stock standard 2, respectively, with water to 1 mL. Prepare the standard solution for measuring the LOD by diluting 50 µL stock standard 2 with water to 1 mL.

Sample preparation

Dilute 1 mL chimeric anti-EGFR mAb solution (sample 1) to 5 mL with formic acid solution (formic acid/water, 2:100, v/v). All samples were provided by customers.

Sample solution for repeatability

Dilute two batches of protein samples (samples 2 and 3), which contain about 600–700 mg/L tenfold with formic acid solution (formic acid/water, 2:100, v/v), resulting in a Tween 80 concentration in the range of 60–70 mg/L.

Chromatographic conditions			
Column:	Mixed-mode anion exchange (2.1 × 20 mm, 30 µm)		
Mobile Phase:	A: Water (containing 2% (v/v) formic acid) B: Isopropanol (containing 2% (v/v) formic acid)		
Gradient:	*Time, min*	*A, %*	*B, %*
	0	90	10
	1	80	20
	3.4	80	20
	3.5	0	100
	4.5	0	100
	4.6	90	10
	10	90	10
Injection Volume:	30 µL		
Flow Rate:	1.0 mL/min		
Temperature:	30 °C		
Detection:	Evaporative temperature: 35 °C; collection frequency: 10 Hz; filter 5 s.; PFV 1.0		

Results and discussion

Chromatographic condition optimized

A chromatographic method reported previously for analyzing Tween 20 was used for analyzing Tween 80.[10] The resulting chromatogram for Tween 80 is shown in Figure 2. A step gradient was used for the elution of Tween 80 to achieve a sharper peak and higher response due to peak compression. However, a step gradient also contributes to a baseline artifact (Figure 2, red trace). To account for this artifact, a baseline subtraction was used (Figure 3). Except for Figure 2, all figures in this manuscript were obtained with baseline subtraction.

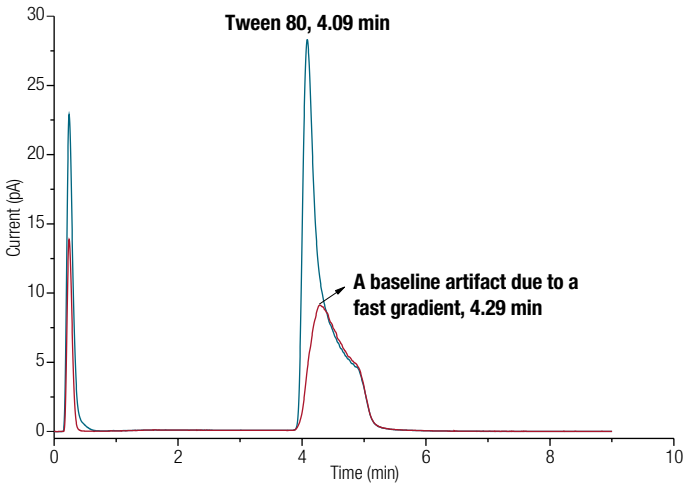

Figure 2. Chromatogram of 50 mg/L Tween 80 (blue trace) and blank (red trace).

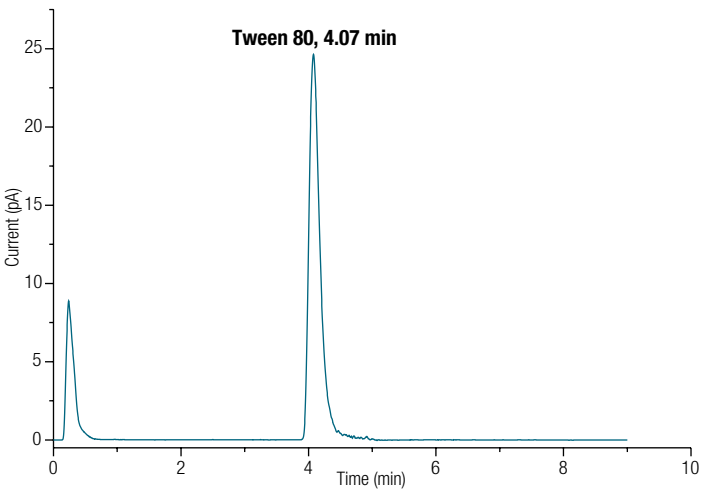

Figure 3. Chromatogram of 50 mg/L Tween 80 with chromatogram subtraction.

Sensitivity and linearity

For the detection of Tween 80 in a narrow concentration range (10–50 mg/L), a linear model can be used to fit to the calibration data. As shown in Figure 4, CAD can provide good linearity ($R^2 > 0.999$) for the detection of Tween 80 with a concentration range from 10–50 mg/L. The LOD and LOQ were taken as the minimum level at which the S/N ratio was above 3 and 10, respectively. The LOD and LOQ of the current method were 5 mg/L (S/N 5.6) and 10 mg/L (S/N 13.6), respectively.

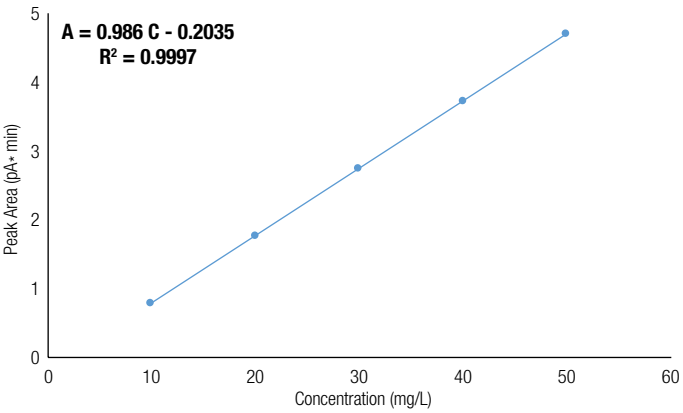

Figure 4. Calibration curve of Tween 80 (10–50 mg/L).

Repeatability

The repeatability of the current method was determined by evaluation of the RSD values of peak areas, which were obtained with five repetitive injections. Two concentrations of polysorbate in protein samples, 67.5 and 70.5 mg/L, were used for testing the repeatability. As shown in Table 1, the RSD values of these two concentrations were no more than 0.7%. This indicates that the current method can provide good repeatability for determining Tween 80 in protein samples.

Table 1. Repeatability (n = 5) of the current method.

Sample	Concentration (mg/L)	Peak Area Repeatability (%)
Sample 2	67.5	0.60%
Sample 3	70.5	0.63%

Sample analysis

Chimeric anti-EGFR mAb sample (Sample 1) was analyzed with the developed method. It can be seen from Figure 5 that Tween 80 can be well separated from the matrix of the protein samples. Almost the entire protein matrix can be eluted close to the dead time of the column due to ion exclusion interactions, since both protein and the column have a cationic group when 2% FA is used as a mobile phase additive. Small molecules such as sorbitol and phosphate, which are commonly used in protein samples, were also eluted close to the dead time due to the very weak hydrophobic retention and ionic repulsive interaction. Thus, many protein formulations can be analyzed by the presented method without any pretreatment. For high concentration samples (greater than 100 mg/L), only dilution was needed before HPLC analysis. The amount of Tween 80 was 105.1 ± 0.06 mg/L in sample 1, which was calculated by the calibration curve described previously. It should also be noted that several complementary approaches using CAD have been described, which provide additional specificity and profiling of polysorbate subspecies.[5,6] These are particularly useful for analysis of more complex formulations, for formulation development and in stability / forced degradation studies.

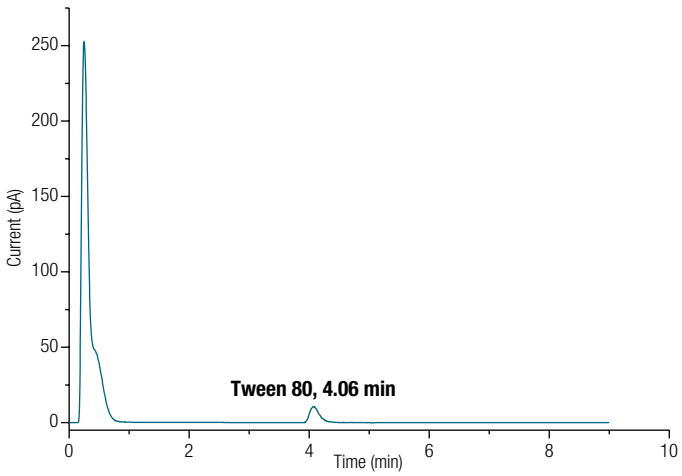

Figure 5. Chromatograms of Sample 1.

Conclusion

An HPLC-CAD method has been developed for the quantitative analysis of Tween 80 in protein formulations. Compared to the ChP 2015 method, the developed method was faster, less toxic, and of higher accuracy. No derivatization and pretreatment was needed and only nine minutes were used for the separation. Thus, the developed method had no pretreatment error. Furthermore, it was more accurate than the ChP 2015 method, since a column separation was used in the current method and there was less matrix disturbance.

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