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Analysis of Polysorbate 80 in Protein Formulations Using 2D LCMS

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Introduction

Polysorbate 80 is commonly used for biotherapeutic products to prevent aggregation and surface adsorption, as well as to increase the solubility of biotherapeutic compounds. A reliable method to quantitate and characterize polysorbates is required to evaluate the quality and stability of biotherapeutic products. Several methods for polysorbate analysis have been reported, but most of them require time-consuming sample pretreatment such as derivatization and alkaline hydrolysis because polysorbates do not have sufficient chromophores. Those methods also require an additional step to remove biotherapeutic compounds. Here we report a simple and reliable method for quantitation and characterization of polysorbate 80 in biotherapeutic products using two-dimensional HPLC.

Materials

Reagents and standards

Reagents: Tween® 80 (Polysorbate 80), IgG from human serum, potassium phosphate monobasic, potassium phosphate dibasic, and ammnonium formate were purchased from Sigma-Aldrich. Water was made in house using a Millipore Milli-Q Advantage A10 Ultrapure Water Purification System. Isopropanol was purchased from Honeywell.

Standard solutions: 10 mmol/L phosphate buffer (pH 6.8) was prepared by dissolving 680 mg of potassium phosphate monobasic and 871 mg of potassium phosphate dibasic in 1 L of water.
Polysorbate 80 was diluted with 10 mmol/L phosphate buffer (pH 6.8) to 200, 100, 50, 20, 10 mg/L and transferred to 1.5 mL vials for analysis.

Sample solutions: A model sample was prepared by dissolving 2 mg of IgG in 0.1 mL of a 100 mg/L polysorbate 80 standard solution. The sample was centrifuged and transferred to a 1.5 mL vial for analysis.

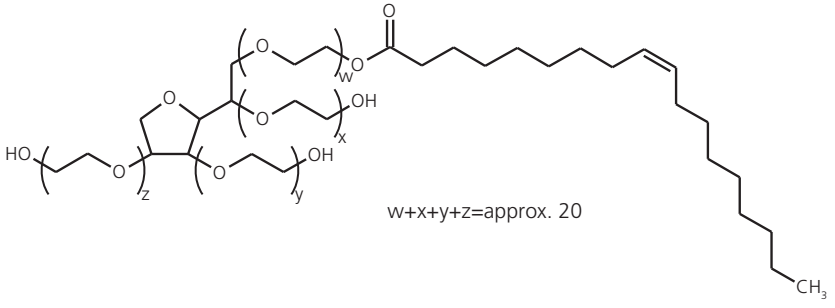
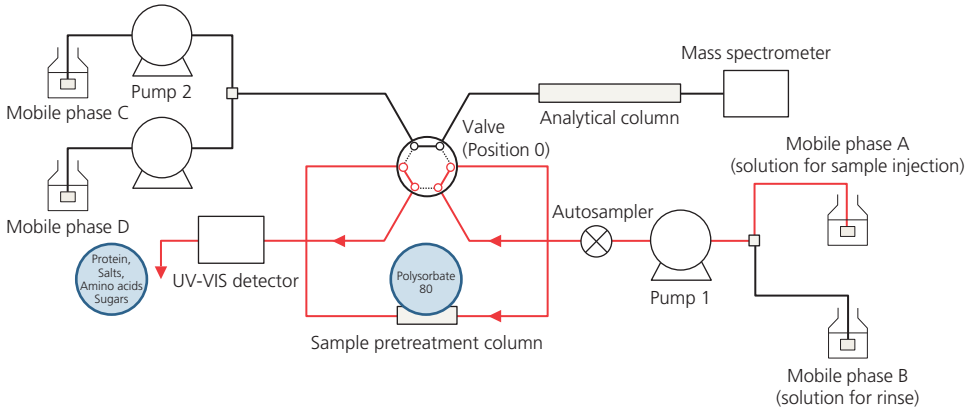


Fig.1 Typical structure of polysorbate 80

System

The standard and sample solutions were injected into a Shimadzu Co-Sense for BA system consisting of two LC-20AD pumps and a LC-20AD pump equipped with a solvent switching valve, DGU-20A5R degassing unit, SIL-20AC autosampler, CTO-20AC column oven equipped with a 6-port 2-position valve, and a CBM-20A system controller. Polysorbate 80 was detected by a LCMS-2020 single quadrupole mass spectrometer or a LCMS-8050 triple quadrupole mass spectrometer because polysorbates do not have any chromophores and are present at low concentrations in antibody drugs. A SPD-20AV UV-VIS detector was used to check protein removal.
Fig. 2 shows the flow diagram of the Co-Sense for BA system. In step 1, a sample pretreatment column "Shim-pack MAYI-ODS" traps polysorbate 80 in the sample. Proteins (antibody) cannot enter the pore interior that is blocked by a hydrophilic polymer bound on the outer surface. Other additives and excipients such as sugars, salts, and amino acids cannot be retained by the ODS phase of the inner surface due to their polarity. In step 2, the trapped polysorbate 80 is introduced to the analytical column by valve switching.

Step 1: Protein removal



Step 2: Analyzing the trapped polysorbate

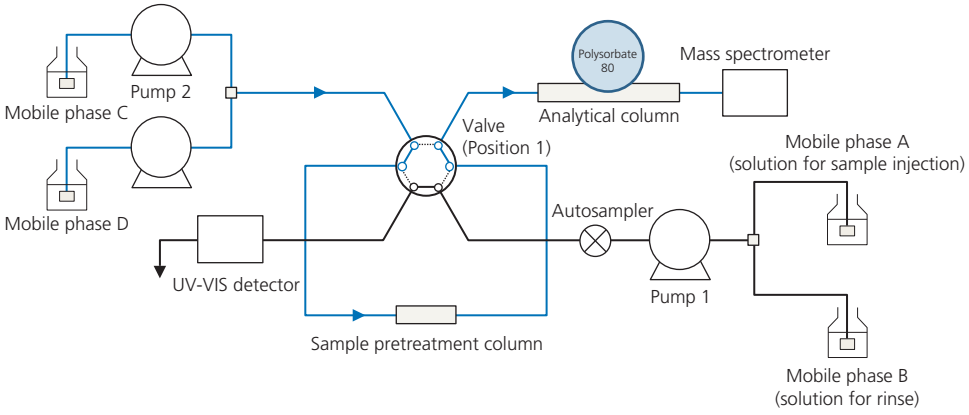


Fig.2 Flow diagram of Co-Sense for BA

Results

Quantitation method

A fast analysis for quantitation will be shown here. Table 1 shows the analytical conditions and Fig. 3 shows the TIC chromatogram of a 100 mg/L polysorbate 80 standard solution and the mass spectrum of the peak at 4.4 min. Polysorbates contain many by-products, so several peaks appeared on the TIC chromatogram. The peak at 4.4 min was identified as polyoxyethylene sorbitan monooleate (typical structure of polysorbate 80) based on E. Hvattum *et al* 2011. The ion at 783 was used as a marker for detection in selected ion mode (SIM). This ion is attributable to the $2NH_4^+$ adduct of polyoxyethylene sorbitan monooleate containing 25 polyoxyethylene groups. Fig. 4 shows the SIM chromatogram of the model sample (20 g/L of IgG, 100 mg/L of polysorbate 80 in 10 mmol/L phosphate buffer pH6.8). Polysorbate 80 in the model sample was successfully analyzed. The peak at 4.4 min was used for quantitation.

Six replicate injections for the model sample were made to evaluate the reproducibility. The relative standard deviations of retention time and peak area were 0.034 % and 1.11 %, respectively. The recovery ratio was obtained by comparing the peak area of the model sample and a 100 mg/L polysorbate 80 standard solution and was 99 %. Five different levels of polysorbate 80 standard solutions ranging from 10 to 200 mg/L were used for the linearity evaluation. The correlation coefficient (R^2) of determination was higher than 0.999.

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Table 1 Analytical Conditions

System	: Co-Sense for BA equipped with LCMS-2020		
[Sample Injection]		**[UV Detection]**	
Column	: Shim-pack MAYI-ODS (5 mm L. x 2.0 mm I.D., 50 µm)	Detection	: 280 nm
Mobile Phase	: A: 10 mmol/L ammonium formate in water	Flow Cell	: Semi-micro cell
	B: Isopropanol	**[MS Detection]**	
Solvent Switching	: A (0-1.5 min), B (1.5-3.5 min), A (3.5-9 min)	Ionization Mode	: ESI Positive
Flow Rate	: 0.6 mL/min	Applied Voltage	: 4.5 kV
Valve Position	: 0 (0-1 min, 7-9 min), 1 (1-7 min)	Nebulizer Gas Flow	: 1.5 mL/min
Injection Volume	: 1 µL	DL Temperature	: 250 °C
[Separation]		Block Heater Temp.	: 400 °C
Column	: Kinetex 5u C18 100A (50 mm L. x 2.1 mm I.D., 5 µm)	Scan	: *m/z* 300-2000
Mobile Phase	: A: 10 mmol/L ammonium formate in water	SIM	: *m/z* 783
	B: Isopropanol		
Time Program	: B. Conc 5 % (0-1 min) - 100 % (6-7 min) -5 % (7.01-9 min)		
Flow Rate	: 0.3 mL/min		
Column Temperature	: 40 °C		

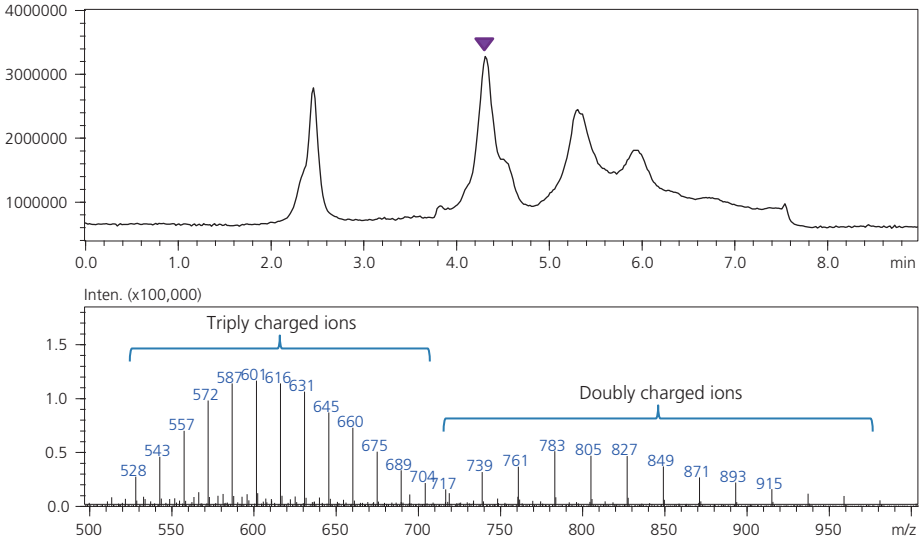

Fig.3 TIC Chromatogram of 100 mg/L polysorbate 80 standard solution and mass spectrum of the peak at 4.4 min

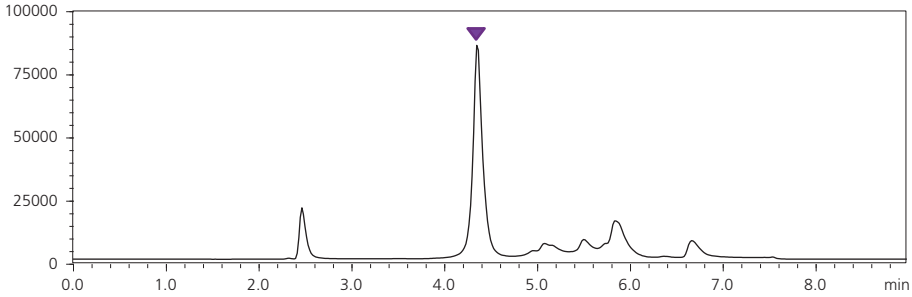

Fig.4 SIM chromatogram of the model sample

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Characterization method

An analysis for characterization will be shown here. Table 2 shows the analytical conditions and Fig. 5 shows the TIC chromatogram of the model sample and mass spectra of the peaks from 10 to 30 min. A longer column and gradient were applied to obtain better resolution. Polysorbate 80 consists of not only monooleate (typical structure of polysorbate 80), but also many by-products such as polyoxyethylene, polyoxyethylene sorbitan, polyoxyethylene isosorbide, dioleate, trioleate, tetraoleate and others. The peaks on the TIC chromatogram are assumed to correspond to those by-products. For example, the peaks from 10 to 22 min correspond to polyoxyethylene and polyoxyethylene isosorbide and the peaks from 22 to 30 min correspond to polyoxyethylene sorbitan. This method is helpful for characterization as well as checking degradation such as auto-oxidation and hydrolysis.

Table 2 Analytical Conditions

System	: Co-Sense for BA equipped with LCMS-8050		
[Sample Injection]		**[UV Detection]**	
Column	: Shim-pack MAYI-ODS (5 mm L. x 2.0 mm I.D., 50 µm)	Detection	: 280 nm
Mobile Phase	: A: 10 mmol/L ammonium formate in water B: Isopropanol	Flow Cell	: Semi-micro cell
		[MS Detection]	
Solvent Switching	: A (0-1.5 min), B (1.5-3.5 min), A (3.5-9 min)	Ionization Mode	: ESI Positive
Flow Rate	: 0.6 mL/min (0-10 min, 95.01-110 min), 0.1 mL/min (10.01-95 min)	Applied Voltage	: 4.5 kV
Valve Position	: 0 (0-3 min, 100-110 min), 1 (3-100 min)	Nebulizer Gas Flow	: 2 mL/min
Injection Volume	: 5 µL	Drying Gas Flow	: 10 mL/min
[Separation]		Heating Gas Flow	: 10 mL/min
Column	: Kinetex 5u C18 100A (100 mm L. x 2.1 mm I.D., 5 µm)	Interface Temperature	: 300 °C
Mobile Phase	: A: 10 mmol/L ammonium formate in water B: Isopropanol	DL Temperature	: 250 °C
		Block Heater Temp.	: 400 °C
Time Program	: B. Conc 5 % % (0-3min) – 35% (15min) – 100% (100min) – 5% (100.01-110min)	Q1 Scan	: *m/z* 300-2000
Flow Rate	: 0.2 mL/min		
Column Temperature	: 40 °C		

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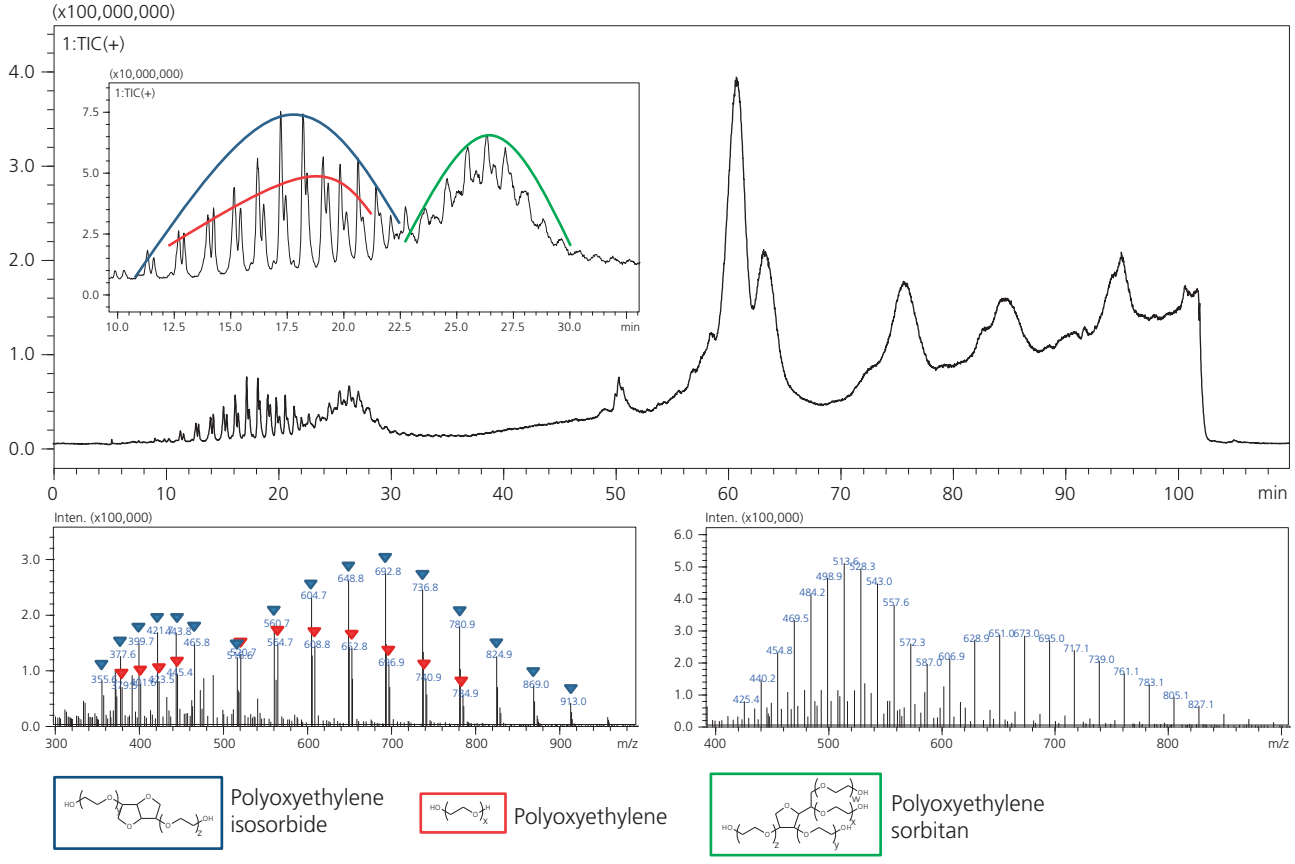

Fig.5 TIC chromatogram of the model sample

Confirmation of protein removal

Fig. 6 shows the chromatogram of elution from the sample pretreatment column. Protein (IgG) was successfully removed from the sample by using the MAYI-ODS column.

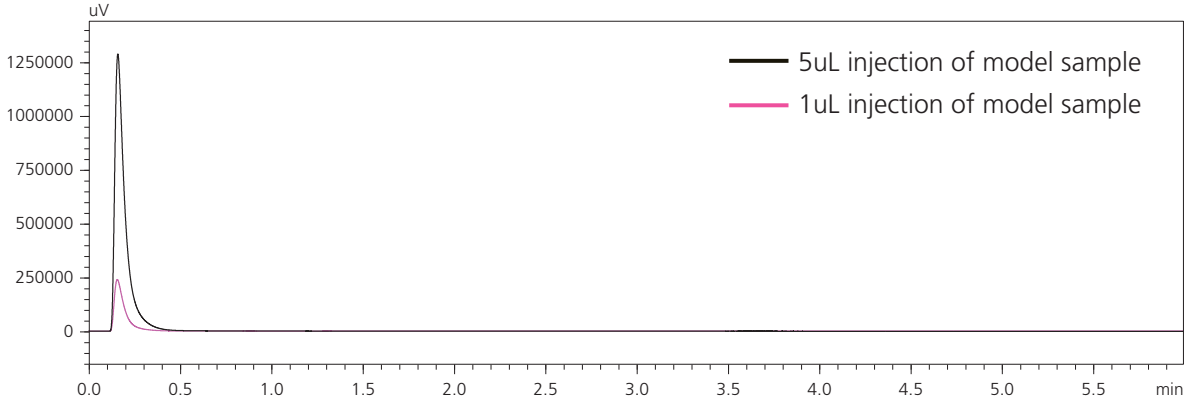

Fig.6 Chromatogram of elution from the sample pretreatment column

Conclusions

1. Co-Sense for BA system automatically removed protein from the sample and enabled quantitation and characterization of polysorbate 80 in a protein formulation.
2. The quantitation method was successfully applied to the model sample with excellent reproducibility and recovery.
3. The high-resolution chromatogram was obtained by the characterization method. This method is helpful for characterization as well as checking degradation such as auto-oxidation and hydrolysis.

Reference

E. Hvattum, W.L. Yip, D. Grace, K. Dyrstad, Characterization of polysorbate 80 with liquid chromatography mass spectrometry and nuclear magnetic resonance spectroscopy: Specific determination of oxidation products of thermally oxidized polysorbate 80, J Pharm Biomed Anal 62, (2012) 7-16

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