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Analytical Products

Application Note

Workflow for the Analysis of Polysorbate 80 in Erbitux® Formulation

Protocol for sample preparation and reversed phase HPLC-ELSD analysis of a nonionic surfactant in a monoclonal antibody formulation

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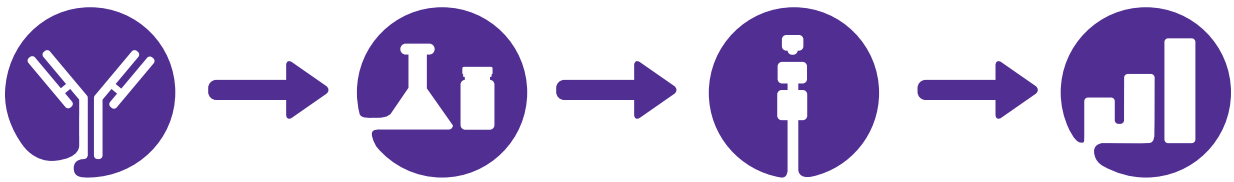
Workflow for the Analysis of Polysorbate 80 in a mAb Formulation



A complete reversed phase HPLC-ELSD workflow has been developed for the quantification of polysorbate 80 (Tween® 80) in antibody formulations.

In detail, it includes:

- Solid phase extraction (SPE) sample preparation procedure
- Preparation of calibration solutions
- Reversed phase HPLC-ELSD method for quantitative analysis of surfactant concentration

Introduction to Polysorbate 80 Analysis in mAb Formulations

Monoclonal antibodies [mAbs or immunoglobulins (IgGs)] are large glycoproteins with a molecular weight of approximately 150 kDa (150,000 g/Mol). They are composed of two identical light chains (LC, molecular weight ca. 25 kDa each) and two identical heavy chains (HC, molecular weight ca. 50 kDa each) linked through covalent inter- and intra-chain disulfide bonds. They are utilized for the treatment of various types of cancer, and other diseases such as multiple sclerosis, Alzheimer's disease, or migraine.

Careful and thorough characterization of therapeutic mAbs is essential for ensuring drug safety and efficacy. mAbs are typically manufactured in mammalian host cell lines in bioreactors, generating a large number of heterogeneous drug molecules.

Establishing a number of critical quality attributes (CQAs) for each mAb and demonstrating that production batches are within acceptable limits is a requirement for both innovator and biosimilar therapeutics.[1,2]

Polysorbate 80 (PS 80; commercial name: Tween® 80) is a nonionic surfactant that is utilized as a stabilizing excipient in protein therapeutics. PS 80 stabilizes proteins, prevents aggregation and nonspecific adsorption of primary and secondary antibodies to surfaces, reduces the rate of protein denaturation and increases the drug solubility and stability.[3,4] In order to ensure product quality, the accurate quantitation of PS 80 in the final drug product is crucial.

Figure 1 displays the chemical structure of polysorbate 80.

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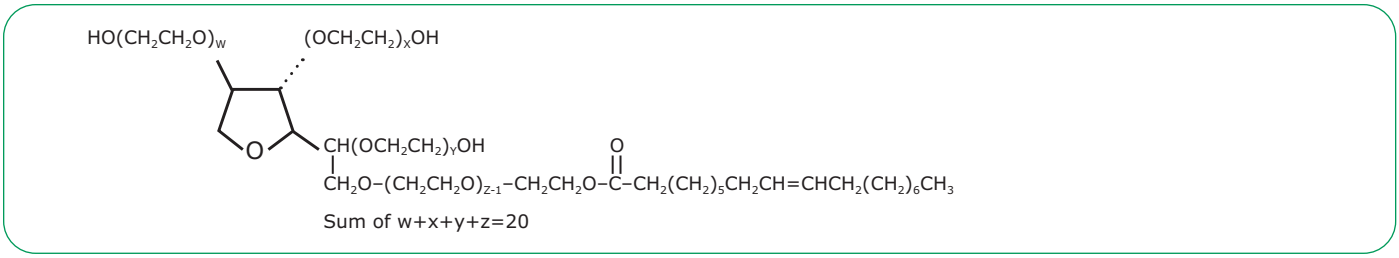

Figure 1. Chemical structure of polysorbate 80 (PS 80; commercial name: Tween® 80).

This report describes the application of reversed phase HPLC-ELSD (high performance liquid chromatography - evaporative light scattering detection) for the quantification of polysorbate 80 in an Erbitux® antibody drug formulation (Erbitux® is the trade name of the drug formulation using the monoclonal antibody cetuximab). Sample preparation is performed using solid phase extraction and a set of seven calibration solutions is prepared for system calibration.

General Procedures – Erbitux® Drug Product Sample Preparation, Polysorbate 80 Standard Preparation, System Setup and Calibration

The samples were received as Erbitux® drug product (DP, formulation of 5 mg/mL cetuximab, excipients: sodium chloride, glycine, polysorbate 80, citric acid monohydrate, sodium hydroxide, water) and were stored at 8 °C. Prior to sample preparation the samples were heated up to room temperature.

A set of two Erbitux® DP batches was analyzed in this work. The polysorbate 80 concentration of the samples was approximately 0.1 mg/mL. No dilution was required before loading onto the solid phase extraction (SPE) cartridge. All solvents applied during sample preparation were of gradient grade HPLC quality or higher.

Sample Preparation

The blank sample was represented by pure water and does not undergo the solid phase extraction process.

The Erbitux® DP samples were purified by SPE. In detail, sample preparation was executed as follows:

1. 4M Guanidinium hydrochloride solution

Dilute 80 mL guanidinium hydrochloride solution 6M with 40 mL water.

2. 10% Methanol

Dilute 10 mL methanol with 90 mL water to obtain a solution of 10% methanol in water.

3. Sample preparation – Solid phase extraction

- Position Supel™ Swift HLB SPE cartridge in Visiprep™ SPE Vacuum Manifold.
- Prime with 1 mL methanol.
- Condition with 1 mL water.
- Load 0.5 mL of an Erbitux® DP sample solution, add 0.5 mL water.
- Wash with 1 mL 4M guanidinium hydrochloride solution.
- Wash with 1 mL 10% methanol.
- Elute with 1 mL acetonitrile and collect eluent in 15 mL centrifuge tube.
- Repeat elution once and collect eluent in the same centrifuge tube.
- Evaporate acetonitrile in vacuum rotary evaporator at 40 °C for 25 minutes.
- Reconstitute sample with 250 µL water, vortex mix well and then transfer to HPLC glass vials.

Standard Preparation

The preparation of calibration standards was performed as follows:

4. PS 80 standard stock solution 1.2 mg/mL

For the preparation of PS 80 stock solution (c = 1.2 mg/mL) weigh approximately 60 mg PS 80 into a 50 mL volumetric flask and fill up to mark with water.

5. PS 80 calibration standards

Prepare dilution series according to **Table 1** to obtain a set of seven calibration standards.

Table 1. Polysorbate 80 calibration standards compositions. Final concentrations resulted from an initial weighed portion of PS 80 of 59.61 mg.

Calibration standard #	Standard stock solution (µL)	Water (µL)	Total volume (µL)	Final concentration (mg/mL)
1	20	980	1000	0.0238
2	40	960	1000	0.0477
3	80	920	1000	0.0954
4	140	860	1000	0.1669
5	200	800	1000	0.2384
6	260	740	1000	0.3099
7	320	680	1000	0.3814

RP-HPLC-ELSD System Setup and Data Analysis

RP-HPLC-ELSD System Setup

The essential settings of the Hitachi Chromaster chromatography system and the gradient conditions applied in the analysis of polysorbate 80 are listed in **Tables 2** and **3** below.

Table 2. HPLC-ELSD settings.

Instrument	Hitachi Chromaster
Software	Chromeleon™ 7.2.10
Column	Supelco® Ascentis® Express C18 5 µm 7.5 cm × 2.1 mm
Column temp	40 °C
Gradient	See Table 3
Flow	0.6 mL/min
Injection volume	50 µL
Run time	18 min
Detection	ELS

Table 3. HPLC-ELSD gradient conditions. A: Water, B: methanol, C: 2-propanol (all solvents LC-MS grade quality).

Minute	% A	% B	% C
0	95	5	0
2.5	95	5	0
5	10	20	70
10	0	10	90
10.5	95	5	0
18	95	5	0

Data Analysis

Data was processed with Chromeleon™ 7.2.10 software; due to application of a gradient profile and the necessity to summarize peak areas in the retention time range from approximately 6 to 9 minutes, the integration was executed manually. Integration of peaks outside the mentioned range was inhibited automatically. The calibration type applied was "Quad with offset".

Calibration data

A total of seven polysorbate 80 calibration standards was prepared. Quadratic regression revealed an excellent fit of the resulting calibration curve over the entire calibration range, with an R2 value of 0.9997 (see **Figure 2**). Experimental data obtained from the calibration experiments are listed in **Table 4. Figure 3** displays an overlay of the chromatograms obtained by the analysis of the seven calibration standards.

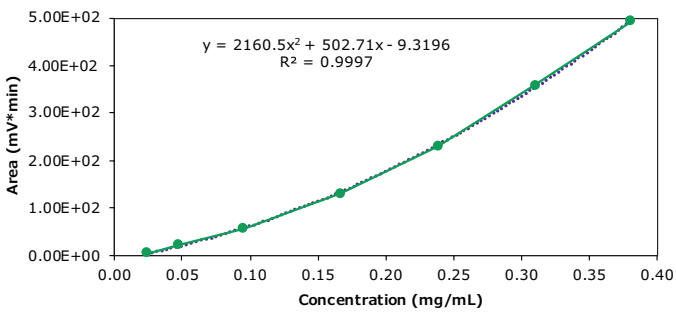

Figure 2. HPLC-ELSD calibration curve obtained by injection of polysorbate 80 calibration standards 1-7.

Table 4. Polysorbate 80 standard solution concentrations, peak areas (median of duplicates) and RSD (%).

Standard solution #	Concentration (mg/mL)	Peak area (mV*min)	RSD(%)
1	0.0238	3.39	0.40
2	0.0477	21.16	1.08
3	0.0954	58.02	0.84
4	0.1669	133.27	2.53
5	0.2384	230.70	3.86
6	0.3099	359.78	2.12
7	0.3814	494.15	2.05

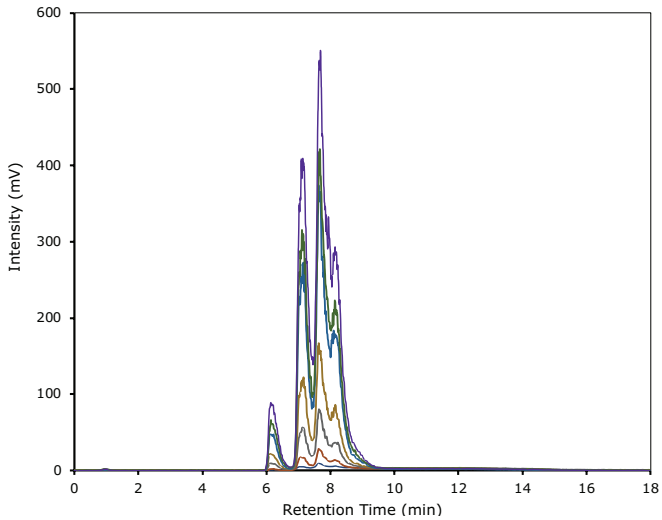

Figure 3. Overlay of chromatograms obtained by the HPLC-ELSD analysis of all seven PS 80 calibration standards.

Results

In this work, an Ascentis® Express C18 HPLC column was utilized for the HPLC-ELSD analysis of polysorbate 80 in two different batches of an Erbitux® antibody drug formulation. The HPLC column applied is comprised of reversed phase-modified, superficially porous silica particles and enables a fast, high-performance analysis.

Sample preparation by hydrophilc-lipophilic balanced solid phase extraction was shown to effectively separate PS 80 from major amounts of drug product excipients.

Duplicates of a total of five samples of each of the batches B4G and BM9 were analyzed to determine their polysorbate 80 content (see also **Table 5**). The corresponding ELSD traces of two representative samples of each batch are shown in **Figure 4**.

The analysis results revealed a PS 80 content of the samples of 0.12 and 0.14 mg/mL, which is in line with typical surfactant concentrations in antibody drug formulations. The calibration curve displayed an excellent quadratic fit over the entire calibration range, with an R2 value of 0.9997, and the LOD for the HPLC-ELSD method was 0.0055 mg/mL.

Table 5. Results of the polysorbate 80 analysis of two Erbitux® DP batches. Concentrations are provided as the median of duplicates.

Batch / sample #	Concentration (mg/mL)	RSD (%)
B4G 1	0.1103	0.0062
B4G 2	0.1117	0.0026
B4G 3	0.1292	0.0031
B4G 4	0.1123	0.0255
B4G 5	0.1488	0.0133
BM9 1	0.1418	0.0002
BM9 2	0.1398	0.0105
BM9 3	0.1455	0.0007
BM9 4	0.1431	0.0233
BM9 5	0.1291	0.0087

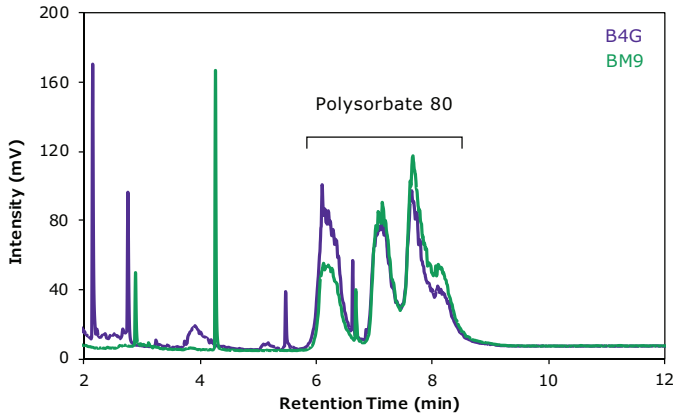

Figure 4. RP-HPLC-ELSD chromatogram of the Erbitux® antibody drug samples B4G (purple trace) and BM9 (green trace). Several polysorbate 80 peaks are visible in the range from approximately 6 to 9 minutes.

Conclusion

This report describes the entire workflow for the quantitative analysis of polysorbate 80 in two Erbitux® antibody drug formulations, using reversed phase HPLC-ELSD analysis. A Supelco® Ascentis® Express C18 HPLC column packed with superficially porous silica particles was applied for the separation of PS 80 and matrix compounds.

The workflow includes a sample purification process using solid phase extraction with HLB cartridges and subsequent analysis of the samples by reversed-phase HPLC-ELSD. HPLC system calibration data was obtained by the preparation and analysis of seven polysorbate 80 standard solutions and allows for a simple quantification of polysorbate 80 content in mAb samples.

The chromatographic method established is suitable for sample separation and analysis of PS 80, an can also be applied in the quantification of similar non-ionic surfactants

References

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3. M. Khossravi, Y.H. Kao, R.J. Mrsny, T.D. Sweeney, Pharm. Res. 19, 634 - 639 (2002).
4. D. Hewitt, T. Zhang, Y.H. Kao, J. Chromatogr. A 1215, 156 - 160 (2008).

Product list

Description	Cat. No.
HPLC columns & sample preparation	
Supelco® Ascentis® Express C18 5 µm 7.5 cm × 2.1 mm	**50511-U**
Supel™ Swift HLB SPE tubes 30 mg (bed), volume 1 mL	**57493-U**
Solvents & reagents	
Ultrapure water from Milli-Q® water purification system or bottled water	**ZIQ7005TOC or 1.15333**
Methanol gradient grade for liquid chromatography LiChrosolv®	**1.06007**
Methanol hypergrade for LC-MS LiChrosolv®	**1.06035**
2-Propanol gradient grade for liquid chromatography LiChrosolv®	**1.01040**
2-Propanol hypergrade for LC-MS LiChrosolv®	**1.02781**
Acetonitrile gradient grade for liquid chromatography LiChrosolv®	**1.00030**
Guanidine hydrochloride solution 6M, manufactured under cGMP controls	**SRE0066**
TWEEN® 80 BioXtra	**P8074-100ML**
Equipment & Consumables	
Visiprep™ SPE Vacuum Manifold, standard, 12-port model Supelco®	**57030-U**
Vacuum centrifuge Eppendorf Concentrator Plus	**EP5305000100-1EA**
Corning® 15 mL centrifuge tubes	**CLS430053-500EA**
Heidolph rotary evaporator Laborota 4003	**Z619094**
Snap Seal™ HPLC vials	**29141-U**

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