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Analysis of Tween 80 by high-performance liquid chromatography with diode array detection

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Abstract

Tween 80 is commonly used as a stabilizing excipient in pharmaceutical medications, or an anticalcification agent for tissues treatment. The quantification of Tween 80 is challenging because of the heterogeneous mixtures and lack of good chromophore. This study developed a simple and fast high-performance liquid chromatography (HPLC) method using an Agilent InfinityLab Poroshell 120 EC-C18 column and max plot diode array detection (DAD) to analyze Tween 80 in 0.02 M phosphate buffer. The quantitative determination is based on the sum of peak areas of peaks in the Tween 80 elution region. Precision, accuracy, linearity, and limit of quantitation/detection experiments gave acceptable results during the evaluation of the method. This method could be applied to various aqueous solutions containing Tween 80 for quality control and stability monitoring.

Introduction

Tween 80, also known as polysorbate 80, is a common nonionic surfactant, emulsifier, stabilizer, and solubilizer widely used in pharmaceutical medications, food products, cosmetics, vitamins, vaccines, intravenous preparations, lotions, and soaps. In protein formulations, Tween 80 and other polysorbates can minimize adsorption to surfaces, reduce the rate of protein denaturation, and increase drug solubility and stability. Tween 80 can be also used in pre- and postglutaraldehyde tissue (fixation) treatments to act as anticalcification agent for tissues prepared for tissue heart valves[1]. Due to these critical roles, accurate quantitation of Tween 80 is needed to ensure product quality.

Tween 80 is a mixture of partial esters of fatty acids, mainly oleic acid, with sorbitol and its anhydrides ethoxylated with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbital anhydrides. Figure 1 shows its molecular structure. As macromolecular compounds, the quantification of polysorbates is difficult because of the heterogeneous mixtures and lack of UV chromophore. Several methods to quantitate polysorbate 80 have been described in literature. Indirect methods based on the chemical transformation of polysorbate 80, such as alkali induced hydrolysis into oleic acid, have been reported[2,3]. These methods are laborious and time-consuming, involving 6 to 18 hours of digestion at an elevated temperature of 40 to 60 °C. A few direct methods for analysis of Tween 80 have been developed using HPLC separation coupled with different detections such as evaporative light scattering detection (ELSD)[4–6], charged aerosol detection (CAD)[7], and mass spectrometry (MS)[8].
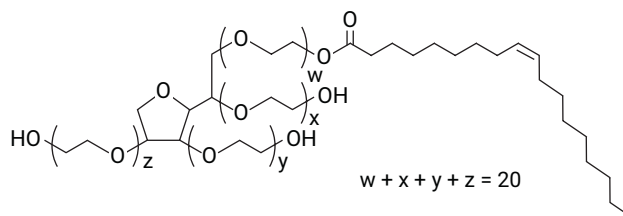

Figure 1. Molecular structure of Tween 80.

Some researchers tried not to separate and use a single chromatographic peak for quantitation[4,9]. These methods may need complex equipment or could not be used to monitor the stability of Tween 80 solutions.

This study introduces a simple and fast method for the determination of Tween 80 in aqueous solution using reversed-phase HPLC separation based on an Agilent InfinityLab Poroshell 120 EC-C18 column with diode array detection. The suitability of the method for the determination of Tween 80 in phosphate buffer has been evaluated by testing its specificity, linearity, precision, sensitivity, and accuracy. This method provides an option for scientists because diode array detection is more commonly used than ELSD, CAD, and MS detectors for HPLC analysis.

Experimental

Materials and instrumentation

Tween 80 standard was obtained from US Pharmacopeial Convention (Rockville, MD). Tween 80 from Fisher Chemical (Fair Lawn, NJ) was used to prepare Tween 80 samples. Acetonitrile (HPLC grade), sodium phosphate monobasic dihydrate (certified crystalline), sodium phosphate dibasic dihydrate (certified crystalline), and phosphoric acid (>85 %) were purchased from Fisher Chemical. Water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA).

Tween 80 samples were analyzed using an HPLC system coupled to a diode array detector (DAD). An Agilent InfinityLab Poroshell 120 EC-C18 column, 3.0 × 100 mm, 2.7 µm (p/n 695975-302A) (Agilent Technologies, Santa Clara, CA) was used to elute Tween 80 peaks. The DAD was set at 1.2 nm resolution, scanning from wavelength 195 to 400 nm. The max plot signal was collected for quantitation. A 20 µL sample amount was injected using an isocratic mobile phase consisting of mobile phase A: water with 0.1 % phosphoric acid, and mobile phase B: acetonitrile with 0.1 % phosphoric acid, at a constant ratio of A:B 20:80. Throughout the analysis, the column was kept at 30 °C, and the mobile phase flow rate was maintained at 0.4 mL/min.

Results and Discussion

Optimization of the analysis

A few HPLC columns and mobile phases such as an HPLC C18 column have been recommended[4] with gradients starting at a water/methanol ratio of 70:30 and, at four minutes, changing to water/methanol 10:90[4]. Neither a single peak nor complete elution of Tween 80 was obtained. The current conditions described above using an InfinityLab Poroshell 120 EC-C18 column and max plot diode array detection resulted in a fast and relatively simple approach to analyze the concentration of Tween 80 in phosphate buffer.

A max plot chromatogram is generated by plotting the maximum spectral absorbance measured at each time point. A max plot chromatogram allows one to see all the chromatographic peaks in a sample regardless of λ_{max} (the wavelength at maximum absorbance). Figure 2 shows a max plot chromatogram of USP Tween 80 standard in 0.02 M phosphate buffer.

Weak UV absorbance of Tween 80 has limited the analysis of Tween 80 by a fixed single wavelength. M. Klein; et al.[10] used a gel filtration chromatography (GFC) column and UV at 245 nm to analyze Tween 80. The sensitivity for Klein's method was poor. When we tried to extract a chromatogram at 245 nm from the DAD data, a surprising chromatogram was obtained. Figure 3 presents the comparison of the max plot of a Tween 80 sample and its chromatogram extracted at 245 nm. The extracted chromatogram at 245 nm had fewer peaks than its original max plot chromatogram, and all peaks in the max plot chromatogram displayed weak UV absorbance at 245 nm. It is clear that max plot diode array detection is a better choice for Tween 80 analysis.

Can one peak or one group of peaks in the max plot chromatogram be used to quantitate Tween 80? Tween 80 solutions at 10 mg/mL prepared from USP Tween 80 standard and Fisher Tween 80 were compared, and their max plot chromatograms are shown in Figures 2 and 3A, respectively. The chromatograms show varying intensity of peaks at similar retention times even though the concentration of Tween 80 was comparable, 10.033 mg/mL of USP Tween 80 against 10.145 mg/mL of Fisher Tween 80.
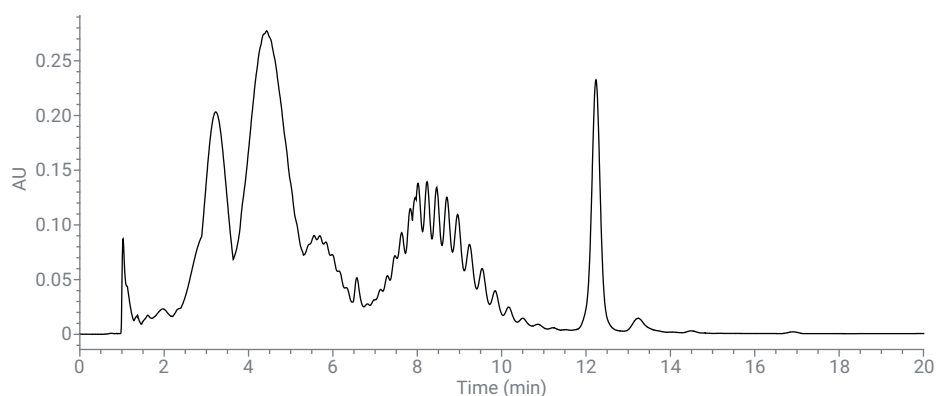

Figure 2. The chromatogram of USP Tween 80 standard at 10 mg/mL in 0.02 M phosphate buffer obtained by max plot diode array detection from 195 to 400 nm.
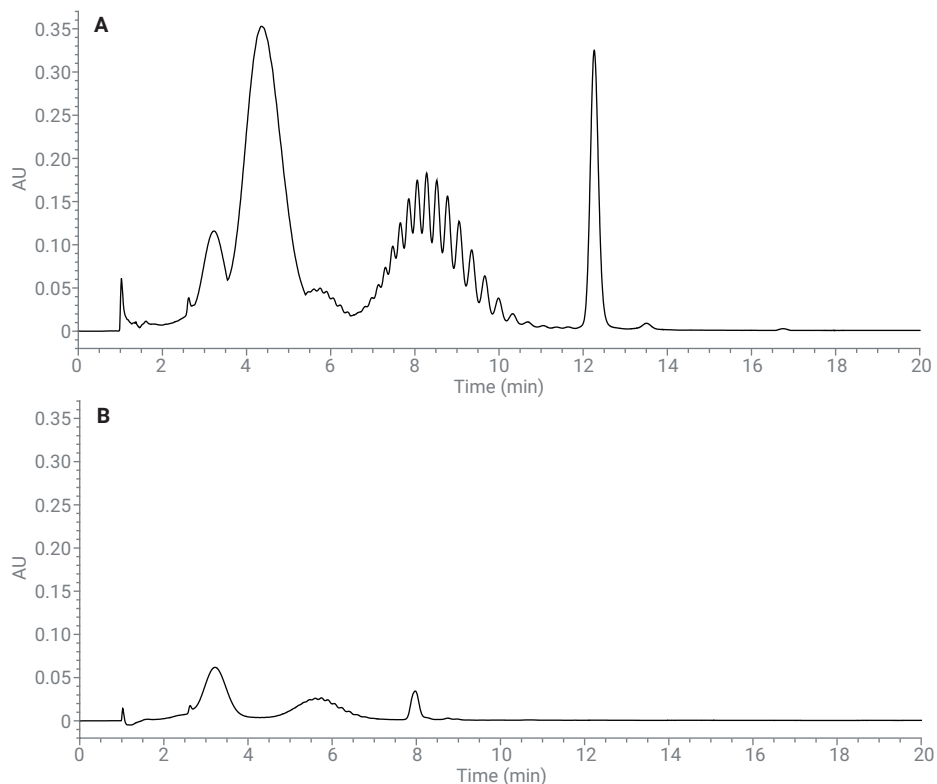

Figure 3. The comparison of the max plot (A) and its extracted chromatogram at 245 nm (B) for a Tween 80 sample at 10 mg/mL (at the same scale) in 0.02 M phosphate buffer.

Table 1 presents the ratio of total peak area of each group if we divided one chromatogram into five groups of peaks:

- **Group 1:** 1.8 to 3.7 minutes
- **Group 2:** 3.7 to 5.3 minutes
- **Group 3:** 5.3 to 6.8 minutes
- **Group 4:** 6.8 to 11.6 minutes
- **Group 5:** 11.6 to 14.2 minutes

The ratio between two Tween 80 materials at similar retention time groups differs from 0.99 (10.033/10.145), indicating that these individual groups of peaks could not represent the whole mixture of Tween 80. The summed peak area from 1.8 to 14.2 minutes was also used as a comparison, with the resulting ratio being 0.98 to 0.99, which is very close to the ratio of the concentration of Tween 80 in these two solutions. Therefore, it is recommended to use sum of peak areas of peaks from 1.8 to 14.2 minutes for the quantitation of Tween 80 in this method.

Analysis of Tween 80 in phosphate buffer

The proposed analytical method was evaluated for the determination of Tween 80 in 0.02 M phosphate buffer used for tissue treatment. The parameters evaluated were linearity, specificity, accuracy, precision, and limit of quantitation (LOQ).

Linearity: The linearity of response was assessed by injecting Tween 80 standard solutions prepared in 0.02 M phosphate buffer. The concentration range of Tween 80 investigated was 10–200 % of the theoretical target Tween 80 content (target = 10 mg/mL, therefore, range = 1 to 20 mg/mL). The sum of all peak areas of each standard solution against Tween 80 concentrations (1.0, 2.5, 5.0, 10, 15, and 20 mg/mL) were plotted by linear regression. The linear equation was $y = (4.57 \times 10^{6})x + (6.06 \times 10^{5})$, with correlation coefficient, $R^2 = 0.999951$ (see Figure 4 for details). The percent deviation of individual levels from low concentration to high concentration was −3.8, −1.0, 0.2, 0.4, 0.8, and −0.5 %. The results for linearity show that the method has excellent linearity in the range of 1 to 20 mg/mL of Tween 80 in 0.02 M phosphate buffer.

Table 1. Ratio of total peak area of each group of peaks.

Group of peaks	Ratio of peak area of Tween 80 from USP/Fisher					%RSD
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	
Group 1 (1.8–3.7 minutes)	1.94	1.95	1.98	1.97	1.97	0.9 %
Group 2 (3.7–5.3 minutes)	0.79	0.80	0.79	0.79	0.80	0.7 %
Group 3 (5.3–6.8 minutes)	2.23	2.19	2.21	2.19	2.19	0.9 %
Group 4 (6.8–11.6 minutes)	0.81	0.82	0.82	0.80	0.82	1.1 %
Group 5 (11.6–14.2 minutes)	0.77	0.78	0.77	0.75	0.77	1.1 %
Sum of peak area (1.8–14.2 minutes)	0.98	0.99	0.99	0.98	0.99	0.6 %

The two solutions contained USP Tween 80 at 10.033 mg/mL and Fisher Tween 80 at 10.145 mg/mL, respectively. The ratio of their concentrations is 0.99.
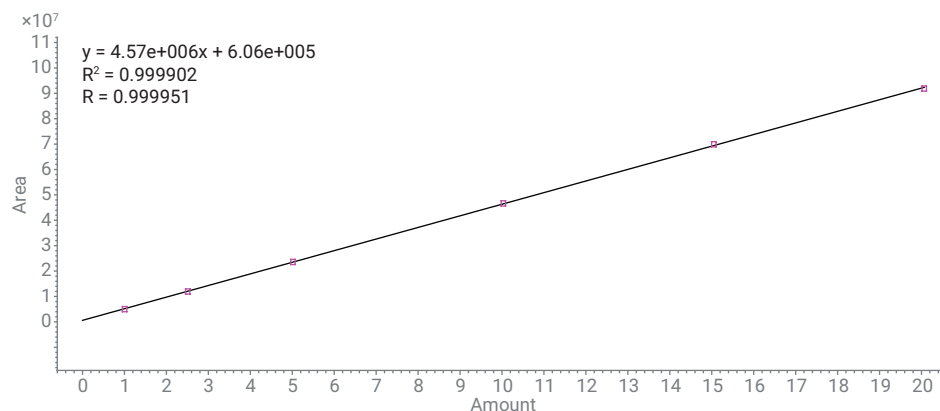

Figure 4. Linear plot and equation.

Accuracy: Accuracy was evaluated by analyzing Tween 80 test solutions prepared at approximately 10 % (low), 100 % (mid), and 160 % (high) of Tween 80 target concentration 10 mg/mL in 0.02 M phosphate buffer. Table 2 shows the measured amount and prepared amount, percent recovery, mean recovery, and %RSD of the replicates for each level. The results show that the %RSD (n = 3) at low, mid, and high levels is 11, 1, and 1 %, respectively.

Specificity: Specificity was assessed by injecting sample solution, 0.02 M phosphate buffer (matrix), and Tween 80 standard solution at 1 mg/mL in 0.02 M phosphate buffer. The injection of the sample matrix had weak and broad peaks that could not be integrated by the software. If they were integrated manually, the sum of peak areas was less than 5 % of the sum peak areas of the lowest calibration standard of Tween 80 (1 mg/mL). This could be caused by carryover due to the stickiness of Tween 80. To reduce material buildup in the column, an acetonitrile injection and column flush (100 % mobile phase B) after each sequence could be helpful. If sample solutions are close to 10 mg/mL, the effect of carryover is negligible.

Method precision
Method precision was evaluated during one analytical run by the preparation and analysis of six replicates from the same homogenous sample solution. The %RSD of the six results was calculated to be 0.3 %, which is much lower than the 2 to 5 % requirement of method precision for a typical HPLC method .

LOQ and limit of detection (LOD)
Since Tween 80 has multiple peaks, it is impossible to estimate the method LOD and LOQ based on signal-to-noise ratio of the peaks. Tween 80 standard solutions were diluted to 0.1 mg/mL in 0.02 M phosphate buffer; the recovery obtained for three replicates was 96, 111, and 143 %, with 20 %RSD. The %RSD is a bit high, therefore, the LOD is estimated to be 0.1 mg/mL. As for LOQ, linearity and accuracy results indicate that the LOQ for the method is approximately 1 mg/mL of Tween 80 in the buffer.

Stability
This method could be used for monitoring the stability of Tween 80 sample and standard solutions. Figure 5 compares a Tween 80 standard solution at 10 mg/mL to the solution being re-injected after five days with a pierced cap at room temperature. The peaks at 6.5, 8, and 12 minutes grew, while the peaks at 2 to 5 minutes decreased. How the solution changed, and what was changed is beyond this study.

Table 2. Accuracy results.

| Levels	Peak area	Measured concentration (mg/mL)	Theoretical concentration (mg/mL)	Recovery (%)	Mean recovery (%)	RSD
Low_1	5,007,741	1.1272	1.0145	111.1 %		
Low_2	5,318,193	1.1971	1.0284	116.4 %	106.9 %	11 %
Low_3	4,167,663	0.9381	1.0073	93.1 %		
Mid_1	44,297,696	9.9714	10.1450	98.3 %		
Mid_2	45,302,502	10.1976	10.2840	99.2 %	99.4 %	1 %
Mid_3	45,041,302	10.1388	10.0730	100.7 %		
High_1	72,116,909	16.2335	16.4400	98.7 %		
High_2	71,863,773	16.1765	16.4630	98.3 %	98.8 %	1 %
High_3	74,398,919	16.7471	16.8730	99.3 %		
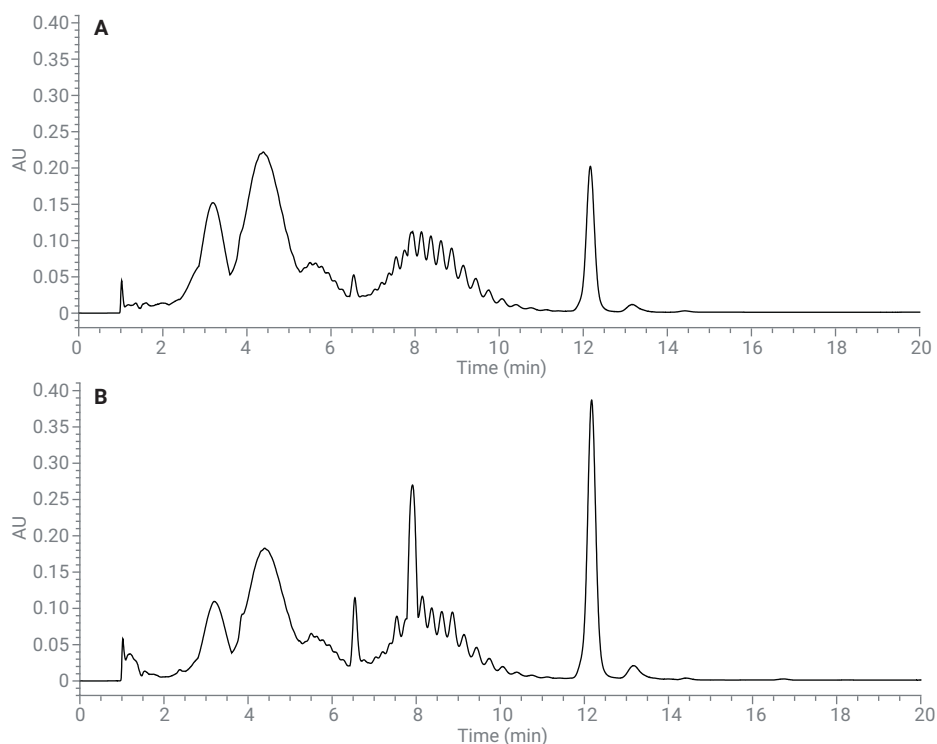
Figure 5. Comparison of a Tween 80 standard solution at 10 mg/mL (A) to the solution being re-injected after five days with a pierced cap at room temperature (B).

Conclusions

A fast and simple HPLC method using an InfinityLab Poroshell 120 EC-C18 column and max plot diode array detection has been developed to analyze Tween 80 in 0.02 M phosphate buffer. The method is accurate, precise, and sensitive. A wide calibration range could apply the method to various aqueous solutions, such as therapeutic protein formulations, if appropriate sample pretreatment were made. In addition, the method can be used to study the stability of Tween 80 solutions.

References

1. Yperman, J. Cell Repopulation of Bioprosthetic Heart Valves, (Leuven University Press, Belgium, **2006**), 77.

2. Hu, M.; *et al. J. Chromatogr. A* **2003**, *984*, 233–236.

3. Adamo, M.; *et al. Chromatogr. B* **2010**, *878*, 1865–1870.

4. Nair, L. M., *et al. J. Chromatogr. A* **2003**, *1012*, 81–86.

5. Nayak, V. S.; *et al. Chromatogr. Sci.* **2012**, *50*, 21–25.

6. Wu, Y.; *et al. AOAC International* **2010**, *93*, 917–921.

7. Shi, S.; *et al. Anal. Bioanal. Tech*. **2015**, *6*, 245–252.

8. Zhang, R.; *et al. Chromatogr. Sci.* **2012**, *50*, 598–607.

9. McCarthy, S. M. *Waters Application Note*, 720005323EN, Mar **2015**.

10. Klein, M.; Preston, J. *Phenomenex Application Note*, App ID 23389.

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