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NMR Method for Accurate Quantification of Polysorbate 80 Copolymer Composition

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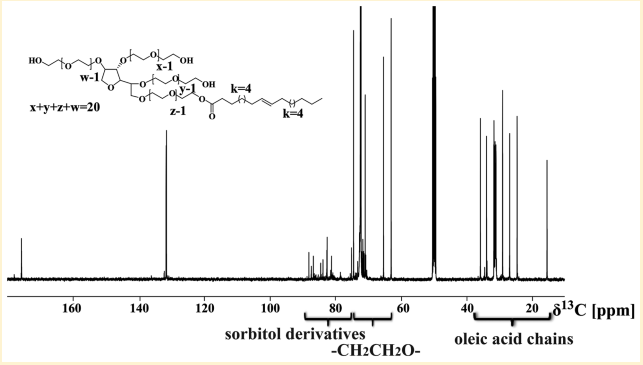
Supporting Information

ABSTRACT: ^{13}C NMR spectroscopic integration employing short relaxation delays and a 30° pulse width was evaluated as a quantitative tool for analyzing the components of polysorbate 80. ^{13}C NMR analysis revealed that commercial polysorbate 80 formulations are a complex oligomeric mixture of sorbitan polyethoxylate esters and other intermediates, such as isosorbide polyethoxylate esters and poly(ethylene glycol) (PEG) esters. This novel approach facilitates the quantification of the component ratios. In this study, the ratios of the three major oligomers in polysorbate 80 were measured and the PEG series was found to be the major component of commercial polysorbate 80. The degree of polymerization of -CH_2CH_2O- groups and the ratio of free to bonded -CH_2CH_2O- end groups, which correlate with the hydrophilic/hydrophobic nature of the polymer, were analyzed, and were suggested to be key factors for assessing the likelihood of adverse biological reactions to polysorbate 80. The ^{13}C NMR data suggest that the feed ratio of raw materials and reaction conditions in the production of polysorbate 80 are not well controlled. Our results demonstrate that ^{13}C NMR is a universal, powerful tool for polysorbate analysis. Such analysis is crucial for the synthesis of a high-quality product, and is difficult to obtain by other methods.



Polysorbate 80 (Tween 80) is a nonionic surfactant consisting primarily of polyethoxy sorbitan oleic acid esters and is widely used in the chemical, cosmetic, food, environmental, and pharmaceutical industries.[1,2] Besides the economic importance of polysorbates, we are interested in the analytical challenge of determining their formulation and monitoring their complex production process. Polysorbates consist primarily of partial oleic acid esters of sorbitol-derived cyclic ethers, polymerized with approximately 20 molecules of ethylene oxide per molecule. In recent years, many adverse clinical events have been reported related to the widespread use of polysorbates in injection formulations. For example, use of polysorbate 80 may cause serious allergic reactions, including breathing difficulties, nausea, vomiting, and skin rashes.[3−7]

Polysorbate 80 is a mixture of oligomers,[8] including poly(ethylene glycols) (PEGs), poly(ethylene glycol) esters, isosorbide polyethoxylates (IPEs), isosorbide polyethoxylate esters, sorbitan polyethoxylates (SPEs), polysorbate monoesters/diesters, and sorbitol polyethoxylate esters. Figure 1 summarizes the major oligomers present in polysorbate 80 formulations. The behavior of polysorbate 80 is dependent on the structure of the sorbitol derivative core, the oleic acid side chains, their degree of esterification, their degree of polymerization, and their distribution within the molecule. Thus, understanding the chemical composition of polysorbate 80 would help formulators not only in predicting the properties of polysorbate 80 emulsions but also in choosing appropriate polysorbate 80 concentrations.

One major challenge faced by polymer scientists is the quantitative determination of polymer components in mixtures. While UV−visible (UV−vis) spectroscopy,[9] high-resolution matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF/MS),[10,11] gas chromatography (GC),[12] and high-performance liquid chromatography (HPLC)[13,14] are typically used for quantitative analysis, such techniques can be time-consuming, can be inaccurate for the measurement of oligomers, or can require specialized instrumentation. The response factors of the detection methods must also be considered. Owing to the absence of standard oligomers for calibration, no suitable method exists for the determination of component ratios using UV−vis, MS, or LC techniques. In addition, quantitative analysis is only appropriate on the basis of indirect observations because of a lack of convenient analytical tools. In contrast, NMR spectroscopy is particularly useful

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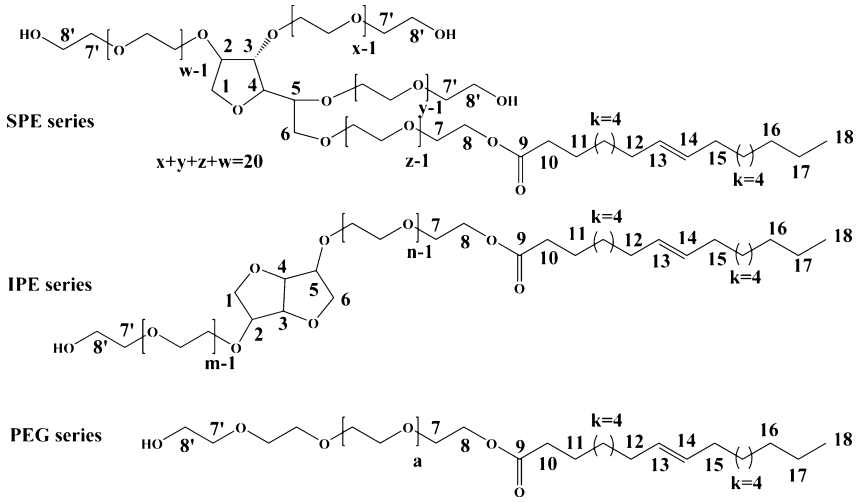

Figure 1. Major polyethoxylate components in polysorbate 80 formulations: sorbitan polyethoxylate (SPE), isosorbide polyethoxylate (IPE), and poly(ethylene glycol) (PEG) ester series. "k = 4" is the number of CH_2 repeat units.

because single pulse experiments give reliable integrations that are directly related to compound ratios.[15,16] However, in the case of oligomers, the compound peaks are highly overlapped and difficult to resolve by ^{1}H NMR spectroscopy (Figure 2), making it unsuitable for analysis of the component ratios in polysorbate 80.

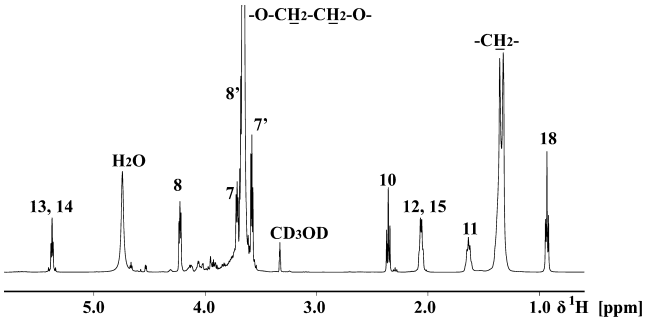

Figure 2. ^{1}H NMR spectrum of polysorbate 80 sample 1 in CD_3OD. Signal numbering corresponds to Figure 1.

The peak dispersion of ^{13}C NMR signals is significantly greater than that of ^{1}H signals (Figure 3). Thus, ^{13}C NMR spectroscopy is suitable for use as a quantitative tool.[17,18] However, quantitative ^{13}C NMR has a number of issues, including the nuclear Overhauser effect, large ^{13}C longitudinal relaxation time (T_1), and low natural abundance of ^{13}C (~1.1%), which necessitates long relaxation delays and results in long measurement times to achieve sufficient signal-to-noise (S/N) ratios. Recent developments from Woerpel's group using broadband proton-decoupled ^{13}C NMR spectroscopy employing short relaxation delays has provided a powerful technique for distinguishing between small molecules and performing polymer end-group analysis at natural abundance levels.[19] We herein report the use of broadband-decoupled ^{13}C NMR spectra to obtain quantification information that cannot be determined by other methods. We aim to investigate the potential of ^{13}C NMR for the quantitative analysis of polysorbate formulations, obtain in-depth molecular information about these formulations, and determine the reasons for adverse reactions in their clinical use.

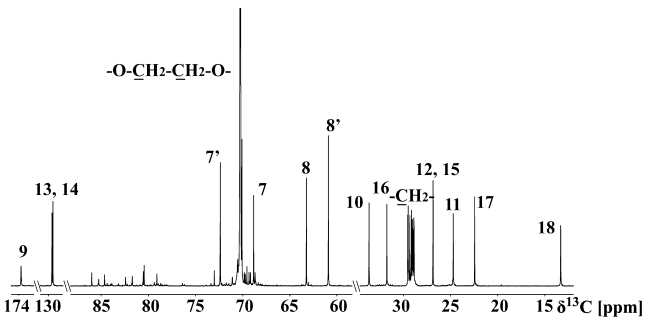

Figure 3. ^{13}C NMR spectrum of polysorbate 80 sample 1 in CD_3OD. For expansion, see Figure 4. Signal numbering corresponds to Figure 1.

EXPERIMENTAL SECTION

Solvents and Reagents. Polysorbate 80 samples were purchased from a number of vendors (detailed information is available in the Supporting Information, Table S1), and CD_3OD was purchased from Sigma–Aldrich (St. Louis, MO, USA). The polysorbate 80 samples (~200 mg) were weighed, dissolved in CD_3OD (0.6 mL), and transferred to 5 mm NMR tubes with a total solution volume of 800 μL.

NMR Experiments. Quantitative ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Avance III HD 500 NMR spectrometer fitted with a 5 mm i.d. BBO probe at 500.15 and 125.77 MHz, respectively. The temperature of the probe was set at 25 °C. ^{13}C NMR spectra were acquired at a spectral width of 25252 Hz (200 ppm) and an acquisition time of 2.59 s. A sufficient S/N ratio was achieved by recording 4096 scans. ^{1}H NMR spectra were acquired at a spectral width of 7500 Hz (15 ppm), an acquisition time of 4.37 s, and a relaxation delay of 20 s. A sufficient S/N ratio was achieved with 128 scans. The ^{1}H and ^{13}C 90° pulse widths were calculated prior to collecting the NMR spectra. The decoupler offset was set at 4.00 ppm for the proton channel. The ^{13}C T_1 values were determined using an inversion recovery pulse sequence available from the pulse sequence library with a d1 value of 60 s and nine different inversion times (τ) ranging from 50 ms to 50 s. The inversion profiles were analyzed using TopSpin 3.2 (Bruker, Billerica, MA, USA) to determine the T_1 values.

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For the acquisition of broadband-decoupled (BBD) ^{13}C NMR spectra, a Waltz-16 decoupling scheme with a pulse of 80 μs was employed.[19] For ^{13}C NMR data processing, the line broadening (LB) was set to 1.0, and zero-filled 2 × 256*k*. A baseline correction was performed using fifth-order polynomial functions from −10 to 240 ppm. All spectra were manually integrated without deconvolution using TopSpin 3.2. The start and end points for integration of all spectra were kept consistent throughout.

RESULTS AND DISCUSSION

NMR Assignment. The three major oligomers in polysorbate 80 formulations (Figure 1) comprise three different groups, namely, oleic acid, polyoxyethylene, and sorbitol groups. The chemical shifts of the ^{1}H and ^{13}C signals from the oleic acid and polyoxyethylene groups were equivalent in the three major oligomers. The NMR data for these groups are compiled in Table 1.

Table 1. NMR Assignment Data for SPE[a] and IPE[b] in Polysorbate 80

	SPE			IPE		
position no.[c]	^{1}H (δ_H)	^{13}C (δ_C)	^{13}C T_1	^{1}H (δ_H)	^{13}C (δ_C)	^{13}C T_1
2	3.95	85.22	0.53	4.14	80.52	1.33
3	4.06	82.39	0.4	4.02	84.61	1.48
4	4.07	81.71	0.48	4.54	85.97	1.31
5	4.23	79.1	0.43	4.67	80.43	1.3
6	3.9	69.51	0.85	3.95	72.69	0.82
	SPE and IPE					
	^{1}H (δ_H)	^{13}C (δ_C)	^{13}C T_1			
-CH_2CH_2O-	3.66	70.2	1.18			
7	3.72	68.79	0.94			
8	4.23	63.2	0.9			
7′	3.58	72.31	2.2			
8′	3.68	60.86	2.18			
9		173.89	6.81			
10	2.36	33.61	1.13			
11	1.64	24.66	1.14			
CH_2	1.34	29.5−28.7	1.2−2.1			
12, 15	2.06	26.77	1.46			
13, 14	5.37	129.49	1.77			
16	1.32	31.7	3.05			
17	1.34	22.37	3.99			
18	0.93	13.14	4.68			

[a]SPE = sorbitan polyethoxylate. [b]IPE = isosorbide polyethoxylate. [c]Position numbering corresponds to Figure 1. The signal of position 1 was not assigned because of overlapping with the CH_2 group.

Figures 2 and 3 show the ^{1}H and ^{13}C NMR spectra for polysorbate 80. The spectra have been normalized by dividing by the area of the CH_3 moiety present at the end of the oleic acid chains. In the ^{1}H spectra, the area of the signals corresponding to the oleic acid side chains (0.8−2.5 ppm) combined with a single - CH═CH- group outside the main envelope, give the average number of protons per oleic acid chain.

The majority of polyoxyethylene proton signals occur in an envelope between 3.5 and 3.8 ppm in the ^{1}H domain (Figure 2) and between 70 and 71 ppm in the ^{13}C domain (Figure 3). The terminal monomer differs from the chain itself, as the two different -CH_2CH_2O- end groups are clearly shifted. One is adjacent to the fatty acid (C7 and C8), whereas the other is the free end group (C7′ and C8′). The identity of this group was confirmed by heteronuclear multiple bond correlation (HMBC) experiments (Supporting Information Figure S1). The carbon signals at 63.20 and 68.79 ppm (C7 and C8) were assigned to the group linked to the fatty acid, whereas the signals at 60.86 and 72.31 ppm (C7′ and C8′) belong to the group without the fatty acid moiety.

Optimization of NMR Parameters. Quantitative ^{13}C NMR spectroscopy generally requires long relaxation delays, which can result in unacceptably long measurement times to achieve sufficient *S*/*N* ratios. Woerpel's group found that, for small molecular weight systems, standard BBD ^{13}C experiments can achieve acceptable results using short relaxation delays (2 s between pulses) and a 90° pulse width. However, polysorbate 80 is a particularly complex system that contains all of the species discussed previously, as well as unreacted materials. Its molecular weight ranges from 200 to 3500 Da, which results in significant differences in NMR behavior.

Quantitative ^{13}C NMR spectroscopy employing various parameter sets can be used to analyze molecules with different molecular weights. ^{13}C NMR spectra of polysorbate 80 sample 1 (as a large molecular weight sample) and oleic acid (as a small molecular weight sample) were collected employing delays of 2.5, 5.0, and 7.5 s with pulse widths of 30° and 90°. The ^{13}C signals corresponding to the -O−(CO)−$\underline{C}H_2$- (33.61 ppm) and - $\underline{C}H_2CH_3$ (22.37 ppm) moieties of the oleic acid side chain were integrated and analyzed. Theoretically, the peak area ratio $((\int_A 22.37/\int_A 33.61)$, where A = area) obtained by integrating two signals from a single molecule should equal 1. A comparison between different experiments is shown in Table 2.

Table 2. Analysis of Peak Area Ratios at Varying Pulse Widths and Relaxation Delays

	90° pulse width		30° pulse width	
relaxation delay	polysorbate 80	oleic acid	polysorbate 80	oleic acid
d1 = 2.5 s	0.726	0.609	0.970	0.928
d1 = 5.0 s	0.869	0.811	0.989	0.979
d1 = 7.5 s	0.938	0.888	1.008	0.994

The accuracy improved for both samples and both pulse width sets using the longer relaxation delay of 7.5 s, although a more significant improvement was observed with the 30° pulse width. The 90° pulse width gave an improved *S*/*N* ratio compared with the 30° pulse width; however, these experiments required a significantly longer relaxation delay to achieve acceptable accuracy. The experiment employing a 30° pulse width and longer relaxation delay (≥5 s) is accurate to <2.5% for strong signals (-CH_2CH_2O- chain and oleic acid side chain, *S*/*N* ∼ 300) and to <10% for weak signals (sorbitol, *S*/*N* ∼ 45). ^{13}C experiments were conducted employing a 30° pulse width and 5 s relaxation delay to obtain reliable results within an acceptable measurement time.

Oleic Acid Impurities. Polysorbate 80 is derived from polyethoxylated sorbitan and oleic acid. Oleic acid is classified as a monounsaturated omega-9 fatty acid and has the formula $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$. It is the most abundant fatty acid in human adipose tissue and is a common monounsaturated fat in the human diet. Its esters play a particularly important role in biological systems. We found that

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Table 3. NMR Data for 19 Commercial Polysorbate 80 Samples

sample no.	oleic acid, %	R^a	SPE[b]/IPE[c]	free/bonded[d]	PEG/(SPE+IPE)	feed ratio[e]	DP[f]
1	0.74	30.53	0.98	1.66	4.04	71.3:4.0:1	6.95
2	0.75	30.12	0.92	1.64	4.00	71.3:4.0:1	6.88
3	0.76	31.18	1.08	1.58	3.08	57.8:3.5:1	6.09
4	0.60	29.42	0.57	1.70	1.81	42.7:2.3:1	6.53
5	0.85	32.68	0.91	1.57	3.37	67.1:3.7:1	6.65
6	1.11	33.37	0.93	1.60	1.82	47.1:2.5:1	6.92
7	0.41	28.51	1.61	1.46	4.91	90.3:5.2:1	6.87
8	0.66	29.66	1.19	1.56	4.10	75.4:4.3:1	6.54
9	0.71	29.52	1.32	1.87	6.22	92.0:5.2:1	6.18
10	0.03	30.86	1.35	2.11	2.10	44.2:2.4:1	5.79
11	0.97	31.66	1.02	1.56	3.33	65.5:3.8:1	6.34
12	0.75	31.75	0.89	1.58	3.20	61.0:3.5:1	6.52
13	0.94	31.80	0.87	1.50	1.16	35.9:2.1:1	6.62
14	0.84	31.35	0.82	1.67	3.27	56.2:3.5:1	5.83
15	0.84	31.74	0.94	1.75	3.80	68.9:3.7:1	6.44
16	0.85	31.13	0.95	1.69	3.70	68.0:3.8:1	6.36
17	0.77	31.37	0.98	1.64	3.75	67.5:3.8:1	6.50
18	0.97	32.09	0.80	1.65	3.21	60.8:3.5:1	6.28
19	0.97	31.95	0.86	1.65	3.19	60.8:3.5:1	6.22

[a]R = number of protons in the oleic acid side chains. [b]SPE = sorbitan polyethoxylate. [c]IPE = isosorbide polyethoxylate. [d]Free/bonded is the ratio of free and oleic acid-bonded -CH_2CH_2O- end groups. [e]Ratio ethylene oxide:oleic acid:sorbitol. [f]DP = degree of polymerization.

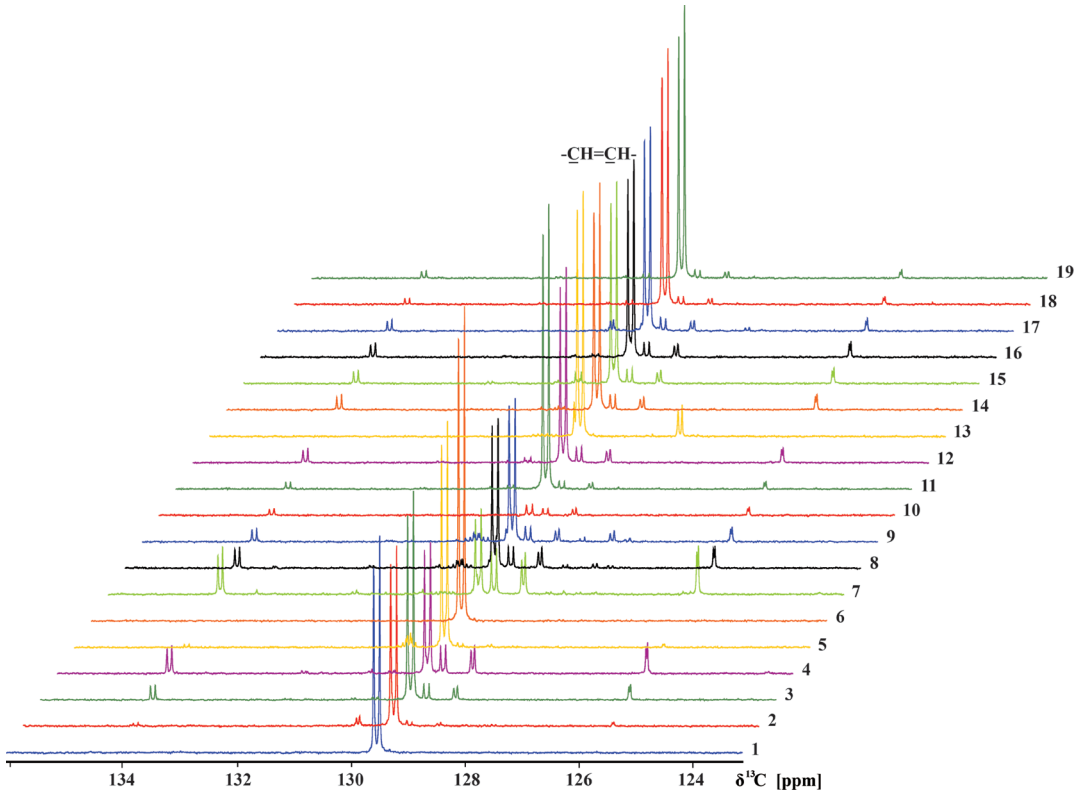

Figure 4. Expanded ^{13}C spectra of 19 commercial polysorbate 80 samples in CD_3OD. The small signals originate from vinyl groups whose position is shifted.

a high level of impurities exist in polysorbate 80 from the oleic acid used in its manufacture.

In the ^1H spectra of all samples, the signals corresponding to the - CH_2- envelope, vinyl group, and CH_3 groups of the oleic acid side chains gave an average number of protons (R) of 29.42−33.37 per chain (Table 3), whereas the calculated value is 33 protons per chain. This difference is due to an impure form of oleic acid being used in the production of polysorbate 80.

In the ^{13}C spectra, the signal at 26.77 ppm was assigned to the - $\underline{C}H_2$- adjacent to the -CH═CH- moiety, whereas that at 33.61 ppm was assigned to the -$\underline{C}H_2$- group adjacent to the C═O moiety. For purely oleic acid side chains, the ratio of these signals would be 1. However, we found that this ratio differed significantly between the 19 samples examined. In

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samples 11, 18, and 19, this ratio was close to 1, suggesting the use of mainly monounsaturated fatty acids in the production of these samples. For sample 10, the ratio was close to zero, indicating the use of mostly saturated fatty acids in its production, meaning that this sample is not, strictly speaking, polysorbate 80. Furthermore, a number of small peaks were present in the vinyl range (125−135 ppm) of the ^{13}C spectrum for all samples (Figure 4), indicating the likely presence of a shifted vinyl position. Thus, ^{13}C NMR data indicate that the quality of oleic acid used in the production of commercial polysorbate 80 is poorly controlled.

SPE/IPE Ratio. In the preliminary steps of polysorbate manufacture, the dehydration of sorbitol yields isomers of sorbitol monoanhydrides (1,4-sorbitan) and sorbitol dianhydrides (1,4:3,6-isosorbide). The resulting mixtures are then reacted with ethylene oxide (polymerization) and oleic acid (esterification) to produce commercial polysorbate formulations. Therefore, although polysorbate 80 should be mainly composed of SPE, it is in fact a complex oligomeric mixture of sorbitan esters and other intermediates, such as IPE and PEG (Figure 1).

Integration of the ^{1}H NMR spectra acquired using multiple scans and longer relaxation delays gave poor results owing to signal overlap. However, quantitative ^{13}C NMR spectroscopy could instead be used to identify molecules with different sorbitol dehydration connectivity. For sample 1, a small difference existed between the SPE/IPE ratios (<10% variation) obtained by comparing different signals (data not shown), caused by a poor S/N ratio for sorbitol in that particular ^{13}C NMR experiment. This technique is unique in measuring the SPE/IPE ratio in commercial samples and afforded comparable results for almost all product ratios analyzed. The SPE/IPE ratios for all 19 samples varied significantly, even for samples originating from the same company. This indicates that the dehydration reaction conditions in polysorbate 80 manufacture are not well controlled.

PEG Quantity. Quantitative ^{13}C NMR spectroscopy can be used to analyze the ratios of PEG and the sorbitol derivatives SPE and IPE in commercial polysorbate 80. The signals at 63.20 and 60.86 ppm were assigned to the carbon atoms of the -$CH_2\underline{C}H_2O$- end group bonded to oleic acid and free, respectively. The total area of the ^{13}C peaks at 63.20 and 60.86 ppm indicates the total number of -$CH_2\underline{C}H_2O$- end groups. In the commercial polysorbate 80 samples, three major oligomers contain polyoxyethylene side chains, namely, SPE, IPE, and PEG (i.e., PEG plus the PEG oleate ester). Hence, the signals corresponding to the -$CH_2\underline{C}H_2O$- end group belong to three different groups. In addition, four polyoxyethylene chains, and thus four -$CH_2\underline{C}H_2O$- end groups, exist in SPE (Figure 1). Furthermore, two polyoxyethylene chains and two -$CH_2\underline{C}H_2O$- end groups are present in IPE, whereas PEG contains two polyoxyethylene chains and two -$CH_2\underline{C}H_2O$- end groups. The areas of the peaks in a 1D quantitative ^{13}C spectrum are directly proportional to the number of carbon atoms present in the active amount of sample. Furthermore, the peak area of the sorbitol ^{13}C NMR signals is related to the amount of SPE and IPE present in the sample. The amount of PEG can be calculated from the peak area of two -$CH_2\underline{C}H_2O$- end groups, excluding the SPE and IPE signals. Hence, the PEG/(SPE + IPE) ratios in this study were determined by a proportional comparison of peak areas according to eq 1 (see Supporting Information for further details):

$$\mathrm{PEG/(SPE + IPE)} = \frac{A_{(\text{-}CH_2CH_2O\text{-})} - 4A_{(\mathrm{SPE})} - 2A_{(\mathrm{IPE})}}{A_{(\mathrm{SPE})} + A_{(\mathrm{IPE})}}/2 \quad (1)$$

where $A_{(\text{-}CH_2CH_2O\text{-})}$ is the total peak area for the -$CH_2\underline{C}H_2O$- end group at 63.20 (C8) and 60.86 ppm (C8′) and $A_{(\mathrm{SPE})}$ and $A_{(\mathrm{IPE})}$ are the areas of the selected peaks for SPE and IPE (C4), respectively.

In the 19 samples examined, the PEG/(SPE + IPE) ratios varied between 1.16 and 6.88. In the majority of samples, this ratio was >3, indicating that that PEG is the major component in these commercial samples. In the polymerization step of polysorbate 80 production, a mixture of anhydrides is condensed with ethylene oxide, which is a process that results in the formation of both ethoxylated sugar oligomers and PEG oligomers. In the case of larger molecular ratios, the quantity of PEG exceeds that of the sorbitol derivatives. This is the first report stating that PEG oligomers are the major component of polysorbate 80.

As previously discussed, polysorbate 80 is a complex mixture of oligomers from three major groups:[11] the SPE series (sorbitan polyethoxylates, polysorbate monoesters/diesters/triesters/tetraesters), the IPE series (isosorbide polyethoxylates, isosorbide polyethoxylate esters), and the PEG series (polyethylene glycols, poly(ethylene glycol) esters). These three series possess similar spectroscopic and chromatographic properties, making qualitative and quantitative analysis, separation, and purification of all components problematic. Although polysorbate 80 used in injection formulations must meet strict criteria for minor components, as detailed in the United States Pharmacopeia (USP) and the European Pharmacopoeia (EP), it has still caused serious allergic reactions. In two case reports,[4] patients developed a reaction following vaccination with pharmaceutical grade polysorbate 80, whereas no reaction occurred with the polysorbate-free vaccine. Thus, the source of the adverse reactions was likely from these three major groups.

In injection formulations, PEGs are generally considered safe surfactants and are commonly used in preclinical *in vivo* pharmacokinetic and efficacy studies because of their solubilizing capabilities and excellent safety profiles.[20] Polysorbate 80 is a surfactant commonly used in parenteral formulations of proteins to minimize denaturation at the air−water interface.[21−23] One problem associated with polysorbate 80 use in injectable products is possible hemolysis,[24] which is inhibited by PEG.[20] Increasing the PEG content in polysorbate 80 could therefore avoid adverse reactions, with the PEG/(SPE + IPE) ratio being the key parameter for polysorbate 80 quality control.

Molecular Feed Ratios. Quantitative ^{13}C NMR spectroscopy can be used to analyze the molecular feed ratio of ethylene oxide, oleic acid, and sorbitol by comparing ^{13}C NMR peak areas. Normally, according to the definitions in the USP and EP, this ratio should be 20:1:1. As shown in Table 3, the feed ratios for the samples studied do not meet the pharmacopoeia definitions. Indeed, our results suggest that during the synthesis of commercial polysorbate 80, approximately 40−90 mol of ethylene oxide and 2−5 mol of oleic acid react with 1 mol of sorbitol. This indicates that the production of polysorbate 80 is poorly controlled.

Degree of Polymerization. The degree of polymerization (DP) of the -CH_2CH_2O- groups refers to the number of repeating units in the chain (n) and gives a measure of molecular weight and size. The use of the term DP is somewhat

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misleading, as it implies that a polymer sample has a uniform formula, i.e., that *n* is the same for all polymer molecules in a sample. However, in the preparation of synthetic polymers, chains of varying lengths are produced, with the product containing a range of formula weights. Therefore, the DP represents an average for the sample. The DP of polysorbate 80 was calculated from the total ^{13}C peak area for -CH_2CH_2O- groups divided by the peak area of the -CH_2CH_2O- end groups. The theoretical DP of polysorbate 80 is 5 (20 mol of ethylene oxide with 4 chains per molecule). However, the ^{13}C NMR data (Table 3) shows that the average DP of the 19 samples analyzed is ~6.5.

Polymer chain length is particularly important because it determines physical properties such as molecule size and viscosity. A change in viscosity occurs with an increase in DP because the increase in length results in the total binding forces between molecules increasing.[20] In this case, an increase in DP leads to an increase in molecular size, viscosity, and intermolecular binding forces. However, it may also cause an increase in hydrophobic interactions between polysorbate and proteins, thus increasing cell hemolysis. Hence, the DP of polysorbate 80 must also be controlled. This issue is currently not addressed in the pharmacopoeias.

Free/Bonded -CH_2CH_2O- End-Group Ratios. Polysorbate 80 has been used extensively to prevent the inhibition of protein surface adsorption and aggregation under various processing conditions. Protein adsorption depends on the affinity of water for the protein surface, with a hydrophilic surface expected to show lower adsorption than a hydrophobic surface.[25] Polysorbates can modulate both protein adsorption and surface-induced structural alterations by their action at interfaces and protein−polysorbate associations. Thus, several liquid pharmaceutical protein products contain polysorbates to minimize protein absorption to surfaces, such as containers and syringes. They also serve to reduce the air−liquid interfacial surface tension and decrease the rate of protein denaturation, thus decreasing aggregation rates.

The signals at 63.20 ppm (C8) and 60.86 ppm (C8′) in the ^{13}C NMR spectra were attributed to the -$CH_2\underline{C}H_2O$- end groups with and without fatty acid side chains, respectively. The -CH_2CH_2O- end group bearing an oleic acid side chain is hydrophobic, whereas the -CH_2CH_2O- end group bearing only a hydrogen atom is hydrophilic.[20] A higher content of the former imparts hydrophobicity to the polymer and causes cell hemolysis. Furthermore, the anaphylactic reactions occasionally caused by polysorbate 80 are likely caused by the degranulation of mast cells and subsequent reduction in cell membrane stability.[26] The ratio of free and bonded -CH_2CH_2O- end groups is directly correlated with hydrophilic and hydrophobic activity. In the USP and EP, the hydroxyl value and saponification value parameters concern the same ratio, but the measurement technique described in the pharmacopoeias is time-consuming and requires specialized instrumentation. In contrast, the ratio derived from our facile ^{13}C NMR method can provide accurate information on both hydrophilic and hydrophobic activity.

CONCLUSION

^{13}C NMR spectra acquired with a short relaxation delay (5 s) and 30° pulse width is a valuable and convenient method for quantifying the component ratios in polysorbate 80. Although the accuracy of our analytical method is not as high as other commonly used techniques, it can provide information that other techniques cannot. ^{13}C NMR analysis revealed that commercial polysorbate 80 formulations are a complex mixture of oligomers, including sorbitan polyethoxylates (SPEs), isosorbide polyethoxylates (IPEs), and poly(ethylene glycols) (PEGs). For the first time, this novel approach has allowed the quantification of the component ratios in commercial polysorbate 80. The ratios of the three major oligomers were determined, with the PEG series being the major component.

The degree of polymerization (DP) of the -CH_2CH_2O- groups and the ratio of free/bonded -CH_2CH_2O- end groups correlate with the hydrophilic and hydrophobic activities of the components. This is important as these key parameters influence whether an adverse reaction is observed in the clinical application of polysorbate 80. Our results also show that the feed ratio of raw materials and reaction conditions in the production of polysorbate 80 are poorly controlled, which is unacceptable in the preparation of injection formulations.

Future work will explore the extension of this $^{1}H/^{13}C$ NMR approach to other polysorbate formulations. A comparison of the results of such studies with previous studies should make it possible to obtain a more complete picture of how polysorbate patterns vary in commercial formulations or under different reaction conditions. Our results can be applied by the pharmaceutical industry to help design and modify polysorbate usage.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b02096.

Sample information for polysorbate 80, comparison of integration methods, HMBC correlation, comparison of BBD/IGD pulse sequences, and explanation of eq 1. (PDF)

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Notes

The authors declare no competing financial interest.

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Anal. Chem. 2015, 87, 9810−9816