Analysis Methods of Polysorbate 20: A New Method to Assess the Stability of Polysorbate 20 and Established Methods that May Overlook Degraded Polysorbate 20

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Purpose. Polysorbate 20 is a commonly used excipient in biopharmaceutical formulations, some of which may have an enzymatic activity. The action(s) of polysorbate 20 in biopharmaceutical formulations as a stabilizer require this surfactant to maintain its intact structure. This manuscript evaluates a new analytic method for the analysis of polysorbate 20 degradation in the format of a biopharmaceutical formulation and makes a comparison with several established methods of analysis.

Methods. Polysorbate 20 samples were degraded in a controlled environment utilizing the enzyme pancreatic lipase to generate degradants that included lauric acid and the sorbitan polyoxyethylene side chain. A new method was developed with sufficient sensitivity to analyze the degraded solutions. Lauric acid was derivatized with the fluorescent reagent 9-anthryldiazomethane to form 9-anthrylmethyl-ethyl ester. The derivatized lauric acid was separated by reversed-phase chromatography and detected by fluorescence or UV spectroscopy. Three established methods utilized to measure polysorbate 20 were evaluated for their ability to detect degraded polysorbate 20. These methods were: (1) fluorescence analysis with N-phenyl-1-naphthylamine fluorescent dye; (2) UV spectroscopy with ammonium cobaltothiocyanate colorimetric reagent; and (3) nuclear magnetic resonance (NMR).

Results. Polysorbate 20 incubation with lipase resulted in degraded polysorbate 20 as determined by the derivatized lauric acid assay. The UV spectroscopy assay utilizing ammonium cobaltothiocyanate reagent was not able to detect the degradation of polysorbate 20 in the samples. The fluorescence method of analysis detected polysorbate 20 degradation as an approximate 50% decrease in micelles in comparison to standard nondegraded polysorbate 20 solutions. NMR analysis resulted in similar proton peak areas for both degraded and nondegraded polysorbate 20 samples. NMR spectra did contain minor differences between the samples.

Conclusions. It is essential to choose the appropriate method of polysorbate 20 evaluation to assess the content, stability, and compatibility of a formulation. Current established methods to assess polysorbate 20 may overlook and do not necessarily monitor the potential degradation of this surfactant, which results in the formation of lauric acid. Because this type of degradation may occur in a formulation by an enzymatically active biopharmaceutical, a new method of analysis has been established.

KEY WORDS: polysorbate 20; stability; analytic method; micelles.

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INTRODUCTION

Excipients are often added to pharmaceutical formulations to aid in the stabilization of the active compound. The compatibility of the excipients with the active compound is crucial for the quality and stability of a formulation. Excipients may cause problems in formulations by various mechanisms. The excipient may degrade and thus lose its mechanism of stabilization or it may produce degradants that interact with the active compound. Excipients also may contain contaminants that may affect formulations.

Surfactants, in particular polysorbates, are commonly utilized excipients in the pharmaceutical industry. Polysorbates are classified as nonionic surfactants and have a wide range of applications in formulations. In small molecule formulations, they may be utilized as wetting agents, emulsifiers, or solubilizers. In protein formulations, polysorbates minimize absorption to surfaces and reduce the air-liquid interfacial surface tension and thus the rate of protein denaturation (1–4). Polysorbates are commonly used in formulations above their critical micelle concentration (CMC) values. For example, recombinant human factor XIII is protected against both agitation and freeze-thaw-induced aggregation in the presence of polysorbate 20 at concentrations corresponding to the CMC (0.007% [w/v]) (3). However, at concentrations below the CMC, it is not stabilized (3). The loss of polysorbate 20 micelles in a formulation may cause instability. Although polysorbate 20 is rather stable in aqueous solution, it can interact with the active compound (2) to undergo degradation in a formulation and can prevent the stabilization of the protein or the degradants could interact with the active compound to pose challenges (such as a loss in activity).

Polysorbate 20 is composed of a hydrophilic head group (sorbitan polyoxyethylene) linked by an ester bond to a hydrophobic tail group (laureate) (Fig. 1). Some active compounds with enzymatic activity (e.g., lipase and esterase) may cleave the ester bond of polysorbate 20 to produce degradants (free lauric acid and sorbitan polyoxyethylene side chain) (Fig. 2). These degradants form micelles less efficiently than polysorbate 20, as is shown in this manuscript. This could impact the stability of the formulation. Therefore, the evaluation of the active compound's potential enzymatic activity prior to formulation is crucial. However, in some cases this may be overlooked because the active compound may have several mechanisms of action, some of which are known and others of which have not yet been investigated.

Another possible mode of polysorbate degradation is mycobacterial contamination. There are reports in the literature of mycobacterial species (e.g., *Candida*, *Staphylococcus aureus,* rapidly growing mycobacteria, etc.) with lypolytic and esterase activity capable of degrading polysorbates (5–7). Due to the potential for the degradation of polysorbate 20 from either the active compound or contaminants, it is important to utilize the proper methods to monitor the stability/compatibility of polysorbate 20.

There are many methods available for measuring polysorbate 20 content (e.g., nuclear magnetic resonance [NMR], fluorescence with N-phenyl-1-naphthylamine [NPN] fluorescent dye [8], and UV with ammonium cobaltothiocyanate colorimetric reagent [9,10]). Not all of these methods can detect the degradation of polysorbate. This manuscript evaluates the

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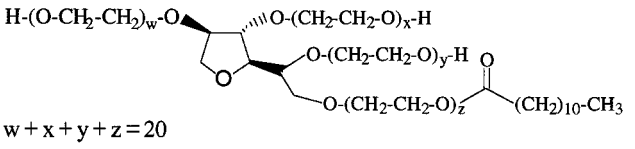


Fig. 1. Chemical structure of polysorbate 20.

methods available to determine whether each method can distinguish between degraded and nondegraded polysorbate 20 in a given formulation. This manuscript also introduces an analytic method that provides the sensitivity needed to detect small quantities of degraded polysorbate 20 in formulations.

MATERIALS AND METHODS

Reagents

Porcine pancreatic lipase (276 U/mg) was obtained from Worthington Biochemical (Lakewood, New Jersey). 9-anthryldiazomethane (ADAM) and NPN were purchased from Molecular Probes (Eugene, OR, USA). Polysorbate 20 was obtained from Abitec (Janesville, Wisconsin). Ammonium thiocyanate, cobalt nitrate hexahydrate, acetonitrile, methanol, and methylene chloride were obtained from Mallinckrodt (Paris, Kentucky). Deuterated methanol was purchased from Sigma (St. Louis, Missouri). Calcium chloride dihydrate and sodium chloride were obtained from Fisher Scientific (Pittsburgh, Pennsylvania). The water used in all studies was from a Millipore MILLI-Q water system (Bedford, Massachusetts).

Sample Preparation

Polysorbate 20 Degradation

Unless otherwise specified, the degradation of polysorbate 20 was performed under the following conditions: solutions contained 5 mM $CaCl_2$, 150 mM NaCl, 1:110 (w/w) ratio of porcine pancreatic lipase to polysorbate 20, and 0.005%, 0.01%, 0.02%, or 0.04% (w/v) polysorbate 20. These solutions were incubated at room temperature (25°C) for 24 h. Methanol was added to each of the solutions after 24 h of reaction to obtain 50/50 (v/v) aqueous/methanol solutions. The solutions were centrifuged at 5000 rpm for 5 min. The supernatant was removed for analysis.

Polysorbate 20 Samples

Varying concentrations of polysorbate 20 samples were prepared to contain 0.04%, 0.02%, 0.01%, or 0.005% (w/v) polysorbate 20, 5 mM $CaCl_2$, and 150 mM NaCl. To these samples, methanol was added to obtain a 50/50 (v/v) aqueous/methanol solution. The final solutions contained 0.02%, 0.01%, 0.005%, or 0.0025% (w/v) polysorbate 20, 75 mM NaCl, and 2.5 mM $CaCl_2$.

Analysis of Degraded Polysorbate 20 by Lauric Acid Derivatization

Polysorbate 20 can undergo degradation to yield lauric acid and sorbitan polyoxyethylene side chain (Fig. 2). Degraded polysorbate 20 samples (prepared as noted above) were analyzed for free lauric acid by derivatization with the fluorescent reagent 9-anthryldiazomethane (ADAM). Three milliliters of methanol containing 0.27 mM ADAM was added to 1 mL of each of the degraded polysorbate 20 solutions. These solutions were incubated in glass vials at room temperature (~ 25°C) for approximately 5 h. They were protected from light since ADAM is photosensitive. Prior to each reaction, fresh stock solutions of ADAM were prepared.

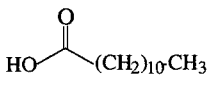
Chromatographic analysis was performed on a Hewlett Packard Series II 1090 liquid chromatograph system (Palo Alto, California) equipped with a diode array detector and autosampler. Turbochrome software (San Jose, California) was utilized for data collection and analysis of the results. Separation was accomplished with a Vydac 218TP C18 reversed-phase column (4.6 × 250 mm) at room temperature (~25°C). Mobile phase A consisted of 100% acetonitrile and, mobile phase B contained 50% methanol and 50% acetonitrile. A flow rate of 1 mL/min for a mixture of 94% mobile phase A and 6% mobile phase B was employed for 20 min. A gradient was not utilized. The detection of derivatized lauric acid was achieved by UV spectroscopy at 254 nm or by fluorescence spectroscopy by excitation at 365 nm and emission at 412 nm.

Colorimetric Analysis of Polysorbate 20 by UV Spectroscopy

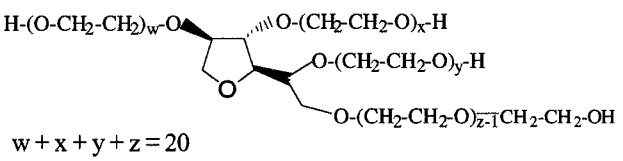
Ammonium cobaltothiocyanate reagent (ACTC reagent) was prepared by dissolving 17.4 g of ammonium thiocyanate and 2.8 g of cobalt nitrate hexahydrate in 100 mL of water (9). Two milliliters each of polysorbate 20, degraded polysorbate 20, and lauric acid samples, prepared as noted above, were evaporated to dryness under nitrogen at 85°C. The samples were allowed to cool, and then 0.4 mL of ACTC reagent and 0.8 mL of methylene chloride were added. The samples then were centrifuged at 2000 rpm for 15 min. The methylene chloride layer was extracted, and absorbance was measured at 620 nm on a Hewlett Packard spectrophotometer (Vectra XM Series 4). The data are averages of duplicate determinations.

Analysis of Polysorbate 20 Micelles by Fluorescence

NPN is a fluorescent dye that partitions into the hydrophobic core of micelle (8). A 5×10^{-6} M NPN in ethanol stock solution was spiked into polysorbate 20, degraded polysorbate 20, and lauric acid samples to a final concentration of



Lauric Acid



Sorbitan Polyoxyethylene Side Chain

Fig. 2. Degradants that may arise from cleavage of the ester bond of polysorbate 20.

10^{-6} M NPN. Fluorescence spectra were obtained using an SLM Aminco 8100 spectrofluorometer (Urbana, Illinois) with a 356-nm excitation wavelength and a 410-nm emission wavelength. The data are averages of duplicate determinations.

NMR Analysis

Polysorbate 20, degraded polysorbate 20, and lauric acid samples were prepared as noted above, except deuterated methanol was utilized instead of methanol. The samples were analyzed on a Varian INOVA (Palo Alto, California) 500-MHz spectrometer equipped with a Nalorac (Martinez, California) 5-mm, triple-resonance gradient probe. Each spectrum is recorded with 32,768 complex points over a spectral width of 6,000 Hz at 25°C. The number of transients recorded for the 1D spectra is 128. The RF transmitter frequency is set to the H_2O (or HDO) line. A low-power saturation pulse was applied to suppress the H_2O signal prior to the excitation pulse.

RESULTS AND DISCUSSION

Detection of the Degradant Lauric Acid as a Novel Method for Determination of Polysorbate 20 Degradation

Pancreatic lipase is an enzyme that cleaves the ester bond of fatty acid residues. Therefore, porcine pancreatic lipase was used to degrade polysorbate 20 to yield lauric acid and sorbitan polyoxyethylene side chain (Fig. 2). The free lauric acid content of the degraded polysorbate 20 samples (polysorbate 20 incubated with lipase) was analyzed. The sorbitan polyoxyethylene side chain was not measured due to the heterogeneous distribution (Fig. 2 chemical formula: $w + x + y + z = 20$); the number of possible structures is greater than 1500 (11). This heterogeneous mixture is not applicable to standard analytic techniques such as reversed-phase high-performance liquid chromatography (HPLC) because of the complexity of the chromatogram. Because lauric acid has a low extinction coefficient and a low detection sensitivity, the low concentrations that may arise from commonly used concentrations of polysorbate 20 (e.g., 0.01% [w/v]) cannot be measured directly. Therefore, for detection sensitivity, it was derivatized with the fluorescent probe ADAM. The ester bond of lauric acid reacts with ADAM to form 9-anthrylmethylethyl ester, which can be detected in nanomolar quantities. Figure 3 shows representative chromatograms of standard lauric acid, polysorbate 20, and polysorbate 20 with lipase. The chromatogram of the degraded polysorbate 20 sample contains a peak with a retention time similar to that of lauric acid. The presence of lauric acid was verified by spiking the sample with free lauric acid and the coelution, and an increase in peak area (Fig. 4). It is clear that this method of analysis provides the sensitivity needed to detect degraded polysorbate 20 at concentrations commonly utilized in pharmaceutical formulations. The method may be useful when initially screening excipients for formulations and compatibility with the active compound. The degraded and nondegraded polysorbate 20 samples were also analyzed for polysorbate 20 content by NMR, colorimetric, and fluorescence methods to determine whether these methods of analysis could detect degradation.

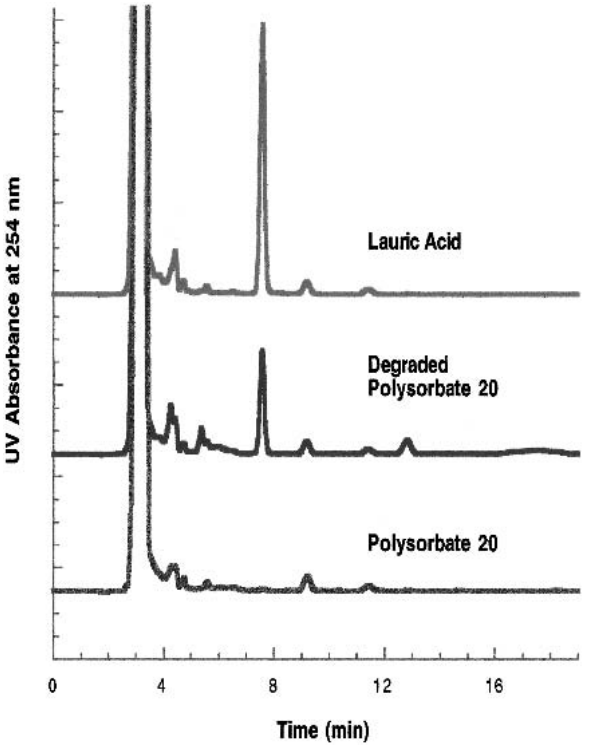


Fig. 3. Representative chromatograms of lauric acid, degraded polysorbate 20, and polysorbate 20. The solutions contained 2.5 mM $CaCl_2$, 75 mM NaCl, and one of the following: lauric acid (equivalent to 0.005% [w/v] degraded polysorbate 20), 0.005% (w/v) polysorbate 20, or 0.005% (w/v) polysorbate 20 with lipase (1:110 [w/w] ratio of enzyme to polysorbate 20). These solutions were reacted with ADAM for ≈5 h at room temperature and were analyzed by reversed-phase HPLC analysis, as described in the Materials and Methods section.

Colorometric Analysis of Polysorbate 20 Samples

The colorimetric method is commonly utilized in the pharmaceutical industry to determine polysorbate content (11). When this method was used to analyze degraded and nondegraded samples of polysorbate 20, no differences were detected (Fig. 5). This method relies on complexation of the polyoxyethylene groups of polysorbate 20 with cobaltothiocayanate reagent to form a colored complex that is detected by UV spectroscopy (10). In essence, the colorimetric method is determining polyoxyethylene content and not the total intact polysorbate 20 content. The lauric acid component of polysorbate 20 analyzed by the colorimetric method has minimal absorption due to the lack of complexation with cobaltothiocayanate reagent (data not shown). This method, if used during excipient screening for formulations, will not detect polysorbate 20 degradation. A different analysis method may thus be necessary. The colorimetric method may also be utilized to determine the compositions of polysorbate 40, 60, and 80, because, like polysorbate 20, they are sorbitan polyoxyethylene esters (9,12). The distinction between these molecules is the length of the fatty acid residue. Therefore, the colorimetric method may be utilized to measure polysorbate content but would not detect degradation.

Fluorescence Analysis of Polysorbate 20 Samples

Fluorescence analysis of polysorbate 20 in the presence of NPN is based on the content of micelles. NPN is a fluores-

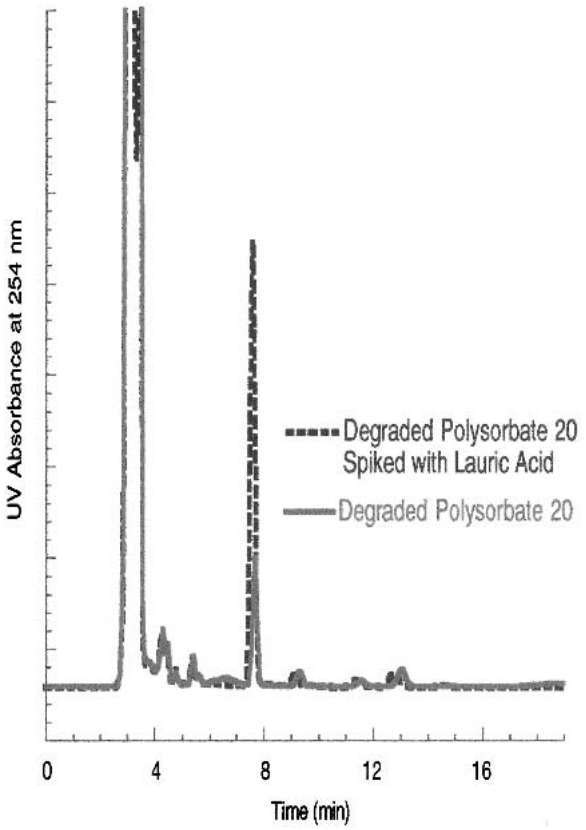


Fig. 4. Representative chromatograms of degraded polysorbate 20 alone and spiked with lauric acid. The degraded polysorbate 20 solution contained 2.5 mM $CaCl_2$, 75 mM NaCl, 0.005% (w/v) polysorbate 20, and lipase (1:110 [w/w] ratio of enzyme to polysorbate 20). This solution was spiked with lauric acid. The degraded solutions of polysorbate 20 alone and that spiked with lauric acid were reacted with ADAM for ≈5 h at room temperature. HPLC analysis was performed, as described in the Materials and Methods section.

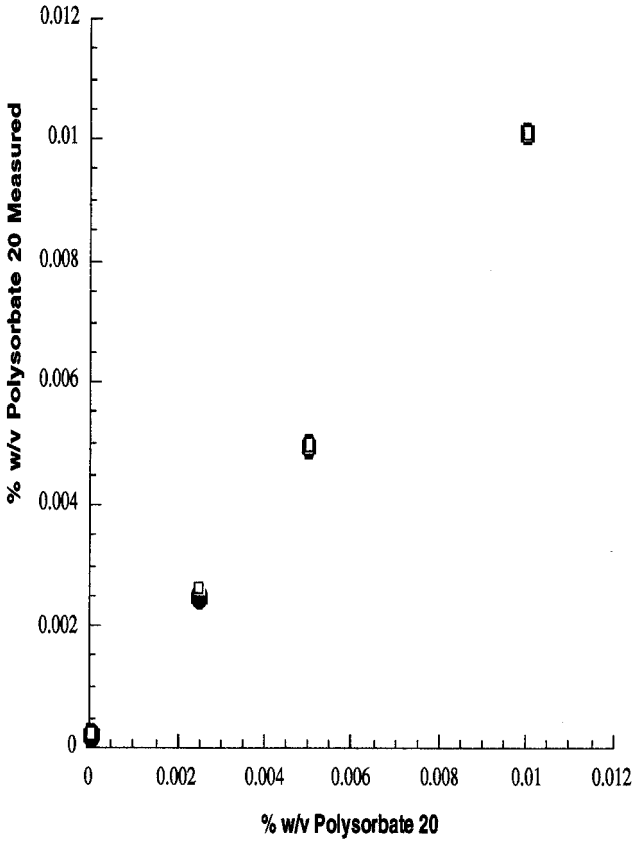


Fig. 5. Determination of polysorbate 20 content using the colorometric method for degraded (□) and nondegraded (●) solutions. The degraded polysorbate 20 solutions contained 2.5 mM $CaCl_2$, 75 mM NaCl, the indicated percentage (w/v) of polysorbate 20, and lipase (1:110 [w/w] ratio of enzyme to polysorbate 20). Polysorbate 20 solutions contained 2.5 mM $CaCl_2$, 75 mM NaCl, and the indicated percentage (w/v) of polysorbate 20. The amount of polysorbate 20 in the solutions was determined by measuring the UV absorbance at 620 nm after complexation with cobaltothiocyanate, as described in the Materials and Methods section.

cent hydrophobic dye that partitions into the nonpolar core of the polysorbate 20 micelle to result in fluorescence. NPN has a low fluorescent quantum yield in an aqueous environment. However, the fluorescent quantum yield of NPN is high in a hydrophobic environment. Degraded and nondegraded polysorbate 20 samples were analyzed by fluorescence spectroscopy in the presence of NPN (Fig. 6). There is a decrease in micelle content for the degraded polysorbate 20 samples, whereas there is no decrease observed in the nondegraded polysorbate 20 samples. It is apparent that the decrease is due to the lack of micelle formation in the degraded samples. Polysorbate 20 micelles are formed by interactions of the fatty acid tail groups to form the hydrophobic core of the micelle. The exterior of the micelle or solvent-exposed portion of the micelle is composed of the polar head group of polysorbate 20 or the sorbitan polyoxyethylene region. The cleavage of polysorbate 20 results in the separation of the polar head group from the nonpolar fatty acid chain. These degradants are not as efficient as intact polysorbate 20 in the formation of micelles. The micelle content of the degraded polysorbate 20 samples had decreased by approximately 50% (Fig. 6).

In formulations, polysorbates are commonly used above their CMC values (3). The loss of polysorbate 20 micelles may cause instability in a formulation (3). Therefore, it may be important to initially monitor micelles in a formulation using this method. The loss of micelles in a given formulation could be due either to degradation or to loss of polysorbate 20 (e.g., binding to filter).

NMR Analysis of Polysorbate 20 Samples

NMR is commonly used to determine the structure of a molecule based on resonance frequencies of nuclei within a molecule, coupling between neighboring nuclei, and signal intensities. It may also be utilized for quantification based on the peak area. Degraded and nondegraded polysorbate 20 samples were analyzed by NMR spectroscopy to quantify polysorbate 20 and to determine whether differences in the spectra could be observed. Figure 7 displays NMR spectra of degraded polysorbate 20, nondegraded polysorbate 20, and lauric acid. The NMR signals arising from the sorbitan portion of the polysorbate 20 appear between 3.5 and 3.7 ppm in the NMR spectrum. The fatty acid (lauric acid) portion has NMR signals at four positions (0.8, 1.3, 1.6, and 2.3 ppm). The differences between the NMR signals from the fatty acid tail portion of polysorbate 20 and from the free lauric acid are subtle. The signals from free lauric acid are sharper and

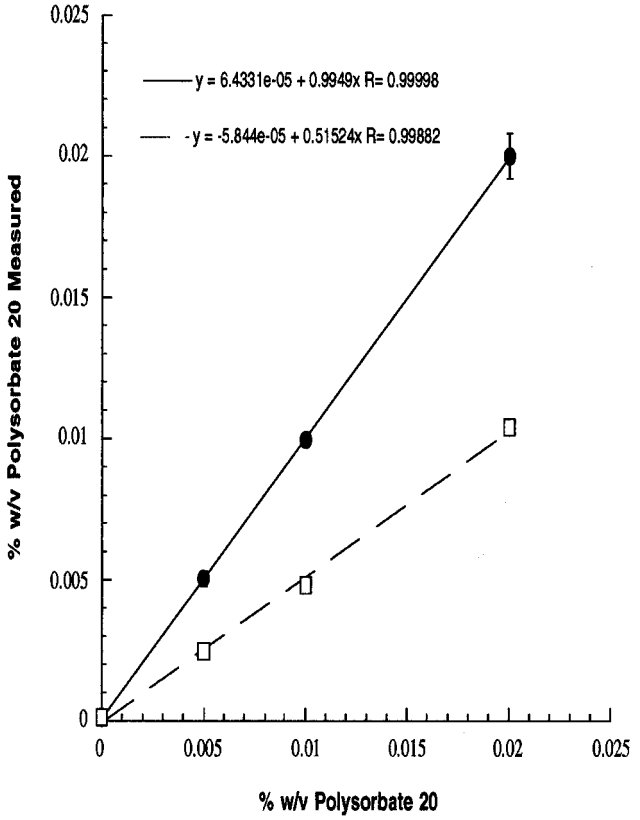


Fig. 6. Determination of polysorbate 20 content using the fluorescence micelle method for degraded (□) and nondegraded (●) samples. The degraded polysorbate 20 solutions were composed of 2.5 mM $CaCl_2$, 75 mM NaCl, the indicated percentage (w/v) of polysorbate 20, and lipase (1:110 [w/w] ratio of enzyme to polysorbate 20). Polysorbate 20 solutions contained 2.5 mM $CaCl_2$, 75 mM NaCl, and the indicated percentage (w/v) of polysorbate 20. The amount of polysorbate 20 in the solutions was determined by measuring the fluorescence emission at 410 nm after incubation with N-phenyl-1-naphthylamine, as described in the Materials and Methods section.

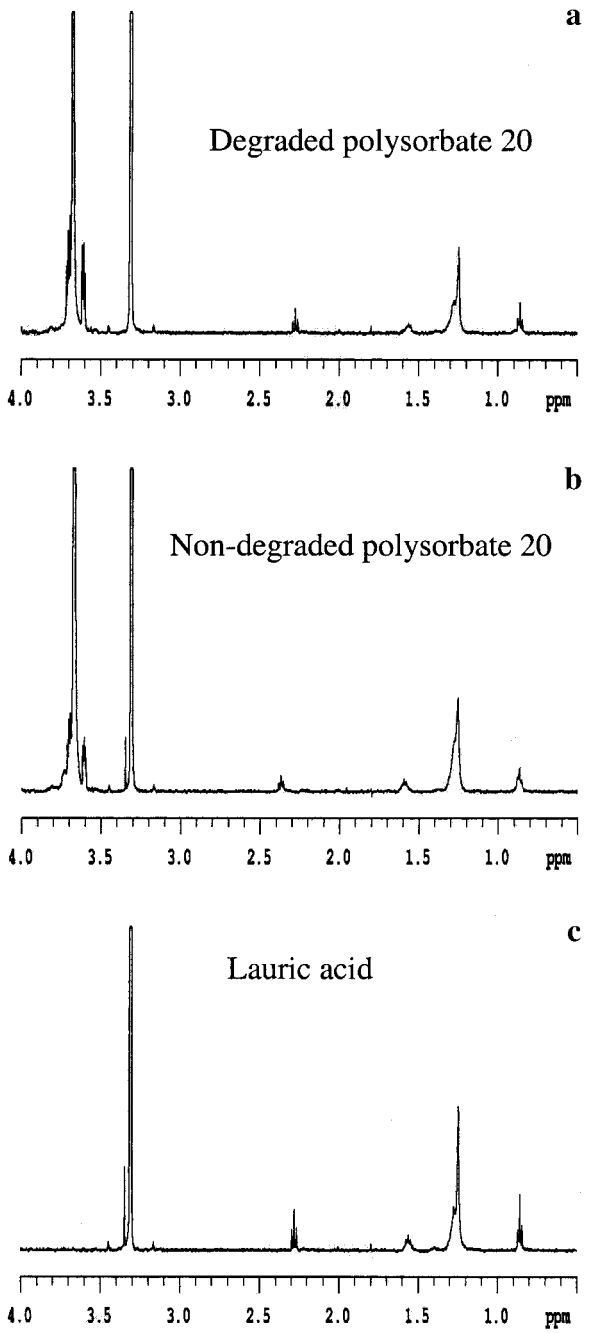


Fig. 7. NMR spectra of 0.2% (w/v) degraded (a) and 0.2% (w/v) nondegraded (b) polysorbate 20 and 0.2% (w/v) lauric acid (c) in 2.5 mM $CaCl_2$, 75 mM NaCl, 50% H_2O, and 50% deuterated methanol. The signals between 3.0 and 3.5 ppm are unrelated to polysorbate 20. They arise from the residual methanol and impurities in the solution.

slightly better resolved. The sharper signals of free lauric acid reflect the change in the dynamics of lauric acid from being attached to a large micelle vs. being present as free molecules in solution. The signals from the sorbitan polyoxyethylene protons are also slightly sharper. This can also be attributed to the change in the dynamics of sorbitan polyoxyethylene. In the NMR spectrum of polysorbate 20 (Fig. 7), the signals from the sorbitan polyoxyethylene protons are more intense than those from the fatty acid (lauric acid) protons because there are more protons on the sorbitan polyoxyethylene side chain. Because the sensitivity of the sorbitan polyoxyethylene signal is much higher, the peak area of this signal is typically used to quantify polysorbate 20. As a result, the measurement of polysorbate 20 based on the NMR signal of sorbitan polyoxyethylene side chain reveals no significant difference in polysorbate 20 content between degraded and nondegraded samples (data not shown). This NMR method only provides the total sorbitan polyoxyethylene side-chain concentration. Because the spectral differences between the degraded and nondegraded polysorbate 20 are subtle, extra care must be taken in the NMR analysis of polysorbate 20, or it is easy to overlook the degradation.

CONCLUSIONS

Polysorbate 20 compatibility with the active compound is not obvious and cannot be taken for granted. The stability of the active compound may be intertwined with that of the excipient. Therefore, it is very important when initially formulating an active compound with polysorbate 20, to monitor the stability of both components to maintain a well-characterized formulation. In this manuscript, several established methods used for polysorbate 20 detection (i.e., color-

imetric, fluorescence, and NMR spectroscopy) were discussed and utilized to analyze polysorbate 20 samples to determine whether they could distinguish between degraded and intact polysorbate 20. The inability and/or difficulty of these methods to detect polysorbate 20 degradation resulted in the development of a new method by which the degradation of polysorbate 20 could be uncovered by the analysis of derivatized lauric acid.

The colorimetric method was not able to distinguish between degraded and intact polysorbate 20. Therefore, when this method is utilized, it is crucial to ensure that polysorbate 20 is intact. The fluorescence micelle method allows for the detection of a decrease in micelle content for the degraded polysorbate 20 samples but not the intact polysorbate 20 samples. Although this method did measure a decrease in micelles, further investigation would be necessary to determine whether the decrease in micelles is due to polysorbate 20 degradation or an actual decrease in polysorbate 20 (e.g., due to binding to the filter or other components that the solution may come into contact with). As demonstrated in this manuscript, the detection of the degradant lauric acid is a novel and useful method in the determination of possible polysorbate 20 degradation in a formulation. It is important to utilize such a method to ensure the stability of the ester bond of polysorbate 20 when initially formulating an active compound. Utilizing NMR as a tool for polysorbate 20 detection may also detect degradation if the spectral differences between the degraded and nondegraded polysorbate 20 samples are carefully examined. In quantifying polysorbate 20 by NMR, the signal used for quantification is normally from the sorbitan polyoxyethylene group and is not the laureate component. Therefore degraded and nondegraded polysorbate 20 will have similar peak areas of protons, and polysorbate 20 content will be comparable, which may be misleading.

Thorough characterization of the stability of all the formulation components is essential for a successful product. The choice of methods to characterize polysorbate 20 provides information on different aspects. It is crucial to choose the appropriate methods to be able to understand the compatibility/stability of polysorbate 20 in a given formulation.

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