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Original Article

ANALYSIS OF POLYSORBATE 80 SOLUTION STABILITY UNDER STRESS CONDITIONS TO ENSURE ITS QUALITY AS A BIOPHARMACEUTICAL EXCIPIENT

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ABSTRACT

Objective: This study aimed to investigated the effect of light, temperature, pH, peroxides, trace metals, and buffer type on the chemical stability of polysorbate 80 (PS80) obtained from the three key manufactures.

Methods: We used a fast liquid chromatography-evaporative light scattering detector that allowed the monitoring of PS80 decay over time. For data analysis, we investigated the change in the peak area percentage of the compound over time.

Results: At pH 6.0 in histidine buffer, PS80-B was more sensitive than PS80-A and PS80-C. The PS80 from the three different sources degraded significantly with varying performance levels when exposed to light, temperature of 40 °C, peroxides, and trace metals over time.

Conclusion: Our results provide an improved understanding of the stability of PS80 obtained from the three different sources under different conditions, which provides a basis for the selection of the appropriate grade of PS80 according to the specific requirements.

Keywords: High-performance/pressure liquid chromatography, Surfactants, Excipients, Stability, Oxidation, Hydrolysis, Degradation product

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INTRODUCTION

Polysorbates (PSs), comprising fatty acid (FA) Esters of polyoxyethylene (POE) sorbitan, are commonly used amphiphilic, nonionic surfactants in biopharmaceutical formulations [1-3]. PSs protect proteins from interfacial stress, likely via the competitive accumulation of the surfactants molecules at interfaces [4-6]. To the best of our knowledge, the quality requirements of PS80 regulated by different pharmacopeias. The United States Pharmacopoeia [7] and Japanese Pharmacopoeia [8] require an oleic acid content of ≥58 %. However, the Chinese Pharmacopoeia has recommended an oleic acid content of ≥98 % since 2015 [9]. Multi-compendial grade-PS80 has been used in commercial products for decades, and a few manufacturers, including NOF Corporation (Tokyo, Japan) and Nanjing Well Chemical (Nanjing, China) have provided high-purity PS80 in recent years that can meet the requirements of the Chinese Pharmacopoeia [10]. However, the differences in the stability and functional properties of the different grades of PS80 prepared by different muanfactures remain unclear.

The factors inducing PS80 degradation are summarized in fig. 1 [11]. In aqueous formulations, light stress, residual hydrogen peroxide (H_2O_2), oxidants, thermal stress, and metal contamination can induce PS80 oxidation. Chemical factors and acidic and basic conditions induce PS80 hydrolysis, and residual hydrolases induce its enzyme-catalyzed hydrolysis. To better understand the degradation profiles of different grades of PS80 from different manufacturers, we applied the current knowledge to pharmaceutically relevant conditions and monitored the course of degradation of PS80 in protein-free solutions. We focused on the performance of different grades of PS80 under the degradation-inducing conditions listed in fig. 1, which give instruction for the selection of the grade of PS80 according to the specific requirements.

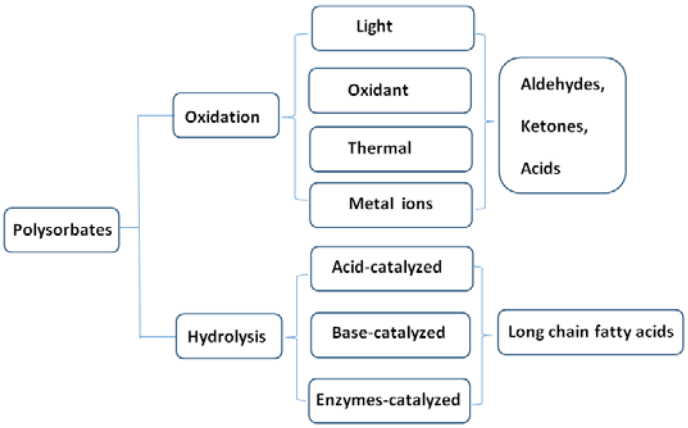

Fig. 1: Polysorbate degradation pathways and the factors causing degradation

MATERIALS AND METHODS

Materials

PS80 was obtained from J. T. Baker (Arnhem, Netherlands) (PS80-A), NOF corporation (Tokyo, Japan) (PS80-B), and Nanjing Well Chemical (Nanjing, China) (PS80-C). H_2O_2 (30 %) was purchased from Thermo Fisher Scientific (Munich, Germany). Sodium phosphate dibasic anhydrous was from Merck KgaA (Darmstadt,

Germany). Ferric chloride anhydrous was purchased from MP Biomedicals (California). Cupric chloride anhydrous was purchased from Sigma-Aldrich (Taufkirchen, Germany) (now Merck KgaA). Nickel (II) chloride hexahydrate was purchased from Acros Organics (Shanghai, China). L-Histidine hydrochloride monohydrate and L-histidine were purchased from J. T. Baker. Acetic Acid was purchased from Avantor (Radnor). Sodium acetate tri-hydrate was purchased from Merck KgaA. An illuminating incubator was purchased from Honeywell (New Jersey).

Liquid chromatography-grade acetic acid was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Acetonitrile was purchased from Merck KgaA. Purified water was obtained using a milli-Q Advantage A10 system (Merck, Millipore).

Solution of PS

PS solutions were prepared at a target concentration of 0.1 % (w/v) in purified water, 0.1 % H_2O_2 (v/v), 10 µM Fe^{3+} aqueous solution, 1 µM Fe^{3+}aqueous solution, 10 µM Ni^{2+}aqueous solution, 10 µM Cu^{2+}aqueous solution, 20 mmol acetate buffer of pH 4.5, 20 mmol histidine buffer of pH 6.0, and 20 mmol phosphate buffer of pH 9.0.

PS80 forced degradation study

Eleven PS80 sample solutions (table 1) were prepared and stored at 25 °C, except for the thermal stress condition (40 °C). Sample 1 (table 1) was the control for other samples under stress conditions. Neat PS80 was dissolved in purified water at 0.1 % (w/v) to prepare samples 1–3. Sample 2 was under light stress condition (5000 lux) and sample 3 was under thermal stress condition (40 °C). For samples 4–8, PS80 was first dissolved in purified water and spiked with different agents evaluated. For samples 9–11, PS80 was directly dissolved in 20 mmol acetate buffer of pH 4.5, 20 mmol histidine buffer of pH 6.0, 20 mmol phosphate buffer at pH 9.0, respectively. The final concentration of PS80 in all samples was 0.1 % (w/v). All solutions (5 ml) were added into 6 ml glass vials, stoppered, and stored at 25 °C. Direct injection liquid chromatography-evaporative light scattering detector (LC-ELSD) was used to analyze the degradation of PS80.

Table 1: Sample list for the PS80 forced degradation study

Sample[#]	Stress type	Condition
1	Control	25 °C
2	Light	5000 lux
3	Thermal	40 °C
4	Oxidative	0.1% H_2O_2
5	Metal-Fe	10 µM Fe^{3+}
6	Metal-Fe	1 µM Fe^{3+}
7	Metal-Ni	10 µM Ni^{2+}
8	Metal-Cu	10 µM Cu^{2+}
9	Buffer-acetate	20 M, pH 4.5
10	Buffer-histidine	20 M, pH 6.0
11	Buffer-phosphate	20 M, pH 9.0

A) All samples contained 0.1% (wt/vol) PS80, B) PS80 manufacturers: J. T. Baker, NOF, Nanjing Well.

LC-ELSD analysis of PS80

The separation of PS80 species was performed using a ZORBAX SB-CN (4.6 mm × 150 mm, 3.5 µm particle size), which was purchased from Agilent Technologies Co., Ltd. (California). The column temperature was maintained at 40 °C. The flow rate was 1.2 ml/min. Two solvents with different elution strengths were used to elute the samples from the column; solvent A contained 0.1 % acetate acid in Milli Q water and solvent B contained 0.1 % acetate acid in acetonitrile. The PS80 species and its degradants were eluted with a starting gradient of 15 % solvent A and 85 % solvent B, which was changed to 60 % solvent A and 40 % solvent B within 4 min. At 49 min, the gradient was changed to 10 % solvent A and 90 % solvent B. At 50 min, the column was equilibrated back to 85 % solvent A and 15 % solvent B for 20 min. The total run time was 70 min.

Data analysis

Major PS80 peaks were identified based on previous reports and comparison with similar experiments in the literature [12-16]. LC-ELSD chromatogram peaks were integrated using Agilent CDS 2.0 software. We integrated all peaks that could be tested by this method at each time point. We investigated the change in the peak area percentage of the compound over time.

RESULTS

LC-ELSD-based monitoring of PS80 hydrolysis or oxidative degradation

According to the previously established method [12-16], the LC-ELSD method was optimized to separate hundreds to thousands of PS species over 70 min. Chromatograms of intact PS80 showed six peak clusters, representing six main classes of PS subspecies as follows: (1) nonesterified free polyethylene glycol, nonesterified free sorbitan-polyethylene glycol, and nonesterified free isosorbide-polyethylene glycol, together known as the POE mix; (2) POE sorbitan monoesters; (3) POE isosorbide monoesters; (4) POE sorbitan diesters; (5) POE isosorbide diesters; and (6) POE sorbitan triesters (fig. 2). Some smaller peaks were not identified with certainty and were, therefore, omitted from the analysis. All six main peaks corresponded to the sorbitan head group, except the nonesterified free polyethylene glycol peak which had no head group attached. LC-ELSD chromatograms (fig. 2A_C) of intact PS80 corresponded to PS80-A, PS80-B, and PS80-C, respectively. The chromatograms and their peak-area percentages of PS80-A, PS80-B, and PS80-C did not differ remarkably among the six main classes of PS subspecies. However, slight differences among PS80-A, PS80-B, and PS80-C were observed at the POE mix peak, which was mainly attributed to the synthetic procedures applied for PS manufacturing, including heterogeneity of the starting materials and harsh process conditions such as high temperature and extreme pH. The actual variety of chemical structures of the different PS species was much more complex. The peak of POE isosorbide diesters and POE sorbitan triesters made up a relatively small percentage of the total peak area among that of the six subspecies, which was less than 10 %; therefore, the changes associated with them were ignored in the subsequent stress study. As a results of which, we mainly focused on the performance changes in the POE mix, POE sorbitan monoesters, POE isosorbide monoesters, and POE sorbitan diesters in subsequent experiments.

Temperature-induced degradation of PS80 from different manufacturers

Time-course experiments were conducted over 12 w on PS80. The PS80 solutions were stored at 25 °C and 40 °C. We used reversed-phase ELSD-high-performance liquid chromatography to study the peak-area change for major esters based on the thermal conditions (fig. 3) at different time points.

Irrespective of the type of manufacturer who prepared PS80, the four main species of PS80 showed no change over 3 mo (fig. 3A1_D1), PS80 was stable in an aqueous form at 25 °C. However, there was a notable degradation of PS80 at 40 °C, and PS80 from all the three sources exhibited the same degradation profile under

thermal stress. The monoesters (sorbitan and isosorbide) and polyesters began degrading after 4 w and were completely degraded at 12 w. Furthermore, the POE Mix peak-area percentages of PS80-A, PS80-B, and PS80-C increased from 14 % to 48 %, 13 % to 47 % and 12 % to 39 %, respectively. These findings indicated that the stability of PS80-A, PS80-B, and PS80-C against thermal stress was similar; the higher the temperature, the more pronounced the degradation.

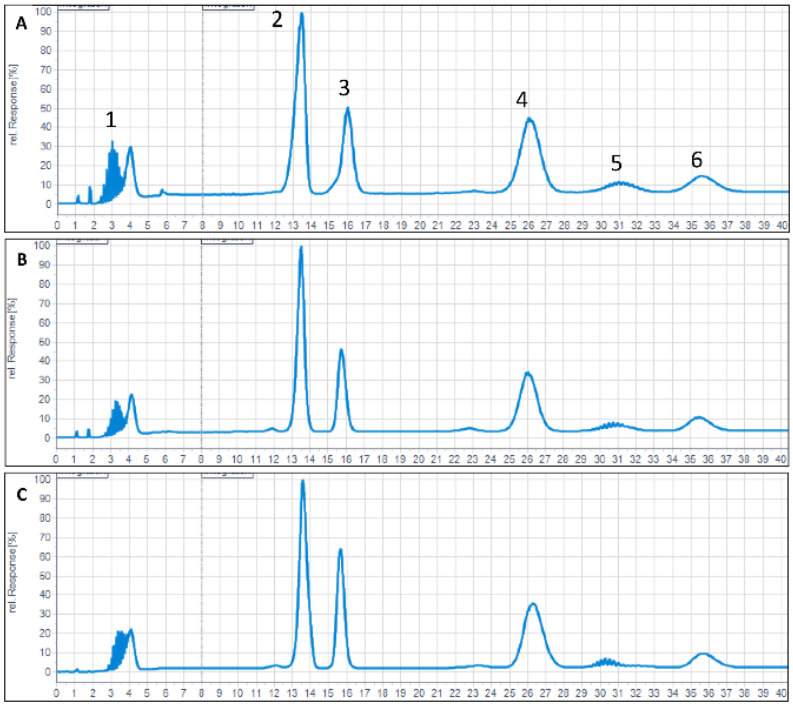

Fig. 2: Representative LC-ELSD chromatograms of (A) PS80-A, (B) PS80-B, and (C) PS80-C. Peak 1: POE mix, Peak 2: sorbitan monoesters, Peak 3: isosorbide monoesters, Peak 4: sorbitan diesters, Peak 5: isosorbide diesters, and Peak 6: sorbitan trimesters

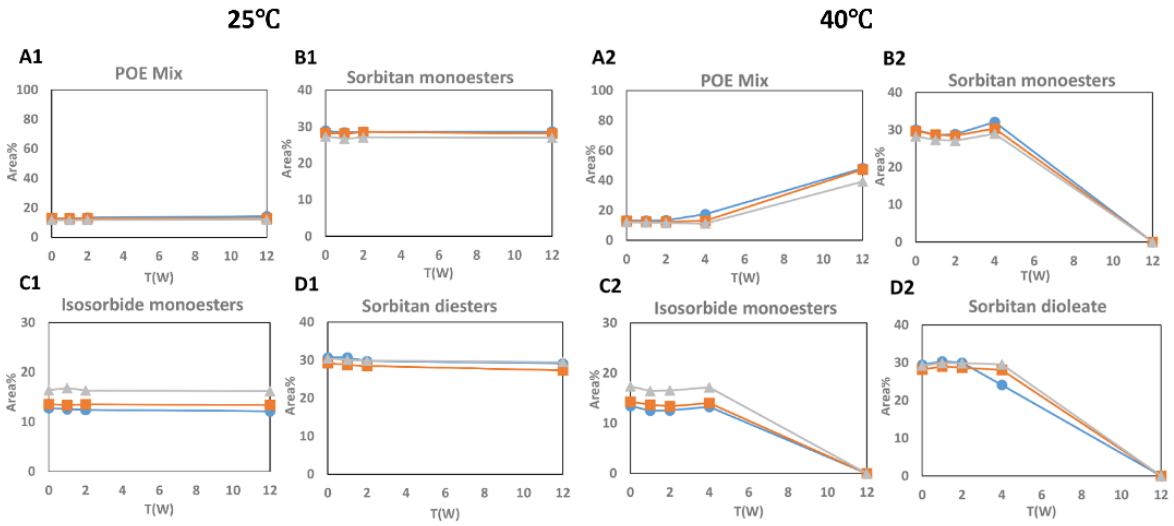

Fig. 3: Effect of temperature on the degradation of the four main classes of PS subspecies: (A) POE Mix, (B) sorbitan monooleate, (C) isosorbide monooleate, and (D) sorbitan dioleate for PS80-A (●), PS80B (■), and PS80C (▲)

Stability of PS80-A, PS80-B, and PS80-C in different buffers

Since acetate, histidine, and phosphate buffers are commonly used in formulation development. We selected these three buffers to study the effect of pH and buffer type on the stability of PS80 at 25 °C. The main components of PS80 from the three sources showed no degradation over 12 w in 20 mmol acetate and at pH 4.5, except for the sorbitan diesters (fig. 4). The sorbitan diesters showed no degradation from T0 to the 4th week, as it began to degrade from the 4th week and remained at 80 % in the 12th week.

Compared with control samples, PS80-A, PS80-B and PS80-C degraded in histidine buffer at pH 6.0 (fig. 4), and degradation profiles of the three PS80 (area percentages) were remarkably different from those of the four main classes of PS subspecies. PS80-A was stable for approximately 10 w, after which it began to degrade slowly from the 10th week. The area percentages of sorbitan monoesters, isosorbide monoesters and sorbitan diesters were 31 %, 16 % and 26 % at the T0 test point and 27 %, 14 % and 25 % during the 12th week, respectively. Compared with PS80-A and PS80-

C, PS80-B began to degrade innediately in the first week, with complete degradation in the 12th week. There was no remarkable degradation of PS80-C until the 8th week, after which the degradation rate increased; the sorbitan monoesters, isosorbide monoesters and sorbitan diesters degraded completely in the 12th week. Overall, the concentration of the POE mix species increased with the degradation of sorbitan monoesters, isosorbide monoesters and sorbitan diesters, and the rate of this increase corresponded with the degradation rate of the other three species. Generally, ester species of different head groups degrade into POE mix [11]. However, despite the main components of PS80 degrading completely, the peak-area percentage of the POE mix was only about 40 %, as many unknown degradation products were also produced (data not shown). Under this stress condition, the stability order was PS80-A>PS80-C>PS80-B.

After exposure to the phosphate buffer at pH 9.0, the degradation profiles of PS80-A and PS80-B were similar but different from that of PS80-C. PS80-C showed a relatively fast degradation in the monosubstituted (sorbitan and isosorbide) species, whereas PS80-A and PS80-B demonstrated only slight changes. POE sorbitan diester species remained almost intact for PS80-A, PS80-B, and PS80-C. Under this stress condition, the stability order was PS80-A=PS80-B>PS80-C.

Overall, PS80 was more stable in 20 mmol acetate at pH 4.5 than in 20 mmol phosphate at pH 9.0, and most unstable in 20 mmol histidine at pH 6.0.

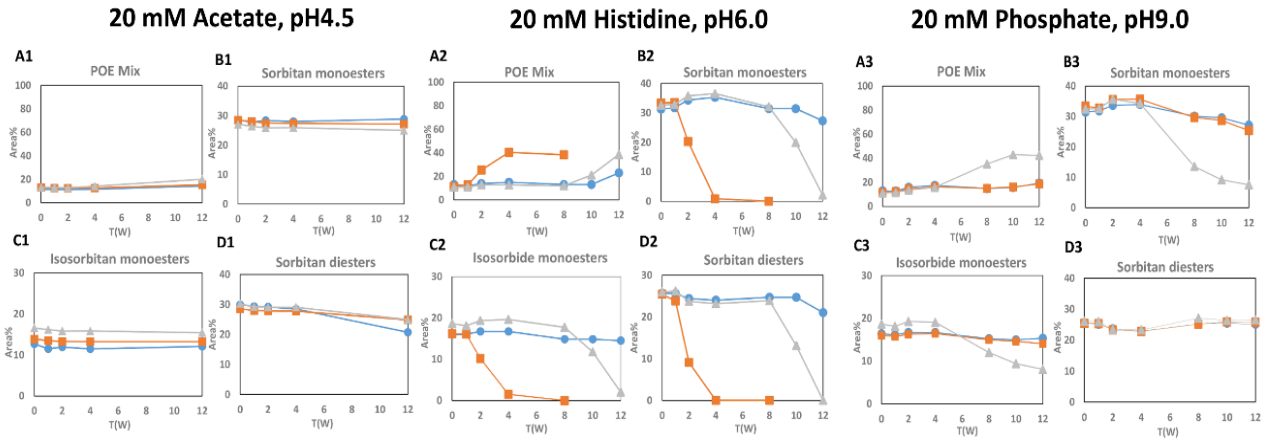

Fig. 4: Effect of pH on the degradation of the four main classes of PS subspecies: (A) POE Mix, (B) sorbitan monooleate, (C) isosorbide monooleate, and (D) sorbitan dioleate for PS80-A (●), PS80B (■), and PS80C (▲)

Forced oxidative degradation of PS80 from different manufacturers

Light, oxidants, and metal ions can induce the degradation of PS80 [11]. We conducted forced oxidative degradation by exposure to light and H_2O_2 or metal ion spiking.

Time-course experiments were conducted over 8 w on PS80-A, PS80-B, and PS80-C exposed to light (fig. 5). Overall, the differences in the sensitivity to degradation of PS80 from all the three sources by light were noticed. PS80-A was most sensitive to light and began to degrade in the first week. PS80-B and PS80-C, which began to degrade during the second week, exhibited the same degradation profile. By the 8th week, all ester species of PS80 from three sources were degraded with the concentration of POE mix species slowly increasing over time. The majority of the ester species in PS80-A and PS80-C degraded into the POE mix, as their area percentage of the mix was nearly 96 % and 88 % in the eighth week, respectively. However, for PS80-B, part of the ester species degraded into POE mix, as the peak-area percentage of the POE mix was only about 40 % at the 8th week. Under this stress condition, the stability order was PS80-B=PS80-C>PS80-A.

Next, H_2O_2-induced oxidation of PS80 was evaluated in aqueous solution. PS80-A, PS80-B, and PS80-C at 0.1 % (w/v) were exposed to 0.1 % (v/v) H_2O_2 at 25 °C (fig. 5). All monoesters and polyesters degraded in the eighth week except for the monoester of PS80-A. Simultaneously, the concentration of the POE mix species slowly increased over time, but their peak area percentage was only 27 % during the 8th week, as many unknown degradation products were produced (data not shown). The sorbitan monoesters of PS80-A degraded by half at the 8th week, whereas the isosorbide monoesters showed no degradation. Interestingly, in 0.1 % H_2O_2, the PS80 polyesters degraded first, and its monoesters began to degrade in the second week. Based on these experimental findings, the stability order against H_2O_2-induced oxidation was monoesters>polyesters and isosorbide monoesters>sorbitan monoesters.

Forced oxidative degradation by H_2O_2 spiking led to unexpected results with respect to the stability of the different grades of PS80. Since transition metal ions are involved in oxidation processes [17-20]. PS80-A, PS80-B and PS80-C were exposed to 10 µM Fe^{3+}, 1 µM Fe^{3+}, 10 µM Ni^{2+}, and 10 µM Cu^{2+}separately at 25 °C for several weeks. The addition of 10 µM $Fe^{3+}$and 1 µM Fe^{3+}resulted in rapid degradation of monoesters from all three sources, which exhibited similar degradation profiles (fig. 5). This suggested that the degradation rate of monoesters was not related to the Fe^{3+}concentration in the range of 1–10 µM. For polyesters, the degradation tendency of PS80-C was similar for two different concentrations of Fe^{3+}; however, the degradation rates of the polyesters in PS80-A and PS80-B were faster at the higher Fe^{3+}concentration. Particularly for PS80-B, the polyesters showed almost no notable degradation during the 8 w of exposure to 1 µM Fe^{3+}. The stability order against 10 µM Fe^{3+}-induced oxidation was PS80-A>PS80-B>PS80-C, while that against 1 µM Fe^{3+}-induced oxidation was PS80-B>PS80-A>PS80-C. Overall, PS80-C was most unstable against Fe^{3+}-induced oxidation.

Next, the impact of 10 µM Ni^{2+}-and 10 µM Cu^{2+}-induced oxidation on PS80-A, PS80-B, and PS80-C was studied. The degradation profiles was similar between Cu^{2+}-and Ni^{2+}-induced oxidation. The monoesters and polyesters degraded simultaneously (fig. 5). The concentration of POE mix species slowly increased over time. The stability order against both 10 µM Ni^{2+}-and 10 µM Cu^{2+}-induced oxidation was PS80-A>PS80-C>PS80-B. However, the degradation rate was different between Cu^{2+}-and Ni^{2+}-induced oxidation; under Cu^{2+}-induced oxidation (fig. 5), the degradation rate was relatively slow during the first week, but that of esters accelerated suddenly from the second week, with complete degradation at the 6th week. However, the area percentage of the POE mix was relatively low, and many other degradation products were generated (data not shown). Overall, PS80 showed notable degradation under 10 µM Ni^{2+}-and 10 µM Cu^{2+}-induced oxidation.

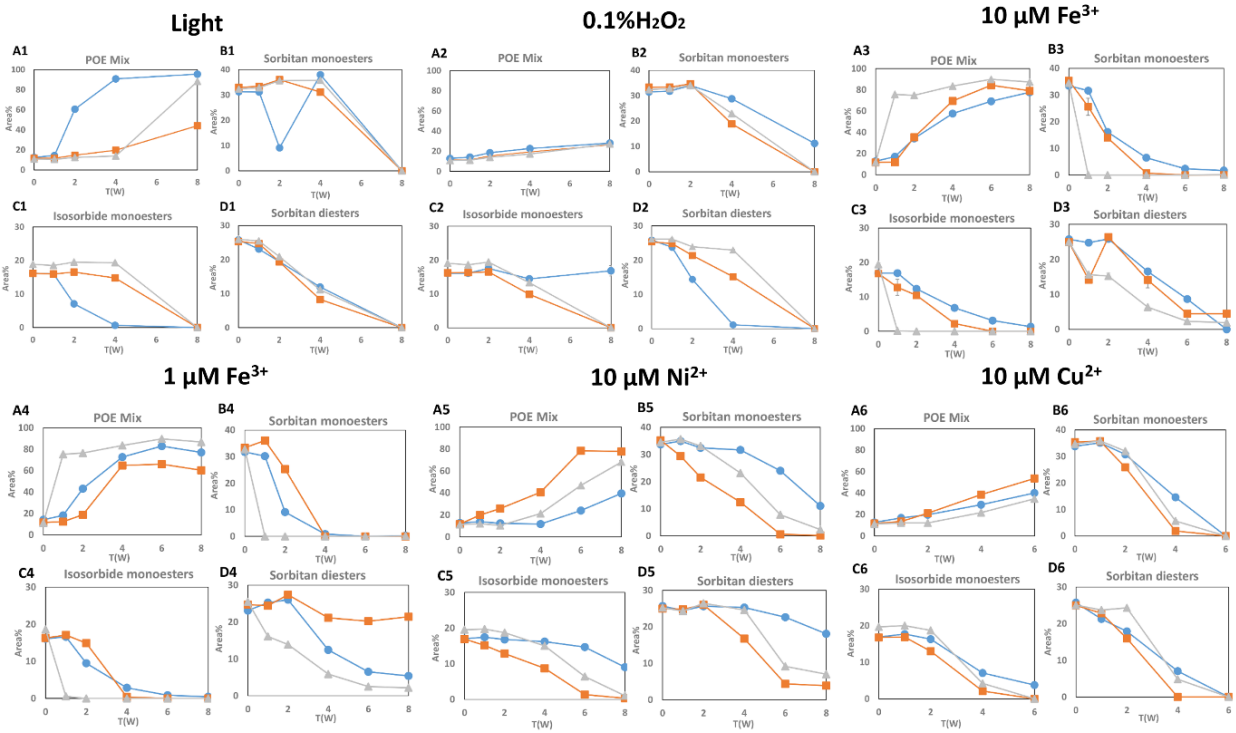

Fig. 5: Forced oxidative degradation of the four main classes of PS subspecies: (A) POE Mix (B) sorbitan monooleate, (C) isosorbide monooleate, and (D) sorbitan dioleate for the PS80-A (●),PS80-B (■), and PS80C (▲)

DISCUSSION

In this study, we analyzed the performance of PS80 from three different manufacturers under different stress conditions. Next, we will analyze and discuss the mechanism behind this performance.

At 25 °C, PS80 from all the three sources was highly stable over 3 mo and degraded remarkably at 40 °C. Kishore *et al.* also reported the stability of PS80 at 25 °C and 40 °C over a 12-month period. PS80 content decreases substantially but very slowly at 25 °C and more quickly at 40 °C [21]. The difference in results compared with our study can be attributed to the different monitoring indications. Kishore *et al.* focused on the PS80 content change and not the peak-area percentage change for the PS subspecies. Overall, PS80 was more unstable at 40 °C. Under thermal stress, the degradation pathway was through oxidation. Kishore *et al.* pointed out a buildup of peroxides of up to 150 µmol/l in liquid formulation samples containing PS stored at 40 °C for up to 5 w [21]. Some other studies also reported that peroxides are produced under oxidative conditions [17, 22].

In our study, in 20 mmol acetate buffer at pH 4.5, the degradation rates of PS80-A, PS80-B and PS80-C were very slow. In pH 4.5 conditions, the mechanism was an acid-catalyzed $A_{AC}2$ reaction, which is a thermodynamically driven reaction with the SN_2 attack of H_2O at the ester carbonyl being a bimolecular rate-determining step [14]. This mechanism has a substantial influence on the overall degradation only at high temperatures (40 °C) and elicits very slow rates at 25 °C. This also explains why the degradation of PS80 in 20 mmol acetate at pH 4.5 was not significant.

In 20 mmol histidine at pH 6.0, there was a notable degradation of PS80-A, PS80-B, and PS80-C, and the degradation pathway was anticipated to be mainly oxidation. First, the degradation product of oleic acid was almost undetectable during the 3 mo of analysis because if the degradation pathway was hydrolysis, the main degradation product was oleic acid. Second, we detected many unknown degradation products that were observed in other oxidative stress conditions. In a previous review, it has been pointed out that in the pH range of 5 to 7, the likelihood of chemical hydrolysis of polysorbates is negligible, especially at low storage temperatures such as 2–8 °C and 25 °C [11]. pH 4.5 and pH 6.0 constitute mildly acidic conditions; although the hydrolysis was negligible, the buffer type caused the actual difference. Wang *et al.* found P188 to be more unstable in 10 mmol histidine than in 10 mmol citrate at pH 6.0; they suggested the degradation pathway to be mainly oxidation [23]. The oxidative degradation was due to the presence of oxygen and reactive oxygen species originating from either the PS raw material or the harsh manufacturing process. The reactive oxygen species initiated radical formation [17], and subsequent radical recombination reaction with molecular oxygen typically led to the formation of peroxides. Stadtman *et al.* pointed out that histidine can be oxidized via Fenton chemistry with H_2O_2 and hydroxyl radicals [24]. Thus, both the free radicals and H_2O_2 generated during PS80 oxidation process could catalyze the oxidation of histidine. Furthermore, the oxidation of histidine has the potential to produce more free radicals and peroxides [25]. Aldehydes (pentanal, hexanal, and heptanal) have been reported as oxidation products of PS80 [21] and suggested to interact with histidine [26, 27]. Thus, it was possible that there was mutual stimulation of PS80 and histidine oxidation in the PS80/histidine sample.

The more the PS80-C was degraded, the more oleic acid was produced over time (fig. S1). In the 12th week, the peak-area percentages for oleic acid in PS80-A, PS80-B, and PS80-C were 0.28 %, 0.21 %, and 1.94 %, respectively. Overall, the results indicated that PS80-C was most sensitive to the basic condition. Generally, the ester species of different head groups, such as POE sorbitan monoester, POE isosorbide monoester, and POE sorbitan diester, degraded in the POE mix, which was a mixture of POE, POE sorbitan, POE isosorbide and other small molecular by-products, causing the increase in area percentage of the POE mix peak. During basic hydrolysis of PS80, PS monoesters hydrolyzed faster than polyesters. This indicated that the hydrolysis rate is mainly dependent on the hydrophobicity of the PS carboxyl ester species; that is, the more the hydrophilicity, the faster the hydrolysis rate [10]. Moreover, the hydrolysis rates correlate with the lengths of the aliphatic chains of the esters [13].

For exposure to metal ions and H_2O_2 under oxidative stress conditions, factors possibly driving the PS80 degradation were closely controlled,

that is, light stress was avoided. PS80-A, PS80-B and PS80-C degraded remarkably under these stress conditions. The mechanism of oxidation followed the classical radical initiation, propagation, and termination reactions [17, 21, 28, 29]. Under H_2O_2 exposure, the PS80 degradation profile was different from that of the other PS80s. The peak-area percentage change for the POE mix was not remarkable as the three main subspecies degraded. It is possible that esters were completely degraded in less than 8 w and most of them degraded in the POE mix, and then the POE mix was further degraded into other compounds by peroxides and hydroperoxides. Under H_2O_2 exposure, the PS80 polyesters exhibited the highest susceptibility to oxidative degradation, previous corroborating findings [18, 30, 31].

Under these 11 stress conditions, the performance of PS80-A, PS80-B, and PS80-C was different except at 25 °C and 40 °C, in 20 mmol acetate at pH 4.5. Under oxidative stress, the total oleic acid-containing component might have led to the differences in the degradation profiles of PS80. PS80-B and PS80-C applied the superior grade of PS80, in which the oleic acid component comprised 99 % pure oleic acid; however, the PS80-A contained about 70 % oleic acid. A previous study reported that the initiation of radical formation occurs at not only the ethylene oxide subunit but also the site of unsaturation (especially linoleate and linolenate moieties) [21]. Yao *et al.* also pointed out that two-thirds of PS80 oxidation occurs owing to the unsaturated FA ester groups [32]. Therefore, the stability order reflects the levels of unsaturated FA moieties; the more unstable the PS80, the higher the levels of unsaturated FA moieties. The observation that PS80-A was more stable under 0.1 % H_2O_2, 10 µM Fe^{3+}, 10 µM Ni^{2+}, and 10 µM Cu^{2+}than PS80-B and PS80-C was in line with our expectations. However, under other oxidative stress conditions, such as exposure to light and 40 °C temperature, the stability order was contradictory to our expectations. Similar results were also reported in another study [10]. Probably, some other factors, such as sources of free radicals, for example, headspace oxygen and leachables from container materials, or different initial content of impurity or contaminant that can catalyze PS80 oxidation, were responsible for the difference. It was shown previously that peroxide content varies greatly (500–8000 nmol/g) among different vendors, grades and lots [33]. Thus, it is important to monitor the H_2O_2 content in PS80 and other common pharmaceutical excipients under different storage conditions. The super-refined PS80 is subjected to an additional chromatographic purification step that facilitates the removal of polar impurities such as formaldehyde and peroxides [34]. Low levels of peroxide impurities may facilitate oxidative degradation of PS80, which might be particularly significant with regard to its photostability. Another study reported that PS80 in an aqueous solution exhibits a faster rate of peroxide formation and a greater amount of peroxides during incubation, which is further promoted/catalyzed by light exposure [22]. Another report suggests that exposure of PS80 aqueous solution to light results in the autoxidation of the alkyl polyoxyethylene chain, leading to the formation of hydroperoxide derivatives [17]. Overall, many potential factors could be responsible for the differences in the degradation profiles of PS80-A, PS80-B and PS80-C. The degradation profile of PS80 from the same manufacturer was remarkably different under the influence of different metal ions, as different metal ions could be specific to the degradation of PS80. The elucidation of the exact mechanism of degradation under exposure to different metal ions at the molecular level was beyond the scope of our study.

Based on the findings of different studies, as discussed above, we are now familiar with the factors that cause the degradation of PS80 and from which PS80 should be protected. For example, PS80 should be stored at sub-ambient temperature (2–8 °C), under nitrogen overlay, and away from light and heat. In addition, it is recommended to select super-refined PS80 as a surfactant in the formulation, avoiding any impurity or contaminant that could catalyze PS80 oxidation. Considering the sensitivity of unsaturated FAs to oxidation, not only should storage container materials be chosen such that they are free of metal ions and other impurities that could lead to the oxidation of PS80 but also the storage conditions of the liquid formulation should be controlled strictly. A PS excipient preferably should not be used again after the initial opening of the containers unless they are protected again with inert gasses.

Irrespective of hydrolysis or oxidation of PS80, the possible concerns arising from PS degradation are two-fold: (1) decreased ability of the surfactant to protect the formulation against interfacial stresses and (2) impact of the degradation products on the stability of the protein [29]. The major criterion for a stable biopharmaceutical drug product is the integrity of the formulation, which can be significantly compromised by particle formation. Martos *et al.* pointed out that particle formation can also occur owing to PS degradation [35]. Degradation product-free FA is an insoluble degradant that not only affects the appearance of the product but also triggers particle formation [11, 29]. In addition, Siska *et al.* demonstrated that FAs in monoclonal antibody formulations could be derived from PSs as raw material impurities that could further trigger faster particle formation over time and promote the mechanistic accumulation of FAs [11, 36]. The acid value reflects the residual FA derived from PSs as a raw material impurity. According to the Certificate of Analysis, the acid values of PS80-A, PS80-B, and PS80-C are 1.0, 0.1, and 0.6, respectively. However, in 20 mmol phosphate at pH 9.0, PS80-C was easier to hydrolyze. Therefore, choosing PS80-C will carry the risk of hydrolysis in a buffer with high pH. Therefore, PS80-B is probably a better choice at high pH.

The oxidation of PS80 results in the generation of hydroperoxide and small molecular compounds, such as aldehydes, ketones, and acids, which may facilitate the oxidation of the active pharmaceutical ingredient and further affect the quality of the product. In our study, since we showed that temperature, light, peroxide, and metal ions induced the oxidation of PS80, it is necessary to protect the product from degradation from these factors. Therefore, throughout the production-to-delivery processes, such as chromatographic purification, bulk storage, fill/finish operations, visual inspections, packaging, and long-term storage, the product needs to be safe, following which the grade of PS80 can be selected according to the specific requirements.

This study only investigated the stability of three sources of PS80 in aqueous solutions under different stress conditions and did not study the stability of PS80 in a protein formulation under different stress conditions. The performance of PS80 in aqueous solutions may be inconsistent with protein formulation solutions. However, the stability of PS80 in protein formulations was more instructive. Therefore, this study was conducted to inspire further investigation into PS80. In the next study, we will further investigate the stability of PS80 obtained from different sources in antibody formulation products.

CONCLUSION

The findings in this study emphasize the importance of PS80 quality to ensure stable and robust formulation development. Temperature, buffer type, peroxide, light, and metal ions affect the stability of PS80. The performance of PS80 from different manufacturers varies according to the conditions. The degradation of PS80 will directly or indirectly affect the quality of the protein. Hence, it is recommended to select PS80 very carefully to ensure robust product quality. In addition to the external environment, the impurities deriving from PS80 affect the stability of PS80 and the protein. Thus, the grade and vendor of PS80 must be carefully screened to ensure robust, stable, and efficacious formulation delivery to patients.

ABBREVIATIONS

ELSD: Evaporative light scattering detector, FA: Fatty acid, H_2O_2: Hydrogen peroxide, LC: Liquid chromatography, POE: Polyoxyethylene, PS: Polysorbate, PS20: Polysorbate 80, PS80: Polysorbate 80, P188: Poloxamer 188

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

REFERENCES

1. Pawestri SA, Saifullah Sulaiman TN. The influence of variation of hydroxypropyl methylcellulose and tween 80 concentrations on physical characteristics and physical stabilities gel of water dry extract of temulawak. Int J Curr Pharm Sci 2019;11:44-8. doi: 10.22159/ijcpr.2019v11i6.36340.
2. Yati K, Srifiana Y, Putra F. Effect of optimization of tween 80 and propylene glycol as a surfactant and cosurfactant on the physical properties of aspirin microemulsion. Int J App Pharm. 2017;9:127-9. doi: 10.22159/ijap.2017.v9s1.71_78.
3. Arianto A, Amelia R, Bangun H. The effect of tween 80, palm kernel oil, and its conversion product on *in vitro* penetration enhancement of indomethacin through rabbit skin. Asian J Pharm Clin Res. 2017;10(7):284-8. doi: 10.22159/ajpcr.2017.v10i7.18608.
4. Chen B, Bautista R, Yu K, Zapata GA, Mulkerrin MG, Chamow SM. Influence of histidine on the stability and physical properties of a fully human antibody in aqueous and solid forms. Pharm Res. 2003;20(12):1952-60. doi: 10.1023/b:pham.0000008042.15988.c0, PMID 14725359.
5. Miskoski S, Garcia NA. Influence of the peptide bond on the singlet molecular oxygen-mediated (O2[1 delta g]) photooxidation of histidine and methionine dipeptides. A kinetic study. Photochem Photobiol. 1993;57(3):447-52. doi: 10.1111/j.1751-1097.1993.tb02317.x, PMID 8475178.
6. Ng DSH, Che Rose L, Suhaimi H, Mohamad H, Rozaini MZH, Taib M. Preliminary evaluation on the antibacterial activities of citrus Hystrix oil emulsions stabilized by tween 80 and span 80. Int J Pharm Sci. 2011;3:209-11.
7. USA Pharmacopoeia Convention. United States Pharmacopeia and national formulary. Rockville, MD: United States pharmacopeial convention; 2018.
8. Pharmaceutical and Medical Devices Agency. Japanese pharmacopoeia. 17th ed. Tokyo: Pharmacopoeia and Medical Devices Agency; 2017.
9. Chinese Pharmacopoeia Commission. Chinese pharmacopoeia. 10th ed. Beijing, China: China Medical Science Press; 2015.
10. Kranz W, Wuchner K, Corradini E, Berger M, Hawe A. Factors influencing polysorbate's Sensitivity against enzymatic hydrolysis and oxidative degradation. J Pharm Sci. 2019;108(6):2022-32. doi: 10.1016/j.xphs.2019.01.006, PMID 30639738.
11. Dwivedi M, Blech M, Presser I, Garidel P. Polysorbate degradation in biotherapeutic formulations: identification and discussion of current root causes. Int J Pharm. 2018;552(1-2):422-36. doi: 10.1016/j.ijpharm.2018.10.008, PMID 30300706.
12. Bhaskar A, Mukhopadhyay K, Kumar M. Effect of polysorbate–80 concentration on G–CSF formulation using liquid chromatography. Int J Pharm Sci. 2014;6:299-302.
13. Hewitt D, Alvarez M, Robinson K, Ji JY, Wang YJ, Kao YH. Mixed-mode and reversed-phase liquid chromatography-tandem mass spectrometry methodologies to study composition and base hydrolysis of polysorbate 20 and 80. J Chromatogr A. 2011;1218(15):2138-45. doi: 10.1016/j.chroma.2010.09.057, PMID 20950816.
14. Li Y, Hewitt D, Lentz YK, Ji JA, Zhang TY, Zhang K. Characterization and stability study of polysorbate 20 in therapeutic monoclonal antibody formulation by multidimensional ultrahigh-performance liquid chromatography-charged aerosol detection-mass spectrometry. Anal Chem. 2014;86(10):5150-7. doi: 10.1021/ac5009628, PMID 24749737.
15. McShan AC, Kei P, Ji JA, Kim DC, Wang YJ. Hydrolysis of polysorbate 20 and 80 by a range of carboxylester hydrolases. PDA J Pharm Sci Technol. 2016;70(4):332-45. doi: 10.5731/pdajpst.2015.005942, PMID 27020650.
16. Hewitt D, Zhang T, Kao YH. Quantitation of polysorbate 20 in protein solutions using mixed-mode chromatography and evaporative light scattering detection. J Chromatogr A. 2008;1215(1-2):156-60. doi: 10.1016/j.chroma.2008.11.017, PMID 19036378.
17. Donbrow M, Azaz E, Pillersdorf A. Autoxidation of polysorbates. J Pharm Sci. 1978;67(12):1676-81. doi: 10.1002/jps.2600671211, PMID 31449.
18. Zhang L, Yadav S, John Wang YJ, Mozziconacci O, Schöneich C. Dual effect of histidine on polysorbate 20 stability: mechanistic studies. Pharm Res. 2018;35(2):33. doi: 10.1007/s11095-017-2321-1, PMID 29368235.
19. Frankel EN. Lipid oxidation: Mechanisms, products and biological significance. J Am Oil Chem Soc. 1984;61(12):1908-17. doi: 10.1007/BF02540830.
20. Wolff SP. Ferrous ion oxidation in the presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. Methods Enzymol. 1994;233:182-89. doi: 10.1016/S0076-6879(94)33021-2.
21. Kishore RSK, Pappenberger A, Dauphin IB, Ross A, Buergi B, Staempfli A. Degradation of polysorbates 20 and 80: studies on thermal autoxidation and hydrolysis. J Pharm Sci. 2011;100(2):721-31. doi: 10.1002/jps.22290, PMID 20803573.
22. Ha E, Wang W, Wang YJ. Peroxide formation in polysorbate 80 and protein stability. J Pharm Sci. 2002;91(10):2252-64. doi: 10.1002/jps.10216, PMID 12226852.
23. Wang TT, Markham A, Thomas SJ, Wang N, Huang LH, Clemens M. Solution stability of Poloxamer 188 under stress conditions. J Pharm Sci. 2019;108(3):1264-71. doi: 10.1016/j.xphs.2018.10.057, PMID 30419275.
24. Stadtman ER. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. Annu Rev Biochem. 1993;62:797-821. doi: 10.1146/annurev.bi.62.070193.004053, PMID 8352601.
25. Zbacnik TJ, Holcomb RE, Katayama DS, Murphy BM, Payne RW, Coccaro RC. Role of buffers in protein formulations. J Pharm Sci. 2017;106(3):713-33. doi: 10.1016/j.xphs.2016.11.014, PMID 27894967.
26. Schmidt A, Koulov A, Huwyler J, Mahler HC, Jahn M. Stabilizing polysorbate 20 and 80 against oxidative degradation. J Pharm Sci. 2020;109(6):1924-32. doi: 10.1016/j.xphs.2020.03.003, PMID 32171713.
27. Neuberger A. The reaction between histidine and formaldehyde. Biochem J. 1944;38(4):309-14. doi: 10.1042/bj0380309, PMID 16747801.
28. Kerwin BA. Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: structure and degradation pathways. J Pharm Sci. 2008;97(8):2924-35. doi: 10.1002/jps.21190, PMID 17973307.
29. Kishore RSK, Kiese S, Fischer S, Pappenberger A, Grauschopf U, Mahler HC. The degradation of polysorbates 20 and 80 and its potential impact on the stability of biotherapeutics. Pharm Res. 2011;28(5):1194-210. doi: 10.1007/s11095-011-0385-x, PMID 21369824.
30. Borisov OV, Ji JA, Wang YJ. Oxidative degradation of polysorbate surfactants studied by liquid chromatography-mass spectrometry. J Pharm Sci. 2015;104(3):1005-18. doi: 10.1002/jps.24314, PMID 25581232.
31. Zhang L, Yadav S, Demeule B, Wang YJ, Mozziconacci O, Schöneich C. Degradation mechanisms of polysorbate 20 differentiated by 18o-labeling and mass spectrometry. Pharm Res. 2017;34(1):84-100. doi: 10.1007/s11095-016-2041-y, PMID 27738952.
32. Yao J, Dokuru DK, Noestheden M, Park SS, Kerwin BA, Jona J. A quantitative kinetic study of polysorbate autoxidation: the role of unsaturated fatty acid ester substituents. Pharm Res. 2009;26(10):2303-13. doi: 10.1007/s11095-009-9946-7, PMID 19669100.
33. Bates TR, Nightingale CH, Dixon E. Kinetics of hydrolysis of polyoxyethylene (20) sorbitan fatty acid ester surfactants. J Pharm Pharmacol. 1973;25(6):470-77. doi: 10.1111/j.2042-7158.1973.tb09135.x, PMID 4146586.
34. Singh SR, Zhang JM, O'Dell C, Hsieh MC, Goldstein J, Liu J. Effect of polysorbate 80 quality on the photostability of a monoclonal antibody. AAPS PharmSciTech. 2012;13(2):422-30. doi: 10.1208/s12249-012-9759-6.

35. Martos A, Koch W, Jiskoot W, Wuchner K, Winter G, Friess W. Trends on analytical characterization of polysorbates and their degradation products in biopharmaceutical formulations. J Pharm Sci. 2017;106(7):1722-35. doi: 10.1016/j.xphs.2017.03.001, PMID 28302541.

36. Siska CC, Pierini CJ, Lau HR, Latypov RF, Fesinmeyer RM, Litowski JR. Free fatty acid particles in protein formulations, part 2: contribution of polysorbate raw material. J Pharm Sci. 2015;104(2):447-56. doi: 10.1002/jps.24144, PMID 25196966.