A Novel Peroxy Radical Based Oxidative Stressing System for Ranking the Oxidizability of Drug Substances

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ABSTRACT: A novel oxidative stressing system is described which generates high levels of peroxy radicals in solution at room temperature, without the use of azonitrile initiators. The oxidative stressing system is composed of a 10% solution of Tween 80 in water to which $FeCl_3 \cdot 6H_2O$ is added. The Tween 80 acts as a solubilizing agent for drug compounds, and also contains substantial amounts of organic hydroperoxides. It is shown that the Fe III/ Fe II couple operates on the hydroperoxide concentration to effectively generate new peroxy radicals, which then propagate in the Tween 80 solution. Key features of the Tween 80/Fe III system are investigated, and the oxidizability of seven known compounds and ten developmental compounds are examined. Relative reaction rates span a 300-fold range, from benzoic acid (nonreactive, defined as $<0.5\%$ reacted per day) to Vitamin D_3 (7% reacted per hour). Oxidizability "rankings" thus generated are shown to agree well with azonitrile initiated oxidative stress. The potential for general correlations between this type of oxidizability data and actual oxidative performance in LFC and solid oral dosage forms is discussed. J Pharm Sci 95:2014–2028, 2006

Keywords: chemical stability; oxidation; HPLC; preformulation; peroxy radical; forced degradation

INTRODUCTION

Understanding the degree to which a drug candidate might be susceptible to oxidation in a solid dosage form, along with the accompanying characteristic HPLC oxidative degradation profile, is critical information for formulation design activities. It would be desirable if such information could be generated prior to formulation activities by simple manipulations of dilute solutions of the drug substance under ambient conditions. However, predicting the oxidative instability and associated chromatographic profiles of drug substances in this manner has remained a challenge for the pharmaceutical industry.[1] There is to some extent a lack of awareness of the oxidants typically responsible for oxidation in solid dosage forms, and as a result there is a general lack of appropriately designed and applied oxidative stressing procedures which aim to create the correct oxidant.

Perhaps the most common oxidative pathway in pharmaceutical solid dosage forms involves free radical chain reactions in which peroxy radical, $ROO^{\bullet}$, is the oxidant.[1–3] This type of oxidation is often referred to as autoxidation or chain oxidation, and has been studied extensively and reviewed.[2–5] Peroxy radicals, being relatively stable and unreactive, are quite selective and preferentially abstract hydrogen atoms only from weak C–H bonds.[2–5] The ubiquitous nature of

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peroxy radical based oxidation in pharmaceutical solid dosage forms is signaled by the common and successful use of very small amounts (typically 0.02% by weight) of phenolic antioxidants such as propyl gallate, BHA and BHT. This effectiveness reflects these antioxidants' well known ability to donate hydrogen atoms to peroxy radicals, thus stopping further peroxy radical propagation steps.[2,5] Thus, the intrinsic reactivity of a drug substance toward peroxy radicals represents a key attribute which will frame the potential for oxidative stability problems during formulation development and early clinical trials.

Despite the central role of peroxy radical oxidation, the majority of pharmaceutical scientists within the industry are in fact not carrying out oxidative stressing procedures which aim to create peroxy radicals as the oxidant. A recent survey of forced stress practices employed by the Pharmaceutical industry[6] shows that by far the most common oxidative stress employed (100% of companies responding) is based on hydrogen peroxide. Hydrogen peroxide is typically used at 1%–3% in water, or water–acetonitrile or water–methanol mixtures with mild heating. While hydrogen peroxide is a facile "two-electron" oxidant for amines and thioethers (to give N-oxides and sulfoxides, respectively) these reactions do not involve peroxy radicals.[3,7] Further, these conditions can produce low level hydroxyl radical activity from homolytic peroxide bond cleavage.[1,2] Hydroxyl radicals are relatively nonselective, strong hydrogen atom abstractors compared to peroxy radicals.[4,5,8,9] It is for this reason that pharmaceutical scientists are increasingly aware that hydrogen peroxide based oxidative stressing procedures are often "not predictive" of oxidation occurring in the solid dosage form.

Alsante et al.'s survey[6] did show, however, that about 25% of the companies surveyed are attempting to generate peroxy radical activity by using azonitrile-based radical initiators. These compounds have the general form $R_3C{-}N{=}N{-}CR_3$. The most common practice in this regard was the use of 2, 2′-azobisisobutyronitrile (AIBN, $R_1{=}R_2{=}CH_3$, $R_3{=}CN$ group) used in acetonitrile/water mixed solvents. Upon heating, azonitriles liberate molecular nitrogen by homolytic cleavage of the C–N bonds to leave two carbon centered radicals, $R_3C^{\bullet}$, which then react with dissolved oxygen to form peroxy radicals, $R_3COO^{\bullet}$. Boccardi[10,11] pioneered the application of AIBN to oxidatively stress pharmaceutical drug substances in solution nearly 15 years ago, and has recently reviewed this topic.[3] It is surprising that since that time few other detailed reports of AIBN initiated oxidation of pharmaceutical drug substances have been reported, and that azonitrile initiated oxidative stressing of drug substances is not more commonly used. Potential causes for this slow uptake by pharmaceutical scientists may be combinations of the scant reporting of specific applications, the use of relatively high drug and initiator concentrations[10,11] in combination with some reports utilizing pressurized oxygen atmospheres,[12] and the "hazardous/explosive" labeling of azonitriles.

In our laboratory, we have been interested in the development and application of simple, "user-friendly" bench-top procedures for creation of peroxy radical based oxidative stressing systems. The goal is to apply these peroxy radical based procedures very early in the developmental process, when drug substance supply is typically limited (tens of mg quantities) and before initiation of formulation development activities. Uniform application of these simple peroxy radical stressing procedures to all drug substances entering the developmental process, as well as for drug substances already developed, enables a "oxidizability ranking" for each drug substance. This ranking can then be leveraged to make more informed decisions concerning the potential for oxidative instability in solid dosage forms. To this end, our efforts have evolved within two areas. One effort has been to investigate if low drug and AIBN concentrations can be used successfully with ambient atmospheres (rather than pressurized oxygen environments). The use of low drug concentrations (sub-millimolar), low initiator concentrations (1–5 mM), in combination with ambient atmospheres has been demonstrated.[13]

The current report describes a parallel effort in which we sought a new, simple, bench-top route to create high levels of peroxy radicals in solution. This system operates at ambient temperature, under ambient atmosphere and does not use azonitrile initiators. The procedure presented here utilizes solutions of 10% Tween 80 in water to which 10 mM Fe III chloride is added. The Tween 80 contains impurity organic hydroperoxide groups, ROOH, at millimolar levels and also acts as a solubilizing agent to dissolve hydrophobic drug substances. The dissolved Fe III initially oxidizes the ROOH groups to directly form peroxy radicals by the well-known reaction

$$\text{Fe III} + \text{ROOH} \xrightarrow{K_{ox}} \text{ROO}^{\bullet} + \text{H}^{+} + \text{Fe II} \qquad (1)$$

The ability of this system to sustain high levels of peroxy radicals for days is demonstrated, and the impact of variation in added Fe III levels and Tween 80 sources is investigated. This Tween 80/Fe III peroxy radical stressing system is then applied similarly to seven known compounds and ten drug substances currently under development. These compounds show a range of ca. 300-fold in relative reaction rates, which are consistent with their expected reactivities toward peroxy radicals. The oxidizability ranking of the 17 compounds determined in the Tween 80/Fe III system is compared to that obtained with AIBN initiated systems.[13] The potential relationship between intrinsic reaction rates with peroxy radicals and relevance to oxidation in pharmaceutical dosage forms is developed and discussed.

MATERIALS AND METHODS

Materials

All chemicals were used as received. Tween 80 was obtained from a variety of vendors (Sigma-Aldrich, St. Louis MO; TCI, Portland OR, Croda, E. Yorkshire U.K., Fisher, Pittsburgh PA, ACROS, Morris Plains NJ, Tab. 1). Fe III chloride hexahydrate, ammonium Fe II sulfate hexahydrate, 2, 2′-Azobisisobutyronitrile (AIBN), benzoic acid, cumeme, benzyl alcohol, and cumic alcohol were obtained from Sigma-Aldrich (St. Louis, PA). Triphenylphosphine (TPP) and triphenylphosphene oxide (TPO) were obtained from ACROS. Vitamin D_3 was obtained from USP. The probe di-ene and Merck developmental compounds 1–12 were obtained from Merck & Co., Inc.

Table 1. Hydroperoxide Content of Random Lots of Tween 80 Available from five Different Manufacturers

Manufacturer	Tween 80 Lot	Number in mM Hydroperoxides in a 10% Solution
Sigma-Aldrich	1	0.45
	2	0.20
	3	0.15
	4	0.35
	5	0.35
	6	0.25
TCI	1	0.09
	2	0.13
	3	0.12
	4	0.10
Croda	1	0.10
	2	0.15
	3	0.08
	4	0.10
	5	0.74
	6	0.10
Fisher	1	0.16
ACROS	1	0.08

Methods

Oxygen Consumption in Solution

Dissolved oxygen in the 10%Tween 80 based solutions was measured with a Corning (Corning, NY) Dissolved Oxygen Sensor (cat. no. 473020). The sensor was calibrated with a zero oxygen solution (cat. no. 473739) and air. Only the relative changes in dissolved oxygen are of importance in this work. In Figure 1, measurements were recorded in 20 s intervals with the unit measuring constantly. In the case of the unstirred solution, the 50 mL volumetric flask containing 25 mL of the solution of interest was slowly rotated to prevent local oxygen depletion at the probe tip. Values are reported as mg dissolved oxygen per liter.

Measurement of Hydroperoxide Content by TPP

The impurity hydroperoxide content of 10% Tween 80 solutions, 10% Tween 80/Fe III reaction

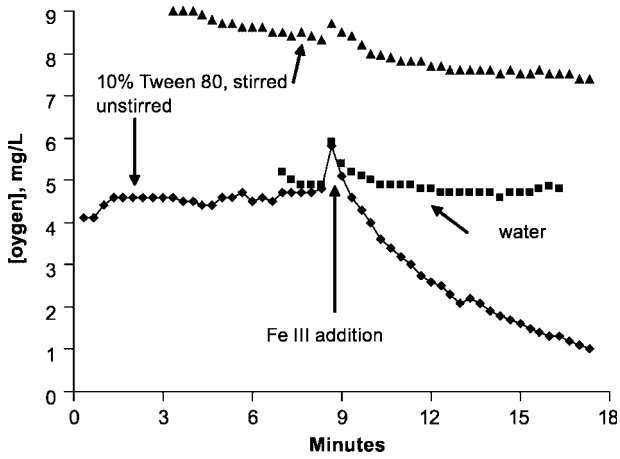

Figure 1. Dissolved oxygen measurements for unstirred water (squares), unstirred 10% Tween 80 (diamonds), and rapidly stirred 10% Tween 80 (triangles). Fe III 10 mM is added at the 9 min timepoint to all three solutions. Note large decrease in dissolved oxygen content after Fe III addition to unstirred 10% Tween 80 solution. Each solution is 25 mL in a 50 mL Erlenmeyer flask open to air.

 DOI 10.1002/jps

samples, and AIBN initiated 50/50 water/acetonitrile solutions was measured by reaction with Tripheylphosphine (TPP). The amount of Triphenylphosphine oxide (TPO) formed by the reaction of the hydroperoxide with the TPP was then determined by HPLC.[14] An Agilent 1100 series HPLC equipped with a quaternary pump, vacuum degasser, Diode Array Detector and autosampler (at ambient temperature) was utilized for all chromatographic measurements reported here. In the case of the 10% Tween 80 solutions and Fe III reaction samples, between 100 and 500 μL of sample was added to a 10.0 mL volumetric flask. The flask was then filled to volume with a TPP stock solution which was 0.10 mg/mL TPP in 100% methanol. For the AIBN initiated sample, 2.0 mL of sample was diluted to 10.0 mL with the TPP standard. Samples were allowed to react for 15 min. TPO eluted near 1.8 min, while TPP eluted near 6.0 min. The concentration (moles/L) of the TPO present was determined by injection of a bracketing TPO standard solution of 0.10 mg/mL. The limit of quantitation is near one micromolar hydroperoxide; a linear response was demonstrated through 500 micromolar hydroperoxide levels.

Percentage Compound/Drug Substance Remaining Measurements

The Tween 80/Fe III stressing sample preparation procedure is as follows. A 10% solution (by weight) of Tween 80 in water is prepared, for example by adding 450 mL water to a 900 mL beaker, followed by addition of 50 g of Tween 80. The solution is stirred until homogeneous. A 25 mL portion of the 10% Tween 80 solution is then taken to a 50 mL Erlenmeyer flask. Drug substance is then dissolved into the solution, at 5× the typical running concentration of the HPLC method to allow subsequent 5× dilution of the sample in the appropriate diluent to reduce the Tween 80 concentration being injected. The solution is then stirred rapidly enough to entrain air bubbles, and 65 mg Fe III chloride hexahydrate is added directly to the flask (while stirring) to give 10 mM Fe III. Samples are typically taken at 1, 2, and 3 day timepoints, diluted in the appropriate mobile phase or diluent, and injected. If quantitative drug loss is being monitored, the evaporation of water from the Erlenmeyer flask at each sampling point should be accounted for by simple weight loss measurements. In addition, it should be noted that addition of 10 mM Fe III chloride to water lowers the pH to near pH 2.5. Any hydrolytic instability (at pH 2.5) occurring over the peroxy radical stressing period would have to be appropriately controlled for. Data is reported as the average percent loss per day over the 1–3 day period examined. A compound is considered "nonreactive" if the percent reacted is $\leq$0.5% (per day) of the initial material present.

AIBN initiated oxidation data in Tables 2 and 3 was carried out according to the general procedures reported by Nelson et al.[13] Dilute drug substance solutions were prepared (typically near 0.1 mg/mL) in 50/50 acetonitrile/water. Portions of this solution are taken and AIBN is added at 5 mM. Ten milliliter portions of these solutions are then placed in a 25 mL volumetric flask, the top closed then the flask placed in a 40°C oven for up to 3 days. Samples are taken at appropriate timepoints, HPLC vials are directly filled and injected. The same nonreactive criterion above is applied.

Table 2. Percent Drug Losses over 24 Hours Oxidative Stressing in 10%Tween 80/Fe III System (Center Column) and in 50/50 Acetonitrile/Water with 5 mM AIBN at 40°C (Right Hand Column), for the Seven Compounds Shown in Figure 8

Compound	Tween 80/ Fe III % loss over 24 h	AIBN, 5 mM, 40°C % loss over 24 h 50/50 ACN/water
Probe di-ene	20% loss in 1 h	5% loss in 1 h
Vitamin D3	7% loss in 1 h	4% loss in 1 h
Cumic alcohol	17	7.0
Benzyl alcohol	8.0	4.0
Merck 7	7.5	12
Cumene	2.0	1.5
Merck 12	No reaction	No reaction
Benzoic acid	No reaction	No reaction

Benzoic acid and Merck 12 are nonreactive in both oxidative systems, while the probe di-ene and Vitamin D_3 are by far the most reactive in both oxidative systems.

Table 3. Percent Loss Data for Compounds in Table II Combined with 10 Additional Merck Compounds Under Development

Compound	Tween 80/Fe III % Loss over 24 Hours	AIBN, 5 mM, 40°C 50/50 ACN/Water % Loss over 24 Hours
Probe di-ene	20% loss in 1 h	5% loss in 1 h
Vitamin D3	7% loss in 1 h	4% loss in 1 h
Merck 1	47	25
Merck 2	24	12
Cumic Alcohol	17	7.0
Merck 3	16	20
Merck 4	15	19
Merck 5	10	12
Benzyl Alcohol	8.0	4.0
Merck 6	8.0	6.0
Merck 7	7.5	12
Merck 8	2.0	No reaction
Cumene	2.0	1.5
Merck 9	No reaction	2.0
Merck 10	No reaction	No reaction
Merck 11	No reaction	No reaction
Merck 12	No reaction	No reaction
Benzoic Acid	No reaction	No reaction

Ranking from top to bottom by greatest to least reactivity in Tween 80/Fe III system. Note wide range of reactivities and excellent agreement between the two oxidative stressing systems.

All percent remaining measurements in Tables 2 and 3 are determined chromatographically. Some details of the chromatographic conditions for the different compounds examined are given below. The primary objective is accurate measurement of loss of the parent compounds, therefore the only critical chromatographic requirement in this work is that expected degradation products do not coelute with the parent species. In all cases bracketing standard injections of each compound were used to quantitate the percent compound remaining using the UV peak area response.

Probe di-ene % Initial Remaining Measurements. The HPLC method used an isocratic mobile phase of 2/1 acetonitrile/water with a flow rate of 1.0 mL/min. Column; 25 cm × 4.6 mm Inertsil ODS-3, 5 micron particle size, ambient column temperature. Detection wavelength was either 205 or 238 nm, injection volume varied depending on sample concentration. Response was linear in the 20%–100% range utilized, degradation products eluted early in the chromatogram while the probe di-ene eluted near 12 min.

Vitamin D_3 % Initial Remaining Measurements. The HPLC method utilized 100% acetonitrile as mobile phase with a flow rate of 1.0 mL/min. Column; 5 cm × 4.6 mm Inertsil ODS-3, 5 micron particle size, ambient column temperature. Detection wavelength was 265 nm, injection volume was 50 μL. Vitamin D_3 elutes near 6.3 min while degradation products eluted much earlier. Response was linear in the 50%–100% range utilized.

Benzoic acid, Cumene, Benzyl Alcohol, and Cumic Alcohol % Initial Remaining Measurements. A general gradient HPLC method was utilized to monitor the loss of these compounds under the stress conditions. Column; Waters Symmetry C18, 25 cm × 4.6 mm. Mobile phase was 90/10 water/acetonitrile at time 0, to 10/90 water/acetonitrile over 40 min followed by re-equilibration. The detection wavelength was 210 nm. The retention times were approximately: benzoic acid, 4.0 min, benzyl alcohol, 12 min, cumic alcohol, 23 min, and cumene at 35 min. In all cases responses were linear in the 50%–100% range utilized.

Merck Developmental Compounds 1–12 % Initial Remaining Measurements. All Merck compound percentage loss data in Tables 2 and 3 was determined by stability indicating HPLC assays. Each chromatographic assay was specific to the drug substance being developed and detailed conditions for each will not be described here.

The HPLC methods were validated showing the typical requirements of linearity, accuracy and recovery from 20% to 150% of the working level concentrations.

RESULTS

Characterization of the Tween 80/Fe III System

Equation (1) shows the oxidation of impurity hydroperoxide groups initially present in the Tween 80 by the added Fe III. This oxidation directly forms peroxy radicals. If Tween 80 is able to be oxidized by these peroxy radicals, then peroxy radical "propagation" will occur, as shown in Eq. 2:

$$\mathrm{ROO}^{\bullet} + \mathrm{Tween} - \mathrm{H} \xrightarrow{K_{\mathrm{prop}}} \mathrm{ROOH} + \mathrm{Tween}^{\bullet} \xrightarrow[fast]{\mathrm{O_2}} \mathrm{Tween} - \mathrm{OO}^{\bullet} \quad (2)$$

The propagation in Eq. 2 is characterized by the consumption of dissolved oxygen in solution and concomitant generation of more hydroperoxide species. Figure 1 shows dissolved oxygen measurements for three different solutions, obtained continuously over a 10–20 min interval. In each case, near the 9 min timepoint 10 mM Fe III chloride was added. The square data points show the dissolved oxygen levels for an unstirred water solution. The dissolved oxygen levels are near 4.5 mg/L and do not change when the Fe III is added except for a brief rise due to the brief swirling of the solution to dissolve the Fe III. In contrast, the diamond data points are for an unstirred 10% Tween 80 solution. The dissolved oxygen levels are fairly steady near 4.5 mg/L until the Fe III is added at which point the oxygen levels in solution begin to steadily decrease. Within 10 min of the Fe III addition, dissolved oxygen levels have been reduced nearly 80% to near 1 mg/L levels, even with the solution open to air. The triangular data points in Figure 1 reflect an identical experiment except that the 10% Tween 80 solution is rapidly stirred during the entire experiment. With rapid stirring the dissolved oxygen levels can be kept high after Fe III addition.

The upper portion of Figure 2 shows the hydroperoxide growth as determined from reaction with TPP. The reaction of TPP with hydroperoxides is rapid and forms Triphenylphosphine oxide (TPO) with 1:1 stoichiometry.[14,15] Thus simple quantitation of the amount of TPP consumed or TPO formed gives the molar amount of hydroperoxides

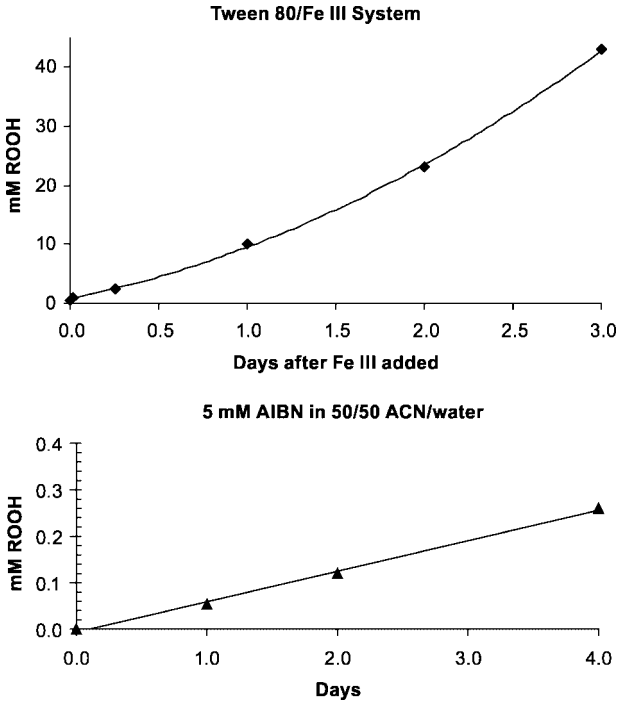

Figure 2. Upper, growth of hydroperoxide levels in 10% Tween 80 system after addition of Fe III. Hydroperoxide levels increase from 0.4 to 40 mM over 3 days. Lower, growth of hydroperoxide levels in 5 mM AIBN initiated 50/50 acetonitrile-water solution. Note the 100-fold difference in Y-axis scaling in lower portion of Figure.

present. The upper portion of Figure 2 shows a very large hydroperoxide growth, from near 0.4 mM at initial to near 40 mM in 3 days; a ~100-fold increase over the 3 day stressing interval. For comparative purposes, the lower portion of Figure 2 shows the same hydroperoxide growth in an azonitrile initiated system. The hydroperoxide levels are nearly 200-fold lower, growing only to near 0.2 mM over 3 days.

Figure 3 shows the structure of a probe di-ene compound which is structurally related to the

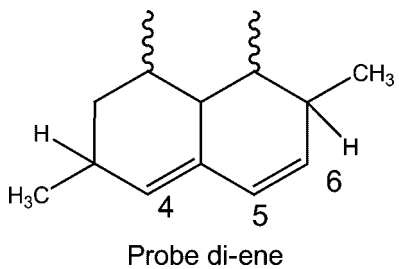

Figure 3. Relevant portion of the probe di-ene structure. Reaction with peroxy radicals can occur by abstraction of the hydrogen atoms from the allylic C–H bonds, or by addition of peroxy radical to C_4 or C_6.

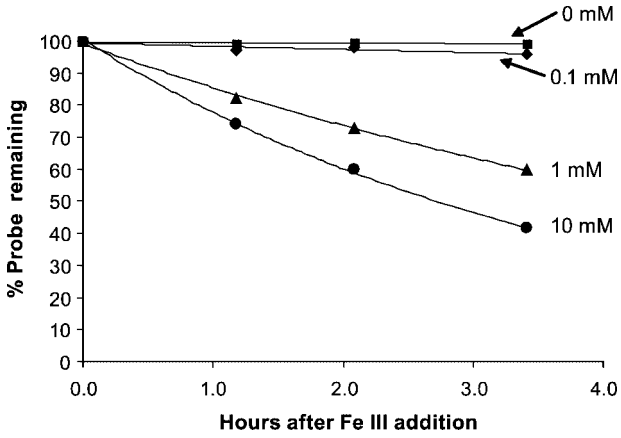

Figure 4. Di-ene probe % remaining curves as a function of added Fe III over 4 h in 10% Tween 80. Squares (no added Fe III), diamonds (0.1 mM Fe III), triangles (1 mM Fe III) and circles (10 mM Fe III). Solid curves to 1 and 10 mM data are best-fit simple exponential decays while the solid curves through the 0 and 0.1 mM data are linear fits.

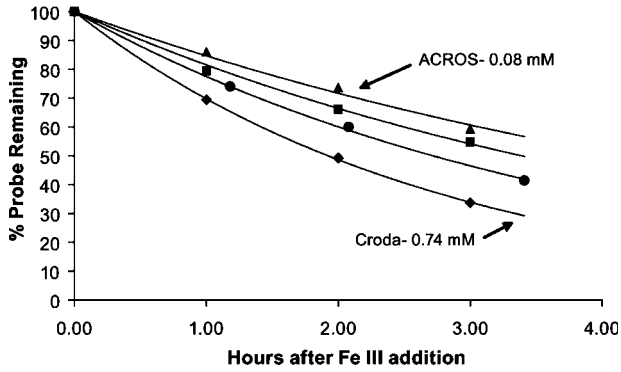

Figure 5. Percent probe di-ene loss curves using Tween 80 from 4 different vendors which span the 0.08–0.74 mM hydroperoxide content range shown in Table 1. From top to bottom: triangles; ACROS (0.08 mM ROOH in 10% solution), squares; Fisher (0.16 mM ROOH), circles; Sigma-Aldrich (0.25 mM ROOH), and diamonds; Croda (0.74 mM ROOH). Solid curves are best-fit simple exponential decays. Trend of faster di-ene loss with higher initial hydroperoxide content is clear.

HMG-CoA reductase inhibitor simvastatin.[16] This compound is expected to be reactive with peroxy radicals,[8,16,17] both by abstraction of hydrogen atoms from the low energy allylic C–H bonds as well as by addition of peroxy radicals to the olefin bonds. The reactivity of this probe di-ene can thus be used as a simple monitor of the peroxy radical activity in the Tween 80/Fe III system. Figure 4 shows four curves which plot the % initial remaining of the di-ene over a 3 h period, following the addition of 0, 0.1, 1.0, and 10 mM Fe III to the 10% Tween 80/water solution in which the di-ene was initially dissolved. In the case of no added Fe III, the di-ene is stable over the 3 h period examined. The remaining data show increasing di-ene loss rates as more Fe III is added, reflecting increased peroxy radical activity. The 1 and 10 mM Fe III added curves in Figure 4 are best fit by a simple exponential decays. The primary products formed all involve either new C–O bonds at C_4 and C_6 (from peroxy radical addition) or new C–O bonds replacing the allylic C–H bonds deriving from initial peroxy radical hydrogen atom abstraction, and are thus consistent with our expectations of peroxy radical mediated oxidation of the di-ene.

Figure 5 examines the effect on di-ene loss rates of using different Tween 80 suppliers with different hydroperoxide contents; all at fixed (10 mM) added Fe III concentration. The hydroperoxide content of Tween 80 from various suppliers has been reported[18] and shows significant variability. Recently, we have examined the lot-to-lot and vendor variability of hydroperoxides in variety of pharmaceutical excipients including Tween 80.[19] Table 1 reviews some of that data along with additional measurements we have undertaken in the context of this work. Table 1 shows Tween 80 hydroperoxide content from 5 different vendors, ranging from 0.08 to 0.7 mM in a 10% Tween 80 solution. The 0.7 mM case appears to be an outlier, in that most values range between about 0.1 and 0.4 mM as shown in Table 1. Figure 5 shows that the di-ene loss rates progressively increase as initial ROOH content is increased through the ca. 0.1–0.7 mM range. Given that the data in Figure 5 derives from four different Tween 80 vendors, this suggests that initial ROOH content alone is the critical attribute of the Tween 80 in terms of the peroxy radical levels which can be generated.

The ability of the 10% Tween 80/Fe III system to sustain the peroxy radical concentrations after the Fe III is added can also be probed with di-ene loss curves as in Figures 4 and 5. In the upper portion of Figure 6, the upper curve is the usual di-ene loss curve, where at "initial" the di-ene is already dissolved in solution and then the 10 mM Fe III is added, and the di-ene loss is monitored over the next 3 h. The lower curves in the upper portion of Figure 6 reflect different experiments, in which the Fe III is added first, followed by longer and longer time intervals until the di-ene is subsequently added to the solution. The upper portion of Figure 6 thus shows that di-ene loss rates actually

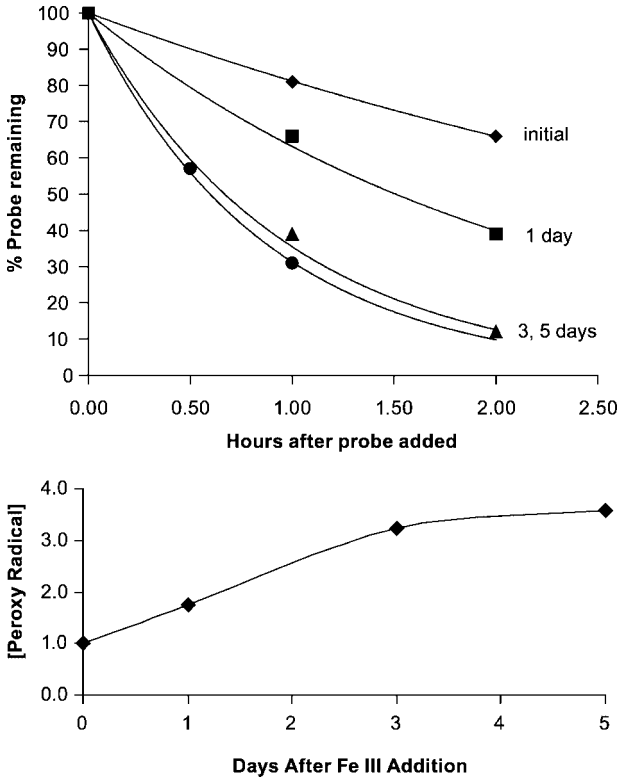

Figure 6. Upper, probe di-ene loss curves obtained just after 10 mM Fe III addition (initial), then 1, 3, and 5 days after Fe III addition. Lower, representation of initial 0–1 h slopes from upper portion of Figure 5. Data show that the relative peroxy radical increases over threefold over 3 days, then sustains the high levels through at least 5 days.

increase more than threefold from initial values over 3 and even 5 days. The lower panel in Figure 6 graphically represents the data in the upper portion of Figure 6 and thus represents the relative growth of the peroxy radical concentration over the 5-day interval examined. The highest peroxy radical activity is sustained for at least 5 days.

Figure 7 explores the effect of adding 1 mM Fe II ammonium sulfate to the Tween 80 solution, instead of Fe III. The conditions are otherwise identical to that in Figure 4; in Figure 7 the 1 mM Fe III added data from Figure 4 is also redisplayed for comparative purposes. Clearly the Fe II di-ene loss data shows a very rapid loss component, followed by a slower di-ene loss. The di-ene product distributions for the 1 mM Fe II and 1 mM Fe III added cases are essentially identical. The Fe II added data in Figure 7 requires fitting by a bi-exponential decay rather than the single exponential decay for the added Fe III cases. This will be discussed below.

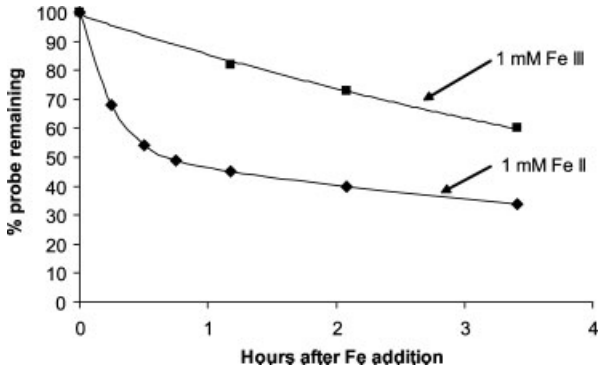

Figure 7. Probe di-ene loss curves obtained just after 1 mM Fe III addition (circles, re-displayed from Fig. 4), and after addition of 1 mM Fe II (diamonds). Note the fast and slow components of the Fe II probe di-ene loss data. Solid curve for Fe III case is a best-fit simple exponential decay, while for Fe II data the solid curve results from a best-fit bi-exponential decay.

Application of Tween 80/Fe III System to Seven Known Compounds

The Tween 80/Fe III oxidative stress procedures outlined here were applied to seven known compounds which are shown in Figure 8. These compounds range widely in their expected reactivity with peroxy radicals. The second column in Table 2 lists the seven compounds in Figure 8 in order from most reactive to least reactive (in the Tween 80 system) by a simple ranking of the % initial material lost over a 24 h period. Benzoic acid is found to be nonreactive (defined as $\leq$0.5% of initial material reacted over 24 h, see Methods section). Merck 12 is also nonreactive. On the other end of the reactivity spectrum, Vitamin D_3 shows very rapid losses, of about 7% per hour. Thus, Vitamin D_3 and Merck 12 span a range of at least $\sim$300-fold in relative reactivities in the Tween 80/ Fe III system.

The third column in Table 2 gives the % initial material reacted using the azonitrile based peroxy radical stressing conditions described in the Methods section.[10,13] Benzoic acid and Merck 12 are again found to be nonreactive; while Vitamin D_3 is again by far the most reactive. Overall, the data in Table 2 show there is good agreement in the ranking of relative reactivities in the Tween 80/Fe III and the azonitrile based systems.

Application to 10 Developmental Merck Compounds: Oxidizability Ranking

The peroxy radical stressing procedures described in Table 2 were applied to ten additional Merck

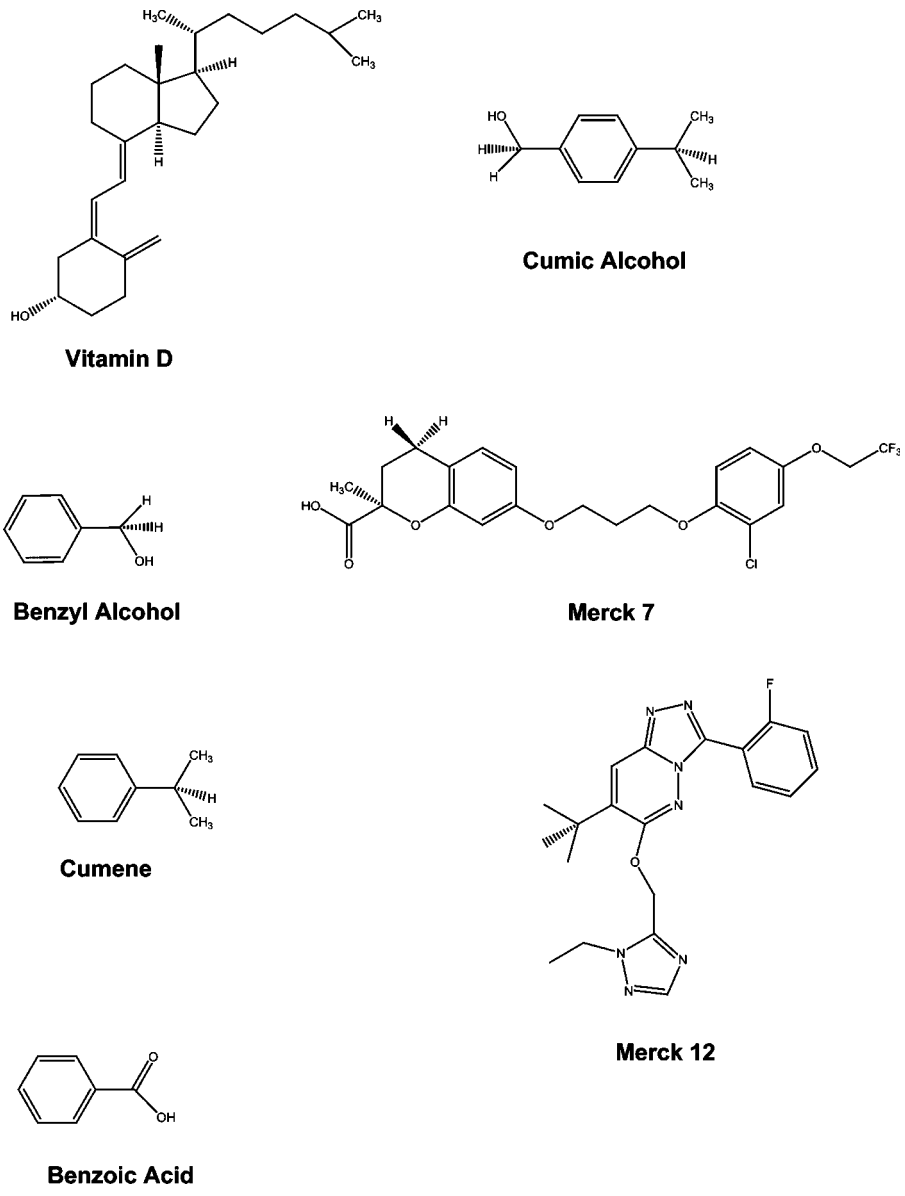

Figure 8. Structures of compounds studied in this work.

compounds under development. The % initial material lost data for those ten compounds, in addition to the seven compounds in Table 2, is shown in Table 3 and ranked according to % loss in the Tween 80/Fe III system. Table 3 shows that 4 of the 12 Merck compounds examined (Merck 9–12) are ranked as nonreactive in the Tween 80/Fe III system. Merck 1 oxidizes nearly 100 times faster than the upper limit of the "nonreactive" criterion of $\leq$0.5% loss per day. The reactivity of all 17 compounds in the azonitrile system is also shown in Table 3. Comparison of the 2nd and 3rd columns of Table 3 again shows good agreement between the oxidizability ranking in the Tween 80/Fe III and the azonitrile based systems.

DISCUSSION

Selection of the Fe III/Fe II Redox Couple

Transition metal ion reactions with hydroperoxides have been well studied.[2,4,5] Transition metal ions such as Mn III, Co III, Fe III, and Cu II all can affect the oxidation of hydroperoxides as in Eq. 1. The reduced forms of these metal ions may also

affect the reduction of hydroperoxides, as shown in Eq. 3:

$$M(red) + ROOH \xrightarrow{K_{red}} RO^{\bullet} + OH^{-} + M(ox) \quad (3)$$

If both the metal ions in the metal ion couple M (ox)/M (red) have comparable stability in solution, then reactions (1) and (3) can achieve a steady state. At steady state, the overall reaction is a metal ion catalyzed decomposition of ROOH which is the summation of Eqs. 1 and 3:

$$2\,ROOH \xrightarrow{M(ox)/M(red)} ROO^{\bullet} + RO^{\bullet} + H_2O \quad (4)$$

In the current context, the reduction reaction shown in Eq. 3 is not desirable, since an alcoxyl radical is generated. Alcoxyl radicals, similar to hydroxyl radicals, are strong nonselective hydrogen atom abstractors[4,5,8,9] which could in principle react with a dissolved drug substance and thus compromise the peroxy radical selectivity of the subject oxidative system. A second important consideration in selecting the metal ion for the subject oxidative system is that the drug substance's themselves should not be able to be oxidized or reduced by the metal ion couple. In earlier stages of this work we investigated the use of the Mn III/Mn II couple in the Tween 80 system. Mn III is a strong oxidant ($E_0 \approx 1.5$ V[20]) and readily affects Eq. 1. We found the Mn II ion to be a poor reductant in that the rate of Eq. 3 was negligible. While this was ideal, ultimately Mn III was found to be able to directly oxidize a fair portion of amine containing drug substances. This direct oxidation would confound assignment of peroxy radical reactivity, which was not considered appropriate for the subject methodology. Cobalt III was found to behave similarly to Mn III ($E_0 \approx 1.8$ V[20]) in that amine containing compounds could be directly oxidized.

We realized a significantly weaker oxidant was needed. Fe III ($E_0 \approx 0.77$ V[20]) was an obvious choice, and has not given a direct reaction with any amine containing dug substances we have examined. However, the lower *E*-value for Fe III means Fe II is a relatively better reductant, and indeed the ability of Fe II to affect Eq. 3 is well known. Fe II agents are used for rapid determination of hydroperoxides[21,22] where the first step is a rapid reduction of the hydroperoxide as in Eq. 3. Thus it is clear in the current application that once Fe III is added, the Fe II formed can achieve a steady state with the Fe III and the hydroperoxide, and Eq. 4 will be operative.

Alcoxyl Radicals Produced Are Quenched by Highly Oxidizable Tween 80

This highlights another important role of the Tween 80 in this system. In the subject oxidative system, drug substances are dissolved at 0.1–0.5 mg/mL, while Tween 80 is present at 100 mg/mL. The nonselective alcoxyl radicals generated by Eq. 3 would be expected to readily react with the oxidizable Tween 80. The result of that reaction is the rapid conversion of the alcoxyl radical to a peroxy radical as shown in Eq. 5:

$$RO^{\bullet} + \text{Tween} - H \xrightarrow{\text{fast}} ROH + \text{Tween}^{\bullet} \xrightarrow{O_2} \text{Tween} - OO^{\bullet} \quad (5)$$

Given the 500–1000 fold higher levels of Tween 80 present, the Tween 80 thus serves to "protect" drug substances from encountering significant alcoxyl radical activity. In fact, under the conditions described here, Eq. 3 followed by Eq. 5 actually provides for a more rapid production of peroxy radicals than the direct oxidation of ROOH by Fe III (Eq. 1). This is demonstrated by two observations. The first is that when Fe II ammonium sulfate is added to the Tween 80 system, the dissolved oxygen levels drop even more rapidly than shown in Figure 1. This signals rapid formation of the alcoxyl radical (Eq. 3), followed by rapid conversion to peroxyl radical with the concomitant consumption of dissolved oxygen in Eq. 5.

The second observation which supports efficient conversion of alcoxyl radicals to peroxy radicals is the unique bi-phasic response of the di-ene loss curve in Figure 7 when Fe II is added. In either the Fe II or Fe III added case, the di-ene product distribution is identical and reflects our expectation for di-ene oxidation by peroxy radical as described in the Results section. We rationalize the faster initial rate of di-ene loss in the Fe II added case as due to transiently higher peroxy radical levels, as follows. In a propagating peroxy radical system such as this, at steadtystate the rate of the initiation reactions (Eqs. 1 and 3 followed rapidly by Eq. 5) is equal to the termination (disproportionation) reactions. The rate of these disproportionation reactions will be proportional to $[ROO^{\bullet}]^2 k_{dis}$, where k_{dis} is the rate constant for peroxy radical termination (range, $\sim 10^3 - 10^7$/M/s[4]). Thus, in order to sustain a doubling (for example) of the peroxy radical concentration, a fourfold increase in the initiation rates would have to occur.

In Eq. 3 the value of k_{red} for Fe II is expected to be much larger than k_{ox} in Eq. 1 for Fe III oxidation[2,4,21,22]; thus at steady-state the value of [Fe III]/[[Fe II] will be large. When Fe II is added to 10% Tween 80 in water, the majority of the Fe II must react to get to steady-state, hydroperoxides are rapidly reduced followed rapidly by Eq. 5, and the much higher initiation rates support a transiently higher peroxy radical concentration. In Figure 7, that happens within the first ~30 min or so, as the solution starts out clear (associated with Fe II) and turns yellow (characteristic of Fe III) in that timeframe. The creation rate of new peroxy radical chains decreases as the Fe II converts to Fe III and the Fe III/Fe II steady-state value is approached; the sustainable peroxy radical concentration lowers. This interpretation is supported by simple bi-exponential fitting the Fe II added data in Figure 7, which gives about 50% of a fast component with a rate near 35-fold faster than the slow component; and a fitted rate for the slow component which is within ~20% of the fitted rate for the Fe III added data in Figure 7. Note the Fe III/Fe II steady-state appears to be reached quickly in the case of Fe III added, as reflected by the good single exponential fits to the di-ene loss curves in Figures 4–7. This is explained by the fact that when Fe III is added initially, the Fe III/Fe II is ratio is already very large, and only a relatively small amount of Fe III oxidation is needed to reach the Fe III/Fe II steady state ratio.

Reactivity Data in Table 3 Consistent with Peroxy Radical Activity

The discussion above argues that use of the Fe III/Fe II ion couple in the 10% Tween 80 system will generate equal portions of new peroxy radical chains via. Eq. 1, and via. Eq. 3 followed rapidly by Eq. 5. The complete conversion of alcoxyl radicals to peroxy radicals given in Eq. 5 is also supported by a simple consideration of the reactivity data in Table 3 and the structures in Figure 8. Three general points can be made.

Consideration of C–H Bond Selectivity

It is well known that for hydrogen atom transfer reactions, a rough correlation exists between exothermicity of reaction and reaction rates.[4] Typical alcohols RO–H have fairly high bond strengths near 105 kcal/mole,[9,26] and thus alcoxyl radicals $RO^{\bullet}$ may have reasonable reaction rates with C–H bonds of equal or lower bond energies. The vast majority of C–H bonds in polyatomic molecules have bond strengths less than 105 kcal/mole, with the exception of aromatic and olefinic C–H bonds with have bond strengths near 112 kcal/mole.[9,26] This is why alcoxyl radicals are considered strong, facile, nonselective hydrogen atom abstractors as noted above. In contrast, a general value for the ROO–H bond strength of peroxy radical is around 89 kcal/mole.[2,4,9] Thus peroxy radicals are known to be much more selective. When reaction can occur, the rate is typically orders of magnitude slower than alcoxyl radicals reactions due to the much lower exothermicity.[4,9,23–26] In Table 3, Merck compounds 9–12 are found to be nonreactive. These four compounds contain 47 distinct C–H bonds. Excluding aromatic and olefinic C–H bonds due to bond strength considerations, this leaves 24 different C–H bonds of methyl, methylene and methine carbon atoms which are not oxidized (at any significant rate) in the Tween 80/Fe III system. This C–H bond selectivity is clearly consistent with peroxy radical, not alcoxyl radical, reactivity.

Reactivity of Tween 80/Fe III System "Turns on" at Cumene

In Table 3, starting at the bottom of the 2nd column and moving up shows that the first C–H bond which is "reactive" in the Tween 80/Fe III system is the benzylic C–H bond of cumene (Fig. 8). This bond energy is known to be about 84 ± 2 kcal/mole[9] and thus can have a significant rate constant for reaction with peroxy radical. Indeed, the rate constants for abstraction of this cumene hydrogen atom by various peroxy radicals have been reported[4,25] and are on the order of 1/M/s. The fact that measurable Tween 80 system reactivity in Table 3 starts at cumene again supports peroxy radical as the active oxidant.

Correlation of Reactivity to Azonitrile Based Systems

A comparison of the 2nd and 3rd columns in Table 3 shows that the reactivity rankings of the 18 species listed is very similar in the Tween 80/Fe III system and the azonitrile initiated system. The azonitrile system generates peroxy radicals in a very different fashion than the Tween 80/Fe III system, and represents a fairly well understood system.[3,10–13] The excellent comparative

agreement between the 2nd and 3rd columns in Table 3 indicates that the oxidants reacting with drug substances in both systems have similar strengths; that is, both are peroxy radical based.

Tween 80/Fe III System as a Simple Peroxy Radical Based Oxidative System

At 10% in water, Tween 80 solubilizes even very hydrophobic drug substances. Addition of 1–10 mM Fe III results in the rapid formation of significant levels of peroxy radicals via. Eqs. 1–3 and 5. The rapid oxygen consumption (Fig. 1), the large hydroperoxide growth (Fig. 2), and the peroxy radical activity as probed by the di-ene in Figures 4–7 and reactivity data in Tables 2 and 3 are consistent with this view. The sustained and growing peroxy radical concentrations (Fig. 6) reflect the rising rate of creation of new peroxy radical chains, as the Fe III/Fe II couple operates on a growing hydroperoxide concentration (as shown in Fig. 2). The peroxy radical levels are thus generated and sustained independently of the presence of added drug substances.

Dissolving drug substances at 0.1–0.5 mg/mL levels in the 10%Tween 80/water and addition of Fe III thus sets up conditions in which drug substances "compete" with Tween 80 in acting as a substrate for the Tween 80 related peroxy radicals. It is interesting to note that dissolved oxygen consumption rates in Figure 2 are much higher than implied by Nelson et al.[13] for 1–5 mM AIBN initiated systems. Thus peroxy radical creation rates are much higher in the subject oxidative system. However, many peroxy radicals react with Tween 80 substrate, and disproportionation rates are likely much higher. In the AIBN initiated systems the solvents are generally not reactive with peroxy radicals and disproportionation rates are lower. The overall effect is that drug substances in each system happen to react at generally similar "absolute" rates (expressed simply as % initial loss/day as in Tab. 3) under the experimental conditions described in Table 3.

Given the general utility of the Tween 80/Fe III system, some discussion of the robustness of this oxidative system is warranted. The starting levels of the impurity hydroperoxides in the Tween 80 is an important factor in being able to quickly and consistently generate significant peroxy radical levels. This factor drove our desire to obtain the hydroperoxide content data in Table 1 and the corresponding effect on relative peroxy radical levels (Fig. 5). Fortunately, even the most "highly pure" (in terms of hydroperoxide content) Tween 80 commercially available gives near 0.1 mM hydroperoxides in a 10% Tween 80 solution (Tab. 1). Tween 80 systems should thus generate the range of peroxy radical activity represented in Figure 5. Another experimental factor to consider is the pH of the solution. The Tween 80/ Fe III sustains higher peroxy radical levels at lower pH due to maximum solubility of Fe III in water at lower pH values. Thus the ambient pH of near 2.5 works optimally, but buffering the system at higher pH values appears to decrease the sustainable peroxy radical levels. (data not shown).

Tween 80/Fe III System: Peroxy Radical Based Oxidizability Rankings

We apply the Tween 80/Fe III oxidative stressing procedure described here, as well as the AIBN initiated oxidative stressing procedures[13] uniformly to all drug substances coming into development. Similar data has also been generated for compounds already developed, for which there is long term stability data for the solid dosage form under ICH conditions. Data from all drug substances tested is compiled as in Table 3. This "oxidizability ranking" provides a framework for understanding the potential reactivity of the current drug candidate with peroxy radicals, in the context of the many developmental compounds which have preceded it. Thus far, such oxidizability rankings appear to have some predictive value. The most powerful predictive ranking is if a drug substance ranks as nonreactive (such as Merck 9–12, Tab. 3). A nonreactive ranking means there is no intrinsic reaction with peroxy radicals, thus no oxidation by peroxy radicals is predicted regardless of formulation components, physical stability of the drug, manufacturing process, or ICH storage condition. We have found this to be true for drug substances formulated as traditional solid oral dosage forms, and even for drug substances formulated in liquid filled capsule formulations (LFC) with solubilizing agents such as Tween 80 and polyethylene glycols. The latter is particularly impressive, as Tween 80 and polyethylene glycols have high hydroperoxide content[18,19] and the catalytic breakdown of the hydroperoxide by trace metals taken advantage of here likely operates at low levels.

As expected, compounds with very high oxidizability rankings in Table 3 have been found to be quite problematic in the type of LFC formulations

discussed above. In contrast, in solid dosage forms such compounds may or may not have an oxidative stability problem. If peroxy radicals are able to form, "propagate" and encounter accessible drug substance, then the high oxidizability ranking indicates oxidation can occur. However, the formulator may have numerous means at hand to prevent this from occurring including a stable crystalline form of the drug substance, careful choice of excipients and processing conditions, and use of antioxidants. It appears at this early stage that in solid dosage forms, use of antioxidants has in fact been more common with compounds with the highest oxidizability rankings.

Developmental candidates that have intermediate oxidizability rankings in Table 3 have been found thus far to have much less pronounced, yet potentially "significant" oxidative degradation in LFC formulations; particularly in view of the common industry practice of applying ICH degradate qualification limits to early phase developmental programs. In solid dosage forms, such intermediately ranked compounds may or may not require antioxidants; in these cases the compositional and process factors discussed above play a large role in determining whether or not an antioxidant will actually be needed.

Product Distributions for Appropriately Selective Stability Indicating HPLC Methods

Specific oxidative degradate peaks generated by the Tween 80/Fe III and AIBN systems can be used to help define selectivity requirements for stability indicating HPLC methods. In this context, it would be most beneficial if the oxidative degradation profile which would occur in the solid dosage form under long-term ICH stability conditions was reproduced by the short term, solution based oxidative stress procedures discussed here. Such an expectation is somewhat naive given the various pathways to final products which are available to the initial drug substance peroxy radical formed. For oxidation of a secondary carbon atom, disproportionation of the peroxy radical could give an alcohol or a carbonyl group. Hydrogen atom abstraction would yield the hydroperoxide. In our experience, hydroperoxides typically decompose faster than they form under long-term ICH stability conditions, and thus do not accumulate. Alcohol groups (and hydroperoxides) may also undergo elimination to form new C=C bonds which extend conjugation. This in turn may activate new C−H bonds which become susceptible to oxidation by peroxy radical. These "intermediate reactions" influence final product distributions. Any solution phase peroxy radical stressing system may have a difficult task in correctly "predicting" accurate relative distributions of peroxy radical oxidation products occurring in a solid dosage form given the very different conditions under which the intermediate reactions are occurring.

Our approach is to apply both AIBN based oxidative stress and the Tween 80/Fe III system and compare their oxidative degradation profiles. While detailed product distribution comparisons are well beyond the scope of the current work, in general the product distributions often have similar species, but can differ significantly in relative intensities. One example of this may be the accumulation of hydroperoxide degradates, which tends to be less pronounced in the Tween 80/Fe III system compared to AIBN systems. This is likely due to high disproportionation rates of drug based peroxy radicals, in combination with the action of the Fe III/II couple on hydroperoxides. Another example of different reactivity is formation of N-oxides.[27] In our experience the low pH of the subject system keeps typical amine groups fully protonated, and thus despite high hydroperoxide levels (Fig. 2) formation of N-oxides is typically lower than in AIBN based stressing. Thioether conversion to sulfoxides can proceed very rapidly. Note that this type of N-oxide and sulfoxide reactivity is readily mimicked by stressing with dilute solutions of hydrogen peroxide in methanol/water mixtures at room temperature.[1,3,7,10] In summary, these differences are why we view the two sets of degradation profiles as complementary, and together they may give a more complete picture of potential degradation profiles in solid dosage forms under longer term stability studies.

CONCLUSIONS

A novel oxidative system has been developed and described which generates significant levels of peroxy radicals at room temperature, under ambient atmosphere, without the use of azonitrile initiators. Significant peroxy radical concentrations can be sustained for days. The alcoxyl radical activity generated from the action of Fe II on hydroperoxides has been shown to be effectively quenched by the Tween 80 via. Eq. 5. Thus, the Tween 80/Fe III system provides an

additional peroxy radical based oxidative stress test, which complements azonitrile initiated oxidative stressing. Eighteen compounds are examined and show relative reaction rates with peroxy radical ranging over 300-fold. Uniform application of these procedures to all drug substances entering development allows for compilation of oxidizability rankings similar to Table 3. As LFC and solid dosage forms are subsequently developed and corresponding long term ICH stability data becomes available, the pharmaceutical scientist can examine potential correlations and leverage them appropriately. The Tween 80/Fe III oxidation system can also be used in combination with AIBN initiated oxidation to help determine potential elution times for peroxy radical mediated degradation products. This facilitates development of appropriately selective stability indicating HPLC methods early in the developmental process.

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