

# Evaluation of Solution Oxygenation Requirements for Azonitrile-Based Oxidative Forced Degradation Studies of Pharmaceutical Compounds

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**ABSTRACT:** AIBN and ACVA oxidative forced degradation models are examined for two drug molecules whose predominant oxidation chemistries arise from different reaction mechanisms (i.e., free radical vs. nucleophilic). Stress was conducted under a variety of initiator concentrations, and under ambient and pressurized oxygen atmospheres. In each case examined, the azonitrile initiator solutions served as a good predictive model of the major oxidative degradation products observed in pharmaceutical formulations. At low to moderate initiator concentrations, the degradation product distributions and degree of reactivity were similar for samples stored in ambient and pressurized oxygen environments. These results are rationalized with reference to the oxygen consumption kinetics of AIBN and ACVA solutions as a function of initiator concentration. The data suggests that ambient air provides sufficient oxygen to enable chain propagation of peroxy radicals in azonitrile solutions of concentrations appropriate to the forced degradation of pharmaceutical compounds. © 2006 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 95:1527–1539, 2006

**Keywords:** AIBN; ACVA; pharmaceutical; oxidation; forced degradation; oxygen

## INTRODUCTION

Pharmaceutical scientists tasked with the development of stability-indicating analytical methods for new drug products face a difficult challenge in that they must develop a method that is selective for all the degradants occurring in the formulation before information is available about what these degradants actually are. The understanding of the degradation chemistry of a pharmaceutical compound is thus an important first step in the development of a stability-indicating method. The

ultimate arbiters of what degradation must be monitored in such methods are the degradation profiles of formulations stored at the intended long-term storage conditions. However, validated stability-indicating methods must be in use well before the availability of long-term stability data for realistic formulations. To bridge this difficulty, forced stress models designed to produce the same qualitative degradation in days that a real formulation generates in months and years serve an important role as a starting point for the development of stability-indicating methods for new chemical entities. When used appropriately, these models can draw powerful insights from limited amounts of drug substance and significantly aid in the rational development of efficient stability-indicating methods. However,

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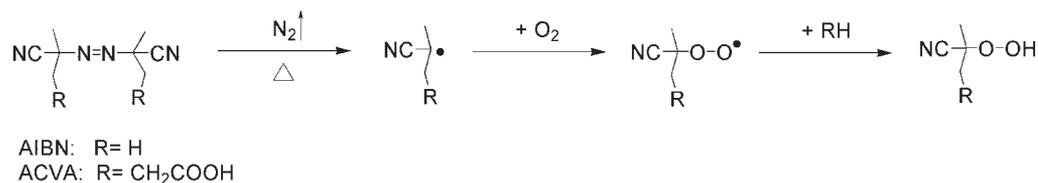
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they carry as well the potential to side-track method development efforts in the fruitless task of designing methods around degradants that are not representative of the degradation actually observed in formulated products. Thus, the ultimate utility of these model systems is a balance between quickly inducing appropriately representative degradation pathways while avoiding nonrepresentative pathways.

Regulatory guidances strongly encourage the use of forced stress systems to challenge stability-indicating methods, but give little specific detail on the subject.<sup>1,2</sup> While it is fairly straightforward to assess the propensity of a drug molecule to react by acid or base-catalyzed hydrolysis, oxidative degradation pathways prove more difficult to predict. Yet these oxidative reactivities are often some of the most important to understand at the early stages of drug development. The special importance of forced degradation experiments in elucidating the potential oxidative degradants of drug molecules is underlined by the often nonlinear growth of such degradates in pharmaceutical products under long-term storage. A recent survey of major pharmaceutical companies by Alsante et al.<sup>3</sup> revealed a fairly wide variety of oxidative stress practices. The only clear consensus across the group surveyed was the use of hydrogen peroxide. However, though hydrogen peroxide is still the most convenient way to force the formation of nitroxide and sulfoxide degradation products of drug molecules with nucleophilic amine and sulfide groups, it provides very little chemical relevance in the more difficult and important task of predicting the products of free radical autoxidation.<sup>4</sup> Autoxidation is mediated by peroxy radicals, which are very selective oxidants, and often produces distinct degradation pathways that are not well reproduced by harsher oxidation chemistries. Determining a drug molecule's intrinsic reactivity toward peroxy radicals should thus be a central component of oxidative stressing procedures purporting to simulate autoxidation chemistry. It is by the intentional formation of peroxy radicals that this is best achieved.

Based on the results of the survey by Alsante et al., only a minority of companies are currently routinely utilizing peroxy radical-based oxidative forced stress procedures.<sup>3</sup> The most widely used peroxy radical-based method is the use of azonitrile-type radical initiators such as 2,2'-azobisisobutyronitrile (AIBN)<sup>5-10</sup> and 4,4'-azobis-4-cyanovaleric acid (ACVA)<sup>4,6</sup> solutions to create the peroxy radical oxidation environment.<sup>11</sup> These azonitrile radical initiators, which have been used for many years in polymer chemistry,<sup>12-14</sup> thermally decompose to expell nitrogen, leaving two cyanoalkyl radicals that can rapidly react with oxygen to form peroxy radicals (Scheme 1). Recently, we have reported another forced degradation procedure which generates a peroxy radical-rich environment in solution, which does not utilize azonitrile-type radical initiators.<sup>15</sup> Our vision is to utilize these two complementary approaches in parallel to more confidently predict the intrinsic peroxy radical reactivities of drug molecules.

The current work focuses on fundamental aspects of the azonitrile radical initiator experiment in the hopes of making these experiments more tractable for general use by pharmaceutical scientists. Despite a small body of literature suggesting that such radical initiators are successful models of autoxidation in pharmaceutical formulations, there is not yet a consensus on the appropriate experimental conditions for the stress experiment. For example, different solvents and reagent concentrations are used, and some investigators advocate that azonitrile stress be conducted in a pressurized oxygen environment<sup>4,8</sup> while others conduct experiments under ambient atmosphere.<sup>5,6,9,10,16</sup> The adequate oxygenation of the stress solutions is a particularly important experimental variable. The assumption underlying the use of pressurized oxygen environments is that this will increase the oxygen concentration in solution and favor quantitative formation of peroxy radicals, improving the oxidative selectivity of the model system.<sup>11,17,18</sup> However, the literature data supporting the impact of this



Scheme 1.

practice is not extensive, and in fact, in his seminal work on this subject Boccardi specifically discourages the practice as unnecessary.<sup>6</sup>

The work reported herein explores the practical implications of azonitrile forced degradation under ambient and pressurized oxygen headspace as a function of initiator concentration. General guidelines are discussed to ensure adequate and efficient practices to provide sufficient solution oxygenation to ensure peroxy radical-dominated chemistry under experimental conditions relevant to preformulation and early method development work in an environment where only milligrams quantities of drug substance are available.

## EXPERIMENTAL

Solutions of compounds **1** and **2** were prepared at ~0.1 mg/mL concentrations (approximately 0.2 mM) together with 1, 5, 25, and 50 mM AIBN or ACVA in 50% water/50% acetonitrile solution. The low drug concentrations used in this study are consistent with the milligrams quantities available in early drug development when initial forced stress studies are most commonly initiated.<sup>3</sup> It should be noted that this low drug concentration maximizes the percent degradation for a given amount of initiator, allowing more moderate stress conditions, and result in differences in product distributions. Aliquots of each solution were stored at 40°C under ambient atmosphere and also at 50 psi oxygen in a pressurized reaction vessel. Samples of **1** and 5 mM solutions were taken at 4, 8, 24, 48, and 72 h for samples stored under both atmospheric environments. Twenty five millimolar and 50 mM solutions were sampled at 4, 8, and 24 h only due to a greater extent of reactivity in these more concentrated solutions. Control samples were stored at 5°C, preventing the decomposition of the radical initiators. Analysis of all samples was conducted without prior quenching or dilution using the stability-indicating HPLC/UV assays developed for each compound and shown to be selective for the oxidative degradants observed in formulations. Quantitation of the active and degradant peaks was by UV peak area and assumed an equal response from the degradant relative to the parent compound. More detailed degradation kinetics were also measured under ambient atmosphere by placing the solutions described above in an HPLC sample tray at 40°C, and measuring active and degradant levels

as a function of time of injection over the course of several days of repeated sample injections. In these latter experiments, care was taken to ensure that a headspace of ~1/2 the vial volume was available, simulating the conditions of the oxygen consumption experiments described below. Selected stress experiments were repeated in the presence of equimolar amounts of BHT to quench peroxy radical reactivity and accentuate alkylhydroperoxide chemistry.

Oxygen consumption kinetics were measured by NIR field modulation spectroscopy with a Lighthouse Instruments FMS-760 headspace gas analyzer.<sup>19</sup> The 1, 5, 25, and 50 mM stock solutions of AIBN and ACVA in 50% acetonitrile/50% water were examined for their intrinsic oxygen consumption behavior. Twenty-five milliliters of each solution was placed in a 50 mL glass vial (Verretubex, Nogent le Roi, France) with teflon rubber stopper and crimp seal (West Pharmaceutical Services, Lionville, PA). These containers have been previously demonstrated to provide an air-tight seal over long periods of storage.<sup>19</sup> Control samples were prepared under nitrogen and ambient atmosphere both empty and with 25 mL of 50% acetonitrile/50% water to further ensure the seal integrity of this container type. Another set of samples was prepared with a magnetic stir bar inside the vial and agitated during the storage period. Oxygen headspace measurements were taken spectrophotometrically through the sealed vials daily for 1 week. All solutions were stored at 40°C between measurements. All results have been normalized to the oxygen measurements for the 50% acetonitrile/50% water control samples, and are presented as fraction of initial.

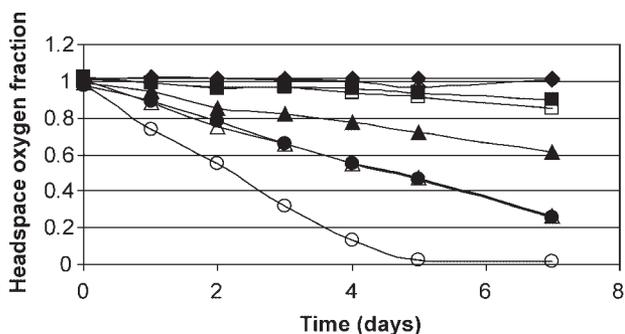
The hydroperoxide content of selected reaction samples was measured by reaction with triphenylphosphine (TPP) followed by HPLC assay.<sup>20,21</sup> The reaction of TPP with H<sub>2</sub>O<sub>2</sub> or ROOH is rapid and forms triphenylphosphine oxide (TPO) with 1:1 stoichiometry. TPP reaction with typical peroxides ROOR is very slow at room temperature.<sup>22</sup> The TPO product is easily resolved from the TPP parent. Quantitation of the amount of TPP consumed gives the amount of hydroperoxide present. TPP was prepared at 0.2 mg/mL in 100% methanol. One or 2 mL of the sample to be measured is then pipetted to a 10.0 mL volumetric flask and the flask brought to mark with the 0.2 mg/mL TPP solution. Reaction is allowed to take place at room temperature for 15 min prior to HPLC chromatographic assay. TPP peak area in

samples is compared to the TPP peak area in the appropriate control. HPLC conditions employed were: column, 5 cm  $\times$  4.6 mm Phenomenex Polar RP; mobile phase 75/25 methanol/water, flow 1.0 mL/min, detection wavelength 203 nm. TPP elution time was near 8.0 min with TPO eluting near 2.0 min.

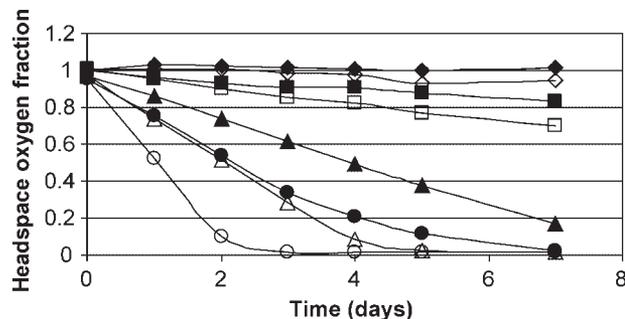
## RESULTS

### Oxygen Consumption Kinetics of AIBN and ACVA Solutions

Figure 1 shows the headspace oxygen concentration for 1–50 mM AIBN solutions in 50/50 acetonitrile/water as a function of time at 40°C. Higher concentrations of initiator give approximately linear increases in oxygen consumption, as expected assuming a first order rate of initiator decomposition. Figure 1 shows that less than 10% of the oxygen in the headspace is consumed over 1 week in the 1 and 5 mM AIBN cases, while nearly 80% of the headspace oxygen is consumed in the 50 mM AIBN case in the same time period. Figure 2 shows the analogous data for ACVA. The oxygen consumption patterns are similar to AIBN, but ACVA solutions consume oxygen approximately twice as fast as AIBN solutions under these conditions. Figures 1 and 2 also show data for stirred AIBN and ACVA solutions. Stirring facilitates more effective mass transfer between the headspace and the solution. The oxygen consumption of stirred solutions increases



**Figure 1.** Headspace oxygen concentration as a function of time for 50% acetonitrile solutions containing (◆) 1 mM AIBN, (◇) 1 mM AIBN with agitation, (■) 5 mM AIBN, (□) 5 mM AIBN with agitation, (▲) 25 mM AIBN, (△) 25 mM AIBN with agitation, (●) 50 mM AIBN, and (○) 50 mM AIBN with agitation. Solutions were stored at 40°C and either stirred or left static in between measurements. Oxygen levels are normalized to the results measured each day for a 50% acetonitrile control sample.

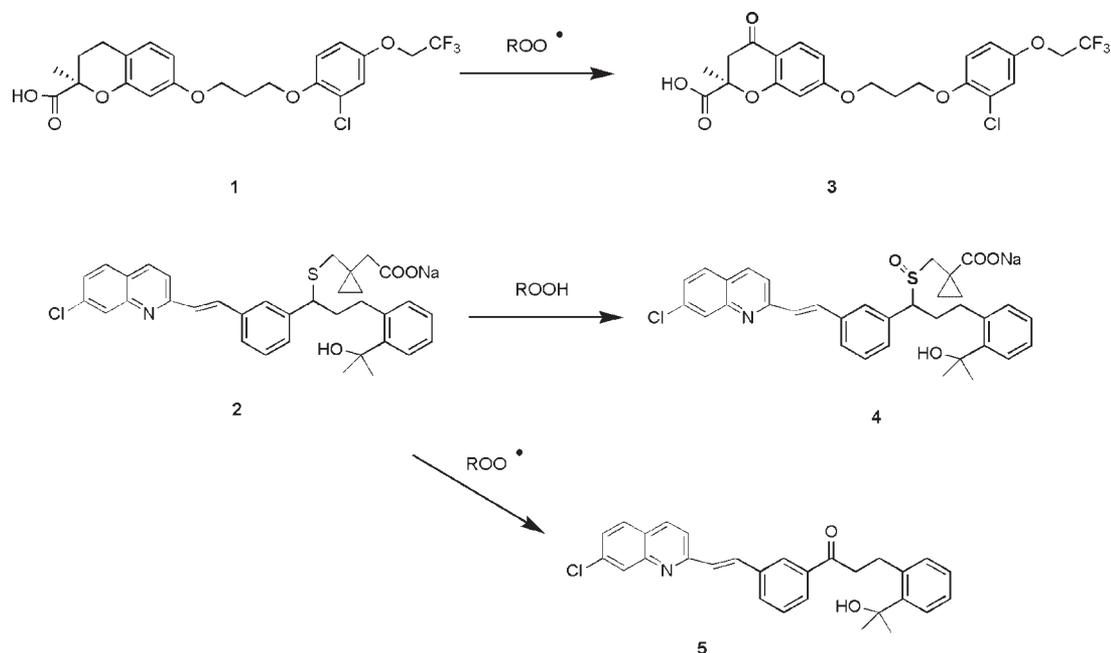


**Figure 2.** Headspace oxygen concentration as a function of time for 50% acetonitrile solutions containing (◆) 1 mM ACVA, (◇) 1 mM ACVA with agitation, (■) 5 mM ACVA, (□) 5 mM ACVA with agitation, (▲) 25 mM ACVA, (△) 25 mM ACVA with agitation, (●) 50 mM ACVA, and (○) 50 mM ACVA with agitation. Solutions were stored at 40°C and either stirred or left static in between measurements. Oxygen levels are normalized to the results measured each day for a 50% acetonitrile control sample.

moderately, most notably for the more concentrated initiator solutions. To our knowledge, the data in Figures 1 and 2 is the first oxygen consumption data obtained in this context. Ambient oxygen levels remain within 10% of their initial values over 3 days at 40°C using either 1 or 5 mM ACVA or AIBN initiators.

### Degradation Profiles under Ambient and Pressurized Oxygen Atmospheres

The implications of the oxygen consumption data presented above were explored by measuring the degradation profiles and degradation rates of two drug molecules as a function of AIBN and ACVA concentration. The two molecules chosen for this investigation are shown in Scheme 2. Compound 1 is a sodium carboxylate salt with several aromatic and ether groups. It has generally been formulated in an amorphous state, accentuating its intrinsic oxidative chemistry. Only a single oxidative degradant has been observed for this compound in a wide variety of tablet formulations. This degradant (3) has been identified as a ketone ( $M + 14$  relative to compound 1), formed by peroxy radical abstraction of hydrogen atom(s) at the single available benzylic position of the molecule. Phenolic antioxidants have been shown to reduce but not eliminate this reactivity. Compound 2 possesses a thioether moiety that has been shown to be the origin of the majority of its oxidative reactivity *via* nucleophilic attack on hydroperoxides present in formulations to result in sulfoxide degradant 4. A minor degradant



Scheme 2.

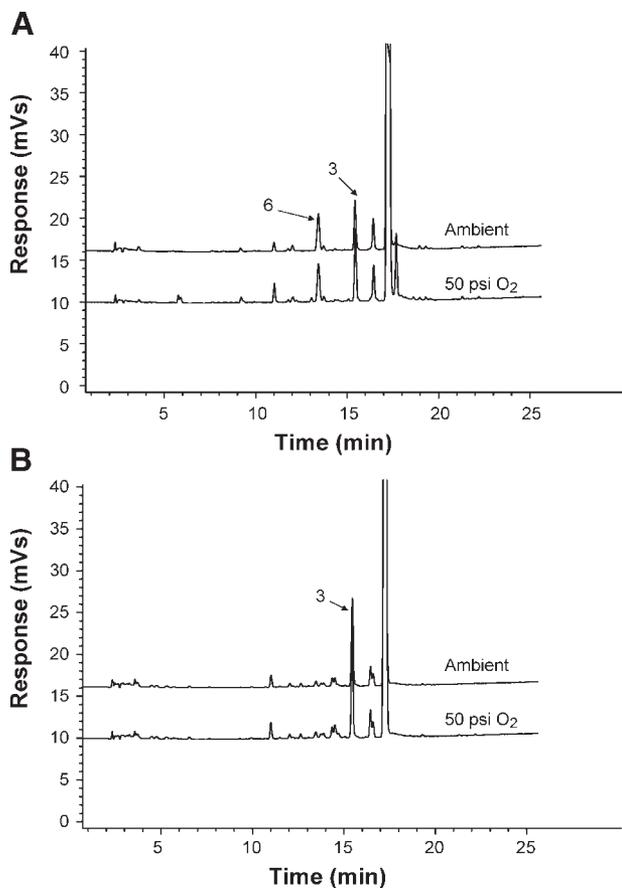
(5) is also observed in some cases. This degradant results from peroxy radical mediated hydroperoxide or alcohol formation at the benzylic position alpha to the thioether functional group with subsequent hydrolysis to form a ketone product.

Figure 3A shows degradation profiles of compound 1 induced by 1 mM AIBN under both ambient and 50 psi oxygen atmospheres after a 48 h stressing period. With the exception of one unknown degradant peak eluting after the active peak, which is present at higher levels in the pressurized oxygen sample, the chromatograms are essentially identical. Figure 3B shows similar chromatograms for ACVA, which are also essentially identical. Figure 4A and B shows chromatograms of the degradation profile for compound 2 under identical conditions as described in Figure 3. Again there is no significant differences in the degradation profiles obtained under ambient versus 50 psi oxygen atmospheres. Figure 5 examines higher initiator concentrations but a shorter stressing period. Figure 5 compares 25 mM AIBN and ACVA induced degradation profiles (compound 1) obtained after a 4 h stressing interval under both ambient and 50 psi oxygen atmospheres. No significant differences in the chromatograms are obvious as a function of oxygen pressure, and the degree of degradation and product distributions observed are very similar to those shown in Figure 3. Figure 6 shows analogous data as shown in Figure 5 for degradation profiles

of compound 2. Figure 6 shows no significant differences in the compound 2 degradation profiles at ambient versus 50 psi oxygen pressures.

#### Kinetics of Formation of Ketone 3 and Sulfoxide 4 in AIBN and ACVA Systems

Figure 7 plots the formation kinetics of the ketone degradant 3 over time in AIBN and ACVA concentrations ranging from 1 to 50 mM under ambient oxygen pressures. The formation rate of degradant 3 increases with initiator concentration, but does not increase linearly. The rate of degradant 3 formation is about twofold faster in AIBN than in ACVA under the same experimental conditions. Figure 8 plots the formation kinetics of sulfoxide degradate 4 under the same experimental conditions. In general, the formation rates of the sulfoxide 4 are more similar in the AIBN and ACVA systems. Repeat of the 5 mM AIBN/ACVA stress of both compounds 1 and 2 in the presence of 5 mM BHT significantly quenches the formation of peroxy radical mediated degradants 3 (>100 fold slower reactivity) and 5 (>50 fold slower reactivity), but has little effect on the formation rates of sulfoxide degradant 4 (Fig. 9). The presence of BHT significantly reduces the peroxy radical mediated reaction rates by sacrificial reactivity that reduces the steady-state peroxy radical concentration in the system. However, this sacrificial reactivity would not be expected to reduce the

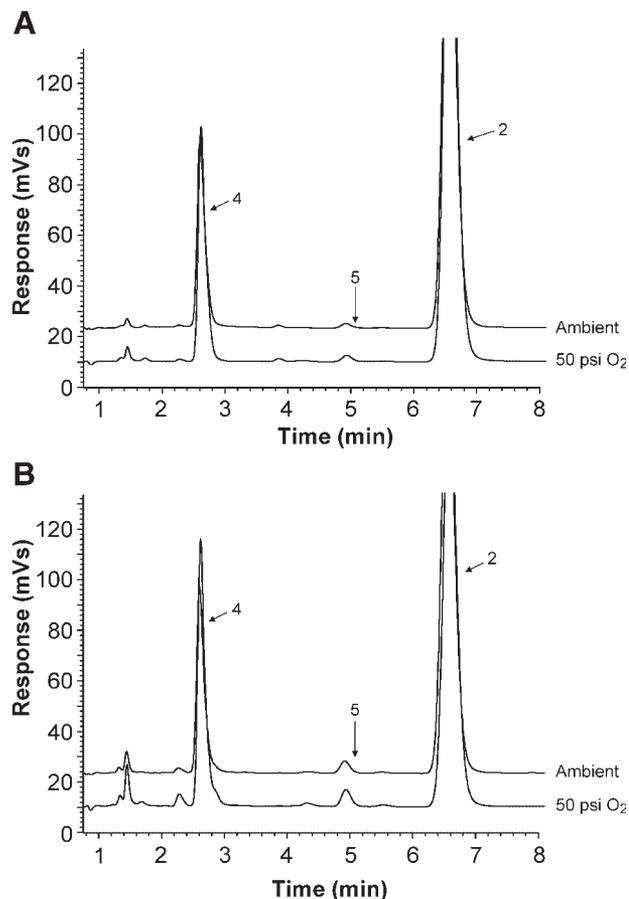


**Figure 3.** Degradation profiles of compound **1** resulting from 48 h of forced degradation at 40°C in 1 mM (A) AIBN and (B) ACVA solutions under ambient and pressurized oxygen headspace.

amounts of alkyl hydroperoxides formed in the reaction, and reactivities arising from nucleophilic reactions toward alkylhydroperoxides are not significantly impacted by the presence of BHT.

#### Hydroperoxide Formation in AIBN and ACVA Initiated Solvent Systems

Even in the absence of added drug substances, AIBN and ACVA initiated solvent systems show measurable levels of hydroperoxide formation (ROOH, where R=H (hydrogen peroxide) or R=alkyl). In the current work, triphenylphosphine was used to measure the hydroperoxide concentrations as a function of stressing time at 40°C in 5 mM AIBN and 5 mM ACVA solutions of 50/50 acetonitrile/water, and 80/20 acetonitrile/water. Hydroperoxide values increase linearly over time, Table 1 below summarizes the slopes of the plots of the mM ROOH found verses days at 40°C. Formation of

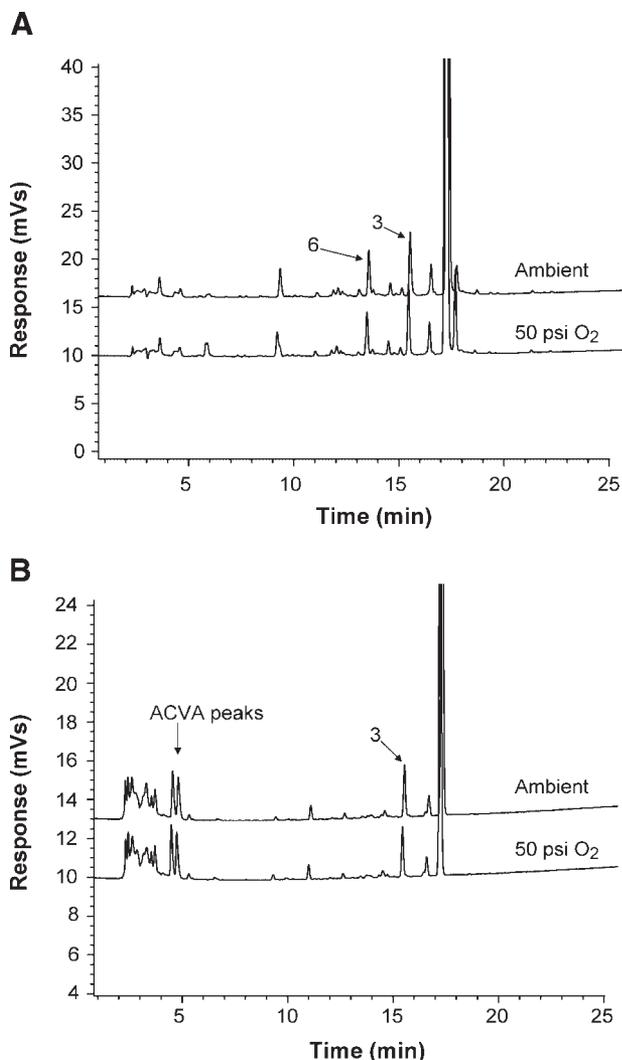


**Figure 4.** Degradation profiles of compound **2** resulting from 48 h of forced degradation at 40°C in 1 mM (A) AIBN and (B) ACVA solutions under ambient and pressurized oxygen headspace.

hydroperoxides in these solvents implies some solvent-related species is able to donate hydrogen atoms to the peroxy radicals formed via Scheme 1. Table 1 shows that ACVA forms twice the amount of hydroperoxides as AIBN under identical solvent conditions. In each case, there is also a solvent dependence, with more hydroperoxides formed at higher acetonitrile content.

#### Solvent Effects in AIBN Degradation Profiles

In Figures 3 and 5, the AIBN degradation profiles show a significant amount of a nonrepresentative species **6**. LC/MS analysis revealed that this degradant has a molecular weight 26 amu less than compound **1**. Significant amounts of this degradant were observed only with AIBN and not with ACVA. This is shown more clearly in Figure 10 by comparison of chromatograms A and C. However, chromatogram B in Figure 9 shows that raising the



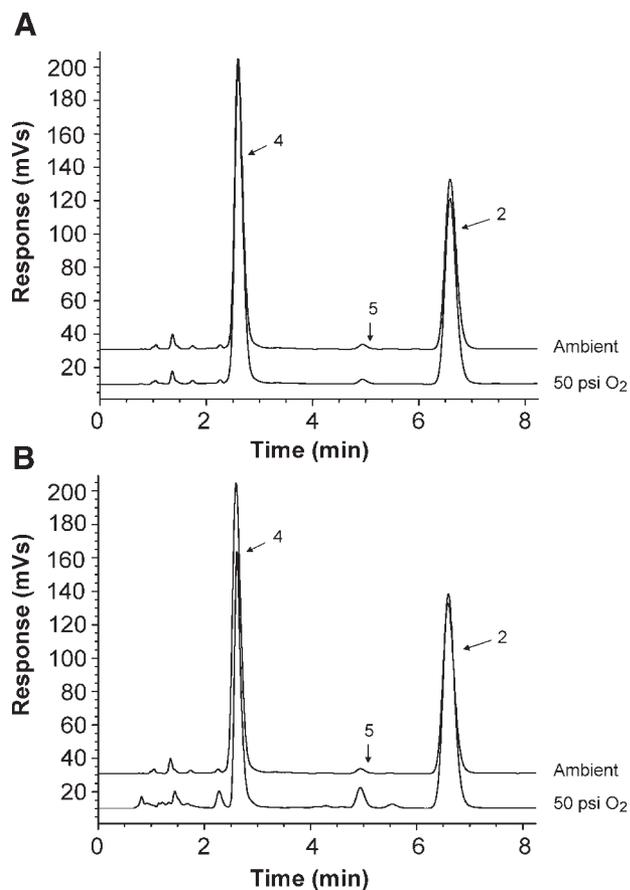
**Figure 5.** Degradation profiles of compound 1 resulting from 4 h of forced degradation at 40°C in 25 mM (A) AIBN and (B) ACVA solutions under ambient and pressurized oxygen headspace.

acetonitrile content in the solvent decreases the relative abundance of species 6 in the case of AIBN. This solvent dependence is explicitly demonstrated in Figure 11 for the AIBN case, in which the intensity of the species 6 peak progressively decreases with increasing acetonitrile content of the solvent.

## DISCUSSION

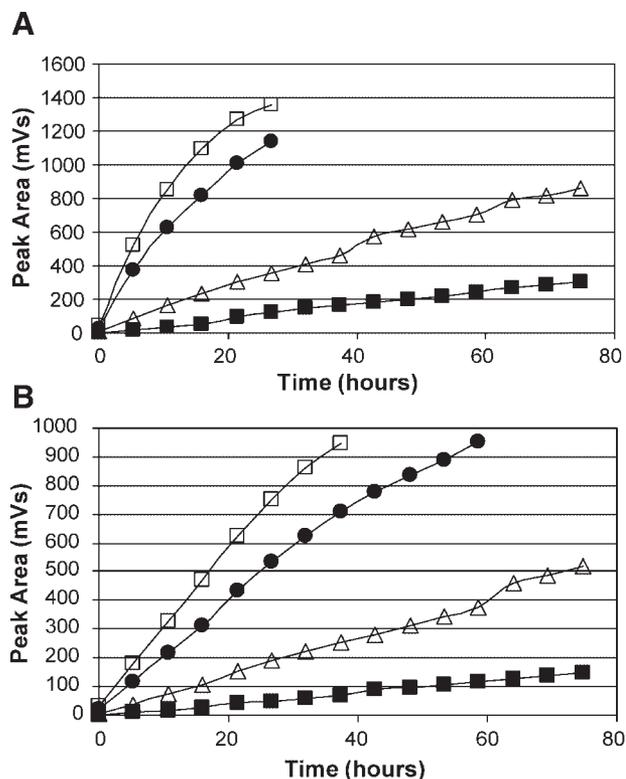
### Use of Ambient Oxygen Atmospheres in AIBN and ACVA Initiated Oxidation

The practice of using pressurized oxygen headspace<sup>4,8</sup> during the azonitrile-initiated stress



**Figure 6.** Degradation profiles of compound 2 resulting from 4 h of forced degradation at 40°C in 25 mM (A) AIBN and (B) ACVA solutions under ambient and pressurized oxygen headspace.

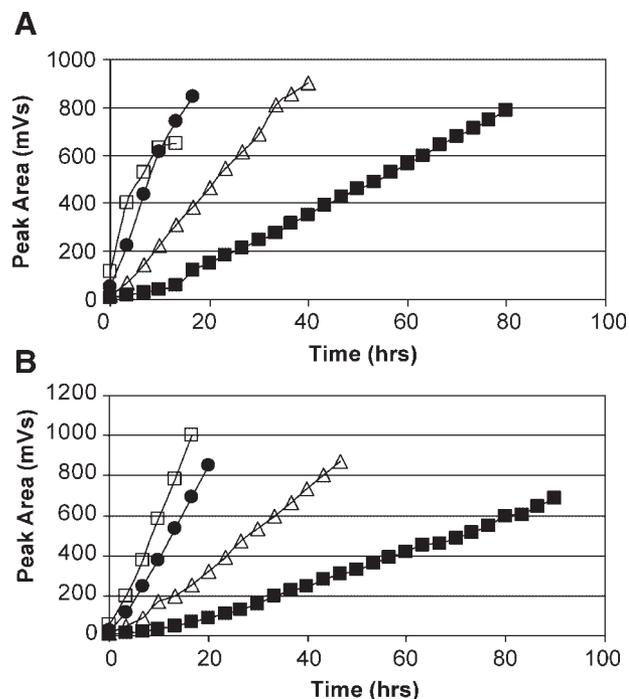
experiment has presumably arisen from a desire to maximize the formation of alkyl hydroperoxy radicals in the belief that such species provide the most representative model of autoxidation. Indeed, this is a reasonable procedure and is consistent with the current understanding of peroxy radicals as the central species involved in solid-state oxidative degradation in pharmaceutical formulations.<sup>11</sup> In this work, the “typical” azonitrile-based oxidative forced degradation experiment we envision is a 2–3 day stressing period, 1–5 mM initiator concentrations, with low concentrations of drug substance (~0.2 mM) reflecting limited drug supply at early stages of drug development. Stopped flasks are filled 50% full of the stressing solution during heating, leaving 50% ambient headspace. Under these conditions, the moles of oxygen in the flask is about 50-fold greater than the moles of drug substance in the flask. Further, the moles of



**Figure 7.** Formation kinetics of ketone degradant **3** at 40°C in 50% acetonitrile solutions containing (■) 1 mM, (△) 5 mM, (●) 25 mM, and (□) 50 mM concentrations of (A) AIBN and (B) ACVA. The time-points shown represent the initial linear portion of the kinetic profiles only.

initiator in the flask is 5–20 fold greater than the moles of drug substance present. Given that most of the drug substance typically remains unreacted over the stressing period, in this limit the observed oxygen consumption is dominated by the initiator decomposition (and subsequent oxygenation as shown in Scheme 1). In this context, the data in Figures 1–6 show there is ample oxygen to conduct such stressing procedures with ambient oxygen atmospheres.

At much higher drug and initiator concentrations, this assumption may not be true and the relevance of the data in Figures 1 and 2 would need to be evaluated. These results show that the use of 25–50 mM initiator concentrations deplete the ambient oxygen levels as the duration of the stressing experiment approaches 24 h. However, it is important to note that in the 25 and 50 mM initiator solutions, compound **1** was near 100% degraded in 24 h, and no attempt was made to monitor the further secondary reactivity once the active had been consumed. Examination of the

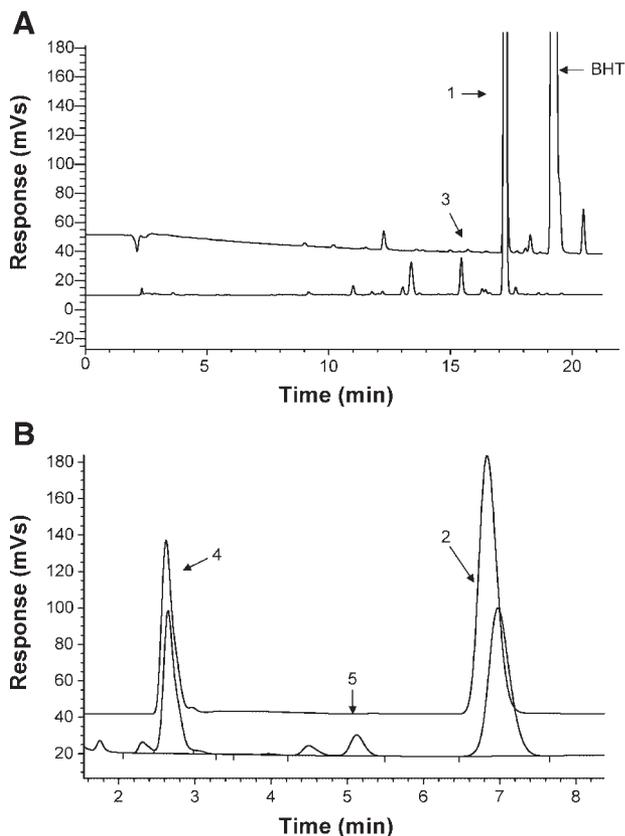


**Figure 8.** Sulfoxide degradant **4** formation kinetics of compound **2** at 40°C in 50% acetonitrile solutions containing (■) 1 mM, (△) 5 mM, (●) 25 mM, and (□) 50 mM concentrations of (A) AIBN and (B) ACVA.

oxygen consumption kinetics presented above for these initiator concentrations reveals that the headspace oxygen is not yet half consumed after 24 h. A more significant divergence between ambient and pressurized O<sub>2</sub> results would have been expected in the more concentrated solutions (Figs. 5 and 6) if the reactivity were carried out over longer times when the oxygen was more fully depleted from the system. Thus, these results illustrate that even moderately concentrated initiator solutions leave a window of acceptable oxygen availability. Because these more concentrated initiator solutions are likewise more reactive, it is likely that the desired extent of reaction will be accomplished during this period of oxygen availability.

### Comparison of AIBN and ACVA Forced Degradation Results

The present study has been conducted with each of the two most popular azonitrile initiators used for oxidative forced degradation,<sup>3</sup> and presents the natural opportunity to draw comparisons between the results obtained for the two initiators under identical experimental conditions.

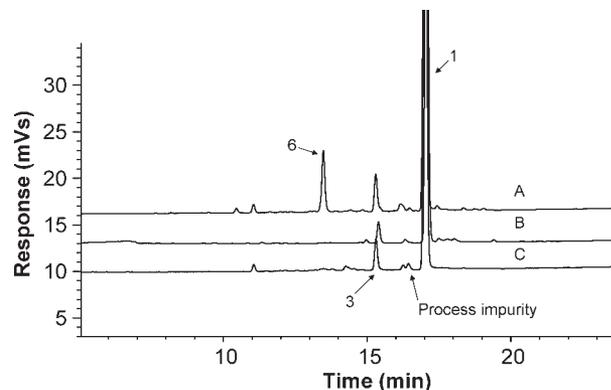


**Figure 9.** Degradation profiles of (A) compound **1** and (B) compound **2** stressed for 2 days at 40°C in 5 mM AIBN solutions with (top) and without (bottom) 5 mM added BHT. All chromatograms are depicted on the same scale. BHT elutes at 19 and 10 min (off scale), respectively.

In general, both radical initiators are successful models of autoxidation and reproduce the oxidation chemistry observed for compounds **1** and **2** in pharmaceutical formulations. However, several key differences stand out between AIBN and ACVA observations. The initial rates of oxygen consumption for the stirred solutions of AIBN and ACVA are linear with initiator concentration ( $r^2 > 0.99$ ). The stirred ACVA solutions consume oxygen approximately twice as fast as the AIBN

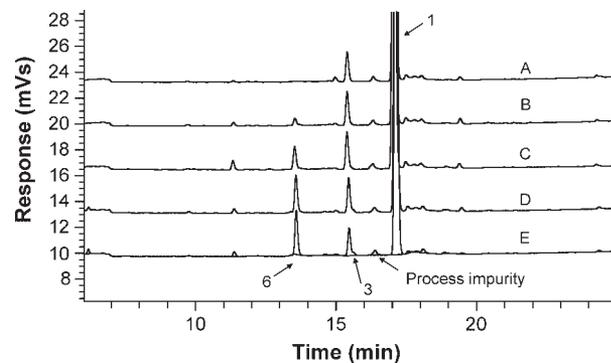
**Table 1.** Measured ROOH Levels in AIBN and ACVA Solutions

Initiator (5 mM)	Solvent System (Acetonitrile/Water)	mM ROOH Formed (Per Day at 40°C)
AIBN	50/50	0.065
AIBN	80/20	0.090
ACVA	50/50	0.130
ACVA	80/20	0.160



**Figure 10.** Comparison of chromatograms of compound **1** stressed at 40°C (A) for 3 days in 50% acetonitrile solution containing 1 mM AIBN, (B) for 4 days in 90% acetonitrile solution containing 0.5 mM AIBN, and (C) for 3 days in 50% acetonitrile solution containing 1 mM ACVA. Starting concentrations of compound **1** were equivalent and all chromatograms are depicted on the same scale.

solutions at a given concentration ( $2.0 \times 10^{-3}$  mmol  $O_2$ /day·mM<sub>ACVA</sub> vs.  $9.1 \times 10^{-4}$  mmol  $O_2$ /day·mM<sub>AIBN</sub>). In this regard, it is interesting to note the data in Table 1, which shows that ACVA also forms hydroperoxides at twice the rate as AIBN under similar conditions. In both cases, the calculated millimoles of oxygen consumed per day is about threefold greater than the millimoles of hydroperoxides formed per day. This agreement is reasonable given system and experimental uncertainties, and the remaining portion of the oxygen mass balance is explained by the formation of alcohol or ketone/aldehyde products via peroxy radical termination reactions.



**Figure 11.** Overlaid chromatograms of compound **1** stressed at 40°C for 4 days in (A) 90%, (B) 80%, (C) 70%, (D) 60%, and (E) 50% acetonitrile solutions, each containing 0.5 mM AIBN.

However, the more rapid oxygen consumption and hydroperoxide formation for ACVA is not translated into more rapid rates of degradation induced for the test compounds of this study. For the peroxy radical mediated oxidation reactivity of compound **1**, reaction rates are two to three times *slower* with ACVA than for AIBN. For example, a 5 mM AIBN solution in 50%acetonitrile stored at 40°C showed 5%/day formation of degradant **3**, 8%/day formation of degradant **6**, and 19%/day collective formation of all other minor degradants. The equivalent ACVA stress solutions resulted in a much cleaner profile with 3%/day formation of degradant **3**, no significant formation of **6**, and 4%/day collective formation of all other minor degradants. In the AIBN and ACVA cases, the formation of degradant **3** is proportional to initiator concentration to the 0.6 power, which is approximately consistent with the theoretical  $\frac{1}{2}$  rate order with respect to initiator concentration. This  $\frac{1}{2}$  rate order with respect to initiator concentration arises from competition between chain propagation reactions (whose rates are directly dependent on initiator concentration) and chain termination reactions (whose rates depend on initiator concentration squared) (Fig. 5).<sup>11,17,23,24</sup> In the AIBN case, the total degradation rate of compound **1**, however, displayed a more linear initiator concentration dependence. If the formation rate of **3** is factored out of the AIBN data, the net rate is essentially linear with respect to initiator concentration. This distinction in rate order between degradant **3** and the other collective degradates serves as a caution that suggests that they may result from different chemical processes. For example, it is plausible to suspect the involvement of alkoxy radicals in the generation of some of the numerous miscellaneous degradant species seen in addition to degradant **3**. Such species arise from the disproportionation of tertiary peroxy radicals (i.e., peroxy radicals unable to undergo Russell termination<sup>25</sup> to produce alcohol/ketone products) such as those produced by oxygen addition to AIBN or ACVA decomposition products (Scheme 1), and are thermodynamically much more aggressive/less selective hydrogen atom abstractors, resulting in more numerous products. Because the formation of alkoxy radicals depends on the bimolecular disproportionation of two tertiary peroxy radicals, these alkoxy radicals would be expected to play a more significant role at higher initiator concentrations, compensating for the diminishing returns of direct peroxy radical reactivity.

The rates of the nucleophilic oxidation reactivity of compound **2** are similar for AIBN and ACVA, despite our observations of faster oxygen consumption and hydroperoxide levels measured by reaction with triphenylphosphine (Tab. 1). In both the AIBN and ACVA cases, the sulfoxide formation rate dependence on initiator concentration is similar to that observed for the ketone **3** described above. This is interesting as the degradation chemistries of **1** and **2** depend on different reactive species (ROO and ROOH, respectively). However, both reactivities depend on the hydrogen abstraction reactivity of peroxy radicals. In the former case, the steady-state concentration of peroxy radicals generated by the azonitrile stress system participate directly in reaction with compound **1**, while in the later case the alkyl hydroperoxides produced by the reactions of peroxy radicals are the reactive species. It is thus reasonable that both compounds **1** and **2** would display initiator concentration dependences proportional to the steady-state concentrations of peroxy radicals in the system.

#### Sulfoxide **4** Formation: Reaction With Hydroperoxides Byproducts of Peroxy Radical Oxidation

It is instructive to consider in some detail the formation of the sulfoxide **4**. The oxidation of the thioether group of compound **2** is quite rapid, with primary oxidation to the sulfoxide **4** occurring quantitatively over several minutes time in 1% hydrogen peroxide solutions even at 5°C. The oxidation of sulfides with hydrogen peroxide has been a common synthetic method for the production of sulfoxides and sulfones since the first reports of this reactivity in 1908 by Gazdar and Smiles<sup>26</sup> and Hinsberg.<sup>27</sup> The related reactivity with alkyl hydroperoxides has also been reported widely in the literature and used as a synthetic method.<sup>28</sup> This latter reactivity of sulfides (and to a lesser extent amines<sup>29–31</sup>) toward hydroperoxides is a key path to sulfoxide and nitroxide degradants in pharmaceutical formulations,<sup>11</sup> and results from the formation of hydrogen peroxide and alkyl hydroperoxides as byproducts of autoxidation. It is generally recognized that the oxidation reaction between hydroperoxides and thioethers proceeds by nucleophilic attack on the peroxide oxidant by the thioether functional group of the oxidized molecule,<sup>32,33</sup> rather than by a free radical process such as that responsible for the autoxidation of compound **1**. Sulfoxide

degradates may also arise from direct reaction with peroxy radicals.<sup>11,34–36</sup> However, control experiments in which the lifetime (and thus steady-state concentrations) of peroxy radical levels have been reduced by the addition of antioxidant demonstrate that the reaction of compound **2** is not sensitive to peroxy radical concentration. In contrast, antioxidant inhibited forced stress experiments conducted under the same conditions reduce the rate of degradant **3** formation by a factor of >100, consistent with the presumption of peroxy radicals involvement in the rate limiting reaction step.

The reactivity of compound **2** in AIBN or ACVA systems is completely dominated by sulfoxide **4** formation. That is, the molar amount of compound **2** lost is essentially equal to the molar amount of the sulfoxide **4** formed. The formation of the peroxy radical mediated degradant **5** for compound **2** is very small in the AIBN and ACVA degradation experiments. If the sulfoxide **4** is formed from nucleophilic attack of the thioether group on ROOH groups, then hydrogen atoms must be donated to peroxy radicals by the solvent. This is the nature of the data shown in Table 1, which highlights the fact that measurable levels of ROOH groups are formed in these solvent systems without added drug substances. The quantitative agreement between the ROOH levels determined in Table 1 and the sulfoxide degradant **4** levels in Figure 8 are reasonable. For example, 5 mM AIBN and ACVA solutions form about 0.03 mM/day sulfoxide **4** degradant, while Table 1 shows 0.065–0.13 mM ROOH formation per day under similar conditions. Thus, the intrinsic reactivity of the solvent toward peroxy radicals formed in Scheme 1 produces enough ROOH species to account for the molar amount of sulfoxide **4** formation.

A cursory examination of the thermochemistry literature would not seem to support the above proposal of peroxy radical reactions with the solvent systems used in this study. Current estimates of the bond dissociation energy of acetonitrile indicates that it is expected to lie several kcal/mol endothermic with respect to hydrogen atom abstraction by peroxy radicals.<sup>37–39</sup> However, some abstraction may take place, driven by the high concentration of solvent molecules and elevated temperature of the experiments. Further, the solvent is present in such a great molar excess that even minor impurities in the solvent could serve as hydrogen atom donors to the extent necessary to explain the observed concentrations of hydroperoxides. A number of such potential

hydrogen atom donor molecules have been reported in the literature as trace impurities in acetonitrile.<sup>40</sup>

### Solvent Dependence of AIBN Degradation Profile

One significant additional nonrepresentative degradant **6** was observed in some AIBN samples (Figs. 3A and 5A). This peak was not observed in any ACVA samples. The presence of this extra peak was found to be strongly associated with solvent composition only for the AIBN case; being larger than **3** in 50% acetonitrile/50% water solutions, and decreasing with increasing acetonitrile concentration until disappearing entirely at 90% acetonitrile (Figs. 10 and 11). Degradant **6** is also significantly reduced in relative abundance when forced degradation is conducted with higher drug concentrations. The qualitative results for AIBN stress in 90% acetonitrile are cleaner and more generally similar to those for ACVA in 50% acetonitrile (i.e., in the absence of the solvent-dependent AIBN degradant **6**, Fig. 10). It is unclear why these two very similar initiators should have these differences. Regardless of the true cause of the differences between AIBN and ACVA, these differences do not stand as a major impediment to the productive use of either initiator for forced stress studies. And in fact, the differing solubility properties of the two initiators make them useful as a complementary pair with generally analogous chemistry.

### CONCLUSIONS

Acetonitrile-type radical initiators hold much promise in their ability to quickly and simply generate relevant oxidative degradation profiles to aid in the development of stability-indicating chromatographic methods. However, clear understanding of the impact of experimental variables is required to achieve the maximum insight from the forced degradation experiment. This work represents a critical evaluation of one of the key experimental variables, the oxygenation of solutions during the forced degradation experiment. This subject is of fundamental importance to the rational pursuit of a forced degradation model simulating autoxidation, because the peroxy radicals formed by the addition of oxygen to the primary cyanoalkyl radicals are best able to mimic the reactivity of the peroxy radicals responsible for autoxidation in pharmaceutical

formulations. Our findings suggest that there is a great deal of flexibility with respect to how this oxygenation is conducted. Further, because ambient air can provide sufficient oxygenation in most cases, the forced degradation experiments can be freed from the rigid constraints of pressurized reaction environments without fear of compromising the quality of the results. Ambient oxygenation of solutions is especially robust at low initiator concentrations (e.g.,  $\leq 5$  mM), and we have found these concentrations to provide a peroxy radical environment sufficient to simulate the autoxidation of pharmaceutical formulations. As the initiator concentration is raised, the yield of peroxy radical reaction products provide a diminishing return of peroxy radicals while simultaneously increasing the potential of undesirable side reactions.

The results presented in this work provide increased clarity about several of the key parameters of the azonitrile forced degradation experiment. However, more work remains to be done before these systems can truly be considered well characterized models of pharmaceutical oxidation. For example, it appears clear that solvent can participate in the oxidation reaction, both by reacting in competition with the drug molecules and by modulating the observed reactivity and rate of reaction. Future investigations will seek to increase understanding this and other aspects of the azonitrile forced degradation experiment.

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