Artifacts Generated During Azoalkane Peroxy Radical Oxidative Stress Testing of Pharmaceuticals Containing Primary and Secondary Amines

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ABSTRACT: We report artifactual degradation of pharmaceutical compounds containing primary and secondary amines during peroxy radical-mediated oxidative stress carried out using azoalkane initiators. Two degradation products were detected when model drug compounds dissolved in methanol/water were heated to 40°C with radical initiators such as 2,2′-azobis(2-methylpropionitrile) (AIBN). The primary artifact was identified as an α-aminonitrile generated from the reaction of the amine group of the model drug with formaldehyde and hydrogen cyanide, generated as byproducts of the stress reaction. A minor artifact was generated from the reaction between the amine group and isocyanic acid, also a byproduct of the stress reaction. We report the effects of pH, initiator/drug molar ratio, and type of azoalkane initiator on the formation of these artifacts. Mass spectrometry and nuclear magnetic resonance were used for structure elucidation, whereas mechanistic studies, including stable isotope labeling experiments, cyanide analysis, and experiments exploring the effects of butylated hydroxyanisole addition, were employed to support the degradation pathways.

Keywords: artifacts; stress testing; forced degradation; oxidation; autoxidation; free radicals; chemical stability; strecker reaction; degradation products; mass spectrometry; stability

INTRODUCTION

A key component of pharmaceutical stress testing is the execution of simple solution-based experiments to reveal intrinsic hydrolytic and oxidative susceptibilities of new drug compounds.[1] Stress testing is used to support various aspects of drug development including development and optimization of stability-indicating methods and relevant control strategies. Identification of potential drug degradation products from solution-based experiments may also facilitate the risk assessment process for the evaluation of degradation products for potential mutagenicity (ICH M7).[2] It is therefore desirable that solution stress testing conditions trigger degradation pathways that are relevant not only to the drug substance, but also to the associated drug product dosage forms. Primary hydrolytic drug degradation products are often predicted via solution-phase chemistry,[3] however, evaluation of propensity of drugs to oxidize is more complex.[4–6] Peroxy radical mediated oxidation, a type of autoxidation, is induced by trace impurities (e.g., in various excipients) and is considered the most common route of drug oxidation.[6] The solution stress test involves peroxy radical formation via thermal decomposition of azo compounds and is designed to accelerate oxidative kinetics so as to induce in a short period of time an observable response for molecules susceptible to autoxidation induced by peroxy radical attack.[7] Because of the complexity of the oxidative system, the test has historically suffered from some selectivity issues in creating the peroxy radical activity, allowing artifactual degradation to be observed.[8] The current study represents another step in refining the test conditions and understanding its limitations.

Peroxy radicals, known to selectively abstract weakly-bound H atoms (bond dissociation enthalpy $< \sim$90 kcal/mol),[4,9] can be generated for the purpose of the stress test through thermal decomposition of radical initiators such as 2,2′-azobis(2-methylpropionitrile) (AIBN)[10,11] or 4,4′-azobis(4-cyanovaleric acid) (ACVA).[7,11] The subsequent, rapid reaction with dissolved oxygen generates the desired peroxy radicals as shown for AIBN in Scheme 1. Usually, the experiment is carried out in dilute drug solutions at 20 mol% or greater of the radical initiator.[12] The long-lived azoalkane-derived peroxy radicals may therefore encounter drug molecules from which they may abstract H atoms, but may also readily undergo recombination/decomposition as shown in Scheme 2. Characteristic to *tertiary* peroxy radicals, the recombination/decomposition of the azoalkane peroxy radicals leads to the undesired formation of alkoxy radicals, estimated to react 10^4–10^5 times faster with organic substrates, than the corresponding peroxy radicals.[13] As a result, the selectivity of the test for weakly bound H atoms is lost, as the drug may preferentially react with alkoxy radicals at a variety of sites within the drug molecule. For drug

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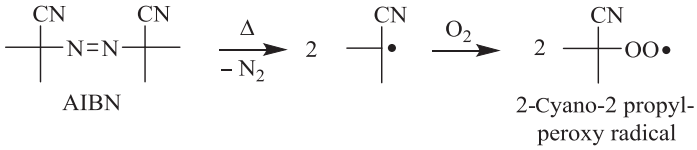


Scheme 1. Formation of peroxy radicals from AIBN.

compounds with little reactivity toward peroxy radicals, the reaction with alkoxy radicals therefore produces false positive test results.

The role of AIBN peroxy radicals in generating artifactual degradation was first indicated by Nelson et al.[8] and recently demonstrated by Watkins et al.[14] The authors showed that addition of a small amount of methanol to the solvent system effectively prevented the alkoxy radicals from encountering and reacting with the dilute drug substance, by providing a sacrificial H atom donor. The use of methanol has been generally adopted in the recent years[12] and has greatly improved the correlation of the azoalkane stress test results with the actual, long term, drug product stability results.

It is within this framework that we report significant non-oxidative, or artifactual degradation even with the use of methanol solvent, for several amine-containing model drug molecules oxidatively stressed using azoalkane initiators. By briefly exploring the effects of several experimental parameters on the artifact yield, including pH, initiator/drug molar ratio, and the type of the azoalkane initiator, we attempted to learn the significance of these findings to the pharmaceutical scientist conducting oxidative stress testing for amine-containing drug molecules. A variety of experiments were also conducted to investigate the degradation mechanisms, including isotopic labeling studies using $^{18}O_2$, methanol-d_3, and [^{13}C,^{15}N]-AIBN, cyanide analysis, and experiments that probed the impact of butylated hydroxyanisole (BHA), a peroxy radical scavenger, on artifact formation. Chemical structures proposed based on tandem and high-resolution MS data were subsequently confirmed using retention time, mass, and fragmentation pattern correlations with compounds that were synthesized for this purpose and characterized by nuclear magnetic resonance (NMR).

Materials

All chemicals were used as received. 2,2′-azobis(2-methylpropionitrile) (AIBN), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), (±) propranolol hydrochloride, (1R,2S)-(−)-2-amino-1,2-diphenylethanol (aminodiphenylethanol), fluvoxamine maleate, (±) baclofen, norfloxacin, carvedilol, potassium cyanate, iodoacetonitrile, BHA, methanol-d_3, $^{18}O_2$, and the following buffer materials: ammonium hydroxide, sodium hydroxide (pellets), hydrochloric acid (concentrated), potassium chloride, and sodium acetate, were obtained from Sigma–Aldrich (St. Louis, Missouri). Diphenhydramine hydrochloride and ethyl ether (anhydrous) were obtained from Fisher Scientific (Pittsburgh, Pennsylvania), methanol and acetic acid were obtained from J.T. Baker (Center Valley, Pennsylvania), and potassium phosphate monobasic, acetonitrile, formic acid, and trifluoroacetic acid, from Fluka (St. Louis, Missouri). [^{13}C,^{15}N]-AIBN (2,2′-azobis(2-methylpropio[^{13}C,^{15}N]nitrile)) was obtained from The Chemistry Research Solution (Bristol, Pennsylvania). Sodium cyanide was obtained from MP Biomedicals (Santa Ana, California), taurine was obtained from TCI America (Portland, Oregon), and naphthalene dialdehyde was obtained from Invitrogen (Carlsbad, California).

EXPERIMENT AND ANALYSIS

Azoalkane-Based Oxidative Stress Testing Conditions

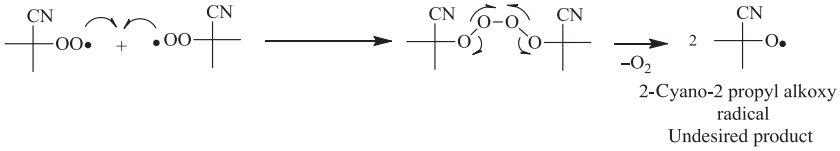
Several oxidative stress experiments were carried out for the purpose of this paper. In general, a methanol/water solution containing the model drug compound and the azoalkane initiator was thermally stressed at 40°C using AIBN or AAPH, and the artifact yield was monitored over a given period of time. Methanol solvent was used to quench the formation of the alkoxy radicals.

In Experiment 1, all seven model drug molecules (fluvoxamine maleate, aminodiphenylethanol, baclofen, norfloxacin, carvedilol, propranolol HCl, or diphenhydramine HCl) shown in Figure 1 were oxidatively stressed using either AIBN or AAPH as the azoalkane initiators. Solutions consisting of 0.2 mg/mL of each compound and 5 mM AIBN or AAPH in 55/45 (v/v) methanol/water or 55/45 (v/v) methanol/0.1 M KCl/HCl buffer (pH 2.0), acetate buffer (pH 4.0), or phosphate buffer (pH 6.0 or 8.0) were transferred to standard HPLC vials and placed on the autosampler tray. The experiment was conveniently performed by heating the sample vials directly on the autosampler tray, which was maintained at 40°C. At the preset time points of 4, 8 (or 12), 24, 48, and 72 h, each sample solution was analyzed by UPLC or HPLC, according to the procedure described below.

Experiment 2 was focused on determining the effect of the initiator excess relative to the model drug. Initiator/drug molar ratios between 2.5 and 50 mol/mol were examined using carvedilol solutions of 0.1, 0.25, 0.5, 1.0, and 2.0 mM, whereas AIBN was maintained at 5 mM. Measurements were conducted as described above, at a pH of 6.0.

In Experiment 3, 25 mM of BHA were added to solutions containing 0.2 mg/mL norfloxacin and 5 mM AIBN, to investigate the effect of the peroxy radical inhibitor on the artifact yield. Solutions were prepared in ACN/MeOH/water 8:1:1 (v/v/v) rather than MeOH/water, to minimize BHA inactivation through H-bonding with the solvent.[15]

Stress test experiments using isotopically labeled reagents are described separately below.



Scheme 2. Recombination/decomposition of AIBN peroxy radicals.

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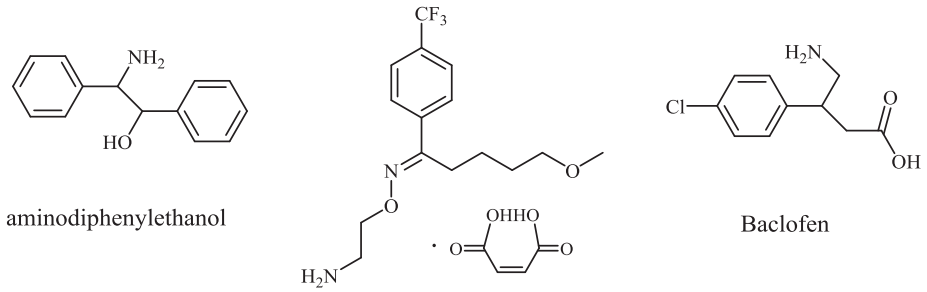
Primary Amine Model Drugs

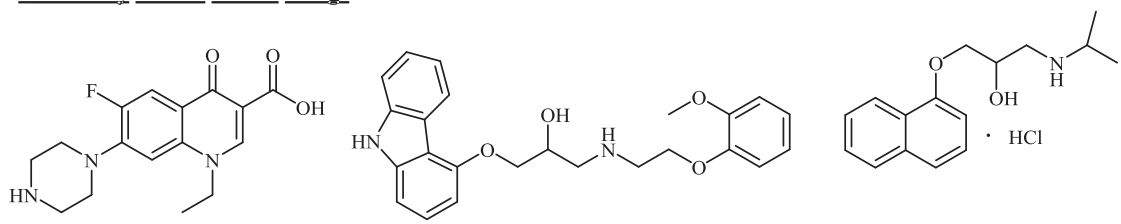


aminodiphenylethanol

Fluvoxamine maleate

Baclofen

Secondary Amine Model Drugs

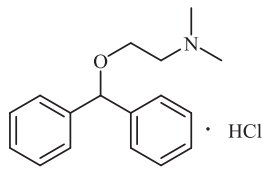


Norfloxacin

Carvedilol

Propanolol HCl

Tertiary Amine Model Drug



Benadryl HCl

Figure 1. Model drug compounds.

HPLC and Liquid Chromatography–Mass Spectrometry Analysis

Reversed-phase chromatography using C18 columns and gradient elution was carried out using either an Acquity UPLC® (Waters Corporation, Milford, Massachusetts) coupled to a single quadrupole mass detector or an Agilent 1200 HPLC (Agilent Technologies, Santa Clara, California) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Electron North America LLC, West Palm Beach, Florida). Both systems were equipped with binary pumps, heated autosampler trays and column compartments, and photodiode detectors. Chromatographic conditions were optimized for each drug and radical initiator and are compiled in Table S1 of the Supporting Information. The autosampler tray was held at 40°C as required for stress testing and the detection wavelength was the extracted λ_{max} for each compound (Table S1, Supporting Information). Artifact yields were calculated as peak area % relative to the sum of API-related peaks by UV, at the characteristic detection wavelengths of the model drug compounds. The mass of the reaction products was monitored by liquid chromatography–mass spectrometry (LC–MS), at unit resolution.

MS Analysis

High-resolution and tandem mass spectrometric experiments for structure characterization were carried out in the positive ion mode using an LTQ Orbitrap XL mass spectrometer (Thermo Electron North America LLC) coupled with an electrospray ionization source. A spray voltage of 4.5 kV, sheath gas flow rate of 45 (in arbitrary units), capillary voltage of 30 V, and capillary temperature of 275°C were used. High-resolution data were acquired using a resolving power of 30,000. Tandem MS experiments were performed using collision-induced dissociation mode with structure-dependent normalized collision energy setting of 20–30 (in arbitrary units). MS fragmentation results can be found in the Supporting Information.

Isotopic Labeling Experiments

Two oxidative stress experiments where either methanol or AIBN were replaced by their isotopically labeled counterparts methanol-d_3 and [^{13}C,^{15}N]-AIBN, respectively, were conducted using aminodiphenylethanol following the procedure described earlier for Experiment 1. In a third experiment, the stress test

was carried out using aminodiphenylethanol in an atmosphere enriched with $^{18}O_2$. The experiment was performed following the general procedure described earlier in Experiment 1 with the following deviations. Deionized water was used in the place of the buffer component of the sample solution. Deionized water, AIBN stock solution, and aminodiphenylethanol stock solution were individually purged with nitrogen, combined and placed into a Parr pressure vessel (Moline, Illinois) resulting in sample concentrations and solvent ratios described in Experiment 1. The Parr vessel was pressurized to approximately 100 psi with nitrogen gas and vented to atmospheric pressure and repeated five times. The Parr vessel was pressurized to approximately 22 psi using $^{18}O_2$ gas and placed into a 40°C oven for 72 h. All samples were analyzed by LC–MS. High-resolution and tandem mass spectrometry measurements were conducted to confirm the incorporation of the isotope labels into the degradation products. The characterization of the labeled products is presented in the Supporting Information.

Cyanide Analysis

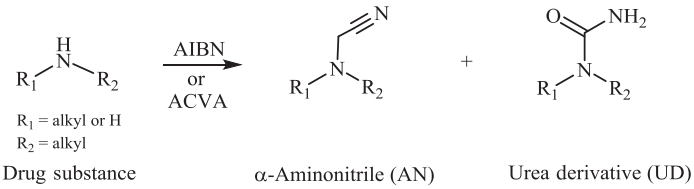
Cyanide levels were measured in thermally stressed samples of AIBN using a derivatization procedure similar to that described by Sano,[16] followed by UPLC® analysis of the resulting 2-cyano-1-ethanesulfonic acid benzoisoindole with UV detection. The amount of AIBN remaining was determined as peak area % relative to peak area at time zero. Details are provided in the Supporting Information.

Preparation of the Oxidative Artifacts for Structure Confirmation: Synthesis and Preparative SFC

Aminodiphenylethanol and propranolol HCl were reacted with iodoacetonitrile and potassium cyanate to produce the corresponding α-aminonitrile (AN) and urea derivative (UD), respectively. Product isolation for structure identification was performed using a Berger Minigram SFC system (Waters Corporation) equipped with a variable wavelength detector. Solvent from collected fractions was removed by centrifugal evaporation using a Genevac EZ-2 Plus solvent evaporator (Genevac, Ipswich, UK). All methods used a CO_2 mobile phase with methanol modifier and gradient elution. Detection wavelength was 215 nm except for the UD of aminodiphenylethanol which was detected at 220 nm. Injection volumes were between 400 and 500 μL. Experimental details on the syntheses of these artifacts and the SFC methods for their individual isolation can be found in the Supporting Information.

Nuclear Magnetic Resonance

All 1D and 2D data were collected at 298 K using a Bruker-Biospin 5 mm BBFO probe on a AVANCE III NMR spectrometer (Bruker-Biospin, Billerica, Massachusetts) operating at 500 MHz for milligram scale samples or using a Bruker-Biospin 5 mm TCI cryoprobe on a AVANCE III NMR spectrometer operating at 600 MHz for a submiligram sample. The following data were collected: 1D proton, 1D carbon, ^{1}H-^{1}H gradient COSY (correlation spectroscopy), ^{1}H-^{13}C multiplicity edited HSQC (heteronuclear single quantum coherence), and ^{1}H-^{13}C HMBC (heteronuclear multiple bond correlation), ^{1}H-^{1}H NOESY (nuclear overhauser effect spectroscopy), or ^{1}H-^{1}H ROESY (rotating-frame overhauser enhancement spectroscopy). A typical sample was dissolved in 0.6 mL of 99.9% deuterated dimethylsulfoxide (DMSO-d_6). Both the 1D proton spectrum and the 1D carbon spectra were referenced using the TMS signal, set to 0.00 ppm. NMR spectra can be found in the Supporting Information.



Scheme 3. Non-oxidative degradation of primary and secondary amines during peroxy radical-mediated stress using AIBN or ACVA.

RESULTS AND DISCUSSION

We found that drug molecules containing primary or secondary amine functionalities undergo significant non-oxidative or artifactual degradation under peroxy radical-mediated oxidative stress using azoalkane initiators, even in the presence of methanol. Several commercially available compounds were selected as model drugs for the purpose of this paper; however, the phenomena described were originally observed on proprietary molecules containing secondary amine functionalities. In these experiments, two degradation products or artifacts were detected, both displaying UV absorbance spectra similar to those of the respective APIs. The major degradant showed a characteristic mass gain of 39 amu, whereas the minor degradant showed a 43 amu gain, resulting from addition of C2HN and CHON, respectively, as calculated based on high-resolution MS. Moreover, fragmentation patterns indicated that the amine groups of the original drug substances had been structurally modified. Given these results and our evolving understanding of the chemistry (*vida infra*), we proposed the structures shown in Scheme 3, corresponding to an α-aminonitrile (AN; the major artifact) and a urea derivative (UD; the minor artifact). For the purpose of structural characterization, both compounds were synthesized from representative primary and secondary amine parents and characterized by LC–MS and NMR. Similarities in the retention times, masses, and fragmentation patterns confirmed that the products synthesized, and shown in Scheme 3, are those formed in the azoalkane experiments. As the mechanism of the artifactual degradation observed during the oxidative stress test was not readily apparent, additional experiments were carried out to understand both the scope of the problem (i.e., false positive reactivity) and the underlying mechanisms.

Product Yields Under Routine Oxidative Stress Conditions and the Effect of Experimental Parameters

Seven model compounds (Fig. 1) containing primary, secondary, and tertiary amine functionalities were chosen to investigate the novel degradation chemistry. Except for aminodiphenylethanol, all of the model compounds are known active pharmaceutical ingredients. In order to determine the generality of the artifactual reaction for amines, the compounds were subjected to AIBN stress under conditions similar to those used in the original experiments (described under Experiment 1). Oxidatively stressed samples were analyzed by LC–MS to observe the appearance of the degradation products with mass gains of 39 and 43 amu, corresponding to the AN and UD,

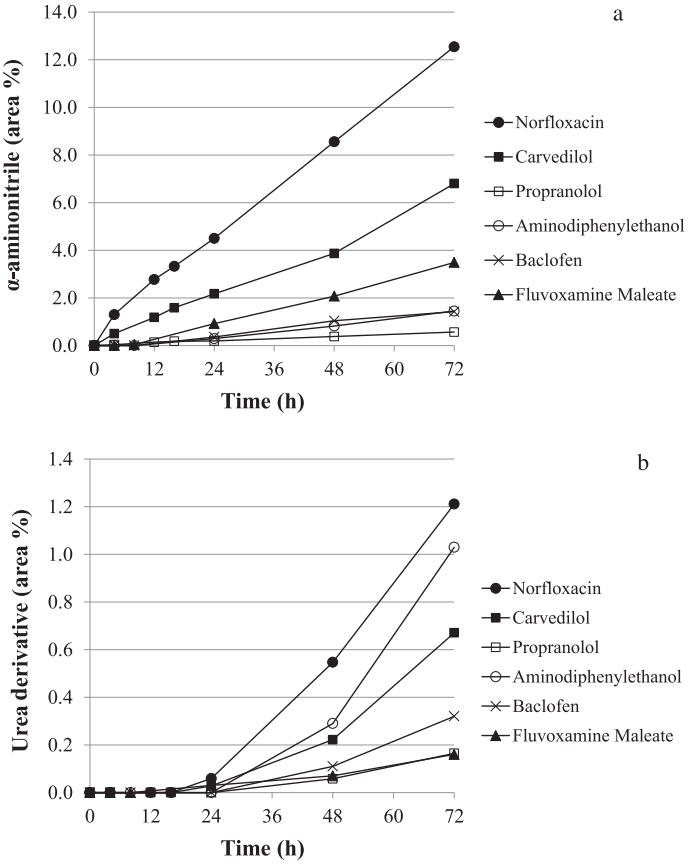


Figure 2. AN (a) and UD (b) yields (as area% relative to the sum of related peaks, by UV) at 40°C in MeOH/water 55:45 (v/v), at 0.2 mg/mL drug and 5 mM AIBN.

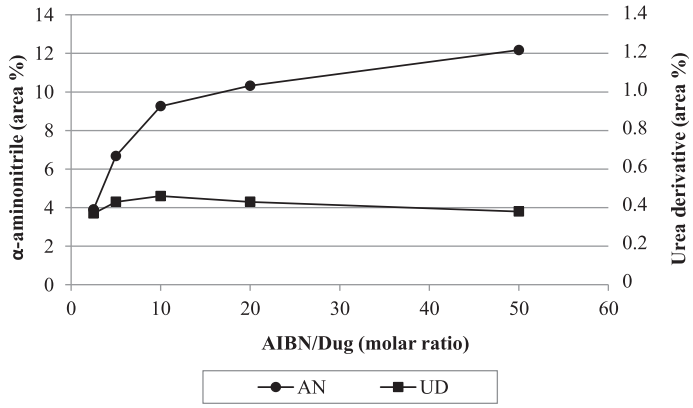


Figure 3. AN and UD yields (as area% relative to the sum of related peaks, by UV) after 72 h, at 40°C in MeOH/buffer (pH 6.0) 55:45 (v/v), at 0.1, 0.25, 0.5, 1, and 2 mM carvedilol and 5 mM AIBN.

respectively. Yields were then calculated as peak area% relative to the sum of the API-related peaks, by UV. Results indicate that artifact formation appears to be a general phenomenon for primary and secondary amines under the conditions specified, but not for tertiary amines. Although reaction rates vary significantly, AN remained by far the major degradant with yields ranging from 1% to 13% after 3 days (Fig. 2a), whereas UD was measured at 0.2%–1.2% (Fig. 2b). In some cases, additional low intensity peaks were observed, but none were investigated.

The effect of the AIBN/drug molar ratio was examined for carvedilol over a range of 2.5–50 mol/mol, at a fixed AIBN concentration of 5 mM (see Experiment 2 for details). For comparison, the molar ratios calculated for the model drug solutions of 0.2 mg/mL, fall between 5 and 10 mol/mol (Table 1). As shown in Figure 3, the artifact yields (especially for the major degradant) decreased significantly when the AIBN/drug molar ratio was less than 10, explaining to some extent the spread in reaction rates observed in Figure 2. Overall however, the artifact yields remained high enough to be problematic over the range of AIBN/drug molar ratios routinely used for the stress test, which is about 3–10 mol/mol.

The effect of varying the pH over a range of 2–8 was investigated by replacing the water component of the diluent, with 0.1 M buffers, as described in Experiment 1. The final pH of the buffered solution containing the model drug and AIBN was measured for each preparation and was used to generate the data plots displayed in Figure 4. The pH of the MeOH/water preparation was also measured and plotted in Figure 4. The complete data set can be found in Tables S4 and S5 of the Supporting Information. The results demonstrate that artifact formation was generally favored by increasing pH, with AN yields reaching maximum levels at pH 7–8. Both degradants remained undetected below a pH of 2 (AN) or 4 (UD). Overall, the pH trend is consistent with the amine groups playing roles of nucleophiles in the degradation pathway, the lower AN yields at higher pHs being attributed to the instability of the intermediate species in the proposed pathways (*vida infra*).

Stable Isotope Labeling Studies

In order to understand the source of the new C, N, and O atoms in the AN and UD degradants, azoalkane oxidative stress tests

Table 1. Summary of Molar Concentration, Native pH in the MeOH/Water Diluent, Molar Ratio AIBN/Drug, Yield of α-Aminonitrile Artifact as Area% Relative to the Sum of Drug-Related Peaks by UV, and Molar Concentration of the α-Aminonitrile Artifact at pH 6, for All Model Drugs

Model Compound	Model Drug (mM)	Native pH[a]	pK_a[b]	AIBN/Drug (mol:mol)	% AN after 72 h[c]	AN (mM)[c]
Aminodiphenylethanol	0.94	8.1	8.9	5.3	2.3	0.022
Fluvoxamine maleate	0.63	6.3	9.4	8.0	3.7	0.023
Baclofen	0.93	6.6	10.3	5.4	1.4	0.013
Norfloxacin	0.63	7.3	8.7	8.0	14.8	0.093
Carvedilol	0.49	7.9	8.2	10.2	9.6	0.047
Propranolol HCl	0.68	5.1	9.5	7.4	0.3	0.002

[a] Measured in the MeOH:water (55:45, v/v) reaction mixture.
[b] Calculated using ACD/ChemSketch, ver 11.02.
[c] Measured in the MeOH:buffer (pH 6) (55:45, v/v) reaction mixture.

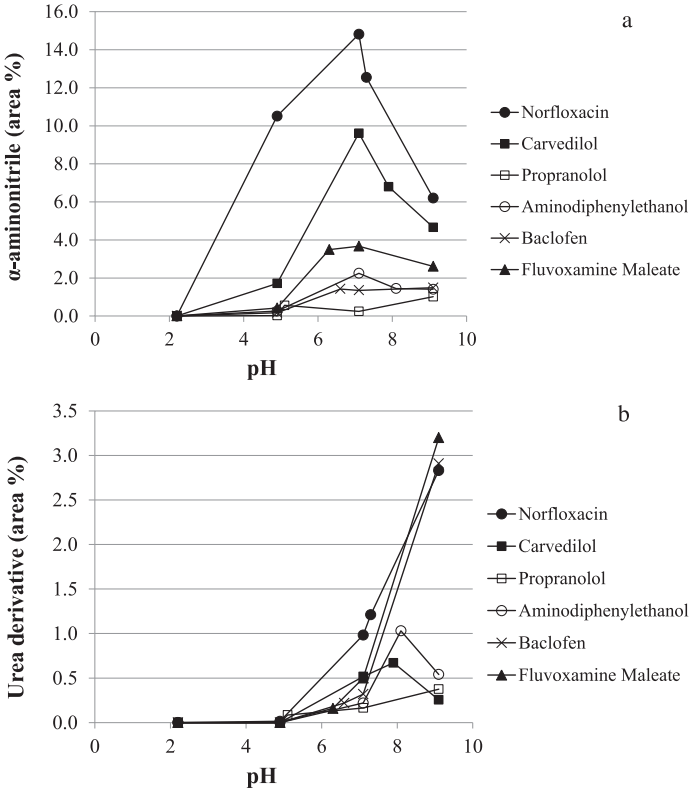
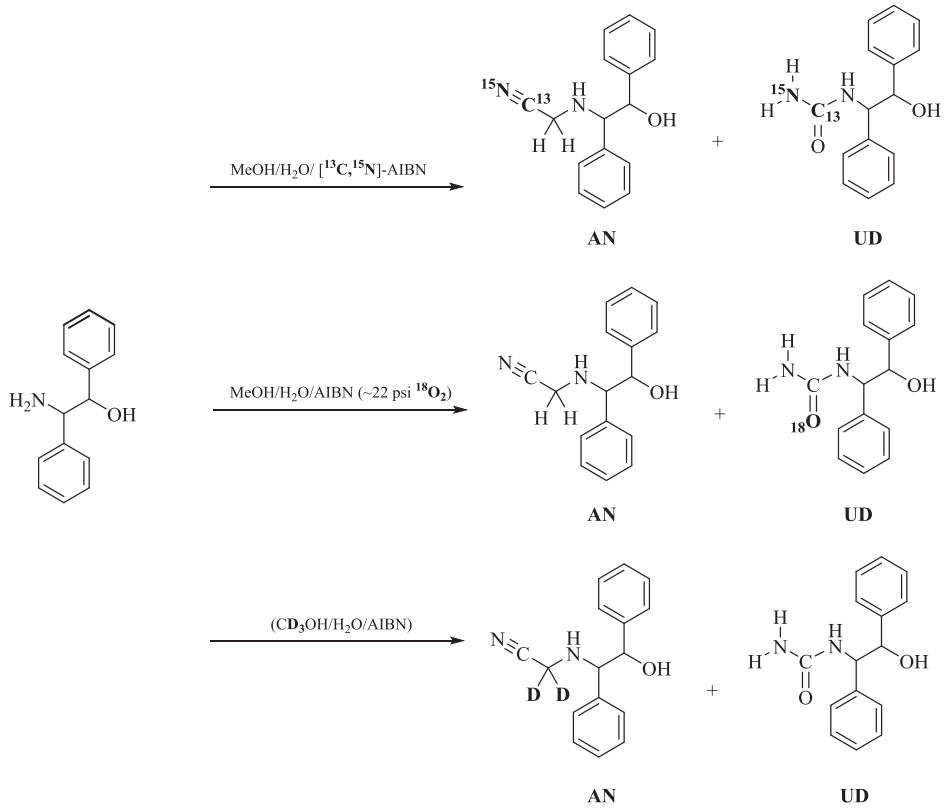


Figure 4. AN (a) and UD (b) yields (as area% relative to the sum of related peaks, by UV) after 72 h, at 40°C in MeOH/buffer 55:45 (v/v), at 0.2 mg/mL substrate and 5 mM AIBN, as a function of pH.

were carried out using isotopically labeled AIBN, methanol, or O_2, whereas the molecular weights of the products were monitored by LC–MS. Experimental details can be found in *Experiment and Analysis* section, whereas the data are summarized in Scheme 4. By replacing AIBN with [^{13}C,^{15}N]-AIBN, a mass gain of 2 amu was observed for both the AN and UD artifacts, indicating that the labeled nitrile group was incorporated into the products. The source of the new O atom in the UD degradant was probed by saturating the sample solution with $^{18}O_2$. The observed mass increase of 2 amu indicated that the new O atom derived from the molecular oxygen dissolved in solution, rather than from water or methanol. The proposed pathway for this incorporation begins with the addition of molecular oxygen to the C-centered AIBN radical generated after nitrogen expulsion, as shown in Scheme 1. Lastly, a mass gain of 2 amu corresponding to two deuterium atoms, was observed for the AN degradant after replacing the methanol with methanol-d_3. Incorporation of two deuterium labels from the –CD_3 group conclusively indicates that the methanol solvent is the origin of the last unknown atoms in the AN degradant, the methylene group.

Formation of AN in AIBN-Stressed Samples

The proposed mechanism for the formation of the AN degradants is essentially an "accidental" Strecker reaction.[17–19] The Strecker reaction is a well-known process in which α-aminonitriles are synthesized from ammonia or amines and carbonyl compounds in aqueous solutions containing alkaline cyanide. During AIBN stress testing of pharmaceutical compounds, the Strecker reagents (cyanide and formaldehyde)



Scheme 4. Isotope labeling results using aminodiphenylethanol.

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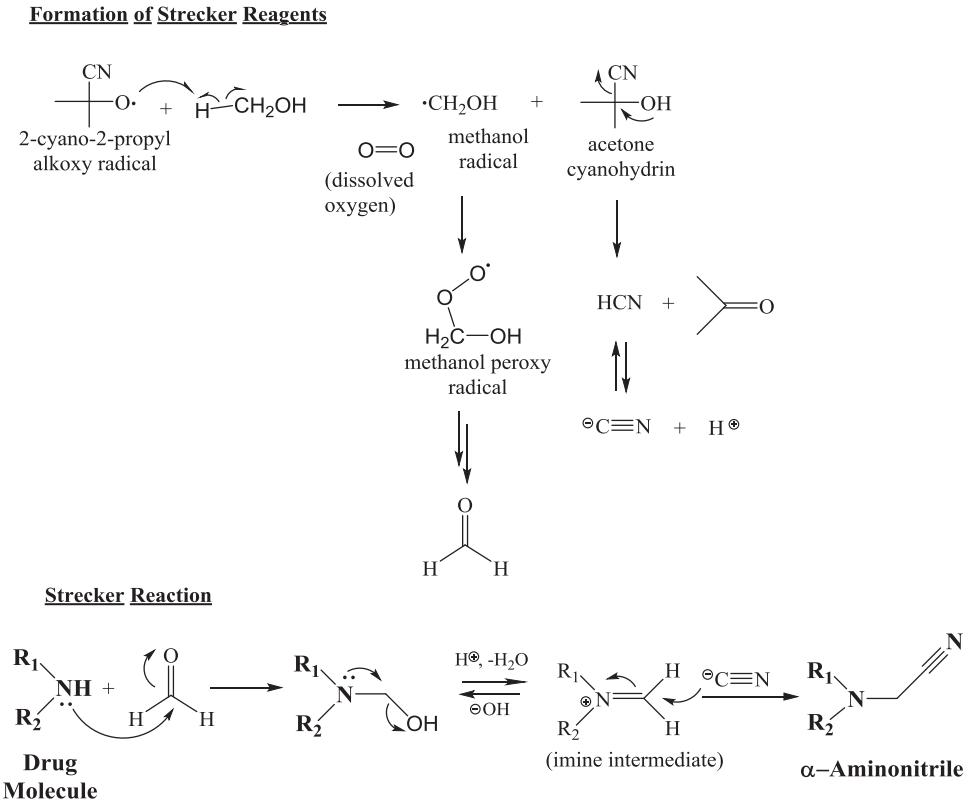


Scheme 5. Formation of AN from reaction of drug molecules with formaldehyde and cyanide (lower) generated *in situ* from reaction of 2-cyano-2-propyl peroxy radicals with methanol.

are generated *in situ* as byproducts of the stress reaction (Scheme 5). We found that methanol, used to quench the AIBN alkoxy radicals, plays a key role in this process. By abstracting H-atoms from methanol, alkoxy radicals form acetone cyanohydrin, which further dissociates into HCN and acetone,[20] the equilibrium being quickly established in the presence of basic compounds.[21] The resultant methanol radicals quickly oxidize to peroxy radicals, which can disproportionate to generate formaldehyde. The drug amine group reacts with formaldehyde to form the imine intermediate, which further reacts with cyanide (Scheme 5), explaining the incorporation of the nitrile and methylene groups observed during the labeling experiments. Previous reports showing formation of cyanide and acetone cyanohydrin during AIBN-initiated oxidation of benzene,[22] as well as formation of formaldehyde during the oxidative stress test carried out in the presence of methanol,[8] support the proposed mechanism. Table 2 shows our own measurements of cyanide levels generated during the thermal stress of AIBN in the absence of the test drug. Data show that the cyanide concentration increases in time and is proportional to the extent of AIBN decomposition. Interestingly, the highest AN concentrations attained during our experiments (norfloxacin, pH 6, Table 1) were equimolar with respect to cyanide, indicating that cyanide is the limiting reagent in the Strecker reaction. The decrease in the AN yields at high pH values (Fig. 4) is also consistent with the proposed mechanism: cyanide and hydroxide ions react competitively with the imine intermediate in Scheme 5. Above pH 9, the hydroxide concentration becomes comparable with that of the cyanide (Table 2), causing a decrease in the yield of the AN product.

Table 2. Summary of the Cyanide Levels Produced in Thermally Stressed AIBN Solution

Time point (h)	AIBN (% Initial)	AIBN (mM)	AIBN Degraded (mM)	CN (mM)	Degraded AIBN Converted to CN[a] (%)
0	100	5.24	–	0.015	–
24	96.4	5.05	0.19	0.042	9.9
48	92.6	4.85	0.38	0.068	9.0
72	88.0	4.61	0.63	0.093	7.5

All values are average of $n = 3$.
[a]Note that each molecule of AIBN can generate two CN ions.

Formation of the UD in AIBN-Stressed Samples

The detailed mechanism for the formation of the UD degradants is more difficult to support. Although the origin of the new C, N, and O atoms in the molecule was determined through isotope labeling studies (Scheme 4), critical information about the reaction pathway was revealed through BHA experiments (see Experiment 3 for details). By conducting the oxidative stress test in the presence of BHA, the AIBN peroxy radicals are expected to be effectively quenched and converted to AIBN hydroperoxide (ROOH). Therefore, any changes in the degradant yields caused by addition of BHA should indicate a direct correlation with the AIBN peroxy radicals, or any of the species deriving from them (Scheme 1). Note that in order to avoid inactivation of BHA through H-bonding with solvent molecules, these experiments were carried out in a

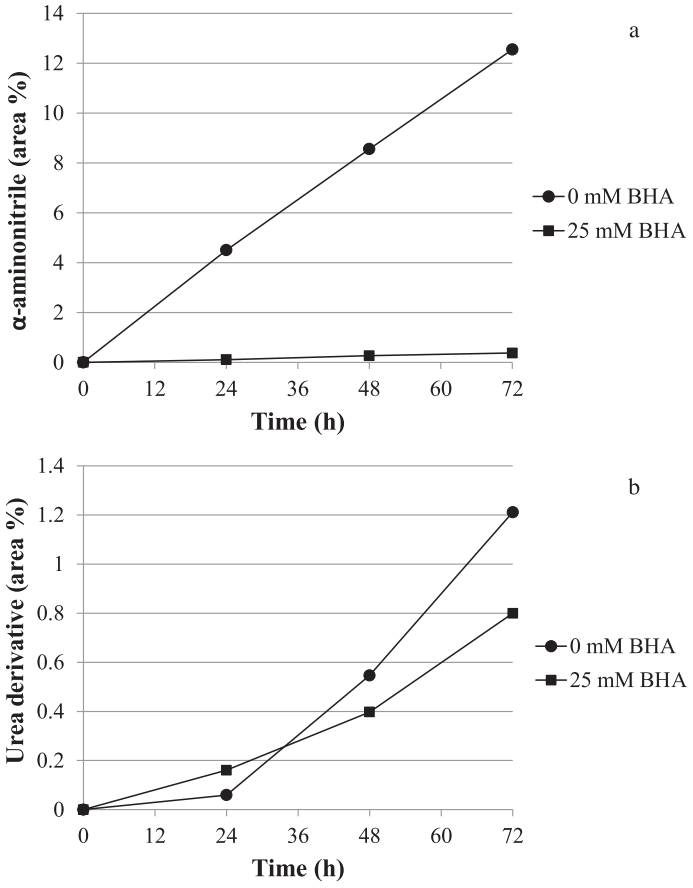
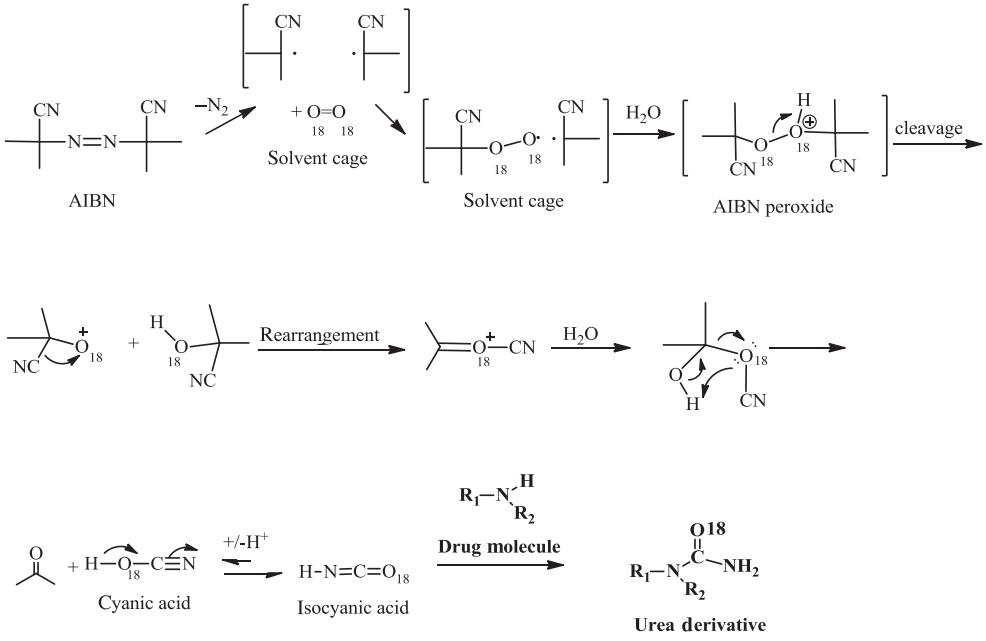


Figure 5. AN (a) and UD (b) yields for norfloxacin (0.2 mg/mL) in the absence of BHA [MeOH/water 55:45 (v/v)] and in the presence of 25 mM BHA [ACN/MeOH/water 8:1:1 (v/v/v)], at 40°C and 5 mM AIBN.

solvent consisting primarily of ACN (80%).[15] The fraction of MeOH was reduced to 10%, which has been previously shown to remain sufficiently effective in quenching alkoxy radicals.[14] Results show that addition of 25 mM of BHA effectively inhibits the formation of the AN artifact, whereas the UD levels remain practically unchanged (Fig. 5). These observations indicate that species deriving from the AIBN peroxy radicals are key to the formation of the AN artifact, consistent with our proposed mechanism. In contrast, the lack of effect on UD levels indicates that the AIBN peroxy radicals are not key to the formation of UD. Recall that the isotope labeling studies showed incorporation of the AIBN nitrile group and molecular oxygen into the UD molecule (Scheme 4), suggesting the participation of AIBN oxidation products to the formation of this artifact. We therefore propose that an alternate oxidation pathway of AIBN accounts for this incorporation, involving the formation of an AIBN peroxide within the AIBN "solvent cage," as shown in the top portion of Scheme 6. Formation of AIBN peroxy radicals (Scheme 1) follows the expulsion of molecular nitrogen and the rapid oxygenation of the carbon-centered radicals at diffusion-controlled rate in oxygen-saturated solutions.[23,24] Geminate recombination between the peroxy radical and the remaining C-centered radical may, however, occur within the solvent cage,[25] producing an AIBN peroxide. We propose that the UD products originate in the AIBN peroxide, which further breaks down to ultimately form isocyanic acid (Scheme 6), known to generate UDs in reaction with amines.[26] This pathway was not affected by the presence of BHA in the bulk solution, consistent with the proposal of rapid reaction within the solvent cage, explaining the lack of correlation between the yield of the UD product and the addition of BHA.

Choice of Azoalkane Initiator to Minimize AN and UD Artifacts

We have shown that primary and secondary amines undergo artifactual degradation reactions that may reach significant



Scheme 6. Proposed formation of UD through AIBN oxidation in the solvent cage to form the AIBN peroxide, followed by conversion to isocyanic acid and reaction with drug molecule.

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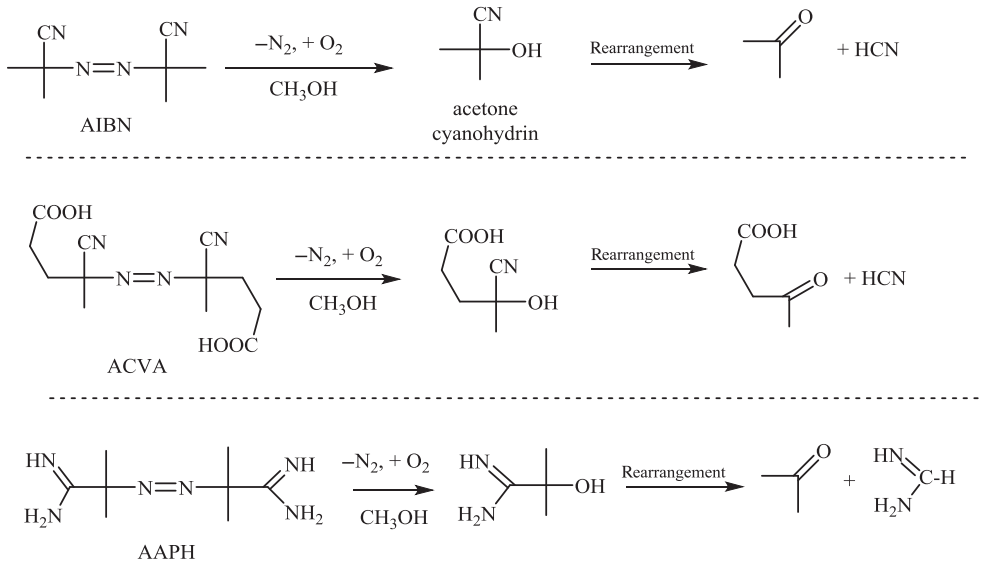


Scheme 7. Oxidative decomposition products of AIBN, ACVA, and AAPH.

yields under the conditions routinely used for oxidative stress testing using AIBN and ACVA. Observing false positive reactivity for drug compounds is undesirable, as it may impact resources and development timelines due to efforts required to address chemical stability issues. Therefore, taking steps to avoid such unrepresentative reactivity may need to be considered, unless mass measurements are routinely carried out for degradant profiling (which would allow a quick way to differentiate these artifacts). As discussed in this paper, decreasing AIBN/drug molar ratio or solution pH may reduce or even inhibit artifact formation. Other options, including the choice of solvent or azoalkane initiator may also be available to the pharmaceutical scientist.

Based on the mechanism described in Scheme 5, azoalkane initiators lacking the nitrile group should not produce cyanide during thermal stress, and therefore would not produce AN artifacts in reaction with amines. For example, the oxidative decomposition of three commonly used initiators, AIBN, ACVA, and AAPH[27] is summarized in Scheme 7. As expected, cyanide is generated from AIBN and ACVA, through decomposition of the corresponding cyanohydrins, but not from AAPH. The inability of AAPH to induce AN formation was confirmed for both primary and secondary amines at pH values ranging between 2 and 8, as described in Experiment 1. In the case of the UD artifacts, ACVA is expected to parallel AIBN and generate isocyanic acid as described in Scheme 6; however, the mechanism cannot be directly translated to AAPH. Although formation of the UD artifacts was observed in this case, the reaction pathway was not investigated.

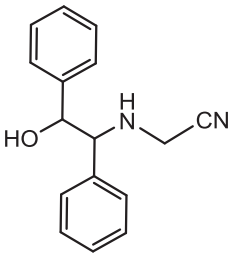
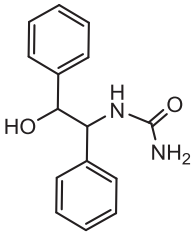
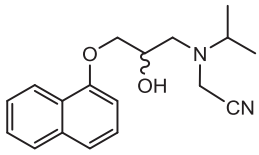
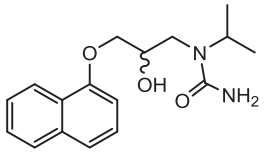
Structure Confirmation—Synthesis of Artifacts, LC–MS, and NMR

For structure confirmation purposes and peak tracking studies, the AN and UD artifacts of representative primary and secondary amine model drug compounds were synthesized as detailed in *Experiment and Analysis* section. For example, aminodiphenylethanol and propranolol were reacted with iodoacetonitrile and potassium cyanate to produce the corresponding AN and UD artifacts, respectively. After reaction completion, the individual products were purified by SFC and characterized by NMR and LC–MS. The retention times, masses, and fragmentation patterns of the synthesized compounds were matched with those of the corresponding artifacts detected in the stressed samples.

Accurate mass measurements of the synthesized artifacts (Table 3) show molecular ions $[M+H]^+$ that correlate to the protonated empirical formulas consistent with the structures shown. Fragmentation patterns, illustrated in Figures S2, S4, S6, and S8 of the Supporting Information section, are also consistent with the proposed structures.

One dimensional and extensive 2D NMR experiments were performed for the assignments of the proton and carbon spectra for the AN and UD artifacts of aminodiphenylethanol and propranolol (Fig. 6). The data are consistent with the structures shown in Table 3 and the proton and carbon assignments for each compound. For instance, the carbon spectrum of the AN artifact of aminodiphenylethanol shows a quaternary signal at 118.5 ppm, indicative of a cyano group carbon. In the HMBC spectrum, this carbon resonance shows correlations to the methylene protons at 3.57 and 3.09 ppm, and a three-bond correlation to the amino group proton at 2.64 ppm. Together with the COSY and HSQC data, the results indicate that the methylene group of the AN is attached to the nitrogen atom of aminodiphenylethanol. The UD product shows characteristic urea carbonyl signal at 157.7 ppm as well as the unique amino protons singlet at 5.52 ppm. The signal of the alkyl urea amino proton appears as a doublet at 6.53 ppm that is coupled to a proton at 4.79 ppm by COSY data. This proton signal, assigned as the nitrogenated methine on the basis of the attached carbon resonating at 58.8 ppm by HSQC experiment, shows a key three-bond HMBC correlation to the urea carbonyl signal, establishing the connectivity between the amino group of the starting material and the urea carbonyl. The other artifacts of propranolol were similarly characterized. The ^{1}H NMR and ^{13}C NMR spectra of the artifacts can be found in Figures S10–S17 of the Supporting Information section.

Table 3. Accurate Mass Measurements for Synthetically Derived α-Aminonitrile (AN) and Urea Derivative (UD) Artifacts of Aminodiphenylethanol and Propranolol

Artifact	Artifact Structure	Mass Measurement (m/z)	Protonated Empirical Formula	Theoretical Protonated Monoisotopic Mass (Da)	Mass Deviation
AN		253.13338	$C_{16}H_{17}ON_2$	253.13354	−0.6
UD		257.12823	$C_{15}H_{17}O_2N_2$	257.12845	−0.9
AN		299.17509	$C_{18}H_{23}O_2N_2$	299.17540	−1.0
UD		303.17044	$C_{17}H_{23}O_3N_2$	303.17032	0.4

CONCLUSIONS

We show that primary and secondary amines undergo artifactual degradation during azoalkane-based oxidative stress testing even when carried out in the presence of significant amounts of methanol. These observations are notable, as methanol is routinely utilized as co-solvent to reduce the occurrence of "false positive" degradants, and improve the quality of the oxidative stress test as a probe for the propensity of molecules to autoxidize via peroxy radical initiated processes.[12] The artifacts, an AN and UD (Scheme 3) are not products of autoxidation, but arise from the reaction between the amine substrate and byproducts of the stress reaction. Mechanistic studies revealed that AN is produced via a Strecker reaction between the amine group of the drug molecule and formaldehyde and HCN generated during the oxidative decomposition of nitrile-containing azoalkane initiators. UD species are believed to arise via a more complex mechanism, from the rearrangement and decomposition of an AIBN peroxide intermediate to yield isocyanic acid, which can easily react with amines to produce urea derivative. We show that under the conditions routinely used in the pharmaceutical industry for oxidative stress testing of drug molecules, significant yields may be observed for these artifacts. These findings should help simplify data interpretation during the peroxy radical-mediated oxidative stress testing by allowing the pharmaceutical scientist to readily understand that degradation products with a mass gain of either 39 amu (AN) or 43 amu (UD) over the amine parent, are most likely artifacts and therefore should be disregarded. Such degradants are not characteristic to the peroxy radical-oxidation chemistry. The most effective strategies to minimize or prevent artifact formation include lowering the reaction pH and switching to alternative azo initiators that do not produce AN artifacts, such as AAPH. Decreasing the initiator/drug ratio was also shown to reduce artifact yields; however, an excess of initiator should be maintained as recommended elsewhere.[12,14] Alternatively, LC–MS can be used to simply identify and exclude the artifacts, following mass identification.

ACKNOWLEDGMENTS

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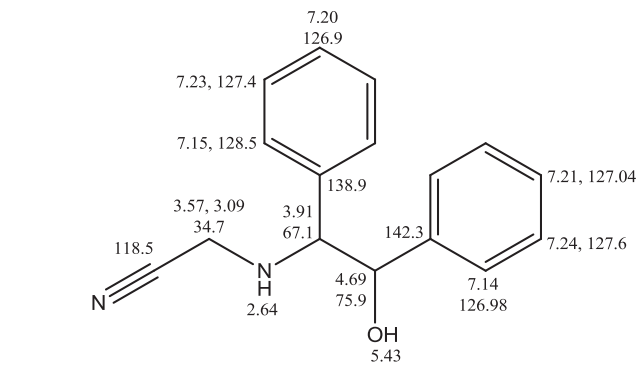
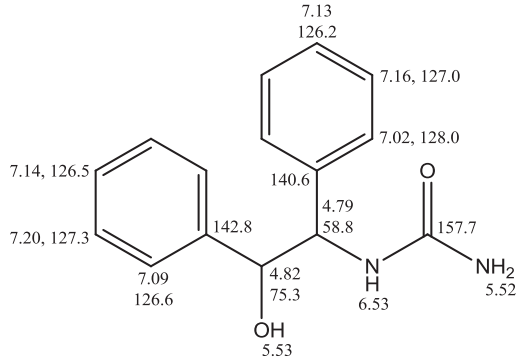
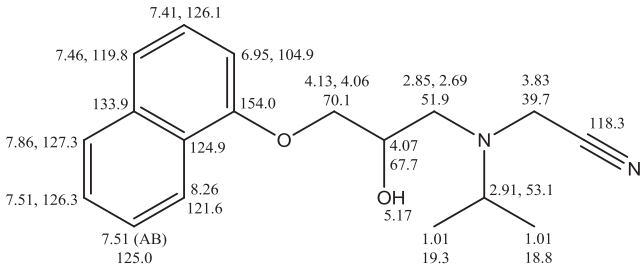


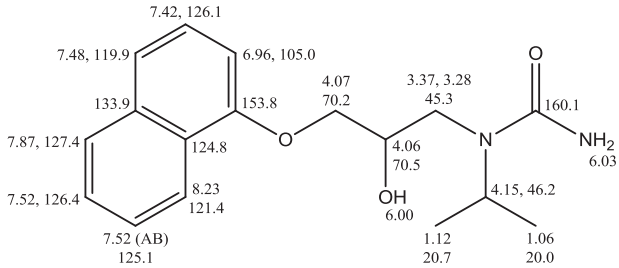
Aminodiphenylethanol Alpha-aminonitrile Artifact



Aminodiphenylethanol Urea Derivative



Propranolol Alpha-Aminonitrile Artifact



Propranolol Urea Derivative

Figure 6. NMR chemical shif assignment data for AN and UD artifacts of aminodiphenylethanol and propranolol.

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