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Research Paper

Use of 2,2′-Azobis(2-Amidinopropane) Dihydrochloride as a Reagent Tool for Evaluation of Oxidative Stability of Drugs

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Purpose. To study the oxidative degradation of drugs using a hydrophilic free radical initiator, 2,2′-Azobis(-amidinopropane) dihydrochloride (AAPH).
Methods. AAPH was used as the free radical initiator to study oxidation of three model compounds (A, B, and C), which represent different oxidizable moieties. In the solution model, the drugs and AAPH were dissolved in a mixture of acetonitrile and aqueous buffer and incubated at elevated temperatures to evaluate oxidative degradation. The effects of pH and drug-AAPH ratio on the kinetics of the reaction were evaluated for compound A. Commonly used antioxidants were also evaluated by addition to solutions of drug and AAPH. In the solid-state model, blends of drug with microcrystalline cellulose were treated with AAPH and placed at elevated temperature and humidity to evaluate solid state oxidation.
Results. Use of AAPH resulted in selective oxidation of the model drugs by a free radical initiated process. The scope of the technique was further investigated in detail using compound A. The rate of oxidation of compound A varied directly with the concentration of AAPH. The pseudo first-order rate constants for the oxidative degradation were calculated from the kinetic data. The antioxidants were rank-ordered based on their quenching activity on the rates of AAPH initiated oxidation for compound A. The concept was extended to oxidation in solid state.
Conclusions. The proposed AAPH model is useful in assessing oxidative stability of drug candidates in development.

KEY WORDS: antioxidant; 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH); oxidation; free radicals.

INTRODUCTION

Apart from hydrolysis, oxidation may be cited as the major cause of drug instability. Although a large body of literature on oxidative degradation in general is well documented, relatively few experimental studies have been reported specific to drugs (1–3). Use of concentrated oxygen atmosphere has been a suggested method to generate oxidative degradation products for chromatographic identification (4). Hydrogen peroxide is often used, but it is a nonspecific reagent that leads to numerous oxidation products and has little predictive value in assessing autoxidation chain processes (5). The use of computer programs has been advocated to predict reactivity, but their usefulness in predicting actual degradation products and reaction rates is not reported (6,7). A non-isothermal differential scanning calorimetric method to evaluate oxidative stability of drugs has been recently reported (8).

Initiation of the oxidation process is unpredictable and is often catalyzed by trace metals and hydroperoxides. Another difficulty in studying oxidation is the induction phase, in which there is no observable chemical change but which may be followed by an unpredictable increase in the rate of degradation. Thus oxidative degradation is difficult to model under laboratory conditions. In model systems, the induction period needs to be judiciously shortened without causing nonspecific and indiscriminate degradation of the compound. Early in the development of a new drug candidate, it is useful to assess its oxidative stability. If a potential oxidative instability is observed at the early development stage, a variety of strategies can be pursued to inhibit the oxidation of the drug (9). There is a need for a simplified model system for studying oxidative stability. Hydrophobic free-radical initiators have been used for studying the oxidation of drugs that, however, necessitates addition of organic solvents or solubilizers to the system (9,10).

Proposed here is an approach using a hydrophilic free-radical initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) for investigating oxidative stability of drugs. AAPH is frequently used in biochemical investigations for studying lipid perooxidations and their inhibition (11). Azo compounds have been used successfully as radical initiators. Whereas mechanism of decomposition of azo-initiators is not well understood, alkyl, alkoxyl, and alkylperoxyl radicals are expected to be formed from their thermolysis. Most studies assume formation of peroxyl radical as the first reactive species (12). Spin trap experiments have shown formation of

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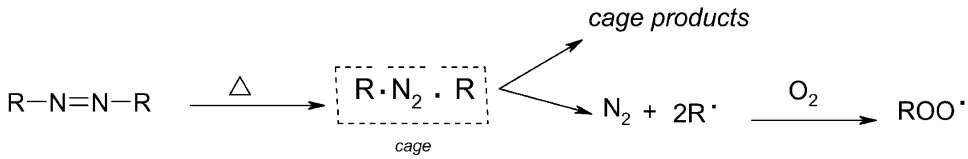

Fig. 1. Unimolecular decomposition of azo-initiators.

AAPH-derived alkoxyl radicals (13). In the absence of oxygen, formation of peroxyl radicals is inhibited and instead the reaction leads to formation of alkyl radicals (14). Notwithstanding the reported complexity of the actual process, a simplified scheme for the unimolecular decomposition of azo-initiators is shown in Fig. 1. The carbon radicals are formed in pairs in close proximity, some recombining to give nonreactive stable products, but many of them diffuse from the solvent cage and react rapidly with oxygen molecules to give reactive peroxyl radicals (15). Because AAPH is water soluble, it is suitable for studying oxidation in aqueous solutions. The solution model enables variation of reaction conditions such as stoichiometric ratio, pH, temperature, and so forth, in a rapid manner. In the examples that were investigated, use of AAPH resulted in clean oxidation in a predictable manner. Because it is a crystalline hygroscopic solid, it is also convenient for studying the solid-state oxidation in drug excipient mixtures where ingress of moisture is often the initial trigger.

The objective of this investigation is to evaluate the utility of AAPH to study oxidative stability of three model drugs. The selected drugs represent different oxidizable moieties in their chemical structures. The drug A bears benzylic hydrogens that are susceptible to oxidation. The drug B bears a diol moiety and compound C represents thioether class of drugs. Treatment with hydrogen peroxide caused significant degradation leading to an intractable mixture of products, and thus was not useful for controlled study (Fig. 2). Preliminary screening studies confirmed that in each case, reaction with AAPH led to clean oxidation forming mechanistically tractable products. Utility of the AAPH model was further investigated in detail with compound A serving as a model substrate for mechanistic and kinetic investigation. The effect of different pH values and drug-AAPH ratios on the kinetics of the reaction was evaluated. The effectiveness of antioxidants in suppressing free radical initiated oxidation was also evaluated. An attempt was also made to extend the concept to excipient-drug interaction in solid state using binary mixtures of drug and microcrystalline cellulose as the diluent.

From the results, it can be concluded that AAPH is a good tool for rapid evaluation of the oxidative stability of drug candidates in both solution and in the solid state.

MATERIALS AND METHODS

The drugs were selected from the development compounds at Bristol-Myers Squibb Company. Their partial structures are shown as A, B, and C (Figs. 3, 4, and 5). The free-radical initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Sigma Chemical Company (St. Louis, MO, USA). Solvents used were of the highest grade commercially available and were used without purification. All other reagents used were of laboratory grade.

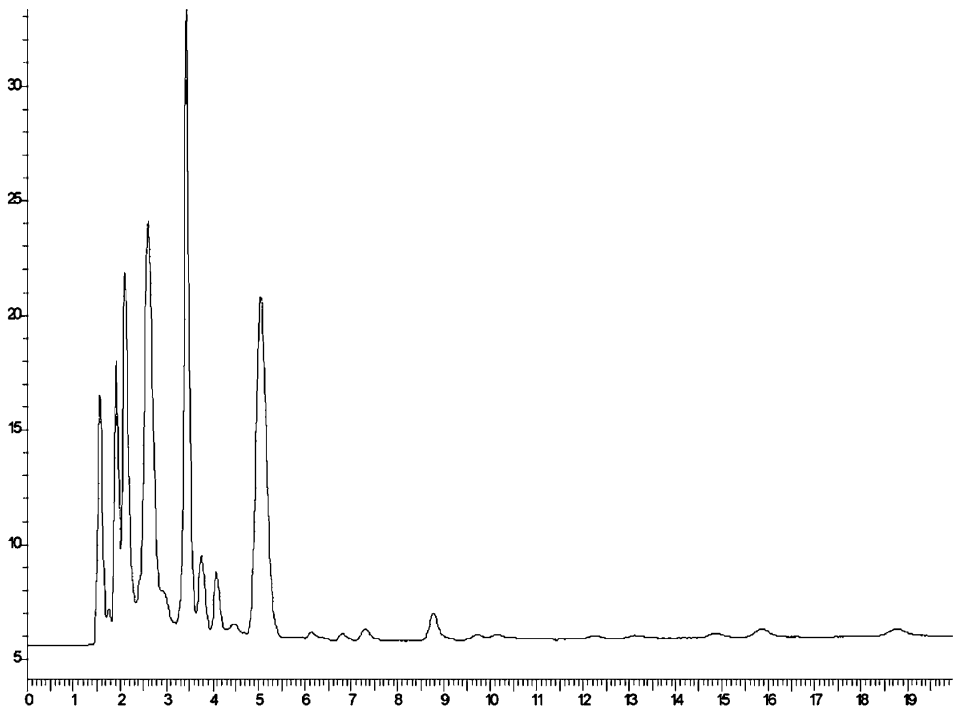

Fig. 2. Chromatogram of BMS-232632 solution in pH 7.5 buffer, oxidized by H_2O_2 at 60°C after 24 h.

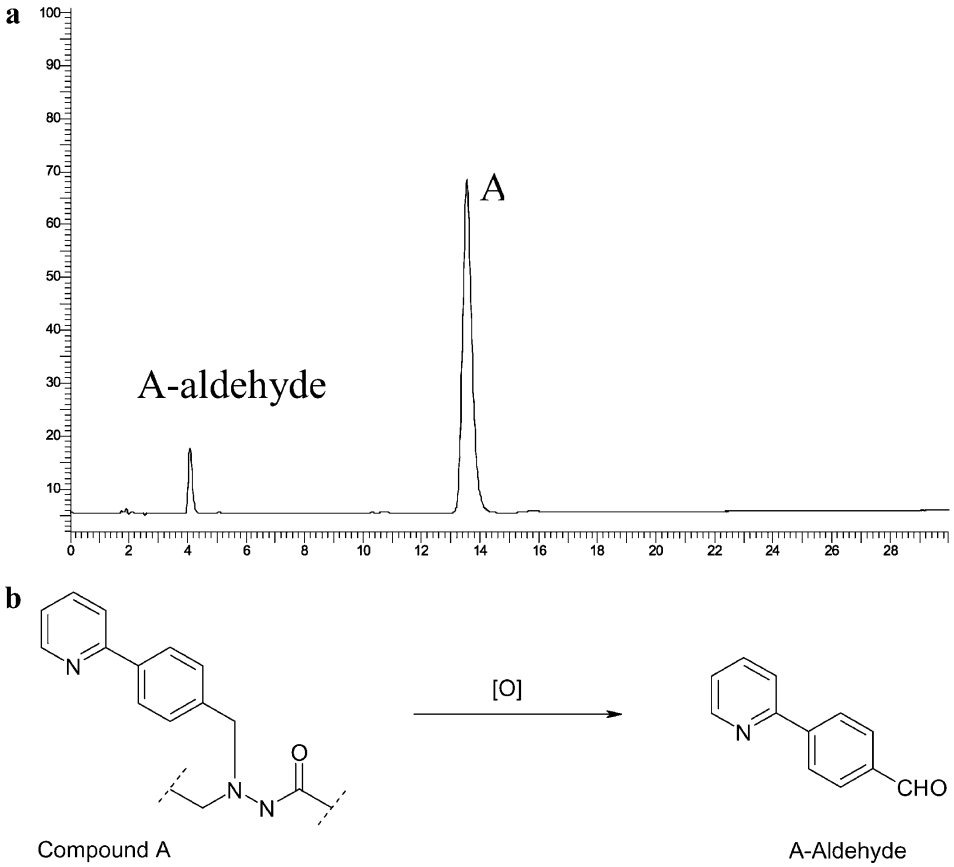

Fig. 3. (a) HPLC chromatogram of a solution of compound A in 50% acetonitrile–pH 7.5 buffer after 24 h at 60°C showing formation of ~7% aldehyde degradation product. Chromatographic conditions were column, 150 mm × 4.6 mm C-18; mobile phase, 40% aqueous acetonitrile containing 0.005 M ion pairing reagent dodecane sulfonic acid; flow rate, 1 ml/min; UV detection wavelength, 300 nm. (b) Partial structures of compound A and A-aldehyde.

Reverse-phase high performance liquid chromatography (HPLC) analysis was used to analyze the drugs and their degradation products using C-18 stationary phase, a mixture of water and acetonitrile as the mobile phase, and a UV detector. The mobile phases were modified by inclusion of appropriate modifiers such as pH control or ion pairing to effect optimum separation of the analytes. The HPLC data was acquired and analyzed using Turbochrome data acquisition system (Perkin Elmer, Shelton, CT, USA). The oxidation products were assigned based on results of liquid chromatography-mass spectrometry (LC-MS) analysis and co-chromatography with available authentic samples of the degradation products.

Solution Model

The drugs were dissolved in aqueous acetonitrile (50% v/v) buffer mixtures at concentrations of 10 mg/ml. The buffers used were pH 1.2 hydrochloric acid, 50 mM sodium acetate, pH 4.5, and 50 mM sodium phosphate, pH 7.5. A stock solution of AAPH (10 mg/ml) was prepared in the acetonitrile-buffer mixture. Typically, varying volumes (0.1 ml to 2 ml) of AAPH solution were added to 1 ml of drug solution and diluted to 10 ml with respective acetonitrile-buffer mixtures in a 20 ml screw-capped glass vial with a Teflon-lined lid. The vials were placed at elevated temperatures. The samples were withdrawn periodically and analyzed by HPLC for drug and degradation products. In each example of the selected drugs, a single oxidation product was detected along with parent peak in the chromatograms. Hence, oxidation was expressed in terms of ratio of peak area (AUC) of the degradant to the parent. In antioxidant screening, varying amounts of each antioxidant in the acetonitrile-buffer mixture were added to the drug-AAPH reaction mixture. In each case, solutions of AAPH and drug served as the controls.

Solid-State Model

Binary mixtures were prepared by mixing drug with microcrystalline cellulose pH 101 (avicel) at a weight ratio of 1:9. Solutions of AAPH were prepared in acetonitrile-buffer mixtures, pH 4.5 and 7.5 at a concentration of 150 mg/ml or in water at 200 mg/ml. Sample blends and controls were prepared by adding appropriate amount of AAPH solution or water/buffers to 1 g of the drug-excipient mixture. The blends were dried at 37°C for 1 h. The drug to AAPH ratio in the dry blend was 1:2 (w/w). The blends were filled in glass vials, and open vials were stored at 40°C/75% RH. Samples were withdrawn at periodic intervals, extracted with 50% acetonitrile-water, and analyzed by HPLC for drug and degradation products.

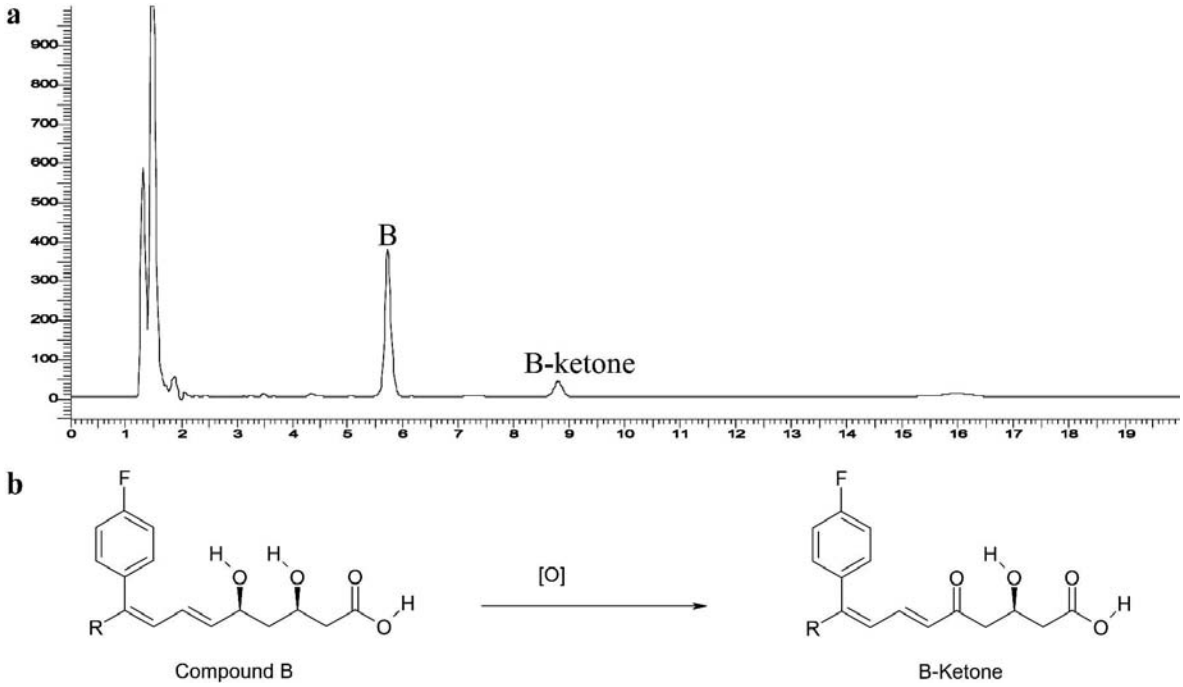

Fig. 4. (a) HPLC chromatogram of a solution of representative compound of series B in 50% acetonitrile–pH 7.5 buffer after 6.5 h at 60°C showing formation of ~12% B-ketone degradation product. Chromatographic conditions were column, 150 mm × 4.6 mm C-18; mobile phase, aqueous mixture of 36% acetonitrile and 4% methanol containing 0.05% trifluoroacetic acid; flow rate, 1 ml/min; UV detection wavelength, 220 nm. (b) Partial structures of representative compound of series B and B-ketone.

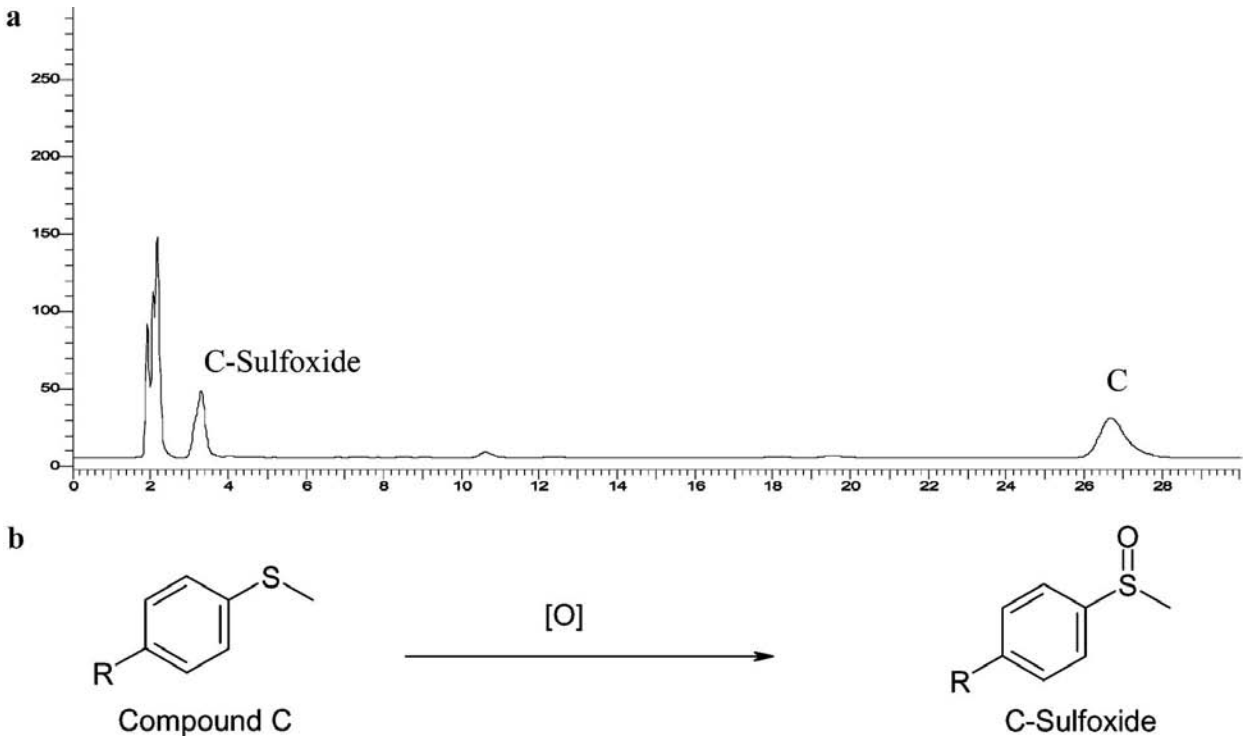

Fig. 5. (a) HPLC chromatogram of solution of compound C in 50% acetonitrile–pH 7.5 buffer after 6.5 h at 60°C showing formation of ~40% C-sulfoxide degradation product. Chromatographic conditions were column, 150 mm × 4.6 mm C-18 basic; mobile phase, aqueous acetonitrile, 25%; flow rate, 1 ml/min; UV detection wavelength, 220 nm. (b) Partial structures of compound C and C-sulfoxide.

RESULTS AND DISCUSSION

Compound A

As shown in the partial structure, compound A has an activated benzylic system that is prone to autoxidation (Fig. 3b). However, under accelerated storage conditions its spontaneous oxidation is very slow, and the corresponding A-aldehyde is formed after prolonged storage. Treatment with hydrogen peroxide solutions led to extensive degradation and formation of a mixture of products and hence was not useful for further study. The solution of A in 50% aqueous acetonitrile treated with AAPH showed progressive loss of parent peak with time and corresponding increase of A-aldehyde peak (Fig. 3a). Identity of aldehyde degradation product was based on LC-mass spectral analysis and confirmed by co-chromatography with the authentic sample that became available later in the drug development. Thus, AAPH offered rapid means of following the oxidation of compound A.

Compound B

The partial structure of B represents a series of congeneric allylic alcohols bearing the same diol side chain but differing in the substituent R (Fig. 4b). The alcohols are generally not autoxidized readily mainly owing to high rate of termination of the hydroxyperoxy radicals (16). Treatment of solutions of compounds of series B with AAPH in aqueous acetonitrile resulted in differing extent of oxidation, but in each case selective formation of B-ketone (Fig. 4a) was observed. The identity of the degradation product was confirmed by LC-MS and NMR analysis. The selective oxidation of the allylic alcohol to ketone is consistent with the vinylic radical intermediate.

Compound C

The compound C is a thioether that is readily oxidized to sulfoxide and sulfone by air under accelerated storage conditions (Fig. 5b). Treatment with AAPH in solutions resulted in selective oxidation of C (Fig. 5a) to give one major degradant. This degradation product was identified as the C-sulfoxide by co-chromatography with the authentic sample. The selective formation of sulfoxide by AAPH was unexpected because the thioethers are known to degrade by multiple mechanisms (17). The formation of sulfoxide as the major oxidation product of compound C is indicative of a two-electron oxidation mechanism similar to that proposed for the oxidation of sulfides by halogenated peroxyl radicals. This mechanism envisages mediacy of a sulfuranyl-type radical adduct in the hydrated state. The oxygen in the sulfoxide product is presumed derived from water rather than from peroxyl oxygen (18).

The above screening results with three representative compounds confirmed that AAPH would serve as a good reagent tool to examine oxidative stability of drugs. The scope of this technique was further investigated in detail by using compound A as the model oxidative substrate.

Although it is generally recognized that heat, light, or metals promote oxidation reactions, the initial radical formation by these methods is difficult to control experimentally. The free radical initiators generate the initial free radicals under a controlled environment at moderately elevated temperatures that lead to large propagation turnovers and resultant degradation product of the drug. Consistent with the

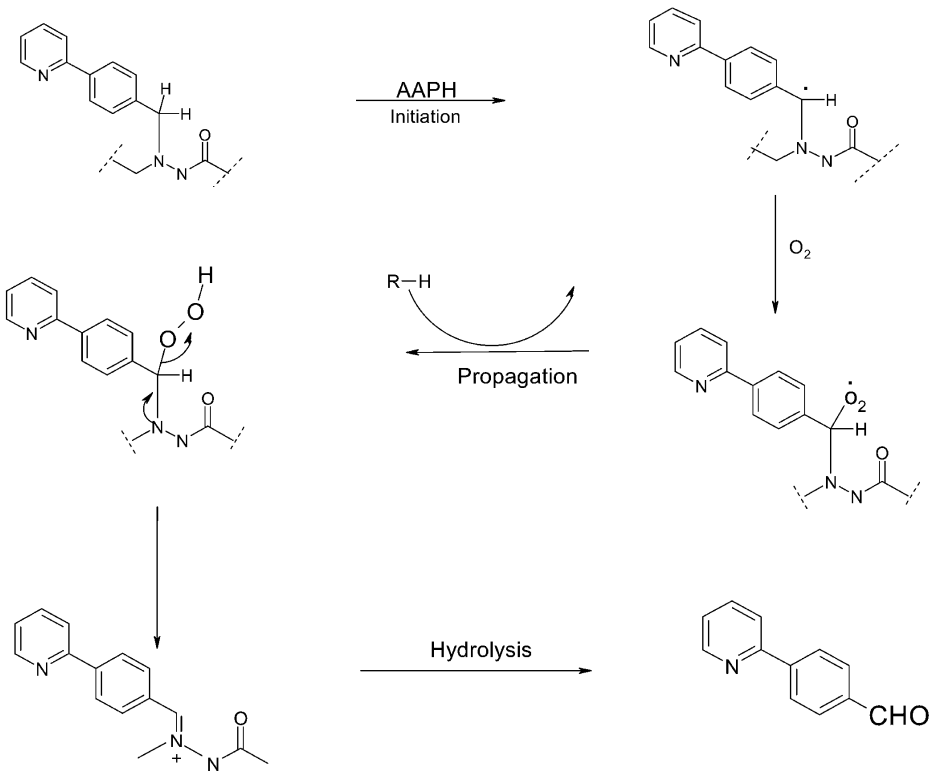

Fig. 6. Proposed mechanism for oxidative degradation of compound A. The identity of A-aldehyde, the primary oxidation product, was confirmed.

Table I. Rates of Degradation for the AAPH-Initiated Oxidation of Compound A in Solutions at 60°C

AAPH:drug ratio (w/w)	Rate constant[a] (h^{-1})	
	pH 1.2	pH 4.5
0	0.0009	0.0005
0.02	—	0.0025
0.1	0.0044	0.005
0.25	0.0091	—
0.5	0.0161	0.008
1.0	0.0294	0.010
2.0	0.0527	—

[a] Obtained from the semilogarithmic plots of % compound A remaining vs. time ($r^2 \geq 0.97$ in all cases).

above, AAPH caused selective oxidation of compound A to the A-aldehyde in a controlled and predictable manner. The postulated mechanistic steps for the oxidation of A by AAPH are depicted in Fig. 6.

The effect of increasing concentration of free radical on the oxidation rate was studied by varying the weight ratio of drug to AAPH in mixtures of 50% (v/v) acetonitrile and aqueous buffer at pH 1.2 and 4.5 (Table I). At each pH condition, at a fixed concentration of drug, the rate of oxidation increased as the concentration of AAPH was increased. The overall kinetics was thus forced to pseudo first order. A representative semilogarithmic plot to depict the kinetics of AAPH initiated degradation of compound A (pH 1.2, AAPH:drug ratio = 0.25:1 w/w) is shown in Fig. 7. In solution, the rate-determining step is hydrogen atom abstraction. It appears that dissolved oxygen in the solutions was sufficient for the initial production of the peroxy radicals and that the

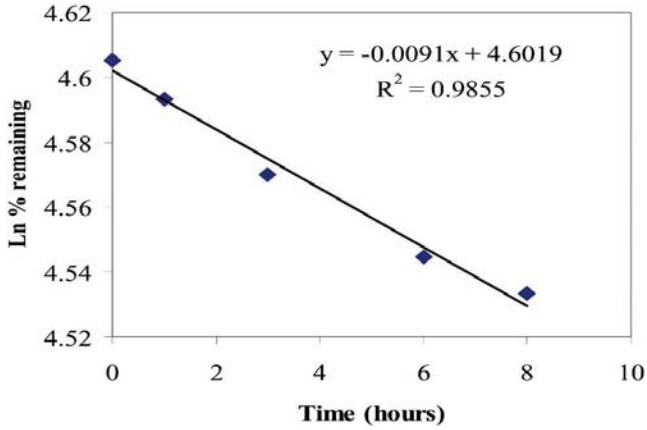

Fig. 7. Representative semilogarithmic plot depicting kinetics of AAPH-initiated degradation of compound A (pH 1.2, AAPH:drug weight ratio of 0.25:1).

rate was not dependent on the concentration of oxygen in the solution (1). The contributions to rate of the reaction by propagation and termination steps are expected to be small and can be neglected. The effect of pH on the oxidation of compound A by AAPH in solutions was studied in mixtures of acetonitrile and aqueous buffer at pH 1.2, 4.5, and 7.5 (Fig. 8a). The reaction rates were found to be in the order pH 1.2 > pH 4.5 > pH 7.5. This was attributed to the greater ease of benzylic hydrogen abstraction from the protonated molecule due to inductive effect (Fig. 8b).

Antioxidant Screening

During formulation development, it is often necessary to select the most effective antioxidant for an oxidatively sensi-

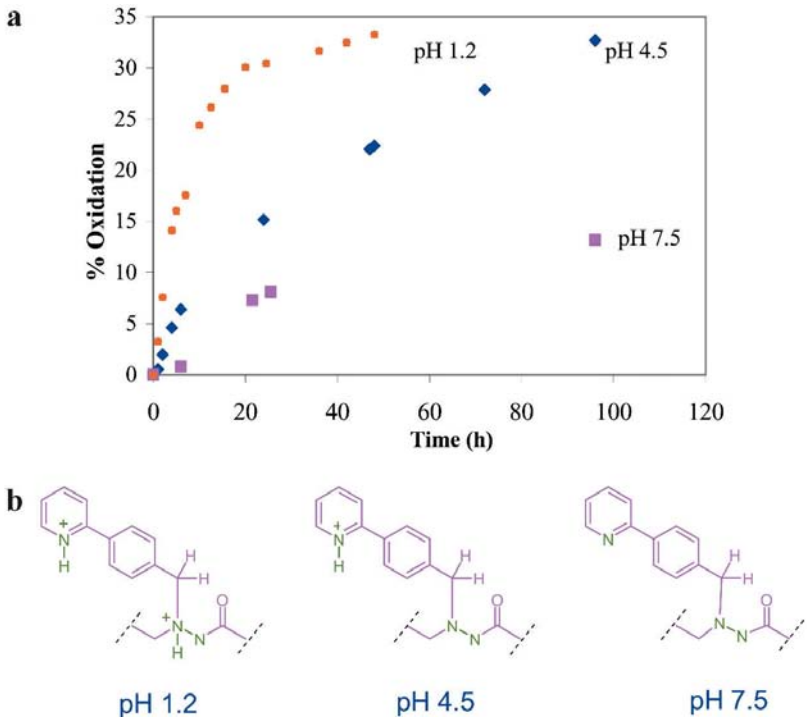

Fig. 8. (a) AAPH-initiated degradation of compound A (0.1 mg/ml) in 50% acetonitrile buffer solutions at 60°C as a function of pH. The weight ratio of AAPH to drug is 1:1. (b) Protonation of compound A at different pH values.

tive drug. Usually the selection of antioxidants is done by empirical means. In contrast to empirical methods, rate constant data can provide a quantitative measure of effectiveness of antioxidants for suppressing oxidation of a drug (9). The effectiveness of different antioxidants was assessed in solution by comparing the rate of AAPH initiated oxidation in 50% (v/v) acetonitrile-pH 4.5 buffer mixture at 60°C. Addition of increasing amounts of antioxidants in solutions of compound A in the acetonitrile-buffer mixture resulted in significantly decreasing the rate of AAPH initiated oxidation at 60°C (Table II). The effectiveness of antioxidants used under these conditions was rank ordered as follows: ascorbic acid > propyl gallate > BHT > BHA > TPGS > SFS > sodium metabisulfite.

Systematic variation in the amount of antioxidant allowed the reaction to be studied in a rapid and predictable manner. The AAPH oxidation model can be used to facilitate the design of inhibiting strategies and the selection of antioxidants.

Solid-State Model

In oxidation reactions in the solid state, the propagation step is hindered by low mobility. As a result, the reaction rates are slower in the solid state. Addition of a free-radical initiator enables initial formation of reactive free radicals that generate enough peroxy species from the drug molecule to increase their overall turnover into the degradation product. The usefulness of AAPH to study the solid-state oxidation of compound A in binary blends with microcrystalline cellulose was evaluated. The oxidation of A in the absence of AAPH was slow (less than 0.1%) after 8 days of storage at accelerated conditions (Table III). Addition of AAPH to the mixtures significantly accelerated the oxidation of compound A (>1%) within 8 days storage at the specified conditions. These results proved that AAPH could be used to screen the potential oxidative stability of drugs in the solid state in a rapid manner.

It is significant that the model compounds did undergo similar oxidative degradation under actual use conditions. As predicted by the AAPH model, A-aldehyde was detected (~0.1%) in the formulated capsules of compound A after prolonged storage at accelerated conditions. This aldehydic impurity in the formulation was found to undergo further degradation in the formulation to acid and other degradation products. The extent of degradation was low and did not necessitate addition of stabilizers in the formulation. The AAPH model confirmed that the degradation pathway for compound A was through benzylic oxidation giving aldehyde as the initial product. Oxidative stability of the B-series of congeneric allylic alcohols bearing the same diol side chain but differing in the substituent R could be rank-ordered based on the extent of their oxidation by AAPH. The oxidation was significantly mitigated by addition of antioxidants. Compound C was found to degrade to sulfoxide as well as sulfone (not detected in the AAPH model) during actual storage. Exclusion of oxygen in the headspace was required to mitigate the oxidative loss of compound C.

Table II. Effect of Antioxidants on the Rates of AAPH-Initiated Oxidation of Compound A

Antioxidant[a]	Rate constant[b] (h^{-1})
Control	0.010
Sodium formaldehyde sulfoxylate (SFS)	0.011
Sodium metabisulfite	0.011
TPGS	0.0071
BHA	0.0008
BHT	0.0005
Propyl gallate	0.0005
Ascorbic acid	0.0003

[a] Drug to antioxidant weight ratio was maintained at 2:1 in aqueous acetonitrile buffer, pH 4.5 at 60°C.

[b] Obtained from the semilogarithmic plots of % Compound A remaining vs. time ($r^2 \geq 0.97$ in all cases, except SFS where r^2 was 0.86).

Table III. Effect of AAPH on Solid-State Oxidation of Compound A in Binary Excipient Blends

		% Oxidation at 40°C/75% RH			
		Control (without AAPH)		Sample (with AAPH)	
Blend	Initital % oxidation	4 day	8 day	4 day	8 day
Drug + avicel (water)	0.0	0.05	0.06	1.65	1.46
Drug + avicel (pH 4.5)	0.0	0.06	0.07	1.17	1.43
Drug + avicel (pH 7.5)	0.0	0.05	0.09	0.93	1.15

CONCLUSIONS

From the results, it can be concluded that AAPH is a good tool for rapid evaluation of the oxidative stability of drug candidates in both solution and in the solid state. The advantage of this simplified model system is that quantitative stability assessments can be made in a short time and the effects of various additives such as antioxidants can be determined. There may be cases where AAPH may give erroneous results. For example, AAPH failed to cause selective oxidation of 5,6-dihydro-4-hydroxy-2-pyrone moiety (19). AAPH may not be suitable for studying the N-oxide formation reaction of tertiary amines. However, our investigations suggest that AAPH is a useful tool for studying the oxidative stability of drug candidates in early development.

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