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Characterization of a Stable 2,2′-Azobis(2-Methylpropanenitrile) Degradant and Its Use to Monitor the Oxidative Environment During Forced Degradation Studies by Liquid Chromatography/Mass Spectrometry

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ABSTRACT

Azo compounds are commonly used to study radical-mediated degradation of pharmaceutical compounds. The favorable chemical and physical properties of 2,2′-azobis(2-methylpropanenitrile) (AIBN) have made it one of the most widely used compound for these type of studies. This article describes the characterization of a stable product, N-(1-cyano-1-methylethyl)-2-methylpropanamide, formed during the decomposition of AIBN. This product is easily detected by liquid chromatography/mass spectrometry and can serve as a marker to confirm the AIBN is working as intended and to monitor the kinetic formation of free radical species.

Introduction

The development of pharmaceutical compounds of interest routinely involves forced degradation studies to characterize products that may form during manufacture and storage. These studies subject the active pharmaceutical ingredient (API) to various pH, temperature, oxidation and light conditions to accelerate the kinetics of degradant formation and concentrations suitable for detection and characterization.[1,2]

Free radical–mediated oxidation of pharmaceutical compounds typically employs azo-compounds that thermally degrade to peroxy radical species.[3] While the number of azo-compounds to choose from is quite large, only a handful are routinely used based on their solubility and stability properties. One of the most commonly used is azobisisobutyronitrile (AIBN),[4] which ultimately forms carbon centered 2-cyanopropyl radicals that react with dissolved oxygen to form the desired 2-cyano-2-propyl peroxy radical intermediates for oxidation of test articles (Fig. 1).

These types of studies rarely incorporate a positive control, so it is rational to wonder if the AIBN is working as intended when no API degradation is detected. In one of our studies utilizing AIBN, a compound was detected by liquid chromatography/mass spectrometry (LC-MS) that increased with time but was unrelated to the API. This work describes the characterization of this product that could be used as a marker to confirm an API has indeed been exposed to an oxidative free radical environment.

Experimental Section

General Experimental Information

All buffers and solvents used in this study were of HPLC grade or of the highest available purity and used without further purification. AIBN was obtained from Sigma Aldrich (St. Louis, MO). ^{1}H NMR and ^{13}C NMR were recorded on a Bruker 400 MHz using d_6-acetone as a solvent.

Abbreviations used: AIBN, 2,2′-azobis(2-methylpropanenitrile); CMMP, N-(1-cyano-1-methylethyl)-2-methylpropanamide; CID, collision induced dissociation; DKI, dimethyl-N-(2-cyano-2-propyl)ketenimine; IBN, isobutyronitrile; LC/MS, liquid chromatography/mass spectrometry; MAN, methacrylonitrile; TMSN, tetramethylsuccinodinitrile; ToF, time-of-flight; UPLC, ultra performance liquid chromatography.

Conflicts of interest: The authors declare no competing financial interest.

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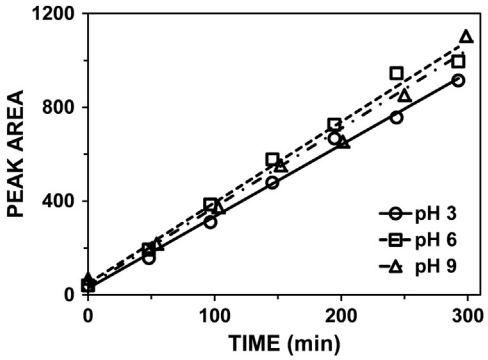

Figure 1. Kinetics for appearance of the AIBN degradation product at pH 3, 6, and 9. Peak areas are derived from selected ion chromatograms (m/z 155.1 + 177.1).

AIBN Incubations

The final concentration of AIBN for all incubations was 2.5 mM in acetonitrile/methanol (40/10, v/v) containing 50 mM sodium phosphate buffer (pH 3 or 6) or 30 mM sodium pyrophosphate (pH 9). The buffer concentration at pH 9 was lower due to the reduced solubility of pyrophosphate buffer with organic solvents. Methanol was present to quench undesired 2-cyano-2-propoxy radical formation.[3] To initiate the formation of oxidative 2-cyano-2-peroxy radicals, the vial was immediately placed in the LC/MS autosampler compartment kept at 40°C. Repeated injections were made at approximately 45 min intervals to evaluate the kinetic formation of AIBN degradant.

Forced Degradation of Solid AIBN

Small amounts of AIBN (<10 mg) were placed in 1.5 mL plastic screw cap microcentrifuge tubes and then incubated for 6 days and 16 days at 45°C (below the 50°C self-accelerating decomposition temperature listed for AIBN). The degraded AIBN samples were placed at 5°C until used for analysis.

Synthesis of N-(1-cyano-1-methylethyl)-2-methylpropanamide (CMMP) [84,213-57-0]

The route used to synthesize CMMP followed a procedure and used a similar compound.[5] All chemicals used were obtained from Sigma Aldrich. Briefly, 340 μL (3.57 mmol) of 2-amino-2-propane nitrile [19,355-69-2], 374 μL (3.57 mmol) of isobutryl chloride [79-30-1] and 498 mg (3.57 mmol) of potassium carbonate were combined in 5 mL of ethyl acetate and stirred for 72 h at room temperature, The reaction was quenched by adding 300 μL of water followed by several washes with saturated brine to remove unreacted compounds, salts, and water from the ethyl acetate layer. The ethyl acetate was evaporated to dryness to yield 463 mg of CMMP (84% yield). ^{1}H NMR (400 MHz, Acetone-d_6) δ 7.45 (s, 1H), 2.51-2.41 (sep, 1H), 1.66 (s, 6H), 1.10-1.08 (d, 6H); ^{13}C NMR (400 MHz, Acetone-d_6) δ 177.1, 122.3, 46.9, 35.4, 27.1, 19.6. HRMS (ion electrospray) m/z calc for $C_8H_{14}N_2ONa$ $[M + Na]^+$ 177.1004, found: 177.0979.

LC/MS Conditions

Samples were injected (5 μL) onto a Waters Acquity ultra performance liquid chromatography system using a Waters HSS T3 column (1.0 × 100 mm, 1.7 μm). The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was 0% B ramped to 7.5% B at 6.0 min, then 20% B at 9.0 min, then 70% B at 10 min. The column was held at 70% B for 1.0 min, then returned to 0% B in 1.0 min and held for 2.0 min to re-equilibrate the ultra-performance liquid chromatography column. The flow rate was 0.15 mL/min, and the column temperature was maintained at 30°C. A divert valve was used to direct flow to waste during the first 0.85 min to minimize contamination of the mass spectrometer source. The kinetic studies were carried out with an autosampler compartment kept at 40°C to promote the degradation of AIBN.

The mass spectrometer was a Waters Synapt G2 HDMS Q-ToF operated in positive ion electrospray mode scanning from 85 to 600 Da using leucine enkephalin (m/z = 556.2771) as a lock mass standard. The parameters used were capillary voltage = 3.25 kV,

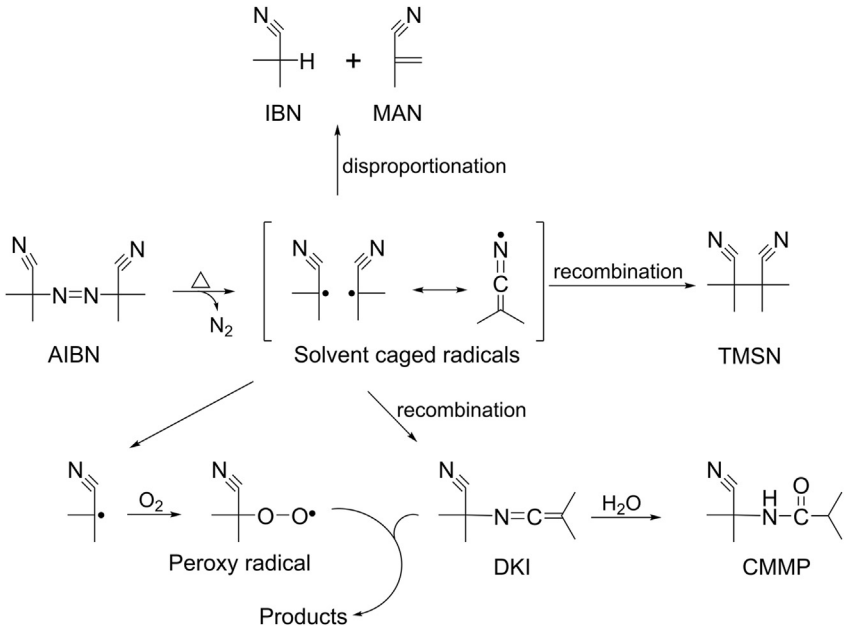

Figure 2. Thermal degradation of AIBN to radical products and various secondary reaction products. MAN, methacrylonitrile; TMSN, tetramethylsuccinodinitrile; IBN, isobutyronitrile.

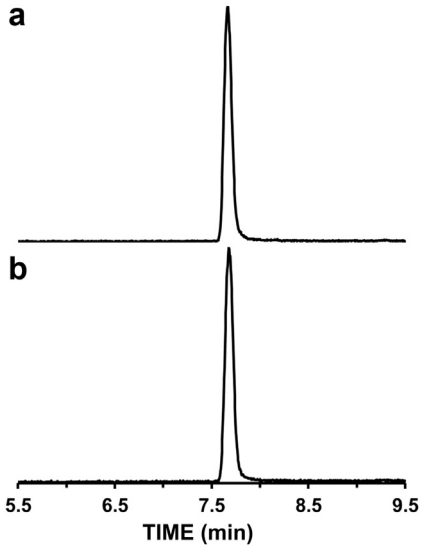

Figure 3. Selected ion chromatograms (m/z 155.1 + 177.1) of (a) AIBN incubation at pH 6 after 18 h and (b) authentic CMMP standard.

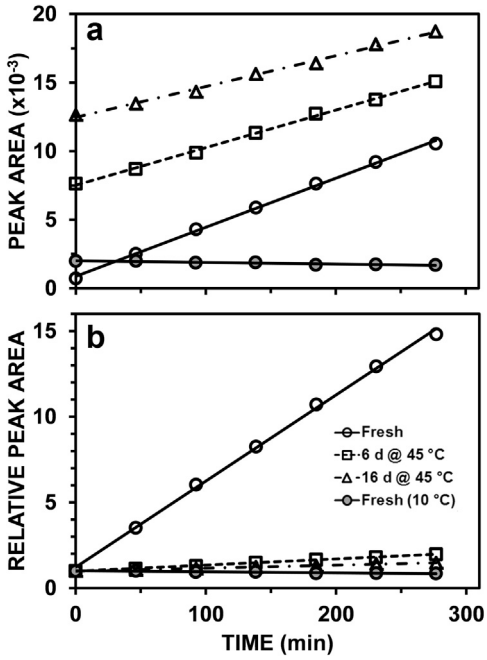

Figure 4. Kinetics for appearance of CMMP from fresh AIBN and degraded AIBN (solid AIBN incubated at 45°C for 6 days and 16 days) when incubated at 40°C in solution at pH 6. Fresh AIBN was also incubated at 10°C in solution at pH 6 as a control. (a) CMMP peak areas derived from selected ion chromatograms (m/z 155.1 + 177.1). (b) CMMP peak areas relative to the initial peak area for each sample.

sampling cone = 25 V, desolvation temperature = 475°C, desolvation gas flow = 800 L/h, source temperature = 120°C, and cone gas flow = 20 L/h. Product ion spectra were obtained with a collision energy = 21 V.

Results and Discussion

The unknown compound formed in the AIBN incubations eluted at 7.8 min with a low intensity $[M + H]^+$ ion of 155.1 and a much larger (~50-fold) relative intensity $[M + Na]^+$ ion of 177.1. Interestingly, the $[M + Na]^+/[M + H]^+$ signal ratio decreased to ~5 by changing the mobile phase additive to 0.1% difluoroacetic acid instead of formic acid (results not shown). High resolution mass spectrometry results suggested an elemental composition of $C_8H_{14}N_2O$ for the unionized molecule. The CID-MS/MS spectrum of the $[M + H]^+$ ion produced 2 major fragments, m/z 128 (neutral loss of HCN) and m/z 86 (neutral loss of isobutyronitrile). Incubations performed at pH 3, 6, and 9 displayed similar kinetics for appearance of this compound out to 5 hours (Fig. 1).

The initial solvent caged radicals formed after release of nitrogen from AIBN are known to produce several products via disproportionation and recombination reactions outlined in Figure 2.[6] In an inert, dry solvent devoid of oxygen, ~55% of AIBN forms dimethyl-N-(2-cyano-2-propyl)ketenimine (DKI). When oxygen is present, no DKI is detected owing to the formation of peroxy radicals that degrade the DKI.[7,8] However, the presence of water allows an additional reaction pathway for DKI that forms CMMP. Early work with DKI did not detect CMMP with the introduction of water because liquid-liquid extractions were performed with dilute hydrochloric acid that hydrolyzed the nitrile group.[9] Rodríguez et al.[10] later detected CMMP in a problematic batch of solid AIBN that was underperforming as a catalyst for radical-mediated synthetic reactions owing to water contamination.

The identity of the AIBN degradation product noted in this article was confirmed to be CMMP after synthesis of the authentic compound which had a similar retention time (Fig. 3), accurate mass and MS/MS spectrum. Identical retention times were maintained when formic acid was substituted with difluoroacetic acid as a mobile phase modifier. To our knowledge, this is the first time that CMMP has been identified in a system that is typical for AIBN-mediated oxidation of pharmaceutical compounds. This suggests peroxy radical concentrations are not high enough to completely ameliorate DKI so it is able to form CMMP. McCarthy and Hegarty reported the hydration of DKI was acid catalyzed; however, the similar kinetics observed in Figure 1 may be explained by the presence of phosphate buffers which are also catalytic.[11] This work shows that CMMP is easily detected with LC/MS conditions commonly used for analysis of pharmaceutical APIs exposed to forced degradation conditions with AIBN. For APIs that exhibit excellent stability, the detection of CMMP could serve as a positive control to confirm the AIBN is behaving as intended, and the API was indeed exposed to a suitable free radical oxidative environment. Indeed, Figure 4a illustrates that when AIBN is compromised, there is a significant amount of CMMP present at t = 0, and the slope is decreased which suggests less radical formation from AIBN. When the CMMP peak area is plotted relative to the area at t = 0 (Fig. 4b), there was an approximately 15-fold increase for the fresh AIBN compared with only a 1.5- to 2-fold increase for the degraded AIBN. No increase in CMMP was observed when fresh AIBN was incubated at 10°C which confirms CMMP is derived from AIBN radical products. The detection of CMMP is also amenable to experiments performed over a wide range pH range. While the present work was specific for AIBN, it may also extend to alternative azonitrile compounds used for forced degradation studies and their respective amide products.

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