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pH control of nucleophilic/electrophilic oxidation

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Abstract

Finding formulations that prevent degradation of the active pharmaceutical ingredient is an essential part of drug development. One of the major mechanisms of degradation is oxidation. Oxidative degradation is complex, and can occur via different mechanisms, such as autoxidation, nucleophilic/electrophilic addition, and electron transfer reactions. This paper uses three model compounds and determines the mechanisms of oxidation and strategies to reduce degradation. The mechanism of oxidation was established by comparing the results of different forced degradation experiments (radical initiation and peroxide addition), computational chemistry to those of formulated drug product stability. The model compounds chosen contained both oxidizable amine and sulfide functional groups. Although, both oxidative forced degradation conditions showed different impurity profiles the peroxide results mirrored those of the actual stability results of the drug product. The major degradation pathway of all compounds tested was nucleophilic/electrophilic oxidation of the amine to form *N*-oxide. Strategies to prevent this oxidation were explored by performing forced degradation experiments of the active pharmaceutical ingredient (API) in solution, in slurries containing standard excipient mixtures, and in solid formulation blends prepared by wet granulation. The reaction was significantly influenced by pH in solvent and excipient slurries, with 100% degradation occurring at basic pH values (>pH 8) and no degradation occurring at pH 2 upon exposure to 0.3% peroxide. Wet granulated blends were also stabilized by lowering the pH during granulation through the addition of citric acid prior to the solution of peroxide, resulting in little (0.02% maximum) or no degradation for the four different blends after 6 week storage at 40 °C/75%RH.

Keywords: Oxidation; *N*-Oxide; Sulfoxide; Excipients; Pharmaceuticals; Preventing degradation

1. Introduction

The stability of drug products are continuously monitored during the lifetime of the manufactured product as outlined by ICH Q1a (ICH, 2003). Degradation can occur from physical or chemical instability of the active pharmaceutical ingredient (API) or by potential reactions of the API with residual water, molecular oxygen, excipients or other impurities present in the final packaged drug product. Oxidation is one of the most important degradation pathways of drug degradation in the case of pharmaceuticals (Crowley and Martini, 2001), second only to hydrolysis as a mode of decomposition. The bulk API can be stable to oxidation under typical storage conditions; however, the formulation and manufacturing process can promote degradation (Crowley and Martini, 2001; Waterman et al., 2002; Kibbe, 2000; Wasylaschuk et al., 2007). Several commonly used excipients have been shown to contain impurities, which are known to facilitate oxidation, such as peroxides or heavy metals (Waterman et al., 2002; Kibbe, 2000). Oxidative degradation is complex and occurs via one or more of the three different mech-

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anisms: autoxidation, nucleophilic/electrophilic addition, and electron transfer. To facilitate rapid drug development a combination of different oxidative forced degradation experiments (Alsante et al., 2007; Nelson, 2006) can be used at an early stage to determine the likelihood of certain degradation pathways and distinguish between mechanisms. In addition, molecular orbital computations can be useful tools to determine potential nucleophilic or electrophilic sites on a molecule. As previously reported, potential selectivity of the nucleophilic/electrophilic process can be understood by the frontier molecular orbital theory where, at a first approximation, the highest occupied molecular orbital (HOMO) can be an indicator of the regiochemistry of oxidation, further elucidating the oxidative mechanism (Reid et al., 2004).

Oxidation can be prevented by controlling impurity levels of peroxides, heavy metals, or by the addition of reducing agents, antioxidants/scavengers (ascorbic acid, vitamin E), or chelating agents (citric acid, EDTA) (Waterman et al., 2002; Hartauer et al., 2000; Hovorka and Schoneich, 2001). However, controlling levels of impurities, such as peroxide (Hartauer et al., 2000), can be very impractical and difficult, and the addition of antioxidants or scavengers may be ineffective depending upon the method of oxidation. If the fundamental mechanism of oxidation is understood before formulation development, a more effective approach to choose excipients and prevent oxidation can be achieved (Waterman et al., 2002; Hovorka and Schoneich, 2001).

In this paper, three model compounds (Fig. 1) possessing multiple oxidation sites were chosen (due to their ease of oxidation) to elucidate potential oxidation mechanisms and determine techniques to prevent oxidation. Exact structures for Compounds A and B are not given due to the proprietary nature of these compounds, but the oxidation sites are clearly shown. The third compound, Raloxifene is a commercially available pharmaceutical used for the prevention of osteoporosis; the oxidation mechanism of Raloxifene has been previously reported (Hartauer et al., 2000).

The comparison of forced degradation experiments using both peroxide and a radical initiator to those of formulated drug products, and results from computational chemistry can elucidate the oxidation degradation pathways (Reid et al., 2004). If an API is susceptible to nucleophilic/electrophilic oxidation it will be shown that oxidation of these compounds can be easily controlled by pH adjustment, as opposed to controlling peroxide levels in the drug product or by the addition of an antioxidant as previously thought (Hartauer et al., 2000). The addition of an antioxidant such as a radical scavenger (ascorbic acid, α-tocopherol), or a chelating agent (citric acid, EDTA) may be ineffective depending on the mechanism of oxidation. In this paper, an alternative method to prevent oxidation is explored and nucleophilic/electrophilic oxidation can be achieved by adjusting the pH of the drug product by addition of citric acid in the solid state. Quantities of citric acid required for stabilization can be determined based on pH degradation profiles of the slurried state in aqueous solutions.

2. Materials and methods

2.1. Reagents and chemicals

Formic acid (90%) was obtained from EM Science. Hydrochloric acid, sodium borate 10-hydrate, sodium hydroxide, and sodium phosphate were purchased from J.T. Baker. HPLC grade acetonitrile and methanol (Mallinckrodt), sodium citrate dihydrate (Spectrum Chemical), 30% hydrogen peroxide (VWR), microcrystalline cellulose (Avicel PH102; FMC), and lactose monohydrate (Fast Flo 316; CHR Hansen) were obtained. Sodium lauryl sulfate (Tensopol USP94; Aceto USA), magnesium stearate (5712; Mallinckr-Baker), crospovidone (Polyplasdone XL; ISP Tech), povidone (Kollidon 30; BASF), anhydrous citric acid (Mutchler Inc.), and calcium carbonate (Mutchler Inc.) were also purchased. Water was purified by A Millipore Milli-Q Gradient A70 water system. L-Ascorbic acid was obtained from Sigma, while 4,4′-azobis-(4-cyanovaleric acid) (ACVA) was an Aldrich product. Raloxifene (hydrochloride salt) was purchased from both Sigma–Aldrich (slurry experiments) and Toronto Research Chemicals (wet granulation experiments). Compounds A (mesylate salt) and B (mesylate salt) were obtained from Pfizer Inc.

2.2. Chromatographic and mass spectrometry conditions

All analyses were conducted on an Agilent 1100 series HPLC instrument (Agilent Technologies). The HPLC method

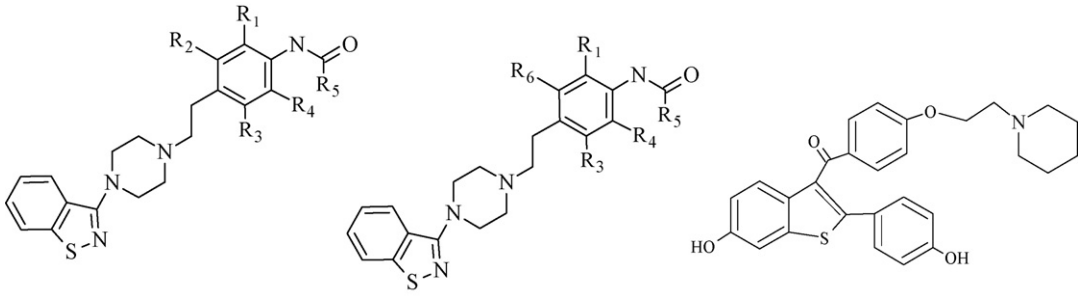

Fig. 1. Structures of the three model compounds: Compound A, Compound B, and Raloxifene. Due to the proprietary nature of Compounds A and B, their exact structures are not given; but the susceptible oxidation sites are clearly shown.

used a YMC ODS AQ column (3 μm particle size, 4.6 mm i.d. × 150 mm length) at 40 °C with a 20-min linear gradient from 10% to 90% mobile phase A (acetonitrile) and 90% to 10% of mobile phase B (0.05% formic acid) at 1.0 mL/min and a 10 μL injection volume. This method was validated for linearity, precision, accuracy, sample preparation (sonication time), stock and diluent solution stability.

In the mechanistic determination and solution pH stability experiments, samples were detected using a diode array detector (DAD) and an in-line QuattroMicro mass spectrometer (MS; Micromass). A 1:5 flow splitter was placed between the DAD and MS so that a flow rate of 0.2 mL/min entered the MS. The DAD scanned from 200 to 400 nm, while the MS acquired data from 100 to 850 amu over 1 s with an interscan delay of 0.05 s. Electrospray ionization with positive polarity was utilized. The cone voltage, capillary voltage, source temperature, and desolvation temperature were 35 V, 3.5 kV, 120 °C, and 400 °C, respectively. In the drug product (Compound A) and remaining pH stability experiments, a variable wavelength detector (VWD) monitored the API and degradants at 254 nm.

2.3. Mechanistic determination

2.3.1. Computational chemistry

The highest occupied molecular orbital (HOMO) was determined for Compound A using the Spartan '02 Windows (Wavefunction, Inc.) software. Geometric minima were located using the conformational Monte Carlo search algorithm with the Merck Molecular Force Field. Once the lowest conformer was chosen the optimized geometric and electronic structure was established using the Hartree-Fock level of theory with a 6-31G* basis set. The 6-31G* basis set puts d functions on each heavy atom to emulate valence electrons.

2.3.2. Autoxidation using a radical initiator

Solutions of Compounds A and B were prepared (0.1 mg/mL) in 50/50 acetonitrile/water. The radical initiator, ACVA, was dissolved (35 mg/mL) in methanol while the antioxidant, ascorbic acid, was prepared (10 mg/mL) in water. ACVA was added to the API solution to yield 5 mol% ACVA (relative to API) and samples were pressurized with oxygen using a Parr general purpose pressure vessel with a gage block assembly and stored at 60 °C. Final pH of this solution was not measured. Sample vials were removed and quenched with an excess of ascorbic acid. The samples and appropriate controls were prepared and analyzed using the HPLC/DAD/MS method as outlined above.

2.3.3. Nucleophilic/electrophilic oxidation using hydrogen peroxide

Ten microliters of 30% hydrogen peroxide was added to 1 mL solutions (40/60 acetonitrile/water) of either Compound A or Compound B (0.1 mg/mL) and analyzed using the HPLC/DAD/MS method as outlined above.

2.3.4. Compound A drug product and drug substance stability under ICH stability conditions

Pure drug substance was placed in double low-density polyethylene bags in cardboard containers. Size 3 gelatin capsules (fill weight 190 mg) containing Compound A (3.2 wt%), microcrystalline cellulose (45.6 wt%), lactose monohydrate (45.6 wt%), sodium lauryl sulfate (1.0 wt%), magnesium stearate (0.5 wt%), and crospovidone (4.0 wt%) were prepared by dry blending. Capsules were stored in HDPE bottles with a heat induction seal and a child-resistant closure. Both the drug substance and drug product supplies were placed in stability chambers (Environmental Specialists, Inc.) at 40 °C/75%RH and 25 °C/60%RH. These conditions correspond with the general case drug substance and drug product accelerated and long-term stability storage conditions, respectively (ICH, 2003).

One bottle of capsules and packet of API was removed from the chambers at each time point. The blend was carefully removed from five capsules and prepared to 0.1 mg/mL active using 40/60 acetonitrile/water with 30-min sonication. The samples were filtered with Gelman Acrodisc syringe filters (25 mm, 0.45 μm pore size, nylon membrane) into HPLC vials and analyzed using the HPLC/VWD method as outlined above. The API was similarly prepared (without filtering) and analyzed.

2.4. Effect of pH on nucleophilic/electrophilic oxidation

2.4.1. Oxidation in solution

The following buffers were prepared to a concentration of 10 mM and their pH adjusted as needed with either hydrochloric acid or sodium hydroxide: sodium citrate (pHs 2, 4, and 6), sodium phosphate (pH 8), and sodium borate (pH 10). Solutions of the three model compounds were prepared to yield a final concentration of 0.1 mg/mL in 50% acetonitrile and 50% of each buffer. Each API solution was aliquotted, 1 mL, into an HPLC vial and 10 μL of 30% hydrogen peroxide was added immediately prior to injection onto the HPLC/DAD/MS. The resulting samples were injected repeatedly in 2 h intervals for a total of 18 h. The autosampler was at ambient temperature and the injections were taken from the same vial with a self-sealing cap preventing evaporation.

2.4.2. Oxidation in an excipient blend suspension

For all three model compounds, an excipient blend (Table 1 Formulation 1) was prepared and divided equally into five bottles. Water, 25 mL, was added to each bottle and 30-min of sonication followed. Using citric acid, the pH values were adjusted to pHs 2, 4 or 6 for three of the bottles. The pH of the fourth bottle was adjusted to a value of 8 with calcium carbonate while the pH of the fifth bottle was not adjusted by addition of either citric acid or calcium carbonate (pH ∼6–7). Both additives were accurately weighed to determine the amount of modifier needed to control the pH of the wet granulated formulations. Acetonitrile, 25 mL, was then added to the suspensions followed by one of the model compounds to yield a final concentration of 0.1 mg/mL. The suspensions were then sonicated for 30-min and subsequently filtered (Gelman Acrodisc syringe filters, 25 mm, 0.45 μm pore size, nylon membrane). Immediately

Table 1
Raloxifene formulations used to determine the effect of citric acid in the presence of hydrogen peroxide

Ingredient	Formulation			
	1[a]	2	3	4
Raloxifene (wt%)	2.83	2.95	2.83	2.98
Microcrystalline cellulose (wt%)	45.83	47.74	45.83	48.24
Lactose monohydrate (wt%)	45.83	47.74	45.83	48.24
Magnesium stearate (wt%)	0.51	0.53	0.51	0.53
Crospovidone (wt%)	4.00	–	–	–
Povidone (wt%)	–	–	4.00	–
Sodium lauryl sulfate (wt%)	1.00	1.04	1.00	–

[a] Excipient mixture used in Compound A drug product stability and for suspension experiments.

prior to injection onto the HPLC/VWD, 10 μL of 30% hydrogen peroxide was added to 1 mL of each filtered solution. The resulting samples were injected repeatedly in 2-h intervals for a total of 18 h.

2.4.3. Raloxifene excipient blend preparation and experimentation

Four different Raloxifene formulations were prepared from the ingredients listed in Table 1. They were combined, sifted (sieve no. 25), mixed for 5 min with a Turbula mixer, and immediately divided into three equal portions. The formulations were then wet granulated using a Bohle-mini-granulator (impeller speed 500 rpm, chopper speed 1000 rpm) for approximately one minute with a granulating solution consisting of either purified water, purified water followed by hydrogen peroxide, or a citric acid solution followed by hydrogen peroxide. Citric acid was added in an amount necessary (as determined in the excipient blend suspension work) to yield pH 4. The amount of hydrogen peroxide added was modeled after an experiment in Hartauer et al. (2000) in order to achieve a level of approximately 800 ppm relative to the crospovidone amount in Formulation 1. The granulated formulations were placed in an oven at 50 °C and removed when the water content was less than or equal to that of the dry granulation measured by a Smart5 CEM (approximately 2.5 h). The granulated formulations were then divided (approximately 20 g per sample) into separate 60 mL white HDPE bottles with child-resistant caps and placed in a 40 °C/75% RH stability chamber (Environmental Specialists, Inc.).

At each time point, one bottle of each formulation was removed and 1.9 g of each blend was weighed to yield 0.1 mg/mL Raloxifene in 50/50 acetonitrile/water. Samples were filtered (Gelman Acrodisc syringe filters; 25 mm, 0.45 μm pore size, nylon membrane) and analyzed by HPLC/VWD.

To determine if citric acid reacted with peroxide we exposed a molar excess of peroxide to citric acid (pH 2) for 4 h and then combined this solution with 0.1 mg/mL Raloxifene to yield a final solution with a pH of 7.5 (while maintaining the same peroxide-to-Raloxifene ratio as used in previous Raloxifene solution experiments). This final solution was injected every 2 h for a total of 18 h.

3. Results

3.1. Mechanistic determination

3.1.1. Computational chemistry

Fig. 2 shows the 6-31G* highest molecular orbital (HOMO) of Compound A. The electron density is centered on both the sulfur and the piperazine nitrogen distal to the benzisothiazole, indicating potential sites of reaction. Establishing where the HOMO is centered is a good way to determine localized reactivity as based on frontier molecular orbital theory. The delocalization on the HOMO of this molecule is a mixture of sp^3 nitrogen lone pair and the pi system of the thiazole indicating both could be the reactive site.

3.1.2. Autoxidation using a radical initiator

Fig. 3 shows the chromatograms from the forced degradation and drug product stability experiments on Compound A. The effect of a 5 mol% radical initiator (ACVA) solution in the presence of pressurized oxygen on Compound A after 5 days of exposure at 60 °C is illustrated in Fig. 3A. The API degraded by 13% and degradants were observed at RRT 1.04 (2%) and RRT 1.35 (11%). The degradant at RRT 1.04 was identified by NMR (^{1}H, HMBC (heteronuclear multiple bond correlation), HMQC (heteronuclear multiple quantum correlation) and COSY (correlation spectroscopy)) as the *N*-oxide (oxidation of the piperazine

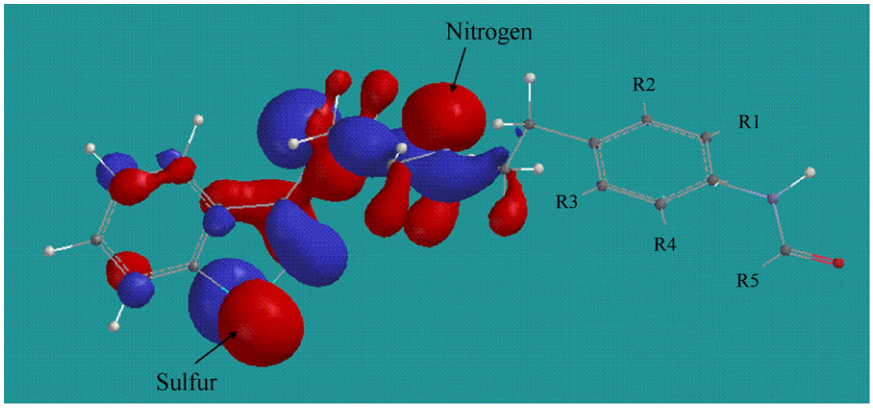

Fig. 2. HOMO from HF/6-31G* optimized geometry of Compound A.

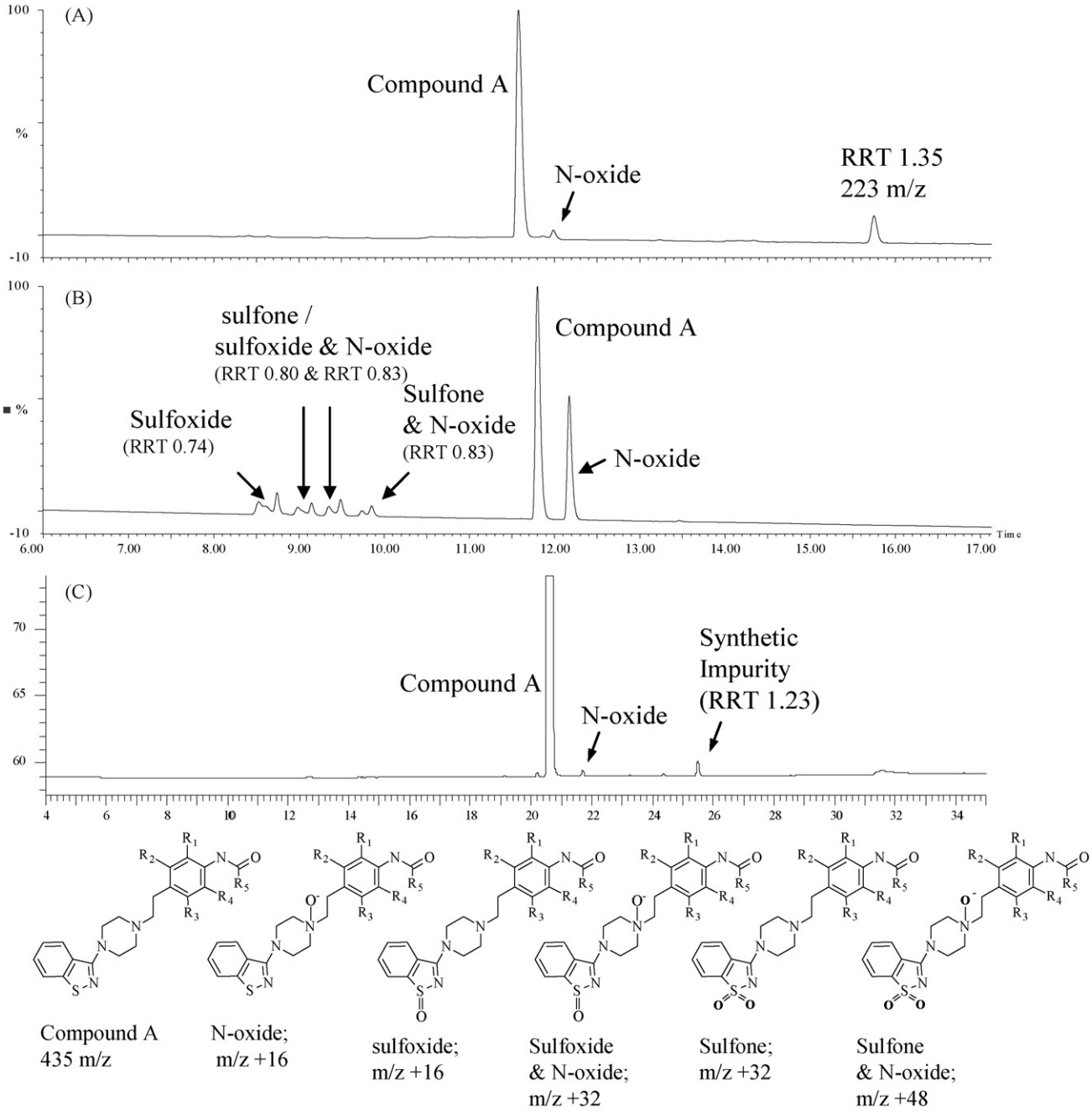

Fig. 3. Chromatographic overlay of Compound A exposed to (A) 5 mole percent ACVA and pressurized oxygen for 5 days at 60 °C; (B) 0.3% hydrogen peroxide for 4 h at room temperature; (C) accelerated (40 °C/75%RH) stability conditions for 6 weeks in formulated capsules. In the latter chromatogram, a 55-min gradient and 0.05% TFA were used in place of the 20-min gradient and 0.05% formic acid, respectively, mentioned in Section 2.2. All other conditions were the same.

nitrogen distal to the benzoisothiozole) and the degradant at RRT 1.35 was characterized by mass spectrometry (223 *m/z*). When Compound B was exposed to a 5 mol% ACVA solution for 5 days a primary degradant at RRT 1.23 (149 *m/z*) was observed. This was also observed under pressurized oxygen conditions.

3.1.3. Nucleophilic/electrophilic oxidation using hydrogen peroxide

Fig. 3B shows the result of Compound A exposed to 0.3% hydrogen peroxide for 4 h at room temperature. Under these conditions, degradation is extensive with 48% of the API being degraded into five degradation products, which were identified by LC/DAD/MS. Some peak splitting was observed for RRT 0.74, 0.78, 0.80, and 0.83 due to poor chromatography, as the diluent was significantly different than the mobile phase. (*Injection Solvent Effects*. http://www.mac-mod.com/cc/cc-10.html Section 10 of the MAC-MOD HPLC Column Companion.)

(Dolan and Snyder, 1989) (40/60 acetonitrile/water vs. gradient elution by acetonitrile and 0.05% formic acid). The major degradation product was the *N*-oxide (parent mass (435 *m/z*) +16; RRT 1.04). The degradation product at RRT 0.72 was iden-

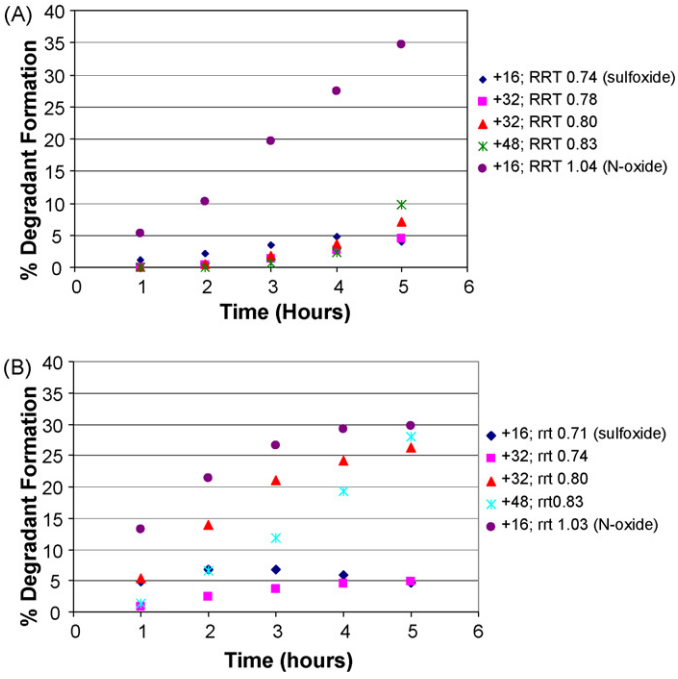

Fig. 4. Degradation profile for the nucleophilic/electrophilic oxidation products of (A) Compound A and (B) Compound B following exposure to 0.3% peroxide at room temperature.

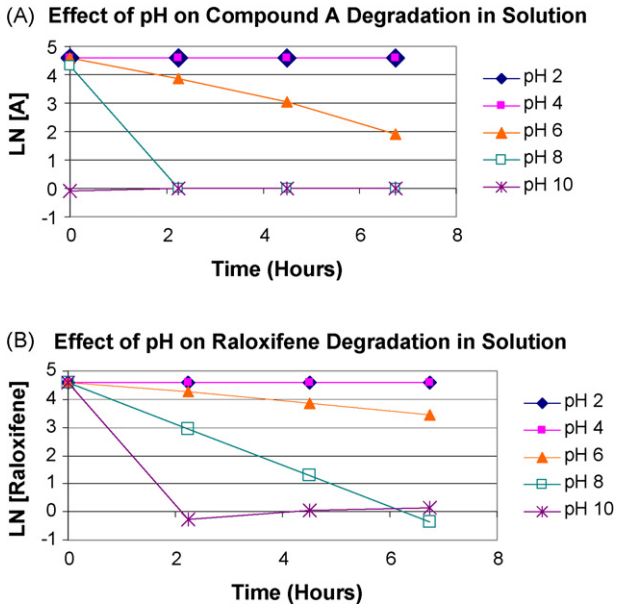

Fig. 6. The effect of pH on the stability of Compound A (6A) and Raloxifene (6B) in solution under forced degradation conditions using 0.3% peroxide at room temperature.

tified as the sulfoxide (parent mass +16) by comparison of its RRT to a reference standard. The other degradation products were identified by mass spectrometry. Two peaks with $m/z + 32$ (RRT 0.78 and 0.80) corresponding to sulfoxide, *N*-oxide, and sulfone were observed. Fig. 4 shows the 5-h degradation profile for these five degradation products of Compounds A and B following exposure to 0.3% hydrogen peroxide at room temperature. The oxidation sites of Compounds A and B were fully oxidized after overnight exposure, resulting in a single degradant with a mass equal to that of the parent +48 (RRT 0.83; data not shown).

The proposed mechanism for nucleophilic/electrophilic oxidation to form *N*-oxide is shown in Fig. 5 on Compound A, with the lone pair of electrons on the piperazine nitrogen distil to the bensoisothiazole acting as an nucleophile. Protonation can occur at this site to retard this reaction.

3.1.4. Compound A drug product and substance stability under ICH stability conditions

The stability of formulated capsules containing 5 mg Compound A, when subjected to ICH general case accelerated stability conditions (40 °C/75%RH) for 6 weeks, is illustrated in Fig. 3C. The peak at 25.5 min (RRT 1.23) is a synthetic impurity present in the drug substance lot used in the capsules (note that this lot of drug substance differs from that used in the forced degradation experiments). The only significant degradation product was the *N*-oxide (RRT 1.04), which was present at 0.1%. Amounts of *N*-oxide decreased slightly upon further storage. Similar results (data not shown) were observed for samples stored under ICH general case long-term storage conditions (25 °C/60% RH). *N*-Oxide formation was not observed in the drug substance stability samples stored under the same conditions.

3.2. Effect of pH on nucleophilic/electrophilic oxidation

3.2.1. Oxidation in solution

Fig. 6a shows the reaction rate of API degradation and pH oxidation profile of Compound A. At pHs 6, 8 and 10, little or no API remained. At high pH (8 and 10), the API rapidly degraded, with several degradants being observed over an 18-h time period. (For clarity only 7 h are shown in Fig. 6.) Over time the *N*-oxide further reacted to form +48 *m/z*, presumably the

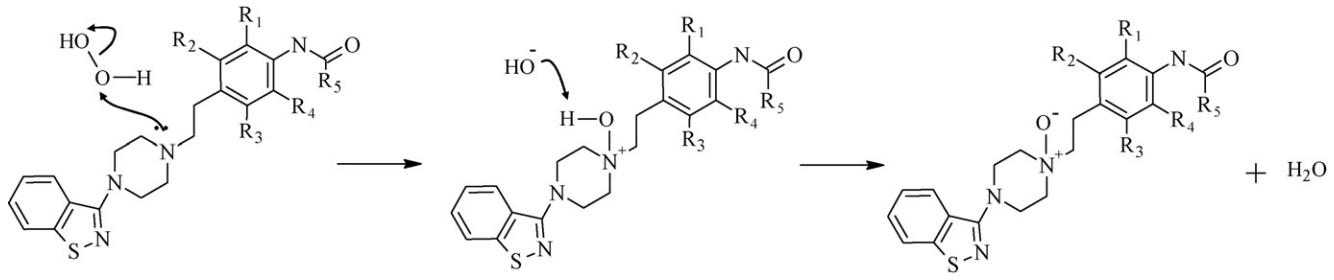

Fig. 5. Proposed mechanism for the nucleophilic/electrophilic oxidation of Compound A to form *N*-oxide.

N-oxide sulfone. These results were mirrored in all three model compounds, as can be seen in Fig. 6b for Raloxifene.

At pH 4, all three model compounds proved to be significantly more stable after an 18-h exposure to peroxide, with only 2.5% of the active (Compounds A and B) degraded to form *N*-oxide. Raloxifene was more stable with only 0.06% *N*-oxide forming. At pH 2, all three model compounds were completely stabilized under the forced degradation conditions after 18 h.

3.2.2. Oxidation in excipient blend suspensions

In suspension, *N*-oxide formation and the overall degradation of API showed a similar trend to the compounds in solution. *N*-Oxide formed quite quickly with all three compounds. At pH 8 little or no API remained 6-h after the addition of hydrogen peroxide. Importantly, samples at pH 6 independent of citric acid showed similar stability. Thus, indicating that pH adjustment is a more important factor to prevent the oxidation than the addition of an antioxidant.

Compound A and Raloxifene were less stable in excipient blend suspension than in solution at pH 4. The sole degradant, *N*-oxide, formed in greater amounts; 29% versus 1.2% for Compound A and 11% versus 0.06% for Raloxifene. There was no significant difference when comparing excipient blend versus solution results for Compound B; *N*-oxide levels for both were 1% after 18 h. *N*-oxide formation was mitigated and all compounds were completely stabilized at pH 2, even after 18-h exposure to hydrogen peroxide in both excipient blend suspension and in solution.

3.2.3. Oxidation in Raloxifene formulated excipient blends

Table 2 shows the *N*-oxide levels determined at various time points in the four different wet granulated Raloxifene formulation blends. *N*-Oxide formation was observed at all time points in those wet granulated with a 0.01 wt% (final blend weight) aqueous hydrogen peroxide solution. Raloxifene was completely stabilized for 6 weeks at 40 °C/75% RH in the formulations acidified with 0.5 wt% citric acid, with the exception of Formulation 3, where 0.02% *N*-oxide formed after 6 weeks.

4. Discussion

There are multiple oxidation sites for the three model compounds shown in Fig. 1. Potential sites of oxidation can be determined using computational chemistry (Reid et al., 2004; Boyd, 2005). Based on the frontier molecular orbital (FMO) theory, the key interactions will be between the nucleophile's highest occupied molecular orbital (HOMO) (e.g. lone pairs on the amine or thiol) and hydrogen peroxide's (electrophile) lowest unoccupied molecular orbital (LUMO). Fig. 2 shows the HF/6-31G* HOMO for Compound A, indicating the most reactive sites are located on the sulfur and the piperazine nitrogen distal to the benzoisothiozole ring. A 6-31G* basis set was necessary to calculate the HOMO of this molecule since the inclusion of d functions was necessary due to the sulfur heteroatom.

The oxidation mechanism of the model compounds was determined by comparing forced degradation experiments to the results of the ICH stability conditions. Nucleophilic/electrophilic mechanisms can be distinguished from autoxidation by the addition of peroxides to the active pharmaceutical ingredient; whereas, autoxidation chain processes can be performed using bubbled oxygen, pressurized oxygen and/or by the addition of a radical initiator to attempt to generate radical oxidation conditions. This can create a complex system of propagation generating various types of radicals. Some nucleophilic/electrophilic reactions, although to a lesser extent, can take place as well using autoxidation conditions, since peroxyl radicals can be one of the by-products of propagation in autoxidative chain processes, i.e. through a propagation reaction of the free radical with molecular oxygen (Waterman et al., 2002; Smith and March, 2000; Beckwith et al., 1983).

Fig. 3 shows that *N*-oxide is formed under all oxidation conditions; autoxidation, the addition of peroxide (nucleophilic/electrophilic), and in the accelerated stability of the drug product. However, overall the autoxidation products for Compound A (Fig. 3A) are different than those formed in the peroxide (Fig. 3B) and accelerated stability experiments (Fig. 3C). *N*-Oxide was not the primary degradant formed when Compound A was subjected to autoxidative conditions. Similarly, no *N*-oxide was formed when 0.5 mg/mL Raloxifene in 80/20 acetonitrile/water was stressed with one equivalent of 2,2′-azobisisobutyronitrile (AIBN) at 40 °C for 1 week (Hartauer et al., 2000). Small pH, and solvent effects have been observed using radical initiation processes affecting reaction rates and types of degradation products. These effects appear to be much smaller for autooxidation than for nucleophilic/electrophilic oxidation. These effects are complex and depend on the site of hydrogen abstraction and the effect of ionization on the XH bond strength. The concentration of OH^- also effects reaction

Table 2
The effect of citric acid on *N*-oxide formation in Raloxifene excipient blends under 40 °C/75%RH stability conditions

Formulation/granulation solution	Area % *N*-oxide (vs. parent)			
	Initial	2 week	4 week	6 week
Formulation 1/H_2O_2	0.02	0.18	0.20	0.21
Formulation 2/H_2O_2	0.05	0.14	0.12	0.17
Formulation 3/H_2O_2	0.05	0.55	0.45	0.50
Formulation 4/H_2O_2	0.05	0.23	0.21	0.27
Formulation 1/H_2O_2 and citric acid	–	–	–	–
Formulation 2/H_2O_2 and citric acid	–	–	–	–
Formulation 3/H_2O_2 and citric acid	–	0.02	0.01	0.01

The results for *N*-oxide are expressed as an area percent relative to the parent peak.

rate due to increased formation of reactive oxide radicals. Solvents can stabilize the radicals and facilitate reactions as well as forming solvent oxide products with the API (Betigeri, 2005; Reichardt, 2004; Kerwin et al., 2006; Nelson, 2006). In our series of molecules we only observed the nucleophilic/electrophilic oxidation products on real time stability. Since, no major autooxidative degradation products were observed in the ICH stability experiments, further work on this pathway was not pursued.

The results for Compounds A and B under forced degradative conditions with hydrogen peroxide are consistent with those predicted from the computational chemistry, as the sites of reaction were the sulfur and piparizine nitrogen. In both, *N*-oxide and sulfoxide formed within 1 h (Fig. 4), with the former being the primary degradant. *N*-Oxide was also reported to be the primary degradant in Raloxifene (in aqueous slurry) upon exposure to 0.3% hydrogen peroxide after 1 week at room temperature (Hartauer et al., 2000).

N-Oxide was the only degradation product observed during the accelerated (40 °C/75% RH) and long-term stability (25 °C/60% RH) studies of formulated Compound A capsules (Fig. 3C). (By itself, the bulk API of all three compounds was stable to oxidation and no degradation occurred at either long term or accelerated conditions.) *N*-Oxide was also the primary degradant in tablets of Raloxifene (Hartauer et al., 2000). Comparison of the accelerated drug product stability, forced degradation, and computational chemistry experiments shows that the primary degradation pathway of all three model compounds is a nucleophilic/electrophilic interaction of peroxide with the piperazine nitrogen to form *N*-oxide (Compound A, Fig. 5).

Oxidation of these compounds can be prevented by avoiding or removing peroxides in the formulation (Waterman et al., 2002; Smith and March, 2000; Hartauer et al., 2000; Reid et al., 2004; Beckwith et al., 1983). During the development of Raloxifene tablets, a strong correlation was observed between total peroxide levels and *N*-oxide formation in tablets stored under accelerated (40 °C/75% RH) conditions (Hartauer et al., 2000). Binary mixtures of Raloxifene and excipients used in formulation development showed that only mixtures with povidone and crospovidone formed the *N*-oxide (Hartauer et al., 2000). This is not surprising since these excipients are known to contain trace levels of peroxides (Crowley and Martini, 2001; Kibbe, 2000; Wasylaschuk et al., 2007). A limit test for peroxide levels in crospovidone and povidone was developed as a strategy to stabilize the drug product (Hartauer et al., 2000). Although an acceptable strategy, peroxide levels in excipients can be difficult to control. The amount of peroxide contamination in excipients is likely to vary from lot to lot based on the manufacturer and the molecular weight (in the case of polymers) (Waterman et al., 2002; Wasylaschuk et al., 2007). As well, it has been shown that peroxide levels in excipients can fluctuate with storage conditions over time (Waterman et al., 2002; Hartauer et al., 2000).

Fig. 6 clearly shows that pH has a significant effect on *N*-oxide formation both in solution and excipient blend suspensions (data not shown). In acidic environments, *N*-oxide is not formed, and the API in solution or suspension is completely stabilized even when exposed to forced degradation conditions of 0.3% peroxide for 18 h. The pK_a value of the piparazine nitrogen at the site of *N*-oxide formation was measured experimentally to be 6.45 and 6.49 for Compounds A and B, respectively. Raloxifene's pK_a was calculated to be 8.67. (Calculated using Advanced Chemistry Development, Inc. (ACD/Labs) Software Solaris V4.67.) At lower pH values, this nitrogen may be protonated, preventing *N*-oxide formation. Under basic conditions, where protonation of the nitrogen would not occur, the API completely degraded within 2 h, initially forming *N*-oxide and further degrading to a fully oxidized degradant with a mass equal to that of the parent +48. Compounds had similar stability at pH 6 with or without addition of citric acid indicating that citric acid mitigated oxidation via pH control rather than acting as a chelating agent. Furthermore, even though all three compounds had acidic counterions (hydrochloric acid for Raloxifene, methanesulfonic acid for Compounds A and B), this did not stabilize the APIs with regard to oxidation, and further pH control with citric acid was necessary. Although other acidic excipients may be used, citric acid is a good choice, as it is relatively inexpensive and has a low pK_a.

Although we were able to stabilize the model compounds in both solutions and in slurries, we examined if adjusting the pH in the solid state could also prevent *N*-oxide formation. The amount of citric acid needed could be estimated from the amount required to reach the desired pH in the excipient blend suspensions. Four different Raloxifene formulations were prepared and are shown in Table 1. All formulations contain Raloxifene, microcrystalline cellulose, lactose monohydrate, and magnesium stearate. The formulations differ by the addition of disintegrants (crospovidone, povidone) or a surfactant (sodium lauryl sulfate). Formulation 1 is the same as that used for the suspension and ICH studies discussed above. Both disintegrants are potential sources of peroxide, whereas, povidone has a slightly lower pH range specified by the USP (3.0–7.0) than crospovidone (5.0–8.0), however, the variation in pH and the small amount used is not the best strategy to prevent *N*-oxide formation (Kibbe, 2000).

As shown in Table 2, all formulations granulated without citric acid degraded to form the *N*-oxide. Small amounts of *N*-oxide were present at $t = 0$ with the formulations granulated with peroxide and water, and levels increased upon storage. At all time points analyzed, the highest *N*-oxide levels were seen in Formulation 3. No other degradation products were observed above the detection limit of the analytical method (0.01%) in any formulation.

The addition of citric acid stabilized all formulations. An acceptably small amount of *N*-oxide formed in Formulation 3 at the 2-, 4-, and 6-week time points (0.02%, 0.01%, and 0.01%, respectively). Amazingly, even when 0.01 wt% peroxide was added to the citric acid (0.5 wt%) wet granulating solution, *N*-oxide formation was prevented.

We also explored the potential of *N*-oxide to re-equilibrate back to the parent compound by completely degrading the API in solution at pH 6 with 0.3% hydrogen peroxide. Once fully degraded, citric acid was added to the solution to lower the pH from 6 to 2. The solution was then analyzed, and no parent com-

pound was found. We also considered the possibility that citric acid could be reacting with peroxide, thereby depleting the levels of peroxide, preventing *N*-oxide formation. It was shown that this was not the case by comparing Raloxifene degradation to unbuffered Raloxifene solution experiments (i.e. without citric acid). Raloxifene degradation for both was identical, indicating that citric acid does not affect peroxide reactivity for this system.

5. Conclusion

Nucleophilic/electrophilic oxidation to yield *N*-oxide was proven to be the prevalent degradation mechanism in the three model compounds by comparing results from forced degradation experiments, formulated drug product ICH stability, and computational chemistry. Lowering the pH by addition of citric acid was shown to stabilize drug products susceptible to nucleophilic oxidation, presumably by protonating the susceptible oxidation site. Stabilization of the model compounds was achieved under forced degradative conditions in solution, excipient blend suspension and formulated blends prepared by wet granulation. Acidifying the formulation in the solid state worked to stabilize the API under accelerated ICH stability conditions, which will allow for a projected shelf life estimation (Parikh, 1981; Waterman and Adami, 2005). The level of the citric acid excipient required can be estimated from slurry solutions of the excipients and API by obtaining pH degradation profiles. In this paper, we have clearly shown that understanding the oxidative mechanism will facilitate the formation of a stable drug product and the addition of an acidic excipient can be a first approach to prevent nucleophilic/electrophilic oxidation.

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