Journal of Pharmaceutical and Biomedical Analysis 149 (2018) 206–213

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Artifactual degradation of secondary amine-containing drugs during accelerated stability testing when saturated sodium nitrite solutions are used for humidity control

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ARTICLE INFO

Article history:
Received 28 June 2017
Received in revised form 10 October 2017
Accepted 28 October 2017
Available online 29 October 2017

Keywords:
Nitrosamine
Nitrite
Stability
Degradation

ABSTRACT

Accelerated stability studies of pharmaceutical products are commonly conducted at various combinations of temperature and relative humidity (RH). The RH of the sample environment can be controlled to set points using humidity-controlled stability chambers or via storage of the sample in a closed container in the presence of a saturated aqueous salt solution. Herein we report an unexpected N-nitrosation reaction that occurs upon storage of carvedilol- or propranolol-excipient blends in a stability chamber in the presence of saturated sodium nitrite ($NaNO_2$) solution to control relative humidity (~60% RH). In both cases, the major products were identified as the corresponding N-nitroso derivatives of the secondary amine drugs based on mass spectrometry, UV–vis and retention time. These degradation products were not observed upon storage of the samples at the same temperature and humidity but in the presence of saturated potassium iodide (KI) solution (~60% RH) for humidity control. The levels of the N-nitrosamine derivatives varied with the pH of various $NaNO_2$ batches. The presence of volatile NOx species in the headspace of a container containing saturated $NaNO_2$ solution was confirmed via the Griess assay. The process for formation of the N-nitrosamine derivatives is proposed to involve volatilization of nitric oxide (NO) from aqueous nitrite solution into the headspace of the container followed by diffusion into the solid drug-excipient blend and subsequent reaction of NOx with the secondary amine.

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1. Introduction

Accelerated stability studies are an important part of the drug development process and are commonly conducted to identify degradation products and reaction pathways to aid in the elucidation of the intrinsic stability characteristics, including development of stability-indicating analytical methods [1]. These studies are usually conducted across a wide range of pH values and at various combinations of temperature and relative humidity (RH). In fact, accelerated stability tests conducted at various temperatures and humidities can be used to predict the degradation rate(s) of drugs utilizing a humidity-modified Arrhenius equation [2,3]. The RH of the sample environment in such accelerated stability tests can be controlled to a particular set point using a humidity-controlled stability chamber or via storage of the sample in a closed container in the presence of various saturated salt solutions [4,5]. Humidity-controlled stability chambers offer advantages of accessing a range of both temperature and humidity conditions as well as provides the ability to change quickly among desired stability conditions; disadvantages include their expense and resource overhead required to maintain and operate such units. On the other hand, saturated salt solutions provide a “fixed” humidity at a given temperature, but are significantly less expensive than stability chambers. Based on the identity of the salt, a range of relative humidity conditions can be achieved with saturated salt solutions (3% –98% at 25 °C) [4]. Despite their advantages, a caveat associated with saturated salt solutions is the potential for non-representative degradation due to reactions induced by the saturated salt solution. This study demonstrates one such non-representative (artifactual) degradation observed in the presence of sodium nitrite ($NaNO_2$), a commonly-used saturated salt solution for accessing relative humidity values in the range of 60% – 65% RH. Use of $NaNO_2$ for this

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https://doi.org/10.1016/j.jpba.2017.10.035
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purpose is described in both the literature [5] and current standards issued by national laboratories [6].

In this report, we describe results observed during storage of a few solid drug substances and formulated products containing secondary amines in the presence of a saturated solution of $NaNO_2$ (~60% RH). Variable amounts of unknown degradation products of the drugs, not seen at higher or lower humidities (using other saturated salt solutions or in humidity controlled chambers), were observed. These unexpected results were further explored using three secondary amines, carvedilol, propranolol and norfloxacin, as model drug compounds (Fig. 1). Thus, the three drug compounds, as drug substances, drug-excipient blends and drug-excipient compacts, were aged in the presence of saturated $NaNO_2$ solutions (~60% RH) and in the presence of saturated potassium iodide (KI) solutions (~60% RH) at 60 °C as a control. In the case of carvedilol and propranolol, two significant degradation products were formed in the presence of the $NaNO_2$ salt solution, which were not observed in the presence of saturated KI. Based on these unusual results, an investigation was initiated, and the results of this investigation are described herein.

2. Experimental

2.1. Caution

The mutagenicity and carcinogenicity of nitrosamines are well known. Care should be exercised in the handling of nitrosamines to avoid exposure.

2.2. Materials and reagents

Carvedilol (certified reference material), (±) propranolol hydrochloride (≥99%), norfloxacin (≥98%), potassium iodide (ReagentPlus®, 99%) and trifluororacetic acid (HPLC grade) were used as received from Sigma-Aldrich (St. Louis, MO, USA). $NaNO_2$ was obtained as various grades from several suppliers including Sigma-Aldrich, Fisher Scientific (Pittsburgh, PA, USA), EMD Millipore (Billerica, MA, USA), Macron Fine Chemicals (Center Valley, PA, USA) and MP Biomedicals (Santa Ana, CA, USA). Microcrystalline cellulose (NF/Ph. Eur./JP grade) and lactose monohydrate (NF grade) were obtained from FMC Biopolymer (Mechanicsburg, PA, USA) and Foremost Farms USA (Rothschild, WI, USA), respectively. HPLC grade acetonitrile was obtained from Honeywell Burdick & Jackson (Muskegon, MI, USA). The sample dissolving solvent, unless otherwise specified, was prepared by combining 1L of acetonitrile with 1L ultrapure water obtained from a Milli-Q water purification system (EMD Millipore, Billerica, MA, USA) and addition of 2.0 mL of concentrated phosphoric acid (85 wt%, Fisher Scientific). The Griess Reagent Kit was obtained from Molecular Probes (Eugene, OR, USA).

2.3. pH measurements of saturated $NaNO_2$ solutions

Saturated $NaNO_2$ solutions were prepared by combining approximately 7.3 g of $NaNO_2$ with 5 mL of water and heating at 70 °C for approximately two hours. After cooling to room temperature, an aliquot of the supernatant was diluted 10X with water and the pH was recorded.

2.4. Manufacture of drug-excipient blends and compacts

Drug-excipient blends were prepared by mixing microcrystalline cellulose (7 g), lactose monohydrate (3 g) and secondary amine drug (0.5 g) in a 60 cc jar using a TURBULA® mixer (Willy A. Bachofen–AG Maschinenfabrik, Muttenz, Switzerland) for approximately 15 min. The blends were then passed through a 500 μm or 600 μm sieve filter and mixed for another 10 min. Carvedilol-excipient and norfloxacin-excipient compacts were prepared by loading 200 mg of each blend into a tablet press (International Crystal Laboratory E-Z Press, Garfield, NJ, USA) and applying approximately 2000 PSI.

2.5. HPLC and HPLC–MS analysis

HPLC and HPLC–MS experiments were carried out using an Acquity HSS T3 C18 column (2.1 mm × 100 mm, 1.8 μm, Waters Corporation, Milford, MA, USA), in conjunction with an Acquity UPLC® coupled to a single quadrupole mass detector (Waters Corporation, Milford, MA, USA) utilizing an electrospray ionization (ESI) source. The system was equipped with binary pumps, a vacuum degasser, an auto-sampler, a heated column compartment and a diode array detector. Trifluoroacetic acid (0.03% in water) and acetonitrile were used as mobile phase solvents and separation was achieved using gradient elution. The percentage of acetonitrile was 5% for one minute then linearly increased to 90% from 1 min to 11 min, held for 0.5 min at 90% and then returned to 5% at 11.6 min with a total run time of 13 min. The flow rate was set at 0.5 mL/min and the column temperature was maintained at 45 °C. Except where noted, drug solutions were prepared at 0.2 mg/mL (nominal) in dissolving solvent. The UV detection wavelengths were 242 nm, 220 nm and 279 nm for carvedilol, propranolol and norfloxacin, respectively. The HPLC injection volumes for each sample type were selected to ensure that the main drug component had an appropriate response (0.9-1.4 AU) and are listed in the corresponding sections that follow. Degradation product levels were calculated as the peak area relative to the sum total of drug-related peaks. The masses of the reaction products were monitored at unit resolution.

2.6. Accelerated stability testing conditions and sample preparation

2.6.1. Saturated salt solutions

Saturated salt solutions were prepared by addition of 10 mL of water to approximately 14.6 g of $NaNO_2$ or 21 g of KI, respectively. The samples were mixed and placed in a 60 °C oven for 30–90 min to facilitate dissolution.

2.6.2. Drug substance stability studies

Approximately 5 mg of each drug substance was transferred to separate uncapped glass vials and placed inside glass jars along with the appropriate uncapped saturated salt solution. The jars were sealed and placed inside a 60 °C stability chamber. Solutions of stressed propranolol hydrochloride were prepared for analysis by addition of 2 mL water/acetonitrile/trifluoroacetic acid (600/400/0.3, v/v/v) to 5 mg of drug. The samples were mixed followed by dilution to 10 mL with the same solvent system to achieve a nominal concentration of 0.5 mg/mL. Carvedilol and norfloxacin solutions were prepared by addition of 2 mL water/acetonitrile/trifluoroacetic acid (600/400/0.3, v/v/v) plus 1 mL 0.1% aqueous trifluoroacetic acid to 5 mg of drug. The samples were mixed and further diluted to 10 mL with the same solvent system to yield a nominal concentration of 0.5 mg/mL. The HPLC injection volumes were 0.4 μL for carvedilol and propranolol hydrochloride and 0.3 μL for norfloxacin solutions.

2.6.3. Drug-excipient blends & compacts stability studies

Approximately 0.5 g of each drug-excipient blend or a single 200 mg drug-excipient compact was transferred to separate uncapped glass vials. The vials were placed inside glass jars along with the appropriate uncapped saturated salt solution, sealed and placed inside a 60 °C stability chamber. Solutions of stressed

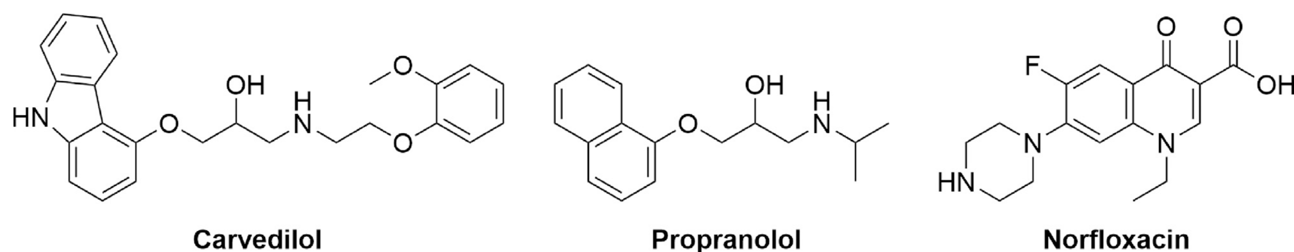

Fig. 1. Model secondary amine-containing drug compounds.

drug-excipient blends were prepared for analysis by transferring approximately 200 mg of the blend to a 50 mL volumetric flask followed by addition of approximately 30 mL of dissolving solvent. The flask was shaken for approximately 20 min on a mechanical shaker. The flask contents were diluted to volume after mixing. An aliquot (~1.2 mL) was centrifuged at 8000 rpm for 5 min and the supernatant was analyzed by HPLC. Solutions of drug-excipient compacts were prepared in the same manner with the exception that ultrasonication was used to disperse the compact pieces into a suspension in the dissolving solvent prior to shaking. The HPLC injection volumes were 1.0 μL for carvedilol and propranolol hydrochloride samples and 0.8 μL for norfloxacin samples.

2.7. In situ generation of N-nitrosamine derivatives

2.7.1. Carvedilol and propranolol hydrochloride

Following modification of a published procedure [7], acidified solutions of carvedilol and propranolol hydrochloride (5 mg/mL in water/acetonitrile/85% phosphoric acid (500/500/1, v/v/v)) were mixed with equal volumes of $NaNO_2$ solution (10 mg/mL in the same solvent system) and stored at room temperature. The reaction solutions were analyzed by HPLC (0.1 μL injection volume). Significant amounts of total N-nitrosamine derivatives (~60%) of carvedilol were formed in approximately 80% chemical yield (relative to carvedilol potency loss) after 45 min. Propranolol afforded approximately 30% of total N-nitrosamine derivatives in quantitative yield after 70 min.

2.7.2. Norfloxacin

A 100 μL aliquot of $NaNO_2$ solution (10 mg/mL in water/acetonitrile/85% phosphoric acid (500/500/1, v/v/v)) was added to 1.5 mL of a solution of norfloxacin (0.2 mg/mL in the same solvent system) and stored at room temperature. The solution was analyzed by HPLC (0.8 μL injection volume). The total N-nitrosamine levels were approximately 25% after 40 min (~85% chemical yield relative to norfloxacin potency loss).

2.8. High-resolution MS analysis

High-resolution and tandem mass spectrometric experiments for structure characterization were carried out in the positive ion mode using a Bruker Solarix XR 7T FTICR mass spectrometer (Bruker Daltonics Inc., Billerica, MA) coupled with a positive electrospray ionization source. High resolution data were acquired using a resolving power of 47,000 and 60,000. Tandem MS experiments were performed using Collision Induced Dissociation (CID) mode with structure-dependent normalized collision energy setting in the range of 15–20 V with a 4 Da window across a mass range of 72–2000 and 150–2000.

2.9. Determination of nitrite levels–Griess assay

Scintillation vials were filled with 10 mL of deionized water (DIW). To minimize evaporation, each scintillation vial was covered with parafilm. Two vials were placed in the headspace of a desiccator with saturated $NaNO_2$ solution and stored in a 40 °C oven. As a control, two vials were placed in a stability chamber set to 40 °C/61% RH. Finally, as a secondary control, one vial was stored at ambient conditions on the benchtop. All samples were stored for two weeks after which the nitrite levels were measured in each sample via the Griess assay, a colorimetric assay utilizing sulfanilic acid and naphthylamine that detected nitrite as a surrogate for reactive nitrogen species which quickly reacted to form nitrite in oxygenated aqueous media [8]. The Griess assay was performed according to the instructions provided with the Griess kit. Calibration solutions were prepared with $NaNO_2$ with concentrations between 1 μM – 100 μM via serial dilutions of nitrite standard solution with DIW. For the colorimetric assay, 100 μL of Griess Reagent, 300 μL of the nitrite-containing samples (calibration standard or sample) and 2.6 mL of DIW were mixed in a 1 cm path length cuvette. The solutions were incubated at ambient conditions for approximately 30 min. Following incubation, the absorbance at 546 nm was measured with a spectrophotometer. A linear calibration was generated with the standard solutions and used to determine the amount of nitrite present in each sample.

3. Results and discussion

3.1. Artifactual degradation of model secondary amine-containing drug compounds

The preliminary observations of variable amounts of two unknown degradation products of secondary amine-containing drugs stored in the presence of saturated $NaNO_2$ solutions for humidity control were further explored using carvedilol, propranolol and norfloxacin as model drug compounds (Fig. 1). Accelerated stability studies of the drug substances, drug-excipient blends and drug-excipient compacts were carried out using saturated $NaNO_2$ and saturated KI solutions. Initial studies in our laboratories indicated that the amount of artifactual degradation varied with the pH of the saturated $NaNO_2$ solution employed with greater degradation observed with lower pH. To probe the variability further, the pH of saturated solutions of ten batches of $NaNO_2$ from five different suppliers was measured and found to range from pH 7.7–8.8. The batches of $NaNO_2$ which yielded the lowest and highest pH values were selected for subsequent accelerated stability studies. The solution pH values did not correlate with the $NaNO_2$ batch purities listed on the manufacturers' certificates of analysis; however, higher degradation levels were observed for compounds that were stored in the presence of $NaNO_2$ solutions with lower pH (*vide infra*).

To study the observation of artifactual degradation of secondary amine drug substances further, three model compounds (Fig. 1) were stored for eight weeks in a 60 °C oven in the presence of saturated solutions of the two above batches of $NaNO_2$. Insignificant degradation (≤0.05%) was observed for propranolol and norfloxacin. Carvedilol drug substance did not exhibit significant degradation (≤0.05%) when stored in the presence of a saturated

solution of the "highest pH" $NaNO_2$; however, when stored in the presence of a saturated solution of the "lowest pH" $NaNO_2$, two degradation products at ~0.4% each were detected after eight weeks. These two peaks appeared to be isomeric based on their identical MS and UV spectra and were subsequently found to be formed at significantly higher levels in aged carvedilol-excipient blends and compacts.

Similar accelerated stability studies were performed using drug-excipient blends and compacts. Carvedilol- and propranolol-excipient blends each exhibited two significant degradation products (**C1**/**C2** and **P1/P2,** see Fig. 2) while norfloxacin-excipient blends did not degrade significantly (≤0.1%) when stored for six weeks at 60 °C in the presence of saturated $NaNO_2$ solutions. The degradation products observed from the carvedilol-excipient blend (**C1**/**C2**) had the same retention times of the two major degradation products observed during the analogous drug substance-only accelerated stability study. **C1**/**C2** and **P1**/**P2** were not generated (≤0.05%) when the samples were stored at 60 °C in the presence of saturated KI solution, suggesting a relationship between the $NaNO_2$ solutions and the formation of these degradation products via a volatile species from the $NaNO_2$ solutions. As shown in Table 1, the **C1**/**C2** and **P1**/**P2** levels increased with storage time and were consistently higher for samples stored in the presence of the more acidic batch of saturated $NaNO_2$ solution. Analogous results were observed upon storage of carvedilol- and norfloxacin-excipient compacts under similar conditions as their respective drug-excipient blends. The carvedilol- and propranolol-excipient blends and compacts were more reactive towards the formation of these degradation products (**C1**/**C2** and **P1**/**P2**) than the corresponding drug substances which is consistent with general observations that solid dosage forms are less stable than their corresponding drug substances due to a variety of potential drug-excipient chemical and/or physical interactions [9–11].

3.2. *Identification of carvedilol and propranolol N-nitrosamine derivatives*

The major degradation products (**C1**/**C2** and **P1**/**P2**) were identified as N-nitrosamine derivatives of carvedilol and propranolol, respectively (Fig. 2), on the basis of mass spectral data (elemental composition and fragmentation), and by comparison of their HPLC retention times, mass spectral data and UV–vis spectra with authentic samples of the N-nitrosamines [7]. The observation of two HPLC peaks in each case is consistent with the known chemistry of N-nitrosamines which possess restricted rotation about the N—N bond leading to the existence of two conformational isomers (atropisomers) [12,13].

High resolution accurate mass measurements of both carvedilol N-nitrosamine peaks (**C1**/**C2**) showed a molecular ion [M + H]+ at *m*/*z* value of 436.186889 that correlated to a protonated empirical formula of $C_{24}H_{26}N_3O_5$ with a deviation of −0.4 ppm from the theoretical mass (Table 2). Collision induced dissociation (CID) of the *m*/*z* 436 ion was not possible, presumably due to the labile nature of the nitroso group. Fragmentation (see supplementary material) was achieved by targeting the ammonium adduct at *m*/*z* value of 453.213435, which correlated to an empirical formula of $C_{24}H_{29}N_4O_5$ with a deviation of −0.4 ppm from the theoretical mass. Base peak *m*/*z* 407 was consistent with a spontaneous source fragment of *m*/*z* 436 and/or *m*/*z* 453 and loss of NO. The MS^2 of *m*/*z* 453 gave fragments *m*/*z* 406 resulting from loss of NO, a characteristic fragmentation pathway for N-nitrosamines [14], *m*/*z* 224 resulting from the loss of NO and carbazolol, an odd electron ion *m*/*z* 223 resulting from the loss of NO and carbazolol, *m*/*z* 210 resulting from the loss of NO and methoxycarbazole and an *m*/*z* 180 ion resulting from the loss of NO and carbazolyloxyethanol (Fig. 3).

High resolution accurate mass measurements of both propranolol N-nitrosamine peaks (**P1**/**P2**) showed a molecular ion [M + H]+ at *m*/*z* value of 289.154598 that correlated to a protonated empirical formula of $C_{16}H_{21}N_2O_3$ with a deviation of 0.2 ppm from the theoretical mass (Table 2). Base peak *m*/*z* 258 (see supplementary material) was consistent with a spontaneous source fragment of *m*/*z* 289 and loss of HNO. The MS^2 of *m*/*z* 289 gave fragments *m*/*z* 259 resulting from the loss of NO [14], *m*/*z* 145 resulting from the loss of naphthalenol, *m*/*z* 128 consistent with a naphthyl fragment ion and an *m*/*z* 215 ion (Fig. 3). A similar *m*/*z* 145 fragment ion has been proposed from electron impact MS analysis of the propranolol N-nitrosamines [7]. The *m*/*z* 215 fragment is proposed to have arisen from rearrangement of an *m*/*z* 259 nitrogen-centered radical cation followed by loss of acetaldehyde (Fig. 3). This type of ion fragment rearrangement has been reported for propranolol and related compounds [15,16].

Authentic samples of N-nitrosamine derivatives of the three model secondary amine-containing drugs were generated *in situ* by established procedures, involving the addition of $NaNO_2$ to acidified solutions of each drug [7]. Comparison of chromatographic and spectral characteristics of the authentic N-nitrosamines to those of **C1**/**C2** and **P1**/**P2** provided additional support for their proposed structures. The HPLC retention times, MS, and UV–vis spectra of the authentic N-nitrosamines of carvedilol and propranolol matched those of the major degradation products (**C1**/**C2** and **P1**/**P2**) from drug-excipient blends stored at 60 °C in the presence of saturated $NaNO_2$ solutions, which was consistent with the proposed structures [7] shown in Fig. 2. It is noted that the two propranolol N-nitrosamine isomers (**P1**, **P2**) were formed in the same ratio of 1:5 from the stressed drug-excipient blends and when synthesized *in situ* [7]. An authentic sample of the norfloxacin N-nitrosamines was generated and detected *in situ* demonstrating specificity of the HPLC method.

3.3. *Determination of nitrite levels in the headspace of desiccators with saturated $NaNO_2$ solutions*

An experiment was designed to determine if the unexpected degradation was indeed a result of reactive nitrogen species generated in the saturated $NaNO_2$ solution entering the headspace of the desiccator in which the saturated $NaNO_2$ solution and samples were stored. As described in the Experimental Section, "nitrite traps" were designed by pipetting 10 mL of DIW into glass scintillation vials, then covering each vial with parafilm. The parafilm was necessary to prevent evaporation over the 2-week storage period. Two vials were placed in the headspace of a desiccator with saturated $NaNO_2$, and stored in an oven at 40 °C for two weeks. As a control, two vials were placed in a separate stability chamber set to 40 °C/61% RH for two weeks. Finally, as a secondary control, one vial was stored at ambient conditions on the benchtop for 2 weeks. The nitrite level in each sample was measured via the Griess assay. No nitrite was detected in either the separate stability chamber or benchtop controls (the lowest calibration standard analyzed was approximately 1 μM nitrite and each control sample produced a signal less than the 1 μM standard). In contrast, significant levels (average concentration of 18.5 μM, across two samples) of nitrite were detected in the "nitrite traps" stored in the desiccator with saturated $NaNO_2$ solution. Detectable levels of nitrite observed in the desiccator indicated that reactive nitrogen species were present in the headspace of the desiccator apparently originating from the saturated nitrite solution, as evidenced by the fact that they were not detected in the stability chamber control where there was no saturated nitrite solution. These reactive nitrogen species in the headspace of the desiccator resulted in the nitrosative degradation of samples stored in the desiccator headspace. Because the Griess assay detects nitrite, which can form from a variety of NOx

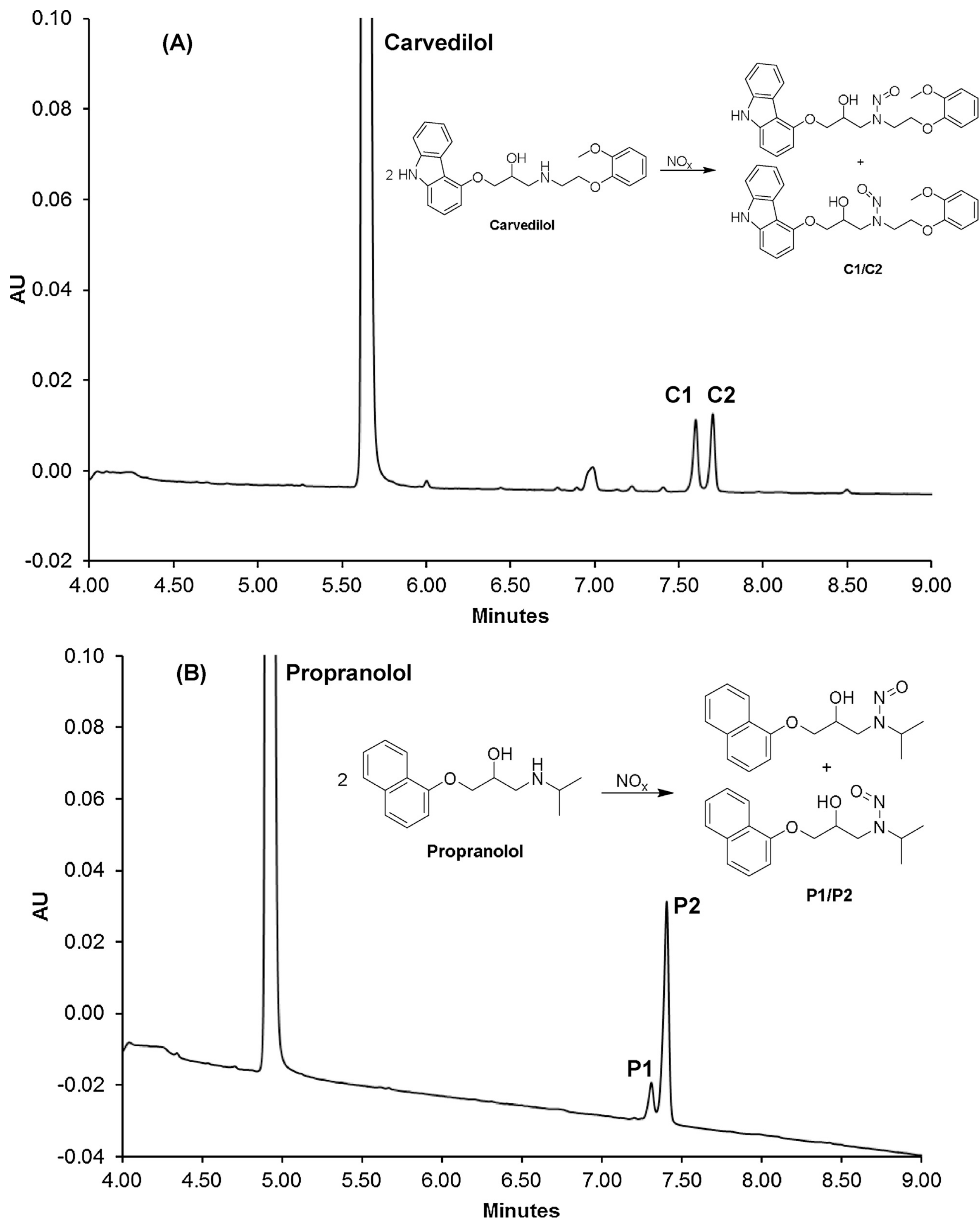

Fig. 2. Proposed degradation product structures and expanded-scale chromatograms generated from HPLC analysis of solid (A) carvedilol- and (B) propranolol-excipient blends that had been stored 6 weeks at 60 °C in the presence of saturated $NaNO_2$ solution (low pH batch).

species in oxygenated aqueous media, it is considered a method capable of detecting reactive nitrogen species in general (i.e., NOx), and not specific nitrogen oxide species. As such, these data do not indicate which reactive nitrogen species enter the headspace, but are consistent with the characterization data indicating that nitrosative degradation occurred with samples stored in the presence of the saturated $NaNO_2$ solution in the desiccator. Given that the "nitrite trap" vials were covered with parafilm to prevent evap-

Table 1
Levels of Major Degradation Products Generated During Storage of Carvedilol and Propranolol Drug Substances, Drug-Excipient Blends and Drug-Excipient Compacts at 60 °C in the Presence of Saturated $NaNO_2$ Solutions (60% RH).

Sample	$NaNO_2$ Batch	Time Point (weeks)	%D1[a]	%D2[a]	%Total Impurities
Carvedilol Drug	High pH	8	≤0.05%	≤0.05%	≤0.05%
Substance	Low pH	8	0.40	0.38	1.6
Carvedilol- Excipient	High pH	3	0.30	0.33	1.2
Blend		6	0.51	0.60	1.8
	Low pH	3	0.86	0.94	2.4
		6	1.3	1.5	3.8
Carvedilol-Excipient	High pH	3	0.29	0.32	1.4
Compact		6	0.59	0.68	2.4
	Low pH	3	1.2	1.3	3.8
		6	2.0	2.3	6.5
Propranolol Drug	High pH	8	≤0.05%	≤0.05%	≤0.05%
Substance	Low pH	8	≤0.05%	≤0.05%	≤0.05%
Propranolol-Excipient	High pH	3	0.19	0.96	1.2
Blend		6	0.38	2.1	2.6
	Low pH	3	0.50	2.6	3.1
		6	0.92	5.1	6.2

[a] D1 & D2 correspond to **C1** & **C2** for carvedilol and to **P1** & **P2** for propranolol samples.

Table 2
Accurate Mass Measurements for N-Nitrosamine Derivatives of Carvedilol and Propranolol.

Drug	N-Nitrosamine Derivatives	Mass Measurement (m/z)	Protonated Empirical Formula	Theoretical Protonated Monoisotopic Mass (Da)	Mass Deviation (ppm)
Carvedilol	C1/C2	436.186889	$C_{24}H_{26}N_3O_5$	436.186697	−0.4
Propranolol	P1/P2	289.154598	$C_{16}H_{21}N_2O_3$	289.154669	0.2

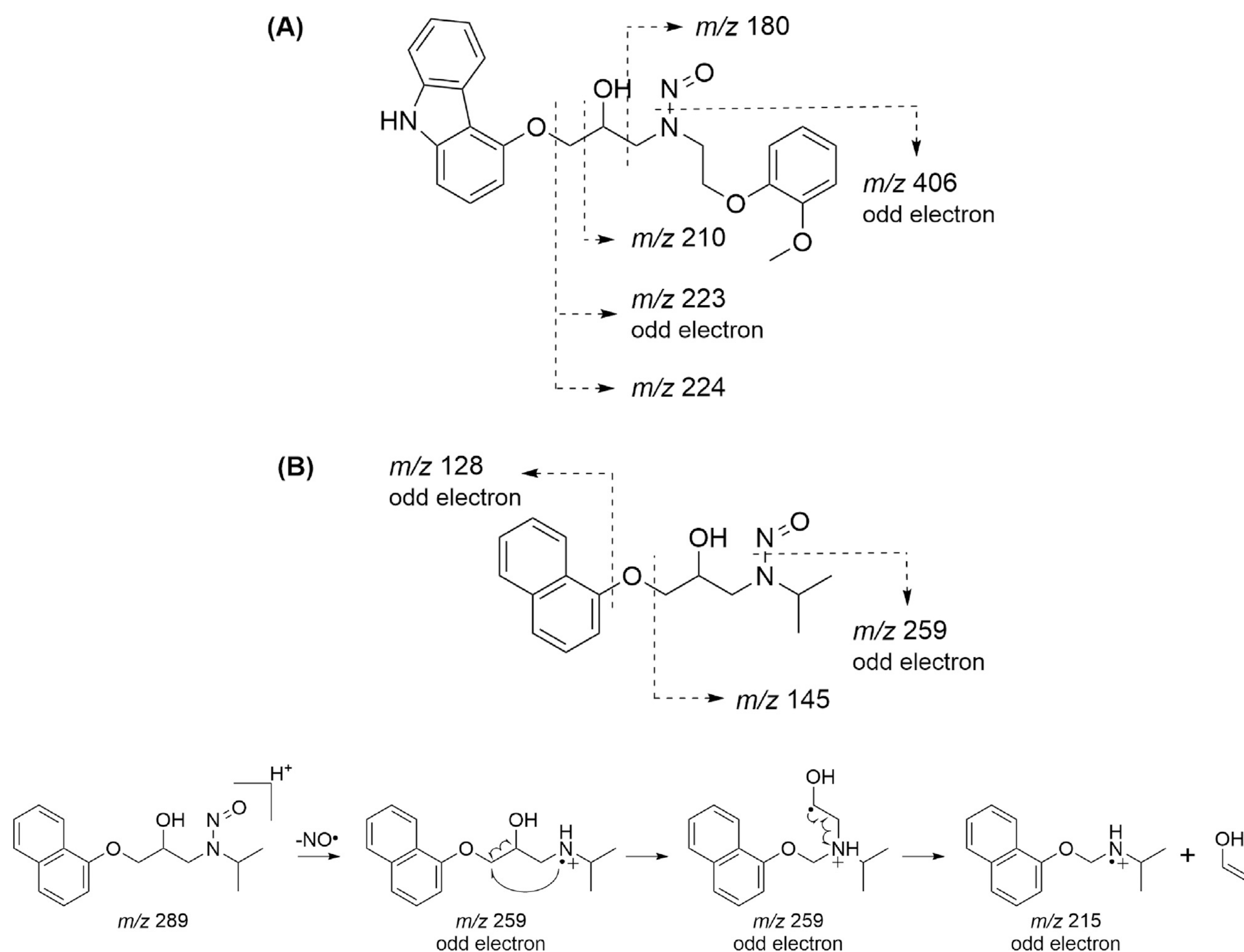

Fig. 3. (A) MS fragmentation pattern for carvedilol N-nitrosamine derivatives **C1** and **C2**. Note that all depicted cleavage sites contain the loss of the NO group. (B) MS fragmentation patterns for propranolol N-nitrosamine derivatives **P1** and **P2**. The proposed pathway for formation of the m/z 215 fragment is shown separately for clarity.

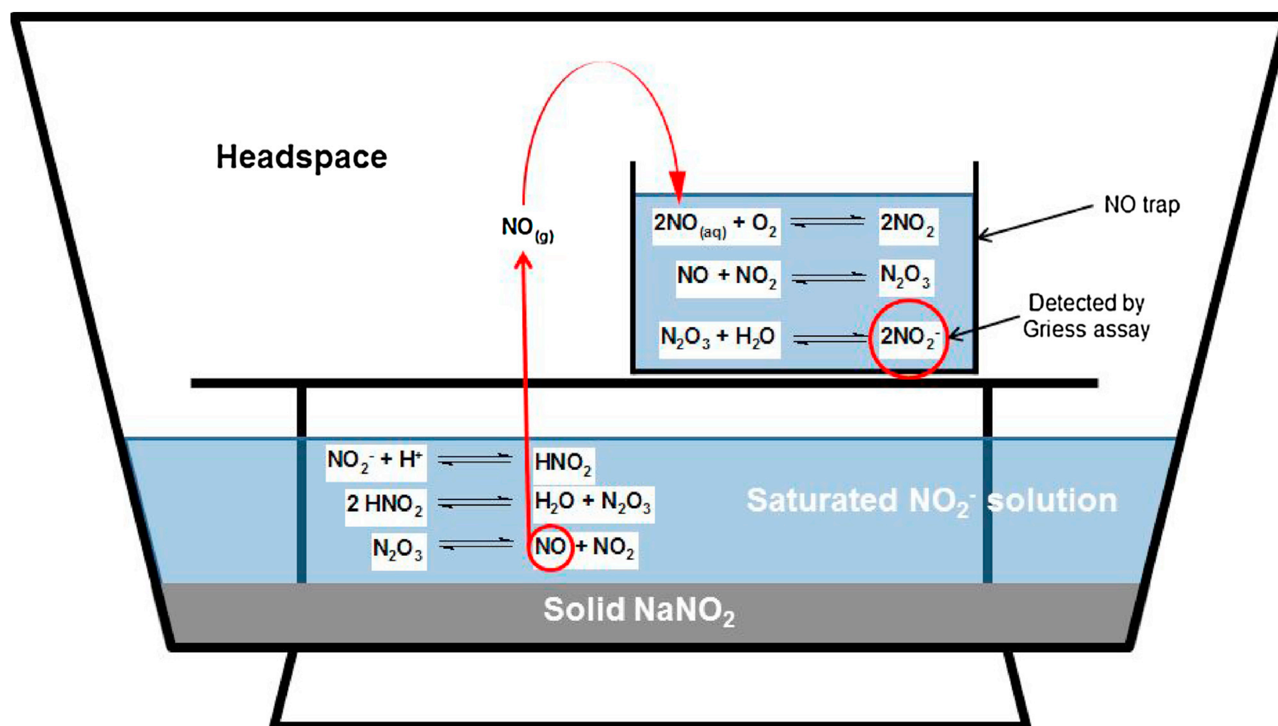

Fig. 4. Volatilization and detection of reactive NOx species from the headspace of saturated $NaNO_2$ solution.

oration, we hypothesize that the species that entered the headspace were lipophilic in nature, permitting their passage through the hydrophobic parafilm [17]. The lipophilicity of certain reactive nitrogen species has been documented with respect to their ability to pass through cell membranes [18]. Thus, the most likely volatile, lipophilic reactive nitrogen species to enter the headspace is nitric oxide (NO).

The observation of elevated nitrosative degradation for amine-excipient blends stored in the presence of the most acidic batch of saturated $NaNO_2$ indicated that higher proportions of volatile NOx species were generated as the $NaNO_2$ solution pH was lowered. Regardless, the Griess assay data demonstrated that reactive nitrogen species entered the headspace of the desiccator with the saturated nitrite solution, and since no nitrite was detected in either of the controls, the origin of the reactive nitrogen species was the saturated nitrite solution (Fig. 4). A plausible mechanism explaining the presence of reactive nitrogen species in the headspace of the desiccator and "nitrite traps" is presented in Fig. 4. The reaction cascade begins with the protonation of nitrite anion in the saturated solution to form nitrous acid (HNO_2). This reaction would be governed by the pKa of HNO_2 [19], thus explaining the higher levels of degradation observed at low pH in Table 1. The reactions associated with the production of the gaseous species nitric oxide (NO) and nitrogen dioxide (NO_2) from HNO_2 are complicated and may proceed via N_2O_3 as described by Komiyama and Inoue [20]. These gaseous species then enter the headspace of the desiccator, where they either react with the secondary amines or partition into the "nitrite trap". The reaction of NO in aqueous solution to form nitrite, which is subsequently detected by the Griess assay, is well-documented in the literature [8]. The observation of higher levels of N-nitrosation with lower solution pH (Table 1) is consistent with the well-known reactions [8] hypothesized in Fig. 4, which initiate with the protonation of aqueous nitrite anion. This reaction would proceed faster at lower pH, resulting in more NOx and greater nitrosative degradation.

4. Conclusions

Amine-containing drugs may be susceptible to artifactual nitrosative degradation when stored in the presence of saturated $NaNO_2$ solutions used for humidity control. The process for formation of the N-nitrosamine derivatives is proposed to involve volatilization of nitric oxide from aqueous nitrite solution into the headspace of the container followed by diffusion into the solid drug or drug-excipient blend and subsequent reaction of NOx with the secondary amine. Use of humidity-controlled chambers or alternative salt solutions for humidity control during accelerated stability studies of secondary amine containing drugs is recommended.

Acknowledgments

The authors thank Ron Morris, Almary Chacon and Victor Soliman for conducting high resolution MS experiments to aid in structure elucidation of the degradation products.

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