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On-column nitrosation of amines observed in liquid chromatography impurity separations employing ammonium hydroxide and acetonitrile as mobile phase

David P. Myers[a,*], Evan M. Hetrick[a], Zhongming Liang[a], Chad E. Hadden[a], Steven Bandy[a], Craig A. Kemp[a], Thomas M. Harris[b], Steven W. Baertschi[a]

[a] Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285, USA
[b] Vanderbilt University, Department of Chemistry, Nashville, TN 37235, USA

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ABSTRACT

The availability of high performance liquid chromatography (HPLC) columns capable of operation at pH values up to 12 has allowed a greater selectivity space to be explored for method development in pharmaceutical analysis. Ammonium hydroxide is of particular value in the mobile phase because it is compatible with direct interfacing to electrospray mass spectrometers. This paper reports an unexpected *N*-nitrosation reaction that occurs with analytes containing primary and secondary amines when ammonium hydroxide is used to achieve the high pH and acetonitrile is used as the organic modifier. The nitrosation reaction has generality. It has been observed on multiple columns from different vendors and with multiple amine-containing analytes. Ammonia was established to be the source of the nitroso nitrogen. The stainless steel column frit and metal ablated from the frit have been shown to be the sites of the reactions. The process is initiated by removal of the chromium oxide protective film from the stainless steel by acetonitrile. It is hypothesized that the highly active, freshly exposed metals catalyze room temperature oxidation of ammonia to NO but that the actual nitrosating agent is likely N_2O_3.

1. Introduction

Robust HPLC stationary phases have been developed in the last decade through hybrid bonding to the silica and/or double end-capping to protect the silica from hydrolysis. These stationary phases are capable of operating at mobile phase pH of greater than 8 without rapid dissolution of the silica support. These columns have excellent stability at pH values up to 12. The operation of these columns in this regime has enabled more selectivity, in particular for aliphatic amines, which typically are poorly behaved at pH values near neutrality where they exist as mixtures of protonated and unprotonated species but pH values above the analyte pK_a give good peak shapes and longer retention times because the analyte is largely unprotonated. Peak shape and loadability of amino compounds are also improved using higher pH mobile phases [1]. Additionally, base deactivation of the silica through hybrid bonding reduces silanol interactions with charged analytes [2].

These base-tolerant columns have been of particular value in drug discovery because basic groups are frequently present in drug candidates. For example, Espada et al. reported use of a range of high pH mobile phases, including ammonium bicarbonate at pH 8–10, for optimization of reversed phase separations of basic compounds [3,4]. A number of column screen space studies employing these higher pH conditions have been reported [5–11]. Stafford et al. [6] evaluated screening conditions utilizing ammonium hydroxide as a means for exploring pH ranges beyond those that can be attained with buffers prepared from mixtures of ammonium hydroxide and ammonium bicarbonate. The volatility of ammonium hydroxide makes it attractive for LC–MS.

The molecular entity employed in the present study, litronesib (**1**, Fig. 1) contains a dialkylamine. Screening of columns and eluents produced an optimal separation for process and degradant impurities on an X-Bridge C18 column utilizing a mobile phase having a pH of approximately 11 that was created with ammonium hydroxide. During the development of these conditions we encountered a novel on-column reaction of the analyte. In this work we describe the investigation into the identification of the on-column impurities and the mechanism of the reaction. Our work has shown the secondary amine moiety on litronesib is converted to an N-nitroso derivative on-column and is dependent on the high pH and the simultaneous presence of ammonia, acetonitrile and adventitious oxygen. Evidence points to the reaction being catalyzed by the stainless steel frit at the head of the column. This reaction is

* Corresponding author at: 1400 W. Raymond St., B110 DC4816, Indianapolis, IN 46221, USA. Tel.: +1 317 276 0023; fax: +1 317 651 6510.
E-mail address: myers_david_patrick@lilly.com (D.P. Myers).

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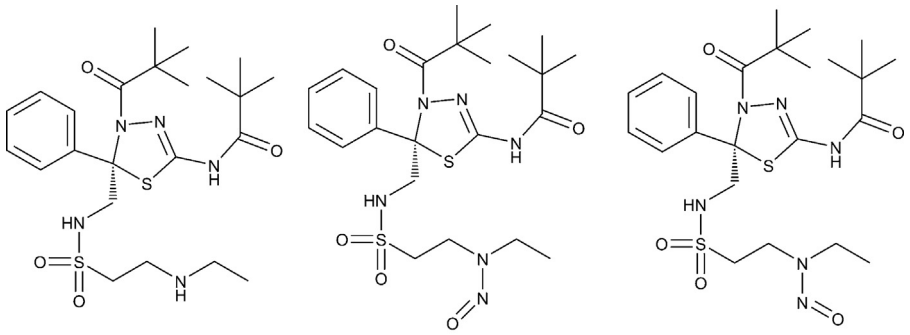

Fig. 1. Structures of litronesib and the N-nitrosamine impurities.

novel in that we have found only one reference in the literature which utilized high pH HPLC conditions and similar columns to the ones studied here [12]. This paper by Wang et al. described detailed studies of on-column oxidative dimerization of anilines to form azo and hydrazo species [12]. Their principal studies were made using an X-Bridge Shield RP18 HPLC column with ammonia as the modifier but could be duplicated on multiple columns capable of higher pH including the Phenomenex Gemini NX column. Their observations are generally consistent with ours although they did not identify the column frits as being the source of the artifact-generating reactions. They concluded that the coupling reactions are most likely the result of oxidative reagents formed by complexation between unknown elements on the surfaces of the X-Bridge stationary phases and ammonia in the high pH aqueous mobile phase; no mechanism was proposed. In this paper we describe the impurities and potential mechanism of the observed on-column reaction and also show the generality to other primary and secondary amines.

2. Materials and methods

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. Solvents and chemicals

Litronesib (LY2523355) was prepared by Lilly Research Laboratories. Acetonitrile (HPLC grade) and methanol (HPLC grade) were obtained from Burdick and Jackson (Muskegon, IL); water (HPLC grade) from a Millipore system (Billerica, MA); ammonium hydroxide, 28%, 99.99+% metals basis from Sigma–Aldrich (St. Louis, MO), LC–MS grade glacial acetic acid from Sigma–Aldrich/Fluka (St. Louis, MO), trifluoroacetic acid from Cambridge Isotopes Laboratories (Andover, MA) and $^{15}N^{-}$ammonium hydroxide (3 N in H_2O, 98 atom % ^{15}N) from Sigma–Aldrich (St. Louis, MO) and all other chemicals were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise noted.

2.3. Sample preparation

Solutions of litronesib were prepared at ~0.3 mg/mL in either 20% acetonitrile/80% water (v/v) or 20% acetonitrile/80% 0.08% acetic acid in water (v/v). Solutions of (a) ~15 mg of [2-(1*H*-pyrrol-1-yl)phenyl]methylamine (Maybridge, Tintagel, UK), (b) ~13 mg of 2-thiophenemethylamine, (c) ~6 mg of *N*-methyl[2-(3-methyl-1,2,4-oxadiazol-5-yl)phenyl]methylamine (Maybridge, Tintagel, UK), (d) ~5 mg of sertraline hydrochloride, (e) ~6 mg of clozapine, and (f) ~6 mg of paroxetine hydrochloride hemihydrate were prepared in 10 mL of 20/80/0.08 (acetonitrile/H_2O/acetic acid).

2.4. LC and LC–MS analysis

LC and LC–MS experiments were performed using an Agilent (Palo Alto, CA) 1200 series liquid chromatography system equipped with a quaternary pump, vacuum degasser, auto-sampler, column thermostat, and diode array detector (DAD). An Agilent G1946D mass spectrometer utilizing an electrospray ionization (ESI) source was connected to the output of the DAD detector with an in-line tee and drain tubing to provide a 50% split at 1 mL/min of LC flow into the tee. Data were collected utilizing ChemStation software (Agilent Technologies, Palo Alto, CA). Accurate mass LCMS experiments were performed on a Thermo Scientific (San Jose, CA) LTQ Orbitrap Discovery Mass Spectrometer.

For experiments on the X-Bridge C18 (4.6 mm × 75 mm, 2.5 μm, Waters Corp., MA) and Gemini C18 columns (4.6 mm × 150 mm, 3 μm, Phenomenex, Torrance, CA), mobile phase A was prepared by adding 1.8 mL of ammonium hydroxide to 1000 mL of water, which yields approximately 0.05% ammonium hydroxide by volume. For the experiments utilizing $^{15}NH_4OH$ in the mobile phase A, 0.9 mL of the $^{15}NH_4OH$ was added to 500 mL of water. Mobile phase B was acetonitrile or methanol. All other instrument parameters are included in Table S1.

For the experiments under acidic conditions, mobile phase A was prepared by adding ~1 mL of trifluoroacetic acid to 1000 mL of water, which yielded ~0.1% (v/v) trifluoroacetic acid. Mobile Phase B was acetonitrile. All other instrument parameters are listed in Table S2. For analysis of the nitrosamine of litronesib on the Agilent Zorbax Bonus-RP (4.6 mm × 75 mm, 1.8 μm, Agilent Technologies) column, mobile phase A was prepared by adding ~1.54 g of ammonium acetate (99.999%) to 2000 mL of water, then adjusting the pH to 5.2 with glacial acetic acid. Mobile Phase B was acetonitrile. Instrument parameters are listed in Table S3.

2.5. Synthesis and NMR analysis of the N-nitroso derivative of litronesib

Hydrochloric acid (3 mL, 1 M) was added to litronesib (37.5 mg) and $NaNO_2$ (333 mg) dissolved in acetonitrile (9 mL) and water (16 mL) in a 250 mL round-bottomed flask to yield two phases with a clear solution over a more dense brown one. The clear solution