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Elizabeth Ann Hardardt for the degree of Master of Science in Pharmacy presented on May 23, 2014.

Title: The Effect of Ascorbate on Enzymatic Nitroglycerin Bioactivation as Measured by Direct LC-MS/MS Determination of Nitrite.

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Dr. Jan Frederik Stevens

This work sought to characterize a link between Vitamin C and nitroglycerin at the level of enzymatic bioactivation. Before we could proceed, we needed to develop a method that sensitively and selectively identified the product of bioactivation, nitrite. We successfully adapted traditional diaminonaphthalene derivatization with the use of stable isotope liquid chromatography tandem mass spectrometry that was free from interferences commonly encountered, including ascorbate and high nitrite background. We then applied our method to enzyme incubations with aldehyde dehydrogenase (ALDH) before moving on to incubations with xanthine oxidase. We successfully determined a link between Vitamin C and GTN bioactivation only in incubations with xanthine oxidase, but saw no evidence of ALDH catalyzed nitrite production.

The Effect of Ascorbate on Enzymatic Nitroglycerin Bioactivation as Measured by Direct LC-MS/MS Determination of Nitrite

by
Elizabeth Ann Hardardt

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Elizabeth Ann Hardardt, Author

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Chapter 1 – Introduction

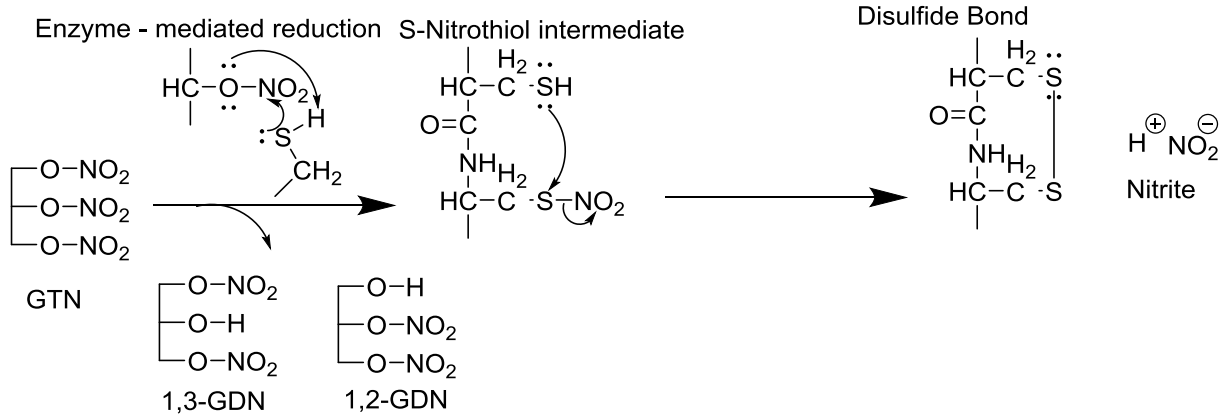
The discovery of cardiovascular effects of nitroglycerin was made shortly after the discovery of the explosive itself. Nitroglycerin (glyceryl trinitrate, GTN) has seen continuous pharmacological use over the past 130 years. Although the most commonly prescribed drug for ischemia and angina, it has always been hampered by the phenomenon of nitrate tolerance. (***1***) Nitrate tolerance refers to a reduction or elimination of vasodilation following nitrate therapy. Although clinical nitrate tolerance generally develops over time, *in vitro* results show reduction in bioactivation and efficacy after just 60 minutes. (***2***)

The elucidation of the GTN bioactivation pathway was initially regarded as a boon toward the elimination of tolerance. There is evidence that inhibition of bioactivation contributes to nitrate tolerance, but it is only one of many factors. (***2***) Physiological vasoconstrictive counterregulation, soluble guanylate cyclase desensitization, increased phosphodiesterase activity and increased oxidative stress have all been linked to nitrate tolerance. Beyond interference with the ·NO vasodilatory pathway, research has shown that nitrate tolerant Sprague-Dawley (male) rats eliminate not only GTN, but also is vasodilatory dinitrate metabolites, 1,2- and 1,3-glyceryl dinitrate (1,2-GDN; 1,3-GDN) more quickly than non-tolerant counterparts. (***3***)

Despite knowledge of other contributors to nitrate tolerance, nitrate tolerance research targeted the initial step in the vasodilation signaling pathway, ·NO production from GTN. But bio*conversion* of GTN is synonymous with bio*activation* to ·NO. While reductive release produces nitrite, which is then further reduced to ·NO, or possibly even ·NO itself, hydrolysis releases nitrate. Nitrate can theoretically be converted to ·NO, but not on a pharmacologically relevant scale. Hydrolysis can either occur solvolytically or enzymatically. Reduction on the other hand appears to be enzyme-mediated by aldehyde dehydrogenase (ALDH).

Aldehyde dehydrogenase has several links to nitrate therapy and tolerance. (***4***) The most common ALDH polymorphism results in impaired activity for approximately 40 % of Asian people. In addition to impaired alcohol tolerance, they experience a reduced GTN efficacy. Disulfram, another ALDH inhibitor also leads to nitrate tolerance.

Reduced ALDH activity has been shown in nitrate tolerance, but the mechanism is unknown. Aldehyde dehydrogenase has been shown to denitrate GTN by S-nitration of its active site cysteine residues (Scheme 1). But S-nitrosation is also implicated in the pathogenicity of aging-related diseases, like cardiovascular disease. (***5***) For example, S-nitrosation in the insulin signaling pathway, the result of either ·NO donor administration or inducible nitric oxide synthase (iNOS) activity, causes insulin resistance in aged mice. Alternately, during hydrolysis, nitrate is released from the S-nitrothiol by the formation of a disulfide bridge with one of the other two cysteine residues in the active site. While the formation of a disulfide bridge is a reversible oxidative modification, those cysteine residues can further oxidize to sulfenic then sulfinic acid, an irreversible modification of the ALDH active site. Additionally, reperfusion upon vasodilation has been shown to correlate with large releases of the reactive aldehyde 4-hydroxynonenal (HNE). Aldehyde dehydrogenase is responsible for the detoxification of HNE and other reactive aldehydes. But in addition to direct competition with GTN for the ALDH active site, HNE inhibits ALDH.



Scheme 1. Bioactivation of nitroglycerin by active-site cysteine of aldehyde dehydrogenase.

Another phenomenon affecting the applicability of GTN in clinical settings is cross tolerance. Cross-tolerance refers to induction of nitrate tolerance following the administration of non-GTN drugs, such as direct ·NO donors. Cross-tolerance mechanisms may or may not be related to the mechanisms of nitrate tolerance as a result of GTN use. One cross-tolerance mechanism is loss of GTN efficacy with high cyclic guanidine monophosphate levels, like those produced upon administration of the ·NO donor diethylamine/NONOate (DEA/NO). (***2***) Irvine

postulates the underlying mechanism is bioactivation related, but provides no direct evidence. Another possible explanation is desensitization to ·NO by feedback inhibition.

Despite the problem of tolerance, GTN has enjoyed continuous use for more than a century because of its relative advantages over alternatives. Its long history of use has provided the accumulation of years of safety data and a reduced cost in comparison to newer drugs produced by the expensive process of modern day drug discovery and approval. There are also pharmacological advantages. Nitroglycerin maintains vasodilation 133% longer than DEA/NO and does not produce the cyclic GMP flood seen with NO donor DEA/NO which may reduce or delay cellular counterregulation. (***2***) These benefits have prompted attempts to resolve nitrate tolerance. It's been shown that intermittent treatment with GTN doesn't lead to tolerance, but limits the benefit of GTN treatment for the patient. (***4***) Another potential resolution is the use of ALDH activators, which increase GTN efficacy in the presence and absence of nitrate tolerance. (***6***) Another potential ALDH activator is Vitamin C (ascorbate, Asc), which actually has its own links to vasodilation and nitrate tolerance.

Vitamin C has reductive potential which can form nitric oxide according to the following equation: $2H^{+}NO_2^{-}$ + Asc → DHA + 2NO + $2H_2O$, where DHA is dehydroascorbic acid. (***7***) Ascorbate can bioactivate GTN non-enzymatically. (***8***) Beyond non-enzymatic nitric oxide production, ascorbate could participate in enzymatic bioactivation by thiol regeneration, i.e. reductive ·NO release from SNO (***9***) Like the dithiol compounds dithiothreitol and dihydro-α-lipoic acid, (***10***) Asc can regenerate cysteine from disulfide bridges. This ability increases significantly with small increases in pH, like those seen between the cytosol and mitochondrial matrix, the location of ALDH2. (***9***)

Beyond mechanistic contributions, Asc and nitrate tolerance have been linked *in vitro* and in clinical settings. After developing an ascorbate-deficient guinea pig model, more GTN was required to achieve vasodialtion in comparison to ascorbate-adequate guinea pigs, but the vasodilatory profile of direct ·NO donors was the same in both groups. (***8***) Furthermore, induction of nitrate tolerance was shown to deplete plasma Asc. (***11***) In the clinic, Vitamin C was shown to suppress increased platelet generation of ·NO and superoxide seen developed

during nitrate therapy. (***12***) Oral Vitamin C was even reported to prevent the development of nitrate tolerance and its symptoms. (***13***) (***14***)

We sought to characterize a link between Vitamin C and nitroglycerin at the level of enzymatic bioactivation. Before we could proceed, we needed to develop a method that sensitively and selectively identified the product of bioactivation, nitrite. We successfully adapted traditional diaminonaphthalene derivatization with the use of stable isotope liquid chromatography tandem mass spectrometry that was free from the interferences commonly encountered, including Asc and high nitrite background. We then applied our method to enzyme incubations with aldehyde dehydrogenase before moving on to incubations with xanthine oxidase. In the end, we successfully determined a link between Vitamin C and GTN bioactivation only in incubations with xanthine oxidase, but saw no evidence of ALDH catalyzed nitrite production.

Chapter 2 – Analytical Methods for Nitroglycerin Bioactivation Measurement

Introduction

Nitroglycerin (glyceryl trinitrate, GTN), like other drugs discovered before the 1900s, experienced over a century of medicinal use before elucidation of its mechanism of action. One reason for the elusive nature of the bioactivation pathway is the difficulty in characterizing the ultimate vasodilator nitric oxide. Initially described as endothelium derived relaxation factor or EDRF, nitric oxide is a small radical species which experiences rapid diffusion and oxidation. (***15***) One example of this problem was shown with incubations of bovine pulmonary vein. (***16***) Sodium nitroprusside (SNP), a direct ·NO donor, showed ·NO production while GTN did not. The authors noted evidence of further transformation and sequestration though, which may account for the lack of product observed with GTN. The short half-life, multiple oxidative end products and delocalization of ·NO all contribute to analytical difficulties which still plague researchers.

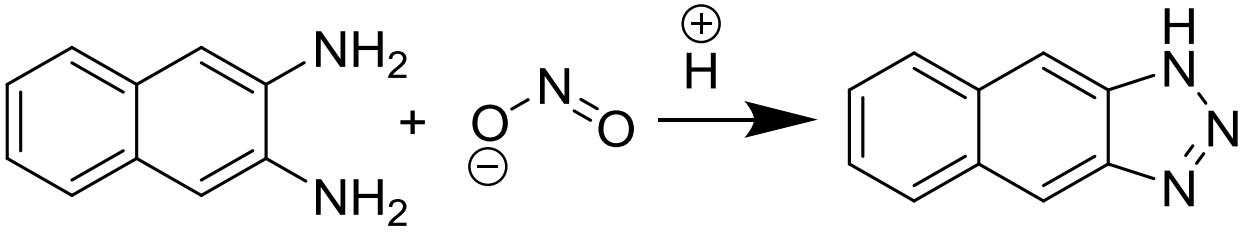
Considerably easier to analyze were the byproducts of nitroglycerin bioactivation, 1,2- and 1,3-glyceryl dinitrate (1,2-GDN, 1,3-GDN), particularly with the use of radioactive GTN. Unfortunately and often overlooked by researchers, there are two potential pathways by which nitroglycerin can yield 1,2- or 1,3-GDN, a hydrolytic pathway and a reductive pathway. Nitric oxide, the ultimate vasodilator, is only released after reductive bioactivation while the hydrolytic pathway produces nitrate. This method is rooted in the assumption that nonspecific denitration of GTN should produce a 2:1 ratio of 1,2-:1,3-GDN and anything greater indicates specific bioactivation. (***17***)

This assumption fails to account for several known characteristics of nitroglycerin bioactivation. Aldehyde dehydrogenase (ALDH), believed to be responsible for GTN bioactivation, has both hydrolytic and reductive catalytic mechanisms. Recent crystallographic structural evidence shows only 1,2-GDN should be released following ALDH catalysis. (***18***) Other known bioactivation mechanisms utilize alternative enzymes, (***19***) but to exemplify the problematic usage of GDN ratios, take the case of ascorbate. Ascorbate can reduce GTN organic nitrate groups, but produces a 1:1 ratio of 1,2-:1,3-GDN. (***20***) Glyceryl dinitrate ratios also differ depending on the route of GTN administration or even the dose. (***21***) In fact, oral nitroglycerin

produces a GDN ratio of 2:1, supposedly indicative of non-specific denitration, but still produces vasodilation. (***17***)

Complicating the application of the assumption that GDN ratios can be used to determine GTN bioactivation, GDNs themselves are vasodilatory prodrugs. Normally bioactivation byproducts can safely be ignored as pharmacological contributors. (***17***) But in this case, the vasodilatory contribution of 1,2- and 1,3-GDN is increased by their long half-lives of 40 minutes, (***2***) in contrast to the 2-3 minute half-life for GTN. Both 1,2- and 1,3-GDN experience similar, even slightly enhanced efficacy when compared to GTN, but with only10 % the potency. (***22***) Despite these concerns, researchers continue to assess nitroglycerin bioactivation using GDN ratios, in part because nitric oxide still presents significant analytical difficulties.

As one would expect, nitric oxide measurement techniques have improved over the decades, but still lack selectivity against nitrite. Some authors have claimed selective derivatization of ·NO by 2,3-diaminonaphthalene (DAN) at neutral pH, as opposed to its conventional use derivatizing nitrite under acidic conditions (Scheme 2). (***23***) One improvement to the sensitivity of this method is the incorporation of DAN into β-cyclodextrin, improving fluorescence of the product, 2,3-naphthalenetriazole (NAT). (***24***) This facilitated detection following derivatization at neutral pH, but the authors did not confirm selectivity.



Scheme 2. Derivatization of nitrite with dimainonaphthalene to yield labeled diaminonaphthalene.

In fact, the utility of DAN lies in its ability to derivatize the more stable ·NO oxidation product nitrite. Under acidic conditions, nitrite exists as nitrous acid, which reacts with itself to produce water and N_2O_3. Dinitrogen trioxide is the species believed to be derivatized by DAN. (***25***) (***24***) It can also form from the combination of nitric oxide and nitrite, allowing some DAN derivatization at neutral pH. Analytical methods that utilize neutral derivatization suffer with respect to sensitivity in comparison with those that use acidic derivatizations. Nitroglycerin produces only small amounts of ·NO. While it is beneficial because ·NO is very potent, able to

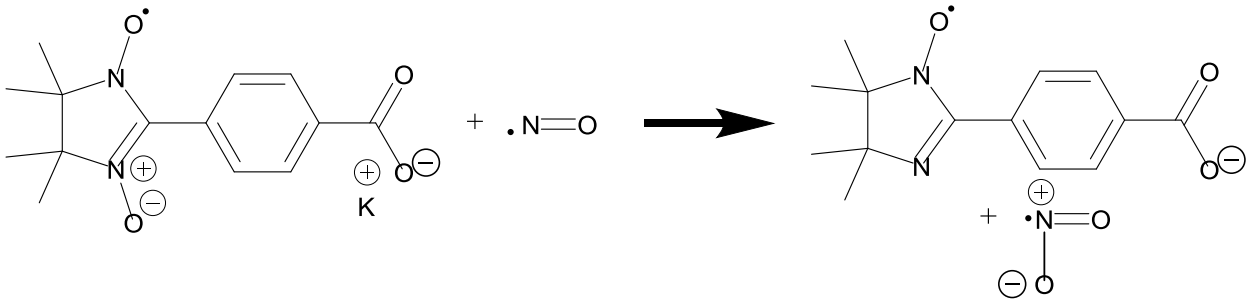
affect significant vasodilation with small amounts, its determinations require increased analytical sensitivity for its study compared to techniques for more abundant analytes.

Another common method used for NO_x determinations is derivatization with the Griess reagent, but like DAN, it is limited to NO_2^- determinations. As nitrite is considered the stable oxidative end product of nitric oxide, so is nitrate considered the stable oxidative product of nitrite. Thus often times both species need to be quantified. This requires two derivatizations and analyses, the initial determination of nitrite followed by the use of nitrate reductase to convert nitrate to nitrite for the second analysis. (***26***)

Despite its continued use, the Griess reagent has somewhat fallen out of favor for more sensitive derivatization reagents like DAN. (***27***) Unfortunately, since both methods rely on spectrophotometry they are sensitive to ascorbate interference and cannot be used in assays containing ascorbate. (***28***) Futhermore, nitrite itself suffers analytical difficulties as a common contaminant of experimental materials and methodologies. (***29***) (***30***) Not only is nitrite present in water, environmental exposure during experiments can introduce nitrite via NO_x species in the air (***31***)

Methodological improvements for selective radical determinations have been applied to nitric oxide. (***32***) These methods combine otherwise simple derivatizations with the use of expensive reagents which generally require additional analytical steps. Those reagents are the stable radical 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (PTIO) or one of its analogs. One such analog, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (carboxy-PTIO) has been used in combination with the Griess reagent and diaminofluorescein diacetate (DAF-2 DA) for selective ·NO measurement (Scheme 3, next page). (***33***) (***34***) The utility of PTIO species is their ability to donate an electron to ·NO, producing nitrite, which is then conventionally derivatized, and a corresponding PTI species. Without accurate determinations of that PTI species, this does little to overcome high background nitrite levels and produces micromolar detection limits. (***35***). Some researchers increase sensitivity reportedly to the picomolar range with evaporation and reconstitution, (***36***), but this increases not only operator time but contamination and thus error. The use of PTIO with traditional

spectrohotometric nitrite determinations still suffer from interferences, such as nitrite contamination or ascorbate.



Scheme 3. CarboxyPTIO reaction with NO to form CarboxyPTI.

Before we could answer any questions about the effect of ascorbate on nitroglycerin reductive or hydrolytic metabolism, we needed improved methods. We first adapted the use of traditional nitroglycerin determinations, (***37***) but as explained above, could not use this method to differentiate between reductive bioactivation and hydrolysis. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is our solution to the selectivity problem inherent in nitrite determinations. The use of stable isotope labeling allows determination of the nitrite source. This has already been used for measurements of nitric oxide synthase activity using $^{15}N_4$-arginine. (***38***) The natural abundance of ^{15}N is 0.366 %, so the contribution of background nitrite to $^{15}NO_2^-$ determinations is significantly reduced. Thus, with the use of $^{15}N_3$-GTN, we could measure the bioactivation product nitrite directly in enzyme incubations, eliminating the mechanistic assumptions GDN determinations rely upon, in the presence of micromolar nitrite background.

Methods

Indirect Measurement – 1,2- and 1,3-GDN

LC-MS/MS method

Analysis of 1,2- and 1,3-GDN was performed on a Shimadzu Prominence LC system coupled to an AB Sciex 3200 QTrap in negative ion mode. Mobile phase A was 100 µM NH_4Cl and mobile phase B was methanol. Separation was achieved on a Phenomenex Luna PFP(2) 5 µm 2.0 x 150 mm column with isocratic elution of 30 % B at a flow rate of 0.2 mL/min for 15 minutes. Flow was directed to the mass spectrometer between 7-10.5 minutes only. Injection volume was 1 µL.

An MRM method monitored the following transitions over 500 ms for 217 → 62 (Da) for GDN and 219 → 62 (Da) for $^{15}N_2$-GDN. The collision energy was set at -18 V. Declustering, entrance, and collision cell exit potential voltages were -20, -3, and -2 V, respectively. The IonSpray voltagewas -4500 and the temperature was 350 °C.

Direct Measurement – Nitrite and Nitric Oxide

Materials

Ethylenediaminetetraacetic acid (EDTA) was obtained from Sigma. Sodium ^{15}N-nitrite was obtained from Cambridge Isotope Laboratories. Sodium nitrite and NH_4Ac were both from Fluka. Ammonium bicarbonate was from J.T. Baker. Ammonium chloride and ascorbic acid (HAsc) were from Mallinckrodt. Sodium nitroprusside (SNP) and CuCl were from Merck. MS-Grade water and methanol (MeOH) were purchased from EMD Millipore. Thioproline and diaminonaphthalene (DAN) were from TCI America. Gases, except where otherwise noted, were from Airgas.

DAN derivatization

A 10 % v/v aliquot of 0.5 mg/mL solution of diaminonaphthalene (DAN) in 50 % EtOH, 0.62 M HCl was used to derivatize NO_2^- in solution at room temperature for one hour before the addition of 10 % v/v 1.4 M NaOH to quench the reaction.

LC-MS/MS detection of ^{15}N-NAT

Mass spectrometric detection of naphthalenetriazole (NAT) was performed on a Shimadzu Prominence LC system coupled to an AB Sciex 3200 Q Trap operated in positive mode. Mobile phase A was 15 mM NH_4HCO_3 and mobile phase B was methanol. The following gradient was used for analysis on an Agilent Extend-C_{18} 5 µm 2.1 x 150 mm column maintained at 40 °C with a flow rate of 0.2 mL/min: 5 % B for 5 minutes, 5-50 % B over 15 minutes, 50-90 % B over 1 minute, hold 90 % B for 4 minutes, 90-5 % B over 1 minute, hold 5 % B for 5 minutes. Flow was directed to MS between 15-20 minutes only. Injections were 10 µL.

A multiple reaction monitoring (MRM) method monitored the following transitions over 100 ms for the analyte indicated: 171 → 115, 142 m/z for ^{15}N-NAT, 159 → 159 m/z for DAN, 170 → 115 for NAT. The collision energies were 33 V for all except DAN, which was 5 V.

Declustering, entrance, and collision cell exit potential voltages were 51, 11.5 and 4 V, respectively. The IonSpray voltage was 5500 V and the temperature was 550 °C.

Construction of a Calibration Curves in the Presence and Absence of Analytical Interferences

An 11-point calibration curve from 0-1 µM was prepared in MilliQ water, derivatized with DAN and analyzed as described previously. The results are shown in Figure 2.1. A calibration curve was prepared from 0-500 nM was prepared in 50 mM pH 7.4 phosphate buffer, derivatized and analyzed; the results are shown in Figure 2.2. An 11-point calibration curve from 0-500 nM was prepared in 50 mM pH 7.4 phosphate buffer containing 10 mM Asc before derivatization and analysis as described. The results are shown in Figure 2.3. Derivatization was performed as described previously on a 1:2 dilution series of 50 nM $^{15}NO_2^-$ containing either 1 mM NADH, NAD^+, NADPH, DTT or nothing and analyzed as described previously; the results are shown in Figure 2.4.

This method was specifically developed to differentiate between the reductase and hydrolase activities of aldehyde dehydrogenase (ALDH) on nitroglycerin, by measuring nitrite which is reductively released. Application of the method to enzyme incubations indicated the presence of additional (non-cofactor) analytical interferences, prompting investigation. Two groups of 15 nM solutions of $^{15}NO_2^-$, with or without 4 µg/mL ALDH2 (ProSpec Bio), were allowed to autooxidize for 0, 3, 9, 27, or 81 minutes in duplicate in their respective autosampler vials prior to derivatization and analysis as described previously. There was no significant loss of $^{15}NO_2^-$ over the course of the hour, but there was a difference of approximately 20 % in signal between samples containing ALDH2 and those without ALDH2 (Figure 2.5).

Alternative Methods for ·NO and NO_2^- Measurement

Synthesis of an N-Nitrosothioproline Standard

N-Nitrosothioproline standards were produced from both $NaNO_2$ and $Na^{15}NO_2$. (***39***) Briefly, 1 M HCl was added to a vial which contained thioproline (final concentration 30 mM) and an excess of nitrite salt. The mixture was stirred magnetically overnight. A 10 % acetic acid solution adjusted with base to pH 3 was used to increase the pH before ethyl acetate extraction and drying under a N_2 stream. Solids were reconstituted in 1 mL of an 80:20 mobile phase B:A mixture and analyzed by LC-MS/MS.

Measurement of N-Nitrosothioproline by LC-MS/MS

N-Nitrosothioproline was measured on a Shimadzu Prominence LC system coupled to an AB Sciex 3200 Q Trap operated in negative ion mode. Mobile phase A was 100 μM NH_4Ac (pH 3.0); mobile phase B was MeOH. Separation was achieved on an Extend-C_{18} column (2.1 x 150 mm, 5 μm, Agilent) with a flow rate of 0.1 mL/min at 95 % B for 50 minutes.

An MRM method monitored the following transitions over 150 ms: for the unlabeled analyte, 161 → 117, 71, and 41 m/z, with collision energies of -10, -16 and -32 V, respectively, and 162 → 118, 72 and 42, with the same collision energies, respectively, for the labeled analyte. The declustering potential was -25 V, the entrance potential -8 V, and the collision cell exit potential was 0 V. The IonSpray voltage was -4500V and the inlet temperature was 450 °C.

Construction of a Nitric Oxide Calibration Curve from a Saturated ·NO solution

Saturated ·NO solution was a gift from Dr. Joe Beckman and was prepared by bubbling ·NO through NaOH solution into water until it ceased to evolve brown gas (NO_2). The solution was stored in an air tight container on ice prior to use. Vials were prepared with pH 7.4, 50 mM phosphate buffer containing 30 mM thioproline and purged with N_2 for a minimum of 15 minutes. Nitric oxide was removed from its airtight container using a gas tight Hamilton syringe and serial dilutions were performed as quickly as possible, again, to minimize the introduction of oxygen.

Following thioproline derivatization for up to 41 hours, the solution was transferred to a test tube, adjusted to pH 3 with a 10 % acetic acid solution adjusted to pH 3, and confirmed with BHD pH indicator strips, and extracted with ethyl acetate into MS autosampler vials. Ethyl acetate was evaporated under N_2 stream to dryness and the samples were reconstituted in 100 μL 50/50 MeOH/H_2O for LC-MS/MS analysis.

Preparation of Standard Solution by Reductive Release of Nitric Oxide from Sodium Nitroprusside

Nitric oxide was released quantitatively from SNP according to the literature. (***40***) Briefly, a saturated solution of CuCl was deoxygenated using either Ar or N_2 for at least 15 minutes. A pH 9 EDTA buffer containing a derivatization reagent was deoxygenated similarly, prior to division into aliquots, including one vial containing $SNP_{(s)}$ to form a 30 mM solution. As quickly as

possible, to minimize introduction of oxygen, the SNP solution was subjected to serial dilution into the buffer aliquots before addition of equivolume $CuCl_{sat}$ solution. Samples were analyzed by LC-MS/MS.

CarboxyPTIO Mass Spectrometric Detection toward Radical Measurement

The stable radical 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxyl-3-oxide (carboxy-PTIO) potassium salt (Sigma) was diluted in H_2O before MS analysis on a Shimadzu Prominence LC system coupled to an AB Sciex 3200 Q Trap operated in positive ion mode. Mobile phase A was 15 mM $NaHCO_3$ and mobile phase B was MeOH. Separation was achieved on an ExtendC_{18} column (2.1 x 150 mm, 5 µm, Agilent) maintained at 40 °C with a flow rate of 0.2 mL/min over a gradient. An MRM method monitored the following transitions over 150 ms: 277→ 247, 149, 148, and 69 m/z. The collision energies were 31, 39, 45 and 57 V, respectively, declustering potential 50 V, entrance potential 10 V and collision cell exit potential 3 V. Curtain gas was set at 40, collision gas at medium, and both ion source gases at 30. The IonSpray voltage was 5500 V and the temperature was 550 °C.

Results

Indirect Measurement – 1,2- and 1,3-GDN

Application of the literature method to our instrumentation was unsuccessful. The high salt content reduced signal with each subsequent run. The reduction of NH_4Cl concentration in the mobile phases and diversion from the mass spectrometer for the majority of the run time allowed retention of chloride in the mobile phase, required for ionization, while preventing build-up in the source over repeated analyses, maintaining sensitivity. A representative chromatogram from an enzymatic incubation of nitroglycerin is shown in Figure 2.6. Peak identification was determined using standards for each dinitrate. A calibration curve in the concentration range of interest for GTN metabolism (nM) was prepared by dilution of true standards and is shown in Figure 2.7.

Direct Measurement – Nitrite and Nitric Oxide

This derivatization method represents the result of several optimizations to literature methods. (***41***) We required reliable and quantifiable results in the nanomolar range for nitrite, corresponding to the level of GTN transformation by aldehyde dehydrogenase determined using

GDN measurements. We found the solubility of diaminonaphthalene in water to be insufficient. We suspect our early lack of reproducibility came from inhomogeneity of the solution during derivatization, but this problem was adequately addressed by the incorporation of ethanol, a miscible organic solvent. Literature reports the use of short derivatization times, which we found similarly insufficient. Although it appears the product of DAN derivatization of NO_2^-, naphthalenetriazole (NAT) continues to increase over time, this is likely the result of NO_x contamination from the environment (air). A one hour derivatization was sufficient to achieve reproducible results.

Following optimization of the method, the relationship between nitrite concentration and ^{15}N-NAT signal was found to be linear over small concentration ranges, but quadratic regression is required for a good model fit over more than two orders of magnitude. The method does reliably produce linear calibration curves in the nanomolar range and thus was suitable to move forward with our measurements.

Tests of interferences were undertaken in response to experimental results. Unexpected results using the method in enzyme incubations (Ch. 3) forced the investigation of each component of the incubations. The good fit of a quadratic model seen for this procedure in pure water (Figure 2.1) was confirmed to hold in phosphate buffer (Figure 2.2). Combining the data from the calibration curves prepared in water and phosphate buffer, there is still excellent agreement with a quadratic model up to 125 nM (Figure 2.8), which is well above our range of interest for GTN metabolism measurements. Although the data was obtained from different experimental conditions on different days and therefore are not comparable, the similarity between their analytical results demonstrates marked improvement of the method from initial trials.

There was also good fit of a quadratic regression model in the presence of Asc (Figure 2.3), which was essential to our use of the method for investigations of Asc effects. Other reducing cofactors used for control incubations were less reliable (Figure 2.4). Across the different cofactors, ^{15}N-NAT peak area integrations were largely comparable at and below 25 nM, but interference increased and peak areas deviated between groups with increasing nitrite concentration. This increasing signal deviation between groups was within our concentration

range of interest. We also determined an approximate 20 % reduction in signal with the inclusion of enzyme (Figure 2.5).

Although we expected measurements to be closer to 10 nM from our GDN determinations, these findings demonstrate the importance of the use of nitrite as an internal standard. The use of stable isotopes as internal standards depends upon the incubation being performed. In the case of unlabeled GTN for incubations that produce enough nitrite to allow determination despite background, like those with xanthine oxidase (Ch. 4), ^{15}N- labeled nitrite should be used; while in the case of lower nitrite production, as seen in ALDH incubations (Ch. 3), labeled GTN and unlabeled nitrite should be used.

Alternative Methods for ·NO and NO_2^- Measurement

Although successful in detection of nitrosothioproline (Figure 2.9), thioproline repeatedly failed to show a correlation between nitric oxide concentration and nitrosothioproline signal. A calibration curve for nitric oxide from SNP using DAN for derivatization was prepared, verifying ·NO release (Figure 2.10). This prompted subsequent optimization of the time required to derivatize using concentrated solutions, which showed a complete lack of signal prior to 27 hours and a lack of significant signal gains after 41 hours. However, upon application to a SNP dilution series, even the optimized method failed to produce a concentration-dependent instrument response.

Figure 2.11 shows a calibration curve obtained from thioproline derivatization of ·NO from a saturated ·NO solution. The lower limit of quantitation (LLOQ) was calculated by scaling the signal-to-noise ratio (S/N) for a 500 μM ·NO dilution (Figure 2.9) to an S/N of 10. The concentration of the solution was determined assuming the concentration for a saturated solution to be 2 mM NO, at 0 °C. (***42***) The LLOQ for this method was approximately 80 μM, the concentration which corresponds to a signal five times that of the noise.

CarboxyPTIO (Figure 2.12) and the product of its reaction with ·NO, CarboxyPTI, were successfully determined using LC-MS/MS, but the method was not developed fully.

Discussion

The successful adaptation, development and optimization of methods to determine both the byproducts and bioactive products of nitroglycerin bioactivation provided a unique opportunity

to research nitroglycerin in ways not previously possible. Importantly, the utility of the method in the presence of Asc, without the analytical problems seen with traditional nitrite determinations, was essential to the investigation of its effect on nitroglycerin bioactivation. The method could be further improved by the inclusion of radical determinations, which has begun for both ascorbate, ascorbyl (Ch. 4) and nitric oxide radicals.

Despite efforts to develop such a method, ultimately we never strictly measured ·NO. Thioproline derivatization was ultimately unsuitable for ·NO detection and determination. The derivatization product, N-nitrosothioproline was detected, but only at low yield after prohibitively long derivatization times. The high pH required for reductive release of ·NO from SNP may have contributed to the failure of this method. Although enzyme incubations and cell culture studies in which we intended to measure ·NO are performed at neutral pH, there were other difficulties developing the method. The relative success seen with the linear response from saturated ·NO solution was likely derivatization of N_2O_3 rather than ·NO, generated in the solution prior to serial dilution and derivatization. Dinitrogen trioxide was formed during bubbling of ·NO to remove O_2. In addition to producing NO_2, the toxic red-brown gas which eluted, HNO_2 is formed, acidifying the solution and promoting the formation of N_2O_3. As mentioned earlier, many ·NO and NO_2^- detection methods actually derivatize N_2O_3, which was likely the case with thioproline as well.

The successful determination of CarboxyPTIO and CarboxyPTI with LC-MS/MS is the first step toward an even more specific detection of ·NO in conjunction with NO_2^- determination. (***43***) Since CarboxyPTIO produces NO_2^- by radical donation, comparison of NO_2^- and CarboxyPTI concentrations could allow differentiation of ·NO and NO_2^- production within GTN bioactivation. CarboxyPTIO inclusion and analysis has been successful in several model systems thus far to measure NO. (***44***) The major limitation with its use is a lack of specificity, but the proposed combined method would help address this issue.

Figures

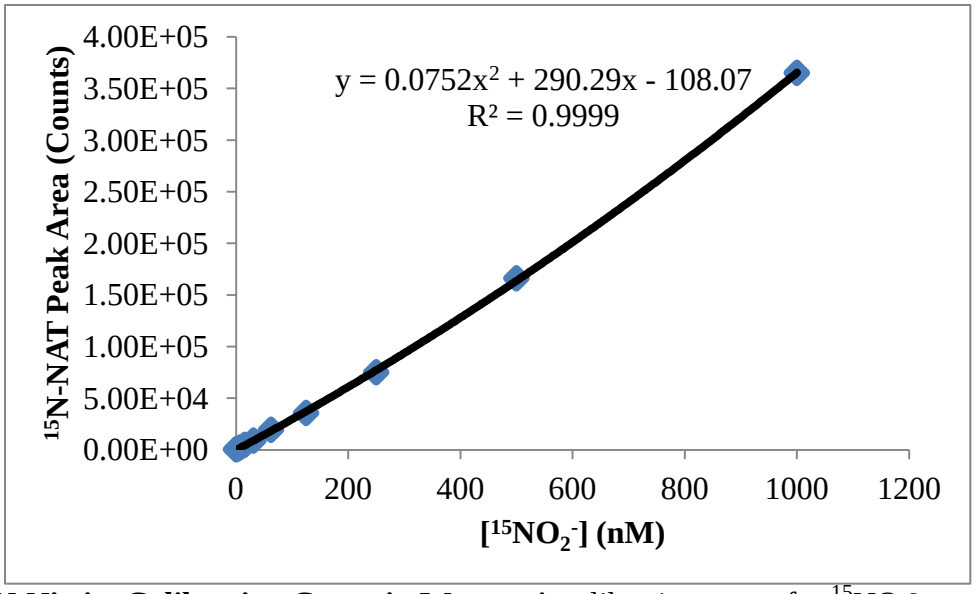


Figure 2.1. ^{15}N-Nitrite Calibration Curve in Water. A calibration curve for $^{15}NO_2^-$, derivatized by 10 % v/v 0.5 mg/mL DAN in 0.62 M HCl, 50 % EtOH for one hour prior to quenching with 1.4 M NaOH, 10 % v/v, analyzed by LC-MS/MS, and quantified on the 171 → 115 m/z transition for ^{15}N-NAT, t_R = 8.6 min.

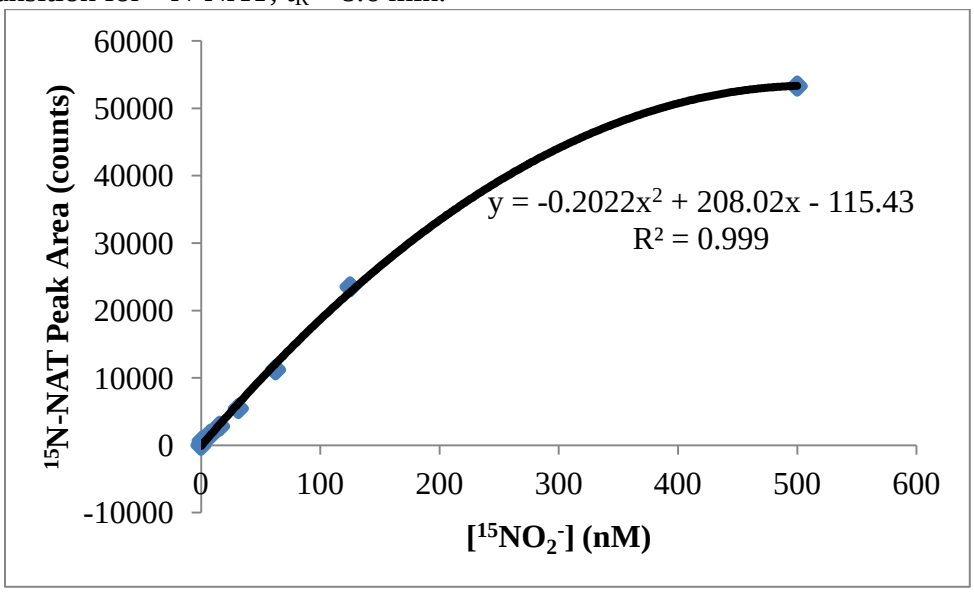


Figure 2.2. ^{15}N-Nitrite Calibration Curve in Phosphate Buffer. A calibration curve for $^{15}NO_2^-$ in 50 mM phosphate buffer, pH 7.4, derivatized by 10 % v/v 0.5 mg/mL DAN in 0.62 M HCl, 50 % EtOH for one hour prior to quench with 10 % v/v 1.4 M NaOH, quantified by LC-MS/MS on the 171 → 115 m/z transition for ^{15}N-NAT.

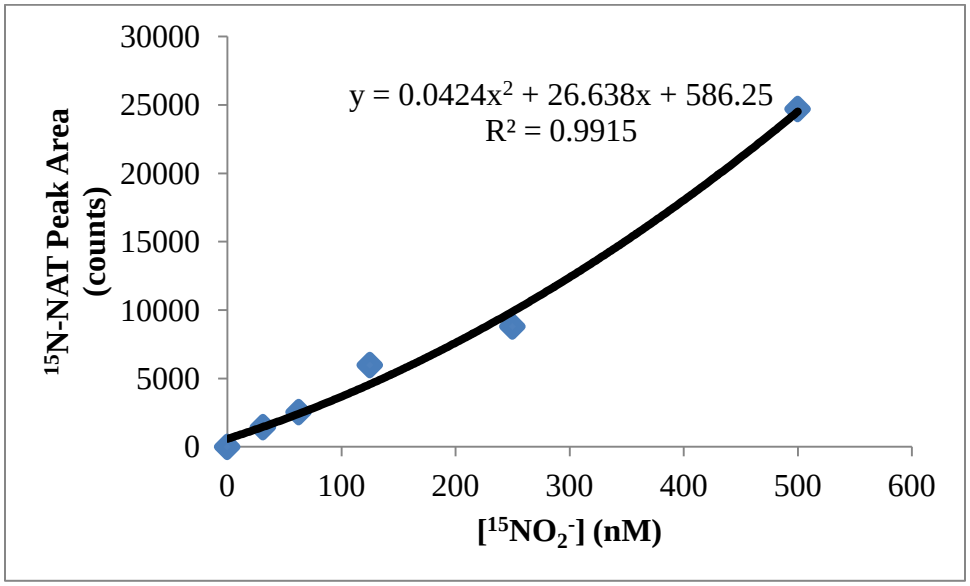


Figure 2.3. DAN Derivatization in the Presence of Asc. A calibration curve for $^{15}NO_2^-$ in 50 mM phosphate buffer, pH 7.4 containing 10 mM Asc was derivatized by 10 % v/v 0.5 mg/mL DAN in 0.62 M HCl, 50 % EtOH for one hour prior to quenching with an equivolume aliquot 1.4 M NaOH, analyzed by LC-MS/MS, and quantified on the 171 → 115 m/z transition for ^{15}N-NAT.

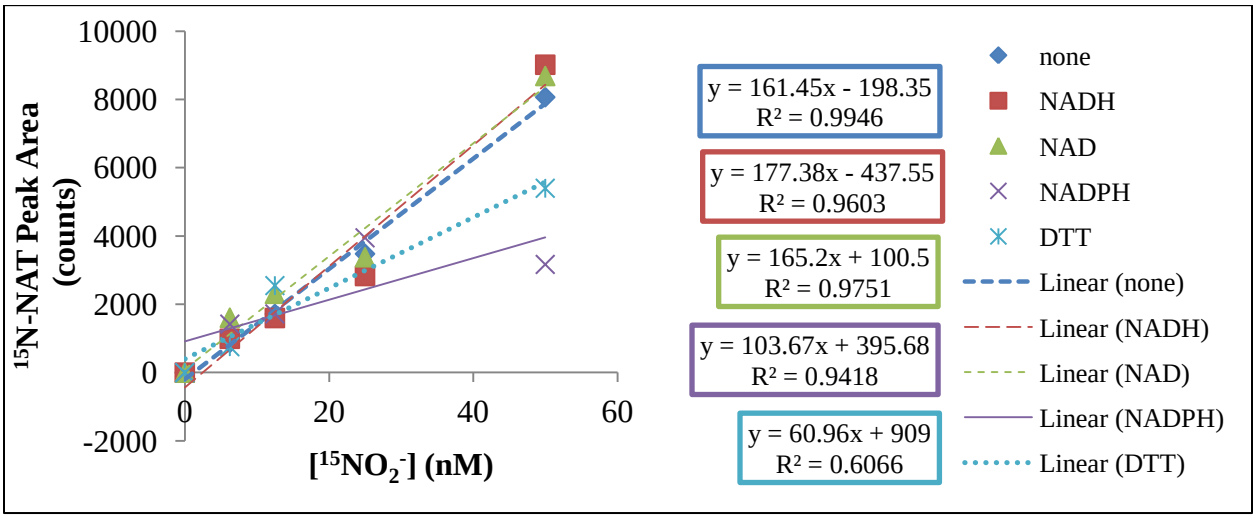


Figure 2.4. DAN Derivatization in the Presence of Various Cofactor Candidates. A calibration curve for $^{15}NO_2^-$ in 50 mM phosphate buffer, pH 7.4, and 1 mM of the indicated cofactor was derivatized by 10 % v/v 0.5 mg/mL DAN in 0.62 M HCl, 50 % EtOH for one hour prior to quenching with 1.4 M NaOH, 10 % v/v, analyzed by LC-MS/MS, and quantified on the 171 → 115 m/z transition for ^{15}N-NAT.

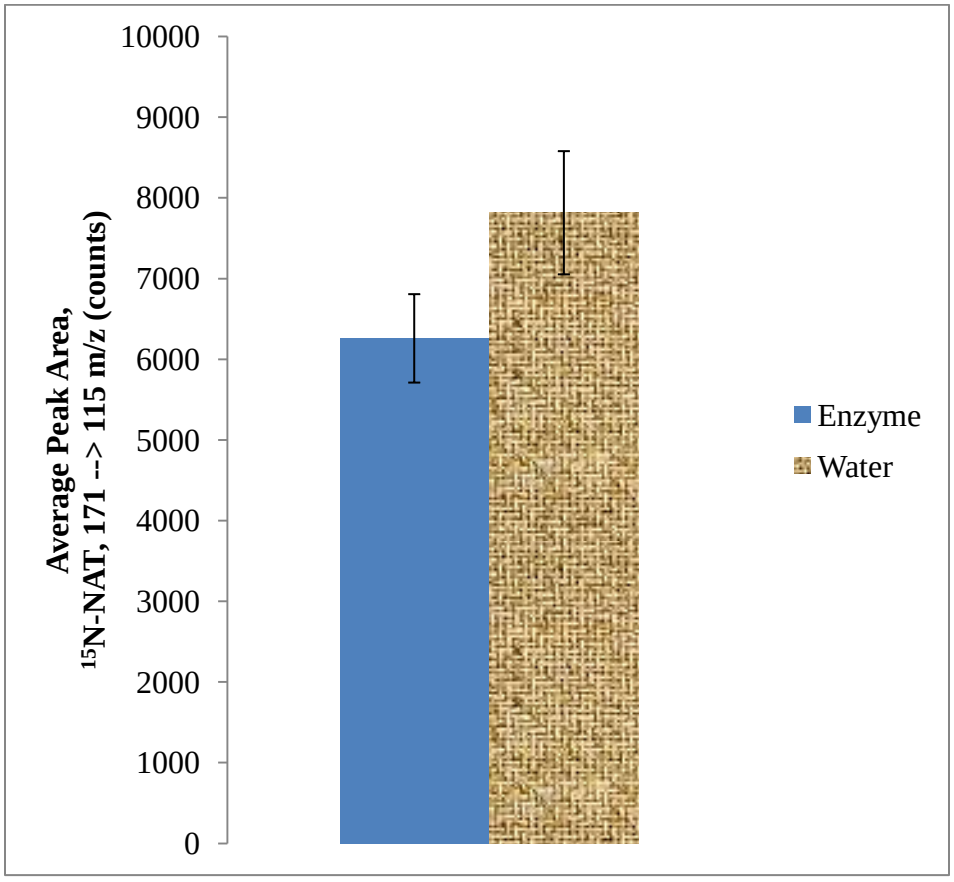


Figure 2.5. Protein Interference in ^{15}N-NAT Determinations. In the presence or absence of ALDH2, 4 µg/mL, 15 nM $^{15}NO_2^-$ in 50 mM phosphate buffer, pH 7.4 was derivatized by 10 % v/v 0.5 mg/mL DAN in 0.62 M HCl, 50 % EtOH for one hour prior to quenching with 1.4 M NaOH, 10 % v/v, analyzed by LC-MS/MS, and quantified on the 171 → 115 m/z transition for ^{15}N-NAT.

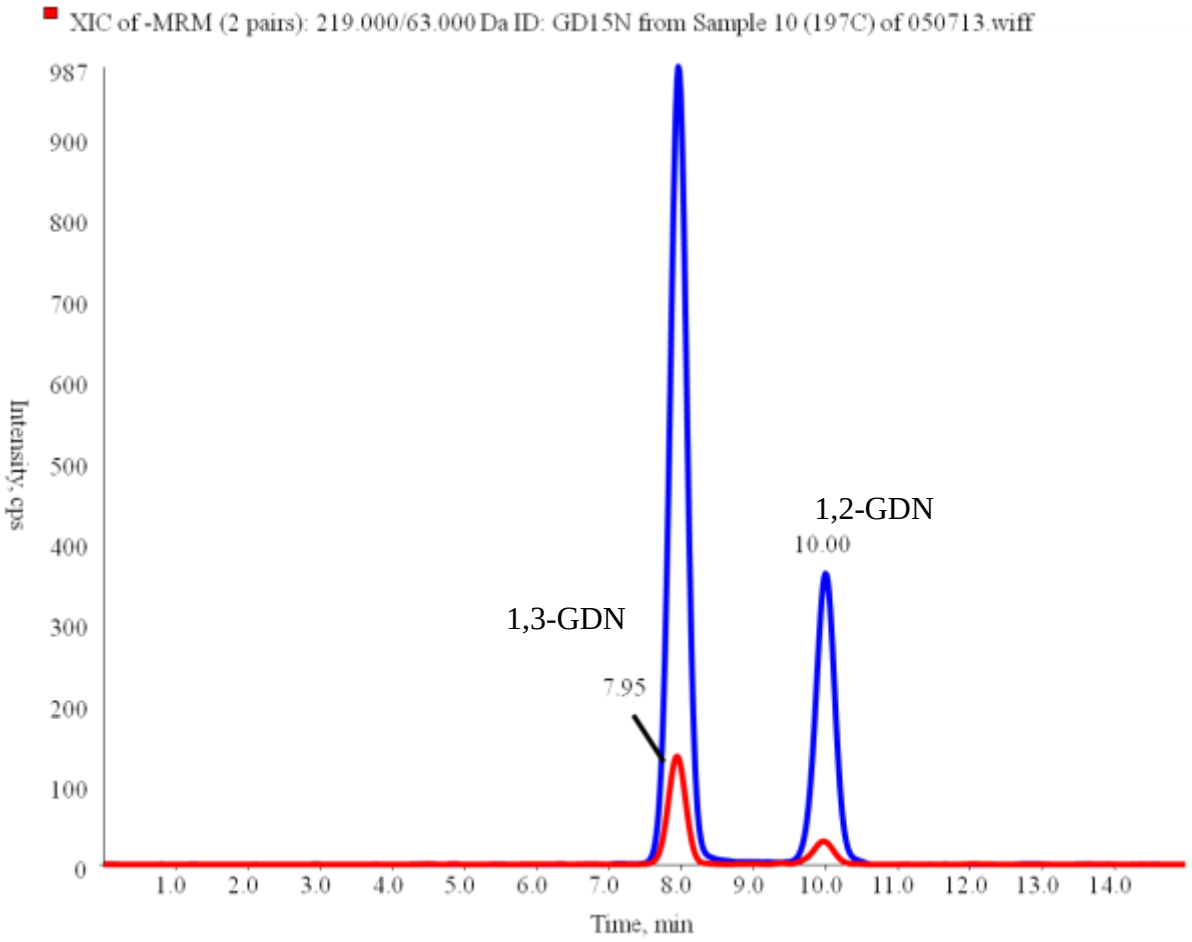


Figure 2.6. Representative Chromatogram for GDN Separations. A chromatogram representative of those seen for GDN analyses separated using 30 % MeOH 100 μM NH_4Cl at 0.2 mL/min on a Luna PFP(2) 5 μm 2.0 x 150 mm column. Flow was directed to MS between 7-10.5 minutes only. An MRM method monitored the following transitions over 500 ms for 217 → 62 (Da) for GDN and 219 → 62 (Da) for $^{15}N_2$-GDN. This sample is an incubation of yeast aldehyde dehydrongenase (yALDH, a model system for human aldehyde dehydrogenase with 95-100 % conservation of the active site) (***45***) with $^{15}N_3$-GTN and Asc, quenched by the addition of 250 μL MeOH and spiked with 1 μL each 549 μM 1,2- and 1,3-GDN.

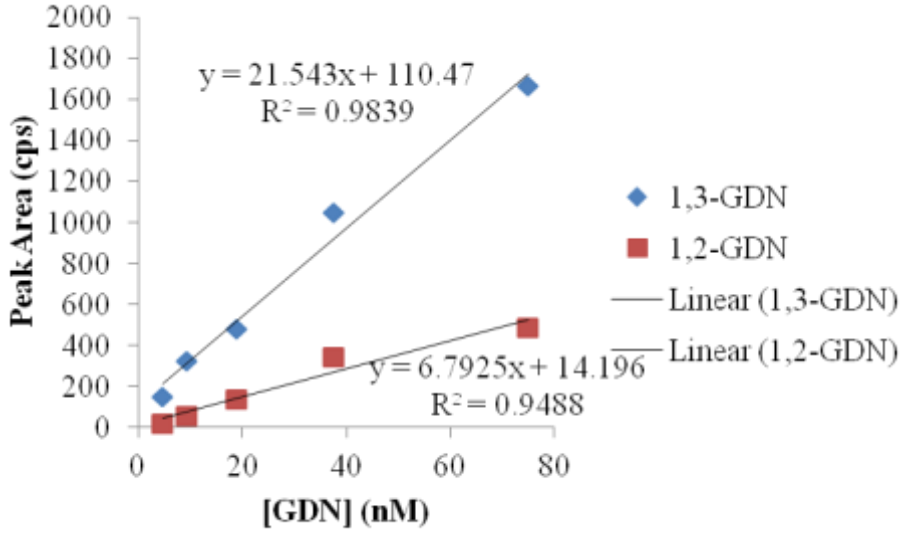


Figure 2.7. Representative GDN Calibration Curve. GDN calibration curves obtained by dilution of true standards and analyzed using separated using 30 % MeOH 100 µM NH_4Cl at 0.2 mL/min on a Luna PFP(2) 5 µm 2.0 x 150 mm column. Flow was directed to MS between 7-10.5 minutes only monitoring the transition 217 → 62 over 500 ms.

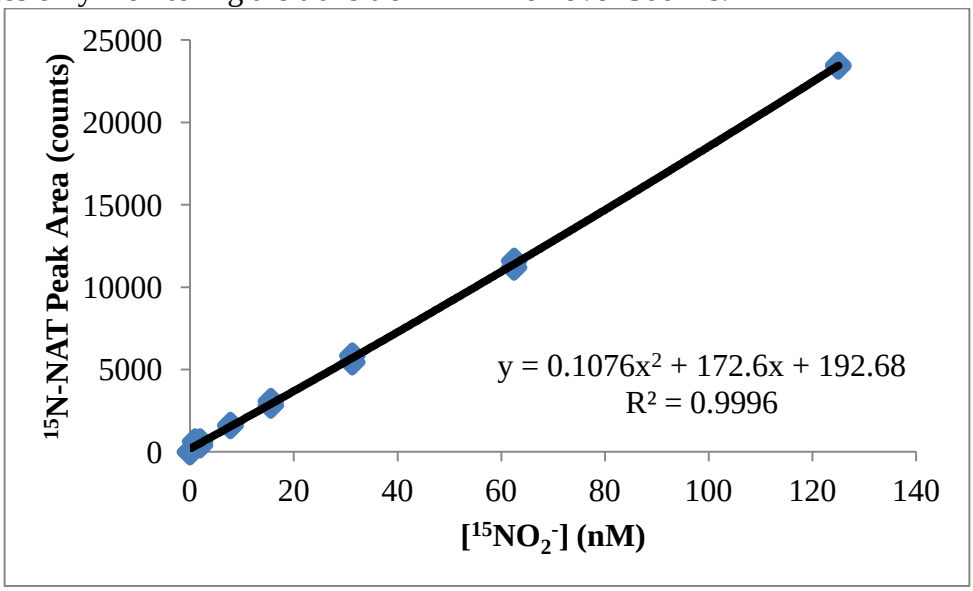


Figure 2.8. Result of Combining Calibration Curve Results Obtained in Pure Water and Phosphate Buffer. Combined $^{15}NO_2^-$ calibration curves in water and in 50 mM pH 7.4 phosphate buffer, derivatized by DAN, analyzed by LC-MS/MS, and quantified on the 171 → 115 m/z transition for ^{15}N-NAT. The good fit of a quadratic regression model to the data from two different experiments illustrates marked improvements in the derivatization method by increasing the derivatization duration and organic composition of the derivatization solution.

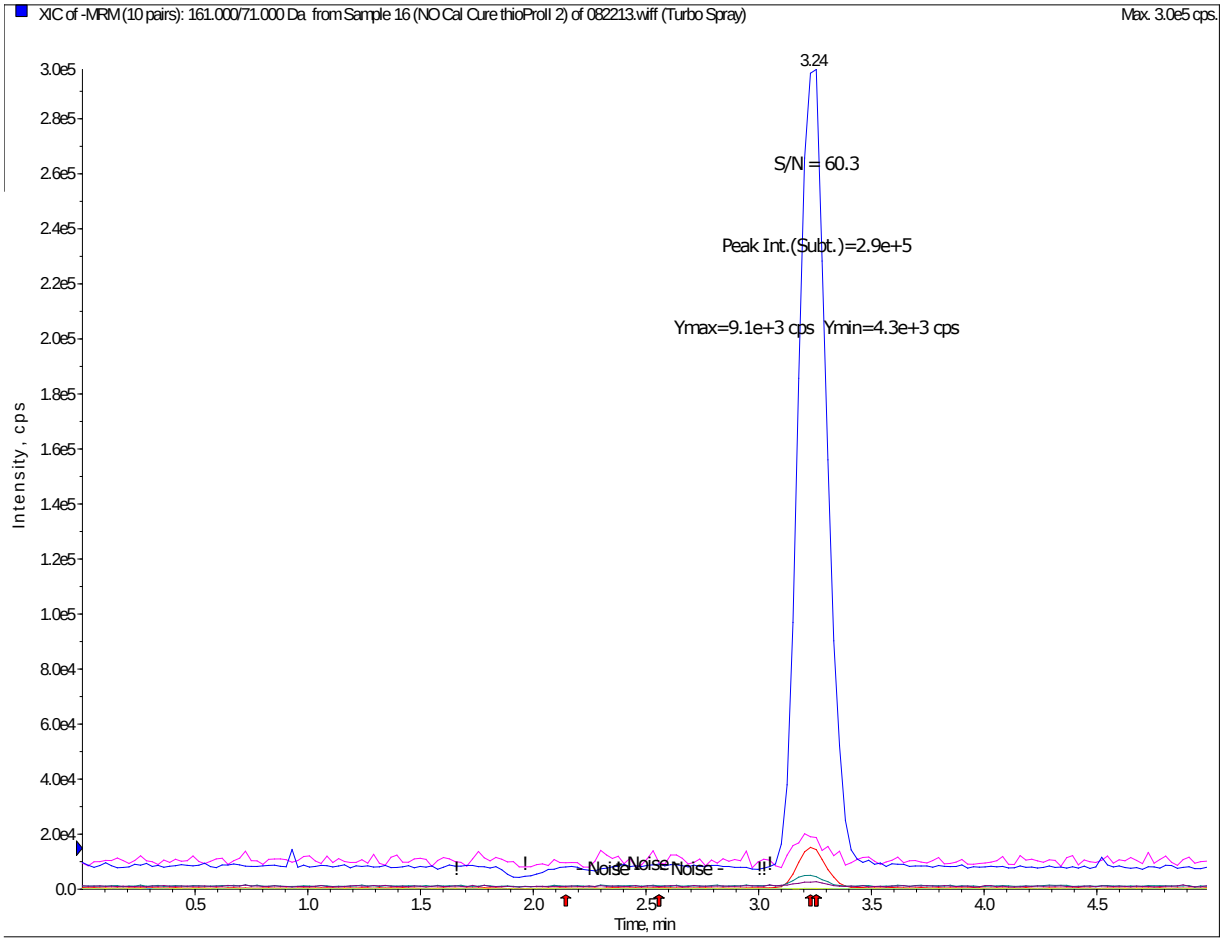


Figure 2.9. Nitrosothioproline Chromatogram· The chromatogram from a 500 µM ·NO solution, derivatized with thioproline, used to calculate the LLOQ for the method. Note the shift in retention time from Fig. 1, which happened with each new batch of mobile phase A, 100 µM NH_4Ac, pH 3.0, and necessitated the use of standards during each analytical run.

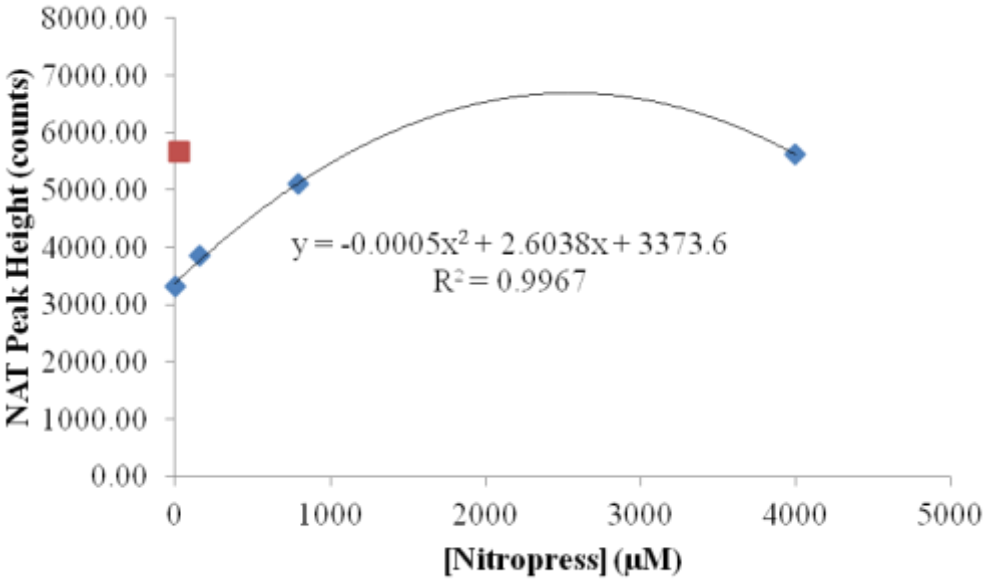


Figure 2.10. ·NO Calibration Curve Obtained by DAN Derivatization. A calibration curve for nitric oxide released from Nitropress by Cu(I) and derivatized with DAN for LC-MS/MS analysis. The inclusion of the data point represented by the red square, corresponding to 32 nM, results in a correlation coefficient of 0.396.

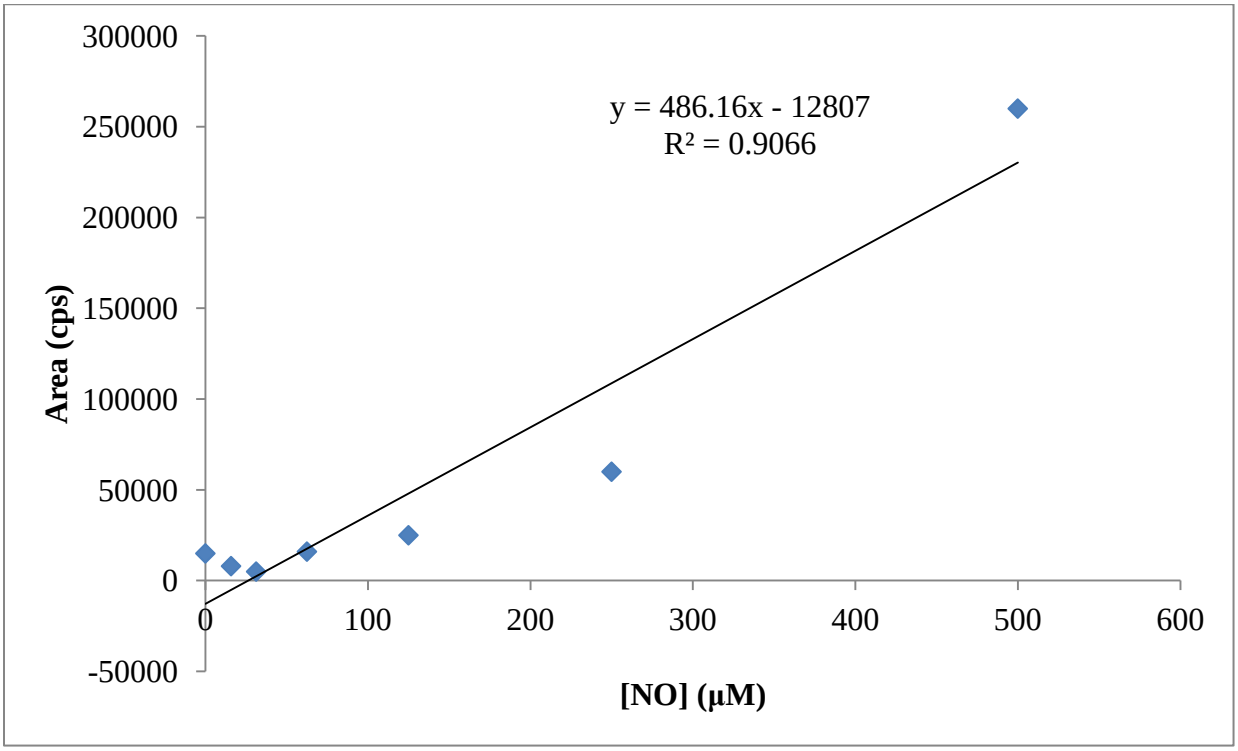


Figure 2.11. ·NO Calibration Curve with Thioproline Derivatization. A calibration curve constructed in Analyst from thioproline-derivatization of a NO_{sat} dilution series.

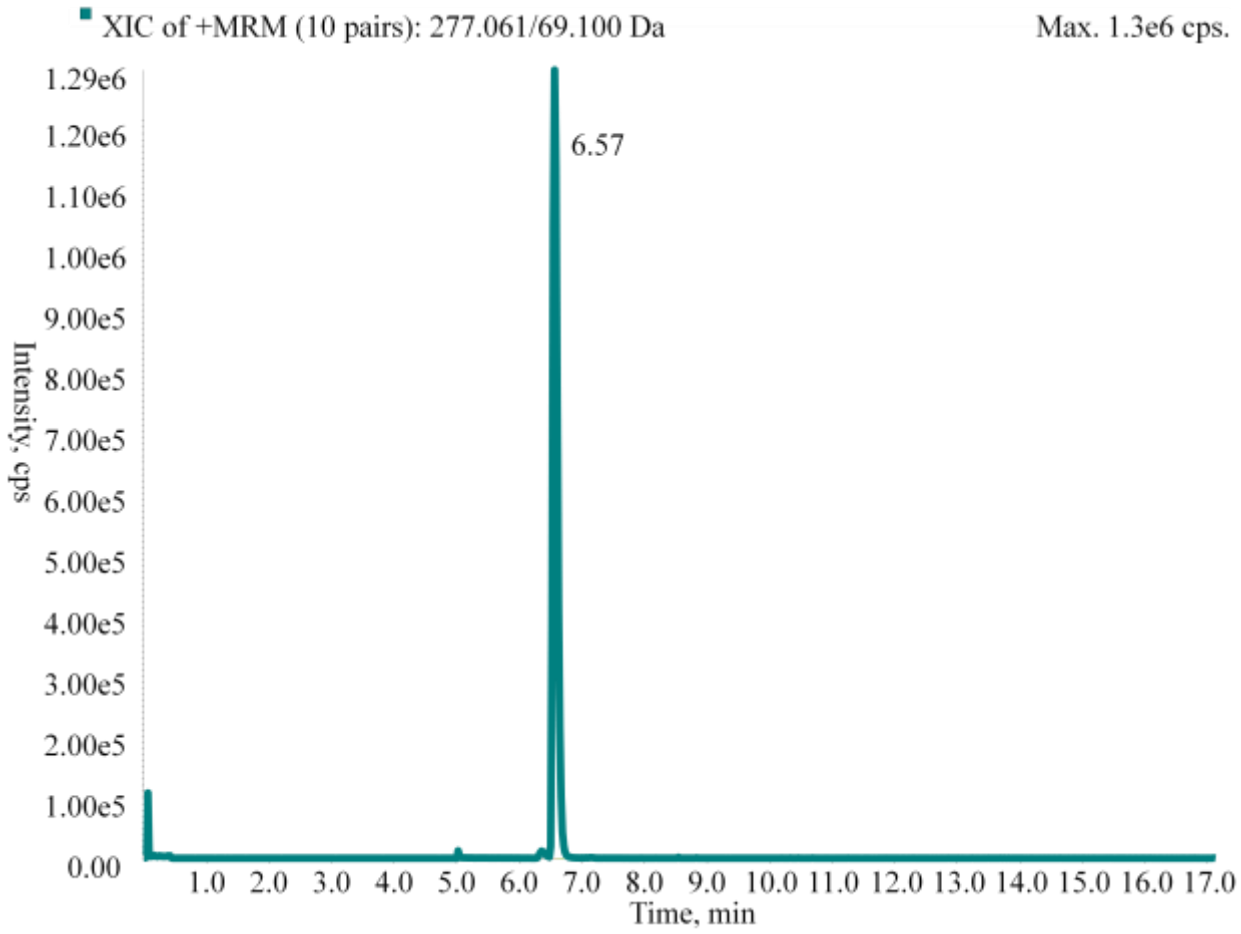


Figure 2.12. Chromatogram for 2-(4-Carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxyl-3-oxide (CarboxyPTIO). Separation was achieved on an Extend-C_{18} column (2.1 x 150 mm, 5 µm, Agilent) maintained at 40 °C with a flow rate of 0.2 mL/min over a gradient with15 mM $NaHCO_3$ and MeOH. An MRM method monitored the following transitions over 150 ms: 277→ 247, 149, 148, and 69 m/z.

Chapter 3 – Aldehyde Dehydrogenase Studies

Introduction

Aldehyde dehydrogenase (ALDH) has an important cardioprotective role in addition to its perhaps better known role in alcohol metabolism and detoxification. In reality, mechanisms overlap between these roles. Following the oxidation of ethanol to acetaldehyde by alcohol dehydrogenase, ALDH further oxidizes it to acetic acid for urinary excretion. Aldehyde dehydrogenase is also responsible for the detoxification of other reactive aldehydes, some of which have shown strong correlations to heart disease. One such aldehyde is 4-hydroxynonenal (HNE), a product of lipid peroxidation.

Protein adduction by HNE has been shown to increase during ischemia-reperfusion injury, as have free HNE levels. The detrimental effects of HNE protein adduction can vary between systems, but several mechanisms have been identified which could contribute to cardiovascular disease. The mechanisms share HNE adduction. The α,β-unsaturated bond allows nucleophilic attack of biomolecules, lending the term "reactive aldehydes" to HNE and related compounds. Adduction then affects different physiological processes such as targeting proteins for proteosomal degradation and enzymatic inhibition. The enzyme needed to detoxify HNE, ALDH, is also inhibited by HNE. HNE is thought to directly promote atherosclerotic progression through this inhibition as well as lipoprotein adduction. (***46***)

The mechanism of ALDH inhibition by HNE is likely covalent modification of the active site. Several mechanisms of ALDH inhibition involving three active site cysteine residues may play a part during nitrate tolerance. One potential mechanism for nitroglycerin bioactivation is the formation of an S-nitrothiol. Cysteine residues have a high affinity to form S-nitrothiols, which would be inaccessible following HNE adduction. (***47***) The formation of a disulfide bond following oxidation of HNE or other molecules would inhibit further ALDH activity, on either nitroglycerin or HNE, increasing their concentrations. Alternately, adduction by HNE may simply block the active site.

The discovery of small molecule activators of ALDH gave hope they may prevent or even reverse nitrate tolerance. The best known small molecule activator of ALDH is Alda-1. (***6***) Alda-1 has been shown to both increase the catalytic turnover of GTN by ALDH and do so under

conditions of nitrate tolerance. α-Lipoic acid (αLA) also activates ALDH and prevents nitrate tolerance. In fact, recent research suggests ALDH activation is responsible for the known cardioprotective effect of αLA. (***48***) In related research performed in Wistar rats, αLA was shown to reverse nitrate tolerance in addition to its prevention, as well as reverse oxidative inhibition of ALDH. (***49***)

Previous work in the Stevens Lab has investigated the role of Vitamin C as an ALDH activator. (***50***) Studies have shown that similar to the effect of Alda-1 on ALDH reductase activity, Vitamin C not only increased oxidase activity in the absence of HNE, but also recovered activity lost to HNE inhibition. These findings raised the question of whether Vitamin C could activate reductase activity as well. If so, similar to the hopes of Alda-1 developers, it could be used in the prevention or treatment of nitrate tolerance. Beyond nitrate tolerance, if similar pathways, such those involving oxidative stress, were involved in both nitrate tolerance and the decreased health outcomes of patients who use nitroglycerin, its use as a pretreatment or cotreatment during heart surgery or cardiovascular emergencies may improve outcomes. Finally, unlike Alda-1, Vitamin C is an inexpensive supplement free of side effects.

Methods

ALDH Oxidase Activity Assays

Enzyme activity was determined by monitoring the formation of nicotinamide adenine dinucleotide (NADH) at 340 nm on a Molecular Devices SpectraMax 190 in kinetic mode using Softmax Pro software. A description of experimental group incubation conditions are described in Table 3.1 for a representative experiment with yeast aldehyde dehydrogenase (yALDH), a model system for human aldehyde dehydrogenase with the triple-cysteine active site conserved.

ALDH Reductase Activity Assays

$^{15}N_3$-GTN incubation with ALDH2 utilizing NADH: Pilot Studies

Ten µg recombinant ALDH2 (human mitochondrial ALDH, ProSpec Bio) was incubated in a total volume of 250 µL for 30 minutes at room temperature under the following conditions: 50 mM phosphate pH 7.4; 17, 35 or 52 µM $^{15}N_3$-GTN; 1 mM NADH. A control was prepared similarly with the exclusion of $^{15}N_3$-GTN and the inclusion of 30 nM $^{15}NO_2^-$. Following incubation, samples were derivatized and analyzed as described previously (Ch. 2).

A second set of incubations with zero, 1 or 2 µg enzyme was incubated in a total volume of 250 µL for 30 minutes at room temperature under the following conditions: 50 mM phosphate pH 7.4; 1, 5, 10, 20 µM $^{15}N_3$-GTN; 0 or 1 mM NADH. A 13-point calibration curve containing 0-1.82 µM $^{15}NO_2^-$ was prepared in 50 mM phosphate, pH 7.4.

Table 3.1. Incubation conditions and results for determination of yALDH oxidase activity.

Well	Treatment	Water (µL)	NaP Buffer, pH 7.4, 0.1 M (µL)	1 mg/mL yALDH (µL)	125 mM ACET (µL)	25 mM NAD (µL)	Mean V_{max} (milliunits/min)
A1-3	Blank Buffer blank	125	125	0	0	0	0
B1-3	No Enz Control ACET, NAD, No yALDH	113	125	0	2	10	-0.4 ± 0.3
C1-3	No NAD Control yALDH, ACET, No NAD	103	125	20	2	0	-0.043 ± 0.004
D1-3	No Sub Control yALDH, NAD, No ACET	95	125	20	0	10	1.2 ± 0.2
E1-3	Complete yALDH, ACET, NAD	93	125	20	2	10	53 ± 1

Pilot $^{15}N_3$-GTN ALDH2 incubations with various reducing agents

Zero or 1 µg ALDH2 was incubated in a total volume of 250 µL for 30 minutes at room temperature under the following conditions: 50 mM phosphate pH 7.4; 0 or 1 µM $^{15}N_3$-GTN; 0 or 1 mM dithiothreitol (DTT), Asc^-, dihydrolipoic acid (DH-αLA), or both Asc and DH-αLA. Dihydrolipoic acid (DH-αLA) was prepared by reduction of αLA, a gift from Dr. Tory Hagen. We assumed 100% yield for this reaction to calculate concentrations and dilution volumes for incubations. A calibration curve was prepared to quantitate $^{15}NO_2^-$ production. Following incubation, samples were split into two aliquots, one of which was derivatized and analyzed as described and the other spiked with 13.5 µM $^{14}NO_2^-$ prior to derivatization and analysis.

Metabolism of $^{15}N_3$-GTN by yALDH with and without DTT and Asc for NAT and GDN analysis

An incubation was performed as described in Table 3.2. After 30 minutes incubation at room temperature with intermittent shaking, 10 μL of 26 μM $NaNO_2$ was added as an internal standard before DAN derivatization for NAT analysis.

Table 3.2. GTN metabolism by yALDH +/- DTT, Asc

Tube	Treatment	Water (μL)	0.1 M NaP, pH 7.4 (μL)	1 mg/mL yALDH (μL)	50 mM Asc or PBS (μL)	1.25 mM $^{15}N_3$-GTN (μL)	25 mM DTT (μL)	25 mM NAD (μL)
A-C	Complete + DTT	53	125	1	50 PBS	1	10	10
D-F	Complete + Asc	63	125	1	50 Asc	1	0	10
G-I	Complete + Asc/DTT	53	125	1	50 Asc	1	10	10
J-L	No Cofactor Control	63	125	1	50 PBS	1	0	10
M-O	No Enz DTT Control	54	125	0	50 PBS	1	10	10
P-R	No Enz Asc Control	64	125	0	50 Asc	1	0	10
S-U	No Enz No Cofactor Control	64	125	0	50 PBS	1	0	10
V-X	Blank	125	125	0	0	0	0	0

An incubation was performed as described in Table 3.2 for 30 minutes at room temperature and quenched by the addition of 250 μL of methanol and 1 μL each 549 μM 1,2- and 1,3-GDN as an internal standards and analyzed for GDN.

Results

$^{15}N_3$-GTN incubation with ALDH2 utilizing NADH Pilot Study

Pilot studies yielded several promising results. There was a dose response relationship in an incubation of ALDH2 between nitroglycerin concentration and nitrite production, indicating reduction (Figure 3.1). It also showed the analytical method was valid for GTN concentrations in this range, facilitating the following experiment which showed the method also worked at lower concentrations (Figure 3.2). The calibration curve used in the latter incubation produced a regression with the equation $y = -0.0042x^2 + 564.88x - 525.69$ and an R^2 value of 0.998, offering additional validation of the analytical method. Unfortunately, despite a linear response between

$^{15}N_3$-GTN and ^{15}N-NAT concentrations, the similarity of response in the presence and absence of enzyme appears to indicate a lack of enzymatic involvement.

Pilot $^{15}N_3$-GTN ALDH2 incubation with various reducing agents

Following the pilot studies, this experiment sought to clarify those results through the identification of an appropriate cofactor for use in incubations. Although the cofactor for ALDH oxidase activity is well known to be NAD^+, the principle of reversibility does not seem to apply. In other words, there is no empirical evidence NADH is the cofactor for ALDH nitrate reductase activity. Dithiothreitol is often used as a positive control in ALDH incubations due to its ability to reduce disulfide bonds, like those seen with oxidative inactivation of the active site. In fact, only the group containing DTT showed reductive enzymatic activity, appearing to produce more $^{15}NO_2^-$in the presence of enzyme (Figure 3.3). However, this pilot study without replicates is insufficient to draw conclusions. The rest of the groups contained $^{15}NO_2^-$levels on par with the no substrate/no cofactor (enzyme only) control.

In an attempt to minimize the effect of different incubation components, unlabeled nitrite was introduced as an internal standard. However, this attempt was made after the method optimization described in Chapter 2 and resulted in comparable signals for both^{15}N-NAT, and the ratio of ^{15}N-/^{14}N-NAT across experimental groups, yielding no additional insights.

Due to the relatively increased cost of ALDH2 in comparison to yALDH, and the repeated lack of success with ALDH2, the decision was made to perform subsequent incubations in yALDH until the identification of experimental conditions which yielded evidence of enzymatic reduction.

Metabolism of GTN by yALDH in the presence and absence of DTT and Asc for NAT analysis

This experiment, detailed in Table 2, suffered wide variation within groups (Figure 3.4). The data was reanalyzed assuming constant $^{14}NO_2^-$background (not shown), but the ratio of the peak area for $^{15}NO_2^-$to that of $^{14}NO_2^-$ failed to improve the variability of the data, consistent with other attempts to use an internal standard to reduce variability. There is however clear separation between groups which included $^{15}N_3$-GTN and those which did not.

Discussion

The second ALDH2 pilot study, represented in Figure 3.2, is exemplary of the problems encountered with ALDH incubations. There appears to be a lack of enzyme involvement, despite continued study of appropriate cofactors, potential analytical interferences, or inferior materials. In the figure, note the clustering of points corresponding to 20 μM GTN. In the case of enzyme involvement, one would expect little or no turnover in the absence of enzyme or cofactor, and double the product in the presence of double the enzyme, unless there is substrate saturation. As you can see, the group without the presumed cofactor in fact produced the most nitrite, while the incubation with double the enzyme produced nitrite on par with the incubation containing half its amount. The linear response seen with higher GTN concentrations (Figure 3.1) seem to indicate substrate saturation is not the problem in this case. The best conclusion which can be drawn at this point is NADH is not the cofactor for the reductase activity of ALDH.

Further work sought to identify a suitable cofactor for use in incubations. Unfortunately, results were similarly disappointing to the pilot studies which used NADH. Researchers commonly use DTT in incubations, but we sought to elucidate enzymatic mechanisms and DTT is incomparable to any known endogenous molecule. The compound with the closest properties is DH-αLA, but as with the other endogenous cofactors tested, incubation results failed to demonstrate any catalytic involvement of ALDH.

One potential analytical interference was $^{15}NO_2^-$ contamination as part of larger nitrite contamination but the results in Figure 3.4 show clearly $^{15}N_3$-GTN is the source of labeled nitrite. The extensive testing and optimization of the analytical method effectively eliminated nitrite contamination as a concern moving forward, but without a cofactor for catalysis, experimental options were limited.

As described previously, ALDH has two catalytic activities relevant to nitroglycerin metabolism, reductase and hydrolase, and thus we compared nitrite results to GDN production. The hope was that if there was no difference in reductase activity, we would see hydrolase activity. Previous research in the field failed to accurately measure reductase activity by relying upon indirect measurements such as GDN ratios or soluble guanylate cyclase activation

measurements. Thus, we expected to see results similar to those of other researchers: an increased ratio of 1,2-GDN:1,3-GDN production in the presence of enzyme. Our findings however showed comparable GTN metabolism with and without enzyme (Figure 3.5), similar to our nitrite production results.

We considered several possible explanations for this. Perhaps nitrite was being lost to our analytical capabilities. We thus worked to develop methods which could trap nitric oxide, preventing loss by protein adduction or diffusion. Although the majority of the nitroglycerin research community believes nitrite to be the product of nitroglycerin bioactivation, there are alternate theories which suggest nitric oxide or S-nitrothiols are the product. In the case of nitric oxide, in addition to the mechanisms already mentioned which could inhibit detection by our method, the rapid formation of peroxynitrite is also a concern. Future incubations could attempt to avoid the formation of peroxynitrite as reported by Oezle et al, whose method includes magnesium and superoxide dismutase toward that end. (***51***) These have been shown to increase nitroglycerin bioactivation, likely thorough inhibition of peroxynitrite formation. In the case of S-nitrothiols, an additional bioactivation step would be required prior to detection, i.e. the release of nitric oxide, which may require components not present our incubations.

Another possibility for the observed lack of activity could be rapid inactivation of the enzyme, either through the formation of S-nitrothiols in the active site, or oxidative inhibition. Total enzymatic hydrolysis of the enzyme could allow measurement of oxidation of the active site by measurement of oxidized cysteine, either as a disulfide or as sulfenic or sulfinic acid. A standard of sulfinic acid was purchased toward this end for future method development, along with cystine.

Following the lack of success with ALDH, we moved to studies with xanthine oxidase, another enzyme known to catalyze the reduction of organic nitrates including nitroglycerin. It is possible that nitroglycerin bioactivation involves multiple enzymes or other components only available in a biological system. Thus, cell culture experiments were undertaken to allow the use of untargeted metabolomics toward the identification of hereto unknown mechanisms which contribute to nitroglycerin tolerance or bioactivation. While the results of cell culture studies

have yet to elucidate additional leads, the move to xanthine oxidase for enzymatic incubation studies was more immediately fruitful and are discussed in Chapter 4.

Figures

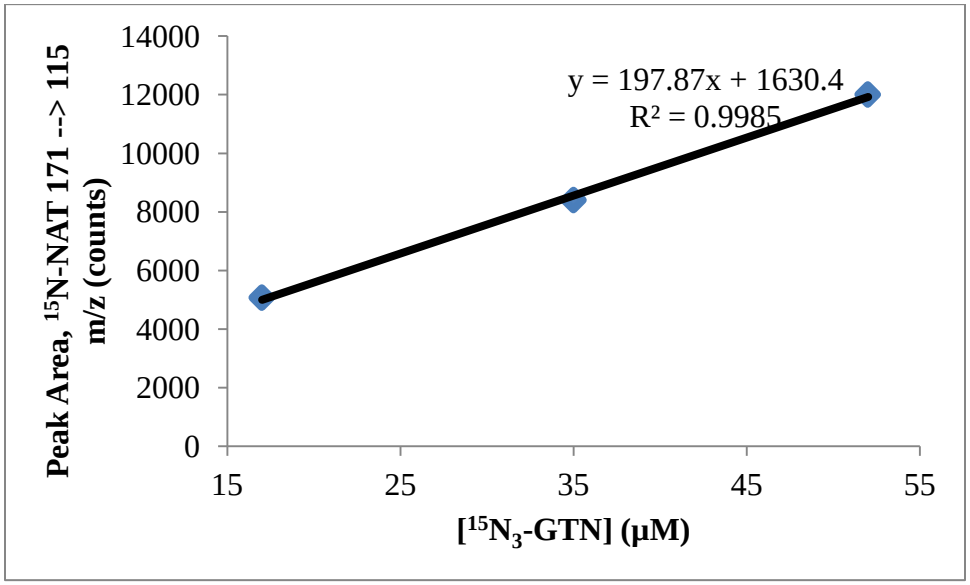


Figure 3.1. ALDH2 Pilot Incubation of GTN. Ten µg of ALDH2 was incubated at room temperature for 30 minutes with 17, 35 or 52 µM $^{15}N_3$-GTN in 50 mM phosphate buffer, 1 mM NADH as a pilot study. Following incubation, samples were derivatized with 10 % v/v 0.5 mg/mL DAN in 0.62 M HCl, 50 % EtOH for one hour prior to quenching with 1.4 M NaOH, 10 % v/v, and analyzed by LC-MS/MS. Peaks were integrated for quantification on the 171 → 115 m/z transition for ^{15}N-NAT using MultiQuant. There was good linear correlation between $^{15}N_3$-GTN concentration and $^{15}NO_2^-$ production.

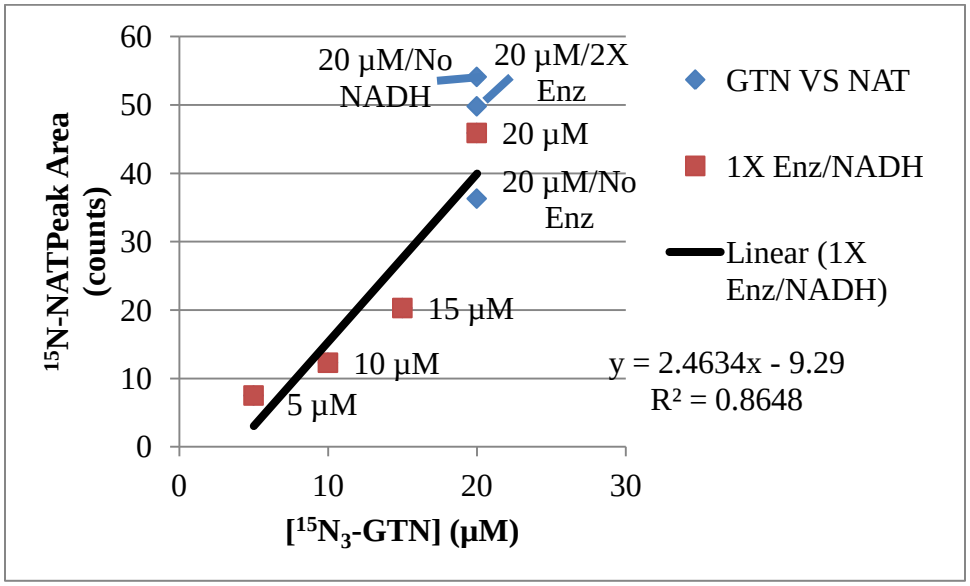


Figure 3.2. Second Pilot Incubation of GTN with ALDH2. Zero, 1 or 2 µg of fresh ALDH2 was incubated at room temperature for 30 minutes with 5, 10, 15 or 20 µM $^{15}N_3$-GTN in 50 mM phosphate buffer, pH 7.4, and 0 or 1 mM NADH before derivatization with 10 % v/v 0.5 mg/mL DAN in 0.62 M HCl, 50 % EtOH for one hour prior to quenching with 1.4 M NaOH, 10 % v/v, analyzed by LC-MS/MS, and quantified on the 171 → 115 m/z transition for 15N-NAT. Peaks were integrated using MultiQuant. Although the linear correlation between $^{15}N_3$-GTN concentration and $^{15}NO_2^-$ concentration was maintained, the lack of significant difference between control groups at 20 µM GTN indicate a lack of involvement for ALDH2.

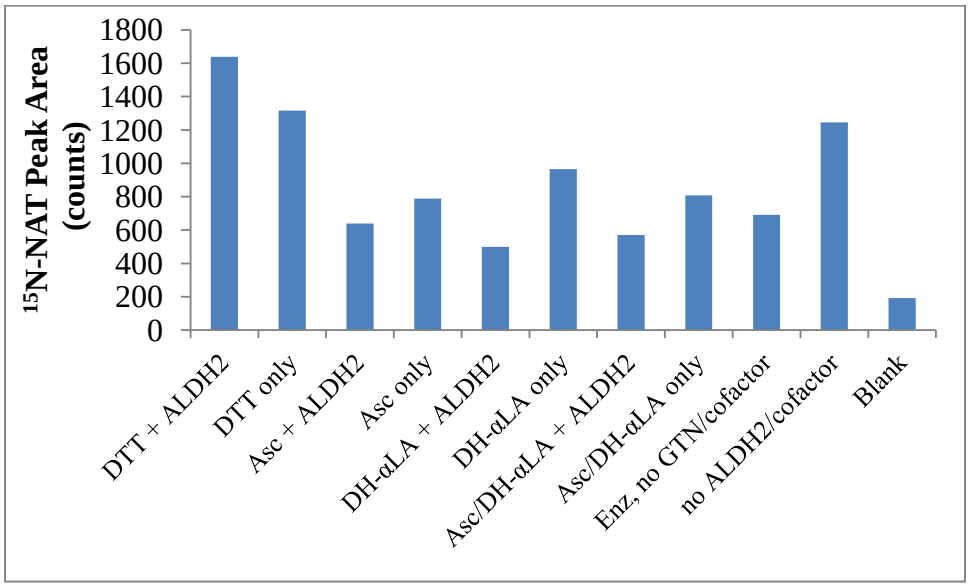


Figure 3.3. Pilot yALDH Incubation of GTN in the Presence and Absence of Various Reducing Cofactors. Zero or 1 µg of ALDH2 was incubated at room temperature for 30 minutes with 0 or 10 µM $^{15}N_3$-GTN in 50 mM phosphate buffer, pH 7.4, and 0 or 1 mM of the reducing cofactor indicated. Samples were derivatized with 10 % v/v 0.5 mg/mL DAN in 0.62 M HCl, 50 % EtOH for one hour prior to quenching with 10 % v/v 1.4 M NaOH, analyzed by LC-MS/MS, and quantified on the 171 → 115 m/z transition for ^{15}N-NAT using MultiQuant for peak integrations. The lack of (significant) $^{15}NO_2^-$ increase in the presence of ALDH2 across of cofactor analytical pairs indicates again a lack of involvement of the enzyme in the conversion of $^{15}N_3$-GTN.

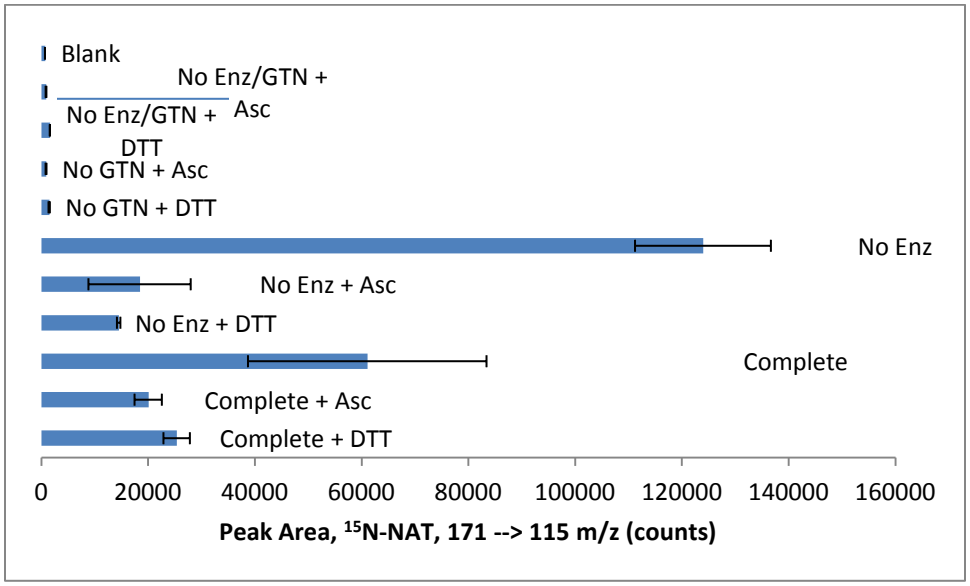


Figure 3.4. NAT Analysis of GTN yALDH Incubations in the Presence and Absence of DTT and Asc. Zero or 1 µg of fresh ALDH2 was incubated at room temperature for 30 minutes with 0 or 10 µM $^{15}N_3$-GTN in 50 mM phosphate buffer and 0 or 1 mM of the reducing cofactor indicated before derivatization with 10 % v/v 0.5 mg/mL DAN in 0.62 M HCl, 50 % EtOH for one hour prior to quenching with 1.4 M NaOH, 10 % v/v, analyzed by LC-MS/MS, and quantified on the 171 → 115 m/z transition for ^{15}N-NAT. Peaks were integrated using MultiQuant. Bars represent the average of triplicate incubations and error bars represent standard deviation. Due to the large within-group variation, little useful comparison can be drawn between groups, but it does appear $^{15}N_3$-GTN is the source of ^{15}N-NAT. The unusually large value seen in the no enzyme, no cofactor incubations is likely the result of interference from DTT, Asc and the enzyme itself with DAN derivatization and prompted further method development (Ch. 2).

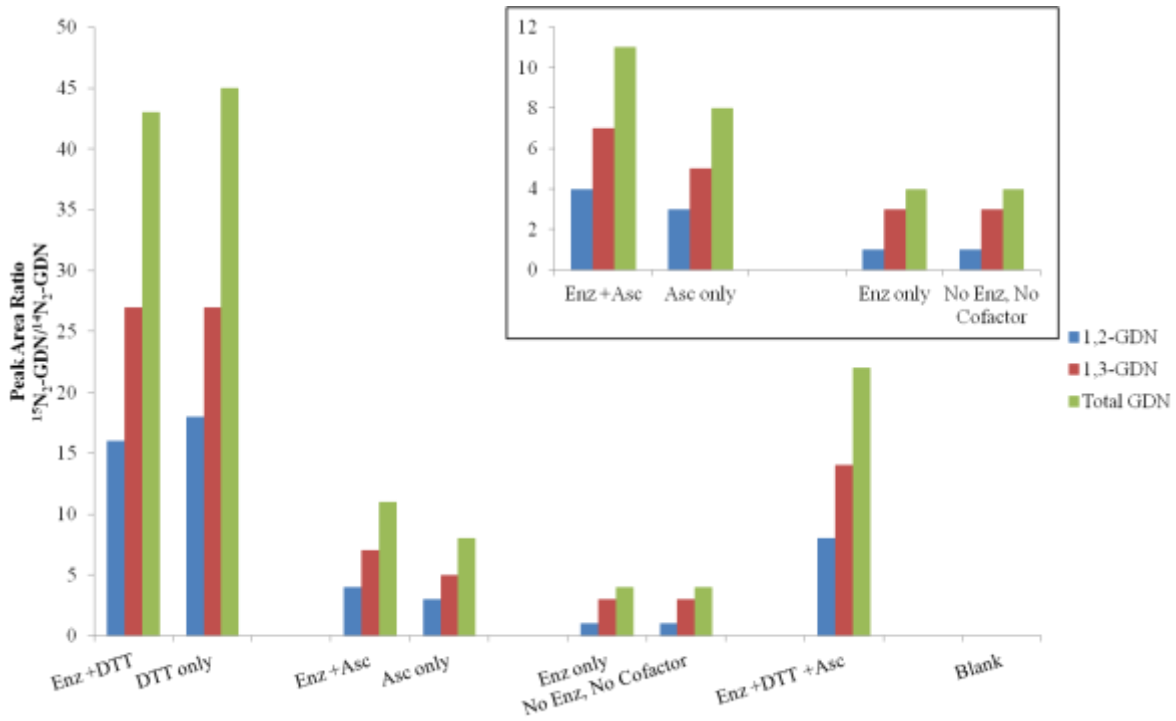


Figure 3.5. GDN Analysis of GTN yALDH Incubations in the Presence and Absence of DTT and Asc. This bar graph shows metabolism of GTN by yALDH in the presence of the indicated cofactor. Incubations of 5 µM $^{15}N_3$-GTN and 1 mM yALDH allosteric modulator NAD^+, with and without 10 µg yALDH and utilizing either no reducing cofactor, 1 mM DTT or 10 mM Asc in a total volume of 250 µL were quenched after 30 minutes with the addition of 250 µL ice-cold MeOH. Samples were spiked with 1 µL each 549 µM 1,2- and 1,3-GDN as an internal standard prior to LC-MS/MS analysis. Bars represent as a percentage the peak area of the ^{15}N-labelled analyte divided by the internal standard for a representative incubation within each experimental group, as determined by results summarized in Figure 3.4,. The group containing Asc as the reducing cofactor was the only group to show an increase in GTN turnover in the presence of enzyme.

Chapter 4 – Xanthine Oxidase Studies

Introduction

Although in recent years aldehyde dehydrogenase (ALDH) has been regarded as the nitroglycerin (GTN) bioactivating enzyme, the nitrate reductase activity of xanthine oxidoreductase (XO) was identified much earlier than that of ALDH. (***52***) (***53***) In contrast to the more limited reductase substrates for ALDH, XO can reduce not only organic nitrates, but organic nitrites as well as inorganic nitrate and nitrite. (***54***) (***55***) (***56***) Similar to ALDH, XO could contribute to nitrate tolerance through multiple mechanisms. The ultimate product of nitrate therapy, nitric oxide, inactivates the very enzyme catalyzing its production by converting XO to its desulfo form. (***57***) Additionally, XO catalysis contributes directly to oxidative stress by producing superoxide. Hydrogen peroxide is also produced during XO catalysis, which ultimately produces the even more reactive hydroxyl radicals *in vivo*. (***58***) Superoxide can also react with nitric oxide forming peroxynitrite, quenching ·NO signaling and contributing to protein nitration.

In many ways the XO nitrate/nitrite reductase activity has been better characterized than that of ALDH. The expression of XO is tightly controlled in respect to this activity. Under hypoxic conditions, like those which occur with ischemia, XO expression is upregulated, while the ·NO it produces to affect vasodilation downregulates XO expression. (***59***) Advantageously, when pH levels drop locally during ischemia, XO prefers to oxidize NADH instead of oxygen, which should reduce superoxide production, though this does not appear to have been confirmed experimentally. (***58***)

Despite these well-established links, like most nitroglycerin researchers, we focused exclusively on ALDH until a recent publication regarding XO inhibition and nitrate tolerance invigorated our interest in XO. (***60***) Researchers found allopurinol, a well-known XO inhibitor, prevented nitrate tolerance.

From this and knowledge of the XO catalytic mechanism, we hypothesized that superoxide production by XO was a significant contributor to nitrate tolerance. If ascorbate (Asc) could quench superoxide, it could not only prevent inactivation of XO, maintaining GTN bioactivation by the enzyme, but also protect the product, ·NO, and perhaps even reperfusion

damage. We sought to show Asc co-incubation with XO and GTN could enhance bioactivation by preventing oxidative inactivation of XO and/or ·NO.

Methods

XO Incubation with GTN

One hundred µg XO was incubated in quadruplicate in a total volume of 250 µL for 30 minutes at room temperature in 50 mM HEPES buffer, pH 7.4 according to Table 4.1. Following incubation, samples were derivatized and analyzed as described previously (Ch. 2).

Results

The results of the incubation described in Table 4 are shown in Figure 4.1 It is immediately apparent there is increased nitrite production by XO in the presence of Asc. Nitrite production is relatively level across other incubations, whether with or without XO.

Table 4. Incubation conditions and results for determination of XO reductase activity.

Treatment	4 mg/mL XO (µL)	5 mM Xanthine (µL)	1mg/mL GTN (µL)	50 mM Asc (µL)
X, GTN	0	1	1.14	0
X, GTN +Asc	0	1	1.14	5
XO, X, GTN	25	1	1.14	0
XO, X, GTN +Asc	25	1	1.14	5
XO, GTN	25	0	1.14	0
XO, GTN, +Asc	25	0	1.14	5
XO, X	25	1		0
XO, X +Asc	25	1		5

Discussion

Considering the relatively level nitrite production seen with and without XO, one might question if the apparent increases in the presence of Asc could be an artifact from Asc inclusion or the product of Asc-mediated GTN reduction. The experimental group which contains Asc without XO dispels this concern. If Asc inclusion was creating analytical interference, it also should have done so in the group without enzyme. Rather, method optimization with ALDH (Ch. 2) revealed a reduced signal in the presence of Asc for naphlathenetriazole (NAT), the product of nitrite derivatization with diaminonaphthalene (DAN), likely from the reaction of ascorbate and DAN. Also shown was decreased NAT signal in the presence of enzyme, likely from DAN association with the protein, further dispelling the possibility of artifactual results for XO/Asc

incubation groups. If the high nitrite seen in XO/Asc groups were the product of Asc-mediated GTN bioactivation alone, the level in the group without XO/with Asc would have been closer in magnitude to the XO/Asc groups.

One question to consider in future research is the similarity of nitrite production with and without XO in the presence of both xanthine and GTN. It is possible xanthine was preferentially metabolized to uric acid, producing hydrogen peroxide and superoxide but not nitrite. Future work could answer this question by determination of either xanthine or uric acid. Similar to the majority of results obtained with ALDH, nitrite production levels seen for all experimental groups except those containing both XO and Asc, is representative of nonspecific GTN bioactivation.

The experiments exemplified in Figure 4 not only represent our first conclusive evidence of enzyme-mediated GTN bioactivation using direct measurement of its product, but also showed increased production in the presence of Asc. But how does Asc accomplish this, mechanistically? Research has shown that XO catalysis in the presence of Asc produces ascorbate and ascborbyl radicals, to the eventual depletion of Asc. Additionally, GTN treatment depletes Asc. The corollary that Asc deficiency blocks vasodilation by GTN has also been shown. Superoxide produced by XO during catalysis reacts with fatty acids, forming lipid peroxidation (LPO) products, like HNE. We have already discussed at length the ALDH metabolism of and inhibition by LPOs, but XO also metabolizes reactive aldehydes; could it also be inhibited by them?

Our findings in conjunction with literature reports lead us to an updated hypothesis: Asc deficiency is a direct partial cause of the development of nitrate tolerance due to a) a lack of superoxide removal, b) competitive XO inhibition toward GTN by aldehydes, and c) could be remediated by Asc repletion.

Under this hypothesis, the increased nitrite produced in XO/Asc could be the result of superoxide quenching by Asc, instead of ·NO. The product of superoxide and ·NO is peroxynitrite. Peroxynitrite cannot react with DAN to form NAT. The thought that Asc repletion could reverse nitrate tolerance contributions relates to oxidative inactivation of XO. Inactive XO can be reactivated, and experimental investigation is required to determine if Asc

can accomplish this. In the case of oxidative inhibition by aldehyde, Asc could regenerate XO from HNE-XO by the formation of ascorbyl-HNE.

Toward elucidation of the mechanism by which Asc increases nitrite production by XO from GTN, method development has begun toward determinations of ascorbate, and its radicals. Literature reports differentiation between Asc oxidative states using diamino derivatization reagents. While two distinct products have been determined by LC-MS/MS, the identity of the ascorbate species derivatized must be determined before additional method development can continue. The advantage of DAN derviatization for these determinations would be their ultimate inclusion in our nitrite determination method, allowing concurrent analysis from GTN incubations.

Toward the elucidation of the clinical relationship between Vitamin C and nitrate tolerance, xanthine oxidase has provided the only evidence of such a relationship on a molecular level. Burgeoning evidence shows nitrate tolerance is a multifactoral phenomenon. It is yet to be determined major and minor contributors, but clarification of the relationship between Vitamin C and nitrate tolerance could be a means to that end. It appears from the research presented here and in Chapter 3, xanthine oxidase is the best candidate on a molecular basis to begin that investigation.

Figures

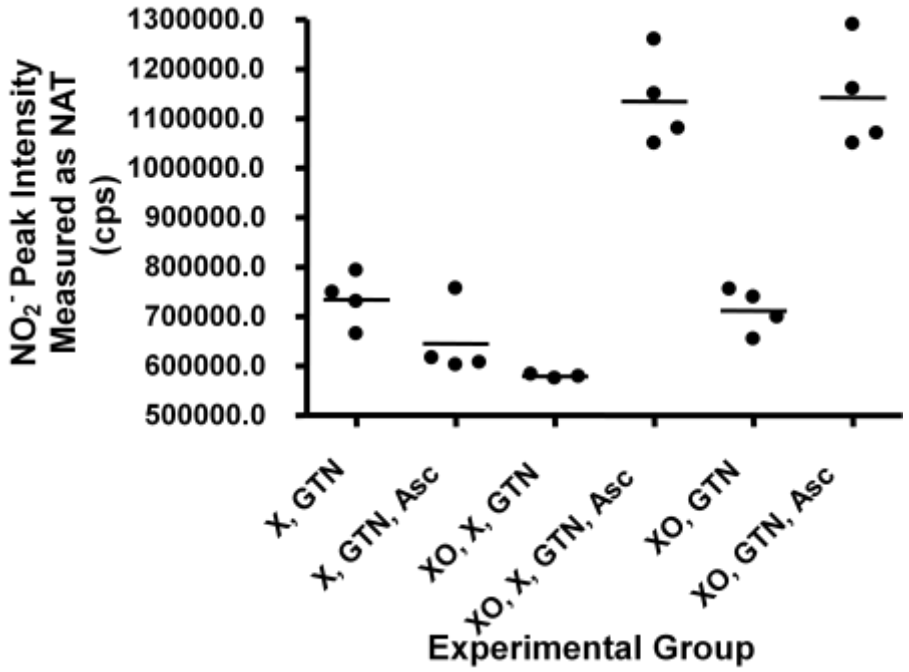


Figure 4.1. XO Reductase Assay. One hundred µg of XO was incubated at 37 °C for 30 minutes at room temperature in 50 mM HEPES buffer, pH 7.4 according to Table 3. Following incubation, samples were derivatized with 10 % v/v 0.5 mg/mL DAN in 0.62 M HCl, 50 % EtOH for one hour prior to quenching with 10 % v/v 1.4 M NaOH, and analyzed by LC-MS/MS. Peaks were integrated for quantification on the 170 → 115 m/z transition for NAT using MultiQuant.

Chapter 5 – Conclusion

This work sought to show Vitamin C, like the small molecule aldehyde dehydrogenase activator Alda-1, is capable of increasing nitroglycerin (GTN) bioactivation, even under conditions of nitrate tolerance. Despite the development of a novel method which allows direct, specific determination of the bioactivation product nitrite, studies with ALDH, widely regarded as the GTN bioactivation enzyme, not only failed to show a Vitamin C effect, but ultimately failed to show any bioactivation by ALDH at all. It is possible that ALDH is just one component of a system required for the bioactivity of nitroglycerin, but without the identification of the other enzymes or cofactors involved, and their subsequent inclusion, there is little utility in continued mechanistic studies with ALDH.

Xanthine oxidase (XO) however, in pure enzyme incubations, not only showed nitroglycerin bioactivation using our nitrite determinations, but also showed increased bioactivation in the presence of Vitamin C. Although XO was more prominent in early nitroglycerin mechanistic research, the repeated identification for a role of ALDH has since overshadowed its potential contribution. The evidence presented here shows a serious need for reconsideration of the evidence and assumptions regarding GTN bioactivation, especially at the intersection with Vitamin C and its deficiency. Although this work should not be interpreted to show XO is responsible for GTN bioactivation and ALDH is not responsible, further work in increasingly complex systems should be performed to elucidate the contributions of these two enzymes, and identify other required components, including relevant cofactors.

Researchers hoped the elucidation of the nitroglycerin bioactivation pathway would help to resolve the problem of nitrate tolerance. Despite long-time wide-spread use of nitroglycerin, the inevitable development of nitrate tolerance limited the patient benefits of nitrate therapy. Although nitrate tolerance is known to be multifactoral, ALDH has been a popular target for intervention as the enzyme thought to be responsible for GTN bioactivation. Through a preponderance of the links between Vitamin C and nitrate tolerance, we sought to describe a relationship between the two at the level of bioactivation. But first, we would need a method.

We developed a method to accurately, selectively and sensitively detect the direct product of nitroglycerin (GTN) bioactivation, nitrite. By using labeled nitrite, our method allows

measurement of the smallest amount of bioactivation, nonspecific bioactivation, as seen in our ALDH incubations. The use of labeled nitroglycerin also allows the unique ability to measure unequivocally the contribution from nitroglycerin directly, and what, if any, indirect contributions the drug may make to the vasodilatory pathway. At this time, the method has been developed to detect nitrite, but there is still some uncertainty nitrite is the bioactivation product, or is always the product. It is the stable oxidation product of nitric oxide, the ultimate vasodilatory species, and thus a great improvement over indirect glyceryl dinitrate determinations. Glyceryl dinitrate determinations are still useful in the study of nitroglycerin bioactivation, however assumptions regarding the meaning of GDN ratios deserves scrutiny with the method developed here.

For further improvements to the method, the inclusion of stable radical reagents could allow even greater insights. Carboxy-PTIO or a similar compound may help to simplify mechanistic elucidation of any ascorbate (Asc) effects, as a radical Asc species may be its mechanistic contribution. Work has begun to incorporate the determinations of various oxidation states of ascorbate using diaminonaphthalene (DAN) into the existing method, but needs additional elucidation of the reactant/product relationships before it will be useful.

Regardless, this work showcases the first use of this method to determine GTN bioactivation. Despite the longtime use of DAN for nitrite determinations, we significantly increased the sensitivity of the method by increasing derivatization time and solubility by the inclusion of organic solvent. This method allows for the first time truly selective direct measurements of GTN bioactivation, when used with stable isotope labeled mass spectrometry. Although the attempted use of unlabeled nitrite as an internal standard failed to improve the results in the presence of interferences such as protein or reducing cofactors, this is only a concern when measuring the extremely low levels of non-specific activation, like those seen with ALDH incubations, and are not seen in experiments with substantial GTN conversion, as seen with XO. We also adapted the published method for GDN determinations, improving reproducibility by decreasing the interference of salt accumulation on mass spectrometric ionization.

Aldehyde dehydrogenase incubations with nitroglycerin repeatedly failed to show any bioactivation, which would have been indicated by increases in the presence of increased enzyme or a reducing cofactor, or decreases in the absence of enzyme and cofactor. Although there was nitrite production, it appears that the levels determined in these incubations are indicative of nonspecific activation. Methods should be developed to measure oxidative inactivation of ALDH to consider possible contribution to nitrate tolerance through inhibited clearance of lipid peroxidation products. The results from isolated enzyme incubations with GTN suggest that perhaps the contribution of ALDH to both nitrate therapy and tolerance may lie with its capacity to regulate oxidative stress, like that seen with ischemia-reperfusion, rather significant reduction of organic nitrates.

Although the results of these incubations could be interpreted to show that ALDH is not the enzyme responsible for GTN bioactivation, more research would be required before such a declaration could be made. Enzyme incubations are the best way to investigate mechanistic questions, but are limited in their comparability to endogenous systems. Most notably for the work described here, there is considerably more oxygen present than would be intracellularly. Since we sought to elucidate a reductive mechanism, the presence of oxygen could have been more problematic than it would have been for other mechanistic studies.

Another major concern for future work to elucidate the mechanism for ALDH reductase activity would be the absolute identification of the reducing cofactor. α-Lipoic acid (αLA), or rather dihydro-αLA (DH-αLA), is one such candidate. α-Lipoic acid has been shown to improve cardiac function during ischemia-reperfusion and reduce HNE adduction, an effect which disappears with ALDH inhibition. (***48***) This particular link does not suggest αLA is the reducing cofactor, rather it suggests a capability to modulate the activity of ALDH. Its structure containing two sulfur atoms suggests a reductive capability and thus potential involvement as a cofactor. Despite our attempt to test DH-αLA, it is known to be extremely difficult to work with and keep reduced, the oxidative state required for the reductase activity we sought to characterize. As currently ALDH reductase studies rely upon dithiothreitol, the dithiol structure of DH-αLA suggests it as the best candidate for ALDH reductase activity.

The reducing power of ascorbate (Asc) may be directly or indirectly involved in GTN bioactivation by ALDH. The mechanism by which Asc activates ALDH oxidase activity has not been elucidated. If Asc is an allosteric modulator of ALDH, similar to NAD^+, the reductase activity could be similarly increased in its presence. (***10***) Whatever the reducing cofactor needed for ALDH reductase activity, Asc may play a role in the cellular redox cycle to that maintains it. It is less likely though, by examination of the results shown here, that Asc could be the reducing cofactor itself. The role of Asc could also be the preservation of the bioactivation product, nitric oxide, from oxidants, or of the enzyme, as suggested with XO. Without a better understanding of the endogenous bioactivation mechanism, the role or roles of Asc will be difficult to determine. The question of whether or not ALDH is the primary bioactivation enzyme, or merely an indispensible part of a larger GTN bioactivation system within the cell should also be explored.

Xanthine oxidase (XO) was once identified as an enzymatic bioactivator of GTN, in addition to S-glutathionyl transferase, cytochrome p450 and others. Aldehyde dehydrogenase became the focus of nitroglycerin research after ALDH knock out model systems and inhibition studies failed to produce vasodilation from GTN. However, our results show clearly XO can bioactivate nitroglycerin. This, coupled with the known nitrite reductase activity of XO supports direct involvement in GTN bioactivation.

Toward an explanation of the evidence which supports an intersection of ascorbate deficiency and nitrate tolerance, XO deserves further consideration. In addition to the significantly increased nitrite comparison to ALDH incubations, GTN bioactivation by XO was increased in the presence of Asc. As mentioned in the discussion of ALDH results however, XO may not be the only cellular component needed for vasodilation from nitrate therapy. Experiments which pointed toward ALDH as the nitroglycerin bioactivation enzyme should be repeated with XO knock out model systems, inhibition and if possible, x-ray crystallography of XO-bound GTN.

In conclusion, this work successfully showed a significant increase in GTN bioactivation in the presence of Asc by XO. Although the impetus for this research was the discovery of Alda-1 and its similar effect on ALDH activity, our research with ALDH was unsuccessful. It is

possible however that our unique detection method for GTN bioactivation contributed to that perceived lack of success by the elimination of analytical assumptions and artifacts. Our work differs from the majority of nitroglycerin research in that we developed a method for the sensitive and selective direct determination of nitrite, the putative product of nitroglycerin bioactivation. Although the method could still be improved toward the determination of radicals with CarboxyPTIO, including those of nitric oxide and ascorbate, it represents a significant improvement over the indirect determinations using GDN, soluble guanylate cyclase and other methods prone to interference, like the use of nitric oxide sensors. This method can be further used in GTN research to provide direct measurements previously unavailable and thus help to answer the questions posed here and elsewhere regarding nitrate therapy and tolerance mechanisms.

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