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Using the Griess colorimetric nitrite assay for measuring aliphatic β -nitroalcohols

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Abstract

Our recent studies suggest that aliphatic β -nitroalcohols (BNAs) may represent a useful class of compounds for topical therapeutic corneoscleral cross-linking agents. Thus, this study was undertaken in order to standardize a simple method for nitroalcohol quantitation based on a denitration step followed by colorimetric Griess nitrite assay. Conditions necessary for denitration included a pH of 7-9 and heating for 1 hour at 100°C. Standard curves for two mono-nitroalcohols (2-nitroethanol and 2-nitro-1-propanol), a nitro-diol (2-methyl-2-nitro-1,3-propanediol), and a nitro-triol (2-hydroxymethyl-2-nitro-1,3-propanediol) showed excellent linearity in the 100-500 M range with absorbance values <1.0 and R^2 values >0.98. The lower limit of detection was ~20 M. Recovery from tissue homogenates (10mg/mL wet weight) of rabbit cornea and sclera as well as solutions of gelatin B (1mg/mL) ranged from 89-103% for scleral tissue, 68-106% for corneal tissue, and 90-99% for gelatin B. The Griess colorimetric nitrite assay can be successfully used for the quantitative determination of BNAs and is simpler to use than conventional chromatographic techniques.

Keywords

nitroalcohols; nitrite; Griess assay; collagen; protein cross-linking; riboflavin photochemistry; cornea; keratoconus; sclera; myopia

Introduction

The ability to selectively enhance the biomechanical properties of a given tissue in vivo could have widespread clinical utility. Our recent studies suggest that aliphatic β -nitro alcohols (BNAs) may represent a useful class of compounds for use as in vivo therapeutic corneoscleral tissue cross-linking agents. Initial studies have shown that BNAs can induce tissue cross-linking effects in both sclera and cornea at physiologic pH (7.4) and body temperature (37°C) [Paik et al 2008, 2009]. In addition, our cell toxicity studies using corneal endothelial cells [Paik et al 2008] and published literature suggests a reasonable safety profile for these agents. Thus, these tissue cross-linking studies have raised the possibility of using BNAs for pharmacologic, therapeutic tissue cross-linking. In particular,

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therapeutic tissue cross-linking with these agents could be applicable to the treatment of destabilizing corneal diseases (i.e. keratoconus and post-LASIK keratectasias) where tissue cross-linking using riboflavin photochemistry has already proven to be efficacious and beneficial [Wollensak 2006, Spoerl et al 2007]. In addition, progressive myopia, a disease of widespread prevalence marked by progressive scleral elongation, is now believed to be potentially treatable through tissue cross-linking of the sclera. Wollensak and Imodina [2008] have recently described the use of a subtenon's injection using glyceraldehyde (a chemical cross-linking agent similar in concept to nitroalcohols) to stiffen the rabbit sclera.

Nitroalcohols have been used extensively in a wide variety of industrial and commercial applications that include their use in cosmetics, rocket fuels, explosives, toilet deodorizers, and plasticizers [Bollmeier 1996]. They have also served as convenient starting compounds as well as chemical intermediates for the synthesis of various classes of organic derivatives [Shvekhgeimer 1998]. The number and scope of industrial applications is vast and includes polymerization chemistry, where nitroalcohols can act as formaldehyde donors to cross-link compounds such as urea, melamine, phenols, resorcinol, etc. in the production of resins, plastics, polyesters, and polyurethane products [Swedo 2003]. In addition, because of their widespread industrial use, the health and safety effects regarding nitroalcohols have been studied extensively, including acute toxicity, teratogenicity, and mutagenicity/carcinogenicity where their profile is quite *favorable* [Bollmeier 1996], and in stark contrast to formaldehyde, whose *unfavorable* toxicity and carcinogenicity profile has been widely publicized [Heck et al 1990].

Quantitative assay of aliphatic β -nitro alcohols (BNAs) has been performed traditionally through the use of chromatographic methods. In particular, gas chromatography (GC) with flame ionization detection (FID) has been used for this purpose [Boneva and Dimov 1982, Dimov and Boneva 1981]. This method, while reliable, requires the availability of a GC unit which may not be practical for the typical research laboratory involved in biomedical research. The use of reversed phase (C18) high pressure liquid chromatography (HPLC) with UV detection has also been reported previously for several of the aliphatic BNAs [Almasy et al 1986]. The detection of bronopol, a brominated BNA with structural similarity to the BNA described in this report, has been measured using various methods that include enzymatic methods [Sanyal et al 1993], thin layer chromatography [Sanyal et al 1996], and HPLC [Challis and Yousaf 1991, Wang et al 2002].

In studies employing ^1H -NMR, we recently confirmed that these BNAs undergo a thermally driven, base catalyzed, retronitroaldol reaction (i.e. reverse Henry reaction) resulting in the formation of an aldehyde which can then react to cross-link tissues. This finding was consistent with previous studies which have underscored the pH and temperature dependency necessary for this reaction to proceed [Challis and Yousaf 1991, Kajimura et al 2008]. Simultaneously, we further noted that a denitration occurs, resulting in the liberation of free nitrite which can be detected colorimetrically [Paik et al 2010]. Denitration of 2-bromonitroethanol (a brominated nitroalcohol similar to those we have studied) to give 2-bromoethanol has been previously noted by Sanyal et al [Sanyal et al 1993] to occur rapidly under alkaline conditions when heated at 100°C. This latter finding (i.e. denitration) prompted us to explore the possibility of using the Griess assay, a simple, well-known, colorimetric nitrite assay as a means to quantitate BNAs in solution.

Thus, the present study was undertaken in order to standardize a simple assay for BNA quantitation in solution and from tissue homogenates using the well known Griess assay. The results indicate that this method can be used to detect BNAs in the concentration range of 100-1000 μM with excellent reproducibility.

Materials and Methods

2-nitroethanol (2ne), 2-nitro-1-propanol (2nprop), 3-nitro-2-pentanol (3n2pent), NaH_2PO_4 , Na_2HPO_4 , sulfanilic acid (SA), N-ethylenediamine hydrochloride (NED), were all obtained from the Sigma-Aldrich Chemical Co (St. Louis, MO). 2-methyl-2-nitro-1,3-propanediol (MNPD) and 2-hydroxymethyl-2-nitro-1,3-propanediol (HNPD) were obtained from TCI America (Portland, OR). Millipore water was used in all the experiments.

Colorimetric nitrite assay protocol (modification of Griess method)

Liberation of free nitrite was monitored using a modification of the colorimetric Griess assay. Briefly, 20 μL of supernatant were applied to a 400 μL well (96-well microtiter plate). Each sample was assayed in triplicate. Fifty microliters of 2N HCl and 50 μL of sulfanilic acid (1mg/mL) were added to the sample and incubated at room temperature for 10 min, followed by 50 μL of N-ethylenediamine hydrochloride (2 mg/mL) and additional incubation for 25 min. The plate was read at 546nm (purple color) on a kinetic microplate spectrophotometer (Benchmark Microplate Reader, Bio-Rad Laboratories, Hercules, CA). A 1 mM stock solution of sodium nitrite was used to create a standard curve by serial dilution and was developed fresh on each day of sampling.

Studies aimed at determining optimal conditions for denitration

Our initial studies were aimed at identifying the optimal conditions (i.e. pH, temperature, and time of heating) for BNA denitration. To this end, we studied three mono-nitroalcohols (2ne, 2nprop, and 3n2pent), a nitrodiol (MNPD), and a nitro-triol (HNPD). For the pH study, the range of pH 2-10 was tested using 0.2M sodium phosphate buffer ($\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ - $\text{pK}_a=2.15, 7.21$). A 1 mM concentration was used in each case. Each sample was heated to 100°C for 60 minutes based on preliminary studies examining the denitration of BNAs under alkaline pH conditions. The heating step was then followed by free nitrite determination using the colorimetric Griess assay. In each case, a minimum of 3 independent determinations were performed. In addition, each sample was assayed in triplicate for a total of 9 values.

The optimal heating temperature and duration of heating was then studied using pH 7.4. The initial pH studies had shown that a neutral or basic pH was optimal for denitration to occur. Thus, the pH of 7.4 was used in the temperature experiments. This pH showed a significantly higher level of denitration as compared with incubation at pH <6 (see Figure 1A). Four different temperatures were studied and included room temperature (25°C), body temperature (37°C), 60°C, and 100°C. In each case a 0.2M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer was used in conjunctions with a BNA concentration of 1mM. The heating time was arbitrarily set at 60min for these experiments and then independently studied as outlined in the next section. In each case, as was done with the pH study, the samples were assayed colorimetrically for liberated nitrite following the heating procedure.

The temperature study was then followed by an evaluation of duration of heating. For this experiment, 100°C was used as a heating temperature since this temperature was found to be optimal for denitration in the temperature study. Using a 0.2M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (pH 7.4), 1mM BNA concentration, and 100°C, heating times of 0, 30, 60, 90, and 120 min were studied in order to determine the duration of time necessary to achieve maximal denitration. As with the previous experiments, the level of liberated nitrite was determined colorimetrically following the heating step.

Creation of standard curves using optimal condition for denitration

From the previous set of experiments using 2ne it was determined that significant denitration could be achieved using a pH of 7-9, 100°C heating temperature, and 120 min duration of heating. Based on these findings, a pH of 7.4 was chosen in order to create standard curves for select BNAs. This pH was chosen for two reasons. First, it was located in a range of pH where optimal denitration was observed to occur. Second, a primary motivation for standardizing this assay has been to use this method for future experimentation with biologic systems, where pH is generally maintained at 7.4. The heating temperature of 100°C and duration of heating of 120 min were used for all experiments related to standard curve creation. Standard curves were created for 4 different BNAs. They were two mono-nitroalcohols (2ne and 2nprop), a nitro-diol (2-methyl-2-nitro-1,3-propanediol), and a nitro-triol (2-hydroxymethyl-2-nitro-1,3-propanediol). 3n2pent was omitted from this analysis due to its poor denitrating capacity at pH 7.4 and its variable tissue cross-linking efficacy (data not shown). In each case, 1mL of solution containing the BNA in a concentration of 10-1000 μ M was heated as described followed by colorimetric nitrite assay. A minimum of 3 independent determinations (i.e. tubes) was made for each data point and each tube was assayed in triplicate.

Recovery studies using collagenous tissue homogenates and gelatin B

In order to determine the recovery of BNAs possible from a tissue preparation, tissue homogenates were produced from corneal and scleral tissues (10mg/mL wet weight). Fresh rabbit globes were obtained from the local abattoir. Wet tissue weights were determined using an analytical balance and the tissues were cut into small pieces and placed into a 50mL conical PTFE centrifuge tube. The corresponding amount of buffer (0.2M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4) was added to the tissues in order to achieve a 10mg/mL preparation. The tissues were then disrupted using a stand mounted handheld homogenizer (Omni International, Kennesaw, GA) for 10 min. One milliliter aliquots of the resulting tissue suspension were placed into 2 mL eppendorf tubes in preparation for the recovery studies. For the gelatin recovery studies, gelatin B in dry powder form (1 and 5 mg/mL) was used to make up a stock solution of proteinaceous solution with the same buffer and heated to 50°C in order to completely solubilize the proteins.

Each tube was then spiked with 100-1000 μ M BNA and the mixture was allowed to incubate for 60 min at room temperature. For the tissue homogenate samples (i.e. tissue suspension), the mixture was centrifuged following the incubation period [12,000rpm (11,270 X g-force)] for 10 min on a microcentrifuge (Eppendorf model 5424). The supernatant was then collected in 100 μ L aliquots in preparation for denitration heating. The sample was then heated at 100°C for 120 min (i.e. the denitration step) as previous described and then assayed for liberated nitrite using the colorimetric assay. For the gelatin B samples, centrifugation was not undertaken since the proteins were solubilized at the time of BNA spiking.

Results and Discussion

Although the colorimetric Greiss nitrite assay was developed many decades ago in 1879 [Ivanov 2004] it has been used extensively in more recent studies related to nitric oxide (NO) biology [Archer 1993, Hevel and Marletta 1994]. Nitrite is an aqueous by-product of NO and accumulates in conditions where NO production is augmented. Thus, it has been used as an indirect measure of NO production. The details of the assay are straightforward. Acidification of nitrite to form nitrous acid allows for diazotization through nitrosation of sulfanilic acid. Subsequent coupling to N-ethylenediamine produces a purple chromophore which is detected at 548nm [Davies et al 2001]. We applied this simple colorimetric nitrite

assay to quantitation of β -nitroalcohols by taking advantage of a denitration that can be accelerated by heating under neutral/alkaline pH.

Studies used to identify optimal conditions for denitration

Necessary conditions for denitration with regard to pH, temperature, and duration of heating were determined. For these studies, a 1mM concentration was used for each compound. These initial experiments were carried out in order to determine conditions necessary for standard curve creation as described below. For pH studies, five different aliphatic BNAs were studied, 2ne, 2nprop, 3n2pent, MNPD, and HNPD.

As shown in figure 1A, 4 of 5 BNAs showed significant elevations in free nitrite beginning at pH 6. In each case, with the exception of 3n2pent, the onset of denitration was observed at pH 6 and was significantly elevated at pH 7. For 2nprop and the nitro-diol, maximal denitration was observed at a pH of 8. For 2ne, the nitro-triol, and 3n2pent, maximal denitration was observed at a pH of 9. 3n2pent showed very little denitration at pH 7 but rose thereafter, becoming maximal at pH 9. The highest level of denitration observed was 20.3% for 2ne, 33.9% for 2nprop, 10.7% for 3n2pent, 28.2% for the nitro-diol, and 22.4% for the nitro-triol. Denitration decreased at pH 10 for all the compounds but was significantly more pronounced for the 2nprop and nitro-diol. Overall, the trends were similar between 2ne and the nitro-triol as well as 2nprop and the nitro-diol. This pH dependency of denitration appears to coincide with the pH dependency for the retro-nitroaldol reaction. We have previously reported that hydrogel formation using these BNAs does not occur below pH 6 [Solomon et al 2010].

The reason for the low levels of denitration and pH shift for 3n2pent is not clear based on the present study but has been observed previously [Paik et al 2010]. 3n2pent contains an ethyl group on the nitro containing carbon as well as an extra methyl group on the hydroxyl containing carbon. The retronitroaldol reaction in this case would produce acetaldehyde as opposed to formaldehyde, which is the expected aldehyde product (i.e. formaldehyde) for the other four BNAs studied. In addition, although increasing pH in the alkaline range resulted in increasing denitration, at pH 10, there was noted to be a decrease in denitration for all five compounds studied (figure 1A). The underlying mechanisms responsible for the lower denitration in these cases is not clear based on the present study but will be the focus of subsequent studies.

Next, in order to determine the optimal necessary temperature to achieve denitration, 4 different temperatures were studied using 5 BNAs at pH 7.4 and 1hr heating time. These experiments were conducted simultaneously with the pH study described above. Thus, the pH of 7.4 was selected based on the finding that denitration was significant at this pH. In addition, developing an assay that functions at pH 7.4 is convenient for studying biological samples. As shown in figure 1B, the level of denitration was temperature dependent, showing little to no effect at 60°C or below and significant elevations at 100°C. From the results of this experiment we determined that heating at 100°C was needed for standard curve formation. It should be noted that in order to obtain consistent results, a more concentrated solution of BNA should be diluted into the working range (i.e. 100-1000uM) *prior* to heating. The heating step is critical in order to achieve maximal denitration. In a similar reaction, denitration of bromonitroethanol (i.e. bronopol) has been previously described by Sanyal et al [15]. In that report, the investigators observed that heating an alkaline solution of bromonitroethanol in a boiling water bath (100°C, duration not specified) resulted in the liberation of nitrite which was detected using the same colorimetric nitrite assay.

The time necessary for heating was also independently studied using 5 BNAs at pH 7.4 and 100°C. As shown in figure 1C, an effect occurred in as little as 30 min. However, heating for up to 120 min resulted in progressively higher amounts of denitration. From this study, we chose the 1 hrs heating time for use in standard curve formation in order to optimize the percent of denitration while providing a logistically reasonable incubation time for experimental purposes.

Similar denitration for 2ne/HNPD and 2nprop/MNPD

As shown in figure 2, the levels of nitrite liberated were equivalent for 2ne and HMPD (triol) but were roughly 10% lower than the other two compounds, 2nprop and MNPD (diol), which were also equivalent to one another. 2ne is the precursor molecule for the nitro-triol, HNPD, and 2nprop is the precursor molecule for the nitro-diol MNPD. In this regard, the free methyl group on the nitro-containing carbon in both 2-nitro-1-propanol and the nitro-diol exhibited a denitration promoting effect. Furthermore, 3-nitro-2-pentanol, which has an ethyl group attached to the nitro-containing carbon and a methyl group attached to the hydroxyl containing carbon, showed significantly less denitration. These findings are also supported by the results of the pH dependency studies (see figure 1A) in which similarities again were noted between 2ne and HMPD (triol) as well as 2nprop and MNPD (diol). The reasons for these differences will require further analysis and experimentation.

Generation of standard curves for 2ne, 2nprop, diol, and triol

Using conditions that were experimentally determined in the preceding section, standard curves were created for 4 different aliphatic BNAs, 2ne, 2nprop, MNPD (nitro-diol), HMPD (nitro-triol). In each case a 10mM NaH₂PO₄/Na₂HPO₄ buffer (pH 7.4) was used and the samples were heated at 100°C for 60 min. As shown in figure 2, a linear increase in the level of denitration was identified for each compound in the defined range (100-500 μM). The lower limit of detection for all of the compounds studied was 20μM (data not shown) at pH 7.4. However, greater sensitivity can likely be achieved for each compound if the heating step were to be conducted at the particular compounds' denitration pH maximum. The linear regression equations for each standard curve showed excellent fit with R²>0.99 for all the curves. The linear fit equations were as follows: Y=(0.000979*X)-0.04335 [R²=0.994] for 2ne, Y=(0.00209*X) -0.0553 [R²=0.984] for 2nprop, Y=(0.00211*X)-0.09319 [R²=0.998] for MNPD, Y=(0.000991*X)-0.02126 [R²=0.990] for HMPD.

Recovery studies using gelatin B and collagenous tissue homogenates

The initial primary reason for standardizing this assay was in order to help us to study transcorneal permeability of BNAs in isolated rabbit corneas. In that setup, a solution of BNA is placed in the upper donor chamber and the lower recipient chamber is serially assayed over the course of two hours in order to determine the flux of the compound through the corneal tissue. The apparent permeability coefficient is then calculated for each compound. The results of that study will be reported separately (manuscript in preparation). Although this assay appears to be well suited for this type of in vitro study where clear solutions are sampled, another foreseeable usage of this assay would be to determine the tissue concentrations achievable following compound application either in vitro or in vivo.

Using gelatin, as a soluble protein surrogate, the recovery of BNAs was very good, with levels consistently greater than 90% (Table 1). We should point out, however, that when using higher concentrations of gelatin (up to 5mg/mL) during the spiking and recovery procedure, the recovery of BNAs dropped to the 50-60% range (data not shown). This observation suggests that the ratio of BNA to protein in a solution can impact the quantitative yield of the assay, a not uncommon occurrence using these types of assays. One

explanation for this difference is that the gelatin serves as a substrate for reaction with the BNAs during the denitration step which involved heating the solution to 100°C for up to 2 hrs. Thus, although our findings indicate that this method should be useful for studying BNAs in proteinaceous solutions, care should be exercised to limit the protein concentration of the fluid being assayed to levels below 1mg/mL or 0.1%.

With regard to the tissue concentrations on scleral and corneal homogenates, the results were more variable although on average (with the exception of the nitrodiol using corneal tissue) the recovery was >85% (see Table 1). The variable result in the case of the tissue homogenate experiments is not fully explained based on the current study. However, one possibility regards differences in protein concentrations between samples. Because fibrillar collagens such as those found in cornea and sclera are poorly soluble in solution, we chose to use a tissue suspension in these studies. However, difficulty in equalizing protein concentrations between samples is a well-known problem that occurs when using such tissue suspensions. Thus, different protein concentrations between samples could have led to variable results.

We attempted to obviate this potential problem by precipitating proteins using trichloroacetic acid (TCA) *prior* to denitration heating. TCA has been used for many years to precipitate proteins. The rationale in this case was to remove the proteins prior to heating and in this way prevent any reaction from occurring between the BNA and the proteins in solution. Several problems, however, arose using this method. First, the addition of TCA, although effective in removing protein, resulted in acidification of our sample, which, as shown in figure 1A, prevents denitration from occurring. We had hoped that simply neutralizing the solution would overcome this issue. However, even poorer recovery occurred with neutralization, leading us to believe that the TCA was interfering with the Griess reaction directly (data not shown). In fact, such interference of the Griess reaction by TCA has been previously reported [Hevel and Marletta 1994]. We next attempted to remove the TCA *prior* to performing the Griess assay using diethyl ether. However, this approach was also unsuccessful since the BNA's are soluble in both water and ether (data not shown). Finally, we tried precipitating proteins with TCA *after* applying the Griess reaction, thinking that sample turbidity could in some way be altering our colorimetric signal. However, this last maneuver was also unsuccessful.

In summary, we have described the use of the colorimetric griess nitrite assay for quantitation of BNAs. This assay can be used as a quick and easy method for studying the concentration of BNAs in solution and/or tissues. However, greater variability and lower quantitative yield occurs when using tissue suspensions. We suggest limiting the protein concentrations of assayed fluids to less than or equal to 1mg/mL or 0.1%. Using this method in applications related to therapeutic corneal and scleral tissue cross-linking provides a useful tool for further developing of these agents for clinical use.

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Highlights for EER

1. Nitroalcohols could be clinically important therapeutic tissue cross-linking agents.
2. Nitroalcohols liberate nitrite during heating at alkaline pH.
3. The Griess colorimetric nitrite assay can be used for quantitation of nitroalcohols.

Figure 1.

Determination of optimal conditions for denitration. **(A) pH:** The percentage of denitration as a function of pH is shown for three mono-nitroalcohols (2ne, 2nprop, 3n2pent), a nitro-diol (MNPd), and a nitro-triol (HNPD). In this case, each sample was heated for 1 hr at 100°C. For 2nprop and the nitro-diol, maximal denitration was observed at a pH of 8. For 2ne, the nitro-triol, and 3n2pent, maximal denitration was observed at a pH of 9. In each case, with the exception of 3n2pent, the onset of denitration was observed at pH 6 and was quite elevated at pH 7. 3n2pent showed very little denitration at pH 7 but rose thereafter, becoming maximal at pH 9. Denitration decreased at pH 10 for all the compounds but was significantly more pronounced for the 2nprop and nitro-diol. **(B) Temperature:** the percentage of denitration as a function of heating temperature is shown for the 5 BNAs (1mM) described. Four heating temperatures are shown; 25, 37, 60, and 100°C. The samples were heated for 1 hour at pH 7.4, and were then assayed colorimetrically for nitrite. A small rise in the percent of denitration was observed in most samples when heating at 60°C, however, the levels were significantly elevated when 100°C was used. 3n2pent was an exception, showing very little denitration even when heated at 100°C. These results supported the findings from the pH studies which indicates that 3n2pent requires a higher pH (i.e. 8) for denitration to occur. **(C) Time of heating:** the percentage of denitration as a function of time of heating is shown for the 5 BNAs (1mM) described. At pH 7.4 and 100°C, denitration occurs as early as 30min and rises steadily up to 120min. Again noted is the similarity between 2ne and the nitro-triol as well as 2nprop and the nitro-diol. In addition, as noted in **(B)** 3n2pent releases very little nitrite under these conditions.

Figure 2.

Standard curves of 4 BNAs are shown, including 2 mono-nitroalcohols, 2ne (A) and 2nprop (B), a nitro-diol (D), and a nitro-triol (C). The curves were created using the Greiss colorimetric nitrite assay following a pre-assay denitration alkaline heating step (pH 7.4, 1hr, 100°C). In a range from 0 to 500uM, the levels of nitrite liberated through neutral heating (pH 7.4, 1hr, 100°C) show a close correlation between 2ne (A) and the nitro-triol (C) as well as between 2nprop (B) and the nitro-diol (D). This likely reflects the structural similarity between these compounds, respectively. The nitro-triol is derived from 2ne and the nitro-diol is derived from 2nprop.

Table 1

Recovery from tissue homogenates for 4 BNAs. All values are percentages (%). Wet weights are given for scleral and corneal tissue and dry weight is given for Gelatin B.

Compound name	recovery sclera (10mg/mL)	recovery cornea (10mg/mL)	recovery gelatin B (1mg/mL)
2-nitroethanol	90.5 +/- 8.4	105.6 +/- 6.5	96.2 +/- 7.3
2-nitro-1-propanol	103.1 +/- 13.5	86.7 +/- 14.0	99.0 +/- 2.7
2-methyl-2-nitro- 1,3-propanediol (MNPD) [nitro-diol]	90.0 +/- 5.2	68.4 +/- 5.4	94.3 +/- 5.1
2-hydroxymethyl-2- nitro-1,3- propanediol (HNPD) [nitro-triol]	88.9 +/- 10.5	94.4 +/- 10.5	90.3 +/- 1.6