Final Report for Collaboratory COIVD-19 research proposal (PI: Schoenfisch)

University of North Carolina at Chapel Hill

Project Title: Nitric oxide-releasing cyclodextrins for treating COVID-19 infections

While nitric oxide's broad-spectrum antibacterial and antiviral activities and ability to prevent clot formation are well known and collectively would benefit treating COVID-19 respiratory infections, there is a scarcity of nitric oxide-based therapeutics due to the challenges associated with delivery into the lungs. For example, delivery of nitric oxide as a gas results in adverse side-effects due to the efficiency with which is crosses into systemic blood circulation. Most chemical nitric oxide donors are characterized by inadequate water solubility, low storage capacity and/or undesirable toxicity. The goal of this project was to develop a new nitric oxide-release platform based on cyclodextrins that would be amenable to nebulization into the lungs to treat COVID-19 respiratory infections. The funding facilitated collaboration between the Schoenfisch lab at UNC-Chapel Hill and Vast Therapeutics, Inc., a preclinical pharmaceutical company in the Research Triangle Park, NC, co-founded by Prof. Schoenfisch, that holds the license to the nitric oxide-releasing cyclodextrin technology, and acceleration of drug development through the initiation of an IND-enabling toxicology program. Below we describe the outcomes of each specific aim of the project. Data and figures are provided in a separate confidential document.

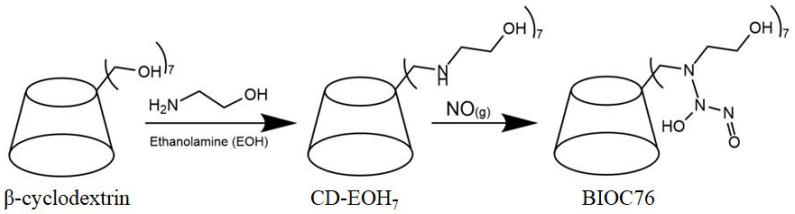
1. Selection and scale-up of a lead drug candidate with acceptable purity for IND-enabling toxicology program

Multiple factors were considered in selecting a lead drug candidate for further development and scale-up of an anti-viral therapeutic against SARS-CoV-2, including 1) theoretical and achievable nitric oxide (NO) loading via *N*-diazeniumdiolate NO donor functional groups; 2) purity of the precursor scaffold; 3) purity of the NO donor-loaded drug candidate; and, 4) the NO-release kinetics of the drug candidate.

To start, both mono- and hepta-substituted β-cyclodextrin (CD) scaffolds were considered. The theoretical NO loadings for all derivatized CDs evaluated varied from approximately 1.6 to 14 μmol NO/mg. To achieve the greatest possible NO doses given that NO and not the biopolymer backbone is responsible for therapeutic action and larger NO doses per mg CD necessitates lower CD levels, our effort focused on developing a hepta-substituted CD candidate capable of accommodating between 7 and 14 NO molecules per CD. Specifically, the following six hepta-substituted CD chemistries were evaluated based on their potential to load NO: ethylenediamine (CD-EDA_7), propylamine (CD-PA_7), hydroxyethyl-ethylenediamine (CD-$HEDA_7$), methoxyethylamine (CD-MA_7), ethanolamine (CD-EOH_7), and propylenediamine (CD-PDA_7). CD-PA_7 and CD-MA_7 were dropped early on due to their limited solubilities in water. CD-$HEDA_7$ was also dropped because even though it has two secondary amines available to react with NO to achieve a high loading, the competing sites significantly complicated the chemistry and array of potential products. The key properties of the three remaining drug candidates are shown in **Slide 1**. In terms of purity, CD-EOH_7/NONO (BIOC76) was selected over CD-EDA_7/NONO and CD-PDA_7/NONO because of its significantly greater NO loading, that inherently translates to greater potential therapeutic activity. A typical chromatogram of BIOC76 is provided on **Slide 2**. The peaks that are present are labeled according to how many *N*-diazeniumdiolate (NONO) functional groups are attached. The single peak at a retention time of 35.5 minutes is the fully loaded compound (7 NONO). Based on FDA precedent, hepta-substituted CDs will be considered complex drug substances in that they form a distribution of closely related compounds rather than a single compound that can be defined by a single molecular weight. While it is theoretically possible to achieve pure forms of these compounds, in practice a distribution of partially reacted regioisomers is produced having a range of loaded *N*-diazeniumdiolate functional groups. An example NO-release profile for BIOC76 at simulated physiological conditions (pH 7.4, 37 °C) shows that BIOC76 does not follow a simple

first-order reaction. The cumulative NO-release curve on **Slide 3** shows a relatively fast NO-release rate within the first half-hour but with sustained release over a 10-hour period. We anticipate such NO release to be ideal clinically in that it will serve as a potent antimicrobial agent initially (with large NO flux) and then maintain sterile-liked conditions for a period thereafter.

The two reactions outlined below show the steps required to derivatize CD to BIOC76. After synthesizing CD-EOH7, the CD-EOH7 is converted to BIOC76 by reaction with high pressures of NO gas in a high-pressure reactor. Once the reaction is complete, BIOC76 is precipitated as a sodium salt, filtered and dried to produce a free-flowing powder for packaging and handling.



We have successfully refined the derivatization of CD to CD-EOH7 to enable scale up suitable for preclinical and clinical studies (~2.1 kilograms of CD-EOH7 per batch). To date, six 2.1-kilogram batches have been successfully manufactured. Details of these batches are provided in the table on **Slide 4**. In parallel with the scale-up of the CD-EOH7 intermediate, the reaction to convert CD-EOH7 to BIOC76 (the NO loaded form) was successfully scaled from ~0.25 kilogram/batch to ~2.5 kilogram/batch. A total of two 0.73-kilogram batches, one 1.93-kilogram batch and one 2.4-kilogram batch have been completed. Batch details (e.g., yields and purity) are summarized in the table on **Slide 5.**

2. Development of an appropriate formulation for delivering drug

To match current standard of care therapies, BIOC76 is being developed as an inhalation solution (i.e., formulation designed to be delivered via a nebulizer). An overview of the finished product is provided in **Slide 6**. Inhalation solutions must have the following attributes to effectively deliver drug to a patient's lungs: API needs to be soluble in the solution vehicle at concentrations relevant to provide the intended dose; the droplet size distribution of the nebulizer aerosol needs to match the size requirements to achieve targeted lung delivery; the pH of the solution must be controlled; the tonicity of the formulation must be controlled; any excipients used need to be qualified for inhalation delivery; and the formulation must be stable with regard to the nebulization process and stable with regard to long term storage.

BIOC76 carries a net overall negative charge at neutral pH which, in combination with its sugar backbone, permits extraordinarily high solubility (>500 mg/mL). BIOC76 formulations with concentrations ranging between 100 and 200 mg/mL are being evaluated for carrying out the IND-enabling toxicology. The finished drug product is intended to be supplied as a lyophilized powder (BIOC76) with a matched diluent (citrate based) that will be used to reconstitute the lyophilized powder at the time of use. A preliminary lyophilization process has been developed for BIOC76 and samples placed on stability. Of note, both sodium citrate and citric acid are qualified for use in approved inhalation products.

The final drug product is being developed to work with Pari's e-Flow nebulizer system (www.pari.com). Formulations are being evaluated as a function of concentration to ensure they will produce aerosols with particle size distributions (PSDs) that have mass median aerodynamic diameters (MMADs) in the 3-5 µm range. The top table on **Slide 7** lists the droplet size distributions of a nebulized BIOC76 formulation via a Pari e-Flow nebulizer. The plot and detailed table on the bottom of **Slide 7** show a graphical representation of the droplet size distribution and the detailed data set from which the data for the 175 mg/mL solution in

the top table were derived. While Dv50 is not strictly equivalent to the MMAD, in this application Dv50 is a good approximation of the aerosol's MMAD.

3. In vitro antimicrobial (antiviral and antibacterial) testing (both because drug has multiple mechanisms of action that may benefit COVID-19 infections beyond antiviral activity alone)

Nitric oxide was previously reported to have antiviral activity against SARS-CoV ("SARS-CoV-1") in tissue culture assays *in vitro* using both low molecular weight NO donors and pure gas (see proposal for references). Precedent for the effectiveness of NO as an antiviral led to the initiation of several clinical trials to evaluate the efficacy of gaseous nitric oxide delivery to SARS-CoV-2 infected patients experiencing symptoms associated with COVID-19. As gas phase delivery of NO is difficult to regulate and may result in severe adverse effects such as methemoglobinemia, our goal was to evaluate the antiviral activity of BIOC76, a proprietary NO-releasing cyclodextrin that facilitates spontaneous delivery of NO directly into the solution phase from a non-toxic, highly water-soluble cyclic oligosaccharide consisting of a macrocyclic ring of glucose subunits.

Upon selecting BIOC76 as our lead compound, we evaluated its antiviral activity against wild-type SARS-CoV-2 at 24- and 48-hours post-infection (one-time treatment at 1 hour following initiation of infection). This work was carried out at MRIGlobal, an independent CRO. Specifically, Vero E6 cells were infected with SARS-CoV-2 at a MOI 0.01 for 1 hour prior to a single treatment with a 2-fold dilution series of BIOC76 or the biopolymer scaffold control (CD-EOH7) without NO attached. At 24 hours post-infection, BIOC76 effectively reduced SARS-CoV-2 ~2 logs at 0.25 mg/ml, >5 logs at 0.5 mg/ml, and to the limit of detection at 1 mg/ml (**Slide 8**). Of note, the CD-EOH7 control did not reduce SARS-CoV-2 at these doses and no loss in cell viability was observed (**Slide 9**). The impact of BIOC76 is most pronounced at 24 hours post-infection since SARS-CoV-2 replication was fully recovered by 48 hours post-infection at 0.25 and 0.5 mg/ml. This result is an indication that the best window to evaluate the antiviral activity of NO donor compounds is shortly after treatment due to the rapid release of NO and NO's short half-life. An approximation of the BIOC76 IC_{50} at 24 hours post-infection was 0.2 mg/ml, resulting in an SI_{50} >10. These data demonstrate that BIOC76 is effective against SARS-CoV-2 *in vitro*.

Conventional SARS-CoV-2 directed antiviral drug therapeutics, such as Remdesivir, have specific mechanisms that target a virus or family of viruses. Viruses such as SARS-CoV-2 may develop resistance to these therapeutics. Nitric oxide donors may impact numerous steps of the virus replication cycle, thereby reducing the likelihood of a virus developing resistance through a single mutation. Moreover, NO donors may have antiviral utility that extend beyond a single family of viruses. Along these lines, we also demonstrated that BIOC76 inhibits replication of influenza A H1N1 (SI_{50} = 8.6), influenza B (SI_{50} = 4.3), human coronavirus OC43 (SI_{50} = 3.1), and human rhinovirus 14 (SI_{50} = 8.9) (**Slide 10**). Overall, our in vitro studies indicate that BIOC76 exhibits antiviral activity against several viruses, including wild-type isolates of SARS-CoV-2.

The antibacterial activity of BIOC76 was also evaluated against common respiratory pathogens, including *Pseudomonas aeruginosa*, both methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) *Staphylococus aureus*, *Burkholderia cenocepacia*, and *Mycobacterium abscessus*. Antibacterial activity was evaluated in vitro by determining the Minimum Inhibitory Concentration (MIC) or concentration of drug required to inhibit bacterial growth. The results indicate that BIOC76 is broad-spectrum and inhibits all species tested (**Slide 11**). We have also begun to investigate the synergy of co-delivering NO-releasing cyclodextrins with conventional antibiotics, particularly for treating antibiotic-resistant infections. Our results indicate that co-

delivery of NO with a conventional antibiotic such as tobramycin or antibiotic alternatives such as gallium is highly synergistic (data not shown).

Respiratory pathogens, especially *P. aeruginosa*, protect themselves by producing thick mucus-like biofilms that are difficult to penetrate by conventional antibiotics or overcome by the native immune response. We thus also evaluated the ability of BIOC76 to eradicate bacteria growing within biofilms. The data indicate that BIOC76 is indeed capable of eradicating *P. aeruginosa* in biofilms, further demonstrating the broader antimicrobial potential of NO-therapies for treating chronic respiratory infections.

4. Bioanalytical method development (to enable tracking in plasma of drug, the NO that is released, and elements of the formulation)

Animal studies to confirm the safety of BIOC76 are required by the FDA prior to initiating human trials. During such in vivo studies, the toxicokinetic and pharmacokinetic effects of the drug product are studied by measuring the concentration of API and related biomarkers in blood plasma and tissue, and relating such data to observed pathology. The robust analytical methods required for these correlations are specific to the formulation being studied and bioanalytical methods must be developed for drug product and byproducts.

It is understood that NO, or drugs using NO to elicit a pharmaceutical effect, can also lead to unintended byproducts and side effects. Nitrate is a useful byproduct formed upon the oxidation of NO in vivo. We have initiated development of methods to quantify nitrate in both rat and dog plasma to support our IND-enabling toxicology program (two species are required by the FDA). This scope of work including validation was carried out at AIT Biosciences, Inc. Briefly, the method relies on conversion of nitrate anion to 3-nitro-4-ethoxybenzoic acid and monitoring the derivatized product via LC-MS/MS (**Slide 12**). The method has a validated range of 500 – 50,000 ng/mL. Due to endogenous nitrate in blood plasma being greater than the lower limit of detection, the calibration curve is created using an isotopically labeled nitrate ($^{15}N0_3^-$) surrogate and a third nitrate isotope, $^{15}N^{18}O_3^-$ as an internal standard.

To track potential pharmacokinetic and toxicokinetic effects of BIOC76 dosing in animals, a bioanalytical method for BIOC76 in plasma is required. As described above, BIOC76 is a complex mixture of NO-releasing chemical species and isomers. Those species release NO and degrade rapidly at physiological temperature and pH to an even wider variety of end-products. Measurement of NO-loaded BIOC76 directly is not viable given the half-life of BIOC76. Likewise, quantifying a specific degradant is also difficult due to their low abundance and the difficulty in synthesizing reference materials for accurate quantitation. The most promising bioanalytical method involves targeting the glucose-EOH monomer derived from fragmentation of the BIOC76 backbone in the mass spectrometer. This fragment is an appealing analytical target because some of the monomeric units remain unadulterated even though many of the monomers of the seven-member ring structure are likely modified in vivo, precluding isolation of any one particular species. By breaking down the ring structure, only one glucose-ethanolamine subunit is required to facilitate BIOC76 molecule detection. The glucose-ethanolamine subunit is both unique to BIOC76 and chemically distinct from other biological sugars or small molecules. A linear response from 0.5 to 50 ppm has been established (**Slide 13**). AIT-Biosciences, Inc. also validated this method for use to analyze plasma collected from animals in the maximum tolerated dose and 7-day repeat dosing toxicology studies (data summarized below).

5. Characterization and stability testing of both drug substance and product.

Several studies were carried out to better characterize the chemical characteristics and stability of both the drug substance and the final drug product. PPD was contracted to develop and implement GLP test

methods to characterize the BIOC76 bulk powder and diluent formulation. As summarized on **Slide 14**, the bulk powder is tested for Appearance, Identification, Assay, Related Substances, Nitric Oxide Loading, Sodium Content, Water Content, Residual Solvents, and Bioburden. Diluent is tested for pH, Osmolality, Foreign Particulate Matter, and Sterility. All tests are in accordance with the FDA's Guidance for Industry - *Nasal Spray and Inhalation Solution, Suspension, and Spray Drug Products – Chemistry, Manufacturing, and Controls Documentation*, July 2002. All of the drug substance tests have been developed and used to release test BIOC76 Batch VT-20-099. Phase-appropriate validation of these test methods is underway and expected to be completed in October to support GLP toxicology studies.

As previously described, BIOC76 is considered a complex API comprised of multiple NO-loaded species and isomers. LC-MS analysis of the product mixture confirms the identity of the cyclodextrin species. Their retention times and abundance are in line with expectations derived from knowledge of the reaction mechanism (**Slide 15)**. Initial studies on the stability of BIOC76 drug substance and the formulation for nebulization have been completed. As shown on **Slide 16**, it has been demonstrated that BIOC76 in the powder form is stable at both frozen and refrigerated conditions for at least 3 months (**Slide 17**). Additionally, room temperature stability of the powder has been shown out to 7 days, ideal for the intended nebulization application.

Lyophilization Technology, Inc. was engaged to determine whether a lyophilization process could be developed to further enhance stability. In this scope of work, the physicochemical behavior of BIOC76 at low temperatures was characterized by electrical resistance, observations under a freeze-drying microscope, and differential scanning calorimetry (DSC). The phase transition temperature acquired from a resistance graph suggests that cooling to temperatures of at least -38 °C would be required for achieving solidification during freezing. Observations under a microscope indicated cooling to below temperatures of at least -49 °C would be required for achieving solidification. Therefore, it was recommended that the material be cooled to temperatures below -49 °C to achieve full solidification. The electrical resistance graph noted a minor change at -55 °C with a full change at -36 °C during warming. DSC indicated a glass transition at an onset of -27 °C. Observations under the microscope revealed void formation and collapse at -29 °C. Process conditions for primary drying should provide a margin of safety. Therefore, product temperatures should be maintained at or below a range of -31 °C to -33 °C for complete drying with retention of the structure formed during freezing and the absence of collapse. The quality of the product was evaluated by HPLC (**Slide 18**) and nitric oxide analyses to confirm that drying above the initial phase transition identified by electrical resistance was not detrimental to the product.

When BIOC76 is dissolved in water, the pH is substantially basic (pH > 10). The compound is stable under these conditions for at least 3 days when refrigerated (**Slide 19**). Before nebulization, the pH is adjusted to be more neutral (7.6-8.0) to trigger NO release. This phenomenon is observed as discrete changes to the HPLC chromatogram. Specifically, Peak J, which is the fully loaded (7 NONO) product, begins to decrease at neutral pH while Peak F, the 6 NONO form, increases by the same degree over the course of 6 hours. This expected instability is exacerbated with lower pH and/or elevated (physiological) temperature. As such, it will be important when dosing BIOC76 to keep all formulations cold and nebulize quickly (within 60 minutes) after formulation.

6. Maximum tolerated dose studies in rodents and canines to support 7-day repeat dosing study.

Covance (Princeton, NJ) was engaged to carry out an Investigational New Drug (IND)-enabling toxicology program using nebulized BIOC76 formulations. Such a program requires testing in two species. Rats and canines are the two most common species for testing inhalation drugs, in part due to cost and ease of

handling during experimentation. Prior to repeat dosing, technical trials are executed to ensure feasibility of delivering intended doses (using proven analytical methods). Following confirmation, dosing of drug is ramped (i.e., increased) to determine a maximum tolerated dose or dose where adverse effects are not observed. For BIOC76, the maximum tolerated doses for rodents and canines were quite different, 1046 mg/kg and 60 mg/kg, respectively (**Slide 20**). Such disparity was surprising as it is not commonly observed in inhalation dosing studies. In fact, no adverse side-effects were observed in rodents even at ~1000 mg/kg. However, vomiting and tremors following dosing were noted in canines above 100 mg/kg.

7. 7-day repeat dosing study for safety assessment and determination of acceptable dose for human clinical trials.

Based on the maximum tolerated doses, five groups including one blank, one control (CD-EOH7) vehicle (not capable of NO release) at the highest BIOC76 dose, and three doses of BIOC76 (low, medium and high) were selected for the 7-day repeat dosing study. Based on the differences in maximum tolerated doses, the rodents and canines received different BIOC76 low, medium, and high doses (**Slide 20**). Both rodents and dogs given medium and medium to high doses of BIOC76, respectively, showed adverse effects by day 3, with the highest doses requiring rest days or revision to a lower dose. These results were unexpected given our hypothesis and prior preclinical data (NTM study) indicating that cyclodextrins are relatively inert. The largest rodent and dog doses that did not result in adverse qualitative assessments in the 7-day repeat study were 600 and 30 mg/kg, respectively. Following termination and organ harvesting, histopathology analysis in the canine groups indicated moderate to marked inflammation of lungs, nodes, and lymph nodes (**Slide 21**). The control group (CD-EOH7) at the highest BIOC76 dose also led to slight inflammation. We are still awaiting completion of tissue histopathology from the rodent subject group, prior to drawing conclusions.

Bioanalytical testing of plasma collected during the studies at discrete time intervals indicated BIOC76 (and the control, CD-EOH7) entered the blood stream either through the lungs or via the stomach if swallowed. As shown in **Slide 22**, no glucose ethanolamine monomer (target) was observed in Group 1 (saline only) samples, because these samples are blanks (containing no glucose ethanol amine monomer fragments). Control Group 2 (CD-EOH7 at the same concentration as high dose BIOC76 (Group 5)) showed signal due to the presence of the glucose ethanol amine monomer), as would be expected. Finally, a dose response was observed for Groups 3-5 (low, medium, and high doses of BIOC76) spiking at roughly 1 hour post treatment, then tails away over the next 24 h. Nitrite trends were similar to those for BIOC76 (**Slide 23**), with both providing plasma half-lives of roughly 2 hours before ultimately being degraded and/or cleared from the body. Of note, bioanalytical testing of blood samples from the 7-day repeat testing from dogs is still underway.

Lead candidate selection

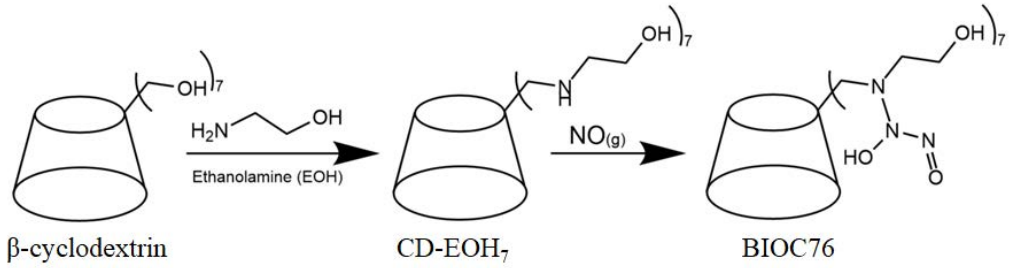
Goal: To identify most suitable NO-releasing cyclodextrin macromolecules for scale-up and anti-viral testing based on synthesis and purity of the precursor, total NO payloads, purity of drug product, and solubility in aqueous solutions.

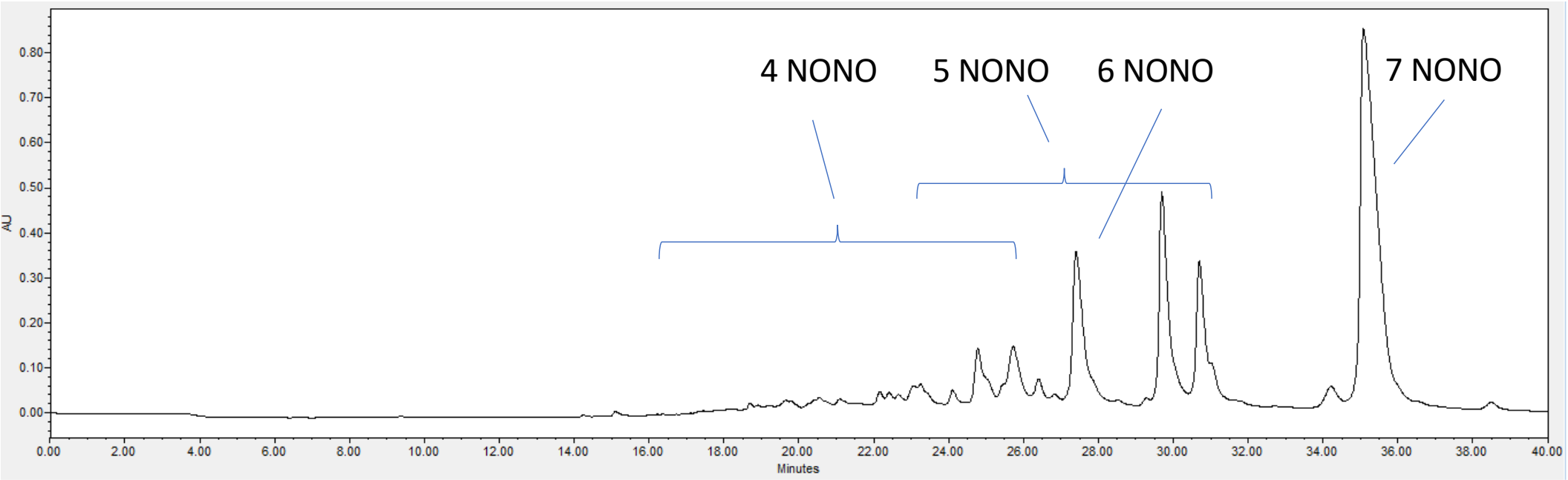
Sample	NO Loading (µmol/mg)[1]	NO Released (µmol/mg)[2]	Scaffold Purity (Area %)	Drug Purity (Area %)	$t_{1/2}$ (h)
BIOC72 (CD-EDA_7)	<3	2.0	60	<20	1.5
BIOC76 (CD-EOH_7)	>5	4.7	86	>90	1.0
BIOC77 (CD-PDA_7)	<3	2.1	72	<50	0.9

[1] as determined by HPLC.
[2] as determined using Chemiluminescent Nitric Oxide Analyzer.

Lead candidate selection: NO loading, purity

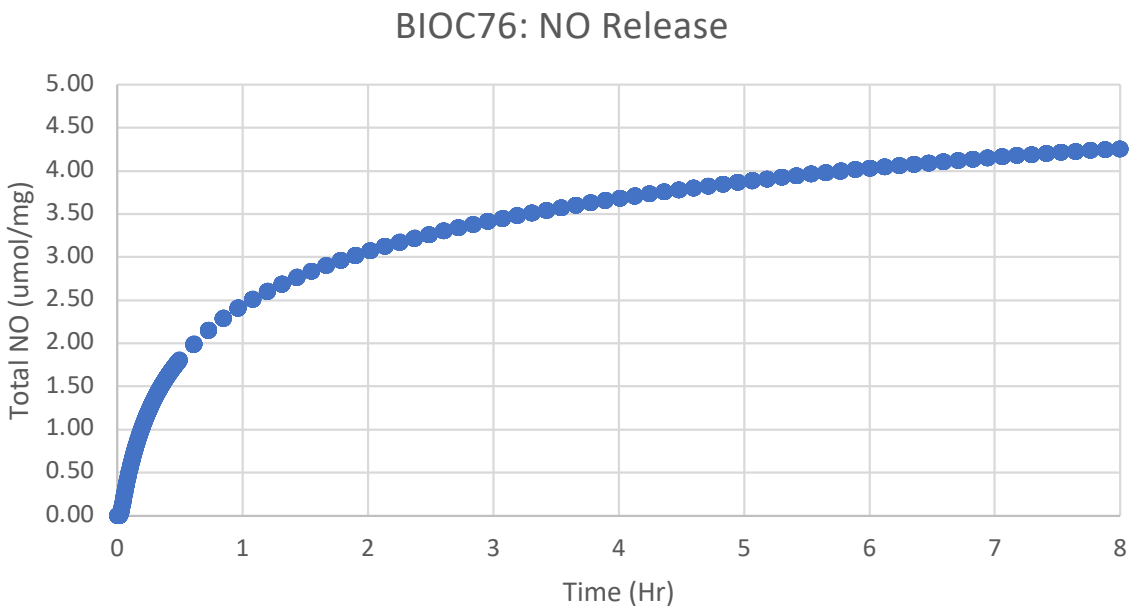
BIOC76 purity (note ability to resolve fully loaded NO donor consisting of two moles of NO at each of the 7 sites)





High Performance Liquid Chromatography (HPLC)

Lead candidate selection: NO Release



Scale up: CD-EOH_7

Goal: To enable manufacturing of both drug precursor (CD-EOH7) and drug (BIOC76) at kilogram scale to facilitate IND-enabling toxicology study.

Lot	Scale (g)	Recovery (g)	Yield (%)	Purity (area %)
494PAL14A	2580	2160	92	95.7
494PAL15A	2631	2160	92	93.1
494PAL16A	2600	2157	91	94.5
494PAL17A	2600	2115	91	90.2
494PAL18A	2575	Underway	Underway	Underway
494PAL19A	2580	Underway	Underway	Underway

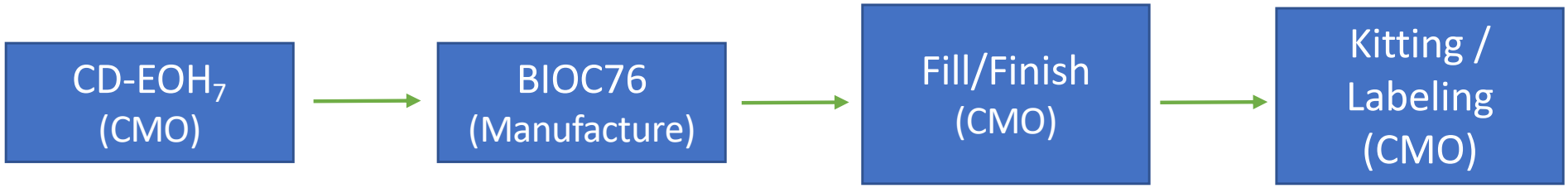
Scale up: BIOC76

Lot	Scale (g)	Recovery (g)	Yield (%)	Purity[2] (area %)
VT-20-094	730	637	87	98.1
VT-20-096[1]	732	440	60	85.4
VT-20-099	1934	1707	88	97.3
VT-20-108	2393	2155	90	95.1

[1] The low yield was due to a lower amount of base being used and a correspondingly slower reaction rate

[2] Combined peak area of all peaks assigned to have 5 or more *N*-diazeniumdiolate functional groups

Finished product overview

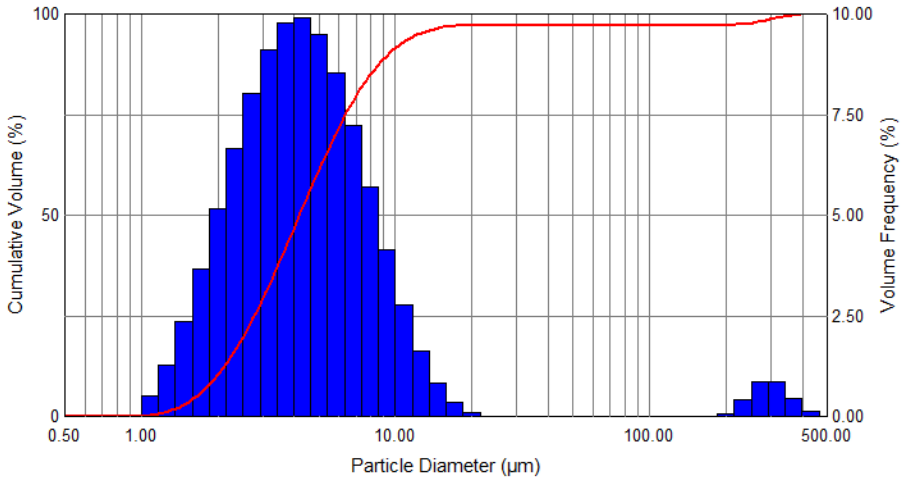


BIOC76 is being formulated as an inhalation solution product (delivered via a nebulizer)

- BIOC76 will be dissolved in water for injection, sterile filtered, filled into a glass vial, and lyophilized. Lyophilized BIOC76 will be reconstituted at time of use with diluent.
- Sodium citrate and citric acid will be used to formulate an appropriate diluent to achieve an appropriate pH when combined with BIOC76. The formulated diluent will be sterile filtered, filled into a glass vial, and capped.
- The lyophilized powder and diluent vials will be kitted together to form the finished drug product.

Droplet size distributions of nebulized formulations

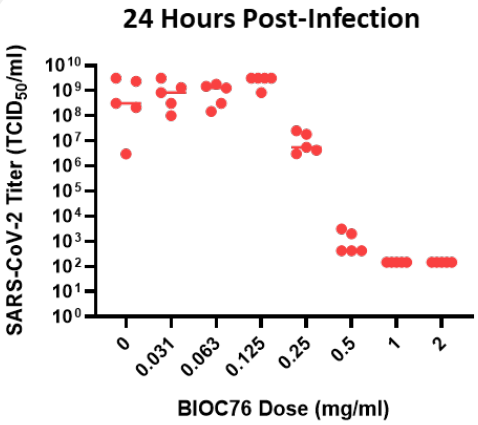
Formulation	Dv10 (µm)	Dv50 (µm)	Dv90 (µm)
BIOC76, 175 mg/mL	1.97	4.15	9.09
BIOC76, 187 mg/mL	2.13	4.34	9.39
BIOC76, 200 mg/mL	1.57	3.17	7.02

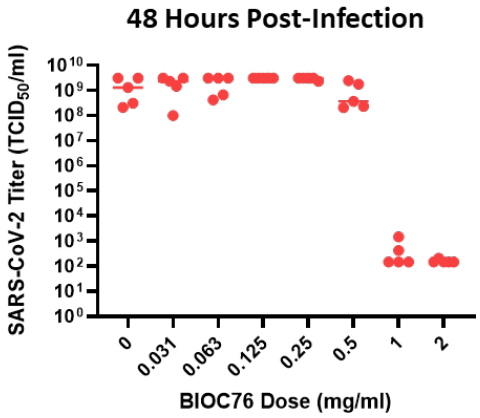


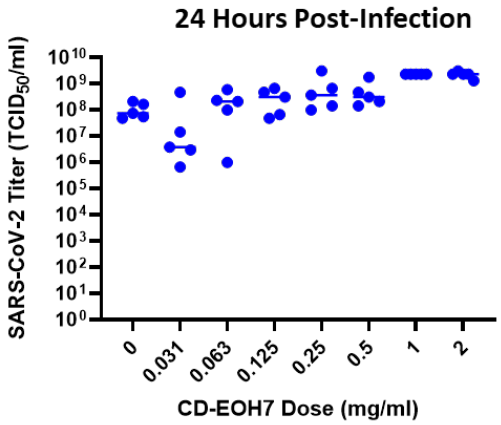
Particle Diameter (µm)

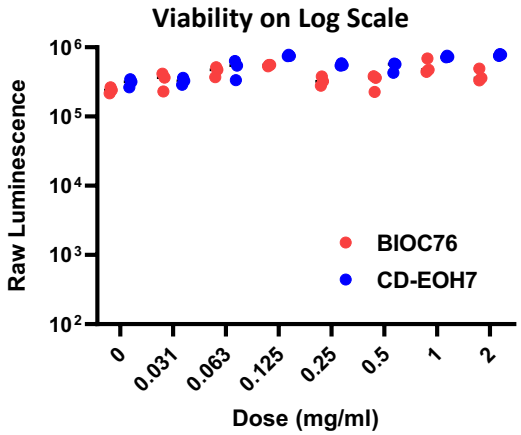
Size (µm)	% V <	% V	Size (µm)	% V <	% V	Size (µm)	% V <	% V
0.117	0.00	0.00	2.51	19.67	6.65	54.12	97.21	0.00
0.136	0.00	0.00	2.93	27.69	8.03	63.10	97.21	0.00
0.158	0.00	0.00	3.41	36.81	9.12	73.56	97.21	0.00
0.185	0.00	0.00	3.98	46.59	9.78	85.77	97.21	0.00
0.215	0.00	0.00	4.64	56.49	9.91	100.00	97.21	0.00
0.251	0.00	0.00	5.41	65.97	9.48	116.59	97.21	0.00
0.293	0.00	0.00	6.31	74.51	8.54	135.94	97.21	0.00
0.341	0.00	0.00	7.36	81.73	7.22	158.49	97.21	0.00
0.398	0.00	0.00	8.58	87.42	5.69	184.79	97.21	0.00
0.464	0.00	0.00	10.00	91.56	4.14	215.44	97.29	0.07
0.541	0.00	0.00	11.66	94.32	2.76	251.19	97.69	0.40
0.631	0.00	0.00	13.59	95.96	1.64	292.87	98.56	0.87
0.736	0.00	0.00	15.85	96.79	0.83	341.46	99.43	0.87
0.858	0.00	0.00	18.48	97.13	0.34	398.11	99.87	0.44
1.00	0.04	0.04	21.54	97.21	0.08	464.16	99.98	0.12
1.17	0.55	0.51	25.12	97.21	0.00	541.17	100.00	0.01
1.36	1.84	1.29	29.29	97.21	0.00	630.96	100.00	0.00
1.58	4.20	2.36	34.15	97.21	0.00	735.64	100.00	0.00
1.85	7.87	3.67	39.81	97.21	0.00	857.70	100.00	0.00
2.15	13.02	5.15	46.42	97.21	0.00	1000.00	100.00	0.00

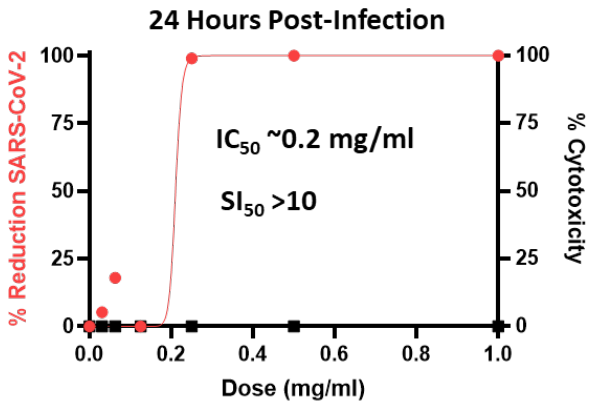
SARS-CoV-2 antiviral activity[1]





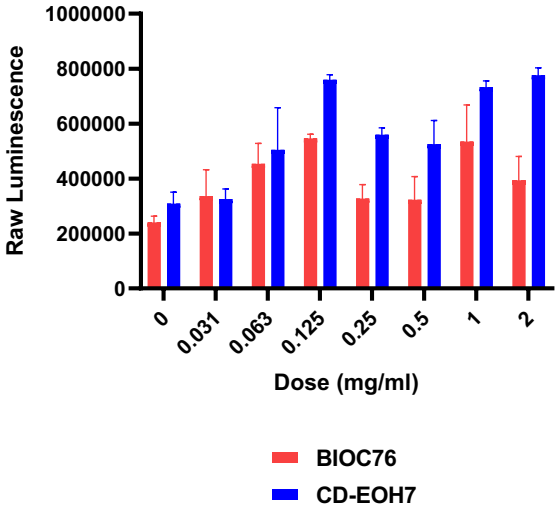


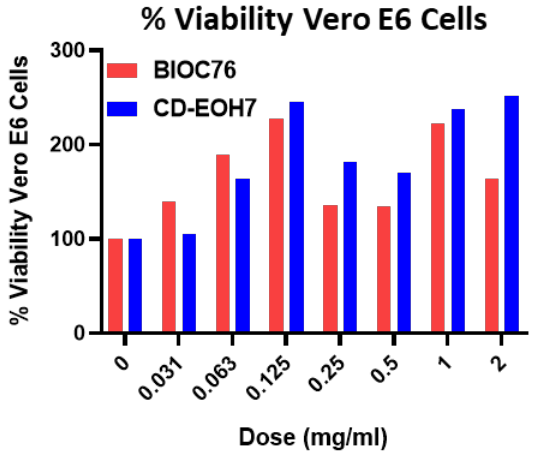




- [1] Independent testing carried out at MRIGlobal.
- The antiviral (SARS-CoV-2) activity of BIOC76 is the result of the nitric oxide and not the cyclodextrin (CD-EOH7).
- No cellular toxicity due to drug was observed even at the highest dose evaluated (2 mg/ml); therefore, % cytotoxicity was set at 0%. As such, the calculated SI_{50} is at least >10.
- With a one-time treatment, the antiviral effect of NO is readily detected at 24-h post-infection, but lessens at 48 h due to NO's limited lifetime (helpful in avoiding adverse side-effects) and the finite duration of NO release (<10 h). Clinically, we envision once or twice daily treatments.

Anti-viral assay toxicity data





- Assay run at MRIGlobal, Inc.
- Values set at 0% cytotoxicity because there was no decrease in raw luminescence values, and thus no decrease in viability according to this assay.

Broader antiviral activity testing

Virus	Compound	Assay	EC_{50}	CC_{50}	SI_{50}
Influenza A H1N1 California/07/2009	BIOC76	Neutral Red (CPE/Tox.)	290 µg/ml	2500 µg/ml	8.6
Influenza A H1N1 California/07/2009	CD-EOH7	Neutral Red (CPE/Tox.)	3400 µg/ml	>10,000 µg/ml	>2.9
Influenza B Florida/4/2006	BIOC76	Neutral Red (CPE/Tox.)	650 µg/ml	2800 µg/ml	4.3
Influenza B Florida/4/2006	CD-EOH7	Neutral Red (CPE/Tox.)	>3500 µg/ml	3500 µg/ml	0
Human Coronavirus OC43	BIOC76	Neutral Red (CPE/Tox.)	98 µg/ml	300 µg/ml	3.1
Human Coronavirus OC43	CD-EOH7	Neutral Red (CPE/Tox.)	2900 µg/ml	3800 µg/ml	1.3
Human Rhinovirus 14	BIOC76	Neutral Red (CPE/Tox.)	360 µg/ml	3200 µg/ml	8.9
Human Rhinovirus 14	CD-EOH7	Neutral Red (CPE/Tox.)	>3200 µg/ml	3200 µg/ml	0

- BIOC76 exhibits selective antiviral activity against Influenza A and B; and, human coronavirus OC43 and human rhinovirus 14, which are associated with the common cold.

Antibacterial activity

Goal: To determine the antibacterial efficacy of BIOC76 using in vitro bacterial cultures and bacterial biofilms.

Minimum Inhibitory Concentration (MIC) = lowest concentration that inhibits growth.

	MIC	
Species	BIOC76 (mg/mL)	NO payload (μg/mL)
P. aeruginosa	2	142
S. aureus (MSSA)	4	284
S. aureus (MRSA)	2	142
B. cenocepacia	0.5	71
M. abscessus	4	284

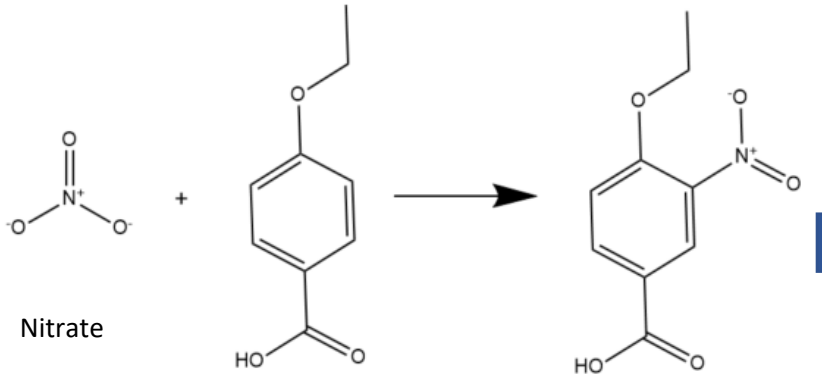
- BIOC76 inhibits 4 major respiratory pathogens, including methicillin-sensitive and methicillin-resistant strains (broad-spectrum antibacterial activity confirmed).

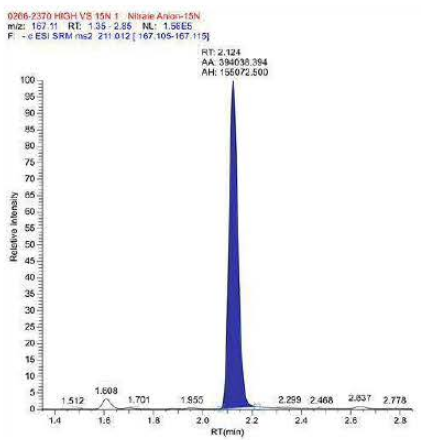
- Respiratory pathogens protect themselves by producing thick mucus-like biofilms that are difficult for immune cells and conventional antibiotics to penetrate. We thus also evaluated the ability of BIOC76 to eradicate *P. aeruginosa* biofilms. Data (not shown) indicate that BIOC76 is capable of eradicating bacteria within biofilms.

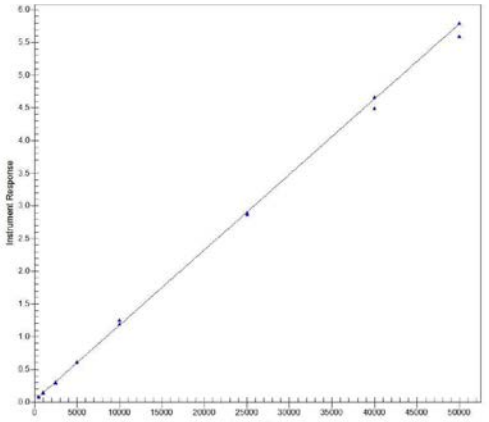
Bioanalytical method development

Goal: To enable assessment of pharmacodynamic effects resulting from BIOC76 dosing.

Nitrate: Plasma nitrate concentrations are determined by derivatizing NO_3^- per the scheme below and analyzing for the product via LC-MS/MS. The assay has a validated linear range from 0.5 to 50 ppm





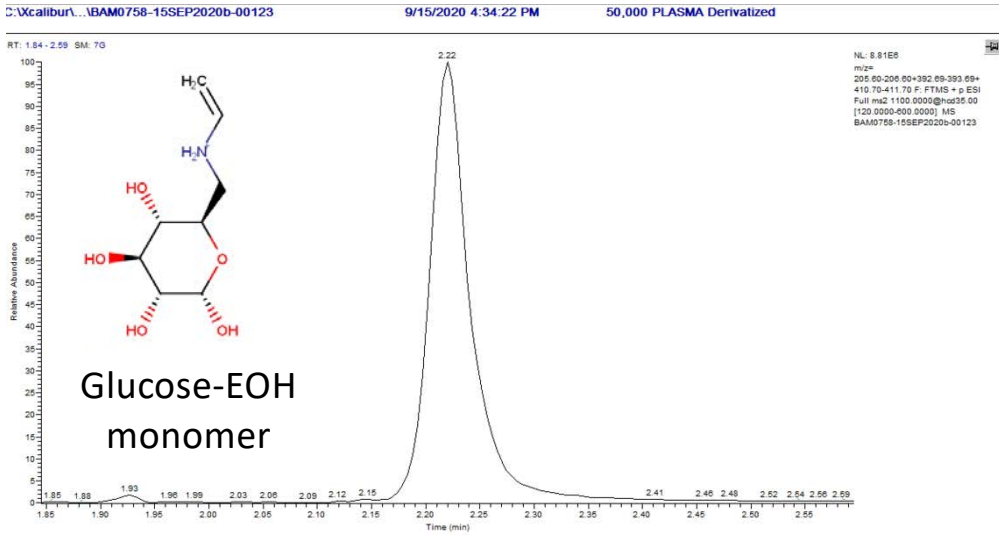


Analyte response is selective and linear from 0.5 to 50 ppm

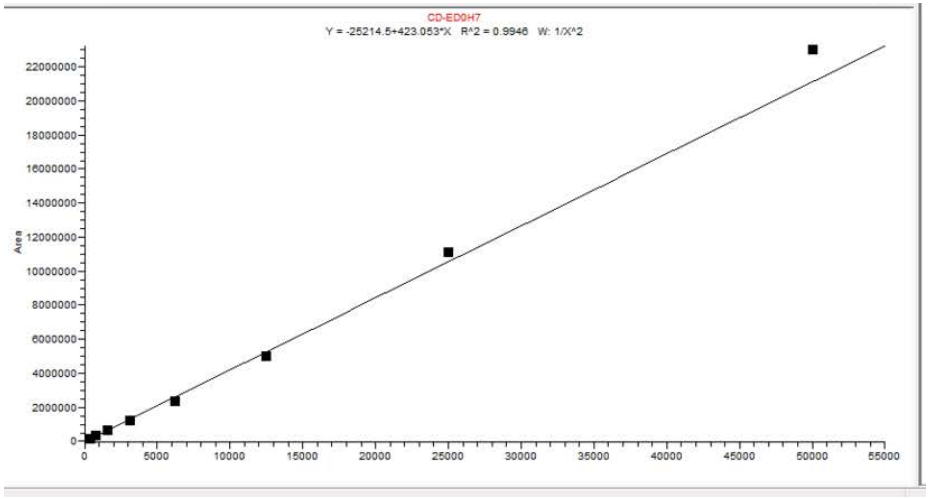
Bioanalytical method development

Goal: To enable pharmacokinetic and toxicokinetic effects of BIOC76 dosing.

BIOC76: After protein precipitation, drug-related products are quantified in plasma by measuring the concentration of glucose-ethanolamine (glucose-EOH); the NO-liberated monomer fragment from BIOC76, via LC-MS/MS.



Detection of glucose-EOH fragment is sensitive and selective via LC-MS/MS



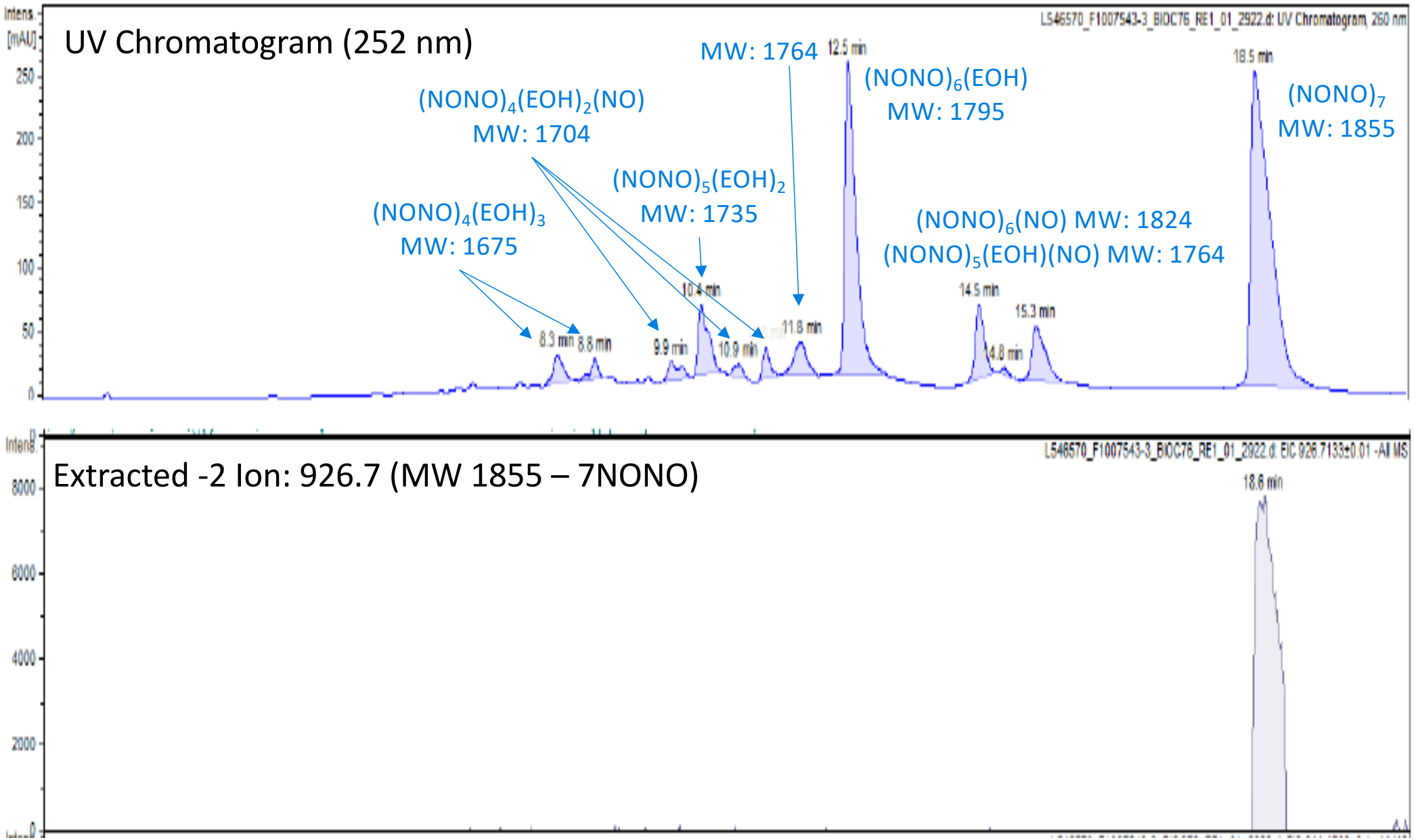
Analyte response is linear from 0.5 to 50 ppm

Drug substance characterizatio

Goal: To establish analytical methods to fully characterize the physiochemical properties of BIOC76 drug substance for GxP release and stability studies.

Property	Status
Appearance	Developed
Identification	Developed
Assay	Developed
Related Substances	Developed
Water Content	Developed
Nitric Oxide Loading	Developed
Sodium Content	Developed
Residual Solvents	Developed
Bioburden	Developed

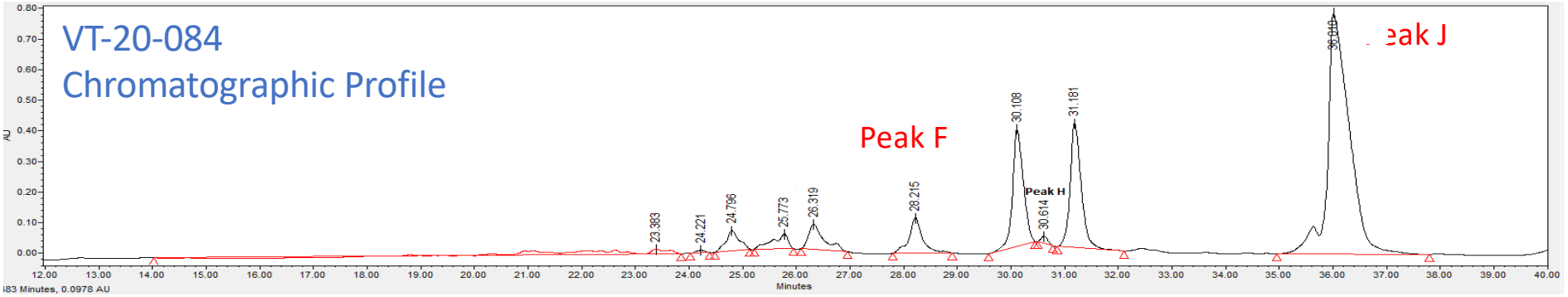
LC-MS Analysis: VT-20-082



Drug substance stability

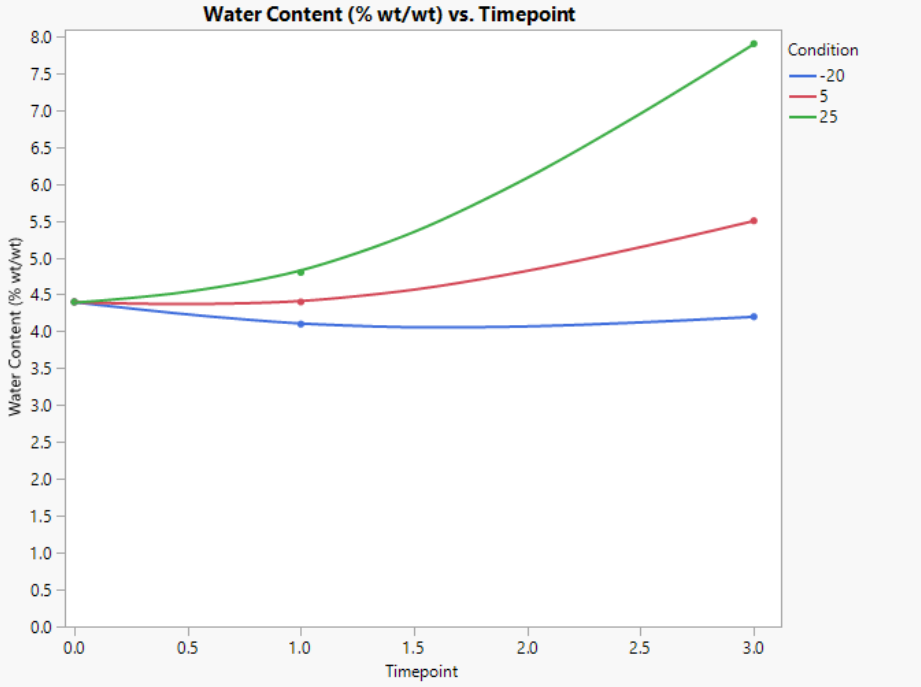
Goal: To ensure stability of API Drug Substance (BIOC76). We are envisioning preparation immediately prior to nebulization.

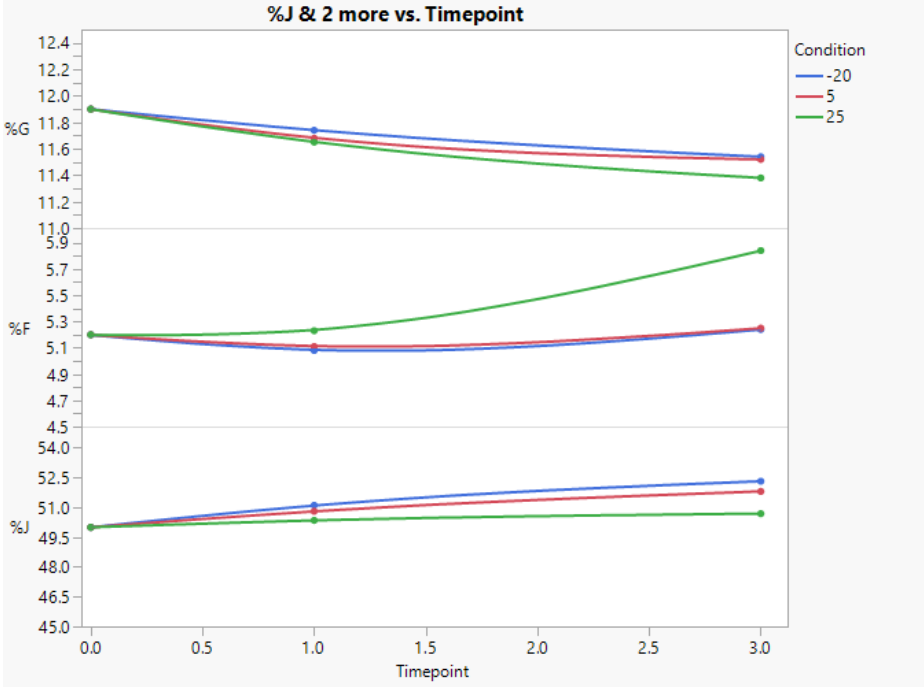
Drug Substance. Studies evaluating changes in the chromatographic profile indicate BIOC76 in powder form is stable for at least 7 days at ambient temperature and at least 3 months at 5 ºC and -20 °C. Formal stability studies evaluating other chemical properties of the drug substance are ongoing.



Peak	Initial	7 days at RT	Initial	1 Month at 5°C	1 Month at -20°C	3 Month at 5°C	3 Month at -20°C
Batch Number	VT-20-084				VT-20-082		
Peak F (Area %)	5%	5%	21%	19%	19%	19%	19%
Peak J (Area %)	53%	58%	48%	41%	41%	40%	41%

BIOC76 3 month stability





Water Content is increasing, would need to explore desiccant inside the sealed foil pouch.

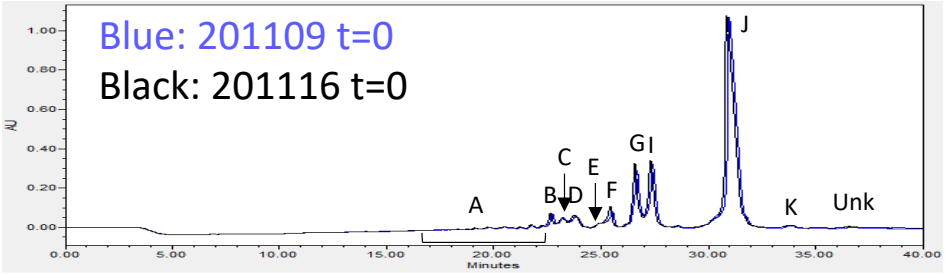
HPLC profile has not changed significantly. Slight loss of Peak G corresponding with increase in Peak F

BIOC76 powder is stable after 3 Mo at -20°C and 5°C. The 25°C / 60% RH condition looks like it will likely pick up too much water and change the HPLC profile to be viable long term in the current packing configuration.

Lyophilization process development

BIOC76 Batch: VT-20-095

Samples dissolve quickly upon reconstitution in water. Resulting pH: 12.7



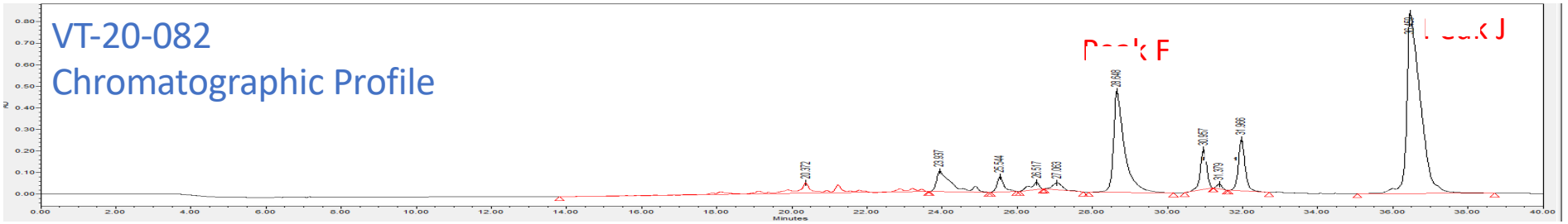
Sample	Storage Condition	Time	%A	%B	%C	%D	%E	%F	%G	%H	%I	%J	%K	%Unk
201109	N/A	0	4.6	1.8	3.1	1.5	1.7	5.5	10.8	0.0	11.7	58.4	0.9	0.1
	5°C	1w	4.2	1.5	3.8	1.0	1.8	5.4	9.7	0.0	13.1	58.6	0.8	0.1
		5w	3.2	2.0	2.3	3.2	0.9	5.1	9.8	0.0	12.7	58.7	1.0	0.1
	RT	1w	3.6	1.4	3.1	1.8	1.6	5.0	9.4	0.0	13.2	60.2	0.5	0.2
		5w	5.1	2.3	1.6	1.6	0.8	5.0	10.2	0.0	12.6	58.5	0.6	1.8
201116	N/A	0	4.5	1.8	4.0	1.1	1.6	5.5	10.7	0.0	12.3	57.7	0.8	0.1
	5°C	1w	4.0	1.5	3.1	1.8	1.8	5.1	9.9	0.0	13.1	59.4	0.3	0.1
		5w	3.3	1.9	2.3	1.6	1.6	5.0	10.4	0.0	12.5	59.2	0.5	1.8
	RT	1w	4.4	1.5	2.7	2.1	2.1	4.9	9.8	0.0	13.2	58.5	0.7	0.2
		5w	3.5	1.8	2.3	1.7	1.6	5.1	10.3	0.0	12.6	58.8	0.5	1.8

No significant changes to the chromatography after 1 Mo at either RT or 5°C

Drug product stability

Goal: To ensure stability of formulation (drug product) once prepared. We are envisioning preparation immediately prior to nebulization.

Drug Product: In basic solution, the drug product is stable for at least 3 days at 5°C. Once neutralized, the product must be used quickly and kept cold as loss of NO is observed in the chromatographic profile at pH 7.4.



Peak	Initial	24h at 5°C pH 11	72h at 5°C pH 11	Initial	1h at 0°C pH 7.4	6h at 0°C pH 7.4
Batch Number		VT-20-082			VT-20-084	
Peak F (Area %)	21%	21%	22%	5%	9%	16%
Peak J (Area %)	47%	47%	48%	53%	52%	44%

7-day toxicology study design: dogs & rats

Dog Study

- Complete maximum tolerated dose study: MTD = 60 mg/Kg for single dose
- Five dosing groups for 7-day repeat dosing
  1. Saline Control
  2. Formulation Control (CD-EOH_7)
  3. Low BIOC76+ (15 mg/kg)
  4. Med BIOC76+ (30 mg/kg)
  5. High BIOC76+ (60 mg/kg)
- 2 male and 2 female dogs per dose group

Rat Study

- Complete maximum tolerated dose study; MTD = 1046 mg/Kg for single dose
- Five dosing groups for 7-day repeat dosing
  1. Saline Control
  2. Formulation Control (CD-EOH_7)
  3. Low BIOC76+ (105 mg/kg)
  4. Mid BIOC76+ (305 mg/kg)
  5. High BIOC76+ (1046 mg/kg)
- 17 male and 17 female rats per dose group.

Histopathology report summary for dogs

- Inflammation of Lungs: Slight to moderate, groups 2-5
- Inflammation of Nose: Moderate, group 5
- Increased Cellularity in Lymph nodes: Moderate to marked, group 3-5
- Inflammation/Necrosis of PPG: Moderate to marked, groups 3-5
- Inflammation/Necrosis of LALT: Moderate, group 5

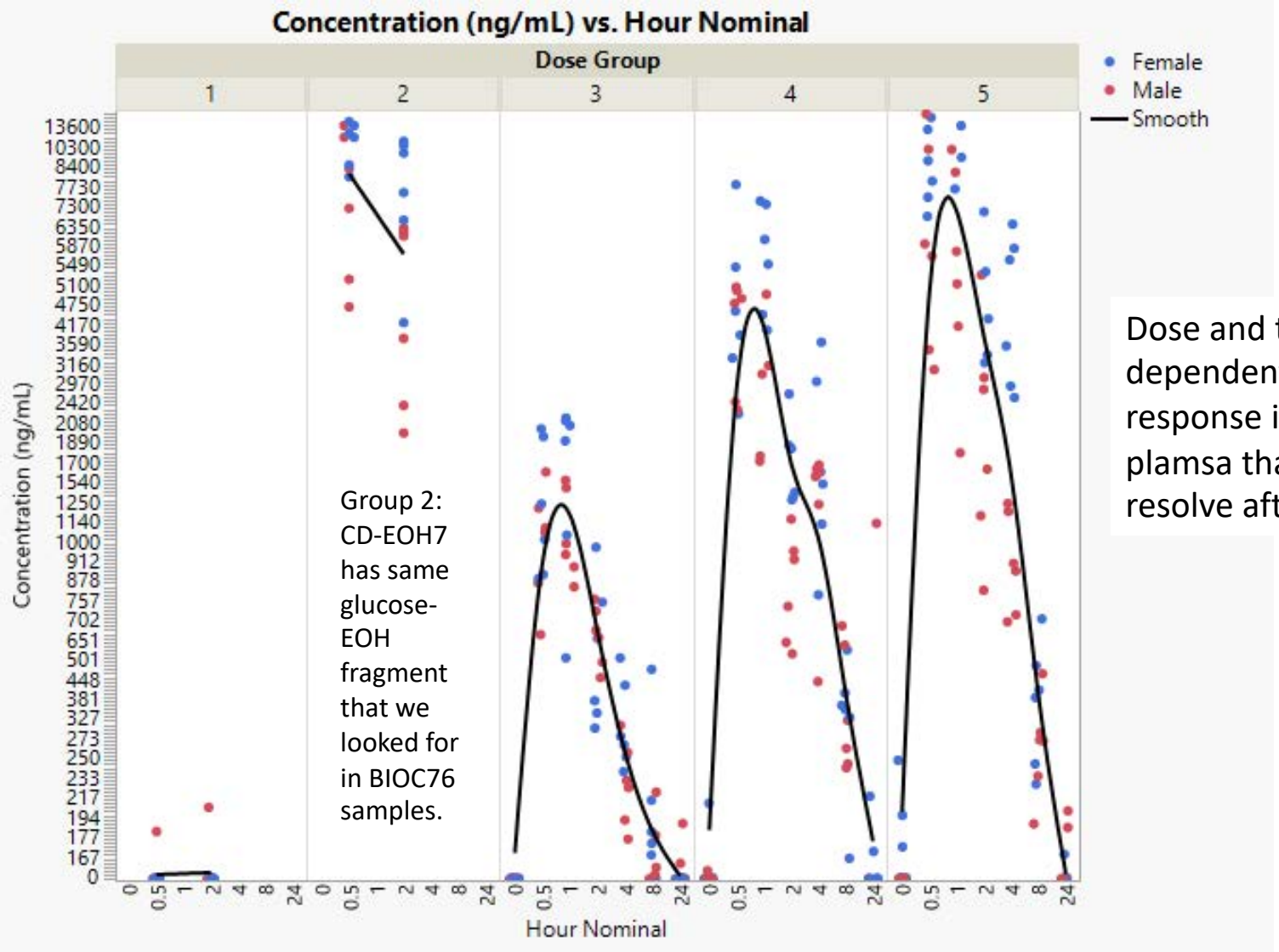
Note: Observations considered adverse because they could compromise organ function or the ability of the organs to respond to additional environmental challenges.

Grades for Severity or Amount

- *Minimal – describes an inconspicuous change*
- *Slight – notice able but not prominent feature*
- *Moderate – prominent feature*
- *Marked – dominant but not overwhelming feature*
- *Severe – overwhelming condition*

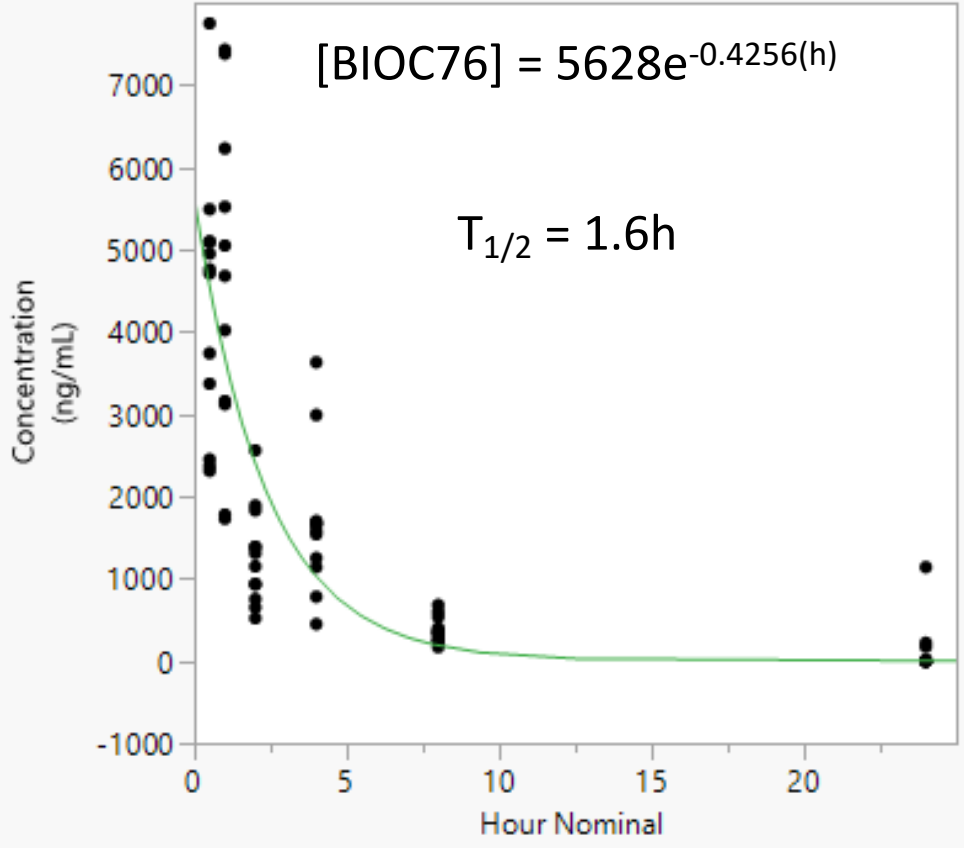
Note: FDA slow to approve anything over minimal

BIOC76 pharmacokinetics (PK) in rats

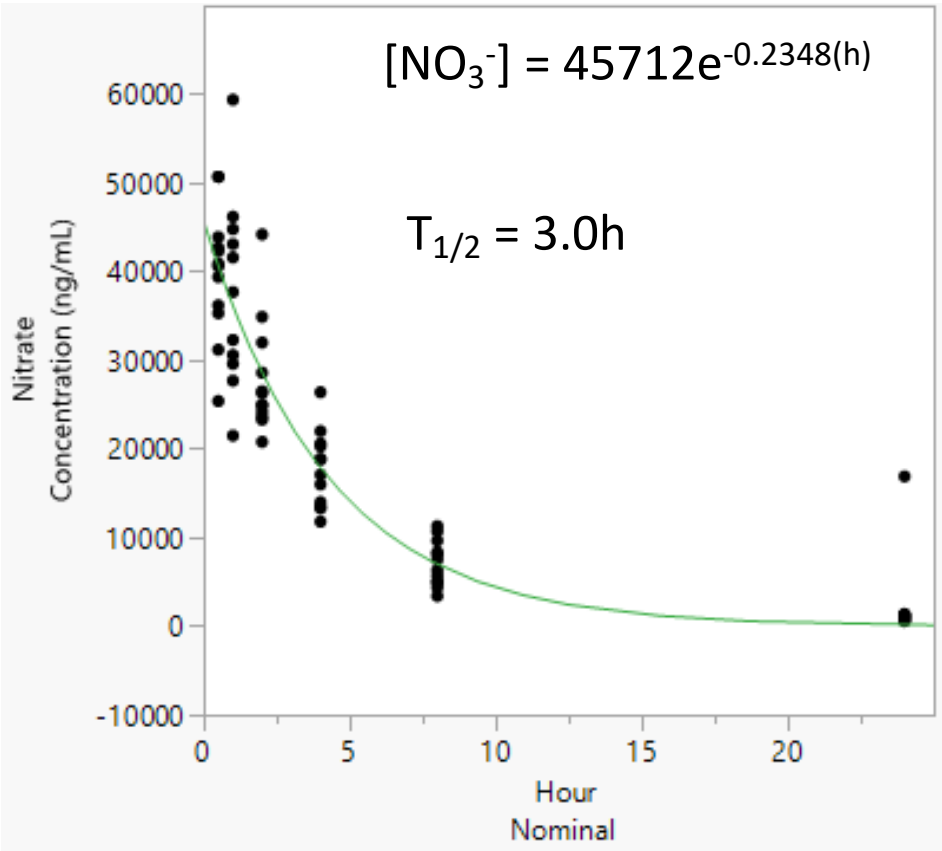


Dose and time-dependent BIOC76 response in Rat plamsa that seems to resolve after 24h

Nitrate vs BIOC76 pharmacokinetics (PK) in rats



BIOC76 Dose 4 PK Data



Nitrate Dose 4 PK Data