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Formation of new bioactive organic nitrites and their identification with gas chromatography-mass spectrometry and liquid chromatography coupled to nitrite reduction.

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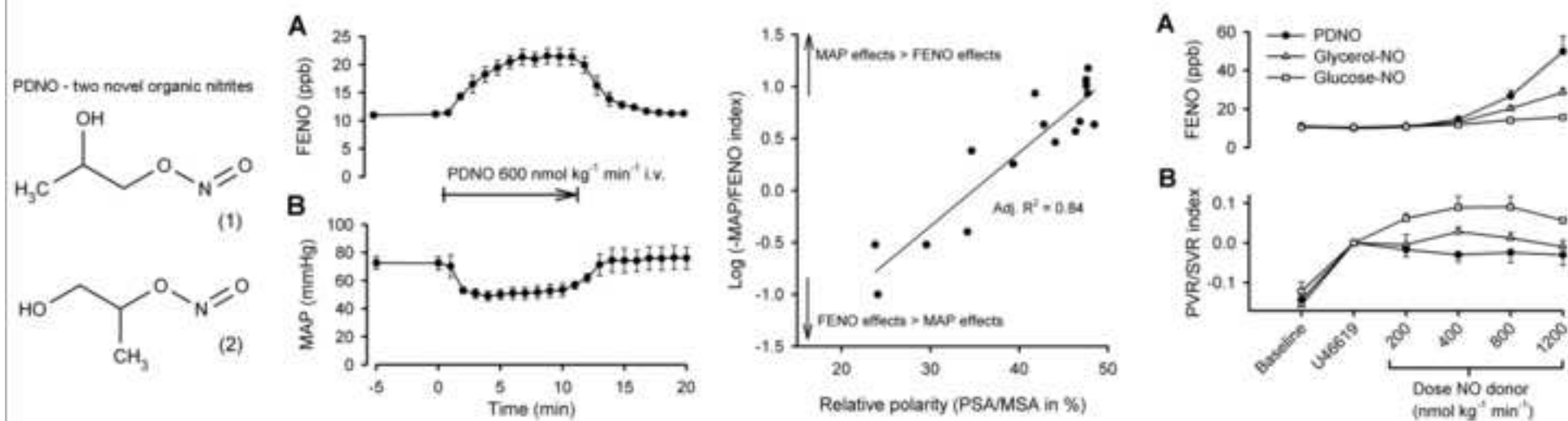
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Development of novel lung selective organic nitrites with effects on exhaled NO, systemic blood pressure and pulmonary hypertension



1 REVISÉD MANUSCRIPT

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3 **Formation of new bioactive organic nitrites and their**
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6 **identification with gas chromatography-mass spectrometry and**
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9 **liquid chromatography coupled to nitrite reduction.**
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Abstract

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6 Nitric oxide (NO) donors, notably organic nitrates and nitrites are used therapeutically but
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8 tolerance develops rapidly, making the use of e.g. nitroglycerin difficult. NO donation in the
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10 pulmonary vascular bed might be useful in critically ill patients. Organic nitrites are not
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12 associated with tachyphylaxis but may induce methaemoglobinemia and systemic
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14 hypotension which might hamper their use. We hypothesised that new lung-selective NO
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16 donors can be identified by utilizing exhaled NO as measure for pulmonary NO donation and
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18 systemic arterial pressure to monitor hypotension and tolerance development. Solutions of
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20 alcohols and carbohydrates were reacted with NO gas and administered to ventilated rabbits
21
22 for evaluation of *in vivo* NO donation. Chemical characterization was made by liquid
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24 chromatography with on-line nitrite reduction (LC-NO) and by gas chromatography-mass
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26 spectrometry (GC-MS). *In vivo* experiments showed that the hydroxyl-containing compounds
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28 treated with NO gas yielded potent NO donors, via nitrosylation to organic nitrites. Analyses
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30 in LC-NO showed that the reaction products were able to release NO *in vitro*. In GC-MS the
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32 reaction products were determined to be the organic nitrites, where some are new chemical
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34 entities. Non-polar donors preferentially increased exhaled NO with less effect on systemic
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36 blood pressure whereas more polar molecules had larger effects on systemic blood pressure
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38 and less on exhaled NO. We conclude that new organic nitrites suitable for intravenous
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40 administration are produced by reacting NO gas and certain hydroxyl-containing compounds
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42 in aqueous solutions. Selectivity of different organic nitrites towards the pulmonary and
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44 systemic circulation, respectively, may be determined by molecular polarity.
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Key words: nitric oxide donors; nitrites; pulmonary circulation; hypertension, pulmonary;
chromatography, high pressure liquid

Accepted Manuscript

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1. Introduction

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6 Nitric oxide donors are used to supply a patient with exogenous nitric oxide (NO) and can be
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8 used in conditions and diseases where increased NO is thought to be beneficial. There is a
9
10 wide range of NO donors with different chemistry and biochemistry available for
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12 experimental use [1]. The list of conditions and diseases is extensive for where experimental
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14 studies have suggested beneficial effects, but only a few compounds are used in clinical
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16 practice [2]. Nitroglycerin and isosorbide mononitrate are used in acute and chronic anginal
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18 pain respectively and nitroglycerin may be used in the treatment for anal fissure [2].
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22 Isosorbide dinitrate together with hydralazine is used for heart failure in African-Americans
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24 [2]. Inhaled amyl nitrite has been used in anginal pain and as an antidote in life-threatening
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26 cyanide intoxication [3]. Organic nitrates and organic nitrites are converted to NO *in vivo*
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28 which can be seen as increased exhaled NO and decreased systemic blood pressure [4, 5]. A
29
30 significant clinical problem with organic nitrates is the rapid tolerance development which
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32 can be demonstrated experimentally with decreased generation of NO to exhaled air [6, 7].
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36 Organic nitrites exhibit little or no tolerance [8], and recently ethyl nitrite has been suggested
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38 as an inhaled therapeutic [9]. However, the use of ethyl nitrite and other organic nitrites have
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40 been questioned due to significant methaemoglobin formation and other serious adverse
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42 effects [10, 11]. Sodium nitroprusside is used in hypertensive crises but is associated with
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44 tolerance development and risk of cyanide intoxication and its use is sometimes therefore
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46 discouraged [12]. Inhaled NO is used in persistent pulmonary hypertension of the newborn
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48 [13] whereas use of inhaled NO in acute lung injury and acute respiratory distress syndrome is
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50 not recommended [14]. Other conditions where treatment with exogenous NO has been
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52 suggested from experimental studies include myocardial ischemia reperfusion [15, 16],
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54 splanchnic ischemia reperfusion and acute intestinal inflammation [17], stroke [18],
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1 pulmonary hypertension [19] and sepsis [20]. The reasons for the lack of development of new
2 NO donors that actually reach clinical practice are complex and may include side-effects in
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4 the form of systemic hypotension, headache, lack of specificity, tolerance development and
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6 toxicity [2]. Altogether there is a quest for new NO donors [2]. We believe that there is a
7
8 specific need for intravenous NO donors with selectivity for the pulmonary circulation and
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10 that can be used, for example, in acute pulmonary embolism, where recent data indicate a
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12 protective role of endogenous NO [21]. We hypothesised that new lung-selective NO donors
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14 can be identified by utilizing exhaled NO as measure for pulmonary NO donation and
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16 systemic arterial pressure to monitor hypotension and tolerance development. We find that
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18 NO donors are formed, in a solution suitable for intravenous delivery, when deoxygenated
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20 aqueous solutions of alcohols and carbohydrates are reacted with NO gas and that these NO
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22 donors have different selectivity towards the pulmonary and systemic circulations.
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32 **2. Material and Methods**

33 **2.1. Preparation of reaction products between compounds and nitric oxide gas.**

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45 Aqueous solutions of alcohols and sugars and related compounds (please refer to Table 1 and
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47 supplementary Table A.1) were prepared and placed in gas tight cylinders (volume 20-150
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49 ml) with taps in both ends and a sideways port with a septum plug (F-145, Alltech
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51 Biotechnology, Ireland) in the middle portion. The cylinders were filled to 75% with solution,
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53 allowing a gaseous head-space. The solutions were deoxygenated by means of helium
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55 bubbling for 10 min and purged with pure nitric oxide gas for 3 min. Samples and infusion
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57 solutions were drawn via the septum plug.
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2.2. The HPLC system

The following reverse phase HPLC systems were used in all the analyses: a C₁₈ column (ReproSil-Pur 120 C₁₈-AQ, 4x150 mm, 5 μm, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany or Nova-Pak C18, Radial-Pak 8x100 mm, 4 μm, Waters Inc., Milford, MA, USA) or a CN column (Nova-Pak CN, 8x100 mm Radial-Pak, 4 μm, Waters Inc.), two pumps (ConstaMetric III; LDC/Milton Roy, Riviera Beach, FL, USA), an injector (9125; Rheodyne, Cotati, CA, USA) with an injector loop of 2.2 ml, a variable UV-absorbance detector measuring at 227 nm or 254 nm (SpectroMonitor III; LDC/Milton Roy), and a scanning UV absorbance detector (2140 Rapid Spectral Detector; LKB, Bromma, Sweden). The absorbance from the SpectroMonitor III detector was recorded and the pumps were controlled by commercial hardware and software (CSW32; DataApex, Prague, The Czech Republic) and the signal from the scanning detector was recorded by Wavescan software (LKB) run in DosBox (open source; www.dosbox.com). Samples and standards were injected at volumes from 2 μl to 2 ml. The flow rate of the mobile phase was 0.5-1 ml min⁻¹ depending on the size of the column and consisted of A) 5-10 mM ammonium formate in water followed by increasing proportions of B) methanol or acetonitrile. Isocratic elution or a linear gradient starting with 100% A and with an increase of 3% min⁻¹ of B was used. The effluent from the HPLC system could then either be sampled or continuously be passed into a nitrite reduction system for online measurement of NO/NO₂⁻/organic nitrites (see section 2.3).

2.3. Online measurement of NO/NO₂⁻ by chemiluminescence.

1 Aliquots or effluent from the HPLC were intermittently respectively continuously injected in
2 a temperature-controlled reaction chamber (total volume 300 ml) containing 75 ml of 1%
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4 (w/v) sodium iodide in hot (89° C) acetic acid. Inorganic and organic nitrites are reduced to
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6 NO in this environment but not inorganic nitrate and organic nitrates [22]. The reaction
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8 solution was purged with N₂-gas (200 ml min⁻¹) to deoxygenate the solution and to carry NO
9
10 gas to a chemiluminescence NO analyzer system. In the NO analyzer ozone was produced in
11
12 an oxygen flow of 100 ml min⁻¹ and made to react in a vacuum chamber with the NO in the
13
14 incoming gas from the reducing chamber (sample flow rate 150-200 ml min⁻¹). In the
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16 NO+ozone reaction, excited NO₂ is generated which emits one photon per molecule. The
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18 photons are counted by a photomultiplier tube and are linearly proportional to the NO content
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20 in the incoming gas. The signal from the photomultiplier tube was collected by a
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22 computerized data acquisition system (MP100 system with AcqKnowledge software,
23
24 BIOPAC Inc., Goleta, CA, USA). The system was intermittently calibrated with aliquots (10-
25
26 500 µl) of known amounts of sodium nitrite either by direct injection or via the HPLC system
27
28 (detection limits: 5 pmol and 10 pmol respectively). The sensitivity of the photomultiplier
29
30 tube was adjusted in order to measure in the linear range. Quantification of NO content was
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32 made by comparing peak area measurements with the peak area of a known amount of
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34 standard. In experiments where the HPLC effluent was introduced into the reaction chamber,
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36 the volume in the reaction chamber increased slowly and intermittent withdrawal of liquid
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38 was necessary. In order to minimise the interference of different volumes in the chamber,
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40 calibration was always done before and after each analysis. These calibrations were very
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42 similar and the mean between the two sets was used in the calculation of quantity. The HPLC
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44 together with the online continuous nitrite reduction is henceforth referred to as LC-NO.
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59 **2.4. Gas chromatography coupled to mass spectrometry**

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2 Eluted peaks from the HPLC with the reaction mixture of propanol and NO gas were
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4 collected in gas tight syringes and extracted by an equal volume of toluene in a small sealed
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6 sample vial, shaken briefly, and quickly separated by centrifugation, if necessary. Fractions
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8 were injected at 1 μ l onto an Agilent DB-5MS gas chromatography column (0.25 mm i.d., 30
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10 m length, 1 μ m phase thickness, Agilent Technologies, Inc., Santa Clara, CA, US) at an initial
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12 temperature of 40° C, thereafter the column temperature was raised by 10° C per min until a
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14 temperature of 200 or 250° C was reached. Detection of eluted compounds was made by an
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16 Agilent 5973 Mass Selective Detector (quadrupole MS, Agilent Technologies, Inc.). The gas
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18 chromatograph was an Agilent 6890 Series GC system with a 7673 autosampler (Agilent
19
20 Technologies, Inc.). Data were collected and analyzed with an Enhanced Chemstation G1701
21
22 BA computer program with a Wiley 7n library of mass spectra (Agilent Technologies, Inc.).
23
24 Alternatively, 1 μ l of reaction mixture consisting of 25-100 % propanol and NO gas was
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26 injected directly in the gas chromatography column thus bypassing the extraction procedure
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28 and the autosampling system. Commercial propyl nitrite was diluted in toluene and injected in
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30 the same way for comparison. In addition, 0.5 μ l samples of 25-100% 1,2-propanediol treated
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32 with NO gas were injected in the gas chromatography-mass spectrometry (GC-MS) system,
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34 now equipped with an Agilent DB-1701 column (0.25 mm i.d., 30 m length, 0.25 μ m phase
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36 thickness, Agilent Technologies, Inc.), in a total He flow of 30 ml min⁻¹, split fraction 1:5.
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38 Temperature gradient was as above. Mass spectrometry was either performed in electron
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40 impact mode, or in chemical ionisation mode using a chemical ionisation interface supplied
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42 with a methane flow.
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57 **2.5. Anaesthesia and surgical preparation**

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1 The experiments were approved by the local animal ethics committee. Male white New
2 Zealand rabbits (body weight: 2.0-3.5 kg, n=34) were anaesthetized with sodium
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4 pentobarbital via an ear vein (6 mg ml^{-1} in saline, $50\text{--}70 \text{ mg kg}^{-1}$). The animals were placed in
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6 the supine position and tracheotomised to allow mechanical ventilation (fraction of inspired
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8 oxygen (FiO_2) = 0.21), using a constant volume ventilator (model 683, Harvard Apparatus,
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10 South Natick, MA, USA). The ventilator was supplied with NO-free air using a charcoal filter
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12 (110 x 11 cm). Respiratory rate (40 min^{-1}) and tidal volumes were adjusted to keep end-tidal
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14 CO_2 at 4.5-5.0% . A heparinised catheter was inserted in the left common carotid artery for
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16 mean arterial blood pressure (MAP) and heart rate (HR) recordings (pressure transducer
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18 PX600P, Edwards Lifescience LLC, Irvine, CA, USA), and for arterial blood sampling.
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21 Another catheter was inserted in the right jugular vein for administration of drugs. In
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23 experiments where the NO donating capabilities of the NO solutions were tested the
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25 preparation was complete at this point and the animals were allowed a 30-60 min intervention
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27 free period. In experiments with pharmacologically induced pulmonary hypertension a mid
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29 thoracotomy was performed and a flow probe around the ascending aorta (T201 system,
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31 Transonic Systems Inc., Ithaca, NY, USA) measured cardiac output. A heparinised catheter
32
33 was introduced via the left jugular vein and advanced to the pulmonary artery for
34
35 measurement of pulmonary artery pressure. Another catheter was inserted through the left
36
37 atrial wall for left atrial pressure measurements, and central venous pressure was measured
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39 via the catheter in the right jugular vein. Pulmonary vascular resistance (PVR), systemic
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41 vascular resistance (SVR) and PVR/SVR index were calculated with the common formulas.
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44 After the surgery, the animals were allowed a 30–60 min intervention-free period to reach
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46 stable circulatory conditions and exhaled NO values. A continuous infusion containing
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48 glucose (26 g l^{-1}), dextran 70 (Macrodex, 28 g l^{-1}) and NaHCO_3 (6.6 g l^{-1}) was administered.
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51 In experiments where the NO donating capabilities of the NO solutions were tested sodium
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pentobarbital (4.2 g l^{-1}) was added to the infusate which was given at a rate of $5 \text{ ml kg}^{-1} \text{ h}^{-1}$. In experiments with induced pulmonary hypertension sodium pentobarbital was 2.1 g l^{-1} in the infusate and the infusion rate was $10 \text{ ml kg}^{-1} \text{ h}^{-1}$.

2.6. NO measurements in exhaled breath

The fraction of NO in mixed exhaled gas (FENO) was continuously measured by means of a chemiluminescence based system (NIOX, Aerocrine AB, Solna, Sweden) sampling at $100\text{--}140 \text{ ml min}^{-1}$ through a Naphion sampling catheter at the end of a mixing chamber (size: 5 tidal volumes) connected to the ventilator exhaust. Calibration was performed using certified NO standard gas in nitrogen (AGA Specialgas, Lidingö, Sweden).

2.7. Experimental protocol for *in vivo* experiments on NO donors

Rabbits received the candidate NO donor solutions (described in section 2.1) intravenously (syringe pump CMA/100, Carnegie Medicin AB, Stockholm, Sweden) at pre-determined flow rates into a standardized carrier flow (864 Syringe Pump, Univentor LTD, Zejtun, Malta) via the right jugular vein catheter. Each rabbit received more than one of the NO donor solutions and was allowed ample intervention-free periods (at least 30 min) between infusions to reach stable baseline conditions and to exclude cross-tolerance. Initial experiments included also trials with NO-saturated isotonic saline and a lipid emulsion purged with NO as means of administering NO parenterally. Arterial blood samples were intermittently drawn from the arterial catheter and analyzed for blood gases, acid–base status, methaemoglobin and total haemoglobin (ABL 520, Radiometer A/S, Copenhagen, Denmark). In some experiments the

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endogenous NO production was inhibited with N^G -nitro-L-arginine methyl ester (L-NAME, 30 mg kg⁻¹). In experiments with pulmonary circulatory measurements (see section 2.5) the thromboxane mimetic U46619 was administered intravenously to the animals in a continuous infusion (10-30 µg ml⁻¹ in saline, 150-750 ng kg⁻¹ min⁻¹) in order to induce a stable pulmonary hypertension (target: increase in PVR by approximately 150%). Thereafter one of the NO donor solutions or nitroglycerin (1 mg ml⁻¹) were administered in cumulatively increasing doses (200-1600 nmol kg⁻¹ min⁻¹, calculated for NO donating groups, 10 min for each dose) while monitoring haemodynamics and respiratory gases, including FENO as well as intermittent checks of blood gases. Several NO donors were investigated in each animal in random order with at least 30 min in between.

2.8. Computation of physicochemical descriptors

The calculator plugin modules of MarvinSketch (version 5.2.6, ChemAxon Kft, Budapest, Hungary), accessed through ChemIDplus at the US National Library of Medicine, were used to obtain calculated molecular descriptors of the NO donor molecules. These descriptors included octanol/water partition coefficient (Log P), molecular size descriptors (molecular weight, MW; molecular surface area, MSA; molar refractivity, maximal and minimal projection radii and projection area, polarizability), molecular shape descriptors (the ratios between maximal and minimal projection radii and projection area respectively), polar and non-polar surface area (PSA and NSA respectively) from which a relative polar surface area of each donor molecule (PSA(%)) was calculated by dividing PSA with MSA. These descriptors were then used as independent variables in multiple linear regression models using the log (-MAP/FENO) as the dependent variable.

2.9. Chemicals

Propyl nitrite was purchased from Karl Industries Inc. (Aurora, OH, USA) and propyl nitrate was from ChemService Inc. (West Chester, PA, USA). Ethyl nitrite, isobutyl nitrite, butyl nitrite, sodium nitrite, L-NAME, methanol, 1-propanol, 2-propanol, 1,2-propanediol, 1,3-propanediol, isobutanol, butanol, 2-deoxyribose, 1-O-methyl-2-deoxyribose, ribose, 6-deoxygalactose, galactose, glucose, fructose, sucrose, lactobionic acid, inulin, fucoidan, dextran (MW 162 000 g mol⁻¹), alanine, glycine, 3-amino-1,2-propanediol, 2-amino-1,3-propanediol, glucosamine and lactate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol was from Kemetyl (Stockholm, Sweden). Glycerol, sorbitol, mannitol, polyethylene glycol 400 and ammonium formate were from VWR International AB (Stockholm, Sweden). U46619 was from Cayman Chemicals (supplied by Larodan Fine Chemicals AB, Malmö, Sweden). Nitroglycerin was from Schwarz Pharma AG (Monheim, Germany). Heparin (Kabi Vitrum, Stockholm, Sweden), dextran 70 (Macrodex, Pharmalink, Spånga, Sweden), Intralipid (20%, Fresenius Kabi, Uppsala, Sweden) and sodium pentobarbital (Apoteksbolaget) were purchased from Apoteksbolaget, Stockholm, Sweden. Pure NO and calibration gases were from AGA gas AB (Lidingö, Sweden).

2.10. Statistics

Descriptive data are presented as mean ± standard error of the mean. Comparisons between groups were made by one way ANOVA and repeated comparisons within one group were made by repeated measures ANOVA or a paired t-test. Multiple linear regressions were made

1 by using the best subsets function. Models including variables with non-significant variables
2 (P>0.05) or with severe multicollinearity (Variance Inflation Factor>4) were excluded. Linear
3 correlations were made by using Pearson Product Moment Correlation. All statistical analyses
4 were made by means of SigmaStat (v. 3.10.0) and SigmaPlot (v. 9.01, Systat Software Inc.,
5 San Jose, CA, USA). P<0.05 was considered as significant.
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11 **3. Results**

12 **3.1. NO treated lipid emulsion and glycerol infused intravenously *in vivo***

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28 Initial experiments showed that bolus infusions intravenously (0.5-2.5 ml) of a lipid emulsion
29 (Intralipid) saturated with pure NO gas transiently increased FENO paralleled with a transient
30 drop in MAP (data not shown). These responses could not be reproduced by only the native
31 lipid emulsion (data not shown). In another experiment a rabbit with inhibited endogenous
32 NO production (L-NAME 30 mg kg⁻¹) first for 30 min intravenously received deoxygenated
33 saline equilibrated with NO gas (0.5 ml kg⁻¹ min⁻¹) and after a recovery period of 3 hours a 30
34 min intravenous infusion of deoxygenated lipid emulsion (0.5 ml kg⁻¹ min⁻¹) equilibrated with
35 NO. Saline with NO gas yielded a small increase in FENO and a very high methaemoglobin
36 concentration whereas the lipid emulsion treated with NO gas gave a large increase in FENO
37 whereas methaemoglobin was essentially not changed (Figure 1). MAP decreased with both
38 infusions (data not shown). However the lipid emulsion turned yellow indicating oxidation.
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¹). This resulted in increased FENO and decreased MAP. Thereafter the glycerol-NO solution was infused intravenously at increasing doses (3-100 $\mu\text{l kg}^{-1} \text{min}^{-1}$) in a group of rabbits (n=4-5). FENO increased and MAP decreased dose-dependently (Figure 2a) during glycerol-NO infusions whereas neither saline treated with NO nor unreacted glycerol in the same doses had these effects (data not shown). Heart rate increased with $36 \pm 2 \text{ beats min}^{-1}$ from $283 \pm 28 \text{ beats min}^{-1}$ during infusion ($100 \mu\text{l kg}^{-1} \text{min}^{-1}$) of glycerol-NO. When studying the pattern of FENO and MAP during the infusion, both these parameters reached a stable plateau, thus indicating absence of tolerance development (Figure 3). It was also found that there was a curvilinear relationship between the increments in FENO and the change in MAP (Figure 2b), a relationship that was linear when FENO increased by less than 20 ppb (this analysis was continued in section 3.4). Methaemoglobin levels remained low (data not shown) and other parameters did not change. These experiments suggested that an NO donor solution was created when deoxygenated solutions of lipid emulsion or glycerol were treated with NO gas.

3.2. Identification of other starting materials exhibiting NO donation when reacted with NO gas

More than 20 different alcohols and carbohydrates (Table 1 and supplementary Table A.1) were dissolved in water, deoxygenated 10 min with helium, bubbled 3 min with pure NO gas, transferred to a syringe and administered intravenously ($100 \mu\text{l kg}^{-1} \text{min}^{-1}$) to rabbits in order to test NO donating capabilities (see following text, Table 1 and supplementary Table A.1). The capacity of NO donation *in vivo* was determined positive if FENO increased and MAP decreased.

1 NO-treated monohydric alcohols (1-propanol, 2-propanol), dihydric alcohols (1,2-propanediol,
2 1,3-propanediol), trihydric alcohols (glycerol) and polymers of alcohol (polyethylene glycol)
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4 donated NO to exhaled air and decreased MAP (Table 1 and supplementary Table A.1). NO-
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6 treated monosaccharides (glucose, galactose, fructose, and ribose), monosaccharide alcohols
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8 (sorbitol and mannitol) and certain modified monosaccharides (fucose, 2-deoxy-ribose, 1-O-
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10 methyl-ribose) all had NO donor capabilities. Also NO-treated disaccharides (sucrose,
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12 lactobionic acid) and carbohydrate polymers (inulin, dextran, fucoidan) increased FENO and
13
14 lowered MAP. Similar with glycerol-NO, all these compounds exhibited a rapid onset and
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16 offset in effects without acute tolerance as illustrated by NO-treated 1,2-propanediol (Figure 3
17
18 and data not shown). A common feature for these compounds and groups are that they contain
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20 at least one hydroxyl group. Some compounds that were tested did not exhibit NO donating
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22 capacity *in vivo* despite NO equilibration: 2-Amino-1,3-propanediol, 3-Amino-1,2-
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24 propanediol, lactic acid, alanine, glycine, and glucosamine (Table 1). This indicates that
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26 amine or carboxyl groups close to the hydroxyl-group in the substrate molecule prevent the
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28 formation of the NO donor compounds.
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40 **3.3. Chemical characterization and identification of NO donors.**

41 42 43 44 45 **3.3.1. Identification of reaction products with liquid chromatography coupled to on-line** 46 47 **nitrite reduction** 48 49

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54 In order to further characterize and identify the NO donating compounds HPLC coupled to
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56 on-line nitrite reduction (LC-NO) was developed. Analyses of water saturated with NO gas
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58 and water with inorganic nitrite showed that the NO gas/inorganic nitrite appeared with the
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1 front of the chromatogram and at the corresponding elution time when the NO/nitrite
2 recording was synchronized by adjusting for the time delay between the two detection
3 methods. This peak was denoted peak I. The same synchronized front peak was seen in the
4 LC-NO of all the analysed NO solutions and was thus regarded as emanating from the NO
5 gas/inorganic nitrite of these solutions. In addition, in all the solutions investigated with
6 HPLC or LC-NO (Table 1) there appeared UV absorbing peaks with UV absorbance maxima
7 near 225 nm, and with faint but for organic nitrites characteristic vibrational spectral peaks in
8 the range 330-370 nm. No other such novel peaks were seen when deaerated but non-NO
9 treated organic starting materials were chromatographed. In the LC-NO it was found that all
10 these chromatographic peaks were capable to generate NO in refluxing conditions (Figure 4-6,
11 Table 1 and data not shown). None of the investigated parent substrate compounds yielded
12 such NO chemiluminescence signals in the nitrite-reducing LC-NO analysis. Furthermore, the
13 number of eluted organic nitrite-like peaks correlated with the number of nitrosylation sites in
14 the form of hydroxyls in the parent compounds. Thus, 1,2-propanediol treated with NO gas
15 (PDNO) exhibited 2 peaks (II and III in Figure 4) and glycerol-NO gave two peaks (II and III
16 in Figure 5) the second of which was several times larger than the other, compatible with
17 glycerol having two equivalent primary hydroxyls and one secondary. NO-treated glucose
18 (glucose-NO) gave 4 organic nitrite-like peaks discernible in the UV recording and which
19 despite considerable coelution, due to peak broadening in the transfer capillary, were still
20 discernible as three peaks when reaching the NO/nitrite analysis (Figure 6). HPLC analysis of
21 sucrose (60% in water, w/v) reacted with NO gas yielded 8 peaks including the front peak
22 (data not shown). LC-NO analyses of these solutions were also made in reversed phase with
23 gradients of methanol and acetonitrile, however no additional peaks were seen (data not
24 shown).

1 Further experiments were performed to verify the formation of organic nitrites. When
2 analyzing commercially available propyl nitrite in methanol gradient elution there was a
3
4 perfect match between the elution time (27.4 min) and UV absorbance between peak II in
5
6 propanol-NO and authentic propyl nitrite. Propyl nitrate analyzed in the same system had a
7
8 retention time of 23.7 min (approximately 55% of methanol, detection limit: 0.5 nmoles) and
9
10 a UV maximum of 205 nm with no other specific UV absorbances. A corresponding peak was
11
12 not found in the analysis of propanol-NO and therefore the content of propyl nitrate, if any, in
13
14 the propanol-NO must be less than 2.5% of the content of propyl nitrite. Further comparisons
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16 with some commercial organic nitrites in HPLC and LC-NO were completely compatible
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18 with formation of the corresponding nitrites in the NO gas treated hydroxyl precursor
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20 solutions (Table 1). In the experiments with authentic organic nitrites it was also found that
21
22 the stability of the authentic organic nitrites was poor in water and was markedly increased by
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24 dissolving them in deoxygenated water or more preferable in deoxygenated NO gas-saturated
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26 solution (data not shown).
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34 In the LC-NO experiments the content of NO donor compounds in the NO donor solutions
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36 could be estimated, by comparison with aliquots of inorganic nitrite. The content was
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38 determined to be 1-5 mM when the concentration of the carrier solutions were 3.3 M (1,2-
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40 propanediol, glycerol and glucose). If the formed compounds were considered reacted with
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42 one NO molecule the relation between reacted carrier molecules to unreacted carrier
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44 molecules would be approximately 1:1000. Detection of the organic nitrites by e.g. NMR
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46 against this background was thus considered nearly futile, and attempts at identification with
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48 LC-MS yielded no conclusive data (unpublished results). The 3 solutions which appeared
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50 having the highest content of organic nitrites were therefore chosen for GC-MS analysis.
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60 3.3.2. Identification of reaction products with gas chromatography-mass spectrometry

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3 For mass spectrometry the C₁₈ HPLC fractions corresponding to the 27.4 min peak from
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5 gradient elution of propanol-NO were injected on the GC-MS system and several peaks eluted.
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7 At the front (1.5 min) the expected air gas peaks (nitrogen, oxygen and CO₂) were observed,
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9 together with a NO peak. At 3 min a peak exhibiting mass spectral characteristics for propyl
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11 nitrite was identified by the mass detector. Propanol was eluted and identified at 3.5 min in
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13 the GC-MS system, followed by small amounts of benzene (breakdown product of toluene) at
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15 5.5 min, and then a large toluene peak at 8 min followed (data not shown). Direct on-column
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17 injection of commercial propyl nitrite confirmed the elution time (3 min) and mass spectral
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19 characteristics of propyl nitrite. Direct on-column injection of propanol-NO, obtained from
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21 the gas-tight reaction vessel, gave similar results but with a very large peak of propanol
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23 followed by toluene (data not shown). A solution of PDNO was similarly analyzed, using
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25 manual injection on an Agilent DB-1701 column (Figures 7-9). Two peaks were identified in
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27 the gas chromatogram (retention times 5.29 min and 6.15 min respectively) in the PDNO
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29 sample that were not found when injecting only 1,2-propanediol (Figure 7). The mass
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31 spectrum of the first of these peaks showed fragments of 30, 45 and 60 which are suggested to
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33 arise from the cleavage of the mononitrite of 1,2-propanediol when the hydroxyl at position 1
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35 is nitrosylated (2-hydroxy propyl nitrite, Figure 8). The mass spectrum of the second peak
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37 showed fragments of 30, 31, 59 and 74 which are suggested to arise from cleavage of the
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39 mononitrite of 1,2-propanediol when the hydroxyl at position 2 is nitrosylated (2-hydroxy-1-
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41 methylethyl nitrite, Figure 9). A major abundance of mass fragment 30, a common fragment
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43 for organic nitrites, was found in the 2 chromatographic peaks for compound 1 and 2 (data not
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45 shown). Mass spectrometry using chemical ionization demonstrated the M-1 and M+1
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47 molecular ions in the mass spectra for both compound 1 and 2 (data not shown), as should be
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49 expected from the structures shown with the mass spectra (Figures 8-9). Attempts at
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1 analyzing glycerol-NO by the same method failed due to chromatographic interference from
2 the large amounts of unreacted glycerol.
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8 **3.4. Determination of important molecular properties for selectivity to the pulmonary** 9 **and systemic circulation respectively.** 10

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16 Infusions of organic nitrites of PDNO, glycerol-NO and glucose-NO were studied in several
17 rabbits and at different doses. By using increases of FENO less than 20 ppb, significant linear
18 correlations were found between the increases in FENO (delta ppb from baseline) and the
19 changes in MAP (% of baseline) (data not shown). Linear regressions (forced to start at 0)
20 showed that the slopes of the lines were different: PDNO -2.4 ± 0.5 , glycerol-NO -2.9 ± 0.5 and
21 glucose-NO -8.6 ± 0.9 . The slope was denoted MAP/FENO-index and was used as a measure
22 of selectivity of NO donation in the pulmonary circulation versus systemic circulation. By
23 dividing the change in MAP (% of baseline) with the increase in FENO from baseline (delta
24 ppb), this index was generated for all NO donor solutions (supplementary Table A.1). A
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26 MAP/FENO index close to zero denoted relatively larger effects on FENO, i.e. pulmonary
27 circulation, and a MAP/FENO index away from zero indicated comparably larger effects on
28 MAP, i.e. the systemic circulation. The MAP/FENO index (i.e. selectivity) differed
29 significantly between the NO donor solutions and it was hypothesized that the selectivity
30 depended on molecular properties of the NO donors. The calculated molecular descriptors are
31 shown in supplementary Table A.2 and the correlations between them are shown in
32 supplementary Table A.3. The MAP/FENO index was made positive and transformed to
33 logarithms ($= \log -\text{FENO}/\text{MAP}$ index) as it was found that this process increased its
34 usefulness in linear regression analyses, due to the nature of the index. In multiple linear
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1 regression, relative polarity of the NO donor molecule, i.e. polar surface area divided with
2 molecular surface area, showed to be the best predictor of the MAP/FENO index, i.e. the
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4 more relative polarity of the NO donor molecule, the more effects on MAP compared to
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6 FENO and vice versa (Figure 10 and supplementary Table A.4). The second best fit showed
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8 that the more non-polar surface area and the higher Log P value of the NO donor molecules
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10 gave more effects on FENO compared with MAP and vice versa (supplementary Table A.4).
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12 The third best fit indicated that the less polar surface area and the more non-polar surface area
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14 of the NO donor molecule predicted more effects on FENO versus MAP (supplementary
15
16 Table A.4). Taken together the results from multiple linear regressions revealed that the
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18 polarity of the NO donor molecules was important for the selectivity towards the pulmonary
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20 versus the systemic circulation. Another important notion was that the very non-polar organic
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22 nitrites (e.g. propyl nitrites) and therefore theoretically the most lung selective compounds
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24 produced significant methaemoglobinemia thus limiting their use as therapeutics.
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35 **3.5. Reversal of pharmacologically induced pulmonary hypertension**

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41 The effects of PDNO, glycerol-NO, glucose-NO and nitroglycerin on pharmacologically
42 induced pulmonary hypertension (obtained by means of i.v. infusion of the thromboxane A₂-
43 mimetic U46619 in 8 rabbits) *in vivo* were investigated. Hemodynamic parameters and blood
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45 gases were normal and essentially similar in each group at baseline and during U46619
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47 infusion. U46619 increased mean pulmonary arterial pressure (mPAP; 15±1 to 23±1 mmHg),
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49 PVR (26±2 to 62±4 mmHg min l⁻¹), SVR (203±14 to 244±16 mmHg min l⁻¹) and PVR/SVR
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51 (0.13±0.1 to 0.26±0.1) and slightly decreased FENO (10.7±0.5 to 10.4±0.4 ppb), cardiac
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53 output (330±20 to 280±10 ml min⁻¹) and arterial partial pressure of oxygen (PaO₂, 12.4±0.4 to
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11.8±0.4 kPa). Other parameters did not change due to U46619 (data not shown), including MAP (70±4 versus 74±3 mmHg). PDNO (n=4, 200-1200 nmol kg⁻¹ min⁻¹, Figure 11), glycerol-NO (n=4, 200-1200 nmol kg⁻¹ min⁻¹), glucose-NO (n=4, 200-1200 nmol kg⁻¹ min⁻¹) and nitroglycerin (n=3, 200-1600 nmol kg⁻¹ min⁻¹ calculated on NO donating groups) dose-dependently reversed the effects of U46619 on mPAP, PVR, cardiac output and PaO₂ (data not shown) to normal or almost normal at the highest doses. The order of efficacy differed and was PDNO>glycerol-NO>nitroglycerin>glucose-NO. All four NO donors also decreased MAP and SVR dose-dependently but on these parameters the efficacy order was the reverse i.e. glucose-NO>nitroglycerin>glycerol-NO>PDNO. Notably, in two out of four experiments the highest infusion rate of glucose-NO (1200 nmol kg⁻¹ min⁻¹) had to be terminated due to extreme systemic hypotension and signs of left heart failure, probably due to inadequate perfusion of the myocardium. All four NO donors also increased FENO dose-dependently (Figure 12a), but their efficacy differed, i.e. PDNO>glycerol-NO>nitroglycerin>glucose-NO. Most importantly the four NO donors differently changed the PVR/SVR index, i.e. an index of selectivity towards the pulmonary and systemic circulations. PDNO decreased the PVR/SVR index (Figure 12b), thus indicating increased selectivity for the pulmonary circulation. Glucose-NO was the most potent in increasing the PVR/SVR index (Figure 12b), thus indicating increased selectivity for the systemic circulation. Glycerol-NO (Figure 12b) and nitroglycerin (data not shown) affected the PVR/SVR index similarly by increasing it slightly. PDNO very slightly increased the methemoglobin level (from 0.4±0.2 to 0.85±0.1%) at the end of the cumulative infusions, whereas the three other NO donors left the methemoglobin concentration unchanged. Taken together these experiments clearly showed that the four investigated NO donors differed in selectivity for the pulmonary and systemic circulations respectively, and that FENO is an adequate marker for this difference.

4. Discussion

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7 For more than a century, organic nitrites have been used as volatile NO donors for relieving
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9 anginal pain. Further use has been limited due to problems with administration, dosing and
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11 adverse effects.

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14 The present study introduces a method for producing potent NO donor solutions, evidenced
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16 by increases in exhaled NO and decreases of systemic blood pressure and by the ability to
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18 deliver NO gas in a nitrite reduction system after HPLC. The NO donor molecules were
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20 identified to be organic nitrites where two of them for the first time are described and
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22 identified with GC-MS. The selectivity of the NO donation towards the pulmonary and
23
24 systemic circulation was dependent on the relative polarities of the NO donor molecules. The
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26 NO donor solutions were also potent in reversing pharmacologically induced pulmonary
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28 hypertension.

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31 The present study provides further evidence that the rabbit *in vivo* model [4, 5, 23], with
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33 measurements of exhaled NO and systemic blood pressure, is an excellent way to explore the
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35 action of NO donors. In the present work we used it as a bioassay system in order to find new
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37 NO donors. Previously the model has been used to study the acute tolerance development to
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39 organic nitrates and other NO donors [6]. In the present study, the response on exhaled NO
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41 and systemic blood pressure to the organic nitrites showed a plateau pattern over time,
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43 indicating no acute tolerance development. This is in contrary to the response to nitroglycerin
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45 where a peak and plateau pattern was observed and taken as an indication of acute
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47 tachyphylaxis [6]. The lack of acute tolerance of other organic nitrites has previously been
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49 shown both *in vivo* and *in vitro* [8], constitutes a very important pharmacological difference
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1 between organic nitrites and organic nitrates and may therefore encourage the use of organic
2 nitrites instead of organic nitrates in clinical settings.
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5 Furthermore, our present experimental data on exhaled NO and systemic blood pressure
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7 indicated that different organic nitrites had differential effects on exhaled NO and systemic
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9 blood pressure. Therefore we used a MAP/FENO index previously used [6] to describe these
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11 differential effects and the data were compared with calculated molecular descriptors of the
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13 NO donor molecules. By screening multiple linear regression models it was found that the
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15 best fits included measures of polarity of the NO donor molecules, i.e. that more polarity of
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17 the NO donor molecules affected systemic blood pressure more and that less polarity of the
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19 donor molecules affected exhaled NO more. This novel structure activity comparison
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21 approach for organic nitrites might be useful in the future in designing organic nitrites with
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23 wanted effects and selectivity as has been suggested for rational design of other classes of
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25 compounds [24] and as used to understand important substructures for organic nitrates [25].
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27 The experiments with pharmacologically induced pulmonary hypertension indicate that
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29 certain organic nitrites will be more selective for the pulmonary circulation (e.g. organic
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31 nitrites of 1,2-propanediol and glycerol) compared to the systemic circulation (e.g. organic
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33 nitrites of glucose). However this observation needs to be confirmed and extended in a future
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35 study.
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39 A study of the exact mechanisms behind the relationship between the selectivity for the
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41 pulmonary circulation and the polarity of the organic nitrite molecules have to be confined to
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43 a future study, but some considerations can be made. Relative polarity can be regarded as a
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45 measure of lipophilicity. Since several measures of compound lipophilicity correlate well with
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47 cell membrane passage [26], one potential mechanism could be that non-polar organic nitrites
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49 have increased permeability through cell membranes and therefore are cleared by the lungs
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51 and reach the systemic circulation in smaller amounts. This idea would also explain why the
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1 most non-polar organic nitrites produced methemoglobin, since a fast passage into the red
2 blood cells should be expected. Other properties which have been shown to differ between
3 organic nitrites, and may affect their half-lives and relative lung selectivity, are differences in
4 efficacy in methaemoglobin formation [28], rate of hydrolysis [29], rate of transnitrosylation
5 [29] and rate of enzymatic bioactivation the lung [30]. A short half-life of organic nitrites
6 seems to be a general property of this group of compounds, although the extent of it may
7 differ between the different individual compounds. In a study of intravenous infusions of
8 isobutyl nitrite in rats the half-life, systematic clearance and distribution volume of this
9 organic nitrite were determined to 1.3 min with a monoexponential decline, clearance being
10 10 times larger than cardiac output and with a distribution volume of 5.8 l kg⁻¹ [31]. In the
11 present study, the effect half-lives of the studied organic nitrites are very short (approximately
12 1.5-3.0 min), as can be seen by the off response for glycerol-NO and PDNO in Figure 3. The
13 short half-lives of organic nitrites simplify the use of them in an intensive care setting, since
14 intravenous infusion of them will be highly controllable and therefore safe and easy to
15 manage.

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37 The present work introduces a method for analyzing inorganic and organic nitrites in HPLC
38 coupled to a nitrite reduction system. The nitrite reduction system alone has previously been
39 used in our laboratory in for example studying NO release from guinea-pig colon [32] and
40 from rabbit corpus cavernosum [33]. Sen *et al.* [34] used a partly similar system (HPLC
41 coupled to nitrite reduction) to analyze inorganic nitrite in food, saliva and urine. One
42 development in the present study compared to Sen *et al.* [34] is the use of gradient elution in
43 the HPLC. The method offers both high sensitivity (at least 10 pmoles, by the
44 chemiluminescence) and specificity (different retention times by HPLC). We used the method
45 for identifying and quantifying formed organic nitrites when NO gas was reacted with
46 alcohols and carbohydrates, especially the method was of great use for the organic nitrites that
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1 could not be identified with GC-MS or LC-MS. When using this method some caution must
2 be taken in that some other compounds will give a signal in the ozone reaction (e.g. ethers)
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4 and that the nitrite reduction system can reduce both inorganic nitrite and O-nitrosylated
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7 compounds, as well as N-nitrosylated and S-nitrosylated compounds [35].
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10 By using GC-MS and LC-NO, the reaction product between propanol and NO gas was
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12 identified to be propyl nitrite, a previously known organic nitrite. By using the same methods
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14 the reaction products between NO gas and 1,2-propanediol were identified to be the
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17 mononitrites of 1,2-propanediol (2-hydroxy propyl nitrite and 2-hydroxy-1-methylethyl
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20 nitrite). Although we were not able to identify the reaction products with GC-MS or LC-MS
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22 of the other NO donor solutions it is most likely that these NO donor compounds also are the
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25 organic mononitrites of the carrier molecules. The evidences are: 1) They were produced with
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27 the same method that produced organic nitrites unequivocally identified by GC-MS; 2) They
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30 showed similar NO donor capabilities *in vivo*; 3) The number of reaction products was
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32 associated with the number of hydroxyl groups in the carrier molecule. 4) The UV absorbance
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35 maxima were around 219-234 nm for all the reaction products which is around the same UV
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38 absorbance as authentic organic nitrites have as shown in this study and previously [36], and
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40 they exhibited characteristic vibrational spectra; 5) They released NO in the nitrite reduction
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42 system similar to authentic organic nitrites. 6) The starting material for the reaction was
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45 known.
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47 Most previously known organic nitrites and that are commercially available include
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50 mononitrites of monohydric alcohols. Furthermore it has been shown that peroxyxynitrite is
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53 capable of nitration (adding NO₂) and nitrosation (adding NO) of glycerol thus forming
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56 glycerol mononitrate and glycerol mononitrite [37]. Similar reactions between peroxyxynitrite
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59 and glucose, fructose and glyceraldehyde have been suggested. Although the reaction
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62 products were not identified they displayed biological activities similar to organic nitrites or
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1 organic nitrates [37, 38]. It has also been suggested that glucose scavenges NO, particularly in
2 hyperglycemia, and that treatment of glucose with acidified sodium nitrite led to
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4 monoaddition of NO to the glucose molecule [39].
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7 The present study does not aim to elucidate the mechanism by which the organic nitrites were
8 formed in the reaction with NO gas. There are several classical ways to generate organic
9 nitrites which include reaction of alcohols with nitrous acid or other nitrosating agents such as
10 nitrosyl chloride and nitrosonium salts [40, 41] or nitrosyl exchange between an alcohol and
11 an alkyl nitrite [29]. The latter principle is interesting for the present study as that means that
12 in a compound with several hydroxyl-groups there will be an equilibrium and thus a mixture
13 between the mononitrites of that compound and therefore it will not be possible to produce
14 one specific organic mononitrite from for example 1,2-propanediol [29]. Grossi and Strazzari
15 [42] produced cycloalkyl nitrites from the reaction of NO gas with parent cycloalkyl alcohols
16 in the presence of oxygen. They suggested that the reaction in this situation is not through the
17 NO molecule itself but rather from nitrous anhydride (N_2O_3) which is formed in the reaction
18 between NO and molecular oxygen ($4 NO + O_2 \rightarrow 2 N_2O_3$). From the present work we can
19 not determine the exact mechanism for the formation of organic nitrites in our system, and it
20 is difficult to exclude trace amounts of oxygen in the reaction chamber. Therefore the reaction
21 may involve a direct action of NO on the hydroxyl groups of the carrier molecules or it may
22 involve a nitrosating agent such as N_2O_3 (formed by the reaction of NO gas and trace amounts
23 of oxygen) or it may be another, for us unknown, mechanism.
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49 We conclude that new organic nitrites suitable for intravenous administration are produced by
50 reacting NO gas and certain hydroxyl-containing compounds in aqueous solutions. In this
51 work we have also developed a new method where liquid chromatography is coupled to nitrite
52 reduction, which can be of great use, due to high sensitivity, in identifying possible
53 endogenous organic nitrites in body fluids. Selectivity of different organic nitrites to the
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1 pulmonary and systemic circulation, respectively, is dependent on the relative polarity of the
2 specific organic nitrite. The nature of organic nitrite will also determine the tendency for
3 methaemoglobin formation. Thus, by careful choice of NO donor molecule methaemoglobin
4 formation may be avoided in therapeutically active doses. The present study shows that
5 PDNO is relatively more selective to the lung compared with one clinically used NO donor,
6 nitroglycerin, which should encourage future investigations on organic nitrites in disease
7 models of pulmonary hypertension. We thus believe that the present study will stimulate
8 further research in the field of organic nitrites as NO donors.
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23 **Acknowledgements and role of funding sources**

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33 support and were not involved in any part of the conduct of the research.
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45 **Statement of conflicts of interest**

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51 The authors (except ML) have filed patent applications on organic nitrites. LEG is a minority
52 shareholder and member of the board of directors of Aerocrine AB (publ.) and is an inventor
53 of patents concerning the use of exhaled nitric oxide.
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1 **Tables**

2 **Table 1** Overview of the formation and the identification of organic nitrites when reacting
 3 deoxygenated aqueous solutions of parent compounds with NO gas.
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6 Parent compounds	NO donation	UV absorbance	NO release	Identified by
	<i>in vivo</i>	in HPLC	in LC-NO	GC-MS
7 1-Propanol	+	+*	+ [#]	+
8 1,2-Propanediol	+	+	+	+
9 Glycerol	+	+	+	
10 Glucose	+	+	+	
11 Ethanol		+*	+ [#]	
12 Isobutanol	+	+*		
13 Butanol		+*		
14 Sucrose	+	+		
15 2-Propanol	+			
16 1,3-Propanediol	+			
17 2-Deoxyribose	+			
18 1-O-Methyl-2-deoxyribose	+			
19 Ribose	+			
20 6-Deoxygalactose	+			
21 Galactose	+			
22 Fructose	+			
23 Sorbitol	+			
24 Mannitol	+			
25 Lactobionic acid	+			
26 Polyethylene glycol	+			
27 Inulin	+			
28 Dextran	+			
29 Fucoidan	+			
30 2-Amino-1,3-propanediol	–			
31 3-Amino-1,2-propanediol	–			
32 Lactic acid	–			
33 Alanine	–			
34 Glycine	–			
35 Glucosamine	–			

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37 The capacity of NO donation *in vivo* was determined positive if both exhaled NO increased
 38 and systemic blood pressure decreased (+). – denotes no NO donation capacity *in vivo*. UV
 39 absorbance in HPLC was denoted (+) if treatment with NO gas of parent compounds resulted
 40 in new chromatographic peaks exhibiting characteristic absorbance around 225 nm and
 41 vibrational absorbance in the range 330-370 nm [36]. *denotes that the new chromatographic
 42 peaks in HPLC had the same retention time and UV characteristics in HPLC as the
 43 corresponding authentic and commercially available organic nitrite. NO release in HPLC
 44 coupled to online nitrite reduction (LC-NO) was indicated (+) if new chromatographic peaks
 45 also were confirmed to release NO in the nitrite reduction system. [#] indicates that authentic
 46 organic nitrite of the parent compound also released NO at identical retention time in the LC-
 47 NO. Some of the formed compounds (+) were identified with gas chromatography-mass
 48 spectrometry (GC-MS). Non-filled spaces in the table indicate that the corresponding analysis
 49 was not performed for that compound in that system. Absence of markers [#] and * means that
 50 commercial reference compounds were not available.
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Figure captions

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5 **Figure 1.** Ventilated and anesthetized rabbit. Exhaled NO (FENO, panel A) and
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7 methaemoglobinemia (Met Hb, panel B) during infusion ($0.5 \text{ ml kg}^{-1} \text{ min}^{-1}$) of first NO gas
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9 dissolved in saline and then NO gas dissolved in lipid emulsion, as indicated by horizontal
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11 bars. Endogenous NO production was inhibited (L-NAME 30 mg kg^{-1}) by an initial infusion
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13 at time 20 min, also indicated by a horizontal bar.
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Figure 2. Ventilated anaesthetized rabbits (n=4-5). Dose-response curves (panel A) of exhaled NO (FENO) and mean systemic arterial pressure (MAP) at several infusion rates of glycerol-NO (25% glycerol in saline, v/v, treated with NO gas and identified by LC-NO to contain glyceryl nitrites). Panel B shows the correlation between the increase in FENO and change in MAP (% of control). The fitted non-linear curve has the format of $y=y_0+ae^{-bx}$, where y is change in MAP (% of control), x is increase in FENO and y_0 , a and b are constants. Note that for infusions that increased FENO with less than 20 ppb, the relationship is linear between increase in FENO and decrease in MAP.

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Figure 3. Ventilated and anesthetized rabbits (n=3-5). Exhaled NO (FENO, panels A and B), mean arterial blood pressure (MAP, panels C and D) and heart rate (HR, panels E and F) during an intravenous infusion for 10 min of glycerol-NO (panels A, C, E; 25% glycerol in saline, v/v, treated with NO gas and identified by LC-NO to contain glyceryl nitrites; 30 $\mu\text{l kg}^{-1} \text{min}^{-1}$) or PDNO (panels B, D, F; 25% 1,2-propanediol in saline, v/v, treated with NO gas and identified by GC-MS and LC-NO to contain the 1,2-propanediol nitrites; the dose was quantified to 600 $\text{nmol kg}^{-1} \text{min}^{-1}$).

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Figure 4. LC-NO, i.e. HPLC (panel A) and reflux NO/nitrites analysis (panel B) for identification of organic nitrites. Chromatogram obtained at 227 nm (panel A) and nitrite content (panel B) of 2.5 μ L of NO-treated 1,2-propanediol solution (25% in saline, v/v), injected at time 0. Isocratic elution with 10 mM ammonium formate on a C₁₈ column (ReproSil-Pur 120 C₁₈-AQ, 150x4 mm, 5 μ m). The three major peaks are marked I, II and III. Peak I corresponded to the retention time of NO/inorganic nitrite. Peak II and III had major UV absorbance maxima at 225 and 226 nm respectively and also exhibited weak but detectable vibrational spectra in the region 330-370 nm.

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Figure 5. LC-NO, i.e. HPLC (panel A) and reflux NO/nitrites analysis (panel B) for identification of organic nitrites. Chromatogram obtained at 227 nm (panel A) and nitrite content (panel B) of 2.5 μ L of NO-treated glycerol solution (25% in saline, v/v), injected at time 0. Isocratic elution with 10 mM ammonium formate on a C₁₈ column (ReproSil-Pur 120 C₁₈-AQ, 150x4 mm, 5 μ m). The three major peaks are marked I, II and III. Peak I corresponded to the retention time of NO/inorganic nitrite. Peak II and III had major UV absorbance maxima at 226 nm and also exhibited weak but detectable vibrational spectra in the region 330-370 nm.

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Figure 6. LC-NO, i.e. HPLC (panel A) and reflux NO/nitrites analysis (panel B) for identification of organic nitrites. Chromatogram obtained at 227 nm (panel A) and nitrite content (panel B) of 2.5 μ L of NO-treated glucose solution (60% in saline, w/v), injected at time 0. Isocratic elution with 10 mM ammonium formate on a C₁₈ column (ReproSil-Pur 120 C₁₈-AQ, 150x4 mm, 5 μ m). The five major peaks are marked I-V. Peak I corresponded to the retention time of NO/inorganic nitrite. Peak II-V had major UV absorbance maxima at 226, 223, 234 and 224 nm respectively and also exhibited weak but detectable vibrational spectra in the region 330-370 nm.

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Figure 7. Gas chromatography with mass detection (Agilent DB-1701 column and electron impact ionisation) after split fraction (1:5) injection at time=0 of 0.5 μ l de-aerated approximately 100% 1,2-propanediol treated with NO gas. Shown is a total ion chromatogram for the m/z range 20-320. Peaks denoted 1 and 2 were further characterized with mass spectra in Figures 8-9. The peak denoted 1,2-propanediol was identical with the corresponding entry in the Wiley mass spectral library, whereas spectra for peak 1 and 2 were not found in the Wiley library or elsewhere.

Figure 8. Electron impact ionisation spectrum of peak 1 from a similar chromatography as in Figure 7, supporting the postulated structure of one of the two 1,2-propanediol nitrites (2-hydroxy propyl nitrite) corresponding to the first of the two peaks in Figure 7.

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Figure 9. Electron impact ionisation spectrum of peak 2 from a similar chromatography as in Figure 7, supporting the postulated structure of one of the two 1,2-propanediol nitrites (2-hydroxy-1-methylethyl nitrite) corresponding to the second of the two peaks in Figure 7.

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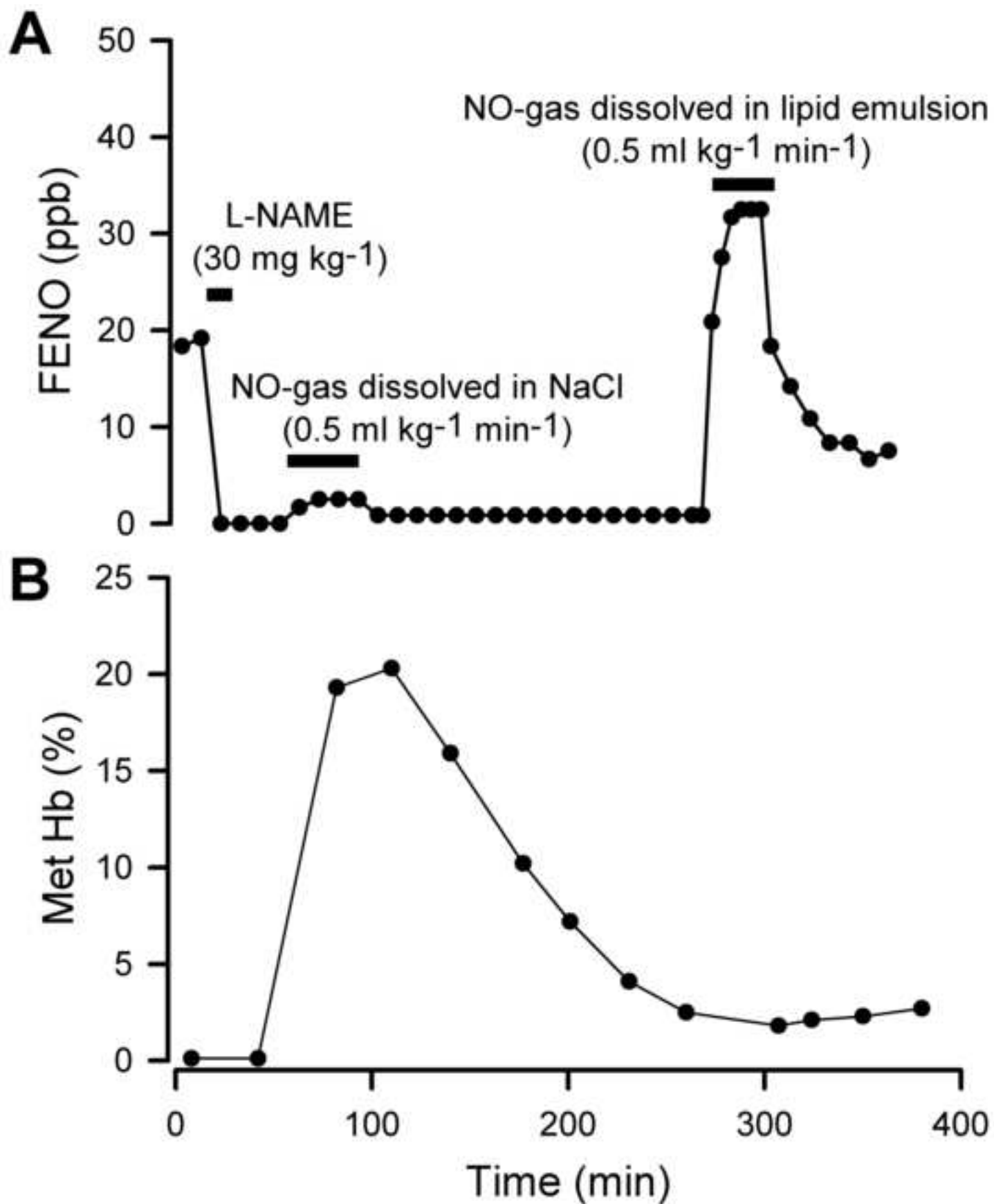
Figure 10. Anesthetized and ventilated rabbits subjected to intravenous infusions of different organic nitrite NO donor solutions. The MAP/FENO indices were calculated from experiments with intravenous infusions of NO donor solutions by dividing the change in MAP (% of baseline) with increase in FENO (delta ppb compared with baseline, supplementary Table A.1). Each point represents one NO donor (Table 1 and supplementary Table A.1). Shown is also the best linear regression found (expression 1 in supplementary Table A.4) by exploring the dependent variable Log (-MAP/FENO index) against the independent variable relative polarity (PSA/MSA in %, calculated as polar surface area divided with molecular surface area) for the different NO donors (Refer to supplementary Table A.2 for the list of compounds included) showing that relative polarity is a strong predictor for MAP versus FENO effects.

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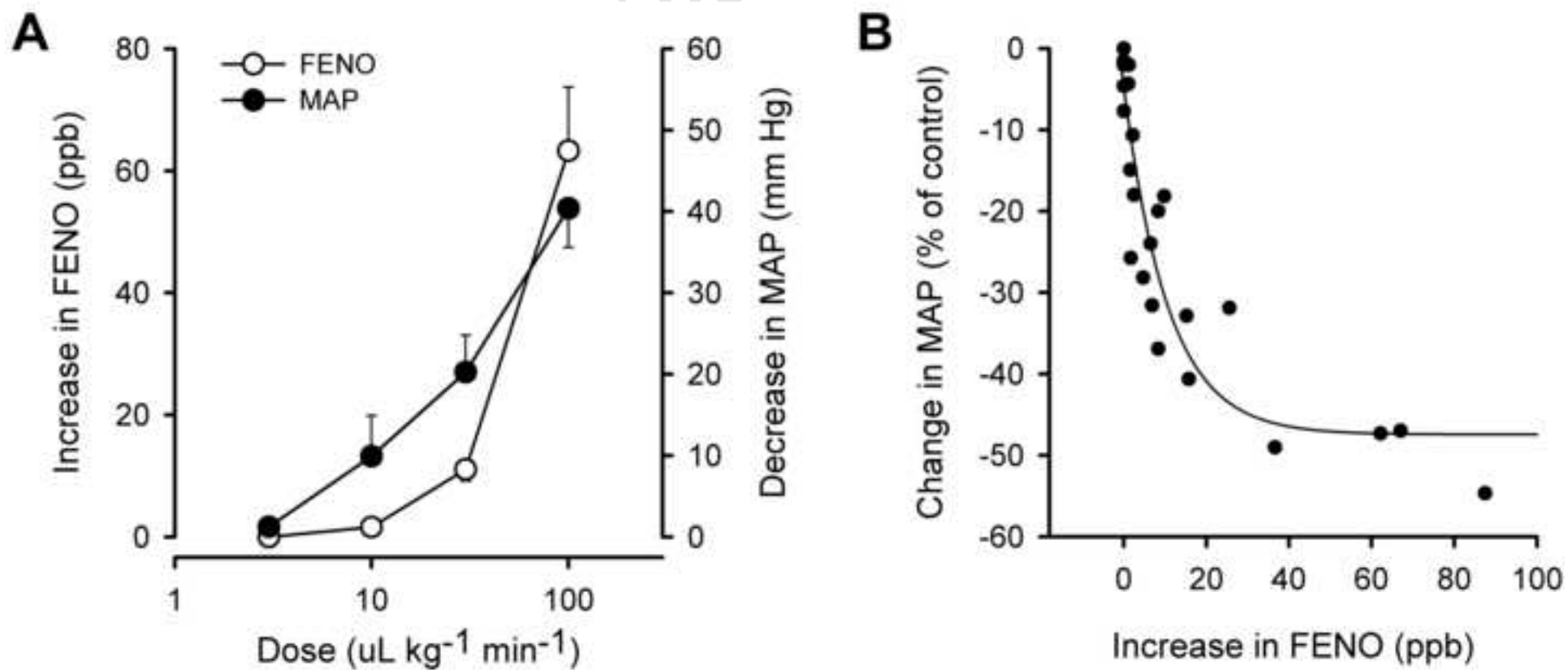
Figure 11. Ventilated and anesthetized rabbit. The effects of intermittent infusions of NO-treated 1,2-propanediol (PDNO, doses of 130 and 450 nmoles kg⁻¹ min⁻¹ respectively) on successively increased U46619-induced pulmonary hypertension. The stippled vertical fields indicate treatment with PDNO. Measured parameters were mean pulmonary artery pressure (mPAP, panel A), mean left atrial pressure (LAP, panel B), pulmonary vascular resistance (PVR, panel C), mean systemic arterial blood pressure (MAP, panel D), exhaled NO (FENO, panel E), cardiac output (CO, panel F).

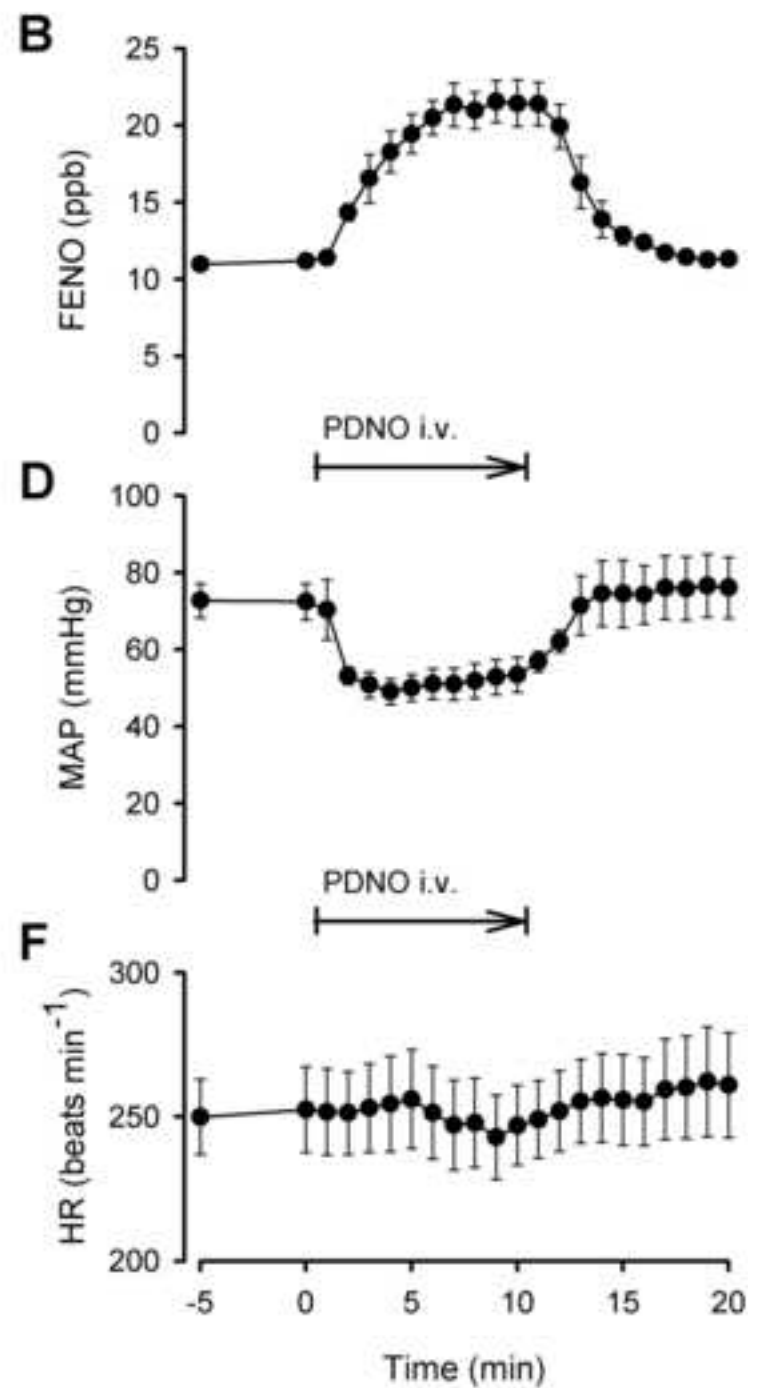
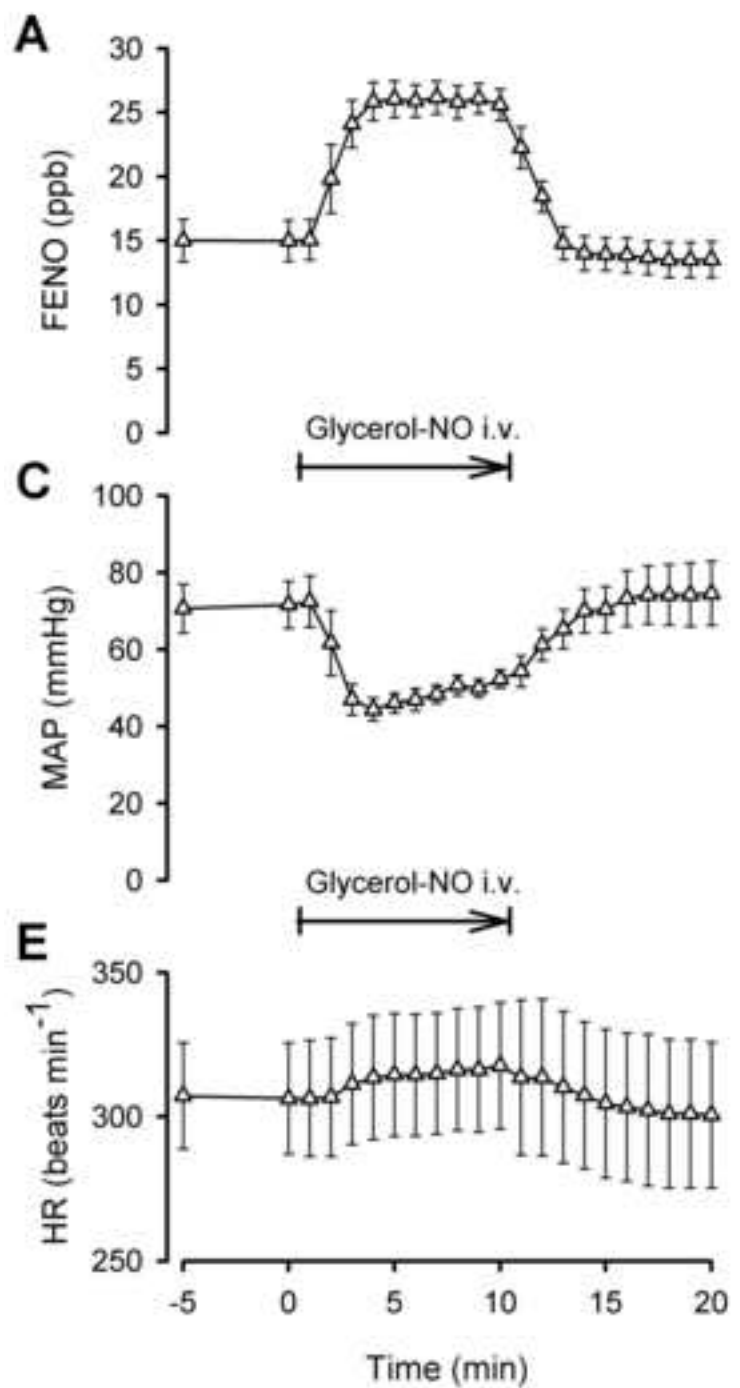
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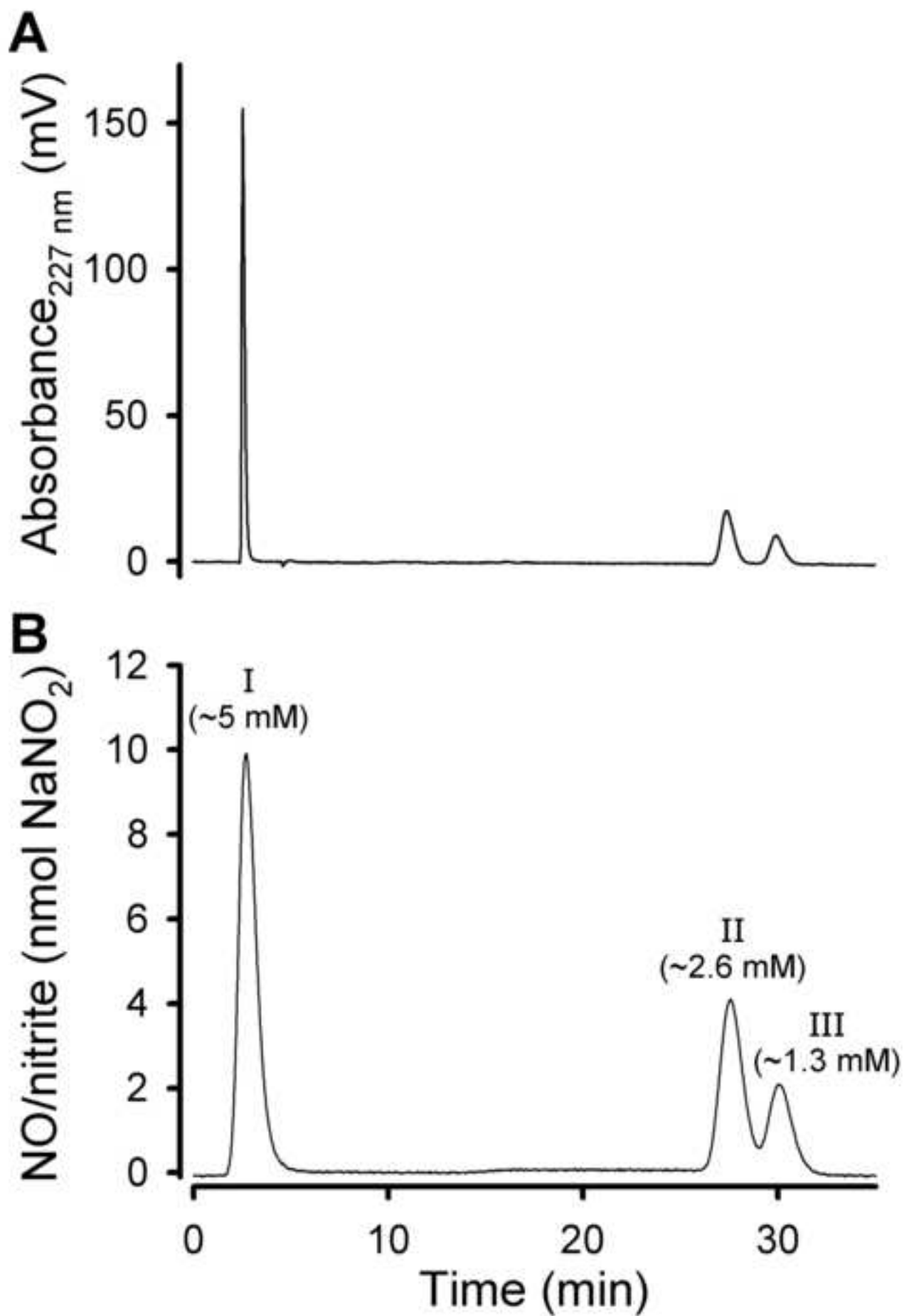
Figure 12. Ventilated and anesthetized rabbits (n=4). The effects of NO-treated 1,2-propanediol (PDNO), NO-treated glycerol (Glycerol-NO) and NO-treated glucose (Glucose-NO) in doses of 200, 400, 800 and 1200 nmoles kg⁻¹ min⁻¹ administered intravenously on U46619-induced pulmonary hypertension. Measured parameters were the concentration of NO in mixed exhaled gas (FENO, panel A) and pulmonary vascular resistance divided with systemic vascular resistance (PVR/SVR, normalised to the PVR/SVR index during U46619 infusion). The effects of nitroglycerin (not shown in the Figure for reasons of clarity) on FENO and PVR/SVR index were similar to the effects of glucose-NO and glycerol-NO respectively. † indicates that n=2 for glucose-NO at 1200 nmoles kg⁻¹ min⁻¹ due to severe systemic hypotension.

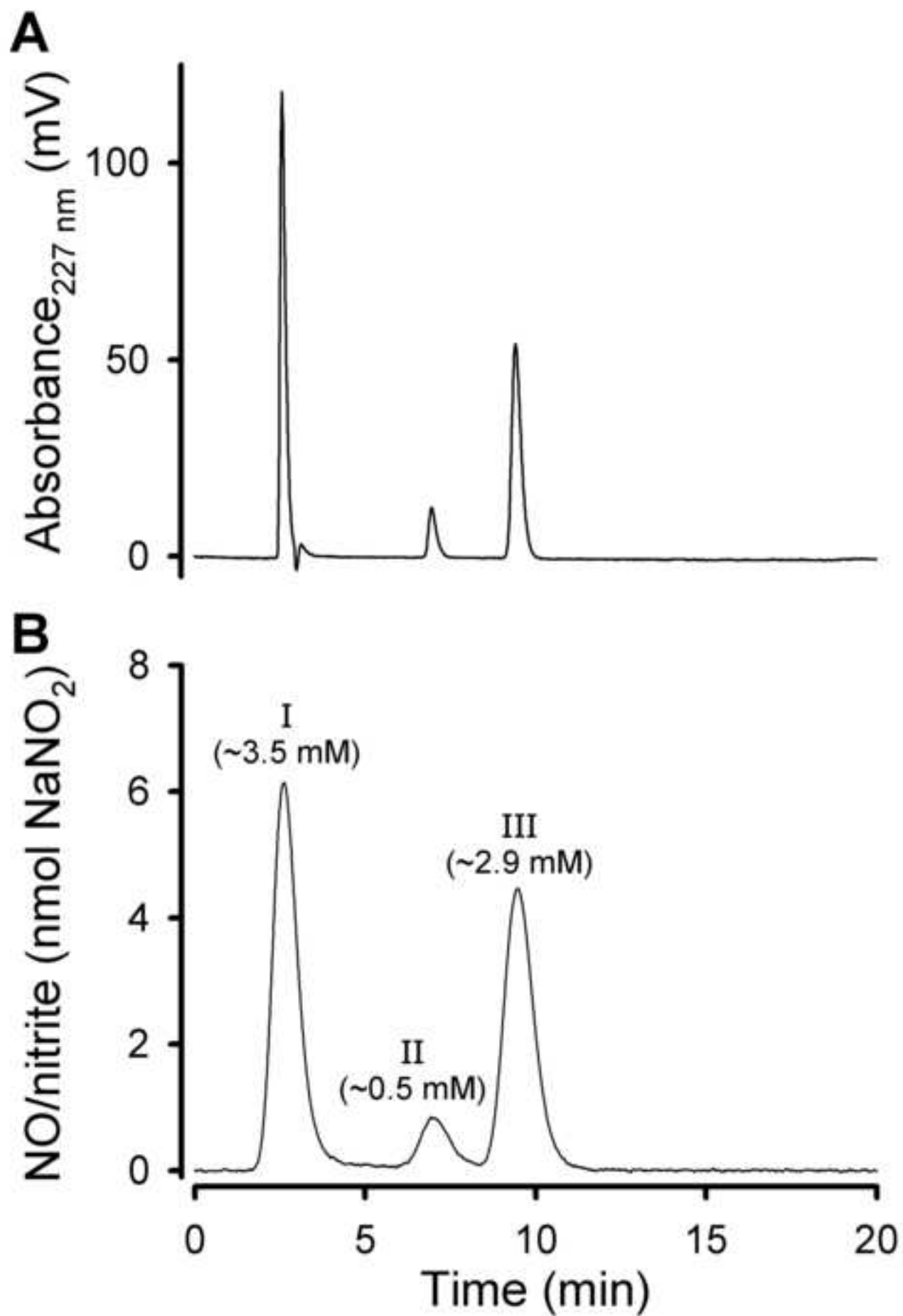


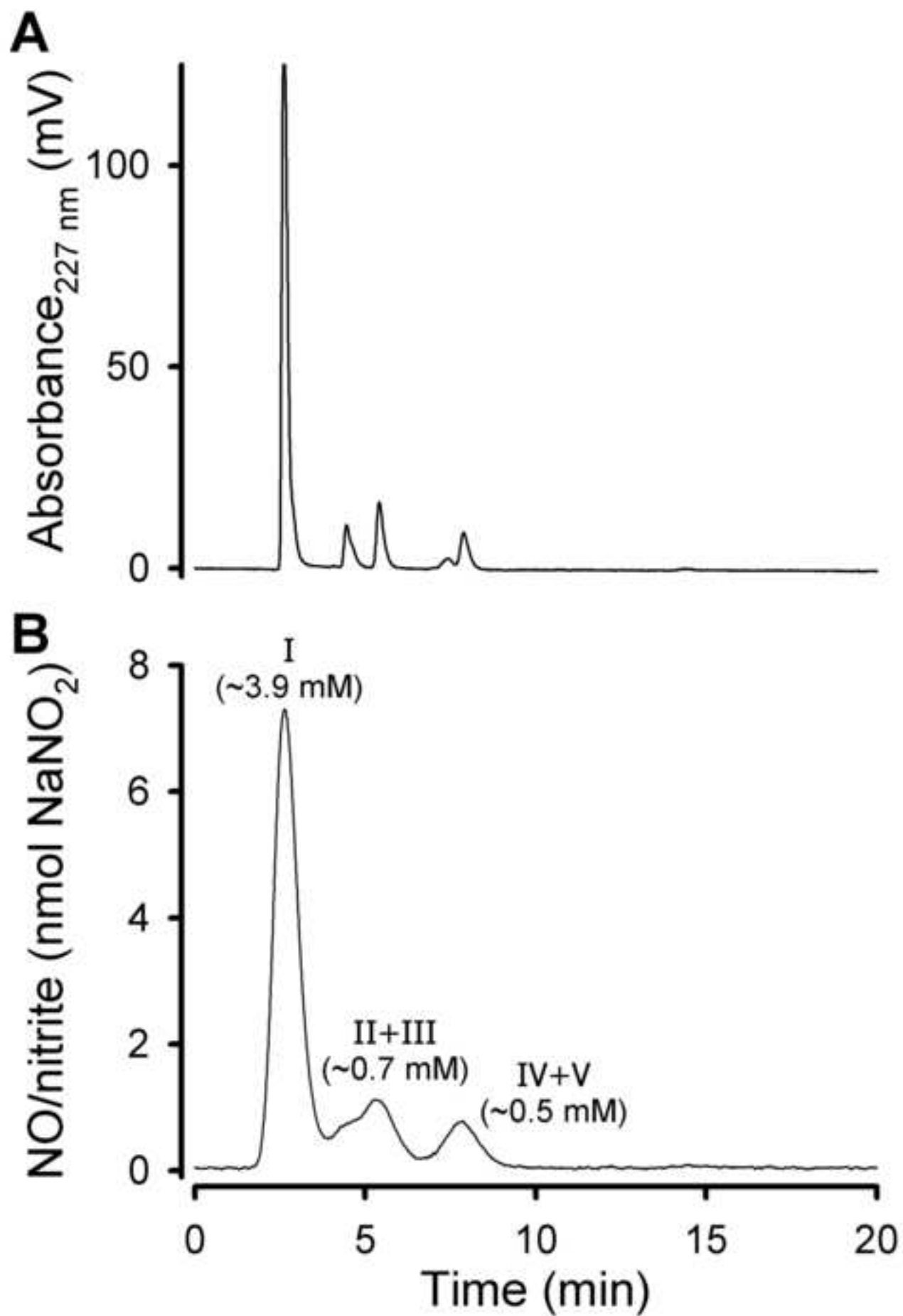
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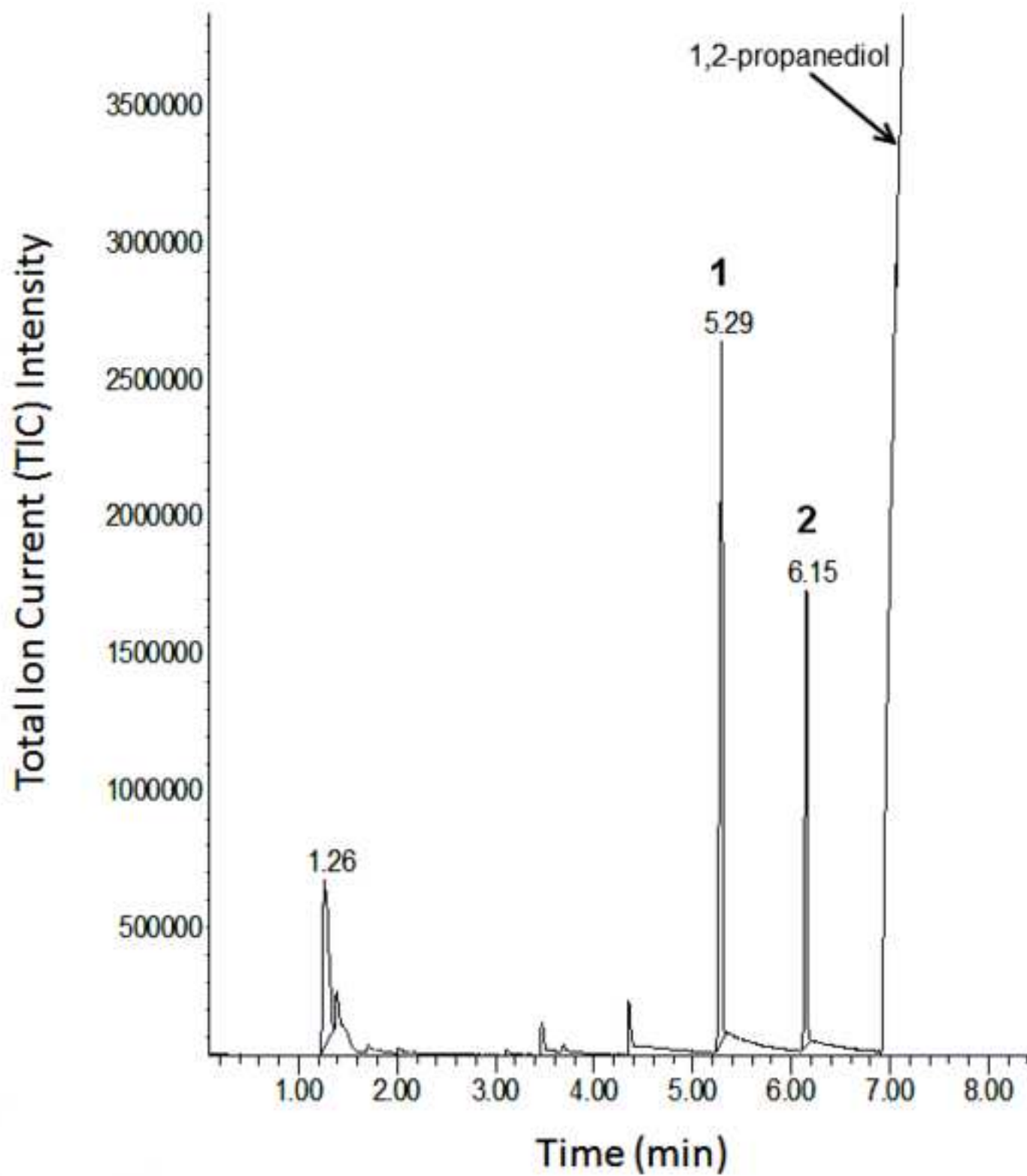


Figure 8

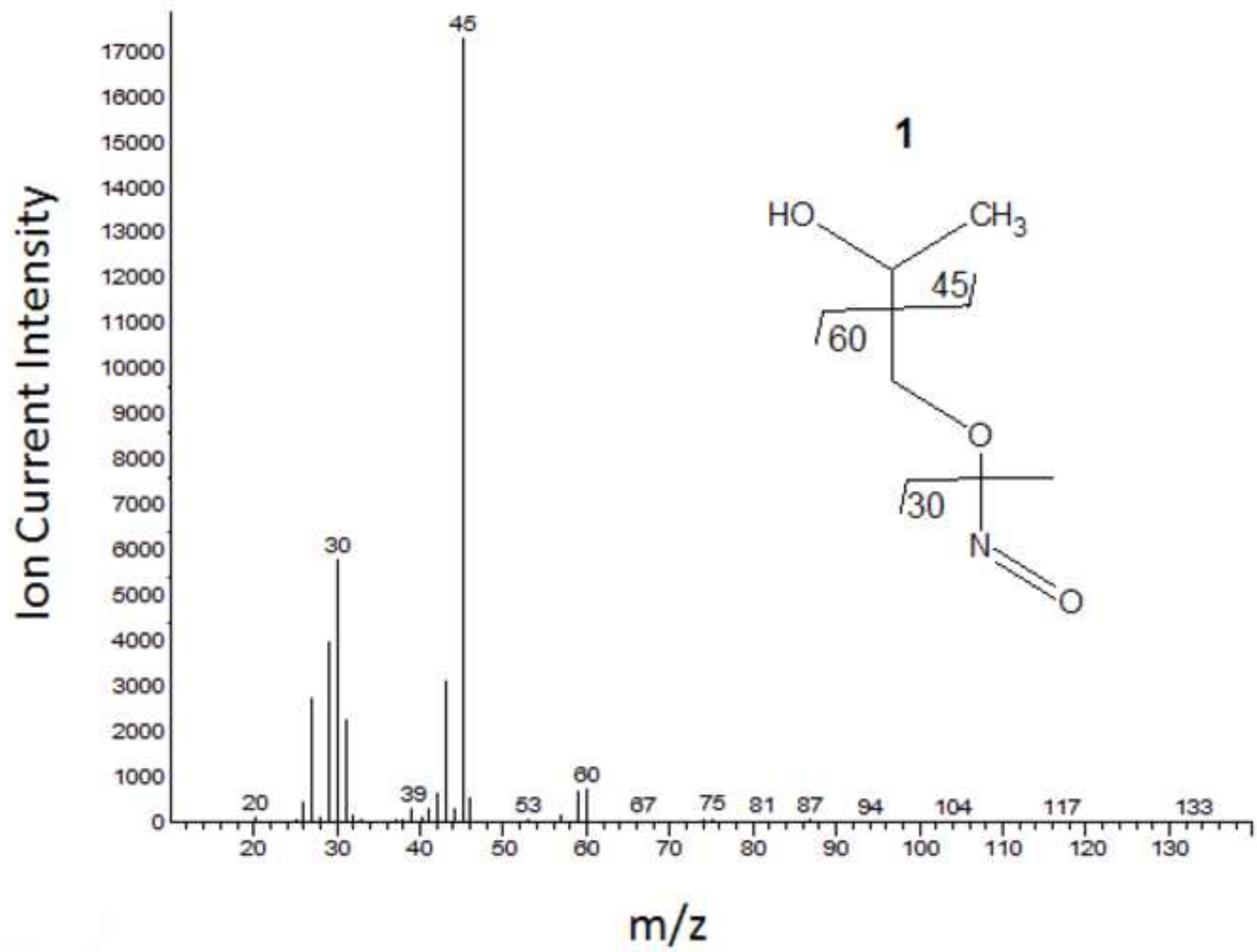


Figure 9

