CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER: NDA 20845

CLINICAL PHARMACOLOGY AND BIOPHARMACEUTICS REVIEW(S)

NOV 10 1999

CLINICAL PHARMACOLOGY AND BIOPHARMACEUTICS REVIEW

NDA: 20845
IND: [redacted]

SUBMISSION DATES: May 24, 1999
August 13, 1999

DRUG NAME: Nitric Oxide gas for inhalation
INOmax™

FORMULATION: 100 & 800 parts per million (ppm)
DOSE: 20 ppm up to 14 days

INDICATION: hypoxemic respiratory failure in the term and near-term newborn in conjunction with mechanical ventilation

SPONSOR: INO Therapeutics, Inc.

REVIEWER: B. Nhi Nguyen, Pharm.D.

SUBMISSION: New Drug Application

SUMMARY

The sponsor is seeking the approval of inhaled nitric oxide (I-NO), 100 and 800 parts per million (ppm), for use in hypoxemic respiratory failure in the term and near-term newborn. The sponsor submitted a paper NDA that cites, for safety and efficacy, one large randomized, controlled, multicenter trial conducted in neonates with hypoxic respiratory failure, one large randomized, double-blind, placebo-controlled trial conducted in neonates with primary pulmonary hypertension (PPHN), and some other studies and case reports that include a total of 188 neonates in whom I-NO was used for PPHN. A randomized, double-blind, placebo-controlled study examining safety in 155 neonates with PPHN was also cited in the label. Additionally, eight studies were cited in the pharmacokinetic section of the label. One of these was the large randomized, double-blind, placebo-controlled trial conducted in neonates with PPHN. In this study, the investigators reported methemoglobin and nitrogen dioxide (NO_2) concentrations. The other seven studies included healthy adults in the treatment arm. One of these seven studies also included a severe heart failure patient treatment arm.

Inhaled NO (I-NO), a gaseous blend of nitric oxide (0.8%) and nitrogen (99.2%), is a potent, local and selective pulmonary vasodilator. I-NO decreases pulmonary artery pressure and increases the partial pressure of arterial oxygen (PaO_2) leading to increased blood flow to the lungs. I-NO enhances V/Q matching by redistributing pulmonary blood flow away from lung regions with low ventilation/perfusion (V/Q) ratios towards regions with normal ratios.

Between 75-90% of I-NO is absorbed by the alveoli. After inhalation, NO immediately binds to hemoglobin. This subsequently produces methemoglobin and nitrate. These are the primary products that enter the systemic circulation. Methemoglobin is primarily metabolized by

methemoglobin reductase to form hemoglobin and nitrate. Thus, nitrate is the final metabolite formed from nitric oxide by all pathways. Nitrates are eliminated principally by the kidney.

RECOMMENDATION

The application does not completely fulfill the requirement of the Office of Clinical Pharmacology and Biopharmaceutics since the pharmacokinetic information in the target population was not submitted. Comments 1-8 and the labeling comment should be forwarded to the sponsor.

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BACKGROUND

The sponsor is seeking the approval of inhaled nitric oxide (I-NO), 100 and 800 parts per million (ppm), for use in hypoxemic respiratory failure in the term and near-term newborn. I-NO is a potent, local and selective pulmonary vasodilator that acts from the outer surface of the pulmonary vessels. I-NO decreases pulmonary artery pressure and increases the partial pressure of arterial oxygen (PaO_2) leading to increased blood flow to the lungs. I-NO enhances ventilation/perfusion (V/Q) matching, the appropriate contact between alveolar gas and pulmonary capillary blood, by redistributing pulmonary blood flow away from lung regions with low V/Q ratios towards regions with normal ratios.

Structure

$\cdot \bar{N}{=}\bar{O} :$ nitric oxide

The recommended safe and effective dose is 20 ppm of INOmax as a constant inhalation for up to 14 days until the underlying oxygen desaturation has resolved and the neonate is ready to be weaned from INOmax therapy.

Formulation

I-NO is a gaseous blend of nitric oxide (0.8%) and nitrogen (99.2%)

Deliver System

The sponsor recommends I-NO be delivered through an FDA approved NO delivery device, such as INOvent™ delivery device. Precise monitoring of inspired NO and NO_2 should be instituted, using a properly calibrated analysis device with alarms. This system should be calibrated using a precisely defined calibration mixture of NO and NO_2, such as INOcal™. Sample gas for analysis should be drawn before the Y-piece, proximal to the patient.

SUMMARY OF BIOAVAILABILITY / PHARMACOKINETICS

Bioavailability

Food effects

It is possible that foods rich in nitrates may interact with I-NO. However, this has not been studied and may not be relevant to the neonatal population. The effect of parenteral nutrition in neonates is unknown.

Pharmacokinetics

Absorption / Uptake and Distribution

Absorption of I-NO has ranged from 66% to 99% in healthy adult volunteers. The majority of I-NO traverses the pulmonary capillary bed where it binds with oxyhemoglobin (60-100% oxygen saturated) to form methemoglobin and nitrate (NO_3-). I-NO can also combine with deoxygenated hemoglobin to form nitrosylhemoglobin which is rapidly converted into nitrogen oxides and methemoglobin upon exposure to oxygen. NO also undergoes direct conversion to nitrite and

nitrogen dioxide with subsequent oxidation to nitrate and methemoglobin in biological fluids. Thus, the end products of I-NO that enter the systemic circulation are predominantly methemoglobin and nitrate.

Metabolism
See Appendix III for full metabolic pathway.

The NADH-methemoglobin reductase system accounts for 67-95% of the conversion of methemoglobin back to hemoglobin in adults. Neonates have reduced NADH-methemoglobin reductase activity compared with adults. Because this is the primary route of metabolism of methemoglobin in humans, caution is advised when extrapolating adult data to neonates. Fetal hemoglobin may also be more prone to oxidation than adult hemoglobin.

Methemoglobin disposition has been measured in neonates with PPHN during the first 12 hours of exposure to 0, 5, 20, and 80 ppm I-NO. All methemoglobin concentrations were significantly elevated above placebo, but only the 80 ppm dose group was clinically elevated and associated with methemoglobinemia.

Elimination
In adults, nitrate is the predominant NO metabolite excreted in the urine, accounting for >70% of the I-NO dose. Nitrates and nitrites are eliminated principally by the kidney at rates approaching the glomerular filtration rate. Thus, serum nitrogen oxide (sNOx) concentrations would be expected to increase when renal function is impaired. The elimination of nitrates in neonates is presently unknown.

Plasma level-dose relationship
In healthy adult volunteers, there is a dose-dependent increase in plasma nitrate and methemoglobin. In adults with severe heart failure, there is a dose-related increase in plasma nitrate in the systemic and pulmonary arteries, with significantly lower levels in the pulmonary arterial plasma compared to the systemic plasma. Twenty minutes after cessation of I-NO, the plasma nitrate had dropped in most patients.

Nitrate plasma concentrations increase with 24 hours of I-NO, while nitrite (NO_2-) plasma concentrations remain unchanged.

Special populations
Renal impairment
No studies have been conducted in renally impaired subjects.

Hepatic impairment
No studies have been conducted in hepatically impaired subjects.

Drug interactions
Drug interactions studies have not been conducted in neonates.

Formulations

The formulation being marketed will be supplied in aluminum cylinders containing nitric oxide gas in 100 or 800 ppm in nitrogen. A constant inhalation of 20 ppm will be the I-NO dose delivered. The supplies used in the cited studies ranged from 100 to 2000 ppm, and the delivered doses ranged from 5 to 512 ppm. None of the studies cited used the supply to be marketed to deliver the dose to be approved. Even the two studies sponsored by INO Therapeutics, Inc. used the 400 ppm cylinder to deliver a dose of I-NO 20 ppm. The impact of the different supply concentrations used to obtain the delivered dose is unknown.

Delivery system

Because most of the studies submitted for this NDA were not sponsored by INO Therapeutics, Inc., different delivery systems were used. Additionally, several studies did not provide details of their delivery system. Of those studies that did provide details, the description of the delivery systems ranged from two mask flow regulators, to a tight fitting mask in a non-rebreathing system to a leak free face mask connected to a non–rebreathing valve. It is unknown if these different delivery systems influence the amount of delivered I-NO.

Assay

COMMENTS TO THE SPONSOR

LABELING COMMENT

1. It should be clearly stated that the uptake, distribution and elimination were determined in primarily healthy adults.

Clinical pharmacology briefing held on November 10, 1999.
(Fadiran, Hepp, Huang, Marroum, Mehta, Nguyen, Robbie, Selen, Venitz were present)

/S/ 11/10/99

B. Nhi Nguyen, Pharm.D.

RD/RT: Patrick Marroum, PhD /S/ 11/10/1999

CC: NDA 20845, HFD-110, HFD-860 (Mehta, Nguyen), CDER document room

APPENDIX II: CITED STUDIES

A SIMULTANEOUS SINGLE BREATH MEASUREMENT OF PULMONARY DIFFUSING CAPACITY WITH NITRIC OXIDE AND CARBON MONOXIDE

INVESTIGATORS: Borland CDR, Higenbottam TW
STUDY SITE: not specified
CITATION: Eur Respir J 1989; 2: 56-63.
STUDY DATES: not specified
OBJECTIVES: To determine the diffusion and rate of combination of carbon monoxide (CO) with hemoglobin and of NO with oxyhemoglobin.
FORMULATION: The inspired gas mixture containing approximately 40 ppm of NO in % helium, % CO, % O_2 in nitrogen was made up in the inspirate bag immediately before each test by adding L of 1000 ppm NO in N_2 to 7L of the standard gas transfer test mixture.
STUDY DESIGN: open label
POPULATION: 13 healthy volunteers

PROCEDURE: No more than four single measurements of DL (maximal diffusing capacity) were made per person each day. The following were measured: forced expiratory volume in one second (FEV1), TLC (whole body plethysmograph), DLCO (pulmonary diffusing capacity measured by carbon monoxide), and DLNO (pulmonary diffusing capacity measured by nitric oxide). On each occasion, studies were performed at least two hours after a meal at the same time of day.

ASSAYS:

Diffusing capacity measurement. Standard single breath gas transfer equipment was used to sample the inhaled and exhaled gases for helium (He), CO, oxygen (O_2) and NO. The equipment was calibrated daily. Measurements of DLCO and DLNO were made simultaneously. A single breath DL measurement was performed during inspiration. During expiration, an alveolar sample was collected and immediately analyzed. The exact NO concentration at the time of the single breath measurement could be calculated knowing the time that had elapsed from filling the inspirate bag (t):

$$1/NO_t = (1/NO_o) \ / \ (1/2\ K(O_2)_t)$$

where k is the rate constant for oxidation of NO.

ANALYSIS: Calculation of DLCO and DLNO. DLCO and DLNO were calculated from inspired and alveolar concentrations by the standard method. Within and between day CV was

6.6% for DLCO and 6.4% for DLNO. The investigators assumed no alveolar back pressure for NO and an exponential rate of decline in alveolar NO in the calculation. These assumptions were later justified.

Measurement of alveolar NO at varying times of breathhold. Combined DLCO and DLNO measurements were carried out on two subjects but at different breath-hold times ranging from 4-10 seconds. The alveolar NO at each time interval was calculated as a fraction of the initial concentration.

Measurement of back pressure of NO and CO in smokers. In eight tobacco smokers and two nonsmokers alveolar concentrations of CO and NO were sampled following a 20 second breath hold at TLC. Alveolar concentrations were also measured in one smoker and one nonsmoker after rebreathing oxygen for 4 minutes. The smokers' daily tobacco consumption and last time smoked were recorded.

Additional measurements. DL at varying levels of lung volumes, alveolar oxygen concentrations and exercise were measured.

PHARMACOKINETIC RESULTS: The response of the NO analyzer was linear from 1-5 ppm but for 5-40 ppm a minor departure from linearity was noted, with a slight underestimate of NO concentration. Repeated analysis of the same concentration of NO was within 10% at 1 ppm and within 1% at 50 ppm. The time to 95% response after a stepwise increase in concentration was 24 seconds. No interference was observed with NO_2 or N_2O. The figure below shows that the decline in alveolar NO and CO concentration over time is logarithmic.

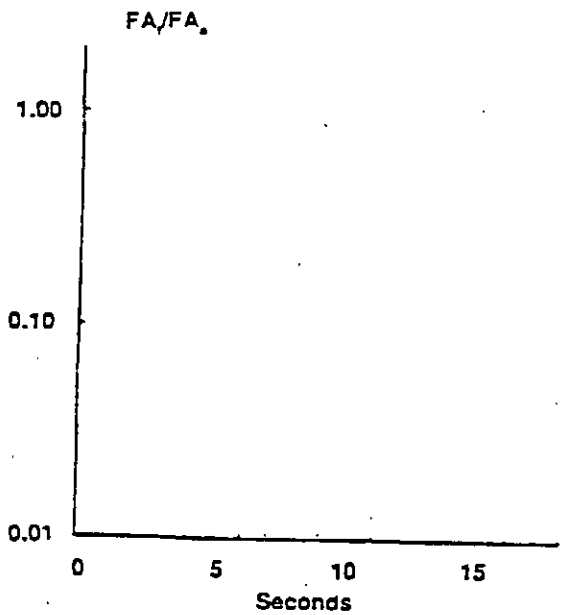


Fig. 1. – Decline in alveolar NO and CO with varying breath-hold times (one subject). Alveolar concentrations at each time (F_A) are expressed as a fraction of the initial alveolar concentration (F_{A_0}).

There was no alveolar back pressure for NO detected in either the two nonsmokers or the eight smokers after a 20 second breath-hold. This was despite the finding of alveolar CO concentrations ranging from 16-34 ppm in the smokers. No alveolar back pressure for NO could be obtained in the one smoker and one nonsmoker after 4 minutes of rebreathing O_2. The smoker's CO back pressure was 70 ppm at an alveolar oxygen concentration of 75%.

DLNO exceeded DLCO by a mean ratio of 4.3 ± 0.3 (SD). Table 1 below shows that the presence of CO or NO in the simultaneous single breath test gas mixture did not interfere with the uptake of either CO or NO.

Table 1. – Comparison of D_{LNO} and D_{LCO} measured together and separately

Subject	D_{LCO} alone*	D_{LCO} with NO	D_{LNO} alone	D_{LNO} with CO
1	13.3 (0.8)	14.3 (0.4)	57.6 (1.0)	59.6 (1.2)
2	11.9 (0.3)	11.9 (0.3)	57.9 (5.6)	55.2 (1.6)
3	13.7 (0.3)	13.5 (0.3)	53.5 (3.6)	61.0 (0.7)
4	12.0 (1.1)	12.0 (1.1)	49.4 (4.7)	50.2 (4.1)
5	8.9 (0.3)	8.7 (0.3)	47.5 (1.6)	42.6 (2.8)

*Breath-hold time of 10 s. The remaining values are measured with a breath-hold time of 7.5 s. Mean values (SD), three replicates ($mmol \cdot min^{-1} \cdot kPa^{-1}$).

The average DLCO was 11.6 ± 2.4 ml/min/mm Hg and the average DLNO was 49.1 ± 10.2 ml/min/mm Hg (table 2).

Table 2. – Average data from which D_{LCO} and D_{LNO} were calculated

	He%	CO% ppm	NO	O_2%	Breath-hold time s
Inhaled	12.3(1)	0.27(0.008)	38.9(13.8)	17.7(1.6)	7.3(0.4)
Exhaled	10.0(1)	0.11(0.02)	2.2 (1)		

V_A l	D_{LCO} $mmol \cdot min^{-1} \cdot kPa^{-1}$	D_{LNO} $mmol \cdot min^{-1} \cdot kPa^{-1}$
6.1 (1.5)	11.6 (2.4)	49.1 (10.2)

Mean values from 13 subjects, 3 replicates were taken (SD).

An increase in alveolar oxygen concentration from an average of 18.6 to 68.5% caused a mean fall in DLCO from 11.3 ± 1.2 to 6.5 ± 1.2 mmol/min/kPa, but DLNO was unchanged.

The reduction in alveolar volume, on average from 7 L to 3.9 L produced a significant mean fall in DLCO of 8%; 12.9 ± 1.6 to 11.9 ± 1.9 mmol/min/kPa. Carbon monoxide transfer coefficient (Kco) rose 48% from 1.9 ± 0.2 to 3.1 ± 0.2 mmol/min/kPa/L. The fall in DLNO was greater; 34% decline from 54.5 ± 7 to 38.6 ± 6.6 mmol/min/kPa ($p < 0.05$). NO transfer coefficient rose 24% from 7.9 to 10.1 mmol/min/kPa/L.

The increase in DLCO and DLNO is shown in Figure 2. With exercise the DLCO significantly increased 45%; 12.3 ± 0.7 to 19.5 ± 1.7 mmol/min/kPa. DLNO significantly increased 26% with exercise; 53.7 ± 2.2 to 70 ± 9.5 mmol/min/kPa.

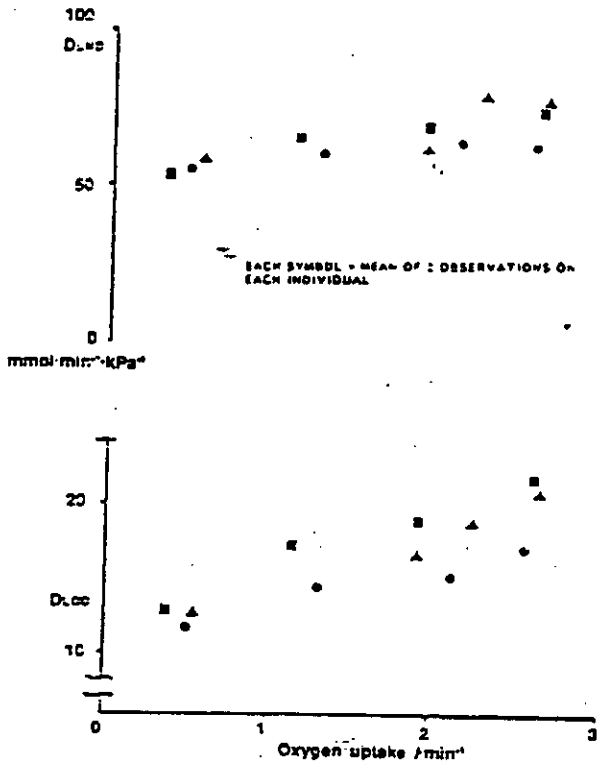


Fig. 2 – Increase in $D_{L_{NO}}$ and D_{LCO} with exercise (three subjects): Rest is represented by the lowest values of oxygen uptake; exercise is represented by the remaining higher values.

The rate of decline in concentration of NO in known oxygen concentrations was 7.1×10^{-10} $ppm^{-2}min^{-1}$. This compares favorably to a previously published figure of 7.6×10^{-10} $ppm^{-2}min^{-1}$.

CONCLUSION: These investigators demonstrated that DLNO is four times greater than DLCO at rest and at normal O_2 levels. Unlike DLCO, DLNO appears to be independent of hyperoxia, but DLNO appears to be more dependent on lung volume. DLCO and DLNO increase with exercise. Since NO reacts much faster with hemoglobin than CO, DLNO should be influenced much less by reacting with hemoglobin. This study supports the sponsor's claim that a large proportion of I-NO is absorbed systemically.

DETERMINATION OF LUNG CAPILLARY BLOOD VOLUME AND MEMBRANE DIFFUSING CAPACITY IN MAN BY THE MEASUREMENTS OF NO AND CO TRANSFER

INVESTIGATORS: Guenard H, Varene N, Vaida P
STUDY SITE: University of Bordeaux, Bordeaux France
CITATION: Respir Physiol. 1987; 70:113-120.
STUDY DATES: not stated
OBJECTIVES: The lung transfer (TL) for a given gas is related to the membrane diffusing capacity for the gas (Dm) and to the capillary blood volume (Qc). The objective of this study was to measure the Dm for NO, and determine Qc.
FORMULATION: The inspired mixture contained either 8 ppm NO or % CO with % He, % O_2 in N_2. To avoid transformation of NO oxidizing to NO_2, the mixture was prepared just before the experiments.
STUDY DESIGN: open-label
POPULATION: 14 healthy subjects (7 men, 7 women) that were either non or moderate smokers

PROCEDURE: NO and CO lung transfer values (TL_{NO} and TL_{CO}, respectively) were measured using the single breath technique. ***TL_{NO} measurement.*** The seated subject breathed through a mouth piece in a volumetric body plethysmograph. Inspired and expired gases were separated by means of a two-way valve outside the body box. A two-way tap was connected to the inspiratory port of the valve. One way was left open to room air, and the other way was connected to a 100 L rubber balloon containing a mixture with 21% O_2, 3% He, $8*10^{-4}$ % NO (8ppm), in N_2. The expiratory branch of the two-way valve was connected to two electric valves set in parallel. One of these was connected to a 1 L rubber balloon and was open during the time of the sample collection, while at the same time the other valve was closed.

During the subject's full expiration , the two-way tap was turned to the mixture. The subject made a full rapid inspired vital capacity and held his/her breath for 3 seconds. Then he/she made a full rapid expiration. The second liter of expirate was collected. The alveolar sample was taken automatically by electrically switching the expiratory valve. TL_{NO} was computed using the equation:

$$TL_{NO} = V_L / (PB-47)t * \ln FA_{NO}(0)/FA_{NO}(t), \text{ where}$$

V_L, lung volume during apnea
t, the effective breath-holding time
$FA_{NO}(0)$, the initial alveolar fraction
$FA_{NO}(t)$, the alveolar fraction of NO at the time of sampling.
The effective breath-holding time was taken to be the sum of the inspirate time, the real breath-holding time and the expirate time to the start of sampling.

TL_{CO} measurements. The inspired mixture contained 21% O_2, 2% He, 0.25 % CO in N_2. The breath holding time was 8 seconds. The sampling and computation of TL_{CO} was similar to that used for TL_{NO}.

SAMPLING: Five successive measurements, at five minute intervals, were made in the seated subjects to determine TL_{NO}. Five measurements were made at one day intervals to avoid underestimation of TL_{CO} due to the back pressure of CO.

ASSAY:

ANALYSIS: NO analysis of the alveolar gas was made immediately after collection from the expiratory valve to avoid any decrease in the NO fraction due to absorption by the balloon rubber wall. The fraction of He was then measured.

RESULTS: Results are summarized in table 1.

TABLE I

Left columns: age, height of the fourteen subjects (Subj.) of both sexes: men (M), women (W). Middle columns: mean values of lung volumes during CO and NO maneuvers ($V_{I_{CO}}$, $V_{I_{NO}}$), TL_{CO}, $\overline{TL}_{NO}$ in $ml \cdot min^{-1} \cdot Torr^{-1}$ and the respective coefficients of variation (standard deviation/mean). Right columns: mean values of membrane conductance in $ml \cdot min^{-1} \cdot Torr^{-1}$ and lung capillary volume (ml) divided by the coefficient $(1 - a/k)$ assumed to be close to 1.

Sex	Subj.	Age years	Height m		$V_{I_{CO}}$ L BTPS	$V_{I_{NO}}$ L BTPS	TL_{CO} mean	SD/m %	TL_{NO} mean	SD/m %	TL_{NO}/TL_{CO}	Dm_{CO}	$Qc/(1 - a/k)$
M	ZT	29	1.61		4.6	5.1	28.3	7.6	153.5	4.8	5.4	77.9	71.9
	PVj	37	1.75		6.5	7.05	30.5	8.1	125.6	11.9	4.1	63.8	95.3
	HG	42	1.80		6.7	7.4	33.0	6.0	158.8	9.6	4.8	80.6	90.0
	G-M.	38	1.68		5.1	5.7	32.7	2.9	168.0	11.9	5.1	85.3	85.4
	PVs	58	1.75		5.4	6.7	26.6	4.3	153.2	13.4	5.8	77.8	65.1
	JFC	32	1.72		5.7	7.0	22.1	2.9	119.2	19.8	5.4	60.5	55.7
	CK	35	1.83		5.8	6.15	34.7	4.3	211.6	16.2	6.1	107.4	82.5
				Mean	5.7	6.4	29.8	5.2	155.7	12.5	5.2	79.0	78.0
W	MCC	31	1.52		3.4	3.9	22.5	2.9	131.4	11.0	5.8	66.7	54.7
	NV	54	1.54		4.2	5.0	24.5	3.2	134.6	3.7	5.5	68.3	61.5
	GF	54	1.54		4.3	4.7	20.3	10.0	90.5	5.2	4.5	45.9	58.6
	AML	26	1.66		4.5	4.65	21.9	6.2	116.0	1.4	5.3	58.9	56.1
	BC	36	1.54		3.9	4.3	23.4	3.2	82.6	8.0	3.5	41.9	85.3
	MLC	33	1.57		3.6	3.4	19.7	5.4	134.0	4.7	6.5	68.0	44.7
	AMC	45	1.66		4.9	5.1	22.4	5.3	124.7	4.8	5.6	63.3	55.8
				Mean	4.1	4.4	22.1	5.1	116.3	5.5	5.3	59.0	59.5

CONCLUSION: The TL_{NO}/TL_{CO} ratio was ~ 5. This suggests that the specific conductance of blood for NO was very high. It may also suggest that NO is not a good indicator for lung transfer measurement. The inspired fraction of NO decreased slightly from one experiment to another at a rate of 0.5 ppm/h, without apparent NO_2 formation. This was possibly due to the absorption of NO and NO_2 in the walls of the rubber balloon.

The Qc for men was 78.0 ± 13.2 mL and that for women was 59.5 ± 11.6 mL. The capillary blood volumes of men and women are significantly different, however after correcting for total lung capacity this difference disappears.

This study also supports the sponsor's claim that a large proportion of I-NO is absorbed systemically.

DEPENDENCE OF THE METABOLISM OF NITRIC OXIDE IN HEALTHY HUMAN WHOLE BLOOD ON THE OXYGENATION OF ITS RED CELL HAEMOGLOBIN

INVESTIGATORS: Wennmalm Å, Benthin G, Peterssen A-S.
STUDY SITE: Gothenburg University, Gothenburg, Sweden
CITATION: British Journal of Pharmacology. 1992;106:507-508.
STUDY DATES: not published
OBJECTIVES: To determine the route by which NO in human blood is converted to nitrate.
FORMULATION: NO (AGA Special Gas, final concentration 50-200mM)
STUDY DESIGN: ex-vivo incubation study
POPULATION: 20 healthy donors

PROCEDURE: Portions of venous blood from healthy donors were incubated with NO with or without previous oxygenation. O_2 saturation was estimated. The incubation was interrupted to separate the blood into cells and plasma. This was followed by freezing the cell fraction at 70K in electron paramagnetic resonance (EPR) tubes. The EPR spectra of the blood cell fraction were recorded for methemoglobin and nitrosylhemoglobin at a microwave frequency of 9.22 GHz and a power of 20mW from about 500 to 3500 gauss with a modulation amplitude of 20 gauss. Plasma levels of nitrite and nitrate were measured as described below.

ASSAY:

RESULTS: Table 1 and figure 1 show the basal concentrations of nitrate, nitrite, methemoglobin, and nitrosylhemoglobin, as well as these concentrations when incubated with arterialized and venous blood (mean ± SE).

	Basal	**Incubation of 200 μM NO with**			
		Arterialized blood, 2 min	Arterialized blood, 15 min	Venous blood, 2 min	Venous blood, 15 min
Plasma nitrate (μM)	44 ± 3.8	203	237	130	260
Plasma nitrite (μM)	< 1 or undetectable	< 1 or undetectable	< 1 or undetectable	< 1 or undetectable	< 1 or undetectable
Methemoglobin (units)	19 ± 2	140	151	135	260
Nitrosylhemoglobin (units)	1.2 ± 0.3	11	10	92	92

Incubation of NO with arterialized blood resulted in a dose-dependent increase in plasma nitrate and methemoglobin, whereas nitrosylhemoglobin increased minimally. Incubation of NO with venous blood for two minutes caused a parallel increase in plasma nitrate and methemoglobin but this increase was of a smaller magnitude than that seen with arterialized blood. In contrast, nitrosylhemoglobin increased markedly. When the incubation was prolonged to 15 minutes, concentrations of nitrate and methemoglobin were similar to that obtained with arterialized blood.

blood. Nitrosylhemoglobin was unaffected. Incubation of plasma with NO for 15 minutes resulted in semiquantative conversion of nitrite to nitrate in a ratio of 5:1.

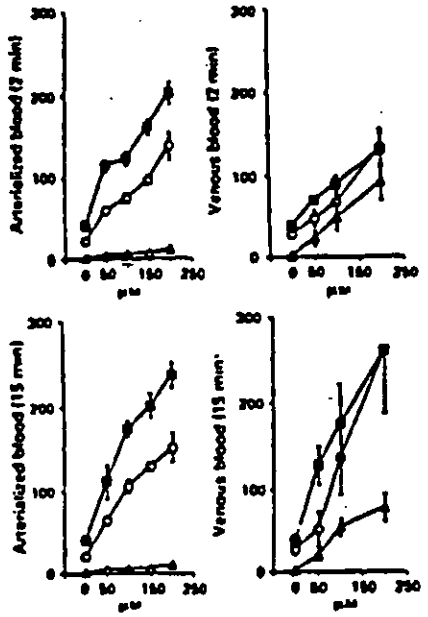


Figure 1. Concentrations of nitrate, methemoglobin, and nitrosylhemoglobin when incubated with arterialized and venous blood (mean ± SE). nitrate (■); methemoglobin (O); nitrosyl hemoglobin (Δ)

CONCLUSION: Plasma, in comparison to whole blood, was inefficient in converting NO to nitrite. This highlights the activity of the blood cell fraction in the inactivation of NO. Since the red cells are the most abundant cells in the body where NO can be produced, and the conversion of NO to nitrate was found to involve hemoglobin, it appears that the conversion of NO to nitrate was more rapid in blood with high compare to low oxygen saturation of hemoglobin. This suggests that HbO_2 acts as an oxygen donor to the NO molecule in it conversion to nitrate.

The present data indicate that NO is readily converted to nitrate and methemoglobin. Nitrate is then eliminated by renal excretion, and methemoglobin is converted to hemoglobin by endogenous mechanisms. If small amounts of nitrosylhemoglobin are formed, this complex can be disintegrated by a high oxygen tension, as in the alveoli capillaries in the lungs.

EFFECTS OF EXPOSURE OF BLOOD HEMOGLOBIN TO NITRIC OXIDE

INVESTIGATORS: Chiodi H, Mohler JG
STUDY SITE: University of Southern California School of Medicine
CITATION: Environmental Research. 1985; 37:355-63.
STUDY DATES: not stated
OBJECTIVES: To describe the reaction between hemoglobin and nitric oxide by an in vitro study.
STUDY DESIGN: in vitro study
POPULATION: humans; no other details provided

PROCEDURE: Heparinized whole human blood kept at 4°C, less than 48 hours old was used. Blood was exposed to NO gas according to a flow-tonometry procedure previously described (Chiodi et al. In vitro methemoglobin formation in human blood exposed to NO_2. Environ Res 1983;30:9-15.). 5.6% CO was added to all gas mixtures to keep blood pH within the physiological range. A total flow of 800 mL/min was maintained throughout the experiments. **Figure 1** shows the amount of methemoglobin, expressed as a percentage of total hemoglobin, plotted against the time of nitrosylhemoglobin exposure to a circulating gas mixture (21% O_2 and 5.6% CO_2 in N_2). Nitrosylhemoglobin was obtained by exposing 10 to 20 mL of deoxygenated blood to a continuous flow of 1000 ppm of NO in N_2 for 3 hours or to 100% NO for 1 hour. The nitrosylhemoglobin containing blood was then exposed to the O_2 and CO gas mixture for 5, 10, 15, 30, 60, 90, and 120 minutes. Anaerobic blood samples for further analysis were taken at the end of each period.

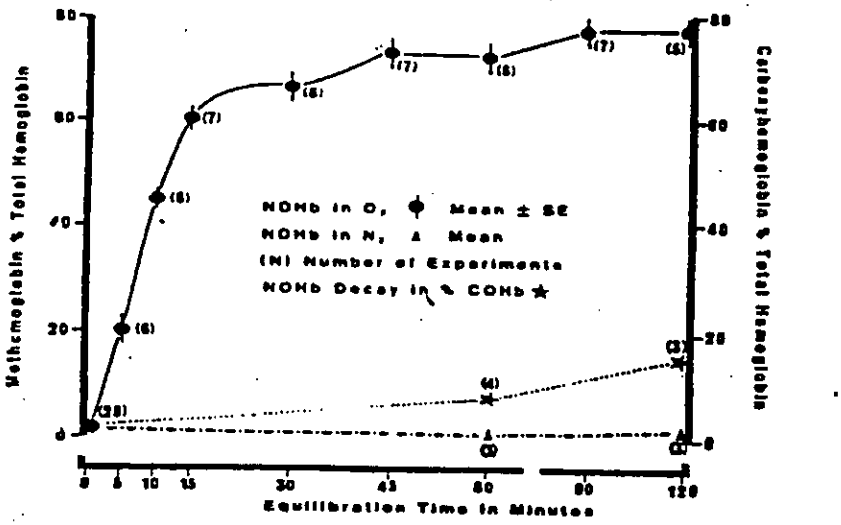


FIG. 1. MetHb formation in blood NOHb equilibrated with 21% O_2, 5.6% CO_2 in N_2. Previously deoxygenated human blood was equilibrated with a continuous flow (800 ml/min) of 1000 ppm NO, 5.6% CO_2 in N_2 for 3 hr followed by a half-hour flush with 5.6% CO_2 in N_2. NOHb-containing blood was then equilibrated for different lengths of time, as indicated on the abcissa, with 21% O_2 and 5.6% CO_2 in N_2 or with 100% CO for 1 hr. MetHb was measured with the anaerobic modification of Evelyn–Malloy method. COHb was measured in the IL-282 CO-oximeter.

ASSAY:

ANALYSIS: A description of the data analysis was not provided.

RESULTS: Nitrosylhemoglobin exposure to oxygen resulted in increased methemoglobin formation. % of the total hemoglobin was oxidized in the first 15 minutes, and % was oxidized after 120 minutes of oxygen exposure. Similar results were obtained when nitrosylhemoglobin was exposed to the oxygen of the air. Blood nitrosylhemoglobin equilibrated with N_2, and did not form any significant amount of methemoglobin (Figure 1). A 15 minute exposure to 100% CO did not induce any changes in the two characteristic peaks at 545 and 575 nm of the nitrosylhemoglobin curve, indicating that all or most of the hemoglobin had combined with NO. The decrease of nitrosylhemoglobin was paralleled by the appearance of the two peaks at 500 and 630 nm characteristic of methemoglobin.

CONCLUSION: Under strict anaerobic conditions, either an N_2 or CO atmosphere, NO will combine with blood hemoglobin forming nitrosylhemoglobin without oxidation of the hemoglobin to methemoglobin. When nitrosylhemoglobin is exposed to O_2 either in the intact red cell or in a buffered solution, the hemoglobin is oxidized to methemoglobin.

METABOLISM AND EXCRETION OF NITRIC OXIDE IN HUMANS: AN EXPERIMENTAL AND CLINICAL STUDY

INVESTIGATORS: Wennmalm Å, Benthin G, Edlund A, et al.
STUDY SITE: Sahlgrenska Hospital, Gothenburg, Sweden
CITATION: Circulation Research. 1993; 73:1121-1127.
STUDY DATES: Not published
OBJECTIVES: To determine the metabolism and excretion of NO in humans
FORMULATION / DOSE:
Inhalation of NO by healthy subjects and in patients with severe heart failure

- 25 ppm in healthy subjects
- 20, 40, 80 ppm in severe heart failure patients

Ex vivo studies of the degradation of nitrite

- A final sodium nitrite concentration of 50, 100, and 200 µmol/L during incubation

STUDY DESIGN: Open label study
POPULATION: Patients participated in only one substudy.
Inhalation of NO by healthy subjects and in patients with severe heart failure

- Eight (four men) healthy nonsmoking hospital staff volunteers
- Eight (six men) heart transplant candidates

Ex vivo studies of the degradation of nitrite

- Eight healthy donors

Studies on the renal elimination of NO metabolite and analysis of nitrate and nitrite in plasma and urine

- Eight healthy volunteers (two men) aged 20 to 33 years.

PROCEDURE:
Inhalation of NO by healthy subjects and in patients with severe heart failure. All volunteers were studied in the morning, while seated. After a stabilization period, NO was administered for 60 minutes in air by mask inhalation. Inhalation depth and frequency were not controlled. Blood samples were drawn into heparinized tubes in the basal state and every 10 minutes during inhalation and were analyzed for nitrate, nitrite, methemoglobin and nitrosylhemoglobin.

Heart transplant candidates were studied in the supine position. Nitrodilators were not allowed at least 12 hours before NO inhalation. NO was administered in air by mask inhalation in subsequent 10 minute periods. Blood samples were drawn similar to that done in volunteers. Additional blood samples were collected during the last two minutes of each inhalation period. Samples were analyzed for nitrate, nitrite, methemoglobin, and nitrosylhemoglobin.

Ex vivo studies of the degradation of nitrite. Venous blood was obtained from eight healthy donors and sampled into heparinized tubes. A fraction of the blood was oxygenated prior to incubation. Eight milliliters of whole blood were incubated at room temperature with sodium nitrite for a final concentration of 50, 100, and 200 µmol/L. The incubation was interrupted after 2 and 15 minutes, respectively, to separate the blood into cells and plasma, and to freeze a portion of the cell fraction and plasma fraction.

Studies on the renal elimination of NO metabolite. Volunteers were instructed to avoid nonsteroidal anti-inflammatory drugs and excessively salted nutrients for five days prior to the study. Voided urine was collected for 24 hours and stored at 4° to 8° C. After the urine collection period and an overnight fast, subjects reported to the laboratory. Subjects were given 400 mL of water to ensure adequate diuresis. After 60 minutes of supine rest, urine was collected using a bladder catheter for 16 to 25 minutes. Arterial blood was collected at the start and end of each sampling period. Urine volume and exact collection time were measured in all cases. Urine and plasma samples were kept frozen at –18°C so that nitrite and nitrate levels remained stable for several months.

ASSAY:

In the inhalation study, methemoglobin and total hemoglobin were measured in an ABL-520 oxygen saturation analyzer (Radiometer).

ANALYSIS: Clearance of nitrate was calculated as the ratio between the nitrate excretion during the respective 16 to 25 minute periods and the arterial plasma concentration at the beginning of each period. The clearance of nitrate was also calculated as the ratio between its 24-hour urinary excretion and the arterial plasma concentration.

Total body water was calculated as 51% and 61% of the body weight, in women and men, respectively.

Data are presented as mean ± SE, unless otherwise stated. For analysis of statistical significance, one-way ANOVA, Friedman's test, or the Wilcoxon signed rank test were used when appropriate. A value of $P < 0.05$ was considered significant.

PHARMACOKINETIC RESULTS:

Plasma levels of nitrate and methemoglobin in eight healthy volunteers in the basal state and during I-NO (25ppm) for 60 minutes are shown in **Table 1.**

TABLE 1. Plasma Levels of Nitrate and Methemoglobin in Eight Healthy Volunteers in the Basal State and During Inhalation of Nitric Oxide (25 ppm) for 60 Minutes

	Plasma Levels, μmol/L													
			Minutes of NO Inhalation											
	Basal		10		20		30		40		50		60	
Subject	Nitrate	MetHb	Nitrate	MetHb	Nitrate	MetHb	Nitrate	MetHb	Nitrate	MetHb	Nitrate	MetHb	Nitrate	MetHb
1	23	2	25	13	30	6	26	8	30	11	33	13	36	17
2	29	6	33	11	38	8	38	11	39	13	36	8	44	8
3	31	4	34	4	36	10	37	13	38	12	39	13	38	12
4	22	4	25	9	25	7	24	13	28	13	29	13	30	13
5	29	9	32	13	33	11	42	11	38	13	43	16	43	14
6	19	11	21	7	25	11	27	7	24	7	35	9	33	9
7	30	9	33	11	33	11	39	14	32	14	45	12	39	. . .
8	27	10	33	10	33	16	40	. . .	38	16	38	. . .	37	16

NO indicates nitric oxide; MetHb, methemoglobin.

The basal level of nitrate before inhalation was 26 ± 2 μmol/L. During inhalation of NO, there was a significant time-related increase in plasma nitrate, reaching μmol/L after 60 minutes.

The concentration of methemoglobin increased, parallel with that of plasma nitrate, from μmol/L to μmol/L (Figure 1). This corresponds to a change from % to % of the total hemoglobin concentration in the blood.

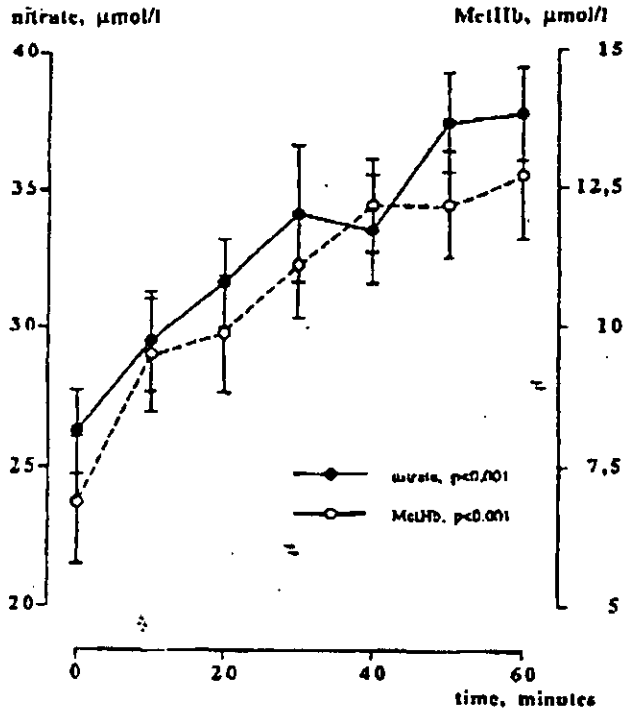


FIG 1. Graph showing plasma levels of nitrate and whole blood concentration of methemoglobin (MetHb) in the basal state and during inhalation of nitric oxide at 25 ppm in eight healthy volunteers. Data are presented as mean+SEM. *P* values refer to changes in the levels of plasma nitrate and whole blood MetHb with time (by one-way ANOVA).

The basal level of nitrite before inhalation was 1.3 ± 0.15 µmol/L. During inhalation of NO, plasma nitrite did not change significantly. No nitrosylhemoglobin levels were detected before or during inhalation of NO.

Individual arterial and pulmonary arterial levels of nitrate in the basal state and during I-NO in the heart failure patients are shown in **Table 2.**

TABLE 2. Arterial and Pulmonary Arterial Levels of Nitrate in the Basal State, During Inhalation of Nitric Oxide (20 to 80 ppm), and 20 Minutes After the End of Nitric Oxide Inhalation in Eight Heart Failure Patients

			Levels of Nitrate, μmol/L									
			Basal		20 ppm NO		40 ppm NO		80 ppm NO		20 Min After NO	
Patient	Sex	Age, y	Art	Pulm Art	Art	Pulm Art	Art	Pulm Art	Art	Pulm Art	Art	Pulm Art
1	M	58	81	71	82	80	88	88	105	97	94	88
2	M	60	62	62	62	62	68	59	90	74	...	...
3	M	51	99	84	97	94	100	95	123	100	114	95
4	F	61	118	120	123	121	130	128	120	137	134	128
5	M	54	27	27	32	30	42	38	58	51	52	47
6	F	23	38	36	47	43	57	44	72	52	52	53
7	M	63	27	26	31	30	48	40	62	61	60	56
8	M	40	177	177	180	186	...	...	...	...	198	196

NO indicates nitric oxide; Art, arterial plasma; and Pulm Art, pulmonary arterial plasma.

Basal level of nitrate in arterial plasma before inhalation ranged from (median, 72) μmol/L; the corresponding range in the pulmonary arterial plasma was from (median, 67) μmol/L. During I-NO, there was a dose-related increase in plasma nitrate in the systemic and pulmonary arteries. The highest levels were observed during inhalation of NO (80 ppm). Twenty minutes after cessation of I-NO, plasma nitrate had dropped in most patients. The levels of nitrate were significantly lower in pulmonary arterial plasma compared with systemic plasma.

Ex vivo studies of the degradation of nitrite. Incubation of arterialized blood with nitrite for 2 minutes resulted in dose-dependent increases in the formation of nitrate and methemoglobin (Figure 2).

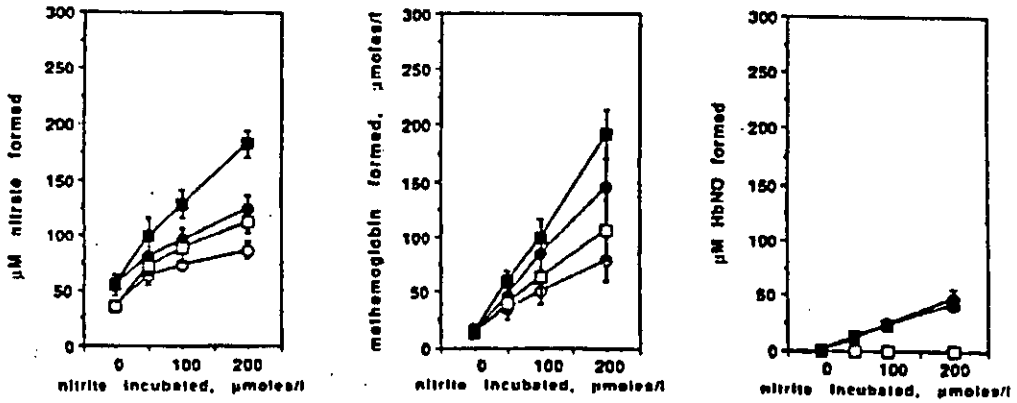


Figure 2. Dose-response curves demonstrating the formation of nitrate (left), methemoglobin (middle) and nitrosohemoglobin (HbNO, right) in arterialized (O_2 saturation, 94-99%, open symbols) or venous (O_2 saturation, 36-85%, filled symbols) blood incubated with nitrite (50-200 μmol/L) for 2 (circles) or 15 (squares) minutes. Symbols indicate mean ± SE of four to six observations. Open circles in right panel are hidden behind open squares.

At the highest nitrite concentration used (200 µmol/L), nitrate in plasma reached a level of about 85 µmol/L, suggesting semiquantitative conversion of nitrite to nitrate. In parallel, methemoglobin was elevated, to about the same level (80 µmol/L). Again, no nitrosohemoglobin was detected. Incubation of venous blood with nitrite for two minutes yielded a different pattern. Nitrate and methemoglobin concentrations increased to 125 and 145 µmol/L, respectively (Figure 2), and a significant formation of nitrosohemoglobin was observed. When the incubations of venous blood with nitrite were prolonged to 15 minutes, plasma nitrate and methemoglobin increased even more (to 185 and 190 µmol/L, respectively). The longer duration did not affect nitrosohemoglobin.

Renal excretion of nitrate. Estimation of nitrate clearance on the basis of urinary excretion during the 16 to 25 minute collection periods and the arterial plasma level yielded clearance values from 6 to 61 mL/min (Table 3). The average clearance was 22 ± 6 mL/min.

TABLE 3. Arterial and Urine Levels of Nitrate, Urine Sample Volumes, Urine Collection Periods, and Calculated Clearance in Eight Healthy Volunteers

Subject	Sex	Age, y	Length, cm	Weight, kg	Arterial Nitrate, µmol/L	Urinary Nitrate, µmol/L	Urine Volume, mL	Collection Time, min	Clearance, mL/min
A	F	28	166	65	30.2	130	50	20.5	10
B	M	27	179	72	21.5	70	250	25.0	34
C	F	33	171	57	15.9	50	43	20.0	6
D	F	31	156	50	15.6	20	166	19.0	13
E	F	26	164	54	31.4	170	52	16.0	18
F	F	20	167	62	23.9	140	67	20.0	20
G	F	24	170	61	56.8	80	213	22.5	13
F	M	24	183	70	17.0	90	250	21.5	61
Mean		27	170	61	27	94	136	20.6	22

From the 24-hour urine collection, the nitrate excretion was 0.47 to 1.13 mmol/L. Based on the nitrate excretion and arterial plasma levels, renal clearance ranged from 12 to 32 mL/min with a mean of 19 ± 4 mL/min (SEM).

CONCLUSION: In healthy subjects, the end products of I-NO that enter the systemic circulation are predominantly methemoglobin and nitrate. The same metabolic pattern was observed in patients with severe heart failure. In vitro incubations indicated conversion of the NO oxidation product nitrite to equal amounts of nitrate and methemoglobin in fully oxygenated blood. In venous blood, the conversion of nitrite also yields nitrosohemoglobin ex vivo. Nitrate in plasma was cleared (20 mL/min) by the kidney.

A DOUBLE-BLIND, RANDOMIZED, PLACEBO-CONTROLLED, DOSE-RESPONSE STUDY OF INHALED NITRIC OXIDE IN THE TREATMENT OF PERSISTENT PULMONARY HYPERTENSION OF THE NEWBORN

STUDY #: INO-01 and INO-02

INVESTIGATORS: Barefield E, Bhutani V, Bifano E, et al.

STUDY SITE: 30 centers in the United States

CITATION: pending

STUDY DATES: April 21, 1994 – July 17, 1996

OBJECTIVES: Primary – To demonstrate a reduction in morbidity and mortality of primary pulmonary hypertension of the newborn (PPHN). Secondary – To study the effective and safe dose range and duration of therapy in the treatment of PPHN

FORMULATION / DOSE: The active drug, inhaled NO, was manufactured by BOC Specialty Gases at Port Allen, Louisiana, from raw NO supplied by Pure NO was diluted with Grade 5 nitrogen gas to achieve the final cylinder concentration. The placebo consisted of 100% Grade 5 nitrogen gas which was manufactured by BOC Specialty Gases at Port Allen , Louisiana. All concentrations of treatment gas were shipped to clinical sites in 30 Liter, H size, aluminum cylinders.

Gas from the cylinder was diluted 1:20 by the inhaled NO delivery device when the device was set to deliver 100% of the treatment gas dose. The dose groups, cylinder concentrations and delivered concentrations at 100% delivery are given below.

Nitric oxide doses

Dose group	Cylinder NO concentration (Balance N2), ppm	Patient NO Dose at 100% Delivery Device Setting, ppm
Placebo	0	0
5 ppm	100	5
20 ppm	400	20
80 ppm	1600	80

CONCOMITANT DRUG EXCLUSIONS: tolazoline, a potent vasodilator, and other intravenous vasodilators

STUDY DESIGN: INO-01 and INO-02 are identical, multicenter, double-blind, randomized, placebo-controlled, multiple dose phase 2/3 trials. The numbers designate the different institutions participating in the study.

DURATION: Until threshold criteria met or 14 days, whichever comes first.

POPULATION: The investigators planned to enroll 320 term infants with echocardiographic evidence of PPHN. Infants enrolled were within 72 hours of birth, full-term (≥ 37 weeks gestational age), an appropriate weight for gestational age (> 2500 grams), and hypoxic (defined as an arterial partial pressure of oxygen between 40 and 100 mm Hg while ventilated with a fraction of inspired oxygen equal to one and a mean airway pressure ≥ 10 cm H_2O). Additionally, infants were excluded if there was evidence of a grade 2-4 intraventricular hemorrhage, significant cardiac lesions, lethal physical or chromosomal abnormalities, and previous treatment with high-frequency ventilation or surfactant therapy.

Forty infants were planned for each arm. The arms in INO-01 and INO-02 consisted of placebo, 5 ppm, 20 ppm, and 80 ppm. 155 patients were enrolled because the trial was halted.

PROCEDURE: After randomization, baseline data were collected and treatment gas was started. For the first 30 minutes after initiation of treatment gas, the ventilator settings were not changed. After 30 minutes, infants were assessed to determine if they were treatment failures. If infants were determined to be treatment failures, the treatment gas was discontinued or, in cases of elevated NO_2 or methemoglobin levels, the NO concentration could be reduced rather than stopped. Otherwise the initial dose of treatment gas was maintained until the patient met gas discontinuation criteria, or for a maximum of 14 days of dosing. Once the patient met these criteria, the treatment gas was rapidly lowered in 20% decrements to 0%, until these criteria were no longer met or the treatment gas has been completely discontinued. Decreases were continued every 30 minutes and within 4 hours as long as the patient continued to meet the gas weaning criteria.

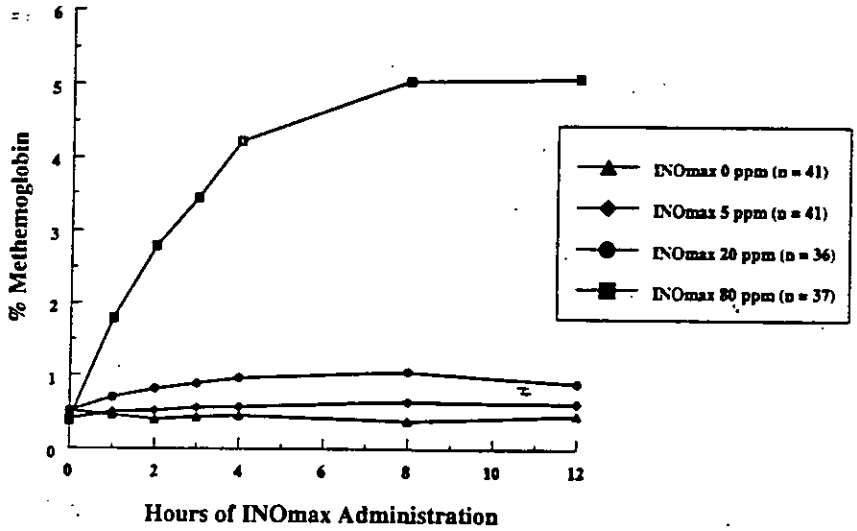
Primary endpoint for the trial was the number of patients in each group who had at least one major PPHN sequela.

SAMPLING: Methemoglobin and inspired NO_2 concentrations were included in the safety assessment.

ASSAY: Description of assay not provided

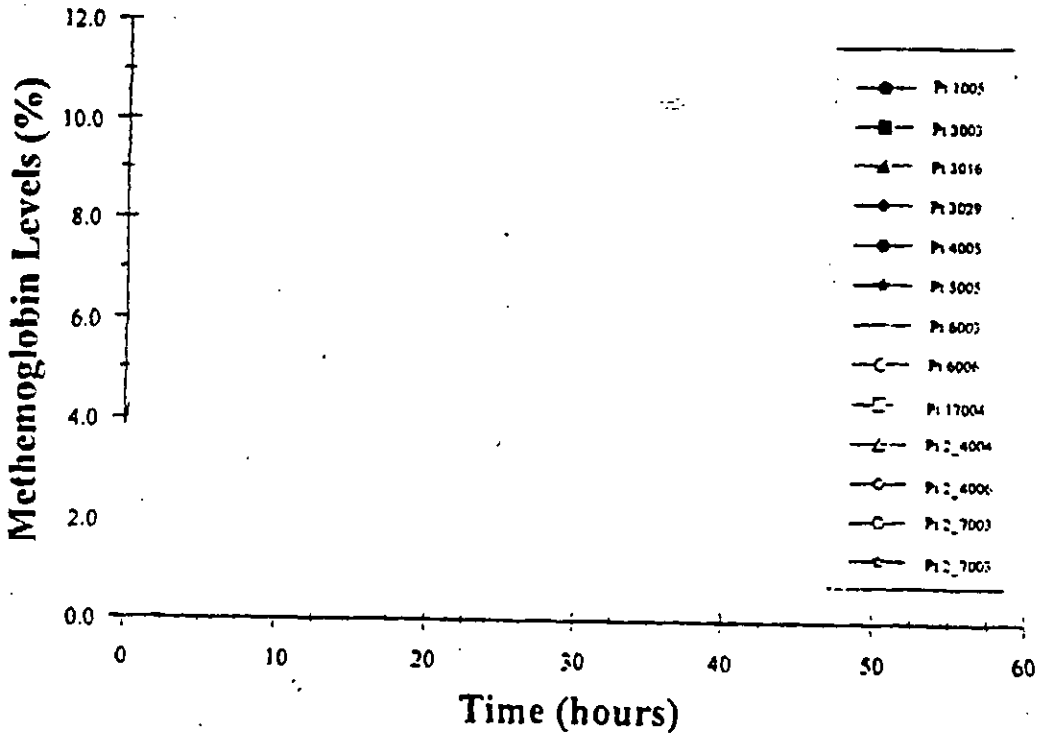
ANALYSIS: No analysis done for methemoglobin or NO_2 concentrations.

PHARMACOKINETIC RESULTS: Percent methemoglobin concentrations during the first 12 hours of exposure are shown below. Although at all doses, concentrations were significantly elevated, only concentrations associated with the 80ppm dose were clinically significant. Thirteen patients developed methemoglobinemia (methemoglobin level >7%); all were in the 80 ppm dose group. The average time to reach the peak methemoglobin level was 10.46 ± 9.46 hours (median 8 hours) in these 13 patients. One patient did not reach their peak concentration until 40 hours later.



Methemoglobin concentrations in patients that developed methemoglobinemia are shown below.

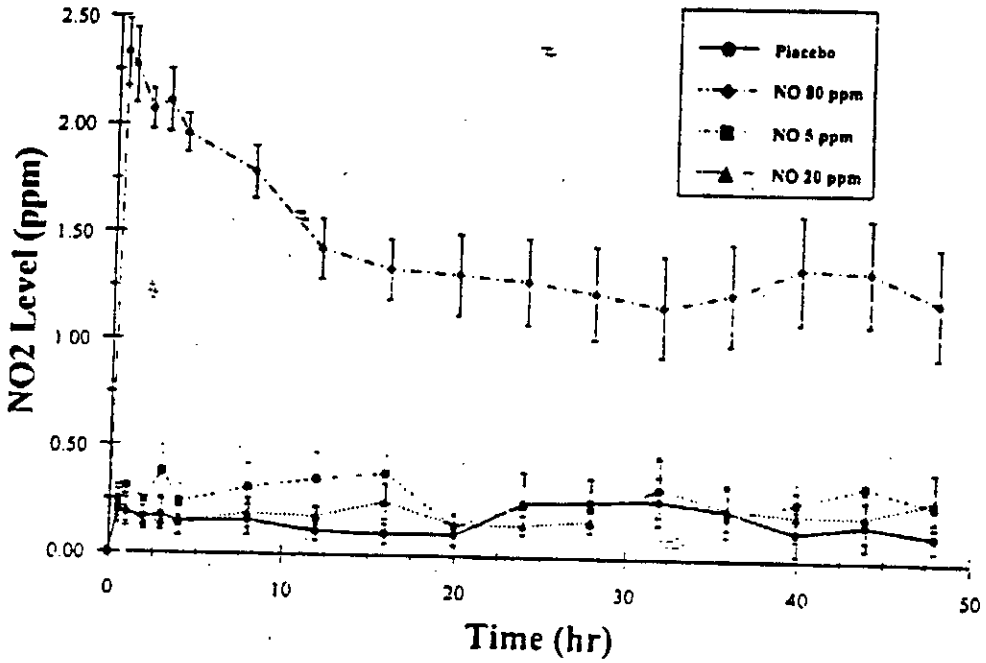
METHEMOGLOBIN LEVELS FOR PATIENTS WITH METHEMOGLOBINEMIA



The mean NO_2 levels over 48 hours are shown below. The NO_2 levels were below 0.5 ppm in the placebo, 5-ppm, and 20 ppm groups, however NO_2 concentrations were substantially higher (~2 ppm) in the 80 ppm I-NO group.

NITROGEN DIOXIDE (NO_2) LEVELS OVER 48 HOURS

(MEAN ± STANDARD ERROR)



CONCLUSION: In neonates with PPHN, the 80 ppm I-NO dose in this study was associated with clinically elevated methemoglobin concentrations. The average time to reach the peak methemoglobin concentration was 10.46 ± 9.46 hours in patients with methemoglobinemia.

CONVERSION OF INHALED NITRIC OXIDE TO NITRATE IN MAN

INVESTIGATORS: Division of Clinical Physiology, Sahlgrenska University Hospital, Goteborg, Sweden.
CITATION: Westfelt UN, Benthin G, Lundin S, Stenqvist O, Wennmalm A. Conversion of inhaled nitric oxide to nitrate in man. British Journal of Pharmacology. 1995; 114: 1621-1624.
STUDY DATES: not published
OBJECTIVES: To assess the quantitative importance of the conversion of inhaled nitric oxide to nitrate in the blood and the subsequent excretion into the urine.
POPULATION: Eight healthy female volunteers, aged 37 ± 3 (range 25-48), were recruited among the hospital staff. Three were smokers. Their height was 166 ± 2 (range 162-172) cm, and their body weight was 62 ± 3 (range 53-74) kg.
STUDY DESIGN: Open-label
STUDY DOSE: ^{15}NO 25 ppm for 1 hour
FORMULATION: Ã final concentration of 25 ppm of ^{15}NO was given at an adjustable flow rate. The system for NO delivery to the breathing gas consisted of two mask flow regulators, one of them controlling the flow of ^{15}NO mixed in N_2 (1000 ppm, AGA Gas AB, Lidingo, Sweden), and the other controlling an O_2/air mixture (Stenqvist et al, 1993). Gas flow and the concentration of ^{15}NO in the mixed expired air throughout the inhalation period. To obtain the volume of alveolar ventilation, mixed and end-tidal expired CO_2 concentrations were measured with an infrared absorption CO_2 analyzer (Normocap, Datex; Helsinki, Finland), which was calibrated with a test gas with 3.0% CO_2 in air.
DIET: Participants were instructed to refrain from intake of nitrate-rich food from two days before and to the end of the study. These included alcoholic beverages, caviar, charcuteries, cheese, herbs, pickled fish, roots, and vegetables.

PROCEDURES: Subjects reported to the laboratory the morning of their study. After emptying the bladder for collection of basal urine, a short catheter was inserted into a medial cubital vein. There was a 10 minute control period of spontaneous respiration by a tight fitting mask in a non-rebreathing system. Subjects inhaled ^{15}NO for 1 hour.

Plasma sampling. Plasma concentrations of $^{15}NO_3$- were followed for 2 hours. A short catheter was used to draw five milliliters of blood through an arm vein at the following time points: the basal state, 20, 40, 60, 80, 100, and 120 minutes after onset of inhalation.

Urine sampling. Urine concentrations of $^{15}NO_3$- were followed for 48 hours. Urine was collected in two 24 hour portions following the start of the inhalation of NO. After volume recording, samples of the collected 24 hour portions were frozen for analysis.

ASSAY: Plasma

ANALYSIS: The total volume of inhaled ^{15}NO was calculated as total ventilation volume * inhaled concentration of ^{15}NO. The uptake of ^{15}NO was calculated as total ventilation volume * (inhaled concentration of ^{15}NO – expired concentration of ^{15}NO). Conversion of gas volume to molar quantity was calculated using the constant 24.2 μL/μmol. CO_2 production was calculated as mixed expired CO_2 concentration * minute ventilation volume and corrected to standard pressure and temperature, dry (STPD). Alveolar ventilation was calculated from Bohr's formula (alveolar ventilation = CO_2 production/end-tidal CO_2 concentration).

RESULTS: Data are presented as mean ± SE.

Table 1. Baseline values

	Plasma level (μmol/L)
Nitrate	31.6 ± 2.7
Nitrite	< 1
	Urine level (μmol/L)
Nitrate	925 ± 217
Nitrite	not detectable

Inhalation of ^{15}NO elicited a rapid linear increase in the plasma concentration of $^{15}NO_3$. (figure 1). The maximum average concentration of $^{15}NO_3$. was reached at the end of the inhalation. Plasma concentrations of $^{15}NO_3$. declined after inhalation ceased. Plasma concentrations of total nitrite taken during inhalation of ^{15}NO remained consistently < 1 μmol/L.

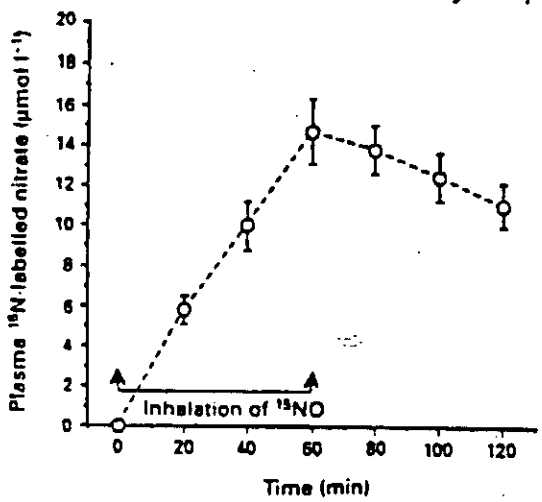


Figure 1. Plasma levels of $^{15}NO_3$. during inhalation of ^{15}NO in eight healthy females.

Individual subject concentration of both plasma and urine are shown in table 2.

Table 2. Total amount of retained ^{15}NO, maximum plasma concentration of $^{15}NO_3$., amount of $^{15}NO_3$. in urine of days 1 and 2 after inhalation of ^{15}NO, and total recovery of inhaled ^{15}NO as $^{15}NO_3$. in urine

Subject	Total amount of ^{15}NO retained (μmol)	Max. $^{15}NO_3^-$ in plasma (μmol l^{-1})	$^{15}NO_3^-$ in urine day 1 (μmol)	day 2 (μmol)	Total recovery of $^{15}NO_3^-$ (% of inhaled ^{15}NO)
1 (S)	206	14.2	189	9	96
2 (NS)	168	8.6	114	8	73
3 (NS)	230	20.7	142	2	63
4 (S)	264	14.8	169	8	67
5 (NS)	242	16.5	135	10	60
6 (NS)	193	19.1	107	5	58
7 (NS)	272	11.9	195	3	73
8 (S)	214	16.5	177	20	92
Mean ± s.e.	224 ± 13	15.3 ± 1.4	154 ± 12	8 ± 2	73 ± 5

(S) and (NS) after subject number indicates smoker and non-smoker, respectively.

The pulmonary ventilation during the 60 minute period of inhalation of ^{15}NO was 6.7 ± 0.3 L/min. The total amount of ^{15}NO inhaled was 399 ± 15 μmol. The total amount of retained ^{15}NO in the lungs was 224 ± 13 μmol. Because alveolar ventilation averaged 62 ± 1% of the pulmonary ventilation, the calculated amount of ^{15}NO reaching the alveoli was 247 ± 12 μmol. This implies that 90 ± 2% of the inhaled ^{15}NO reaching the alveoli was retained.

Plasma $^{15}NO_3^-$ increased during the inhalation of ^{15}NO to about 15 μmol/L, and fell when inhalation of ^{15}NO was terminated. Urinary excretion of $^{15}NO_3^-$ during the first 24 hours after inhalation was 154 ± 12 μmol. During the following 24 hours another 8 ± 2 μmol of $^{15}NO_3^-$ appeared in the urine. No nitrite was detectable in urine samples taken during the 48 hours following inhalation of ^{15}NO.

CONCLUSION: Following inhalation of ^{15}NO in healthy female subjects, ~90% is absorbed in the alveoli, and NO is rapidly converted to nitrate. $^{15}NO_3^-$ plasma concentrations increase linearly, and immediately decrease when ^{15}NO inhalation ceases. Almost 70% of inhaled ^{15}NO is excreted into the urine as $^{15}NO_3^-$ within 24 hours. Nitrite was undetectable during or after inhalation of ^{15}NO.

KINETICS OF METHAEMOGLOBIN AND SERUM NITROGEN OXIDE PRODUCTION DURING INHALATION OF NITRIC OXIDE IN VOLUNTEERS

INVESTIGATORS: Young JD, Sear JW, Valvini EM
CITATION: British Journal of Anaesthesia. 1996; 76: 652-56.
STUDY DATES: Not published
OBJECTIVES:

- To determine the amount of inhaled nitric oxide that reacts with oxyhemoglobin to form nitrates and methemoglobin.
- To determine if direct conversion to nitrate also occurs.
- To determine the pharmacokinetics of the nitrate produced.

STUDY DESIGN: open label
POPULATION: 6 healthy male volunteers, ages 30-38, weighing 67-105 kg
FORMULATION: Nitric oxide was stored as a mixture of 2000 volumes per million (vpm) in nitrogen and mixed with medical grade air to achieve a final inspired concentration of 100 vpm
ASSAY:

PROCEDURES: A 16 gauge cannula was used to obtain blood for measurement of baseline methemoglobin, total hemoglobin, serum nitrate and nitrite concentrations. Subjects were fitted with a leak-free face mask connected to a non-rebreathing valve. Subjects inhaled NO for 3 hours and then room air. Venous blood was obtained at 0, 15, 30, 45, 60, 90, 120, 180 min during inhalation of nitric oxide. After inhalation ceased, blood was also sampled at 15, 30, 45, 60, 90, 120, 180, 240, 300, 540 and 1260 min. Because processed meats may contain added nitrites, subjects were asked to avoid them on the day of the experiment; no other diet or fluid restrictions were placed.

ANALYSIS: Data were analyzed using an analysis/graphics package (Software). Inspired minute volume was determined from a dry gas meter fitted to the inspiratory limb of the circuit. For each subject, the inspired and expired NO concentrations and minute volume were corrected for temperature, ambient pressure and water vapor, and used to calculate NO uptake (VNO) at STPD (standard temperature and pressure dry). Methemoglobin production during inhalation of NO at a fixed inspired concentration followed simple first order kinetics. The methemoglobin concentration at time, t, is described by this equation:

$$\text{Methemoglobin concentration at time } t = B + A * (1 - e^{-\pi})$$

B= baseline methemoglobin concentration (% of total hemoglobin)
A= plateau concentration of methemoglobin above baseline that would be reached if inhalation continued (% of total hemoglobin)

τ = time constant (minutes)

This equation was fitted to the methemoglobin concentration results during inhalation of NO for each subject using least squares regression to give estimates of A and τ. The maximum rate of increase in methemoglobin concentration was determined by differentiating this equation and solving for $\tau = 0$.

Assuming that all of the NO absorbed initially forms methemoglobin, estimates of blood volume were obtainable because the rate of methemoglobin production and initial increase were known.

$$\text{Estimated blood volume} = \frac{\text{Methemoglobin production (g/min)}}{\text{initial rate of increase in blood methemoglobin (g/L/min)}}$$

$$\text{Thus, estimated blood volume is given by } \frac{(V_{NO} * \tau)}{(1.39 * A)}$$

V_{NO} = nitric oxide uptake (mL/min)
A = plateau concentration of methemoglobin above baseline that would be reached if inhalation continued (gm of methemoglobin/L blood)
1.39 = affinity of hemoglobin for nitric oxide (ml of nitric oxide / gm of hemoglobin)

Estimates of blood volume were compared with other estimates calculated from regression equations using the subjects' heights and weights. Two different equations were used and the mean of the estimates taken. Neither the regression equations nor the calculations based on nitric oxide uptake have a correction for the difference between central and peripheral packed cell volume.

To determine if NO initially forms methemoglobin the Vd of methemoglobin using continuous inhalation of gas was determined. The increase in serum nitrogen oxides during inhalation of NO was best described by a one compartment model. The Vd was calculated after assuming all inhaled NO formed nitrogen oxides, and clearance was calculated as the volume of distribution divided by the time constant.

PHARMACOKINETIC RESULTS: The mean I-NO concentration was 100 ± 3.9 (SD) vpm. and mixed exhaled I-NO concentration was 0.49 ± 0.08 mL/min STPD (standard temperature and pressure dry). The first subject had inspired nitrogen dioxide concentrations measured; they were less than 0.25 vpm. The mean methemoglobin concentration before inhalation of NO was 0.87 ± 0.04 %, and the baseline serum nitrogen oxide concentration was 36.7 ± 7.6 µmol/L.

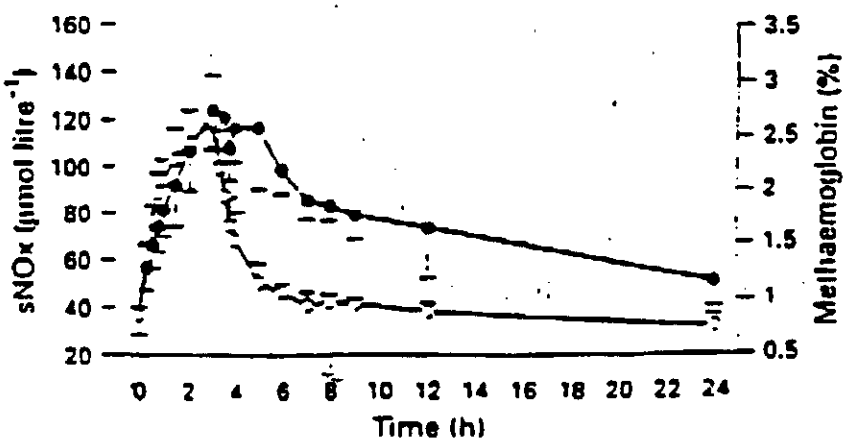


Figure 1. Changes in methemoglobin (o) and sNOx (•) concentrations (mean ± SD) in volunteers breathing I-NO 100 vpm during the study

Table 1. Uptake of NO and calculated results of methemoglobin kinetics for all subjects during inhalation of NO

Subject No	Height (cm)	Weight (kg)	Haemoglobin (g)	Mean nitric oxide uptake (ml min⁻¹ STPD)	τ (min)	A (% of total haemoglobin)	from regression equations (litre)	blood volume from nitric oxide uptake (litre)	Difference between estimates (%)
1	187	105	14.5	0.54 (0.07)	46.4	1.81	6.67	6.81	2.2
2	184	91	15.8	0.50 (0.04)	34.4	1.23	6.04	6.32	4.6
3	175	77	14.4	0.47 (0.07)	53.1	2.05	5.20	6.16	18.4
4	180	75	14.9	0.42 (0.01)	41.5	1.26	5.31	6.62	24.6
5	168	67	14.7	0.47 (0.05)	65.3	2.47	4.6	6.15	33.8
6	178	70	13.9	0.50 (0.07)	33	1.68	5.06	5.18	2.4
Mean (SD)				0.49 (0.08)	45.6 (11.1)	1.77 (0.47)			(X.X)%

The mean peak serum nitrogen oxide concentration was 124.2 ± 17.0 µmol/L and highest value recorded was 151.1 µmol/L reported in one subject. The mean peak methemoglobin concentration was 2.65 ± 0.46% and the peak was 3.3%, obtained in one subject. Serum nitrogen oxide concentrations showed a double peak; at the end of inhalation of NO and between 45 min to 3 hour after cessation of inhalation of NO.

Calculated mean ± SD pharmacokinetic variables are: A of 133 ± 29.9 µmol/L, Vd of 27.6 ± 11.6 L, (331 ± 104 ml/kg), and Cl of 169 ± 32.7 ml/min (2.15 ±0.53 ml/min/kg). Individual results are shown below.

Subject No.	(minutes)	A (μmol litre^{-1})	Volume of distribution (litre)	Volume of distribution (ml kg^{-1})	Clearance (ml min^{-1})	Clearance (ml min^{-1} kg^{-1})
1						
2						
3						
4						
5						
6						
Mean (SD)	172 (91.4	133 (29.9)	27.6 (11.6)	331 (104)	169 (32.7)	2.15 (0.53)

CONCLUSION: Seventy-four percent of I-NO was the mean uptake. Most of the initially absorbed NO reacts with hemoglobin and forms methemoglobin and nitrogen oxides. During inhalation, methemoglobin increases monoexponentially. Approximately 14% undergoes direct conversion to nitrogen oxides. The nitrogen oxides produced have a volume of distribution of one-third of body weight and a clearance similar to glomerular filtration rate.

REVIEWER'S COMMENTS: This study was performed with exhaled nitric oxide measurements to determine the fraction of adsorbed nitric oxide that reacts with hemoglobin to form methemoglobin, and to determine the peak of the serum nitrogen oxides formed during inhalation of nitric oxide.

Because the calculated and measured blood volumes were similar, this suggests that the majority of the inhaled nitric oxide that is absorbed forms methemoglobin, although in all cases blood volume calculated from nitric oxide uptake was greater than the expected value. Thus 14% of the nitric oxide may have been absorbed without forming methemoglobin. This could be by forming nitrates and nitrates, or nitrosyl hemoglobin.

Vd, clearances and time constants for serum nitrogen oxides were greater than previously reported values where oral doses of nitrate were given to volunteers. Vd 21.1, 19L, total Cl 48.3, 25.8 ml/min and elimination constant of 429, 740 min. These differences may reflect the route of administration. A reduced bioavailability of nitrates by the oral route would cause an apparent increase in the Vd, but delayed absorption would explain the difference in clearance between this study and others.

Clearance of nitrogen oxides in this study were close to the expected values for GFR, suggesting that nitrates are filtered, but reabsorbed in the kidney. The kidney is known to be a major elimination pathway for nitrates, and serum nitrates increased markedly in a patient with renal failure who received inhaled nitric oxide.

APPENDIX II: OTHER STUDY

METHAEMOGLOBIN PRODUCTION IN NORMAL ADULTS INHALING LOW CONCENTRATIONS OF NITRIC OXIDE

INVESTIGATORS: Young JD, Dyar O, Xiong L, Howell S.

CITATION: Intensive Care Medicine. 1994; 20:581-584.
STUDY DATES: not published
OBJECTIVES: To determine the changes in blood methemoglobin level during the inhalation of nitric oxide.
STUDY DESIGN: This was an unblinded dose-response study conducted on four occasions, with each occasion separated by one week.
POPULATION: Five healthy, non-smoking adults (4 males, 1 female) ages 30-36 were studied.
STUDY DOSE: Nitric oxide was inhaled at inspired concentrations of 32, 64, 128, and 512 volumes per million (vpm) in air. For subjects receiving 32, 64 and 128 vpm, inhalation continued for 3 hours. Subjects inhaling 512 vpm continued inhalation until the methemoglobin exceeded 5% of the total hemoglobin. All gas flows were measured with precision rotameters.
FORMULATION: NO was stored as 2000 vpm in nitrogen and was added to the breathing circuit to give a final inhaled concentration of 32, 64, 128 or 512 vpm in air.

PROCEDURES: A 16 G cannula was placed in a vein in the forearm to allow blood sampling. Subjects were fitted with a leak-free face mask connected to a breathing circuit. Air was delivered to the circuit at 5 L/min. Inspired oxygen fraction was measured with a paramagnetic oxygen analyzer, and in the experiments using 512 vpm a small amount of oxygen was added to the circuit to overcome the reduction in inspired oxygen caused by the nitrogen carrier gas.

Sampling. Blood samples for methemoglobin levels were taken at 0, 3, 5, 10 min and at 10 minute intervals after the beginning of inhalation. Venous blood was drawn every 10 minutes for methemoglobin determination.

ASSAY:

ANALYSIS: The increase in methemoglobin fraction during NO inhalation and the decay after ceasing inhalation was best described by a first order elimination model. The increase in methemoglobin fraction during NO inhalation were fitted to a simple single compartment model, where it is assumed that methemoglobin is produced at a constant rate during NO inhalation, that methemoglobin remains in the blood, and that the rate of methemoglobin elimination from the blood depends on the methemoglobin concentration (the model is virtually identical to a single compartment drug infusion model with first order pharmacokinetics). The equation used was:

$$\text{Methemoglobin concentration at time } t = B + A * (1 - e^{-(t/\tau)})$$

B= baseline methemoglobin concentration (% of total hemoglobin)
A= plateau concentration of methemoglobin
τ = time constant (minutes)

For the decrease in methemoglobin concentrations after discontinuing 512 vpm, the model used was,

$$\text{Methemoglobin concentration at time } t = B + A * (e^{-(t/\tau)})$$

B in this equation was the asymptotic value for methemoglobin.

The curves were fitted to the averaged data using a least squares regression method (Asystant Plus software version: 1.1, Asyst Technologies, Rochester, NY). The value for B in both cases was taken as the baseline mean methemoglobin level prior to NO inhalation, the values for A and τ were then obtained from the fitted curve. To obtain the maximum rate of increase in methemoglobin concentration, which corresponds to the rate at which methemoglobin is formed, the fitted equations were differentiated and solved for t-0. This simplifies to A/τ.

PHARMACOKINETIC RESULTS:

The mean methemoglobin level before NO inhalation was 0.64% (0.09%). The increase in methemoglobin level over 3 hours during inhalation are shown in figure 1.

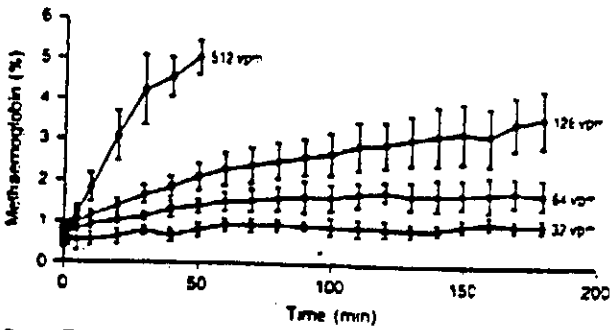


Fig. 1. The increase in methaemoglobin level over 3 h during inhalation of 32, 64, 128 and 512 vpm nitric oxide. Means ± SD shown ($n = 5$)

The decline in methemoglobin levels after discontinuing 512 vpm NO when the methemoglobin reached 5% are shown in figure 2.

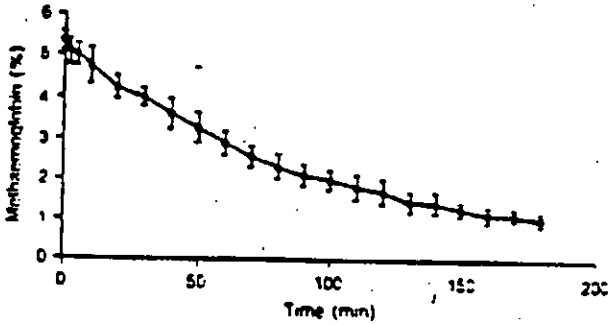


Fig. 2. The decline in methaemoglobin levels after discontinuing 512 vpm nitric oxide when the methaemoglobin reached 5%. Means ± SD shown ($n = 5$)

The calculated time constants for the elimination of methemoglobin were between 39-91 minutes. The predicted mean maximum methemoglobin levels that would be achieved during inhalation at the doses used are shown in Table1. This table lists the parameters calculated from the fitted curves.

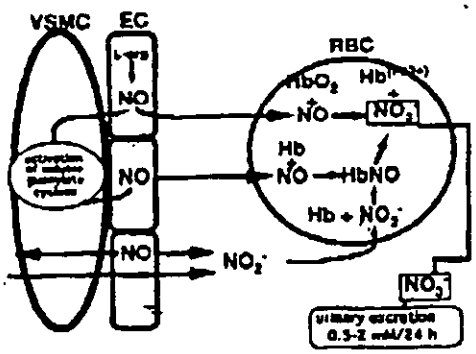
Nitric oxide dose	Calculated plateau concentration as % (A + B)	95% confidence interval for plateau	Calculated time constant in minutes (τ)	95% Confifidence interval for time constant	Calculated maximum rate of increase in methaemoglobin at t = 0 (% per min)
32 vpm	1.04	0.92 – 1.16	73.9	33.1 – 114.7	0.0065
64 vpm	1.75	1.60 – 1.90	54.9	48.4 – 61.4	0.021
128 vpm	3.75	3.58 – 4.05	87.7	74.2 – 101.2	0.036
512 vpm	6.93	5.70 – 8.16	39.2	27.0 – 51.4	0.164
decay from 5% methaemoglobin	N/A	N/A	90.9	87.0 – 94.9	N/A

For all curve fitting $r^2 > 0.995$, $p < 0.0001$

CONCLUSION: Normal individuals that inhale up to 128 vpm of NO, greater than any dose used clinically to date, does not exhibit clinically significant methemoglobinemia. Maximum methemoglobin levels are likely to be reached 3-5 hours after inhalation begins in normal individuals.

Dose of NO up to 128 vpm exhibit first order kinetics. Because only the initial steep portion of the curve for 512 vpm was determined, first order kinetics is not definitive. However, the decay from 5% methemoglobin contains many points and follows first order kinetics.

APPENDIX III: METABOLIC PATHWAY FOR I-NO



NO is generated from L-arginine (L-arg) in endothelial cells (EC) and diffuses to the vascular lumen, from which it is taken up in the red blood cells (RBC) or abluminally to the vascular smooth muscle cells (VSMC). In the RBC, NO may react with oxyhemoglobin (HbO_2) to form methemoglobin (Methb) and NO_3- or with hemoglobin (Hb) to form nitrosohemoglobin (HbNO). HbNO may then also be converted to methemoglobin and NO_3-. If NO is converted to nitrite (NO_2-) before being taken up in the RBC, the same products are formed, i.e., HbNO, NO_3-, and methb. NO_3- diffuses to the plasma from which it is cleared via the kidneys.

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