**NATIONAL INSTITUTE OF
PUBLIC HEALTH AND THE ENVIRONMENT
BILTHOVEN, THE NETHERLANDS**

Report no. 235802 011

Intravenous administration of sodium nitrite to healthy volunteers: a single ascending dose study

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May 1998

This investigation has been performed on behalf of the Health Protection Inspectorate and the Directorate for Food and Product Safety in the Netherlands under project number 235802.
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ABBREVIATIONS

ADI	Acceptable Daily Intake
AE	Adverse Experience
Alk. Phos.	Alkaline phophatase
ALAT	Alanine amino tranferase
ARO	Laboratory of Residue Analyses
ASAT	Aspartate amino transferase
AUC_{0-24}	Area under the curve of a 24 hour interval
AZU	Utrecht University Hospital
b.p.m.	Beats per minute
bw	Body weight
cm	Centimetres
C_{max}	maximum concentration
dL	deciliters
ECG	Electrocardigram
exp9	times 10^9
F	female
g	gravity
gr	gram
GCP	Good Clinical Practice
G6PD	Glucose-6-Phosphate Dehydrogenase
γGT	Gamma Glutamyl Transpeptidase
GLP	Good Laboratory Practice
Hb	Hemoglobin
HPIC	High Performance Ion Chromatography
hr	hour
hrs	hours
Ht	Hematocrit
kg	kilogram
L	litres
LDH	lactate dehydrogenase
ln2	natural logarhythm
M	Male
metHb	Methemoglobin
min	minutes
mg	milligram
mmHg	millimeters mercury pressure
mmol	millimolar
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
$NaNO_2$	Sodium nitrite
NMN	N-methylnicotinamide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOEL	No Observed Effect Level
NVIC	National Poisons Control Centre
RIVM	National Institute of Public Health and the Environment

SD	Standard Deviation
SOP	Standard Operating Procedure
t_{max}	Time to maximum concentration
$t_{1/2}$	Time needed to reach half of the initial plasma concentration
U/L	Units per liter
V_z	Volume of distribution
WHO	World Health Organization
WOM	Medical Ethics Committee of the Utrecht University Hospital
yrs	years
y/m/d	year/month/day

ABSTRACT

There is widespread concern about exposure to nitrate from dietary sources and its potential risk to human health. The toxicity of nitrate is low. However, in the proximal part of the gastro-intestinal tract, part of the nitrate is converted to nitrite, a more toxic compound. In considering the health risks of ingested nitrate, therefore, it is important to take into account the formation, oral bioavailability and toxicity of nitrite. A healthy volunteer study was planned to investigate the oral bioavailability of nitrite from an aqueous solution. However, in blood nitrite is rapidly oxidized by hemoglobin to yield methemoglobin and nitrate. Calculating the oral bioavailability of nitrite from the plasma nitrite concentration is, therefore, hampered and requires a substantial nitrite dose to be administered to healthy volunteers. Because of the vasodilating and methemoglobin inducing potential of nitrite there are limitations to the nitrite dose which can safely be administered to volunteers in a bioavailability study. The bioavailability study was, therefore, preceded by a single , intravenous, ascending dose study to investigate the maximum nitrite dose which can safely be administered in vivo; i.e. inducing approximately 10 to 15% metHb. This study demonstrates that a sodium nitrite dose of 0.12 mmol $NaNO_2$ per mmol Hb (between 290 and 370 mg) and inducing approximately 10.8% methemoglobin in the blood, is the maximum tolerated dose for adult volunteers. At this dose level all volunteers reported complaints, although of mild intensity. In addition lowering of the blood pressure and a compensatory increase of the heart rate was evident at all dose levels but stayed within acceptable limits. The peak plasma nitrite concentrations were between 3.3 and 4.8 mg/kg. Given the limit of quantification for nitrite in plasma these concentrations are acceptable for performing a bioavailability study.

SUMMARY

This report describes the results of a healthy volunteer study, investigating the maximum sodium nitrite dose which can safely be administered to adult volunteers; i.e. inducing approximately 10 to 15% metHb. The study was conducted at the National Poisons Control Centre of the Netherlands. The study was designed as a single, ascending, intravenous dose study. Three adult volunteers (1 male and 2 females) participated in this study. They received 4 times an intravenous infusion with a different sodium nitrite dose with a wash out period of one week between the administrations. All sodium nitrite doses were administered after an overnight fast. Adverse experiences, blood pressure and heart rate, hemoglobin concentration, the percentage of methemoglobin in the blood and the plasma nitrite and nitrate concentration were frequently determined over a period of 24 hours following the intravenous administration of the sodium nitrite.

The first dose of 0.04 mmol $NaNO_2$ per mmol Hb (between 90 and 130 mg) induced on average 3.4% metHb. All volunteers reported complaints, although of mild intensity. In two out of three volunteers a rapid and substantial drop of the diastolic blood pressure was observed.

After adjusting the time of infusion from 10 to 30 minutes in the second treatment session, with the same dose , the decrease of the blood pressure was less substantial and not so rapidly. Only one of the volunteers reported complaints.

In the third treatment session the sodium nitrite dose was doubled to 0.08 mmol $NaNO_2$ per mmol Hb (between 190 and 250 mg). The metHb reached a maximum of 6.6%. One volunteer reported complaints. The blood pressure decreased in all volunteers to an extent comparable to the second treatment session.

In the fourth and last treatment session a sodium nitrite dose of 0.12 mmol $NaNO_2$ per mmol Hb (between 290 ans 370 mg)was administered. The metHb reached a maximum of 10.8%. All volunteers reported complaints. The decrease of the blood pressure was comparable to the first treatment session.

The plasma nitrite concentration increased approximately proportional to the sodium nitrite dose. The peak plasma nitrite concentrations were between 3.3 and 4.8 mg/kg at the highest dose level. Nitrite rapidly disappeared from the plasma with a terminal elimination half life of approximately 30 minutes.

The formation of metHb was not proportional to the sodium nitrite dose. The ratio AUC_{metHb} / $AUC_{nitrite}$ increased with the administered dose.

The results of the present study will be used to design an absolute oral bioavailability study of nitrite in healthy volunteers. From the peak plasma nitrite concentrations that were observed in the present study it is concluded that the highest dose must be considered for the bioavailability study.

SAMENVATTING (SUMMARY IN DUTCH)

Dit rapport beschrijft de resultaten van een vrijwilligers-onderzoek dat als doel had in kaart te brengen wat de maximale hoeveelheid natriumnitriet is die nog veilig aan proefpersonen kan worden toegediend; dat is een dosis die ongeveer 10 tot 15% methemoglobine induceerd. Het onderzoek werd uitgevoerd door het Nationaal Vergiftigingen Informatie Centrum in Nederland. Het betrof een studie met enkelvoudige, intraveneuze toedieningen van natriumnitriet in opklimmende dosering. Drie vrijwilligers (2 vrouwen en 1 man) ontvingen 4 intraveneuze infusies met natriumnitriet met een tussenpoos van een week. De natriumnitriet werd steeds toegediend na een nachtelijke periode van vasten. Eventuele klachten en symptomen, de bloeddruk en de hartslag, het hemoglobine gehalte, het percentage methemoglobine in het bloed en de plasma nitriet en nitraat concentraties werden frequent bepaald over een periode van 24 uur na toediening van de natriumnitriet.
Tijdens de eerste behandelperiode werd een natriumnitriet dosis van 0.04 mmol $NaNO_2$ per mmol Hb (tussen 90 en 130 mg) aan de vrijwilligers toegediend. Alle drie vrijwilligers hadden hierop klachten die weliswaar mild van aard waren. Twee van de drie vrijwilligers toonden bovendien een snelle en substantiele daling van de bloeddruk.
Nadat de tijd waarover de natriumnitriet werd toegediend was aangepast van 10 naar 30 minuten werd met dezelfde dosis in de tweede behandelperiode een minder snelle en minder grote daling van de bloeddruk waargenomen. Bovendien had maar één van de drie vrijwilligers klachten.
In de derde behandelperiode werd de dosis verdubbeld tot 0.08 mmol $NaNO_2$ per mmol Hb (tussen 190 en 250 mg). Het metHb gehalte bereikte een maximum van 6.6%. Een vrijwilliger rapporteerde klachten. De bloeddrukdaling was vergelijkbaar met de tweede behandelperiode.
In de vierde en laatste periode werd een natriumnitriet dosis van 0.12 mmol $NaNO_2$ per mmol Hb (tussen 290 en 370 mg) toegediend. Het metHb gehalte bereikte een maximum van 10.8%. Alle vrijwilligers hadden klachten op deze dosering. De bloeddrukdaling was vergelijkbaar met de eerste behandelperiode.
De toename van de plasma nitriet concentratie was ongeveer proportioneel met de dosis.De maximale nitriet concentraties varieerden tussen 3.3 en 4.8 mg/kg op het hoogste doseringsniveau.
The vorming van methemoglobine verliep niet proportioneel met de natriumnitriet dosis. De ratio AUC_{metHb} / $AUC_{nitriet}$ nam toe met de dosis.

De resultaten van deze studie worden gebruikt bij het ontwerpen van een orale biobeschikbaarheids-studie van nitriet. Uit de waargenomen maximale plasma nitriet concentraties tijdens deze studie kan worden geconcludeerd dat de hoogste dosis overwogen moet worden voor de biobeschikbaarheids-studie.

1. INTRODUCTION

Setting adequate standards for nitrate in vegetables and drinking water under the guidance of the present Acceptable Daily Intake (ADI) is becoming increasingly problematic, since nitrate levels in food products and drinking water tend to increase continuously in Western Countries (1). In some areas, cultivating certain vegetables will encounter problems because growers cannot meet the requirements set for nitrate based on the present ADI. The present ADI, allocated to nitrate by the WHO, is 5 mg sodium nitrate/kg bw per day and is based on the no-adverse effect level in rats (2,3). Nitrate itself is considered to be of low toxicity. Generally, the toxicity of nitrate has been ascribed to the conversion of nitrate into nitrite by bacterial reduction in the oral cavity (4,5) and gastro-intestinal tract (6,7). In rats, however, nitrate conversion into nitrite is low. Healthy volunteer studies indicate that, in humans, approximately 7 % of a nitrate-dose is converted into nitrite in the proximal part of the gastro-intestinal tract (8,9,10). The rat, therefore, seems less suitable to extrapolate the toxicity of nitrate to man. Since the toxicity of nitrate is mainly determined by the amount of nitrate converted to nitrite, the ADI for nitrate may be calculated from the conversion rate of nitrate to nitrite in humans and the no-toxic effect level for *nitrite* in rats.

The amount of nitrate converted to nitrite in the oral cavity provides information on the exposure of the gastro-intestinal tract to nitrite. This information may be useful when the reactivity of nitrite with amines in the food, to form carcinogenic N-nitroso compounds, appears to be relevant for public health (i.e. increased gastric cancer risk). This has not been supported by epidemiological data yet (11,12).
However, besides the gastro-intestinal exposure, the nitrite absorbed, is leading to a systemic exposure to nitrite. At present no data are available on the systemic exposure to nitrite from an oral nitrite dose. Since the present ADI for nitrite is derived from the NOEL observed in rats which is based on systemic toxicity parameters, and since the formation of endogenous N-nitroso compounds in gastric cancer etiology has never unambiguously been shown, it is highly relevant to perform an oral nitrite bioavailability study in healthy volunteers.

In blood nitrite is rapidly oxidised by hemoglobin to yield methemoglobin (metHb) and nitrate. Calculating the oral bioavailability from the plasma-nitrite concentration is, therefore, hampered and requires a substantial nitrite dose to be administered to healthy volunteers.

The acute effects of nitrite, when large doses are ingested, are: methemoglobinemia, vasodilatation (facial redness, headache, dizziness, hypotension and palpitations) and gastro-intestinal disturbances (nausea, vomiting, abdominal cramps and diarrhoea). At a metHb percentage of 15 % or more cyanosis occurs, at a metHb percentage between 30 and 40 % symptoms like fatigue, weakness, tachycardia, headache and dizziness occur. In more serious cases hypoxic convulsions and coma can appear.

Thus, there are limitations to the nitrite dose which can safely be administered to volunteers, in a bioavailability study (13). The aim of the present study was, therefore, to find a suitable dose for intravenous administration to healthy volunteers during the oral nitrite bioavailability study; i.e. a safe dose which is also adequate to perform bioavailability calculations.
In the present study, four ascending, intravenous, doses of sodium nitrite were investigated in healthy volunteers. The first sodium nitrite dose was selected on bases of the results of an in vitro experiment

testing the relationship between the nitrite dose and the induced methemoglobin level in human blood samples (14). A nitrite dose inducing approximately 5 % metHb in vitro, was selected as starting dose for the present study.
A sodium nitrite dose, inducing approximately 10% methemoglobin in vivo, was chosen as the upper limit of the dose range to be tested in the present study.

This report provides a detailed description of the clinical effects, the metHb concentration in the blood and the nitrite and nitrate concentrations in plasma, in relationship to the administered sodium nitrite dose.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Study Population

Participants for the study were recruited by announcements on the bulletin boards in the building of the Utrecht University and through direct mailing to former participants or individuals who had notified us to be willing to participate in healthy volunteer studies.
One month to one week before the start of the study, volunteers, willing to participate, were screened for enrolment by a pre-study medical examination. The medical examination consisted of a standard medical history questionnaire, a physical examination, 12 lead electrocardigraphy (using a Hewlett Packard cardiograph, type 4700 A) and non-invasive automated blood pressure measurement (using a Passport Monitor of Datascope®). Furthermore, blood en urine samples were drawn for routine laboratory analyses.
The pre-study medical examinations were performed by physicians other then the medical investigators involved in this study.
The participants were finally selected for enrolment in the study on basis of the results of the medical screening and the selection criteria as summarised in Appendix 5.
The General Practitioner was informed when his patient was participating in the study and was asked to inform the medical investigator when he disagreed on the decision to enrol the subject in the study.
If the routine blood and urine analyses revealed abnormal values, the clinical relevance was determined. If the results were considered to be of no clinical relevance the volunteer was included in the study. Otherwise, a new blood or urine sample was drawn and analysed until normal results were obtained or the volunteer was excluded from participation in the study.
After the study was completed the volunteers underwent a post-study medical examination. The volunteers were asked to report any change in their health status as compared to the pre-study medical screening. Furthermore, the physical examination and routine blood and urine investigations were repeated.

2.1.2. Products under Study

Sodium nitrite (CAS 7632-00-0) was dissolved in distilled water in an isotonic concentration of 10 mg/ml. The sodium nitrite formulation was prepared by the Pharmacy of the University Hospital in Utrecht and delivered in bottles of 100 ml each. For each volunteer and each session a new bottle was used.
All nitrite studies (the in vitro nitrite study (14), the present study, the oral dose finding study (15) and the bioavailability study (13)) were performed with sodium nitrite originating from the same batch (identification number: 99950619 42614).
During the study the bottles were stored in the dark, at room temperature.

After completion of the study one bottle of the batch was analysed by the Laboratory for Residue Analyses for the actual sodium nitrite content. The results indicated an actual sodium nitrite content of 9.7 ± 0.2 mg/ml in the bottles. The remainder of the batch will be returned to the Pharmacy.

2.2. Study Sites

The clinical part of the study was performed at the Clinical Research Unit of the National Poisons Control Centre in Utrecht, the Netherlands.
The analyses of the blood samples for the hemoglobin concentration and the percentage of methemoglobin were performed at the Clinical Research Unit of the National Poisons Control Centre in Utrecht, the Netherlands.
The routine laboratory analyses of blood and urine specimen obtained during pre and post-study screening and the determination of liver enzymes in blood samples collected during the study were performed by the Department of Clinical Chemistry of the Utrecht University Hospital (AZU), the Netherlands.
The analyses of the plasma samples for nitrate and nitrite were performed by the Laboratory for Residue Analyses (ARO) of the National Institute of Public Health and the Environment (RIVM) in the Netherlands.

2.3. Methods

2.3.1. Study Protocol

The present study was carried out in accordance with the protocol entitled "Intravenous administration of sodium nitrite to healthy volunteers: a single ascending dose study (dated 18-04-1995, revised 08-06-1995)". The study protocol was approved by the Medical Ethics Committee of the University Hospital in Utrecht, the Netherlands.
The study was performed under the recommended principles of Good Clinical Practice for studies with medicine in the European Community (Appendix 1 and 2).
The participants in the study were fully informed, both orally and in writing, about the purpose of the study, the study design and the possible risks involved. The volunteers signed a written statement of consent (Appendix 4).
During the first treatment session, sodium nitrite was infused over 10 minutes, as stated in the protocol. Because a decrease of the blood pressure that was observed during the first treatment session, the infusion time was prolonged form 10 to 30 minutes, and the same dose level was administered again as a 30 minutes infusion. All subsequent sodium nitrite doses were then administered over 30 minutes. The volunteers were orally informed about the changes in the study procedures and the purpose of these changes before the changes in the study procedures were implemented. The volunteers signed a written statement of consent (Appendix 4A).
The Medical Ethics Committee was informed as soon as possible after the changes in the study procedures were implemented.

2.3.2. Study Design

The study was designed as a single dose, intravenous study with ascending sodium nitrite doses. Three healthy, adult volunteers received four intravenous infusions with sodium nitrite with a washout period of one week between the treatments.
The dose level of the first dose of sodium nitrite to be administered to the volunteers, was based on the results of an in vitro study, that was previously performed, and in which the methemoglobin inducing potential of nitrite was investigated in human blood samples. A sodium nitrite dose of 0.04 mmol $NaNO_2$ per mmol hemoglobin, which induced, in vitro, on average 5.3 % methemoglobin in the blood, was chosen as starting dose for the present in vivo study. To extrapolate the in vitro dose to a sodium nitrite dose to be administered in vivo the sodium nitrite dose of 0.04 mmol/ mmol Hb was multiplied by the estimated total amount of hemoglobin in the body. It was assumed that the distribution of nitrite within the body is restricted to the blood compartment.

The following formulas were used to calculate the total amount of hemoglobin (Hb) in the body:
1) Blood volume (L) = 0.07 * BW (kg)
2) Total amount of Hb in the circulation (mmol) = Hb (mmol/L) * Blood volume

in which:
0.07 = factor to derive the blood volume from the body weight. On average, 70 ± 10 ml blood per kg body weight is present in the human body.
BW = Body weight of the volunteer as determined each session before administration of the sodium nitrite dose
Hb = baseline hemoglobin concentration as determined each session before administration of the sodium nitrite dose

The sodium nitrite dose was increased in subsequent treatment session until approximately 10 % methemoglobinemia was induced in the volunteers.

The volunteers received the following treatments in subsequent order:
- 0.04 mmol $NaNO_2$ per mmol Hemoglobin infused over 10 minutes,
- 0.04 mmol $NaNO_2$ per mmol Hemoglobin infused over 30 minutes,
- 0.08 mmol $NaNO_2$ per mmol Hemoglobin infused over 30 minutes,
- 0.12 mmol $NaNO_2$ per mmol Hemoglobin infused over 30 minutes.

For the intravenous administration of the sodium nitrite a volumetric infusion pump, type IVAC® 560, was used. The infusion pump was connected to an intravenous cannula inserted in a forearm vein. The cannula was inserted in the forearm opposite to the arm in which the cannula for blood sampling was inserted. The infusion speed and the infusion volume were dependent on the time of infusion and the calculated sodium nitrite dose to be administered to the volunteer (see previous page).

The sodium nitrite was administered in fasting state and fasting was maintained until approximately 4 hours post dose.
Clinical observation of the participants took place from approximately 1.5 hours before until 10 hours after administration of sodium nitrite. During this period blood samples were taken at timed intervals, adverse experiences were recorded, the blood pressure and heart rate were determined and continuous telemetric monitoring was performed. Additionally, the volunteers were requested to visit the Clinical

Research Unit 24 hours following the administration of the sodium nitrite dose for the last blood sample and blood pressure measurement.
At each session, before administration of the sodium nitrite, a pregnancy test (TestPack Plus^{tm} hCG-urine, ABBOTT) was performed in female volunteers and the body weight of all volunteers was determined using a Seca balance of Schinkel Medical Instruments, the Netherlands.

Diet
Three days prior to and on each investigation day the volunteers consumed a nitrite and nitrate low diet (Appendix 7). The actual food intake was registered in a diary.
On each study day the same meals were consumed during clinical observation. Between 4 and 4.5 hours after administration of the sodium nitrite dose, lunch was served, consisting of 3 slices of bread (two with jam and one with cheese) and one glass of milk.
Between 6 and 6.5 hours after administration of the sodium nitrite dose, tea, two slices of bread with jam and one banana was served.
Distilled water was taken ad libitum.

Adverse Experiences
During the study days the volunteers were frequently asked about adverse experiences, in general terms. The participants were requested to report all side effects, whether related to the sodium nitrite or not.

Blood pressure and heart rate
Blood pressure and heart rate were measured with a non-invasive automated blood pressure meter (Passport Monitor® of Datascope) at timed intervals during the day. Blood pressure and heart rate were determined with the participant being in bed, in a slightly upright position. The volunteers remained in this position until 4 hours post dose. The time points on which the blood pressure and the heart rate were determined were specified in advance, following the scheduled procedures as presented in Appendix 6. When clinically indicated more frequent blood pressure measurements were performed.

Continuous heart rate monitoring
Participants were under continuous ECG-surveillance (lead II or III) for 4 hours following the start of the sodium nitrite infusion (using the Passport Monitor® of Datascope).
Print outs of rhythm strips were made, each session, before dosing and if clinically indicated thereafter.

Blood sampling
Blood samples of 5 ml were taken from a cannula inserted in a forearm vein. The blood sampling was performed following Standard Operating Procedures (16) with the volunteer being in supine position and without using tourniquet pressure. The 24 hour blood sample was obtained by vein puncture in the fossa cubiti, using tourniquet pressure.
The blood samples were collected in heparinized tubes and immediately placed in the IL 282 Co-oximeter to determine the percentage of methemoglobin and the hemoglobin concentration.
Thereafter, the samples were centrifuged for 10 minutes at 3000 g and the supernatant (plasma) transferred into polypropylene tubes as soon as possible. The plasma samples were immediately frozen in liquid nitrogen and stored at -80°C until analysed for the nitrate and nitrite content. The scheduled time points for blood sample collection are presented in Appendix 6. The volunteers were

requested to come in a fasting state when visiting the Clinical Research Unit for the 24.00 hour blood sample.
The total amount of blood taken from the participant during the investigation (including hematology and clinical chemistry tests) was approximately 500 ml.

Liver enzymes
In dogs elevated liver enzymes were observed after administration of sodium nitrite (17). No data were found concerning the liver dysfunction in healthy volunteers during administration of sodium nitrite. Therefore, in the present study, the liver enzymes of the volunteers were determined during the pre- and post-study screening and during the third and fourth study day, just before and 24 hours following the sodium nitrite dose. Blood samples of 5 ml were collected in heparinized tubes and analysed for ALAT, ASAT, LDH and γGT activity.

2.3.3 Sample Analyses

Routine laboratory analyses
During the pre and post study screening blood and urine samples were taken for routine laboratory investigations. The following parameters were determined: Blood analyses on Hb, Ht, methemoglobin, leukocytes (+ differentiation), sodium, potassium, urea, calcium, creatinin, bilirubin, ASAT, ALAT, LDH, γGT and dipstick urine analyses on glucose, protein, blood and leukocytes. A microscopic analyses of the urine was only performed if indicated by the results of the qualitative urine analyses. During the third and fourth study day, blood samples were taken for the analyses of the ALAT, ASAT, LDH and γGT-activity. The precision of the test-results was assured by interlaboratory surveillance procedures ("ring validation" method).

(Met)hemoglobin measurements
The percentage of methemoglobin and the hemoglobin concentration (gr/dL) were determined with an IL 282 Co-oximeter (Instrumentation Laboratory BV, the Netherlands) according to Standard Operating Procedures (18). The Hb concentration was measured in gr/dL and converted to mmol/l by multiplying with 0.62.
The IL 282 Co-oximeter measures the absorbance spectra of diluted and hemolyzed blood samples at four specific wavelengths to enable differentiation between four species of hemoglobin: oxyhemoglobin, methemoglobin, reduced hemoglobin and carboxyhemoglobin. The total hemoglobin concentration is calculated subsequently.
Within the range of measurement, the stated 95% confidence limits of accuracy are ± 0.2 gr/dL for the Hb and ± 1% for the metHb, respectively. The stated precision is 0.2 gr/dL and 0.5%, respectively.
The Co-oximeter was daily calibrated on the total hemoglobin value with IL CalDye^{R} solution (lot no. 7412203).

Nitrate and nitrite analyses
Concentrations of nitrate and nitrite in plasma were determined by the Laboratory of Residue Analyses by means of a previously established method based on High Performance Ion Chromatography and UV detection at 208 nm (19). The method was optimised to ensure a limit of quantification for plasma nitrite which was sufficiently low. Analyses of the samples took place according to SOP ARO/414 (20). For quality assurance about 1 out of 10 samples was analysed in

duplicate. Recovery experiments and analyses of blanc samples took place daily. Additional quality assurance included frequent analyses of home made quality control plasma samples with a nitrite concentration of 3.00 mg/kg and a nitrate concentration of 13.8 mg/kg.
The results of all but one of the duplicate samples were within 10% of the mean concentration of the samples. The recovery experiments showed an average recovery of 100 ± 10% for all but one of the experiments. The lower limit of quantification was 2 mg/kg for plasma nitrate and 0.3 mg/kg for plasma nitrite.

2.3.4 Data Analyses

Toxicokinetic analyses were performed on the percentage of metHb and the plasma nitrite concentration. For toxicokinetic analyses the computer programme Topfit 2.0 was used.
The area under the curve (AUC), terminal elimination half-life ($t_{1/2}$), maximum concentration (C_{max}), time to maximum concentration (t_{max}) and the volume of distribution (V_z) were calculated on the basis of a model independent analyses.
The AUC was calculated from the data, using the linear trapezoid rule and extrapolation to infinity from the lowest reliable data point onwards. The lowest reliable data point being 1.5% for metHb and 0.3 mg/kg for the plasma nitrite concentration. The percentage of the AUC that was obtained via extrapolation, is indicated in the tables (Extrapolation AUC (%), Appendix 15c and 16c). For treatment comparison the AUC and C_{max} were adjusted to a standard sodium nitrite dose of 220 mg (C_{max} adjusted*, Appendix 15c and 16c).
Under normal circumstances, small amounts of methemoglobin are present in the blood. The percentage of metHb, as observed after administration of sodium nitrite, was, therefore, corrected for the baseline concentration. It was assumed that the baseline percentage of metHb in the blood reflects a steady state situation. The baseline metHb values of the four treatment periods, of each individual volunteer were, therefore, averaged and the data of every treatment period were corrected for this mean baseline metHb value.
The baseline plasma nitrite concentrations were in all but two cases below the detection limit of 0.1 mg/kg. The plasma nitrite concentrations observed after administration of sodium nitrite were not corrected for a baseline concentration since there are as yet no evident data on measurable amounts of nitrite being present in the blood under normal circumstances.
The volume of distribution of nitrite was calculated applying the formula
$V_z = NaNO_2$ dose / AUC * (ln2 / $t_{1/2}$).
The terminal elimination half-life of metHb was calculated from a time point of approximately twice t_{max} onwards, being from 1.50 hrs post dose onwards for treatments 1 and 2 and from 2.00 hrs and 2.50 hrs post dose onwards for treatments 3 and 4, respectively. MetHb values below 1.5% were not included in the calculation of the terminal elimination half-life.
The terminal elimination half-life of plasma nitrite was calculated from a time point of twice t_{max} onwards, being from 0.50 hrs post dose onwards for treatment 1 and from 1.00 hrs post dose onwards for treatments 2, 3 and 4.

2.4 Archiving

All study documents, including this report will be kept on file in the GCP-archives of the Department of Medical Toxicology of the National Poisons Control Centre of the Netherlands for a period of at least 15 years.

3. RESULTS

3.1 General

No major protocol violations occurred. In cases the study procedures were not in full compliance with the protocol this was registered. The deviations to the protocol are summarised in Appendix 3. The deviations to the protocol had no impact on the outcome of the study.

3.2 Pre-Study Medical Screening

Six volunteers underwent a medical screening between June 27 and July 21, 1995. The demographic characteristics of these volunteers are summarised in Appendix 8. Volunteer 01 was not eligible because of an abnormal ECG that was diagnosed as a wandering pacemaker. Volunteer 02 was found eligible but withdrew before the first treatment session. Volunteer 4 entered the study as a possible replacement. She did not receive treatment because all participants completed the study.
Two female and one male, Caucasian, volunteers entered and completed the study. They were non-smokers and did not take any concurrent medication except for subject 3 who took Tryginon® as an oral contraceptive. The medical history, the physical examination and the routine blood and urine investigations of the participating volunteers did not reveal abnormalities which were considered to have implications for their participation in the study. The results of the vital signs and routine laboratory tests are tabulated in Appendix 10.

- Volunteer 1 was 25 years old, his weight was 70 kg and his height 179 cm. The medical history and the physical examination of the volunteer did not reveal abnormalities apart from low vision for which he wore spectacles. The routine laboratory test results were all within normal range.

- Volunteer 2 was 21 years old, her weight was 66 kg and her height 166.5 cm. Her medical history showed two episodes of eczema, once during childhood and once during adulthood. The routine laboratory test results of the blood were all within normal range. The dipstick urine analyses was positive for blood. The microscopic analyses of the urine was normal apart from the presence of 0 - 4 leukocytes per field. The results were judged by the physician as of no clinical relevance.

- Volunteer 3 was 19 years old, her weight was 66.5 kg and her height 174.5 cm. The medical history showed surgery of the tympanic membrane at the age of 8 and 11; chiropractic treatment for low back pain due to mild scoliosis; alcohol intolerance, for which she practised abstinence; abdominal pain during the menstruation period; on rare occasions short lasting vascular collapse after prolonged fasting or during menstruation and episodes of nose bleed, approximately once a month. The routine laboratory test results of the blood showed a mildly decreased alkaline phosphatase and the dipstick urine analyses was weakly positive for blood and leukocytes. The results were judged by the physician as of no clinical relevance.

3.3 Post-Study Medical Screening

The post-study medical examinations were performed between August 11 and August 31, 1995. The results are presented in Appendix 10. No changes in health were observed. The routine laboratory test results of the blood showed minor abnormalities: a slightly elevated percentage of monocytes in the blood of volunteer 1 and a mildly decreased urea in volunteer 2. The dipstick urine analyses of volunteers 2 and 3 yielded abnormal results: for volunteer 2 the test was weakly positive for blood and leukocytes and for volunteer 3 the test was weakly positive for albumin and leukocytes and strongly positive for blood. In general, dipstick urine analyses yielding negative or weakly positive test results were considered of no clinical importance. The strongly positive test result for blood of volunteer 3 was considered to be due to the menstruation and therefore of no clinical relevance.

3.4 Study Dates and Treatments

The clinical part of the study was performed between July 18 and August 22, 1995. The actual dates on which sodium nitrite was administered and the administered doses are reported in Appendix 9.
The sodium nitrite doses that were administered depended on the body weight and the blood-hemoglobin concentration of the volunteer that was measured at each treatment session just before the dosing (see section 2.3.2). The weight and the baseline hemoglobin concentration of the volunteers are therefore presented in Appendix 9 as well.
Four sodium nitrite doses were administered intravenously. The first sodium nitrite dose was 0.04 mmol $NaNO_2$ per mmol Hb, i.e. between 90 and 130 mg. The sodium nitrite was infused over 10 minutes.
In the second treatment session the same dose level as in the first treatment session was now infused over 30 minutes.
In the third and fourth treatment session, respectively, sodium nitrite doses of twice and three times the starting dose were administered, i.e. between 190 and 250 mg in the third treatment session and between 290 and 370 mg in the fourth treatment session.. These doses were administered over 30 minutes.
For volunteer 3, during the first treatment session, on July 24, the infusion of the sodium nitrite was temporarily stopped for 8 minutes because of improper placement of the infusion line in the infusion pump. After resolving the problem the pump was restarted (see also Appendix 3).

3.5. Adverse Experiences

The adverse experiences (AEs) as reported by the individual volunteers, during each treatment session, are summarised in Appendix 12. In general, AEs which are known to be related to sodium nitrite were classified as possibly related. AEs reported by the volunteer in more then one treatment session were classified as definitely (yes) related to the testproduct. Adverse experiences present before administration of the testproduct were classified as not related. With respect to the treatment related AEs, no distinction was made between nitrite and its metabolite, nitrate.
No serious adverse events were observed during any of the treatment sessions. All AEs were of a mild intensity. During the first treatment session all three volunteers reported complaints: volunteer 1 experienced head discomfort and eye accommodation disorder; volunteer 2 experienced dizziness and tiredness; volunteer 3 experienced headache. After adjusting the infusion speed in the second

treatment session less complaints were reported by the volunteers: only volunteer 3 had complaints of dizziness.
In the third treatment session the sodium nitrite dose was doubled. This did not lead to an increase of the number of complaints. Only volunteer 1 had complaints of a tingling sensation at the infusion site.
During the last treatment session a sodium nitrite dose equal to three times the starting dose was administered. All volunteers reported complaints, resembling the complaints as observed in the first treatment session: volunteer 1 complaint of head discomfort, eye accommodation disorder, a cold feeling and a tingling sensation at the infusion site; volunteer 2 experienced dizziness; volunteer 3 experienced headache.
Except for the headache reported by volunteer 3 all adverse experiences started within one hour after the start of the infusion. Most of the AEs were short lasting (< 1 hour).
During the third and fourth study period volunteer 1 reported a tingling sensation at the infusion site, starting within 5 minutes after the start of the infusion and subsiding within 10 minutes after the infusion was stopped. This was classified as definitely related to the testproduct.
Volunteer 1 also complaint of an eye accommodation disorder. This is not an adverse experience known to sodium nitrite. Since the eye accommodation disorder occurred, both during the first and the fourth treatment session, it was classifies as definitely related to sodium nitrite.

3.6. Vital Signs

Nitrites are known to lower the blood pressure through vasodilatation (21, 22, 23). To prevent complaints of orthostatic hypotension the volunteers were kept in a recumbent position for 4 hours following the administration of the sodium nitrite.
The blood pressure and the heart rate data of the individual volunteers are tabulated in Appendix 13a. Appendix 13b and 13c graphically present the diastolic blood pressure and the heart rate of the individual volunteers, respectively.
During the first treatment session the blood pressure of volunteer 1 dropped from 124 / 70 mmHg (baseline) to 121 / 46 mmHg at 5 minutes after the start of the infusion. Directly after the infusion was stopped a blood pressure of 109 / 55 mmHg was measured. The blood pressure normalised to 126 / 72 mmHg at 1.55 hours post dose. Volunteer 2 also experienced a substantial drop in the blood pressure from 109 / 59 mmHg (baseline) to 97 / 45 mmHg directly after the end of the infusion. The blood pressure normalised to 111 / 60 mmHg at 1.27 hours post dose. Volunteer 3 showed a less substantial decrease of the blood pressure from 116 / 68 mmHg (baseline) to 112 / 63 mmHg directly after the end of the infusion. At 0.70 hours post dose the blood pressure normalised to 117 / 70 mmHg.
During the second treatment session the same sodium nitrite dose as in the first treatment session was now infused over 30 instead of 10 minutes All volunteers showed a decrease of the diastolic blood pressure. The decrease was less substantial then during the first treatment session. The blood pressure of volunteer 1 dropped from 113 / 65 mmHg (baseline) to 98 / 47 mmHg after the end of the infusion. The blood pressure of volunteer 2 dropped from 110 / 62 mmHg (baseline) to 105 / 52 mmHg during the infusion and was 101 / 59 mmHg after the end of the infusion. The blood pressure of volunteer 3 dropped from 106 / 63 mmHg (baseline) to 102 / 56 mmHg at 5 minutes after the end of the infusion.
During the third and fourth treatment session the sodium nitrite dose was increased to twice and three times the starting dose, respectively. The decrease of the blood pressure was comparable to the decrease as observed during the first treatment session.

In most instances a compensatory increase of the heart rate could be observed as the blood pressure decreased. During the first treatment session the heart rate increased on average 14 b.p.m. within the first half hour after the start of the infusion. During the second treatment session the average increase of the heart rate was 6 b.p.m.. within the first hour after the start of the infusion. During the third and fourth treatment session, respectively, the heart rate increased on average 11 and 12 b.p.m.. within the first hour after the start of the infusion.

3.7 Liver function parameters

The test results of the liver function parameters, which were tested prior to and 24 hours following the administration of the sodium nitrite in the third and fourth study period, are tabulated in Appendix 11. Elevated liver enzymes or a trend towards an increase of the liver function parameters was not observed.

3.8 Hemoglobin Concentrations

The hemoglobin concentrations (Hb) of the individual volunteers, as observed on timed intervals following the administration of the sodium nitrite are tabulated in Appendix 14a and graphically presented in Appendix 14b. Despite standard procedures for the blood sampling, considerable variation in the Hb concentration was observed. For volunteer 1 the largest within-day variability in the measured Hb concentrations was observed during the third treatment day. During this day the Hb concentration ranged from 8.49 to 9.55 mmol/l. For volunteer 2 the largest within-day variability in the Hb concentration was observed during the fourth treatment day. The Hb concentration ranged from 7.81 to 8.43 mmol/l on this day. For volunteer 3 the largest within-day variability in the measured Hb concentrations was observed during the second treatment day. During this day the Hb concentration ranged from 7.01 to 7.75 mmol/l (measurements performed under tourniquet pressure were excluded).
The graphics from the individual volunteers consistently show that, during the day, the measured Hb concentrations are lowest between 1 and 4 hours after administration of the sodium nitrite. We regard the observed variability as posture related. The volunteers continuously remained in supine position from one hour before until 4 hours following each sodium nitrite treatment. It is well known that the hydrostatic pressure acting on the circulatory system of the legs, in upright position, can cause fluid filtration to the tissues and reduce the blood volume by as much as 15 to 20 percent (24). This fluid filtration can raise the haematocrit and, therefore, the hemoglobin concentration of the blood to a considerable extent. In supine position the fluid is reabsorbed into the circulation and thus lowering the hemoglobin concentration again.
The individual volunteers also showed a considerable day-to-day variability in the Hb concentration. For volunteer 1 the average Hb concentration on a particular day ranged from 8.41 mmol/l on the fourth treatment day to 9.20 on the first treatment day. For volunteer 2 the average Hb concentration ranged from 7.88 mmol/l on the fourth treatment day to 8.97 mmol/l on the first treatment day. For volunteer 3 the average Hb concentration ranged from 6.83 mmol/l on the third treatment day to 7.17 on the second treatment day (= comparison of the average Hb concentration observed between 1 and 4 hours post dose).
Both, the two female and the male volunteers, showed day-to-day variability in the Hb. The menstruation was, therefore, not (solely) responsible for the effect. The day-to-day variability might

be a treatment or period effect. Both, volunteers 1 and 2, showed a decreasing trend in the Hb concentration during the four study periods. For volunteer 3 this trend was not apparent.

3.9 Methemoglobin Data

The percentage of methemoglobin (metHb) in the blood of the individual volunteers, as observed on timed intervals following each of the treatments, is tabulated in Appendix 15a and graphically presented in Appendix 15b. The percentage of metHb was measured twice prior to each intravenous administration of sodium nitrite.
The baseline metHb levels observed during the treatment periods ranged from 0.3 to 0.5% in volunteer 1, from 0.0 to 0.4% in volunteer 2 and from 0.4 to 0.7% in volunteer 3.
During the intravenous infusion of the sodium nitrite the percentage of metHb gradually increased and continued to increase for some time after the infusion stopped. During the first treatment period the percentage of metHb reached a maximum of 3.1 and 3.2% in volunteers 1 and 2, respectively, and a maximum of 2.0% in volunteer 3 (metHb corrected for the baseline value, see 2.3.4.).

In the second treatment period the volunteers received the same sodium nitrite dose as in the first treatment period, only this time the sodium nitrite was infused over 30 minutes instead of 10 minutes. The percentage of metHb reached a maximum of 3.0 and 3.2% in volunteer 1 and 2, respectively, and a maximum of 2.8% in volunteer 3.
For volunteer 3 a pump dysfunction occurred during the intravenous infusion of sodium nitrite in the first treatment period. The infusion of the sodium nitrite stopped after three minutes. After solving the problem the pump was restarted again after 8 minutes (see Appendix 3). It cannot be ruled out that the pump dysfunction caused a discrepancy between the infused volume on the display of the pump and the actual volume that was infused. This could explain the lower maximum metHb level in the first treatment period as compared to the second treatment period for this volunteer.
After doubling the sodium nitrite dose in the third treatment period the maximum percentages of metHb were 6.5, 6.6 and 6.7% in volunteers 1, 2 and 3, respectively. In the fourth treatment period a sodium nitrite dose of three times the starting dose was administered. The maximum percentage of metHb increased to 11.0% in volunteer 1 and to 10.6 and 10.8% in volunteers 2 and 3, respectively.
The time to reach the maximum percentage of metHb in the blood increased with the dose. During the first treatment period the t_{max} was observed at 0.42 and 0.50 hr after the start of the sodium nitrite infusion for volunteers 1 and 2, respectively, and at 0.85 hr post dose for volunteer 3. As the infusion time was prolonged in the second treatment session the t_{max} was observed at 0.75 hr post dose for volunteers 1 and 2 and at 1.00 hr post dose for volunteer 3. After doubling the dose in the third treatment period the t_{max} was reached at 1.00 hr, 1.02 hr and 1.17 hr post dose for volunteers 1, 2 and 3, respectively. In the fourth treatment period the t_{max} was observed at 1.00 hr, 1.17 hr and 1.17 hr post dose, respectively.
The kinetic parameters for metHb are summarised in Appendix 15c. Note that all kinetic parameters were determined from the concentration-time data which were corrected for the mean baseline percentage of metHb observed over the 4 treatment periods.
The percentage of metHb in the blood decreased approximately exponentially with time. The terminal elimination half life of the metHb, observed in each of the treatment periods, ranged between 1.19 and 1.47 hrs for volunteer 1, between 0.86 and 1.14 hrs for volunteer 2 and between 1.20 and 1.30 hrs for volunteer 3.

3.10 Plasma Nitrite Concentrations

The plasma nitrite concentrations of the individual volunteers, as observed on timed intervals following each of the treatments are tabulated and graphically presented in Appendix 16a and 16b, respectively. The kinetic parameters derived from the plasma nitrite concentrations are summarised in Appendix 16c. The baseline plasma nitrite concentration was measured twice before each treatment session. The baseline plasma nitrite concentrations were below 0.1 mg/kg except for two measurements that were 0.1 mg/kg.
The maximum nitrite concentration in the plasma was observed during or directly after the end of the infusion. In the first treatment session maximum plasma nitrite concentrations of 1.8, 2.8 and 1.4 mg/kg were observed. After increasing the infusion time from 10 to 30 minutes in the second treatment session maximum nitrite concentrations of 1.2, 1.3 and 0.9 mg/kg were observed. Doubling of the sodium nitrite dose in the third treatment session resulted in maximum nitrite concentrations of 3.3, 3.1 and 2.1 mg/kg. In the fourth treatment session a sodium nitrite dose of three times the starting dose was administered resulting in maximum nitrite concentrations in the plasma of 4.8, 3.3 and 4.1 mg/kg, respectively.
The plasma nitrite concentration decreased approximately exponentially with time. However, the terminal elimination half life of nitrite could not be established accurately. The most accurate estimations of the half life were made from the data of third and fourth treatment session. For these study periods the calculation of the half life was based on 5 and 6 concentration-time-points, respectively. The correlation coefficient of the regression analyses was however low, ranging between 0.81 to 0.99 (except volunteer 3, treatment session 3: 0.65). The elimination half life as observed in the third and fourth treatment session, was, respectively, 0.51 and 0.52 hrs for volunteer 1, 0.48 and 0.51 hrs for volunteer 2 and 0.68 and 0.60 hrs for volunteer 3.
The $AUC_{(0-\infty)}$ increased approximately linear with the administered sodium nitrite dose. The AUCs as calculated from the concentration-time data of each treatment session and adjusted to a standard sodium nitrite dose of 220 mg ranged between 1.846 and 2.373 mg*hr/l for volunteer 1, between 2.187 and 2.616 mg*hr/l for volunteer 2 and between 2.476 and 2.811 mg*hr/l for volunteer 3 (Appendix 16c).
The volume of distribution of nitrite was calculated applying the formula $V_z = NaNO_2$ dose / AUC * $(\ln 2/t_{1/2})$. The terminal elimination half life is part of the formula and was estimated best in the third and fourth treatment session. The V_z as observed in the third and fourth treatment period are therefore most reliable. For volunteer 1 the V_z was 73 and 83 litres in treatment session 3 and 4, respectively. For volunteer 2 the V_z was 60 and 66 litres, respectively, and for volunteer 3 the V_z was 77 and 68 litres, respectively.

3.11 Plasma Nitrate Concentrations

The plasma nitrate concentrations of the individual volunteers, as observed on timed intervals following each of the treatments are tabulated and graphically presented in Appendix 17a and 17b, respectively.
The fasting plasma nitrate concentration was measured twice before each treatment session. Nitrate is normally present in the blood. The baseline nitrate concentrations were mostly below the limit of quantification of 2 mg/kg. In volunteer 1, the baseline plasma nitrate concentrations were below 2 mg/kg and once 2.0 mg/kg. In volunteer 2 the baseline plasma nitrate concentration was below 2 mg/kg and once 2.0 mg/kg in periods 1, 2 and 4 and 3.5 and 3.2 mg/kg in period 3. In volunteer 3 the

baseline plasma nitrate concentration was below 2 mg/kg in treatment period 2, below 2 mg/kg and 2.2 mg/kg in period 1, 2.4 and 2.2 mg/kg in period 3 and 3.7 and 3.8 mg/kg in period 4.
During the intravenous infusion of sodium nitrite the plasma nitrate concentration gradually increased and continued to increase for some time after the sodium nitrite infusion stopped. During the first treatment period a maximum nitrate concentration of 6.1, 6.2 and 4.6 mg/kg was observed in volunteers 1, 2 and 3, respectively. In the second treatment period, with the same sodium nitrite dose but now infused over 30 instead of 10 minutes, a maximum nitrate concentration of 5.8, 6.8 and 5.2 mg/kg, respectively, was observed. After doubling the sodium nitrite dose in the third treatment period a maximum nitrate concentration of 10.3, 11.9 and 9.1 mg/kg was observed. In the fourth treatment period a sodium nitrite dose of three times the starting dose was administered leading to maximum nitrate concentrations in the plasma of 14.2, 14.0 and 14.0 mg/kg, respectively.
Especially for the highest sodium nitrite dose it appeared that the concentration-time profile of nitrate showed two or three peaks around its maximum concentration (see Appendix 17b).
The time to reach the maximum nitrate concentration in the plasma increased with the dose. During the first treatment period the t_{max} occurred between 0.83 and 1.02 hrs post dose. During the second treatment period the t_{max} was reached between 0.92 and 1.50 hrs post dose. In the third and fourth treatment period the t_{max} was reached between 1.33 and 1.50 hrs post dose and between 1.50 and 2.00 hrs post dose, respectively.
The plasma nitrate concentration decreased approximately exponentially with time. In all but one cases the nitrate concentration that was measured 24 hours post dose had decreased to values below 4 mg/kg. During the second treatment session a plasma nitrate concentration of 9.8 mg/kg was measured 24 hours post dose in volunteer 3. Volunteer 3 consumed beetroot in the evening of the second treatment day. It is very likely that the elevated nitrate concentration is caused by the beetroot since in the months of May through July the nitrate content of the beetroot can be very high (> 3000 mg/kg product).

4. DISCUSSION

Nitrite is rapidly eliminated from the blood due to its reaction with hemoglobin. In vitro studies have shown a terminal elimination rate for nitrite of 45 minutes (25). To enable oral bioavailability calculations for nitrite plasma concentrations well above the limit of quantification of 0.3 mg/kg are necessary. This means that high sodium nitrite doses must be administered to achieve this goal.
The acute effects of nitrite are well known (21). Nitrite can react with hemoglobin in the blood leading to the formation of methemoglobin and nitrate. In addition, nitrites are known to lower the blood pressure through vasodilatation. The dose level at which the effects of nitrite become ill tolerated is not well documented. Fatal cases of nitrite intoxication have occurred (26, 27).
The aim of the present ascending dose study was to find the maximum sodium nitrite dose that could safely be administered to volunteers. That means a sodium nitrite dose inducing less then 10 to 15% metHb since it is well known that metHb levels beyond 15% lead to adverse effects, starting with cyanosis, fatigue tachycardia, dizziness etc.
The first dose level for the present study was selected on basis of an in vitro experiment investigating the metHb inducing potential of nitrite in human blood samples (14). A dose level of 0.04 mmol $NaNO_2$ per mmol Hb was chosen from the in vitro study to be the starting dose for the present study. This dose level caused in vitro on average 5.3 ± 0.6 % metHb in the blood samples. To extrapolate this dose to the in vivo situation it was assumed that the volume of distribution of nitrite is restricted to the blood compartment. In that case the in vivo dose is derived from the in vitro experiment by substituting the total hemoglobin content of the blood sample by the total Hb calculated to be present in the blood (see section 2.3.2.). The dose level of 0.04 mmol $NaNO_2$ per mmol Hb caused on average 3.0 ± 0.2% metHb in vivo. The lower metHb levels in vivo as compared to the in vitro experiment may be explained by inaccuracy in the estimated blood volume and hence the total amount of Hb in the blood. The estimate of the blood volume was based on the body weight of the volunteer. The most accurate method to determine the blood volume is by radioactive labelled chromium. Wennesland et al. converted the blood volumes that were measured by the method of radioactive labelled chromium to a regression equation involving height, weight or surface area (28). The estimate of the blood volume, based on the weight of the volunteer, showed a coefficient of variation (CV) of 9% to the "true" values i.e. the blood volumes as measured by radioactive labelled chromium. The magnitude of the variation may be less in a, with respect to height and weight, more homogenic group of volunteers as in this study.
A second explanation for the lower metHb levels in vivo may be that the volume of distribution of nitrite is not restricted to the blood compartment. The distribution volumes of nitrite as observed in this study, i.e. between 60 and 83 litres, suggest that this is indeed the case. Part of the nitrite may then react with other molecules in the body i.e. myoglobin to form metmyoglobin, and is therefore not available for the induction of metHb.
Furthermore, the higher in vitro metHb levels may be due to the fact that the in vitro the erythrocytes are less effective in reducing the metHb back to Hb. The reduction of metHb requires NADPH which is formed during the G6PD catalysed utilisation of glucose. However, the in vitro system lacks a continuous input of glucose and may therefore exhibit a diminished reduction of metHb. This may lead to a higher overall metHb level in vitro as compared to the in vivo situation.
The formation of metHb was not the only factor that determined the administration of higher dose levels of sodium nitrite to the volunteers. In two out of three volunteers a rapid and substantial drop in the blood pressure was observed after the first dose level. The lowest blood pressure values occurred approximately at the end of the infusion as the highest nitrite concentrations in the blood are reached. Since the blood pressure dropped so rapidly it was assumed to be directly related to the plasma nitrite

concentration. In volunteer 3, due to a pump dysfunction, the infusion of the sodium nitrite was temporarily stopped for 8 minutes during the first treatment session (see Appendix 3). This was probably the reason why in this volunteer only a mild decrease of the blood pressure was observed as compared to the other volunteers.
Prolongation of the infusion time from 10 to 30 minutes would increase the plasma concentrations of nitrite more gradually and would lower the peak plasma nitrite concentration to some extent. In the second treatment session, therefore, the same dose level was tested again, now with an infusion-time of 30 instead of 10 minutes. The decrease in the blood pressure was less substantial and not so rapid and in addition the volunteers reported less complaints. The peak plasma nitrite concentrations dropped from 2.0 ± 0.7 mg/kg in the first treatment session to 1.1 ± 0.2 mg/kg in the second treatment session. Based on the observed adverse events in the second treatment session and the metHb level that was far below 10% (3.2%) it was considered justified to double the sodium nitrite dose in the third treatment session. In this treatment session one volunteer reported complaints and the decrease of the blood pressure was comparable to the second treatment session. The maximum metHb level increased to 6.6 ± 0.1%. To aim for a metHb level of 10% the sodium nitrite dose was increased to three times the starting dose in the fourth and last treatment session. This sodium nitrite dose was administered over 30 minutes and therefore the infusion speed was equal to the infusion speed that was used in the first treatment session. All volunteers reported complaints, resembling the complaints as observed in the first treatment session. The decrease in blood pressure was also comparable to the first treatment session.
These data indicate that sodium nitrite related effects on the blood pressure and the adverse experiences that were reported in this study are related to the speed at which the sodium nitrite is infused into the blood circulation rather then to the dose of the sodium nitrite. The speed-related effects of nitrite on the blood pressure indicate that the mechanism of vasodilatation induced by nitrite occurs almost instantaneously. Because of the rapid vasodilatation compensating mechanisms to counteract the decrease of the blood pressure may be less adequate at a higher infusion speed. Furthermore the effect of nitrite on the blood pressure is either short lasting or the maximum fall of the blood pressure is already reached at the first dose level. Since higher nitrite doses do not lead to an accumulation of the effect of nitrite on the blood pressure. For the bioavailability study that is to be performed in the near future this means that the nitrite should be infused slowly into the blood circulation.
The formation of metHb on the other hand is dose-related. A dose level of 0.04 mmol $NaNO_2$ per mmol Hb caused approximately 3 % metHb, regardless of the fact that the sodium nitrite was infused over 10 or 30 minutes. The formation of metHb increased with the dose. In the fourth treatment session the metHb was 10.8 ± 0.2%. Because the metHb reached a level beyond 10% this sodium nitrite dose was considered to be the maximum tolerated dose. Thus, the sodium nitrite dose that is selected for the future bioavailability study should not exceed this dose.
The apparent volume of distribution of nitrite ranged between 60 and 83 litres. The volumes of distribution as observed in the third treatment period were confirmed by the data of the fourth treatment session. The V_z of nitrite is a virtual volume of distribution, i.e. not matching a physiological body compartment as the extracellular fluid compartment (approximately 20 litres) or total body water (approximately 40 litres). The high apparent volume of distribution of nitrite may be explained by rapid accumulation and/or biotransformation of nitrite in the body, i.e. in the red blood cells. In addition, because nitrite is a highly reactive compound its apparent volume of distribution is easily overestimated. In the present study the reaction of nitrite with hemoglobin continued in the blood samples until the blood was centrifuged and the plasma separated from the blood cells. It is obvious that the ongoing elimination of nitrite may result in decreased nitrite concentrations in the

samples. This may have contributed to the high apparent volume of distribution of nitrite, between 60 and 83 litres. When the $AUC_{nitrite}$ is underestimated, due to the decreased plasma nitrite concentrations, the V_z will be overestimated (13).
The plasma nitrite concentration increased approximately proportional to the sodium nitrite dose. The metHb level, however, did not increase proportional to the nitrite dose. In the third and fourth treatment session the sodium nitrite dose was doubled and tripled, respectively, in comparison to the second treatment session. The AUC and the C_{max} of the metHb in the third and fourth treatment session were however higher then twice and three times the AUC and C_{max} as observed in the second treatment session. The fact that the $AUC_{(0-\infty)}$ of metHb increased disproportional to the sodium nitrite dose is also apparent from the AUCs that were adjusted to a standard dose of 220 mg (Appendix 15c). In the second treatment session the average adjusted $AUC_{(0-\infty)}$ was 13.03 %*hr/l, compared to an adjusted $AUC_{(0-\infty)}$ of 18.64 and 24.40 %*hr/l in the third and fourth treatment period, respectively. The graphical presentation of the sodium nitrite dose versus the $AUC_{(0-\infty)}$ and the C_{max} of metHb suggests a linear relationship between the nitrite dose and the maximum metHb level but a non-linear relationship between the nitrite dose and the $AUC_{(0-\infty)}$, respectively (Appendix 18). This is the first report that describes the relationship between increasing doses of sodium nitrite and the formation of metHb in vivo. Prior to this study we performed an in vitro experiment in which ascending sodium nitrite doses were tested for their metHb inducing potential in human blood samples (14). Similar to the in vivo situation a linear relationship was established in vitro between the nitrite dose and the maximum metHb level. However, in vitro, also, the maximum metHb level increased disproportional to the dose. Where a nitrite dose of 0.2 mmol $NaNO_2$ per mmol Hb induced 2.4% metHb a dose of 0.4 and 0.8 mmol $NaNO_2$ per mmol Hb induced more then two and four times that percentage of metHb; i.e. 5.3% and 12.3% metHb, respectively.
Two explanations can be brought forward for the disproportional relationship between the nitrite dose and the metHb level. First, Rodkey et al. showed that the rate at which metHb is formed is dependent on the nitrite and metHb concentrations in the blood (32). The rate increases as the nitrite and metHb concentrations in the blood increase, which is the case at higher nitrite doses. Considering a second elimination pathway for nitrite, this second pathway may be more dominant at lower nitrite doses as the rate of metHb formation is low and the elimination via this pathway may be smaller as the rate of metHb formation increases at higher nitrite doses. Consequently, the formation of metHb will increase non proportional to the nitrite dose. A second explanation for the disproportional increase of the metHb level with the nitrite dose could be that the stoichiometry of the reaction between nitrite and hemoglobin changes with the concentrations of nitrite and metHb present in the blood. The reaction equation of nitrite with hemoglobin is still not fully clarified. Several molar ratios for the amount of nitrite utilised versus metHb formed have been suggested; Kosaka reported a molar ratio of 3:2, Rodkey reported a ratio of 1:1 and Greenberg a ratio of 1:2 (31,32,33). The conflicting data may indicate that the reaction equation of nitrite with hemoglobin is dose dependent.
For the bioavailability study that is to be performed in the near future the disproportional relationship between the nitrite dose and the metHb level in the blood means that the metHb level cannot be used as a substitute parameter for the plasma nitrite concentration to determine the oral bioavailability of nitrite.

5. CONCLUSIONS

The present ascending dose study was set up to investigate the maximum sodium nitrite dose that could safely be administered to adult volunteers, i.e. inducing less then 10 to 15% methemoglobineamia and inducing a decrease of the blood pressure that is within acceptable limits. Three dose levels were tested.

It can be concluded that a sodium nitrite dose of 0.12 mmol $NaNO_2$ per mmol Hb (between 290 and 370 mg $NaNO_2$), inducing approximately 10.8% methemoglobin in the blood, is the maximum tolerated sodium nitrite dose for adult volunteers without considerable adverse effects. At this dose level all volunteers reported adverse experiences, although of mild intensity. Lowering of the blood pressure and a compensatory increase of the heart rate was present at all dose levels but stayed within acceptable limits.
The plasma nitrite concentration increased approximately proportional to the sodium nitrite dose. The peak plasma nitrite concentrations were between 3.3 and 4.8 mg/kg at the highest dose level. Nitrite rapidly disappeared from the plasma with a terminal elimination half life of approximately 30 minutes. The increase in the metHb formation at higher dose levels was not proportional to the dose. The ratio $AUC_{metHb}/AUC_{nitrite}$ increased with the administered dose.

The results of the present study will be used to design an absolute oral bioavailability study of nitrite in healthy volunteers. From the peak plasma nitrite concentrations that were observed in the present study it is concluded that the highest dose level must be considered for the bioavailability study. The plasma nitrite concentrations measured at this dose level are sufficient for a bioavailability study.
It is recommended to infuse the sodium nitrite slowly (over 30 minutes) into the blood circulation during the bioavailability study to prevent severe hypotension. It may be that the volunteers in the bioavailability study will experience adverse events at this dose level. This must be communicated to the volunteers prior to their participation in the study. From the results of the present study it is clear that metHb cannot be used as a substitute parameter for plasma nitrite to determine the oral bioavailability of nitrite from since the formation of metHb is not proportional to the dose. MetHb should however be monitored during the study for safety reasons as well as the blood pressure.

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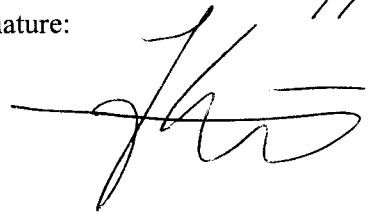
APPENDIXES

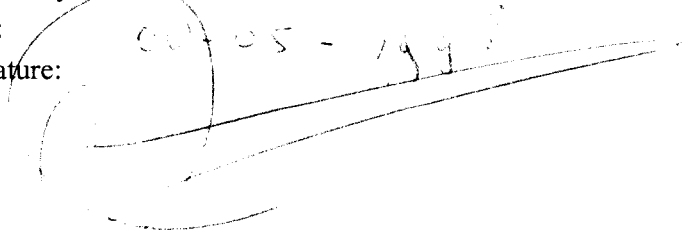
Appendix 1. Statement of GCP Compliance

The undersigned hereby certify that the clinical phase of the study, as described herein, was performed in accordance with the recommended principles of Good Clinical Practice for studies with medicine in the European Community (EC doc. III/3976/88 FINAL).
This report provides a complete, correct and faithful record of the results obtained. The study was performed by the principal investigator J.M. Kortboyer, MD.

Name: J.M. Kortboyer, MD (principal investigator)
Laboratory: Unit National Poisons Control Centre, RIVM
Date: 08-05-1998
Signature:



Name: J. Meulenbelt, MD, PhD (project leader)
Laboratory: Unit National Poisons Control Centre, RIVM
Date:
Signature:



Appendix 2. Quality Assurance Statement

The undersigned hereby certifies that the clinical phase of the study, as described herein, was performed in accordance with the recommended principles of Good Clinical Practice for studies with medicine in the European Community (EC doc. III/3976/88 FINAL).
This report provides a complete, correct and faithful record of the results obtained.

QA inspections were performed on the following dates:

1995: July 18, 19 and 25
July 31
August 2, 3, 8 and 11
August 15
August 16

QA inspection reports to the study director and management are dated:

1995: July 26
July 31
August 1
August 11
August 15
August 16

QA audits of this report were performed on the following dates:

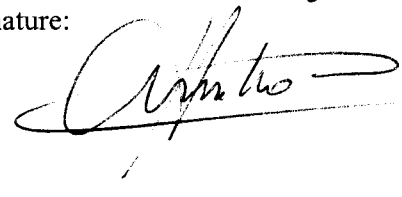
1998: April 21 and 22

QA audits of this report were reported to the study director and management on:

1998: April 22

Quality Assurance Manager

Name: A.W.M. Hofstee
Laboratory: National Poisons Control Centre, RIVM
Date: 08-05-1998
Signature:



Appendix 3. Deviations to the Protocol

• During the pre-study medical screening it was omitted to measure the percentage of metHb in the blood of the volunteers. Since the metHb level was measured at the first treatment session, prior to treatment, and within normal range for all volunteers the omission during the pre-study medical screening had no consequences.

• The nitrate low diet was not followed accurately prior to all treatment sessions. In one case this might have influenced the baseline nitrate concentration that was measured in the plasma just prior to the treatment: Volunteer 3 consumed Chinese food at 21.15 hr on the day before the third treatment session. The baseline plasma nitrate concentration in the third treatment session was slightly elevated being 2.4 and 2.2 mg/kg. During the fourth treatment session baseline plasma nitrate concentrations of 3.7 and 3.8 mg/kg were observed for volunteer 3. The diet was accurately followed. An explanation for the high baseline nitrate levels was not found.
Volunteer 3 consumed beetroot in the evening of the second treatment day. The blood sample that was taken 24 hours post dose showed a plasma nitrate concentration of 9.8 mg/kg. It is very likely that the elevated nitrate concentration is caused by the beetroot.
In a number of cases the diet during the investigation days was slightly adjusted: The deviations were minor and considered not have influenced the study results.
Volunteer 2 had breakfast before the 24 hr blood sample during the first treatment period. This might have increased the nitrate concentration of the blood to some extent.

• Volunteer 2 experienced an intercurrent illness prior to the third treatment session. It started with headache and fever 4 days prior to the treatment session. She took 2 tablets of paracetamol (acetominophen) that day. Three and two days prior to the treatment session she took another 3 and 2 tablets of paracetamol, respectively, after which the complaints subsided. The illness might have had a slight influence on the baseline plasma nitrate concentration that was measured prior to the treatment session (plasma nitrate 3.5 and 3.2 mg/kg). From the paracetamol no influence on the study results is expected.

• Volunteer 3 could not produce urine for a pregnancy test during the first and fourth treatment period. Because she took oral contraceptives and stated that she had practised abstinence in the month prior to the treatment session the sodium nitrite infusion was not postponed. The pregnancy test was performed with delay after the treatments were administered. The pregnancy test was both times negative.

• During the first treatment session of volunteer 3 a pump dysfunction occurred. The infusion of the sodium nitrite stopped after 3 minutes. The display of the pump indicated that 1.3 ml (13 mg) sodium nitrite was infused. It turned out that the infusion line was not properly placed into the pump. After correction, the infusion of the sodium nitrite was restarted again 8 minutes later. A blood sample was taken 5 minutes after the restart and directly after the end of the infusion. The temporal discontinuation of the infusion of sodium nitrite might have caused a slight discrepancy between the infused volume on the display of the pump and the actual volume that was infused.

• In the fourth treatment period the infusion of the sodium nitrite was stopped after 2 minutes in volunteer 3 because of subcutaneous administration. The display of the pump showed that 1.7 ml (17 mg) sodium nitrite had been infused already. The volunteer was kept for clinical observation for one

hour. A local reaction to the subcutaneous infusion was not observed and the volunteer was dismissed form the clinical research unit. The volunteer returned one week later to repeat the treatment session.

• During the first treatment session it was omitted to measure the blood pressure and the heart rate of volunteer 1 at 24 hours post dose.

• For volunteer 1 , the blood sample that was obtained 4 hours post dose during the second treatment session and 3 hours post dose during the third treatment session was left in the centrifuge (4 oC) for one hour and 30 minutes, respectively, before the plasma was transferred to another tube and frozen in liquid nitrogen. For volunteer 3, during the first treatment session, the blood sample of 6 hours post dose was left in the centrifuge for two hours before the plasma was transferred to liquid nitrogen. Since nitrate is stable in blood and the nitrite level was below the limit of quantification at this time point the results of the nitrite/nitrate analyses were considered not to have been influenced.
A number of blood samples were obtained somewhat later or earlier then the scheduled time. The actual times on which the blood samples were drawn are mentioned in the relevant Appendices.
For volunteer 2, the blood sample of 0.50 hr post dose in period 1, was centrifuged twice because the sample fell down after the first centrifugation. Furthermore, in the blood sample obtained 1.00 hr post dose during the first treatment session a trace of blood was present in the plasma that was frozen for nitrite/nitrate analyses. From the concentration-time curve of plasma nitrite it seems that these deviations did not influence the nitrite level in the plasma.
At 09.50 hr, during the second treatment period, due to inattention, a second unscheduled blood sample was taken from volunteer 1, directly after the blood sampling that took place at 45 minutes post dose. The results of the plasma nitrate/nitrite analyses of this blood sample were disregarded.

Appendix 4. Written Informed Consent Form (in Dutch)

Appendix II

INFORMED CONSENT FORMULIER

Afd. IC1 en Klinische Toxicologie van het Academisch Ziekenhuis Utrecht
Nationaal Vergiftigingen Informatie Centrum van het RIVM

Ondergetekende verklaart een exemplaar te hebben ontvangen van de "Informatie voor Deelnemers" betreffende het onderzoek getiteld:
"Onderzoek naar het verband tussen inname van natrium nitriet en de afname van de zuurstof-transport capaciteit van het bloed (datum 18-04-1995/ revisiedatum 08-06-1995)".

Ondergetekende heeft een mondelinge toelichting op dit informatieformulier gekregen en is in de gelegenheid geweest iedere gewenste vraag te stellen.

Ondergetekende verklaart dat zij/hij, na kennis te hebben genomen van bovengenoemde informatie, als vrijwilliger wil deelnemen aan bovengenoemd onderzoek.
Ondergetekende verklaart dat hij/zij naar eer en geweten alle vragen gesteld tijdens de medische keuring zal beantwoorden en geen informatie bewust achterhouden.
Afgesproken is dat zij/hij zich gedurende de totale onderzoeksperiode zal houden aan de opzet van het onderzoek en aan de restricties zoals beschreven in de schriftelijke informatie voor vrijwilligers.

Voorts verklaart de vrijwilliger dat zij/hij in een periode van 30 dagen voorafgaand aan en tijdens dit onderzoek niet deelneemt aan enig ander vrijwilligersonderzoek.

Aan ondergetekende is medegedeeld dat bij vragen en problemen gedurende 24 uur per dag, 7 dagen per week direkt kontakt kan worden opgenomen met de onderzoekers via het Academisch Ziekenhuis Utrecht, afdeling Intensice Care-I en Klinische Toxicologie, tel. 030-507330 (tijdens kantooruren) en het Nationaal Vergiftigingen Informatiecentrum, tel. 030-748888 (buiten kantooruren).

Voorts is ondergetekende medegedeeld dat zij/hij zonder opgave van reden op elk moment deelname aan het onderzoek kan staken.

Ondergetekende gaat accoord met inzagerecht van persoonlijke (medische) gegevens door medewerkers van de afdeling IC-1 en Klinische Toxicologie en medewerkers van inspecterende instanties

Naam : .....

Voornamen : .....

Geboortedatum : .....

Adres : .....

Postcode + Woonplaats : .....

Telefoonnummer : .....

Utrecht, ...(datum en jaar)

Handtekening vrijwilliger: Naam en handtekening onderzoeker:

....

Appendix 4A. Written Informed Consent Form (Addendum)

INFORMATIE VOOR VRIJWILLIGERS

Er is een kleine wijziging aangebracht in het onderzoeksvoorstel, getiteld:

"Onderzoek naar het verband tussen inname van natriumnitriet en de afname van de zuurstof-transportcapaciteit van het bloed (projectnummer 348801/03; protocoldatum 18-04-1995; revisie-datum 08-06-1995)"

Hiermee verklaar ik, dat ik vooraf, mondeling, op de hoogte gesteld ben van de wijziging in de onderzoeksopzet, betreffende de verlenging van de infuusduur (inlooptijd van de natrium nitriet doses in de onderarm) van 10 naar 30 minuten.

Mij is vooraf meegedeeld dat ik de tweede onderzoeksdag dezelfde natriumnitriet dosering heb ontvangen als de eerste onderzoeksdag. De infuusduur was de eerste onderzoeksdag 10 minuten en de tweede onderzoeksdag 30 minuten.
Mij is verteld dat deze wijziging in de onderzoeksopzet is doorgevoerd omdat er een lichte bloeddrukdaling was waargenomen bij 2 van de 3 vrijwilligers tijdens de eerste onderzoeksdag. Deze bloeddrukdaling zou mogelijk minder zijn als de infuusduur tot 30 minuten zou worden verlengd.

In principe zal de infuusduur 30 minuten bedragen tijdens de nog volgende onderzoeksdagen.

Naam: .....

Adres: .....

Postcode+ woonplaats: .....

Geboortedatum: .....

Utrecht, ...(datum en jaar)

Handtekening Vrijwilliger: Handtekening onderzoeker:

....

Appendix 5. Selection Criteria

Selection criteria as applied for enrolment of the volunteers in the intravenous, ascending dose study of sodium nitrite

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Inclusion criteria

- female/male
- age 18-35 years
- weight between 60 and 70 kg and within the normal range of the Metropolitan Height and Mass Tables (Appendix V)
- willing to give Written Informed Consent
- healthy as judged by a physician from the medical history, physical examination, electrocardiography and routine laboratory blood and urine analyses

Exclusion critera

- smoking
- chronic drug treatment (especially antacids) or any drug treatment within one week before the start of the study. Except for oral contraceptives.
- drug abuse
- more than 3 units of alcohol per day
- blood donation within 30 days before the start of the study
- participation in another drug study within 30 days before the experiment or during the study
- pregnant or lactating females
- mental illness
- any chronic illness
- family history positive for pernicious anaemia
- family history positive for G6PD-deficiency or original inhabitants of the mediterannee
- excessive pyrosis or history of gastritis or gastric or duodenal ulcer
- abnormal dietary habits as judged by a physician
- anaemia

Appendix 6. Flow Chart of Scheduled Study Procedures

Scheduled procedures during the intravenous administration of sodium nitrite over 10 minutes

Clock-time	Scheme-time	Scheduled Procedure
07.30 h	-1.50	Pregnancy test, Body Weight, Inserting intravenous cannula
08.00 h	-1.00	Blood sampling
08..00- 08.55 h	-1.00 - -0.08	Connecting Chest-electrodes for heart rate monitoring Inserting second intravenous cannula, Blood Pressure, Adverse Experiences
08.55 h	-0.08	Blood sampling
09.00- 09.10 h	0.00 - 0.17	Administration of Sodium Nitrite
09.05 h	0.08	Blood sampling, Blood Pressure, Adverse Experiences
09.10 h	0.17	Blood sampling, Blood Pressure, Adverse Experiences Removing intravenous cannula
09.15 h	0.25	Blood sampling, Blood Pressure, Adverse Experiences
09.20 h	0.33	Blood sampling, Blood Pressure, Adverse Experiences
09.25 h	0.42	Blood sampling, Blood Pressure, Adverse Experiences
09.30 h	0.50	Blood sampling, Blood Pressure, Adverse Experiences
09.40 h	0.67	Blood sampling, Blood Pressure, Adverse Experiences
09.50 h	0.83	Blood sampling, Blood Pressure, Adverse Experiences
10.00 h	1.00	Blood sampling, Blood Pressure, Adverse Experiences
10.15 h	1.25	Blood sampling, Blood Pressure, Adverse Experiences
10.30 h	1.50	Blood sampling, Blood Pressure, Adverse Experiences
10.45 h	1.75	Blood sampling, Blood Pressure, Adverse Experiences
11.00 h	2.00	Blood sampling, Blood Pressure, Adverse Experiences
11.30 h	2.50	Blood sampling, Blood Pressure, Adverse Experiences
12.00 h	3.00	Blood sampling, Blood Pressure, Adverse Experiences
13.00 h	4.00	Blood sampling, Blood Pressure, Adverse Experiences Removing chest-electrodes
13.00- 13.30 h	4.00-4.50	Lunch
14.00 h	5.00	Blood sampling, Blood Pressure, Adverse Experiences
15.00 h	6.00	Blood sampling, Blood Pressure, Adverse Experiences
15.00- 15.30 h	6.00-6.50	Tea
17.00 h	8.00	Blood sampling, Blood Pressure, Adverse Experiences
19.00 h	10.00	Blood sampling, Blood Pressure, Adverse Experiences Removing intravenous cannula Departure from Research Unit
09.00 h	24.00	Blood sampling, Blood Pressure, Adverse Experiences

Appendix 6. Flow Chart of Scheduled Study Procedures

(continued)

Scheduled procedures during the intravenous administration of sodium nitrite over 30 minutes

Clock-time	Scheme-time	Scheduled Procedure
07.30 h	-1.50	Pregnancy test, Body Weight, Inserting intravenous cannula
08.00 h	-1.00	Blood sampling
08..00- 08.55 h	-1.00 - -0.08	Connecting Chest-electrodes for heart rate monitoring Inserting second intravenous cannula, Blood Pressure, Adverse Experiences
08.55 h	-0.08	Blood sampling
09.00- 09.30 h	0.00 - 0.50	Administration of Sodium Nitrite
09.10 h	0.17	Blood sampling, Blood Pressure, Adverse Experiences
09.20 h	0.33	Blood sampling, Blood Pressure, Adverse Experiences
09.30 h	0.50	Blood sampling, Blood Pressure, Adverse Experiences Removing intravenous cannula
09.35 h	0.58	Blood sampling, Blood Pressure, Adverse Experiences
09.40 h	0.67	Blood sampling, Blood Pressure, Adverse Experiences
09.45 h	0.75	Blood sampling, Blood Pressure, Adverse Experiences
09.50 h	0.83	Blood sampling, Blood Pressure, Adverse Experiences
10.00 h	1.00	Blood sampling, Blood Pressure, Adverse Experiences
10.10 h	1.17	Blood sampling, Blood Pressure, Adverse Experiences
10.20 h	1.33	Blood sampling, Blood Pressure, Adverse Experiences
10.30 h	1.50	Blood sampling, Blood Pressure, Adverse Experiences
10.45 h	1.75	Blood sampling, Blood Pressure, Adverse Experiences
11.00 h	2.00	Blood sampling, Blood Pressure, Adverse Experiences
11.30 h	2.50	Blood sampling, Blood Pressure, Adverse Experiences
12.00 h	3.00	Blood sampling, Blood Pressure, Adverse Experiences
13.00 h	4.00	Blood sampling, Blood Pressure, Adverse Experiences Removing chest-electrodes
13.00- 13.30 h	4.00-4.50	Lunch
14.00 h	5.00	Blood sampling, Blood Pressure, Adverse Experiences
15.00 h	6.00	Blood sampling, Blood Pressure, Adverse Experiences
15.00- 15.30 h	6.00-6.50	Tea
17.00 h	8.00	Blood sampling, Blood Pressure, Adverse Experiences
19.00 h	10.00	Blood sampling, Blood Pressure, Adverse Experiences Removing intravenous cannula Departure from Research Unit
09.00 h	24.00	Blood sampling, Blood Pressure, Adverse Experiences

Appendix 7. Nitrate and Nitrite Low Diet

The study participants have to adhere to the following diet instructions for three days prior to every nitrite administration.

VEGETABLES

A large part of daily nitrate is provided by vegetables. The nitrate-content of the vegetables can vary enormously. The diet instructions given here are based on mean nitrate concentrations in various vegetables. Because the nitrate content of the vegetables can vary enormously it is always important to eat different vegetables every day.

A Vegetables containing more than 1000 mg nitrate per kg product. These vegetables are **forbidden**

endive
celery
chinese cabbage
chervil
turnip cabbage
paksoi
purslain
turnip tops
radish
black radish
red beetroots
lettuce
beetroot
spinach
cabbage
fennel
watercress

B Vegetables containing between 500 and 1000 mg nitrate per kg product. **Limited use** of these vegetables is permitted. The day before administration of sodiumnitrite these vegetables should not be eaten.

cauliflower
curly kale
paprika
leek
sliced beans

C Vegetables containing less than 500 mg nitrate per kg product. These vegetables can be eaten **without restriction**.

artichokes
asparagus
aubergine
broccoli
courgette
green peas
peas
cucumber
turnip rooted celery
maize
mushrooms
padded peas
rhubarb
red cabbage
tauge
tomato
savoy cabbage
butter beans
sprouts
broad beans
onions
carrots
white cabbage
belgian endive
sauer kraut

POTATOES

Limited use of potatoes is permitted. This also accounts for other potatoe-products such as chips and french fries. The day before administration of sodiumnitrite these products should not be eaten.

RICE AND PASTE
These products can be eaten **without restriction**.

BREAD
Bread and bread-products can be eaten **without restriction**.

MEAT AND FISH
Fish and fresh meat can be eaten **without restriction**.

MEAT-PRODUCTS/MEAT-DELICACIES
In these products nitrate and nitrite are used as preservatives. Therefore, only limited use of these products is permitted.The day before administration of sodiumnitrite the following products should not be eaten.

bacon
ham
smoked beef
smoked sausage

MILK, EGGS AND DAIRY-PRODUCTS
These products can be eaten **without restriction**.

FRUIT
Fruit can be eaten **without restriction**.

DRINKS
The use of potable water from a private well is **forbidden**.
Consumption of vegetable juices is **forbidden**.
All other drinks can be consumed **without restriction**.

For the duration of the experiment alcoholconsumption, with a maximum of three glasses a day, is permitted.

Adapted from: "Nitraatinname van de Nederlandse bevolking op basis van de Voedselconsumptiepeiling". van Loon A.J.M. and van Klaveren J.D., RIVM-report 90.18, April 1990, National Institute of Public Health and the Environment, the Netherlands.
"Nitrates and nitrites in foodstuffs: health protection of consumers", Schuddeboom L.J., Coucel of Europe Press, 1993.

Appendix 8. Table of Demographic Data

Demographic data obtained during the pre-study medical screening

Volunteernr.	Gender (M/F)	Age (yrs)	Height (cm)	Weight (kg)	Comment
101	F	27	175.5	61.0	Excluded
102	F	29	167.0	67.5	Excluded
1	M	25	179.0	70.0	
2	F	21	166.5	66.0	
3	F	19	174.5	66.5	
4	F	21	167.0	60.0	Replacement

Appendix 9. Table of Treatments and Study Dates

Study dates and administered treatments to the volunteers participating in the single dose, intravenous, ascending dose study of sodium nitrite

Treatment 1= single dose, intravenous administration of 0.04 mmol sodium nitrite per mmol Hb infused over 10 minutes,

Treatment 2= single dose, intravenous administration of 0.04 mmol sodium nitrite per mmol Hb infused over 30 minutes,

Treatment 3= single dose, intravenous administration of 0.08 mmol sodium nitrite per mmol Hb infused over 30 minutes,

Treatment 4= single dose, intravenous administration of 0.12 mmol sodium nitrite per mmol Hb infused over 30 minutes,

Volunteernr.	Treatment	Date (d/m/y)	Body Weight (kg)	Hb (mmol/L)	$NaNO_2$ dose (mg)
1	1	18-07-1995	70.5	9.4	130
	2	25-07-1995			130
	3	01-08-1995	69.5	9.4	250
	4	08-08-1995	70.0	9.0	370
2	1	19-07-1995	65.5	9.3	120
	2	26-07-1995			120
	3	02-08-1995	66.0	8.8	220
	4	09-08-1995	65.5	8.4	320
3	1	24-07-1995	66.0	7.3	90
	2	31-07-1995			90
	3	07-08-1995	66.0	7.3	190
	4	22-08-1995	65.5	7.6	290

Appendix 10. Test results of the Pre- and Post-study Medical Screening

PHYSICAL EXAMINATION

Volunteer	1		2		3		
Parameter	Pre-study Screening 04-07-1995	Post-study Screening 11-08-1995	Pre-study Screening 27-06-1995	Post-study Screening 15-08-1995	Pre-study Screening 13-07-1995	Post-study Screening 31-08-1995	Normal Range
Supine:							
Blood pressure	136/78	119/66	124/66	117/69	120/63	127/66	(mmHg)
Heartrate	88	71	72	70	53	72	(b.p.m.)
Standing:							
Blood pressure	120/78	131/69	118/66	115/67	127/69	131/69	(mmHg)
Heartrate	102	90	84	85	67	107	(b.p.m.)
ECG-registration (normal/abnormal)	normal	normal	normal	normal	normal	normal	

BLOOD ANALYSES

Hemoglobin	9.7	9.1	9.3	8.7	7.8	7.8	(M: 8.6-10.7 mmol/L) (F: 7.4-9.6 mmol/L)
Hematocrite	0.47	0.44	0.42	0.4	0.39	0.39	(M: 0.41-0.55 L/L) (F: 0.36-0.46 L/L)
Leucocytes	5.7	4.9	6.8	6.6	6.1	5.7	(4.0-10.0* Exp9/L)
Eosinophiles	3	1	1	1	2	2	(< 5 %)
Basophiles	1	0	0	1	0	0	(< 2 %)
Granulocytes	63	61	70	69	71	71	(40-72 %)
Lymfocytes	25	26	24	24	24	23	(20-45 %)
Monocytes	8	* **12**	5	5	3	4	(3-10 %)
Urea	4.6	4.2	3.7	* **2.6**	3.6	3.7	(3.0-7.5 mmol/L)
Creatinine	83	84	74	71	70	68	(50-120 micromol/L)
Sodium	144	145	142	144	144	143	(136-146 mmol/L)
Potassium	4	3.9	3.9	3.9	4	4	(3.8-5.0 mmol/L)
Calcium	2.29	2.24	2.40	2.35	2.34	2.35	(2.20-2.60 mmol/L)
Bilirubin	9	8	12	10	7	9	(< 17 micromol/L)
AF	85	81	47	50	* **38**	42	(40-130 U/L)
γGT	18	16	20	19	21	20	(M: 15-70 U/L) (F: 15-45 U/L)
ASAT	16	21	18	17	18	19	(15-45 U/L)
ALAT	20	23	26	24	10	17	(10-50 U/L)

URINE ANALYSES

Acidity (pH)	5	5	6	6	7	5	(4.5-7.8)
Albumin	neg	neg	neg	neg	neg	w.pos	Negative
Glucose	neg	neg	neg	neg	neg	neg	Negative
Leucocytes	neg	neg	* **pos**	* **pos**	w.pos	w.pos	Negative
Blood	neg	neg	neg	neg	w.pos	* **st.pos**	Negative
Microscopic Analyses							
Erythrocyten				0-4		not done	(0-4)
Leucocyten			0-4	0-4			(0-4)
Amorf zout							
Epitheelcellen							(none)
Bacteria				many			(none)

* = out of normal range

neg = negative; pos = positive; w.pos = weakly positive; st.pos = strongly positive

Appendix 11 Test results of the Liver Function Parameters

Test results of the liver function parameters as observed prior to and 24 hours following single dose intravenous administration of sodium nitrite to healthy adult volunteers

Treatment 3 = administration of 0.08 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
Treatment 4 = admininstration of 0.12 mmol $NaNO_2$ per mmol Hb infused over 30 minutes

Parameter	Treatment 3 pre-dose	Treatment 3 post-dose	Treatment 4 pre-dose	Treatment 4 post-dose	Normal Range
Volunteer 1:					
ASAT (U/L)	18	21	15	18	15 - 45
ALAT (U/L)	21	20	18	19	10 - 50
γGT (U/L)	18	19	<u>14</u>	16	M: 15 - 70 F: 15 - 45
LDH (U/L)	387	414	351	369	300 - 620
Volunteer 2:					
ASAT (U/L)	17	17	16	<u>14</u>	15 - 45
ALAT (U/L)	27	26	22	19	10 - 50
γGT (U/L)	19	20	19	17	M: 15 - 70 F: 15 - 45
LDH U/L)	518	464	557	439	300 - 620
Volunteer 3:					
ASAT (U/L)	19	21	23	19	15 - 45
ALAT (U/L)	22	16	18	10	10 - 50
γGT (U/L)	22	22	22	22	M: 15 - 70 F: 15 - 45
LDH (U/L)	328	341	328	310	300 - 620

underline = out of range

Appendix 12. Table of Adverse Experiences

Adverse Experiences as reported after single dose, intravenous, administration of sodium nitrite to three healthy adult volunteers.

Treatment 1: Administration of 0.04 mmol $NaNO_2$ per mmol Hb infused over 10 minutes

Volunteer number	Adverse Experience	Time started after infusion (hrs.min)	Duration (hrs.min)	Intensity	Related to testproduct
1	Head discomfort	0.13	5.39	mild	possible
	Eye accomodation disorder	0.52	0.18	mild	possible
2	Dizziness	0.16	0.30	mild	yes
	Tiredness	0.16	0.30	mild	yes
3	Headache	6.36	5.00	mild	possible

Treatment 2: Administration of 0.04 mmol $NaNO_2$ per mmol Hb infused over 30 minutes

Volunteer number	Adverse Experience	Time started after infusion (hrs.min)	Duration (hrs.min)	Intensity	Related to testproduct
3	Dizziness	0.54	1.14	mild	possible
	Burning sensation both arms	-1.00	0.55	mild	no

Appendix 12. **Table of Adverse Experiences**
(continued)

Adverse Experiences as reported after single dose, intravenous, administration of sodium nitrite to three healthy adult volunteers.

Treatment 3: Administration of 0.08 mmol $NaNO_2$ per mmol Hb infused over 30 minutes

Volunteer number	Adverse Experience	Time started after infusion (hrs.min)	Duration (hrs.min)	Intensity	Related to testproduct
1	Tingling sensation at infusion site	0.5	0.40	mild	yes

Treatment 4: Administration of 0.12 mmol $NaNO_2$ per kg body weight over 30 minutes

Volunteer number	Adverse Experience	Time started after infusion (hrs.min)	Duration (hrs.min)	Intensity	Related to testproduct
1	Head discomfort	0.25	10.19	mild	possible
	Eye accomodation disorder	0.25	0.59	mild	possible
	Cold feeling	0.30	2.14	mild	possible
	Tingling sensating at infusion site	0.04	0.35	mild	yes
2	Dizziness	0.21	0.34	mild	yes
3	Headache	2.14	8.32	mild	possible

Appendix 13a Table of Vital Signs

Vital Signs as observed after intravenous administration of ascending sodium nitrite doses to volunteers

Treatment 1 = 0.04 mmol $NaNO_2$ per mmol Hb infused over 10 minutes

	Volunteer 1			Volunteer 2			Volunteer 3		
Scheme Time (hr)	Actual Time (hr)	Blood Pressure Systolic / Diastolic (mmHg)	Heart rate (b.p.m.)	Actual Time (hr)	Blood Pressure Systolic / Diastolic (mmHg)	Heart rate (b.p.m.)	Actual Time (hr)	Blood Pressure Systolic / Diastolic (mmHg)	Heart rate (b.p.m.)
-0.08	-0.63	124 / 70	60	-0.62	109 / 59	55	-0.30	116 / 68	59
0.08	0.10	121 / 46	66	0.10	105 / 59	70	0.30	122 / 66	56
0.17	0.20	109 / 55	63	0.18	97 / 45	73	0.37	112 / 63	60
0.25	0.27	108 / 50	68	0.27	107 / 43	84	0.45	113 / 64	58
0.33	0.33	98 / 65	66	0.35	99 / 41	79	0.53	111 / 64	58
0.42	0.43	110 / 55	71	0.43	100 / 48	67	0.63	115 / 66	58
0.50	0.52	117 / 54	67	0.53	106 / 51	71	0.70	117 / 70	61
0.67	0.68	115 / 65	61	0.67	112 / 55	63	0.87	106 / 64	57
0.83	0.87	108 / 74	75	0.85	103 / 50	62	1.03	107 / 63	58
1.00	1.02	107 / 57	64	1.00	103 / 52	64	1.20	109 / 61	58
1.25	1.27	107 / 65	69	1.27	111 / 60	66	1.47	106 / 62	51
1.50	1.55	126 / 72	66	1.52	105 / 60	55	1.70	104 / 66	54
1.75	1.77	120 / 68	71	1.78	112 / 62	56	1.95	109 / 66	56
2.00	2.02	114 / 72	72	2.08	101 / 63	66	2.20	115 / 61	56
2.50	2.52	113 / 76	69	2.52	106 / 59	60	2.72	107 / 65	60
3.00	3.02	117 / 72	63	3.00	106 / 63	59	3.22	121 / 68	56
4.00	4.05	117 / 72	76	4.02	100 / 60	54	4.25	114 / 66	59
5.00	5.10	128 / 78	76	5.07	111 / 59	60	5.30	125 / 72	65
6.00	6.05	128 / 64	85	5.98	106 / 55	61	6.20	116 / 80	55
8.00	8.00	117 / 68	89	7.95	111 / 65	72	8.18	110 / 78	59
10.00	9.98	116 / 64	76	9.88	119 / 65	65	10.97	130 / 76	78
24.00	-	- -	-	23.85	119 / 67	66	22.83	121 / 73	72

Treatment 2 = 0.04 mmol $NaNO_2$ per mmol Hb infused over 30 minutes

	Volunteer 1			Volunteer 2			Volunteer 3		
Scheme Time (hr)	Actual Time (hr)	Blood Pressure Systolic / Diastolic (mmHg)	Heart rate (b.p.m.)	Actual Time (hr)	Blood Pressure Systolic / Diastolic (mmHg)	Heart rate (b.p.m.)	Actual Time (hr)	Blood Pressure Systolic / Diastolic (mmHg)	Heart rate (b.p.m.)
-0.08	-0.43	113 / 65	63	-0.65	110 / 62	62	-0.07	106 / 63	58
0.17	0.12	105 / 53	61	0.18	103 / 60	65	0.18	102 / 60	55
0.33	0.37	102 / 57	64	0.35	105 / 52	62	0.35	112 / 61	53
0.50	0.52	98 / 47	61	0.53	101 / 59	68	0.50	112 / 63	53
0.58	0.60	100 / 62	73	0.60	101 / 54	59	0.62	102 / 56	50
0.67	0.68	106 / 46	63	0.68	102 / 55	60	0.68	126 / 59	56
0.75	0.78	109 / 74	72	0.77	106 / 61	57	0.77	102 / 60	60
0.83	0.93	116 / 53	63	0.88	104 / 58	57	0.85	101 / 63	59
1.00	1.02	112 / 57	68	1.02	105 / 58	56	1.02	107 / 60	56
1.17	1.18	108 / 62	65	1.18	102 / 56	56	1.18	114 / 59	50
1.33	1.35	105 / 57	61	1.35	105 / 60	51	1.33	104 / 64	58
1.50	1.53	110 / 60	57	1.52	111 / 65	60	1.50	106 / 66	56
1.75	1.83	108 / 58	61	1.77	111 / 62	59	1.77	133 / 59	52
2.00	2.03	113 / 61	72	2.02	104 / 46	51	2.03	106 / 64	60
2.50	2.53	116 / 67	56	2.50	105 / 54	53	2.52	107 / 58	54
3.00	3.12	119 / 74	62	2.98	110 / 52	59	3.02	106 / 68	57
4.00	4.02	114 / 66	70	4.05	101 / 53	55	4.02	128 / 62	60
5.00	5.07	117 / 70	75	4.98	102 / 53	56	5.03	115 / 66	67
6.00	6.03	114 / 70	78	6.02	108 / 56	55	6.03	124 / 70	69
8.00	8.05	112 / 67	74	8.02	104 / 56	55	8.03	112 / 60	54
10.00	10.03	118 / 70	71	9.98	109 / 55	58	9.90	134 / 72	71
24.00	23.68	121 / 67	81	24.03	108 / 59	54	23.80	130 / 72	77

- = not performed

Appendix 13a Table of Vital Signs

(continued)

Vital Signs as observed after intravenous administration of ascending sodium nitrite doses to volunteers

Treatment 3 = 0.08 mmol $NaNO_2$ per mmol Hb infused over 30 minutes

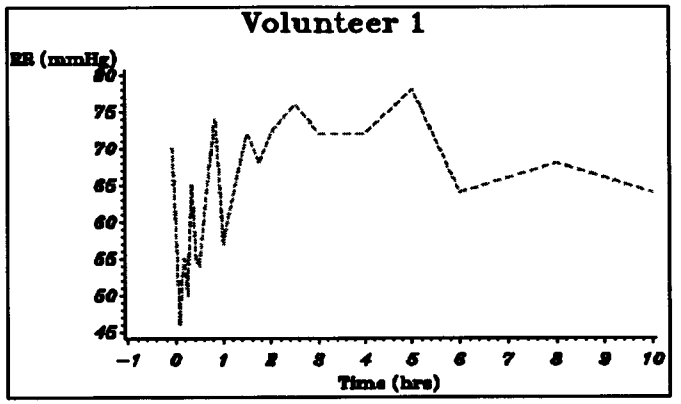
	Volunteer 1				Volunteer 2				Volunteer 3			
Scheme Time (hr)	Actual Time (hr)	Blood Pressure Systolic / (mmHg)	Diastolic (mmHg)	Heart rate (b.p.m.)	Actual Time (hr)	Blood Pressure Systolic / (mmHg)	Diastolic (mmHg)	Heart rate (b.p.m.)	Actual Time (hr)	Blood Pressure Systolic / (mmHg)	Diastolic (mmHg)	Heart rate (b.p.m.
-0.08	-0.07	117 /	66	73	-0.77	108 /	55	64	-0.10	116 /	60	60
0.17	0.18	110 /	69	79	0.18	106 /	48	67	0.18	115 /	62	60
0.33	0.35	107 /	67	73	0.35	104 /	53	80	0.35	115 /	62	62
0.50	0.53	110 /	61	85	0.52	100 /	45	73	0.53	106 /	59	63
0.58	0.60	110 /	70	73	0.60	104 /	47	74	0.60	111 /	55	61
0.67	0.70	110 /	56	68	0.70	103 /	46	66	0.68	109 /	59	61
0.75	0.75	105 /	59	66	0.77	103 /	51	68	0.77	108 /	62	59
0.83	0.83	110 /	54	84	0.85	109 /	42	62	0.85	105 /	54	61
1.00	1.02	107 /	58	79	1.03	101 /	49	65	1.00	112 /	62	65
1.17	1.17	104 /	67	70	1.18	109 /	51	65	1.18	109 /	64	75
1.33	1.33	111 /	53	70	1.35	102 /	47	67	1.37	108 /	62	62
1.50	1.50	115 /	59	76	1,52	109 /	54	66	1.52	106 /	63	56
1.75	1.77	105 /	68	67	1.80	107 /	55	63	1.78	106 /	62	57
2.00	2.02	106 /	65	74	2.08	106 /	50	62	2.02	115 /	70	66
2.50	2.52	113 /	68	79	2.53	105 /	57	61	2.52	110 /	66	60
3.00	3.00	109 /	70	72	3.02	104 /	58	65	3.00	117 /	63	65
4.00	4.02	111 /	59	61	4.03	95 /	56	58	4.00	125 /	67	54
5.00	5.00	111 /	63	80	5.02	107 /	60	66	5.07	117 /	65	67
6.00	6.03	111 /	68	79	6.02	104 /	60	61	6.02	117 /	67	62
8.00	8.03	105 /	74	75	8.00	105 /	57	61	8.05	115 /	68	61
10.00	10.03	124 /	68	73	9.98	111 /	58	62	9.95	122 /	73	61
24.00	23.20	123 /	74	84	24.27	110 /	57	66	23.37	126 /	70	62

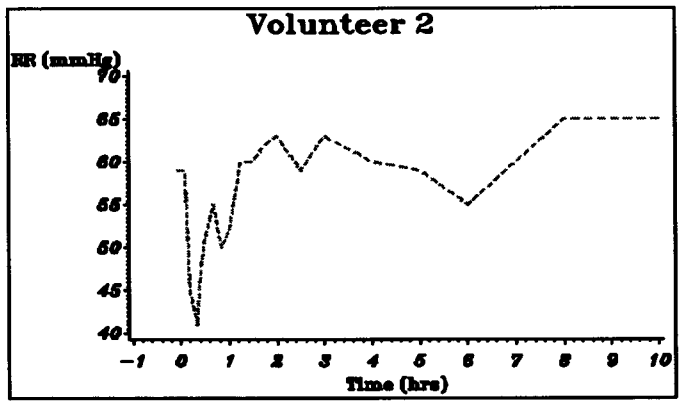
Treatment 4 = 0.12 mmol $NaNO_2$ per mmol Hb infused over 30 minutes

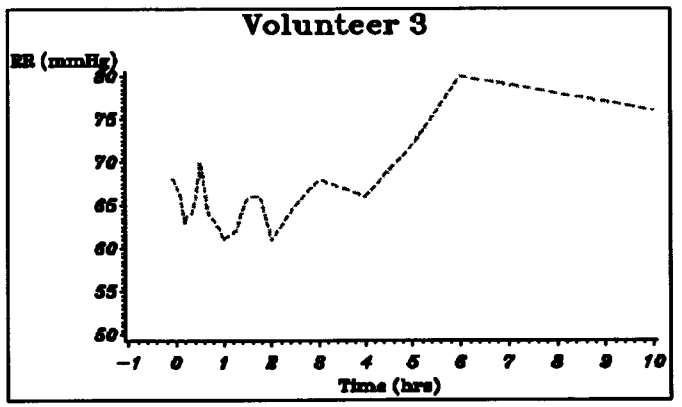
	Volunteer 1				Volunteer 2				Volunteer 3			
Scheme Time (hr)	Actual Time (hr)	Blood Pressure Systolic / (mmHg)	Diastolic (mmHg)	Heart rate (b.p.m.)	Actual Time (hr)	Blood Pressure Systolic / (mmHg)	Diastolic (mmHg)	Heart rate (b.p.m.)	Actual Time (hr)	Blood Pressure Systolic / (mmHg)	Diastolic (mmHg)	Heart rate (b.p.m.)
-0.08	-0.07	113 /	54	58	-0.07	103 /	65	58	-0.40	135 /	69	70
0.17	0.18	98 /	51	73	0.17	109 /	54	75	0.18	115 /	59	64
0.33	0.33	101 /	58	72	0.33	101 /	47	63	0.35	117 /	55	66
0.50	0.52	98 /	51	77	0.53	103 /	44	65	0.53	112 /	56	67
0.58	0.62	96 /	61	61	0.60	98 /	42	65	0.60	106 /	59	69
0.67	0.68	101 /	51	76	0.68	99 /	45	65	0.70	110 /	72	70
0.75	0.75	101 /	57	69	0.77	103 /	41	60	0.77	105 /	54	66
0.83	0.85	98 /	72	59	0.85	101 /	44	62	0.85	108 /	60	72
1.00	1.02	103 /	62	68	1.02	98 /	44	70	1.02	112 /	61	66
1.17	1.17	102 /	67	74	1.18	102 /	43	58	1.18	110 /	64	60
1.33	1.33	99 /	58	62	1.35	103 /	50	55	1.35	110 /	57	65
1.50	1.52	102 /	55	67	1.55	101 /	52	60	1.55	106 /	65	66
1.75	1.80	108 /	69	63	1.75	105 /	46	63	1.78	108 /	63	67
2.00	2.02	106 /	60	60	2.02	102 /	58	56	2.02	111 /	59	63
2.50	2.55	101 /	64	80	2.52	100 /	64	62	2.53	110 /	63	68
3.00	3.02	109 /	70	86	3.00	118 /	68	67	3.13	119 /	65	71
4.00	4.02	108 /	67	60	4.02	92 /	53	53	4.00	117 /	74	60
5.00	5.00	111 /	61	72	5.02	102 /	53	61	5.00	129 /	70	67
6.00	5.98	117 /	68	71	6.00	107 /	64	58	6.05	113 /	67	64
8.00	8.00	108 /	69	77	8.08	106 /	62	58	8.03	121 /	63	65
10.00	9.95	116 /	59	58	9.97	117 /	65	58	9.92	120 /	71	77
24.00	23.87	125 /	69	71	24.18	112 /	61	58	23.92	123 /	66	93

Appendix 13 b ***Diastolic Blood Pressure as observed after intravenous administration of sodium nitrite to adult volunteers***

---------- = 0.04 mmol $NaNO_2$ per mmol Hb infused over 10 minutes



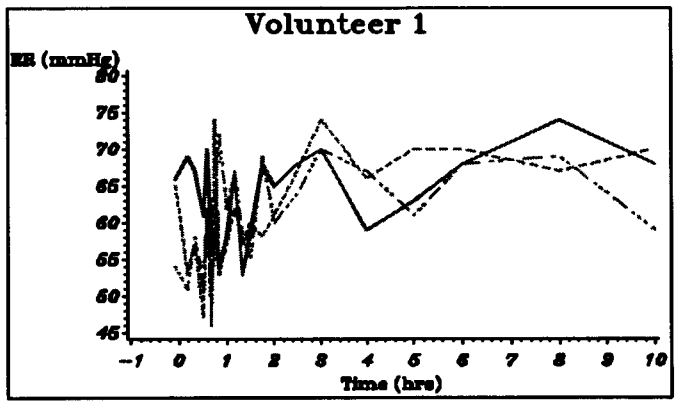


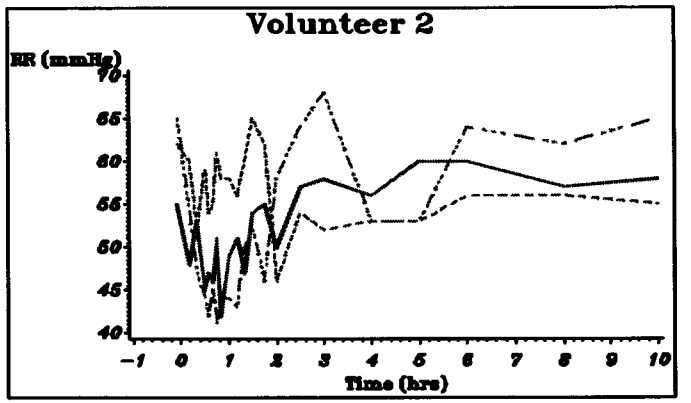


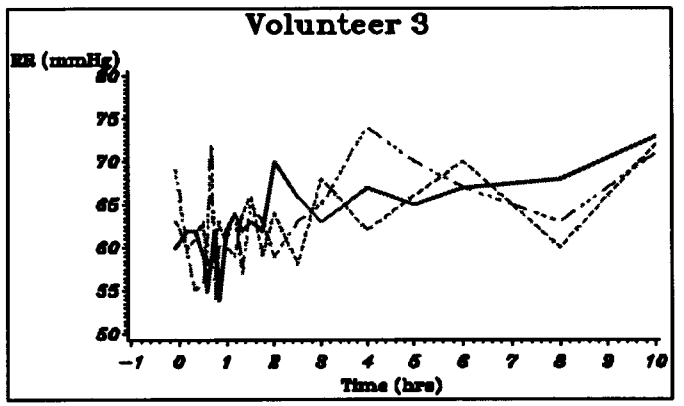
Appendix 13 b (continued)

Diastolic Blood Pressure as observed after intravenous administration of sodium nitrite to adult volunteers

---------- = 0.04 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
———— = 0.08 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
— --- — = 0.12 mmol $NaNO_2$ per mmol Hb infused over 30 minutes

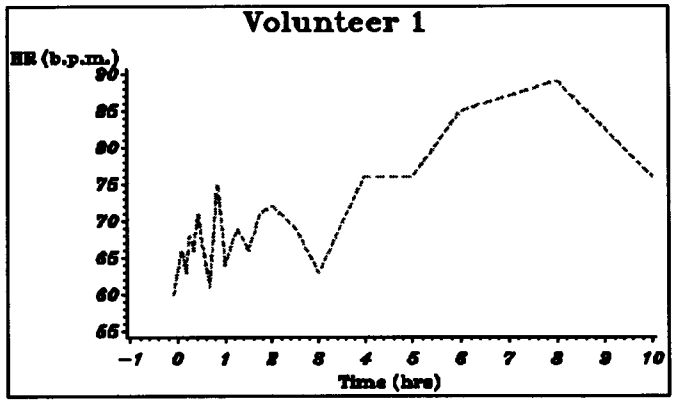


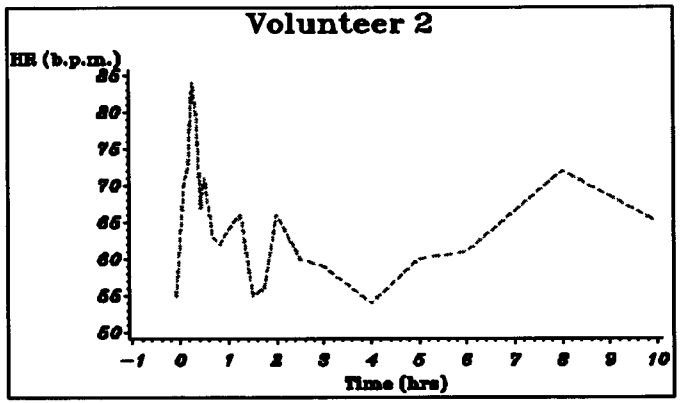


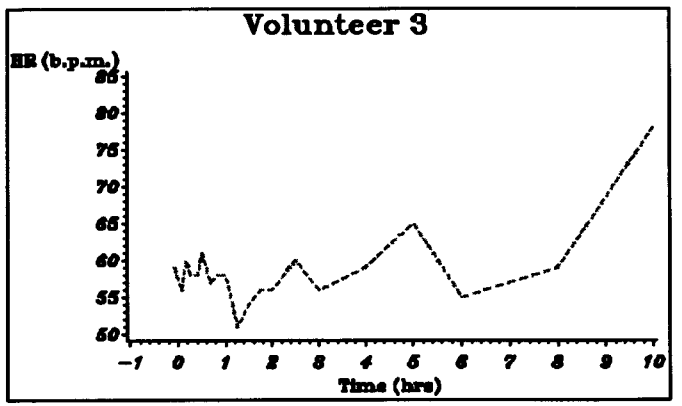


Appendix 13 c ***Heart Rate as observed after intravenous administration of sodium nitrite to adult volunteers***

---------- = 0.04 mmol $NaNO_2$ per mmol Hb infused over 10 minutes



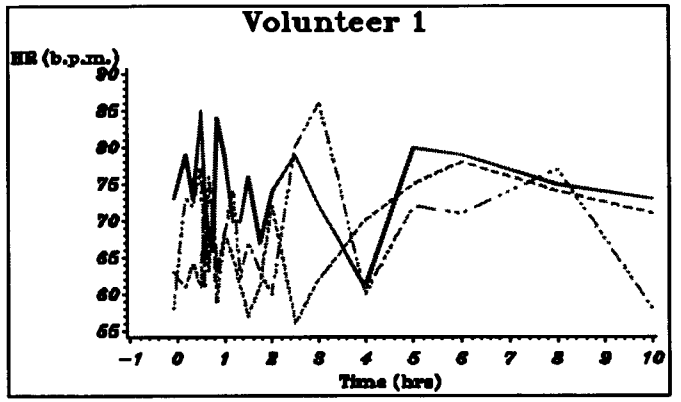


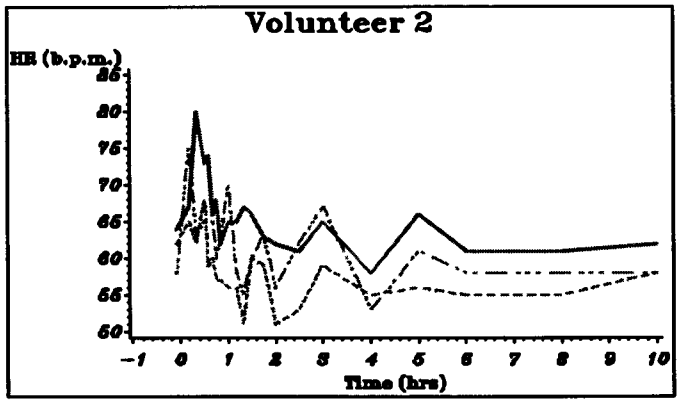


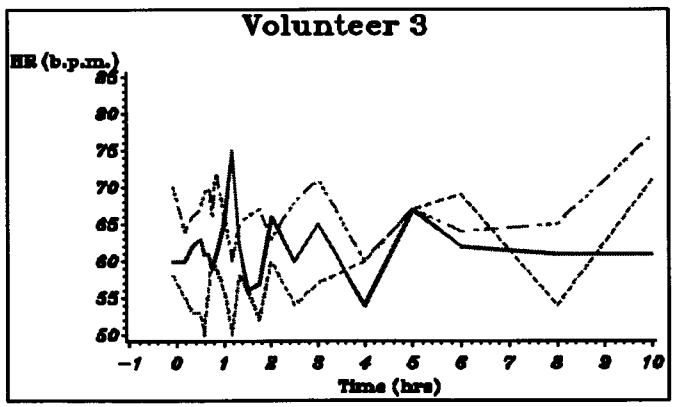
Appendix 13 c (continued)

Heart Rate as observed after intravenous administration of sodium nitrite to adult volunteers

---------- = 0.04 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
———— = 0.08 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
— --- — = 0.12 mmol $NaNO_2$ per mmol Hb infused over 30 minutes







Appendix 14a Table of Hemoglobin Concentrations

Hemoglobin Concentrations as observed after intravenous administration of $NaNO_2$ doses to volunteers

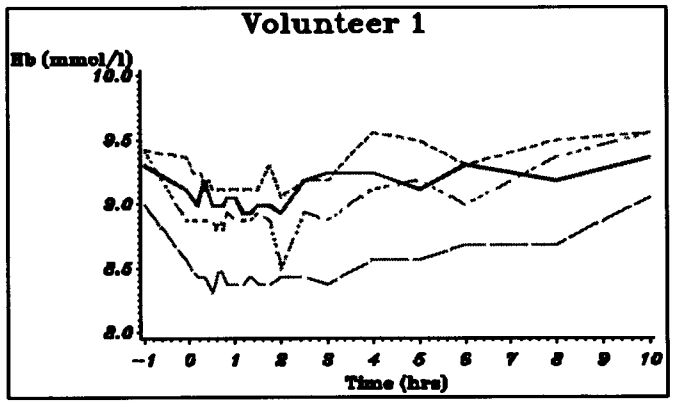
TREATMENT			1				2					
VOLUNTEER	1		2		3		1		2		3	
Scheme Time (hr)	Actual Time (hr)	Hb (mmol/l)	Actual Time (hr)	Hb (mmol/l)	Actual Time (hr)	Hb (mmol/l)	Actual Time (hr)	Hb (mmol/l)	Actual Time (hr)	Hb (mmol/l)	Actual Time (hr)	Hb (mmol/l)
8.00	-0.93	9.42	-1.32	9.30	-1.38	7.38	-0.67	9.30	-0.95	9.11	-0.97	7.32
8.55	-0.07	9.36	-0.32	9.05	-0.05	7.13	-0.03	9.11	-0.08	8.68	-0.12	7.01
9.05	0.08	9.24	0.08	8.93	0.27	7.19	0.08	8.99	0.17	8.68	0.17	7.07
9.10	0.17	9.24	0.17	8.99	0.35	7.13	0.33	9.18	0.33	8.62	0.33	7.07
9.15	0.25	9.24	0.25	8.93	0.43	7.07	0.50	8.99	0.52	8.56	0.48	7.13
9.20	0.33	9.11	0.33	8.93	0.52	6.88	0.58	8.99	0.58	8.68	0.58	7.07
9.25	0.42	9.18	0.42	8.99	0.60	7.07	0.67	8.99	0.67	8.74	0.67	7.19
9.30	0.47	9.11	0.50	8.99	0.68	6.88	0.75	8.99	0.75	8.68	0.75	7.13
9.40	-	-	0.67	8.99	0.85	7.13	0.92	9.05	0.83	8.80	0.83	7.13
9.50	0.83	9.11	0.83	8.87	1.02	6.94	1.00	9.05	1.00	8.74	1.00	7.13
10.00	1.00	9.11	1.00	9.05	1.18	6.88	1.17	8.93	1.17	8.68	1.17	7.13
10.15	1.25	9.11	1.25	8.99	1.45	6.88	1.33	8.93	1.33	8.68	1.33	7.19
10.30	1.52	9.11	1.50	8.93	1.68	6.94	1.50	8.99	1.50	8.68	1.50	7.13
10.45	1.75	9.30	1.75	8.93	1.93	6.76	1.78	8.99	1.75	8.68	1.75	7.25
11.00	2.00	9.05	2.05	8.93	2.18	6.94	2.02	8.93	2.00	8.74	2.00	7.19
11.30	2.50	9.18	2.50	8.99	2.68	6.76	2.50	9.18	2.50	8.74	2.50	7.13
12.00	3.00	9.18	2.98	8.99	3.18	6.88	3.03	9.24	3.00	8.74	3.00	7.13
13.00	4.00	9.55	4.00	8.93	4.23	6.94	4.00	9.24	4.00	8.68	4.00	7.25
14.00	5.08	9.49	5.00	9.05	5.25	7.19	5.03	9.11	5.00	8.68	5.00	7.56
15.00	6.02	9.30	5.97	9.05	6.18	7.07	6.00	9.30	6.00	8.62	6.00	7.75
17.00	7.98	9.49	8.00	8.99	8.18	7.25	8.00	9.18	8.00	8.87	8.00	7.38
19.00	9.98	9.55	9.93	9.30	10.12	7.63	10.00	9.36	10.00	9.11	9.88	7.81
24.00	22.55	9.98	23.82	8.87	22.78	7.50	23.67	9.30	24.05	8.99	23.72	7.63

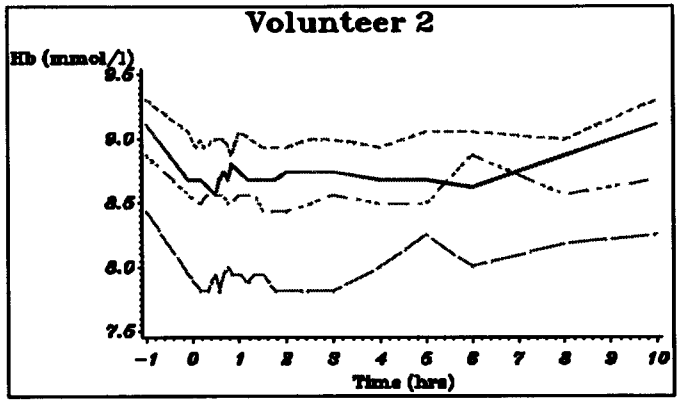
TREATMENT			3				4					
VOLUNTEER	1		2		3		1		2		3	
Scheme Time (hr)	Actual Time (hr)	Hb (mmol/l)	Actual Time (hr)	Hb (mmol/l)	Actual Time (hr)	Hb (mmol/l)	Actual Time (hr)	Hb (mmol/l)	Actual Time (hr)	Hb (mmol/l)	Actual Time (hr)	Hb (mmol/l)
8.00	-0.50	9.42	-0.98	8.87	-0.72	7.32	-0.77	8.99	-0.92	8.43	-1.07	7.63
8.55	-0.08	8.87	-0.33	8.56	-0.10	7.01	-0.07	8.56	-0.25	7.94	-0.10	7.32
9.10	0.17	8.87	0.17	8.49	0.17	6.88	0.17	8.43	0.17	7.81	0.17	7.19
9.20	0.33	8.87	0.33	8.56	0.33	6.76	0.33	8.43	0.33	7.81	0.33	7.01
9.30	0.52	8.87	0.50	8.56	0.52	6.82	0.50	8.31	0.50	7.94	0.52	7.25
9.35	0.58	8.80	0.58	8.56	0.58	6.82	0.58	8.37	0.57	7.81	0.58	7.19
9.40	0.67	8.87	0.67	8.56	0.67	6.82	0.67	8.49	0.67	7.94	0.67	7.19
9.45	0.75	8.80	0.75	8.49	0.75	6.82	0.73	8.43	0.75	8.00	0.75	7.25
9.50	0.83	8.93	0.83	8.49	0.83	6.82	0.83	8.37	0.83	7.94	0.83	7.13
10.00	1.00	8.87	1.02	8.56	0.98	6.82	1.00	8.37	1.00	7.94	1.00	7.13
10.10	1.17	8.87	1.17	8.56	1.17	6.82	1.17	8.37	1.17	7.87	1.17	7.19
10.20	1.33	8.87	1.33	8.56	1.35	6.76	1.33	8.43	1.33	7.94	1.33	7.07
10.30	1.50	8.93	1.50	8.43	1.50	6.88	1.50	8.37	1.53	7.94	1.53	7.19
10.45	1.75	8.87	1.77	8.43	1.75	6.76	1.75	8.37	1.75	7.81	1.75	7.13
11.00	2.00	8.49	2.08	8.43	2.00	6.70	2.00	8.43	2.00	7.81	2.00	7.13
11.30	2.50	8.93	2.50	8.49	2.50	6.88	2.53	8.43	2.50	7.81	2.52	7.25
12.00	3.00	8.87	3.00	8.56	3.00	6.94	3.00	8.37	3.00	7.81	3.10	7.25
13.00	4.00	9.11	4.00	8.49	4.00	6.94	4.00	8.56	4.00	8.00	4.00	7.13
14.00	4.98	9.18	5.00	8.49	5.03	7.32	5.00	8.56	5.00	8.25	5.00	7.50
15.00	6.02	8.99	6.00	8.87	6.00	7.01	5.97	8.68	6.00	8.00	6.02	7.07
17.00	8.00	9.36	7.98	8.56	8.03	7.13	8.00	8.68	8.05	8.18	8.00	7.44
19.00	10.00	9.55	9.97	8.68	9.92	7.56	9.98	9.05	9.95	8.25	9.97	7.44
24.00	-	-	24.17	8.68	23.42	7.44	23.85	9.36	24.13	8.56	24.02	7.75

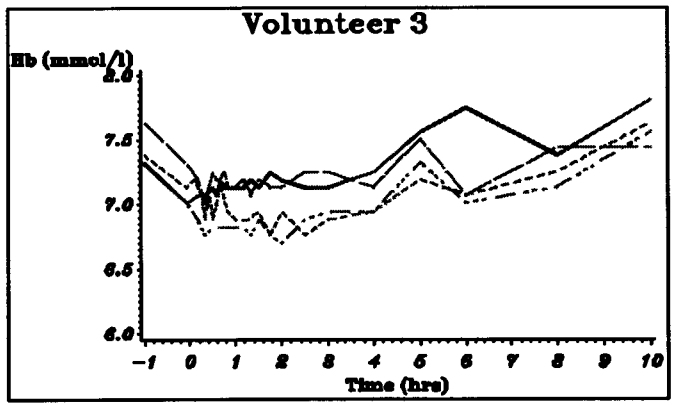
- = missing data underline = blood sampling performed under tourniquet pressure

Appendix 14 b ***Hemoglobin concentration as observed after intravenous administration of sodium nitrite doses to adult volunteers***

---------- = 0.04 mmol $NaNO_2$ per mmol Hb infused over 10 minutes
———— = 0.04 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
— --- — = 0.08 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
—— —— = 0.12 mmol $NaNO_2$ per mmol Hb infused over 30 minutes







Appendix 15a Table of Methemoglobin Data

Percentage of Methemoglobin as observed after intravenous administration of $NaNO_2$ doses to volunteers

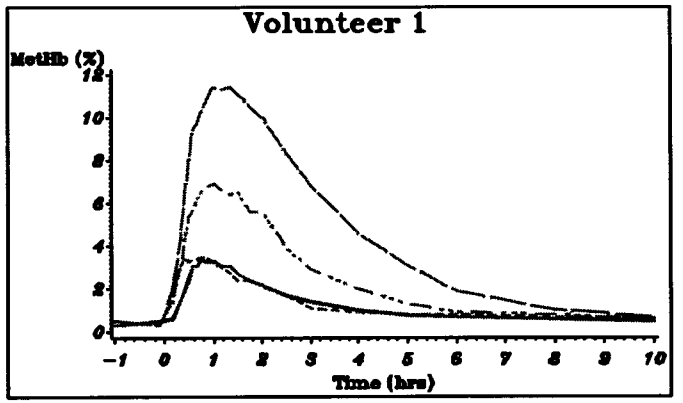
TREATMENT	1						2					
VOLUNTEER	1		2		3		1		2		3	
Scheme Time (hr)	Actual Time (hr)	MetHb (%)	Actual Time (hr)	MetHb (%)	Actual Time (hr)	MetHb (%)	Actual Time (hr)	MetHb (%)	Actual Time (hr)	MetHb (%)	Actual Time (hr)	MetHb (%)
8.00	-0.93	0.3	-1.32	0.2	-1.38	0.4	-0.67	0.3	-0.95	0.2	-0.97	0.6
8.55	-0.07	0.4	-0.32	0.2	-0.05	0.5	-0.03	0.5	-0.08	0.1	-0.12	0.5
9.05	0.08	1.1	0.08	1.0	0.27	0.5	0.08	0.6	0.17	0.7	0.17	1.0
9.10	0.17	1.8	0.17	2.2	0.35	1.4	0.33	1.5	0.33	1.6	0.33	1.7
9.15	0.25	2.5	0.25	2.7	0.43	1.7	0.50	2.5	0.52	2.6	0.48	2.5
9.20	0.33	3.1	0.33	2.9	0.52	2.3	0.58	3.1	0.58	3.0	0.58	2.9
9.25	0.42	3.4	0.42	3.2	0.60	2.4	0.67	3.1	0.67	3.2	0.67	3.1
9.30	0.47	3.3	0.50	3.4	0.68	2.4	0.75	3.4	0.75	3.4	0.75	3.2
9.40	-	-	0.67	3.4	0.85	2.6	0.92	3.3	0.83	3.4	0.83	3.3
9.50	0.83	3.5	0.83	3.4	1.02	2.5	1.00	3.3	1.00	3.3	1.00	3.4
10.00	1.00	3.2	1.00	3.0	1.18	2.2	1.17	3.1	1.17	3.0	1.17	3.0
10.15	1.25	2.9	1.25	2.6	1.45	2.0	1.33	3.1	1.33	2.8	1.33	2.9
10.30	1.52	2.4	1.50	2.2	1.68	1.9	1.50	2.7	1.50	2.3	1.50	2.7
10.45	1.75	2.4	1.75	1.8	1.93	1.8	1.78	2.4	1.75	2.0	1.75	2.3
11.00	2.00	2.1	2.05	1.6	2.18	1.5	2.02	2.2	2.00	1.6	2.00	2.0
11.30	2.50	1.7	2.50	1.1	2.68	1.0	2.50	1.7	2.50	1.3	2.50	1.7
12.00	3.00	1.1	2.98	0.7	3.18	1.1	3.03	1.4	3.00	0.9	3.00	1.5
13.00	4.00	0.9	4.00	0.4	4.23	0.9	4.00	1.0	4.00	0.5	4.00	0.9
14.00	5.08	0.8	5.00	0.3	5.25	0.6	5.03	0.8	5.00	0.6	5.00	0.5
15.00	6.02	0.8	5.97	0.3	6.18	0.7	6.00	0.7	6.00	0.2	6.00	0.4
17.00	7.98	0.7	8.00	0.3	8.18	0.9	8.00	0.6	8.00	0.4	8.00	0.7
19.00	9.98	0.6	9.93	0.4	10.12	0.4	10.00	0.5	10.00	0.5	9.88	0.5
24.00	22.55	0.7	23.82	0.3	22.78	0.5	23.67	0.4	24.05	0.0	23.72	0.3

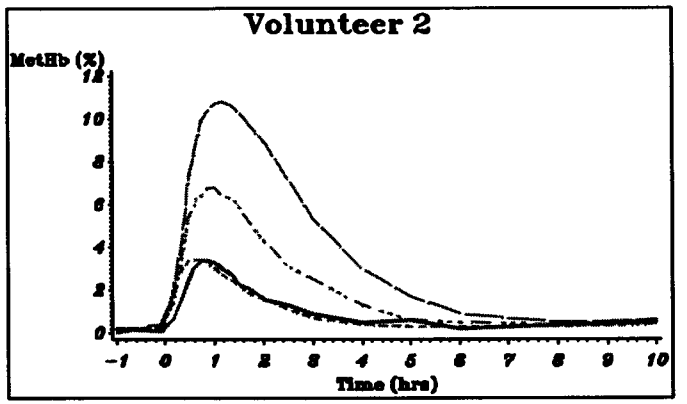
TREATMENT	3						4					
VOLUNTEER	1		2		3		1		2		3	
Scheme Time (hr)	Actual Time (hr)	MetHb (%)	Actual Time (hr)	MetHb (%)	Actual Time (hr)	MetHb (%)	Actual Time (hr)	MetHb (%)	Actual Time (hr)	MetHb (%)	Actual Time (hr)	MetHb (%)
8.00	-0.50	0.3	-0.98	0.1	-0.72	0.6	-0.77	0.5	-0.92	0.0	-1.07	0.7
8.55	-0.08	0.4	-0.33	0.4	-0.10	0.6	-0.07	0.3	-0.25	0.4	-0.10	0.6
9.10	0.17	1.4	0.17	1.4	0.17	1.4	0.17	1.9	0.17	1.8	0.17	1.8
9.20	0.33	3.0	0.33	3.3	0.33	3.0	0.33	4.4	0.33	4.2	0.33	3.8
9.30	0.52	5.4	0.50	5.5	0.52	5.2	0.50	7.9	0.50	7.4	0.52	7.5
9.35	0.58	5.7	0.58	5.8	0.58	5.5	0.58	9.4	0.57	8.2	0.58	8.4
9.40	0.67	6.2	0.67	6.4	0.67	6.5	0.67	9.8	0.67	9.1	0.67	9.5
9.45	0.75	6.5	0.75	6.4	0.75	6.5	0.73	10.3	0.75	9.9	0.75	10.2
9.50	0.83	6.7	0.83	6.7	0.83	6.8	0.83	10.7	0.83	10.2	0.83	10.4
10.00	1.00	6.9	1.02	6.8	0.98	7.2	1.00	11.4	1.00	10.7	1.00	11.2
10.10	1.17	6.6	1.17	6.4	1.17	7.3	1.17	11.3	1.17	10.8	1.17	11.4
10.20	1.33	6.4	1.33	6.3	1.35	7.3	1.33	11.4	1.33	10.6	1.33	11.2
10.30	1.50	6.5	1.50	5.9	1.50	6.7	1.50	11.1	1.53	10.3	1.53	11.3
10.45	1.75	5.6	1.77	5.0	1.75	6.6	1.75	10.5	1.75	9.6	1.75	10.9
11.00	2.00	5.6	2.08	4.3	2.00	5.8	2.00	10.0	2.00	8.9	2.00	10.4
11.30	2.50	3.9	2.50	3.1	2.50	4.7	2.53	8.3	2.50	7.1	2.52	8.8
12.00	3.00	2.9	3.00	2.5	3.00	3.9	3.00	6.8	3.00	5.3	3.10	6.9
13.00	4.00	2.0	4.00	1.3	4.00	2.5	4.00	4.6	4.00	3.0	4.00	4.8
14.00	4.98	1.3	5.00	0.6	5.03	1.6	5.00	3.1	5.00	1.7	5.00	2.8
15.00	6.02	0.9	6.00	0.5	6.00	1.3	5.97	1.9	6.00	0.9	6.02	1.9
17.00	8.00	0.8	7.98	0.4	8.03	0.7	8.00	1.0	8.05	0.5	8.00	1.0
19.00	10.00	0.6	9.97	0.6	9.92	0.5	9.98	0.7	9.95	0.6	9.97	0.9
24.00	-	-	24.17	0.3	23.42	0.5	23.85	0.3	24.13	0.3	24.02	1.0

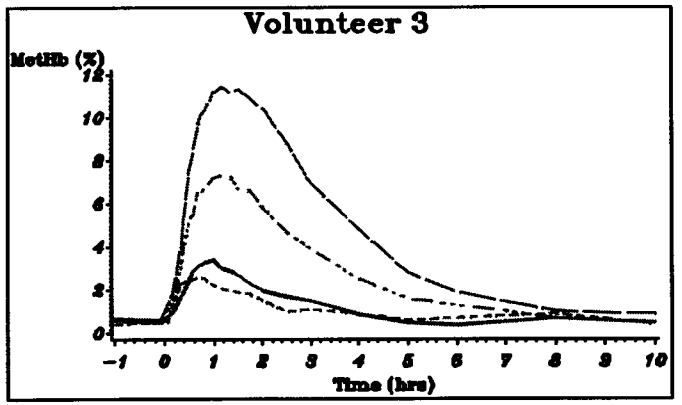
- = missing data

Appendix 15 b ***Percentage of Methemoglobin as observed after intravenous administration of sodium nitrite to adult volunteers***

---------- = 0.04 mmol $NaNO_2$ per mmol Hb infused over 10 minutes
———— = 0.04 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
— --- — = 0.08 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
—— —— = 0.12 mmol $NaNO_2$ per mmol Hb infused over 30 minutes







Appendix 15 c ***Table of Kinetic Parameters derived from the Methemoglobin data***

Treatment 1 = 0.04 mmol $NaNO_2$ per mmol Hb infused over 10 minutes
Treatment 2 = 0.04 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
Treatment 3 = 0.08 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
Treatment 4 = 0.12 mmol $NaNO_2$ per mmol Hb infused over 30 minutes

	TREATMENT 1							
Volunteer	Nitrite dose (mg)	C-max (%)	C-max adjusted*	t-max (hrs)	t-1/2 (hrs)	AUC(0-∞) (%*hr/l)	Extrapolation AUC (%)	AUC(0-∞) adjusted*
1	130	3.1	5.2	0.83	1.47	8.14	34	13.78
2	120	3.2	5.9	0.50	1.08	6.90	32	12.65
3	90	2.0	4.9	0.85	1.20	4.30	36	10.51

	TREATMENT 2							
Volunteer	Nitrite dose (mg)	C-max (%)	C-max adjusted*	t-max (hrs)	t-1/2 (hrs)	AUC(0-∞) (%*hr/l)	Extrapolation AUC (%)	AUC(0-∞) adjusted*
1	130	3.0	5.1	0.75	1.22	7.25	31	12.28
2	120	3.2	5.9	0.75	0.86	5.96	29	10.92
3	90	2.8	6.8	1.00	1.25	6.50	25	15.88

	TREATMENT 3							
Volunteer	nitrite dose (mg)	C-max (%)	C-max adjusted*	t-max (hrs)	t-1/2 (hrs)	AUC(0-∞) (%*hr/l)	Extrapolation AUC (%)	AUC(0-∞) adjusted*
1	250	6.5	5.7	1.00	1.19	18.24	15	16.05
2	220	6.6	6.6	1.02	1.11	16.58	22	16.58
3	190	6.7	7.8	1.17	1.27	20.13	9	23.30

	TREATMENT 4							
Volunteer	Nitrite dose (mg)	C-max (%)	C-max adjusted*	t-max (hrs)	t-1/2 (hrs)	AUC(0-∞) (%*hr/l)	Extrapolation AUC (%)	AUC(0-∞) adjusted*
1	370	11.0	6.5	1.00	1.47	38.91	8	23.14
2	320	10.6	7.3	1.17	1.14	31.58	8	21.71
3	290	10.8	8.2	1.17	1.30	37.37	7	28.35

* = results adjusted to a standard dose of 220 mg sodium nitrite

Appendix 16a Table of Plasma Nitrite Concentrations

Plasma Nitrite Concentration as observed after intravenous administration of $NaNO_2$ to adult volunteers

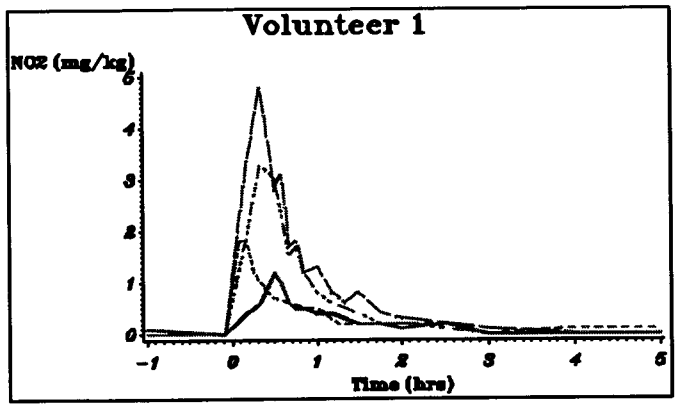
TREATMENT	1						2					
VOLNTEER	1		2		3		1		2		3	
Scheme Time (hr)	Actual Time (hr)	Nitrite (mg/kg)	Actual Time (hr)	Nitrite (mg/kg)	Actual Time (hr)	Nitrite (mg/kg)	Actual Time (hr)	Nitrite (mg/kg)	Actual Time (hr)	Nitrite (mg/kg)	Actual Time (hr)	Nitrite (mg/kg)
8.00	-0.93	0.1	-1.32	<0.1	-1.38	0.1	-0.67	0.1	-0.95	<0.1	-0.97	<0.1
8.55	-0.07	<0.1	-0.32	<0.1	-0.05	<0.1	-0.03	<0.1	-0.08	<0.1	-0.12	<0.1
9.05	0.08	1.8	0.08	1.9	0.27	0.2	0.08	0.4	0.17	0.9	0.17	0.8
9.10	0.17	1.8	0.17	2.8	0.35	1.4	0.33	0.6	0.33	0.8	0.33	0.8
9.15	0.25	1.2	0.25	1.7	0.43	1.2	0.50	1.2	0.52	1.3	0.48	0.9
9.20	0.33	1.0	0.33	1.2	0.52	0.8	0.58	1.0	0.58	1.2	0.58	0.8
9.25	0.42	0.8	0.42	1.2	0.60	0.8	0.67	0.6	0.67	1.0	0.67	0.7
9.30	0.47	0.7	0.50	1.0	0.68	0.6	0.75	0.5	0.75	0.7	0.75	0.7
9.40	0.67	0.6	0.67	0.8	0.85	0.5	0.92	0.5	0.83	0.7	0.83	0.5
9.50	0.83	0.5	0.83	0.6	1.02	0.6	1.00	0.4	1.00	0.5	1.00	0.4
10.00	1.00	0.5	1.00	0.5	1.18	0.3	1.17	0.4	1.17	0.4	1.17	0.4
10.15	1.25	0.2	1.25	0.3	1.45	0.3	1.33	0.3	1.33	0.2	1.33	0.2
10.30	1.52	0.2	1.50	0.2	1.68	0.2	1.50	0.2	1.50	0.2	1.50	0.2
10.45	1.75	0.2	1.75	0.2	1.93	0.2	1.78	0.2	1.75	0.1	1.75	0.2
11.00	2.00	0.2	2.05	0.1	2.18	0.2	2.02	0.1	2.00	0.2	2.00	0.1
11.30	2.50	0.2	2.50	0.2	2.68	0.1	2.50	0.2	2.50	<0.1	2.50	0.1
12.00	3.00	<0.1	2.98	0.1	3.18	0.1	3.03	<0.1	3.00	<0.1	3.00	<0.1
13.00	4.00	0.1	4.00	0.1	4.23	<0.1	4.00	<0.1	4.00	<0.1	4.00	<0.1
14.00	5.08	0.1	5.00	<0.1	5.25	<0.1	5.03	<0.1	5.00	<0.1	5.00	<0.1
15.00	6.02	0.1	5.97	<0.1	6.18	0.1	6.00	<0.1	6.00	<0.1	6.00	<0.1
17.00	7.98	0.1	8.00	0.1	8.18	<0.1	8.00	0.1	8.00	<0.1	8.00	<0.1
19.00	9.98	<0.1	9.93	<0.1	10.12	<0.1	10.00	<0.1	10.00	<0.1	9.88	<0.1
24.00	22.55	0.1	23.82	0.1	22.78	<0.1	23.67	<0.1	24.05	<0.1	23.72	<0.1

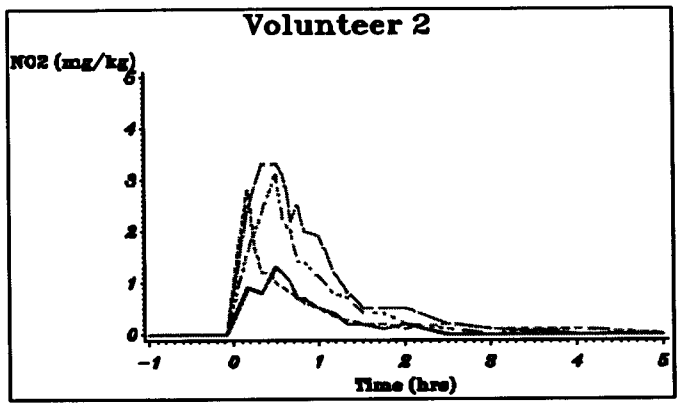
TREATMENT	3						4					
VOLUNTEER	1		2		3		1		2		3	
Scheme Time (hr)	Actual Time (hr)	Nitrite (mg/kg)	Actual Time (hr)	Nitrite (mg/kg)	Actual Time (hr)	Nitrite (mg/kg)	Actual Time (hr)	Nitrite (mg/kg)	Actual Time (hr)	Nitrite (mg/kg)	Actual Time (hr)	Nitrite (mg/kg)
8.00	-0.50	<0.1	-0.98	<0.1	-0.72	<u>≤0.1</u>	-0.77	<0.1	-0.92	<0.1	-1.07	<0.1
8.55	-0.08	<0.1	-0.33	<0.1	-0.10	<0.1	-0.07	<0.1	-0.25	<0.1	-0.10	<0.1
9.10	0.17	1.9	0.17	1.6	0.17	1.3	0.17	3.3	0.17	2.4	0.17	2.1
9.20	0.33	3.3	0.33	2.3	0.33	2.0	0.33	4.8	0.33	3.3	0.33	2.4
9.30	0.52	3.0	0.50	3.1	0.52	2.1	0.50	2.8	0.50	3.3	0.52	4.1
9.35	0.58	2.3	0.58	2.2	0.58	1.8	0.58	3.1	0.57	3.1	0.58	3.0
9.40	0.67	1.5	0.67	2.0	0.67	1.5	0.67	1.7	0.67	2.2	0.67	3.0
9.45	0.75	1.7	0.75	1.4	0.75	1.5	0.73	1.8	0.75	2.5	0.75	2.4
9.50	0.83	1.2	0.83	1.4	0.83	1.1	0.83	1.2	0.83	2.0	0.83	1.8
10.00	1.00	0.8	1.02	1.1	0.98	<u>0.9</u>	1.00	1.3	1.00	1.9	1.00	1.8
10.10	1.17	0.6	1.17	0.8	1.17	0.9	1.17	0.8	1.17	1.2	1.17	1.1
10.20	1.33	0.5	1.33	0.7	1.35	0.8	1.33	0.6	1.33	0.8	1.33	1.0
10.30	1.50	0.4	1.50	0.4	1.50	0.4	1.50	0.8	1.53	0.5	1.53	0.7
10.45	1.75	0.2	1.77	0.4	1.75	0.5	1.75	0.4	1.75	0.5	1.75	0.5
11.00	2.00	0.2	2.08	0.2	2.00	0.2	2.00	0.3	2.00	0.5	2.00	0.6
11.30	2.50	0.1	2.50	0.1	2.50	0.1	2.53	0.2	2.50	0.2	2.52	0.2
12.00	3.00	<0.1	3.00	<0.1	3.00	<0.1	3.00	0.1	3.00	0.1	3.10	0.1
13.00	4.00	<0.1	4.00	0.1	4.00	<0.1	4.00	<0.1	4.00	<0.1	4.00	<0.1
14.00	4.98	<0.1	5.00	<0.1	5.03	<0.1	5.00	<0.1	5.00	<0.1	5.00	<0.1
15.00	6.02	<0.1	6.00	<0.1	6.00	<0.1	5.97	<0.1	6.00	<0.1	6.02	<0.1
17.00	8.00	<0.1	7.98	<0.1	8.03	<0.1	8.00	<0.1	8.05	<0.1	8.00	<0.1
19.00	10.00	<0.1	9.97	<0.1	9.92	<0.1	9.98	<0.1	9.95	<0.1	9.97	<0.1
24.00	23.08	<0.1	24.17	<0.1	23.42	<0.1	23.85	<0.1	24.13	<0.1	24.02	<0.1

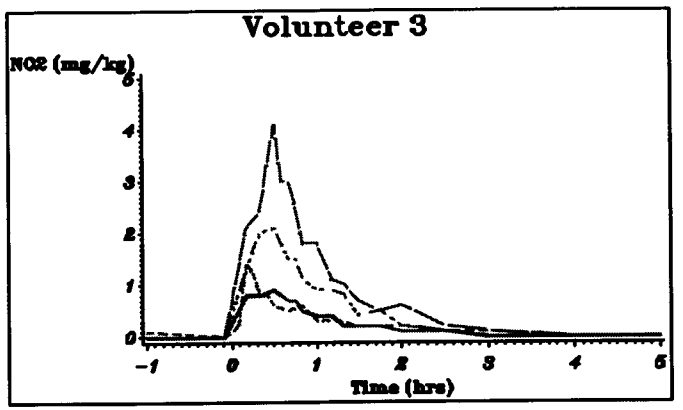
underline = hemolytic plasma sample

Appendix 16 b ***Plasma Nitrite Concentration as observed after intravenous administration of sodium nitrite to adult volunteers***

---------- =0.04 mmol $NaNO_2$ per mmol Hb infused over 10 minutes
———— = 0.04 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
— --- — = 0.08 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
—— —— = 0.12 mmol $NaNO_2$ per mmol Hb infused over 30 minutes







Appendix 16 c ***Table fo Kinetic Parameters derived from the Plasma Nitrite Concentration***

Treatment 1 = 0.04 mmol $NaNO_2$ per mmol Hb infused over 10 minutes
Treatment 2 = 0.04 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
Treatment 3 = 0.08 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
Treatment 4 = 0.12 mmol $NaNO_2$ per mmol Hb infused over 30 minutes

	Treatment 1								
Volunteer	Nitrite dose (mg)	C-max (mg/kg)	C-max adjusted*	t-max (hrs)	t-1/2 (hrs)	AUC(0-∞) (mg*hr/l)	Extrapolation AUC (%)	AUC(0-∞) adjusted*	Vz (L)
1	130	1.8	3.0	0.08	0.75	1.402	38	2.373	100
2	120	2.8	5.1	0.17	0.44	1.427	13	2.616	53
3	90	1.4	3.4	0.35	0.62	1.013	26	2.476	79

	Treatment 2								
Volunteer	Nitrite dose (mg)	C-max (mg/kg)	C-max adjusted*	t-max (hrs)	t-1/2 (hrs)	AUC(0-∞) (mg*hr/l)	Extrapolation AUC (%)	AUC(0-∞) adjusted*	Vz (L)
1	130	1.2	2.0	0.50	0.80	1.091	32	1.846	138
2	120	1.3	2.4	0.52	0.53	1.193	26	2.187	77
3	90	0.9	2.2	0.48	0.56	1.049	31	2.564	70

	Treatment 3								
Volunteer	nitrite dose (mg)	C-max (mg/kg)	C-max adjusted*	t-max (hrs)	t-1/2 (hrs)	AUC(0-∞) (mg*hr/l)	Extrapolation AUC (%)	AUC(0-∞) adjusted*	Vz (L)
1	250	3.3	2.9	0.33	0.51	2.497	12	2.197	73
2	220	3.1	3.1	0.50	0.48	2.536	11	2.536	60
3	190	2.1	2.4	0.52	0.68	2.423	20	2.806	77

	Treatment 4								
Volunteer	Nitrite dose (mg)	C-max (mg/kg)	C-max adjusted*	t-max (hrs)	t-1/2 (hrs)	AUC(0-∞) (mg*hr/l)	Extrapolation AUC (%)	AUC(0-∞) adjusted*	Vz (L)
1	370	4.8	2.9	0.33	0.52	3.366	7	2.001	83
2	320	3.3	2.3	0.33	0.51	3.567	10	2.452	66
3	290	4.1	3.1	0.52	0.60	3.705	14	2.811	68

* = results adjusted to a standard dose of 220 mg sodium nitrite

Appendix 17a Table of Plasma Nitrate Concentrations

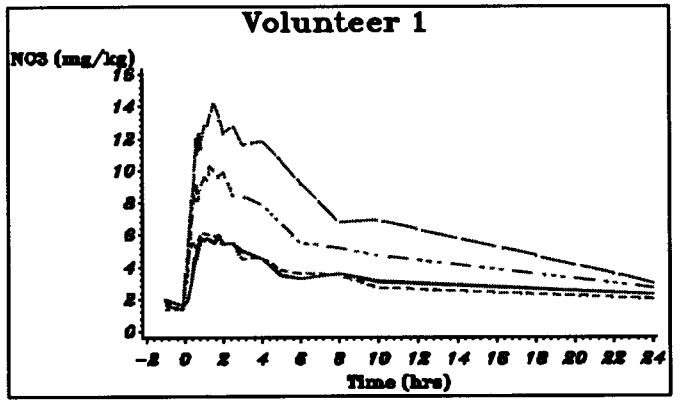
Plasma Nitrate Concentration as observed after intravenous administration of $NaNO_2$ to adult volunteers

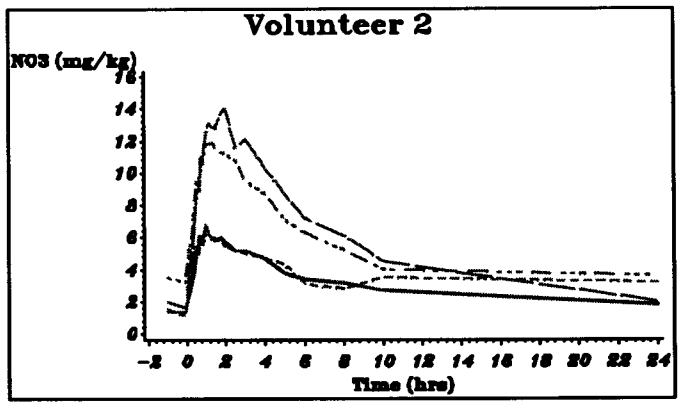
TREATMENT			1				2			
VOLUNTEER	1		2		3		1		2		3	
Scheme Time (hr)	Actual Time (hr)	Nitrate (mg/kg)	Actual Time (hr)	Nitrate (mg/kg)	Actual Time (hr)	Nitrate (mg/kg)	Actual Time (hr)	Nitrate (mg/kg)	Actual Time (hr)	Nitrate (mg/kg)	Actual Time (hr)	Nitrate (mg/kg)
8.00	-0.93	<2	-1.32	<2	-1.38	<2	-0.67	2.0	-0.95	2.0	-0.97	<2
8.55	-0.07	<2	-0.32	<2	-0.05	2.2	-0.03	<2	-0.08	<2	-0.12	<2
9.05	0.08	3.5	0.08	3.3	0.27	<2	0.08	<2	0.17	2.6	0.17	2.3
9.10	0.17	5.0	0.17	4.8	0.35	3.3	0.33	2.9	0.33	3.8	0.33	2.5
9.15	0.25	5.1	0.25	4.4	0.43	2.9	0.50	4.6	0.52	5.2	0.48	3.4
9.20	0.33	5.2	0.33	5.0	0.52	3.5	0.58	4.5	0.58	5.0	0.58	3.8
9.25	0.42	5.5	0.42	5.4	0.60	3.5	0.67	5.1	0.67	6.1	0.67	4.2
9.30	0.47	5.3	0.50	5.4	0.68	3.4	0.75	5.4	0.75	5.7	0.75	4.7
9.40	0.67	5.3	0.67	5.6	0.85	3.7	0.92	5.8	0.83	6.0	0.83	4.8
9.50	0.83	6.1	0.83	5.6	1.02	4.6	1.00	5.7	1.00	6.8	1.00	4.9
10.00	1.00	6.1	1.00	6.2	1.18	4.3	1.17	5.8	1.17	6.2	1.17	4.9
10.15	1.25	6.0	1.25	6.2	1.45	4.4	1.33	5.7	1.33	5.9	1.33	4.7
10.30	1.52	6.0	1.50	5.9	1.68	4.3	1.50	5.5	1.50	5.8	1.50	5.2
10.45	1.75	5.7	1.75	5.8	1.93	3.9	1.78	6.0	1.75	6.0	1.75	4.9
11.00	2.00	5.4	2.05	5.4	2.18	3.9	2.02	5.5	2.00	5.7	2.00	4.7
11.30	2.50	5.5	2.50	5.2	2.68	4.0	2.50	5.5	2.50	5.2	2.50	4.7
12.00	3.00	4.5	2.98	5.0	3.18	3.9	3.03	5.0	3.00	5.2	3.00	4.1
13.00	4.00	4.6	4.00	4.7	4.23	3.8	4.00	4.5	4.00	4.7	4.00	4.7
14.00	5.08	3.8	5.00	4.3	5.25	3.1	5.03	3.5	5.00	3.8	5.00	3.7
15.00	6.02	3.6	5.97	3.1	6.18	3.1	6.00	3.3	6.00	3.4	6.00	3.1
17.00	7.98	3.5	8.00	2.8	8.18	3.2	8.00	3.6	8.00	3.2	8.00	3.5
19.00	9.98	2.7	9.93	3.5	10.12	2.7	10.00	3.1	10.00	2.7	9.88	3.2
24.00	22.55	<2	23.82	3.2	22.78	3.5	23.67	2.3	24.05	<2	23.72	9.8

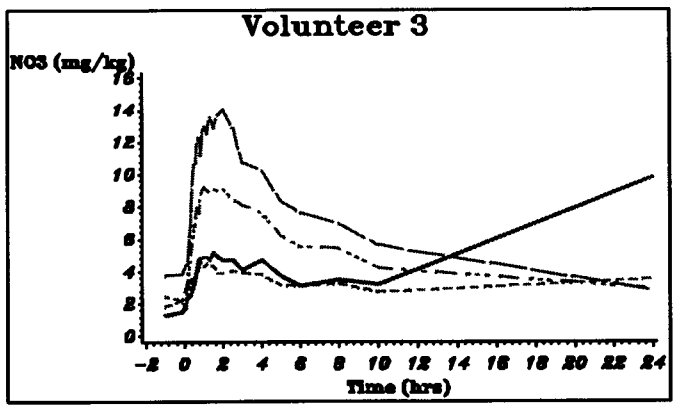
TREATMENT			3				4			
VOLUNTEER	1		2		3		1		2		3	
Scheme Time (hr)	Actual Time (hr)	Nitrate (mg/kg)	Actual Time (hr)	Nitrate (mg/kg)	Actual Time (hr)	Nitrate (mg/kg)	Actual Time (hr)	Nitrate (mg/kg)	Actual Time (hr)	Nitrate (mg/kg)	Actual Time (hr)	Nitrate (mg/kg)
8.00	-0.50	<2	-0.98	3.5	-0.72	2.4	-0.77	<2	-0.92	<2	-1.07	3.7
8.55	-0.08	<2	-0.33	3.2	-0.10	2.2	-0.07	<2	-0.25	<2	-0.10	3.8
9.10	0.17	3.8	0.17	5.6	0.17	3.4	0.17	4.8	0.17	3.7	0.17	4.6
9.20	0.33	6.4	0.33	6.8	0.33	4.9	0.33	7.6	0.33	5.7	0.33	7.0
9.30	0.52	8.6	0.50	9.5	0.52	6.6	0.50	9.6	0.50	8.4	0.52	10.8
9.35	0.58	9.1	0.58	9.3	0.58	7.0	0.58	12.0	0.57	9.0	0.58	10.7
9.40	0.67	8.1	0.67	9.9	0.67	7.9	0.67	11.0	0.67	8.9	0.67	11.9
9.45	0.75	8.7	0.75	11.0	0.75	7.5	0.73	12.3	0.75	10.2	0.75	12.3
9.50	0.83	9.1	0.83	10.6	0.83	8.6	0.83	11.4	0.83	11.3	0.83	11.2
10.00	1.00	9.6	1.02	11.8	0.98	9.2	1.00	12.8	1.00	12.4	1.00	13.0
10.10	1.17	9.4	1.17	11.8	1.17	9.0	1.17	12.8	1.17	13.1	1.17	12.5
10.20	1.33	10.3	1.33	11.9	1.35	8.9	1.33	13.5	1.33	12.9	1.33	13.6
10.30	1.50	10.0	1.50	11.5	1.50	9.1	1.50	14.2	1.53	12.8	1.53	12.9
10.45	1.75	9.6	1.77	11.3	1.75	9.0	1.75	13.4	1.75	13.6	1.75	13.7
11.00	2.00	9.9	2.08	11.2	2.00	9.1	2.00	12.3	2.00	14.0	2.00	14.0
11.30	2.50	8.5	2.50	10.8	2.50	8.4	2.53	12.8	2.50	11.6	2.52	12.9
12.00	3.00	8.4	3.00	9.5	3.00	8.1	3.00	11.6	3.00	12.1	3.10	10.7
13.00	4.00	7.9	4.00	8.7	4.00	7.6	4.00	11.8	4.00	10.3	4.00	10.3
14.00	4.98	6.7	5.00	7.1	5.03	6.2	5.00	10.6	5.00	8.7	5.00	8.3
15.00	6.02	5.5	6.00	6.3	6.00	5.5	5.97	9.2	6.00	7.2	6.02	7.6
17.00	8.00	5.2	7.98	5.2	8.03	5.4	8.00	6.8	8.05	6.1	8.00	6.9
19.00	10.00	4.7	9.97	4.0	9.92	4.2	9.98	6.9	9.95	4.5	9.97	5.6
24.00	23.08	2.7	24.17	3.6	23.42	2.9	23.85	3.0	24.13	2.0	24.02	2.8

Appendix 17 b ***Plasma Nitrate Concentration as observed after intravenous administration of sodium nitrite to adult volunteers***

---------- = 0.04 mmol $NaNO_2$ per mmol Hb infused over 10 minutes
———— = 0.04 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
— --- — = 0.08 mmol $NaNO_2$ per mmol Hb infused over 30 minutes
—— —— = 0.12 mmol $NaNO_2$ per mmol Hb infused over 30 minutes







Appendix 18 ***Graph of the $AUC_{(0-\infty)}$ and the C_{max} of MetHb versus the administered Sodium Nitrite Dose***

Sodium nitrite dose expressed as an absolute amount in mg and as an amount in mmol per mmol Hb

---------- = volunteer 1
———— = volunteer 2
— --- — = volunteer 3