

ON THE SAFETY OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE (NADH)

THE MAXIMUM TOLERATED DOSE (MTD) IN DOGS IS 500 MG PER KG*

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Running title: Maximum tolerated Dose (MTD) of NADH

Abstract

At dose levels of between 100 and 1000 mg/Kg/day changes that identified the cardiovascular system as a target system were observed. There was also some evidence of an effect in the central nervous system and the adrenals. At doses of 500 mg/Kg/day and above food consumption and body weight were reduced. On the basis of the observed changes, the maximum intravenous dose of NADH that was tolerated by beagle dogs was considered to be 500 mg/Kg/day. The MTD for a 70 kg heavy human individual would be 35000 mg or 35 grams. Based on this remarkably high dose NADH can generally be-regarded as safe (GRAS).

Summary:

The objective of the study was to determine the maximum tolerated intravenous dose (MTD) of RNADH (reduced form of nicotinamide adenine dinucleotide) in beagle dogs and then to evaluate the potential toxicity of this dose level in these animals for 14 days. Two male and two female dogs received 100 mg NADH/kg/day, for 4 days followed by 200 mg NADH/kg/day, for 3 days followed by 500 mg NADH/kg/day, for 4 days and on the final day 1000 mg NADH/kg/day.

At the end of the MTD phase the control animals which have received saline solution in the MTD phase were used for the fixed dose phase in which they received 500 mg NADH/kg/day for 14 days. There were no deaths.

Introduction:

The objective of the study was to determine the maximum tolerated intravenous dose/MTD of the test article NADH in beagle dogs, and then to evaluate the toxicity of this dose level in naive beagles for 14 days. The route of administration was chosen because the intravenous route is a possible human therapeutic route. Dogs were selected as they are one of the non-rodent species recommended by various regulatory authorities.

Materials and Methods

1. Test and control articles

The test article, NADH, was a white powdered substance. When not in use, NADH was stored at about 4°C, protected from light and moisture prior to solution preparation. The control article and vehicle for the test article was physiological saline. The test and control articles were administered intravenously at a rate of 0.5 ml-/minute, into the cephalic veins, alternating where possible, between left and right.

2. Experimental design and dose levels Maximum Tolerated Dose (MTD) For the MTD Phase, two groups (2 males and 2 females per group) received intravenously either the physiological saline or NADH solutions of varying concentrations (Table 1). The NADH concentrations were based on a prior rat study.

The control group received the saline control for 14 days, while the test group received the intravenous NADH in saline solution at varying doses at different times during the investigation.

Table 1: Study Schedule

Group Number	prescription	Dose Level (mg/Kg/day)	Day of Study	Animals Group	
				Male	Female
1	Control	0.00	1-14	2	2
2	Test	100	1-4	2	2
		200	5-7		
		500	8-11		
		1000	12		
		0.0	13		
		750	14		

3. Fixed Dose Phase

On completion of the MTD Phase, the control beagles, which have received placebo in the MTD phase, were assigned to a Fixed Dose Phase. The animals were treated at 500 mg NADH/Kg/day on the basis of findings from the MTD Phase. A constant dose volume of 5 mg was used for both the IVITD and Fixed Dose Phases of the study. The required volume was adjusted daily during the IVITD Phase and twice weekly during the Fixed Dose Phase, calculated from the most recently recorded individual body weight.

4. Frequency and Duration of Administration

During the MTD Phase, the test article NADH was administered once daily, excluding Day 13 and the day of necropsy. During the Fixed Dose Phase, the test article was administered once daily for a minimum of 14 days, excluding the day of necropsy.

5. Test Article Formulation

Dosing of solutions of the test article was prepared daily for each group. The formulations were stable for two hours. Before dosing, the formulations were stored at ambient temperature, in the dark.

6. Test System

Four male and four female pure-bred beagles were obtained from Hazleton Research Products, Inc. Before delivery, they received a course of treatment at the supplier's premises for endoparasites and were vaccinated against distemper virus, canine infectious hepatitis virus, parvo virus, rabies, Bordetella, parainfluenza virus, *Leptospira canicola*, and *Leptospira icterohaemorrhagiae*. Documentation provided by the supplier included date and birth, litter identification, vaccination dates, and details of treatment given. Shortly after arrival, the beagles were inspected for ill health, re-vaccinated against distemper virus, canine infectious hepatitis virus, parvo virus, Bordetella, parainfluenza virus, *Leptospira canicola*, and *Leptospira icterohaemorrhagiae*, and received oral anthelmintic treatment.

The beagles were held in stock about three weeks before the start of the MTD Phase. Towards the end of this period, their health status was reassessed and their suitability for experimental purposes confirmed.

At the start of treatment, males weighed 7.85 Kg to 12.30 Kg while the females weighed from 8.20 Kg to 9.65 Kg. All were between 9 and 11 months old.

The beagles were housed in a single room, singly during the working day and in pairs of the same sex and group overnight. The room was air-conditioned to provide a minimum of ten air changes per hour and routinely maintained at a temperature of 16°C to 22°C and a relative humidity of 40% to 80%³/₀. Fluorescent lighting was controlled automatically to give a cycle of 12 hours light (0700 to 1900 h) and 12 hours darkness.

Daily throughout this investigation, each beagle was offered 400 g of SQC Laboratory Diet A, Expanded (Special Diets Services Ltd. Witham). Any uneaten diet was removed and weighed during each afternoon, then discarded. Filtered drinking water was available ad libitum from an automatic watering system. The diet and water were considered not to have contained any contaminant at a level which might have affected the integrity or outcome of the study. The beagles were arbitrarily assigned to treatment groups. After allocation to a treatment group, each beagle was individually identified by subcutaneous electronic implant. A color-coded card on each kennel gave information including study number and animal number.

7. Experimental Observations

All beagles were observed in the morning, before feeding and again in the afternoon. In addition, all beagles were given a detailed clinical examination at weekly intervals. An individual record of the condition of each beagle was maintained. Additional observations were made throughout the working day as necessary, particularly between two and four hours after dosing during the MTD Phase. On days when the dose level was increased and Day 1 of the Fixed Dose Phase, the beagles were also observed between 8 and 12 hours post dosing.

Individual body weights were recorded once weekly during the pre-dose period. Body weights were recorded daily during the MTD Phase and twice weekly during the Fixed Dose Phase. The beagles were also weighed before necropsy.

Individual food consumption was determined daily throughout the study by subtracting the amount of food left or discarded from the quantity offered. Blood pressure recordings (diastolic, systolic, and mean arterial) were made from all beagles before dosing, and 10 minutes, and 1, 2, and 4 hours after dosing on Day 1 of the Fixed Dose Phase. Blood pressure was measured by cannulation of the ear artery. On Day 1 of the Fixed Dose Phase, the heart rate of each beagle was determined from the blood pressure traces.

8. Laboratory Investigations

Blood samples were obtained from all beagles pre-dose and at the end of both the MTD and Fixed Dose Phases. Blood samples were collected from the jugular vein after an overnight fast. Samples taken during the treatment periods were collected before daily dosing.

(a) Hematology

Blood was collected into EDTA anticoagulant and the following parameters were measured:

- Hemoglobin Concentration
- Mean Cell Volume
- Red Blood Cell Count and Indices (including Mean Cell Hemoglobin, Mean Cell Hemoglobin Concentration, Packed Cell Volume)
- Total and Differential White Blood Cell Count
- Platelet Count.

Further blood samples were collected into 3.13% trisodium citrate anticoagulant and prothrombin and activated partial thromboplastin times measured according to Babson and Babson (1974).

(b) Clinical Chemist

Blood was collected into lithium heparin anticoagulant and the following parameters were measured:

- Aspartate Aminotransferase (Kommissionen for Enzymdiagnostic und Standardisierung, 1972)
- Alanine Aminotransferase (Kommissionen for Enzymdiagnostic und Standardisierung, 1972)
- Gamma Glutamyl Transferase (Szasz et al., 1974)
- Alkaline Phosphatase (Kommissionen for Enzymdiagnostic und Standardisierung, 1972)
- Potassium (Eisenmann et al., 1957)
- Glucose (Schmidt, 1981)
- Total Bilirubin (Pearlman et al., 1974)
- Total Protein (Spencer et al., 1977)
- Albumin/Globulin Ratio
- Calcium (Moorehead et al., 1974)
- Urea (Gutmann et al., 1974)
- Creatinine (Jaffb, 1886)
- Albumin (Spencer et al., 1977)
- Total Cholesterol (Allain et al., 1974).

9. Pathology

The beagles were killed by an intravenous injection of sodium thiopentone, following an overnight period without food. The beagles were exsanguinated, a full internal and external examination was made under the general supervision of a pathologist, and all lesions were recorded. The necropsies for each phase of the study were carried out on a single day.

(a) Organ Weights

The following organs were dissected free from fat and other contiguous tissue and weighed before fixation:

- Adrenals
- Brain (including Brain Stem)
- Heart
- Kidneys
- Liver
- Lungs
- Ovaries
- Pancreas
- Pituitary

- Prostate
- Spleen
- Testes and Epididymides
- Thymus
- Thyroids
- Uterus.

(b) Histology

Samples of the following tissues were fixed in 10% neutral buffered formalin, with the exception of the eyes and optic nerves which were fixed in Davidson's Fluid:

- Adrenals
- Aorta
- Brain (including Brain Stem)
- Caecum
- Colon
- Duodenum
- Epididymides
- Eyes (With Optic Nerves)
- Femur
- Gall Bladder
- Heart
- Ileum
- Injection Sites
- Jejunum
- Kidneys
- Lachrymal Glands
- Liver
- Lungs (with Mainstem Bronchi)
- Lymph Nodes (Mandibular and Mesenteric)
- Esophagus
- Ovaries
- All Gross Lesions
- Pancreas
- Pituitary
- Prostate
- Rectum (with Anus)
- Salivary Gland (Submandibular)
- Sciatic Nerves
- Skeletal Muscle (Quadriceps)
- Skin and Mammary Gland
- Spinal Cord (Lumbar, Cervical, Thoracic)
- Spleen
- Sternum (with Bone Marrow)
- Stomach
- Testes
- Thymus
- Thyroids (with Parathyroids)
- Tongue
- Trachea
- Urinary Bladder

- Uterus (Corpus and Cervix)
- Vagina.

Tissues specified above from all fixed dose beagles were embedded in paraffin wax BP, sectioned at a nominal thickness of 5 µm, stained with hematoxylin and eosin, and evaluated using light microscopy.

Results

1. Mortality and Clinical Observations

All Beagles survived the phases of the study to which they were assigned.

2. Maximum Tolerated Dose/MTD Phase

During dosing at all dose levels administered during the MTD Phase the treated Beagles frequently became subdued, and the gums often became pale. At dose levels of 200 mg/Kg/day and greater, the Beagles frequently had tremors immediately after dosing and occasionally during dosing. From Day 10 onwards, in Beagles receiving 500 mg/Kg/day and greater, the pads of the feet became pale after dosing, and those of the males were also cold to touch.

During dosing at 750 and 1000 mg/Kg/day the beagles often had warm ears and a dry nose. After dosing the ears were cold and the beagles were described as being restless and having an arched back or hunched posture. The respiratory rate of female number 4088 increased during dosing at both levels. The respiratory rate of both males increased after dosing at 1000 mg/Kg/day. However, after dosing at 750 mg/Kg/day this was only exhibited by one animal. The beagles also had an awkward gait, ataxia and were unsteady on their feet after dosing at these levels. All treated beagles vomited during the MTD Phase, but the incidence showed no clear relationship with dose. Other signs exhibited by treated beagles included salivation, retching, lip licking, soft or mucoid feces which were often pale or yellow, red eyes, and the appearance of the third eyelid during dosing.

On several occasions, one male (number 4084) was clearly agitated and vocalized during dosing. On Days 3 and 4 the infusion was suspended for a short time during which the signs regressed, but once the infusion was continued similar signs were again exhibited. On Days 5 and 6 the beagle was not dosed, but on Day 7 the beagle was returned to dose at 200 mg/Kg/day.

3. Fixed Dose Phase

The clinical signs observed during the Fixed Dose Phase were similar to those seen during the MTD Phase.

Treated beagles became subdued, and had pale gums, cold (and less frequently warm) ears, blood shot eyes, dry nose and increased respiratory rate during dose administration.' After dosing all beagles had tremors and the pads of their feet were cold. All beagles except one male vocalized and occasionally appeared agitated during dosing.

Other signs seen included salivation, lip licking, red eyes, awkward gait, increased heart rate, panting and the appearance of the third eyelid during dosing. Only one female (number 4087) vomited during the Fixed Dose Phase.

There were no other clinical observations that were associated with the administration of the test article.

4. Body Weigh

During-the MTD Phase, at dose levels of up to 200 mg/Kg/day there was little change in the body weight of treated beagles, but at doses of 500 mg/Kg/day and greater, the weight of these beagles decreased. On Day 13, the day after the administration of 1000 mg/Kg/day, the body weights of the males were 4 and 6% lower and the weights of the females were 6 and 8% lower than before treatment started on Day 1. The body weights of all controls on Day 14 were within 2% of their weights on Day 1. On Day 14 of the Fixed Dose Phase the weight of female number 4086 was 3% lower and the weight of female number 4087 was 6% lower than on Day 1. The weights of both males were slightly lower (2%) on Day 14 than on Day -1.

5. Food Consumption

In the MTD Phase, at dose levels of 500 mg/Kg/day and greater, the food consumption of treated beagles tended to be slightly low compared with pre-dose and control values. Neither of the males ate more than 260 g/day during treatment at these levels, whereas pretreatment both beagles ate up to 400 g/day. Similarly, during the same period females ate up to 210 g/day, compared with up to 400 g for number 4085 and 370 g for #4088 pre-treatment. At lower dose levels the food intake was similar to that pre-treatment.

Throughout the Fixed Dose Phase, the food consumption of one male (number 4081) and both females was generally lower than pre-dose. Prior to treatment the male frequently consumed all of the food offered (400 g) and the females ate up to 370 or 380g.

The only occasions during treatment when consumption approached similar levels was when the food was left available to the beagles overnight to encourage eating. In Week 2 of the Fixed Dose Phase the amount eaten by male number 4082 also decreased. Previously, this beagle almost consistently ate all 400 g offered, but in Week 2 consumption became as low as 250 g/day.

6. Blood Pressure

One hour after dosing on Day 1 of the Fixed Dose Phase, the systolic and mean arterial blood pressure was lower than before dosing in all beagles except female (#4086) for which the values were similar to those before dosing. However, there was no consistent pattern of change in blood pressure at the other time points at which measurements were taken.

7. Heart Rate

On Day 1 of the Fixed Dose Phase the heart rate~ of all beagles except one male (#4082) were higher at all time points that measurements were taken after dosing when compared with the predose rates. The heart rate of dog #4082 generally remained similar to the pre-treatment rate.

8. Laboratory Investigations

(a) Hematology

On Day 15 of the MTD Phase, the treated beagles showed changes in a number of the hematology parameters measured when compared with the pre-dose values. However, similar changes were generally apparent in controls, suggesting that the changes were not associated with treatment.

On Day 14 of the Fixed Dose Phase, the hemoglobin concentration, red blood cell count and packed cell column in male #4081 and female #4086 were high, but the mean cell hemoglobin and mean cell hemoglobin concentration were low compared with pre-dose and Day 15 of the MTD Phase values.

Similar changes were evident in female number 4087 except for the hemoglobin concentration which was higher than at the end of the MTD Phase but lower than predose.

The other parameters measured were considered to be unaffected by treatment.

(b) Clinical Chemistry

On Day 15 of the MTD Phase the activities of alkaline phosphatase in all treated beagles and alanine aminotransferase in one female (#4085) were higher than pretreatment anIJ. control values. The creatinine concentrations in one male (#4085) and both treated females, and the cholesterol concentrations in all treated beagles were also high compared with pre-treatment and control values. Other changes apparent in treated beagles were generally also evident in the controls.

Pre-dose: the activities of aspartate aminotransferase and alanine aminotransferase in beagle #4087 were unusually high. The beagle was re-bled before dosing on Day I of the study and both parameters were considered to be within the ranges normally expected.

On Day 14 of the Fixed Dose Phase the plasma calcium concentration in all beagles was high compared with pre-dose and Day 15 of the MTD Phase values. The activity of alkaline phosphatase in one female (#4086) was higher than on both previous sampling occasions, and the activity of this enzyme in one female (#4087) was also higher than on Day 15 of the MTD Phase, but lower than pre-treatment.

The total protein and cholesterol concentrations in all beagles were higher, but the total bilirubin concentration in both females was lower than pre-dose and Day 15 of the MTD Phase.

9. Pathology

(a) Organ Weights

At termination of the MTD Phase, the relative adrenal weights of the beagles were generally higher than normally expected for beagles of this age and strain at this laboratory." Two beagles (male # 4083 and female #4088) had relative brain and lung weights which were considered to be slightly higher than normally found in beagles of the same age and strain. The relative heart weight of both females was also considered to be high.

At termination of the Fixed Dose Phase of the study, all beagles were also found to have relative adrenal weights higher than normally expected. The relative lung and heart weights of one male (#4081) and the relative lung weight of one female (#4087) were considered to be high. The weights of the other organs were considered to be unaffected by treatment.

(b) Macroscopic Pathology

Most tissues were unremarkable at the necropsies of both maximum tolerated dose (MTD) and Fixed Dose Phase beagles.

Macroscopic observations at injection sites were consistent with those expected with repeated venepuncture. All other findings were consistent with the expected pattern of background observations in dogs of this strain and age.

(c) Microscopic Pathology

The majority of microscopic findings were infrequent, of a minor nature, and consistent with the expected background pathology in dogs of this strain and age and were not considered to be related to test article administration. The only unusual microscopic findings were a mixed, mainly mononuclear, inflammatory cell perivascular cuffing of blood vessels in the medulla oblongata of the brain in all beagles together with a focus of inflammatory cells in the thalamus of one female.

The microscopic findings at injection sites (low grade dermatitis, phlebitis/periphlebitis and subcutaneous hemorrhage) were considered to be consistent with the physical procedure of repeat intravenous injections with no evidence of local irritation or other toxicity of the test article.

Discussion

The intravenous administration of NADH to beagle dogs at dose levels of between 100 and 1000 mg/Kg/day resulted in a number of findings that demonstrated an effect on the cardiovascular system. Clinically these included pale gums, pale and cold pads of the feet, bloodshot eyes, and changes in the temperature of the ears. The subdued behavior of the beagles during dosing may also have been associated with cardiovascular changes. Data from the Fixed Dose Phase showed that blood pressure was reduced 1 hour after the administration of 500 mg/Kg/day and there was an increase in heart rate for at least four hours after dosing.

The heart weights of both MTD Phase females and one Fixed Dose Phase male were higher than normally expected. Although this may have been an indication of an adaptive response to increased workload, there were no histopathological findings in the Fixed Dose Phase male to support this.

An increase in adrenal weight was evident in beagles from both phases of the study. Changes in the adrenals could be related to the increase in heart rate or may have been a response to stress, but no histopathological changes in the adrenals were found.

There was also evidence of an effect on the Central Nervous System. At dose levels of 200 mg/Kg/day and above the beagles had tremors after dosing and the relative brain weights of two MTD Phase beagles were considered to be higher than normally expected. Microscopic findings in Fixed Dose Phase beagles included an inflammatory cell perivascular cuffing of blood vessels in the medulla oblongata of the brain. However, these histopathological findings could not be unequivocally attributed to treatment.

Treatment with NADH at dose levels of 500 mg/Kg/day and higher resulted in a reduction in food intake, with a corresponding decrease in body weight.

There were apparent changes in the activities of alkaline phosphatase and alanine aminotransferase, and in the plasma concentrations of creatinine, cholesterol, calcium, total protein, and total bilirubin, but the significance of these changes was unclear.

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It was performed in accordance under the UK Principles of Good Laboratory Practice

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