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INITIAL SUBMISSION: NINOX HCDO - TWENTY-EIGHT DAY REPEATED DOSE ORAL (GAVAGE) TOXICITY STUDY IN THE RAT, WITH COVER LETTER DATED 10/20/2000		
Chemical Category		
HYDROGENATED COCOAMIDOPROPYLDIETHYLAMINE-N-OXIDE		

A 03 Stepan

Stepan Company

Northfield, Illinois 60093
Telephone 847 446 7500

8E HQ-1000-14804



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October 20, 2000

Document Processing Center (TS-790)
Office of Pollution and Toxic Substances
U.S. Environmental Protection Agency
401 M Street, SW
Washington, DC 20460-0001
Attn: Section 8(e) Coordinator

MR 40528



88010000016

Subject: Notice of Substantial Risk under TSCA Section 8(e)
EPA Document Control No.: None

Dear Sir or Madam:

The listed study is being submitted to the U.S. EPA in accordance with Section 8(e) of Toxic Substances Control Act (TSCA) by:

Stepan Company
22 West Frontage Road
Northfield, IL 60093
Telephone: 847/446-7500

Study Title: Twenty-eight day repeated does oral (gavage) toxicity study in the rat.

Stepan Document No.: 99-009F

Product tested (chemical name and the CAS number is known): Hydrogenated
cocoamidopropyl diethylamine-N-oxide (CAS No. not available).

Summary of the reportable adverse effect(s): The twenty eight day repeated dose study in rats was carried out with the above material at three different doses: 1000 mg/kg/day, 150 mg/kg/day and 15 mg/kg/day. The most significant changes occurred at 1000 mg/kg/day. Most observations seen were consistent with those commonly associated with oral administration of irritant materials such as noisy respiration, increased salivation, hunched posture and tiptoe gate (often associated with gastric irritation). The enhanced startle reflex may simply reflect a heightened response due to the underlying, painful gastric condition. The gastric irritation was supported histologically. Animals in this group showed marked increase in water consumption, however, there was no evidence of any effect on kidney function. Histopathological examination of the urinary bladder demonstrated hyperplasia of the transitional cell epithelium, suggesting urinary excretion of an irritant substance.

No adverse effects were demonstrated in animals of either sex treated with 15 mg/kg/day and the "No observed Effect Level" (NOEL) was therefore considered to 15 mg/kg/day. We are submitting 3 copies of draft report since final report has not yet been issued.

Sincerely,

Lela Jovanovich, Ph.D.
Research Toxicologist
Telephone: 847/501-2272

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**NINOX® HCDO:
TWENTY-EIGHT DAY REPEATED DOSE
ORAL (GAVAGE) TOXICITY STUDY IN THE RAT
SPL PROJECT NUMBER: 625/051**

AUTHORS: L J Jones
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QUALITY ASSURANCE REPORT

The conduct of this study has been subjected to periodic inspections by Safepharma Quality Assurance Unit. Inspection findings are reported to Management/Study Directors on the day of inspection in each case.

This report has been audited by Safepharma Quality Assurance Unit. It is considered to be an accurate account of the data generated and of the procedures followed.

Inspection and audit occasions relevant to this study are as follows:

20 September 1999	Protocol Compliance Audit
07 March 2000	Test Material Preparation
08 October 1999, 07 March 2000	Chemical Analysis
04 April 2000	Dosing Procedure
04 April 2000	Clinical Observations
05 October 1999, 07 April 2000	Post Mortem Proceedings
25 September 2000	Draft Report Audit
Date of QA Signature	Final Report Audit

..... DATE:

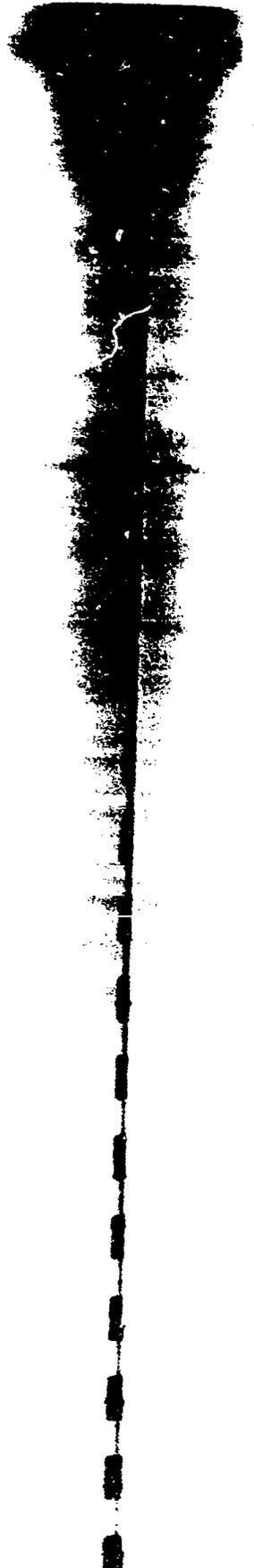
For Safepharma Quality Assurance Unit

*** Authorised QA Signatures:**

Head of Department: JR Pateman CBiol MIBiol DipRQA
 Deputy Head of Department: JM Crowther MiScT
 Senior Audit Staff: JV-Johnson BSc; G Wren ONC; RJ Gilbert BSc

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GLP COMPLIANCE STATEMENT

I, the undersigned, hereby declare that the objectives laid down in the protocol were achieved and as nothing occurred to adversely affect the quality or integrity of the study, I consider the data generated to be valid. This report fully and accurately reflects the procedures used and data generated.

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1997 (SI 1997/654)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (Revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 87/18/EEC and 88/320/EEC.

These international standards are acceptable to the United States Environmental Protection Agency and Food and Drug Administration, and fulfil the requirements of 40 CFR Part 792 and 21 CFR Part 58 (as amended).

..... DATE:

L J Jones BSc (Hons)
Study Director

The following scientific and supervisory personnel were involved in the study under the overall supervision of the Study Director:

- D Harris HNC
- M Trussell HNC
- J Kemp
- N Doleman HNC

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AUTHENTICATION

I, the undersigned, hereby declare that the microscopic pathology data presented in this report were compiled by me and that the results reported herein accurately reflect the data obtained.

..... DATE:

P N Brooks MSc BSc EurBiol CBiol MIBiol
EUROTOX Registered Toxicologist
Study Pathologist

I, the undersigned, hereby declare that the analytical data presented in this report were compiled by me or under my supervision and that the results reported herein accurately reflect the data obtained.

..... DATE:

D Mullee CChem MRSC
Head of Analytical Chemistry

Approved for issue:

..... DATE:

M P Blackwell BSc PhD CBiol MIBiol FIAT
EUROTOX Registered Toxicologist
Director of Mammalian Toxicology

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ORAL (GAVAGE) TOXICITY STUDY
IN THE RAT**

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SPL PROJECT NUMBER: 625/051

PART I

**NINOX® HCDO:
TWENTY-EIGHT DAY REPEATED DOSE
ORAL (GAVAGE) TOXICITY STUDY
IN THE RAT**

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SUMMARY

STUDY SPONSOR : STEPAN COMPANY

STUDY TITLE : TWENTY-EIGHT DAY REPEATED
DOSE ORAL (GAVAGE) TOXICITY
STUDY IN THE RAT

TEST MATERIAL : NINOX® HCDO

1. The study was designed to investigate the systemic toxicity of the test material. It complies with the requirements for notification of a new chemical substance in the EC and follows the testing method described in Commission Directive 96/54/EC (Method B7) and OECD Guidelines for Testing of Chemicals No. 407 "Repeated Dose 28 Day Oral Toxicity Study in Rodents" (Adopted 27 July 1995).

The test material was administered by gavage to three groups, each of five male and five female Sprague-Dawley CrI:CD®BR strain rats, for up to twenty-eight consecutive days, at dose levels of 15, 150 and 1000 mg/kg/day (incorporating a correction factor for 81.47% purity). A control group of five males and five females was dosed with vehicle alone (distilled water).

Clinical signs, functional observations, bodyweight development and food and water consumption were monitored during the study. Haematology and blood chemistry were evaluated for all animals at the end of the study.

All animals were subjected to a gross necropsy examination and histopathological evaluation of selected tissues was performed.

The results are summarised as follows:

2. Mortality

One female treated with 1000 mg/kg/day was found dead on Day 12 of the study.

3. Clinical Observations

Animals treated with 1000 mg/kg/day showed clinically observable signs of toxicity from Day 3 onwards. Signs included fur loss, noisy respiration, increased salivation, red/brown staining of the fur, wet fur and hunched posture. Sporadic signs of diuresis, staining around the ano-genital region, tiptoe gait, gasping respiration and pallor of the extremities were also noted. The female found dead at the start of Day 12 showed a substantial deterioration in condition on Day 11 with additional signs including pilo-erection, respiratory pattern changes, staining around the snout and mouth and dehydration.

No clinically observable signs of toxicity were detected in animals treated with 150 or 15 mg/kg/day throughout the study period.

4. Functional Observations

4.1 Behavioural Assessments

Weekly behavioural assessments supported the clinical observations recorded during the study with incidents of tiptoe gait, hunched posture, noisy respiration and increased salivation being recorded for 1000 mg/kg/day animals.

No such effects were detected at 150 or 15 mg/kg/day.

4.2 Functional Performance Tests

No treatment-related effects were detected.

4.3 Sensory Reactivity Assessments

Males treated with 1000 mg/kg/day showed an enhanced startle reflex compared with controls.

No such changes were detected for 1000 mg/kg/day females or animals of either sex treated with 150 or 15 mg/kg/day.

5. Bodyweight

Males and females treated with 1000 mg/kg/day showed a substantially reduced bodyweight gain during Week 1 of the study only.

No adverse effect on body weight development was detected for animals of either sex from the 150 or 15 mg/kg/day dose groups.

6. Food Consumption

Males and females treated with 1000 mg/kg/day showed reduced food consumption and reduced food efficiency during the first week of the study only. Males continued to show a reduced dietary intake throughout the study period.

No such effects were detected in animals of either sex treated with 150 or 15 mg/kg/day.

7. Water Consumption

Water consumption measurement, initiated on Day 15, revealed a substantial increase for animals of either sex treated with 1000 mg/kg/day, which persisted until the end of the study.

There was no convincing effect on water consumption in animals from the 150 or 15 mg/kg/day dose groups.

8. Haematology

Haematological changes consistent with haemolytic anaemia were detected in animals of either sex treated with 1000 or 150 mg/kg/day. Males and females from the 1000 mg/kg/day dose group also showed a significant elevation in neutrophil numbers together with a slightly elevated prothrombin time.

No such effects were detected at 15 mg/kg/day.

9. Blood Chemistry

Males and females treated with 1000 mg/kg/day showed elevations in plasma aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and bilirubin compared with those of controls.

ASAT and ALAT were also elevated for 150 mg/kg/day animals.

No such effects were detected at 15 mg/kg/day.

10. Organ Weights

Males and females treated with 1000 mg/kg/day showed a statistically significant increase in spleen weight, both absolute and relative to bodyweight, compared with controls. The effect extended to the 150 mg/kg/day dose group although statistical significance was not always achieved.

There were no toxicologically significant organ weight changes detected at 15 mg/kg/day.

11. Necropsy

One female treated with 1000 mg/kg/day showed thickening of the non-glandular gastric epithelium but there were no other macroscopic findings at necropsy that could be considered treatment-related.

12. Histopathology

Treatment-related changes were detected in liver, spleen, kidneys, urinary bladder and stomach. Centrilobular hepatocyte enlargement was detected in three males from the 1000 mg/kg/day dose group and hepatic haemosiderin pigment deposition was noted in both sexes from this dose group and in one female treated with 150 mg/kg/day. Increased severities of splenic extramedullary haemopoiesis and haemosiderin pigment accumulation were detected in animals of either sex treated with 1000 or 150 mg/kg/day. At a dose level of 15 mg/kg/day splenic changes were confined to pigment deposition in females. Haemosiderin deposition was also apparent in the kidneys of males and females treated with 1000 mg/kg/day and hyperplasia of the urinary bladder epithelium was noted in these animals. The non-glandular (forestomach) epithelium of animals from the 1000 mg/kg/day dose group showed acanthosis and hyperkeratosis occasionally with associated subepithelial inflammatory cell infiltrates.

13. Conclusion

Oral administration of the test material, NINOX[®] HCDO, to rats for a period of up to twenty-eight consecutive days at dose levels of up to 1000 mg/kg/day resulted in toxicologically significant effects at dose levels of 1000 and 150 mg/kg/day. No such effects were demonstrated in animals of either sex treated with 15 mg/kg/day and the "No Observed Effect Level" (NOEL) was, therefore, considered to be 15 mg/kg/day.

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**NINOX® HCDO:
TWENTY-EIGHT DAY REPEATED DOSE
ORAL (GAVAGE) TOXICITY STUDY IN THE RAT**

1. INTRODUCTION

The study was performed according to the protocol presented in Appendix XVI and was designed to investigate the systemic toxicity of the test material, by repeated oral administration to the Sprague-Dawley Crl:CD®BR strain rat for a period of up to twenty-eight consecutive days at dose levels of 15, 150 and 1000 mg/kg/day (incorporating a correction factor for 81.47% purity).

The study was designed to comply with the requirements for notification of a new chemical substance in the EC and follows the test method described in Commission Directive 96/54/EC (Method B7) and the OECD Guidelines for Testing of Chemicals No. 407 "Repeated Dose 28 Day Oral Toxicity Study in Rodents" (Adopted 27 July 1995).

The rat was selected for this study as it is a readily available rodent species historically used in safety evaluation studies and is acceptable to appropriate regulatory authorities.

The dose levels were chosen based on the results of the range-finding study presented in Part II of this report. The oral route was selected as the most appropriate route of exposure, based on the physical properties of the test material, and the results of the study are believed to be of value in predicting the likely toxicity of the test material to man.

The study was performed between 17 September 1999 and 30 June 2000.

2. TEST MATERIAL AND EXPERIMENTAL PREPARATION**2.1 Description, Identification and Storage Conditions**

Sponsor's identification	:	NINOX® HCDO
Description	:	off white paste
Batch number	:	876TK
Purity	:	81.47%
Sample number	:	IX
Date received	:	10 May 1999
Storage conditions	:	room temperature in the dark

Data relating to the identity, purity and stability of the test material are the responsibility of the Sponsor.

A Certificate of Purity is included as Appendix XXIII.

2.2 Experimental Preparation

For the purpose of this study the test material was prepared at the appropriate concentrations as a solution in distilled water. The stability and homogeneity of the test material formulations were determined by Safeparm Analytical Laboratory. Results are given in Appendix XVIII and show the formulations to be stable for at least fourteen days. Formulations were therefore prepared weekly and stored at approximately +4°C in the dark.

Samples were taken of each test material formulation and were analysed for concentration of NINOX® HCDO at Safeparm Analytical Laboratory. The method used for analysis of formulations and the results obtained are given in Appendix XVIII. The results indicate that the prepared formulations were within $\pm 10\%$ of the nominal concentration.

3. METHODS

3.1 Animals and Animal Husbandry

A sufficient number of male and female Sprague-Dawley Crl:CD®BR strain rats were obtained from Charles River (UK) Limited, Margate, Kent. On receipt the animals were examined for signs of ill-health or injury. The animals were acclimatised for nine days during which time their health status was assessed. A total of forty animals (twenty males and twenty females) were accepted into the study. At the start of treatment the males weighed 139 to 173g, and the females weighed 127 to 155g, and were approximately five to seven weeks old.

The animals were housed in groups of five by sex in polypropylene grid-floor cages suspended over trays lined with absorbent paper. The animals were allowed free access to food and water. A pelleted diet (Rat and Mouse SQC Expanded Diet No. 1, Special Diets Services Limited, Witham, Essex, UK) was used. Certificates of analysis of the batches of diet are given in Appendix XVII. Mains water was supplied from polycarbonate bottles attached to the cage. The diet and drinking water were considered not to contain any contaminant at a level that might have affected the purpose or integrity of the study.

The animals were housed in a single air-conditioned room within the Safepharm Barrier Maintained Rodent Facility. The rate of air exchange was at least fifteen air changes per hour and the low intensity fluorescent lighting was controlled to give twelve hours continuous light and twelve hours darkness. Environmental conditions were continuously monitored by a computerised system, and print-outs of hourly mean temperatures and humidities were included in the study records. The temperature and relative humidity were controlled to remain within target ranges of $21 \pm 2^{\circ}\text{C}$ and $55 \pm 15\%$ respectively. Occasional deviations from the humidity targets were considered not to have affected the purpose or integrity of the study.

The animals were randomly allocated to treatment groups using random letter tables, and the group mean bodyweights were then determined to ensure similarity between the treatment groups. The cage distribution within the holding rack was also randomised. The animals were uniquely identified within the study by an ear punching system routinely used in these laboratories.

3.2 Procedure

Animals were allocated to treatment groups as follows:

TREATMENT GROUP	DOSE LEVEL OF TEST MATERIAL# (mg/kg/day)	DOSE LEVEL OF ACTIVE INGREDIENT (mg/kg/day)	TREATMENT VOLUME (ml/kg)	CONCENTRATION OF TEST MATERIAL# (mg/ml)	ANIMAL NUMBERS	
					MALE	FEMALE
Control	0	0	5	0	5 (1 - 5)	5 (6 - 10)
Low	18.4	15	5	3.68	5 (11 - 15)	5 (16 - 20)
Intermediate	184	150	5	36.8	5 (21 - 25)	5 (26 - 30)
High	1227	1000	5	245	5 (31 - 35)	5 (36 - 40)

incorporating a correction factor for 81.47% purity

The numbers in parentheses () show the individual animal numbers allocated to each treatment group.

The test material was administered daily, for up to twenty-eight consecutive days, by gavage using a stainless steel cannula attached to a disposable plastic syringe. Control animals were treated in an identical manner with 5 ml/kg/day of distilled water.

The volume of test and control material administered to each animal was based on the most recent bodyweight and was adjusted at weekly intervals.

3.3 Observations

3.3.1 Clinical Observations

All animals were examined for overt signs of toxicity, ill-health or behavioural change immediately before dosing and one and five hours after dosing during the working week. Animals were observed immediately before dosing and one hour after dosing at weekends. All observations were recorded.

3.3.2 Functional Observations

Prior to the start of treatment and on Days 5, 12, 21 and 26 all animals were observed for signs of functional/behavioural toxicity. During Week 4 functional performance tests were also performed on all animals together with an assessment of sensory reactivity to different stimuli. Observations were carried out from approximately two hours after dosing on each occasion.

3.3.2.1 Behavioural Assessments

Detailed individual clinical observations were performed for each animal using a purpose built arena. The following parameters were observed:

Gait	Hyper/Hypothermia
Tremors	Skin colour
Twitches	Respiration
Convulsions	Palpebral closure
Bizarre/Abnormal/Stereotypic behaviour	Urination
Salivation	Defecation
Pilo-erection	Transfer arousal
Exophthalmia	Tail elevation
Lachrymation	

The scoring system used is outlined in Appendix I.

3.3.2.2 Functional Performance Tests**a) Motor Activity**

Twenty purpose-built 44 infra-red beam automated activity monitors were used to assess motor activity. Animals of one sex were tested at each occasion and were randomly allocated to the activity monitors.

The tests were performed at approximately the same time of the day, under similar laboratory conditions. The evaluation period was sixteen hours for each animal. The percentage of time each animal was active and mobile was recorded for the overall sixteen hour period and also during the final three hour twelve minute period (considered to be the asymptotic period).

b) Forelimb/Hindlimb Grip Strength

An automated grip strength meter was used. Each animal was allowed to grip the proximal metal bar of the meter with its forepaws. The animal was pulled by the base of the tail until its grip was broken. The animal was drawn along the trough of the meter by the tail until its hind paws gripped the distal metal bar. The animal was pulled by the base of the tail until its grip was broken. A record of the force required to break the grip of each animal was made. Three consecutive trials were performed for each animal.

3.3.2.3 Sensory Reactivity

Each animal was individually assessed for sensory reactivity to auditory, visual and proprioceptive stimuli. The scoring system used is outlined in Appendix I. The following parameters were observed:

Grasp response	Touch escape
Vocalisation	Pupil reflex
Toe pinch	Startle reflex*
Tail pinch	Blink reflex
Finger approach	

- * Startle reflex was measured using the ST1058 Startle Test Meter (Benwick Electronics). Each animal was placed on the force platform and allowed to settle. An audible tone was activated and any change in the force exerted by the animal on the force platform, as a result of startle induced tremor, was measured. The percentage average response, root of the mean square and peak response were recorded for each animal and two consecutive trials were performed.

3.3.3 Bodyweight

Individual bodyweights were recorded on Day 1 (the start of treatment) and on Days 7, 14, 21 and 28. Bodyweights were also recorded at terminal kill.

3.3.4 Food Consumption

Food consumption was recorded for each cage group at weekly intervals throughout the study.

3.3.5 Water Consumption

Water intake was observed daily, for each cage group, by visual inspection of the water bottles for any overt changes. Daily visual inspection of water bottles during Week 2 revealed possible intergroup differences. Water consumption was, therefore, measured and recorded for each cage group from Day 15 onwards.

3.3.6 Laboratory Investigations

Haematological and blood chemical investigations were performed on all surviving animals from each test and control group at the end of the study (Day 28). Blood samples were obtained from the lateral tail vein. Where necessary repeat samples were obtained by cardiac puncture prior to necropsy on Day 29. Animals were not fasted prior to sampling.

The methods used for haematological and blood chemical investigations are given in Appendix XIX and normal ranges are shown in Appendix XXI.

3.3.6.1 Haematology

The following parameters were measured on blood collected into tubes containing potassium EDTA anti-coagulant:

Haemoglobin (Hb)

Erythrocyte count (RBC)

Haematocrit (Hct)

Erythrocyte indices

- mean corpuscular haemoglobin (MCH)
- mean corpuscular volume (MCV)
- mean corpuscular haemoglobin concentration (MCHC)

Total leucocyte count (WBC)

Differential leucocyte count

- neutrophils (Neut)
- lymphocytes (Lymph)
- monocytes (Mono)
- eosinophils (Eos)
- basophils (Bas)

Platelet count (PLT)

Reticulocyte count (Retic)

Prothrombin time (CT) was assessed by 'Hepato Quick' and Activated partial thromboplastin time (APTT) was assessed by 'Preci Clot' using samples collected into sodium citrate solution (0.11 mol/l).

3.3.6.2 Blood Chemistry

The following parameters were measured on plasma from blood collected into tubes containing lithium heparin anti-coagulant:

Urea

Calcium (Ca⁺⁺)

Glucose

Inorganic phosphorus (P)

Total protein (Tot.Prof.)

Aspartate aminotransferase (ASAT)

Albumin	Alanine aminotransferase (ALAT)
Albumin/Globulin (A/G) ratio (by calculation)	Alkaline phosphatase (AP)
Sodium (Na+)	Creatinine (Creat)
Potassium (K+)	Total cholesterol (Chol)
Chloride (Cl-)	Total bilirubin (Bili)

3.3.7 Pathology

On completion of the dosing period all surviving animals were killed by intravenous overdose of sodium pentobarbitone (Sagatal, 60 mg/ml; May and Baker Limited, Dagenham, Essex, UK) followed by exsanguination.

All animals were subjected to a full external and internal examination, and any macroscopic abnormalities were recorded.

3.3.7.1 Organ Weights

The following organs removed from animals that were killed at the end of the study, were dissected free from fat and weighed before fixation:

Adrenals	Brain	Epididymides	Heart
Kidneys	Liver	Ovaries	Spleen
Testes	Thymus		

Normal ranges for these organ weights are given in Appendix XXII.

3.3.7.2 Histopathology

Samples of the following tissues were removed from all animals and preserved in buffered 10% formalin:

Adrenals	Muscle (skeletal)
Aorta (thoracic)	Oesophagus
Bone & bone marrow (femur including stifle joint)	Ovaries
Bone & bone marrow (sternum)	Pancreas
Brain (including cerebrum, cerebellum and pons)	Pituitary
Caecum:	Prostate
Colon	Rectum
Duodenum	Salivary glands (submaxillary)
Epididymides	Sciatic nerve
Eyes	Seminal vesicles
Gross lesions	Skin (hind limb)
Heart	Spinal cord (cervical)
Ileum	Spleen
Jejunum	Stomach
Kidneys	Testes
Liver	Thymus
Lungs (with bronchi) #	Thyroid/parathyroid
Lymph nodes (cervical and mesenteric)	Trachea
	Urinary bladder
	Uterus

Lungs were inflated to approximately normal inspiratory volume with buffered 10% formalin before immersion in fixative

All tissues were despatched to Propath UK Ltd, Willow Court, Netherwood Road, Rotherwas, Hereford, UK for processing. The tissues shown in **bold** from all control and 1000 mg/kg/day dose group animals were prepared as paraffin blocks, sectioned at nominal thickness of 5 μ m and stained with haematoxylin and eosin for subsequent microscopic examination. The liver and spleen from all 15 and 150 mg/kg/day dose group animals were also processed.

Since there were indications of treatment-related kidney, liver, spleen, stomach and urinary bladder changes, examination was subsequently extended to include similarly prepared sections of these tissues from all animals in the other treatment groups.

Microscopic examination was conducted by the Study Pathologist. All findings were entered into the ROELEE 84 pathology computerisation system for tabulation and report production.

3.4 Evaluation of Data

Data were processed to give group mean values and standard deviations where appropriate.

Haematological, blood chemical, organ weight (absolute and relative to terminal bodyweight), weekly bodyweight gain and quantitative functional performance and sensory reactivity data were assessed for dose response relationships by linear regression analysis followed by one way analysis of variance (ANOVA) incorporating Levene's test for homogeneity of variance. Where variances were shown to be homogenous pairwise comparisons were conducted using Dunnett's test. Where Levene's test showed unequal variances the data were analysed using non-parametric methods: Kruskal-Wallis ANOVA and Mann-Whitney "U" test.

The haematology variable basophils was not analysed since consistently greater than 30% of the data were recorded as the same value.

Probability values (p) are presented as follows:

$p < 0.001$ ***

$p < 0.01$ **

$p < 0.05$ *

$p \geq 0.05$ (not significant)

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safeparm archives for a period of five years. After this period, the Sponsor's instructions will be sought.

5. RESULTS

5.1 Mortality

One female treated with 1000 mg/kg/day was found dead on Day 12 of the study. This death was probably related to treatment but no specific cause of death was established.

There were no other interim mortalities.

5.2 Clinical Observations

A summary incidence of daily clinical observations is given in Tables 1 and 2.

Animals treated with 1000 mg/kg/day began to show clinically observable signs of toxicity from Day 3. Symptoms gradually worsened and became more prevalent during Weeks 1 and 2 before stabilising during the second half the study. Signs seen in most animals included fur loss, noisy respiration, increased salivation, red/brown staining of the fur, wet fur and hunched posture. Sporadic signs of diuresis, staining around the ano-genital region, tiptoe gait, gasping respiration and pallor of the extremities were also noted. The female found dead at the start of Day 12 showed a substantial deterioration in condition on Day 11 with additional signs of pilo-erection, respiratory pattern changes, staining around the snout and mouth and dehydration.

The only observations seen in animals from the 150 or 15 mg/kg/day dose groups were incidents of fur loss, which is a common finding in group housed rats and, in isolation, is not toxicologically significant and a single incident of increased salivation entirely associated with the dosing procedure and not indicative of toxicity.

5.3 Functional Observations

A summary incidence of behavioural assessments is given in Tables 3 to 12. Individual observations are given in Appendices II to VI. Group mean functional

test values and standard deviations are given in Tables 13 and 14. Individual values are given in Appendix VII. A summary incidence of sensory reactivity assessments is given in Tables 15 and 16 together with group mean startle reflex values and standard deviations. Individual responses are given in Appendix VIII.

5.3.1 Behavioural Assessments

Weekly behavioural assessments supported the clinical observations recorded during the study with incidents of tiptoe gait, hunched posture and noisy respiration seen at each observation time, for animals treated with 1000 mg/kg/day. Animals from this group also showed increased salivation during the study.

No such effects were detected at 150 or 15 mg/kg/day.

All remaining inter and intra group differences in behavioural scores were considered to be a result of normal variation for rats of the strain and age used and were, therefore, of no toxicological importance.

5.3.2 Functional Performance Tests

There were no treatment-related changes in the functional performance parameters measured.

Statistical analysis of the data revealed no significant intergroup differences.

5.3.3 Sensory Reactivity Assessments

Males treated with 1000 mg/kg/day showed an enhanced startle reflex compared with controls. The percentage peak response was statistically significantly elevated and the percentage average response and RMS response were also higher than controls, although the differences did not achieve statistical significance.

Females dosed at 1000 mg/kg/day and animals of either sex treated with 150 or 15 mg/kg/day were not similarly affected.

All remaining inter and intra group differences in sensory reactivity scores were considered to be a result of normal variation for rats of the strain and age used and were, therefore, of no toxicological importance.

5.4 Bodyweight

Group mean weekly bodyweights and standard deviations are given in Tables 17 and 18 and are presented graphically in Figures I and II. Group mean weekly bodyweight gains and standard deviations are given in Tables 19 and 20 (statistically significant differences are indicated). Individual data are given in Appendices IX and X.

Males and females treated with 1000 mg/kg/day showed a substantially reduced bodyweight gain during Week 1 of the study only. In subsequent weeks bodyweight gain in these animals was similar to controls but a full recovery was not apparent in males with terminal bodyweight remaining notably lower than controls.

No adverse effect on bodyweight development was detected for animals of either sex from the 150 or 15 mg/kg/day dose groups.

A statistically significant increase or reduction in bodyweight development was noted for 15 mg/kg/day males, during Week 2, or females, during Week 1, respectively compared with controls but, in the absence of a dose response relationship, the intergroup differences were considered to be fortuitous.

5.5 Food Consumption

Group mean weekly food consumptions are given in Tables 21 and 22 and are presented graphically in Figures III and IV. Weekly food efficiencies are given in Tables 23 and 24.

Males and females treated with 1000 mg/kg/day showed reduced food consumption and reduced food efficiency (the ratio of bodyweight gain to dietary intake) during the first week of the study. Dietary intake and food efficiency recovered in females thereafter but dietary intake remained reduced in the males throughout the study period.

No adverse effects were detected in animals of either sex treated with 150 or 15 mg/kg/day.

5.6 Water Consumption

Group mean daily water consumptions are given in Tables 25 and 26.

Water consumption measurement, initiated on Day 15, revealed a substantial increase for animals of either sex treated with 1000 mg/kg/day, which persisted until the end of the study.

There was no convincing effect on water consumption in animals from the 150 or 15 mg/kg/day dose groups.

5.7 Laboratory Investigations

5.7.1 Haematology

Group mean values and standard deviations for test and control group animals are given in Tables 27 and 28 (statistically significant differences are indicated). Individual data are given in Appendix XI.

Haematological changes consistent with haemolytic anaemia were detected in animals of either sex treated with 1000 or 150 mg/kg/day. The condition was dose related in severity and involved statistically significant reductions in erythrocyte count, haemoglobin, haematocrit and mean corpuscular haemoglobin concentration (MCHC); and increases in mean corpuscular volume, mean corpuscular haemoglobin (MCH) and reticulocyte count; the majority of 1000 mg/kg/day reticulocyte values were outside the normally expected range for rats of the strain and age used. Males and females from the 1000 mg/kg/day dose group also showed a statistically significant increase in neutrophil numbers, but not in the total leucocyte count, together with a slightly elevated prothrombin time.

Animals of either sex treated with 15 mg/kg/day showed no toxicologically or statistically significant haematological changes.

5.7.2 Blood Chemistry

Group mean values and standard deviations for test and control group animals are given in Tables 29 and 30 (statistically significant differences are indicated). Individual data are given in Appendix XII.

Males and females treated with 1000 mg/kg/day showed statistically significant elevations in plasma aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) including individual values outside the normally expected range for rats of the strain and age used, particularly for ALAT. The group mean ALAT concentration for males from the 150 mg/kg/day dose group was also significantly elevated and there was a slight, but not statistically significant increase in this and ASAT levels for animals of either sex at this dose level. Plasma bilirubin levels were increased for animals of either sex from the 1000 mg/kg/day dose group but statistical significance was confined to the males.

Animals of either sex treated with 15 mg/kg/day showed no toxicologically or statistically significant blood chemical changes.

5.8 Pathology

5.8.1 Organ Weights

Group mean absolute and relative organ weights and standard deviations for test and control group animals are presented in Tables 31 to 34 (statistically significant differences are indicated). Individual data are given in Appendix XIII.

Males and females treated with 1000 mg/kg/day showed a statistically significant increase in spleen weight, both absolute and relative to bodyweight, compared with controls. Many of the individual values were outside the normally expected range for rats of the strain and age used and the effect extended to either sex from the 150 mg/kg/day dose group, although statistical significance was not always achieved at this dose level.

A statistically significantly higher relative testes weight for 1000 mg/kg/day males was considered to result from the lower terminal bodyweight for these animals and was not indicative of a target organ effect. Relative brain weights were statistically significantly higher than controls in all male treatment groups but in the absence of any histopathological brain changes or any effect on the absolute weight, these intergroup differences were probably of no toxicological significance. Similarly, reductions in absolute liver and kidney weight for 1000 mg/kg/day males were not reflected in the relative weights and were considered to be of no toxicological importance.

There were no toxicologically significant effects on organ weight for animals of either sex treated with 15 mg/kg/day.

5.8.2 Necropsy

A summary incidence of necropsy findings is given in Tables 35 and 36. Individual data are given in Appendix XIV.

One female treated with 1000 mg/kg/day showed thickening of the non-glandular gastric epithelium but there were no other macroscopic findings at necropsy that could be considered treatment-related. The 1000 mg/kg/day female that died on Day 12 showed normal post-mortem changes and there was no indication of a cause of death.

The incidental findings recorded for animals from various dose groups, including controls (identified as misshapen kidney, mammary mass, small testis and hydronephrosis), whilst showing no convincing dose-related response, were consistent with normally expected low incidence findings in laboratory maintained rats and were, therefore, of no toxicological importance.

5.8.3 Histopathology

A summary incidence of histopathological findings is given in Tables 37 and 38. Details of the grading system used and all individual data are given in Appendix XV.

The following treatment-related changes were observed:

LIVER: Centrilobular hepatocyte enlargement was observed for three males and scattered deposits of pigment were seen in a few animals of either sex dosed at 1000 mg/kg/day. One female dosed at 150 mg/kg/day similarly demonstrated hepatic pigment accumulation. The accumulated pigment was Perl's positive and therefore probably haemosiderin.

Hepatocyte enlargement is commonly observed in the rodent liver following the administration of xenobiotics and, in the absence of associated inflammatory or degenerative changes, is generally considered to be adaptive in nature.

SPLEEN: Increased severities of extramedullary haemopoiesis and pigment accumulation were observed in relation to treatment for rats of either sex dosed at 1000 or 150 mg/kg/day. Female rats receiving 15 mg/kg/day demonstrated increased severities of splenic pigment accumulation but no alteration in extramedullary haemopoiesis compared with control animals. The accumulated pigment was Perl's positive and therefore probably haemosiderin.

KIDNEYS: An increased severity of pigment accumulation was observed in the renal tubular epithelium of animals of either sex dosed at 1000 mg/kg/day. The accumulated pigment was Perl's positive and therefore probably haemosiderin.

URINARY BLADDER: Hyperplasia of the transitional cell epithelial lining of the urinary bladder was seen for males and females dosed at 1000 mg/kg/day.

STOMACH: Acanthosis and hyperkeratosis, occasionally with associated subepithelial inflammatory cell infiltrates, were observed in relation to treatment in the forestomach of animals of either sex receiving 1000 mg/kg/day of the test material.

All remaining morphological changes were those commonly observed in laboratory maintained rats of the strain and age employed, and there were no differences in incidence or severity between control and treatment groups that were considered to be of toxicological significance.

6. DISCUSSION

The administration of NINOX® HCDO by oral gavage, to rats for a period of up to twenty-eight consecutive days resulted in toxicologically significant effects at dose levels of 1000 and 150 mg/kg/day.

Adverse clinical signs developed in 1000 mg/kg/day animals from Day 3 onwards with observations reflected in the weekly behavioural data. Sensory reactivity determinations showed an enhanced startle reflex response in these animals. The observations seen were consistent with those commonly associated with oral administration of irritant materials. In particular signs such as noisy respiration, increased salivation, hunched posture and tiptoe gait are often associated with gastric irritation. The enhanced startle reflex may simply reflect a heightened response due to the underlying, painful gastric condition. The gastric irritation effect was supported histologically by the observation of acanthosis, hyperkeratosis and associated subepithelial inflammatory cell infiltrates in the forestomach epithelium. A substantial reduction in bodyweight gain was noted in 1000 mg/kg/day animals during the first week of treatment, together with reduced food consumption. These findings could be associated with the gastric irritation and an adaptation to the effect may be indicated by recovery, particularly in the females, during the rest of the study. Food efficiency was also reduced in the first week of the study, suggesting impaired food utilisation. Animals from this group also showed a marked increase in water consumption, which was associated with diuresis observed clinically. There was no evidence of any effect on kidney function so this was considered to be also associated with the oral administration of an irritant substance resulting in a behavioural response to drink more. Interestingly, histopathological examination of the urinary bladder demonstrated hyperplasia of the transitional cell epithelium, which suggests urinary excretion of an irritant substance. In view of the water solubility of the test material, this may indicate excretion of the unchanged parent substance.

Haematological investigations identified haemolytic anaemia in animals treated with 1000 or 150 mg/kg/day and this was associated with deposition of haemosiderin pigment in liver, spleen and kidneys. Histopathology also revealed splenic extramedullary haemopoiesis, which represents a normal compensatory response to the haemolytic condition, and 1000 mg/kg/day males showed increased levels of plasma bilirubin. The haemopoietic response in the spleen was considered to account for the increased weight of this organ.

Blood chemistry determinations revealed elevations in plasma levels of aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) enzymes. The increase in ASAT can probably be explained as a consequence of the haemolytic effect since erythrocytes contain high levels of this enzyme. The reasons for the increase in the other enzymes, however, are not clear. Elevations in AP could have an intestinal origin associated with the irritant effect, but ALAT is a liver specific enzyme and there was no evidence of liver changes that could be associated with this. Liver histopathology showed only haemosiderin deposition and centrilobular hepatocyte enlargement in a few animals, neither of which is indicative of cell damage that could result in the increased plasma ALAT.

The only treatment-related change seen in animals treated with 15 mg/kg/day was an increased severity of splenic haemosiderin accumulation in females. This may indicate that some haemolysis occurred at this dose level but was so minimal that it was not detected by haematology and resulted in no perceptible increase in extramedullary haemopoiesis. This slight increase in pigment deposition was, therefore, considered not to be toxicologically significant.

7. CONCLUSION

Oral administration of the test material, NINOX[®] HCDO, to rats for a period of up to twenty-eight consecutive days at dose levels of up to 1000 mg/kg/day resulted in toxicologically significant effects at dose levels of 1000 and 150 mg/kg/day. No such effects were demonstrated in animals of either sex treated with 15 mg/kg/day and the "No Observed Effect Level" (NOEL) was, therefore, considered to be 15 mg/kg/day.

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NINOX® HCDO : TWENTY-EIGHT DAY REPEATED DOSE ORAL (GAVAGE) TOXICITY STUDY
IN THE RAT

APPENDIX XV (continued)
INDIVIDUAL HISTOPATHOLOGICAL FINDINGS

1000 mg active ingredient/kg/day Male

Animal	Tissue	Observation
35	Heart	Mode of death : Terminal kill
	Liver	Focal myocarditis (minimal) Mononuclear cell foci (minimal) Centrilobular hepatocyte enlargement (minimal) Pigment deposition (minimal)
	Lungs	Perivascular/peribronchiolar lymphoid aggregations (minimal) Groups of alveolar macrophages (slight)
	Spleen	Extramedullary haemopoiesis (moderate) Pigment deposition (slight)
	Stomach	Acanthosis (minimal)
	Urinary bladder	Hyperplasia transitional epithelium (minimal)

**NINOX® HCDO : TWENTY-EIGHT DAY REPEATED DOSE ORAL (GAVAGE) TOXICITY STUDY
IN THE RAT
APPENDIX XV (continued)
INDIVIDUAL HISTOPATHOLOGICAL FINDINGS**

Animal	Tissue	Observation
1000 mg active ingredient/kg/day Female		
36	Heart	Mode of death : Terminal kill
	Kidneys	Focal myocarditis (minimal)
	Liver	Pigment deposition (minimal)
		Mononuclear cell foci (minimal)
		Pigment deposition (minimal)
	Lungs	Perivascular/peribronchiolar lymphoid aggregations (minimal)
		Groups of alveolar macrophages (minimal)
	Spleen	Extramedullary haemopoiesis (slight)
		Pigment deposition (slight)
Stomach		Acanthosis (moderate)
		Hyperkeratosis (moderate)
	Urinary bladder	Hyperplasia transitional epithelium (minimal)
37	Liver	Mode of death : Terminal kill
		Mononuclear cell foci (minimal)
		Pigment deposition (slight)
	Lungs	Perivascular/peribronchiolar lymphoid aggregations (minimal)
	Spleen	Extramedullary haemopoiesis (moderate)
		Pigment deposition (moderate)
	Stomach	Acanthosis (moderate)
	Hyperkeratosis (moderate)	
Urinary bladder		Subepithelial inflammatory cell infiltrates (minimal)
		Hyperplasia transitional epithelium (minimal)
	38	Cervical lymph nodes
Heart		Lymphadenitis
Kidneys		Focal myocarditis (minimal)
Liver		Pigment deposition (minimal)
		Mononuclear cell foci (minimal)
Lungs		Perivascular/peribronchiolar lymphoid aggregations (minimal)
		Groups of alveolar macrophages (minimal)
Spleen		Extramedullary haemopoiesis (slight)
		Pigment deposition (moderate)
Stomach		Acanthosis (slight)
Urinary bladder		Hyperplasia transitional epithelium (minimal)
Uterus/cervix	Dilatation horn (minimal)	
39	Cervical lymph nodes	Mode of death : Terminal kill
	Kidneys	Lymphadenitis
	Liver	Pigment deposition (minimal)
		Mononuclear cell foci (minimal)
		Pigment deposition (minimal)
	Lungs	Perivascular/peribronchiolar lymphoid aggregations (minimal)
		Groups of alveolar macrophages (minimal)
Spleen	Extramedullary haemopoiesis (slight)	
	Pigment deposition (moderate)	

NINOX® HCDO : TWENTY-EIGHT DAY REPEATED DOSE ORAL (GAVAGE) TOXICITY STUDY
IN THE RAT

APPENDIX XV (continued)
INDIVIDUAL HISTOPATHOLOGICAL FINDINGS

Animal	Tissue	Observation
39 (cont)	Stomach	Acanthosis (moderate) Hyperkeratosis (slight) Subepithelial inflammatory cell infiltrates (slight) Epithelial ulceration
	Urinary bladder	Hyperplasia transitional epithelium (minimal)
40		Mode of death : Interim death
	Bone marrow	Autolysis (minimal)
	Cervical lymph nodes	Autolysis (minimal)
	Heart	Focal myocarditis (minimal)
	Kidneys	Vacuolation tubular epithelium
	Liver	Mononuclear cell foci (minimal) Generalised hepatocyte enlargement (minimal) Cytoplasmic basophilia
	Mesenteric lymph nodes	Autolysis (slight)
	Spleen	Pigment deposition (slight) Lymphoid atrophy (minimal)
	Stomach	Autolysis (minimal) Acanthosis (minimal) Hyperkeratosis (minimal)
	Thymus	Autolysis (minimal)
	Thyroids	Autolysis (minimal)
	Urinary bladder	Hyperplasia transitional epithelium (minimal)

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SPL PROJECT NUMBER: 625/051

NINOX® HCDO : TWENTY-EIGHT DAY REPEATED DOSE ORAL (GAVAGE) TOXICITY STUDY
IN THE RAT
APPENDIX XVI
PROTOCOL

SafePharm
Laboratories

PROTOCOL

TEST MATERIAL : NINOX® HCDO
STUDY TYPE : Twenty-Eight Day Repeated Dose Oral (Gavage)
Toxicity Study in the Rat
PROJECT NUMBER : 625/051
PROPOSED START DATE : October 1999
PROPOSED COMPLETION DATE : October 1999
TARGET (DRAFT) REPORT DATE : March 2000
SPONSOR : Stepan Company
22 West Frontage Road
Northfield
ILLINOIS 60093
UNITED STATES OF AMERICA

AUTHORISED BY: H. Cormack DATE: 03 SEP 1999
H Cormack BSc (Hons)
STUDY DIRECTOR

AUTHORISED FOR SPONSOR BY: Jim Austin DATE: 14 Sep 99
60093

A105

NINOX® HCDO : TWENTY-EIGHT DAY REPEATED DOSE ORAL (GAVAGE) TOXICITY STUDY
IN THE RAT
APPENDIX XVI (continued)
PROTOCOL

TWENTY-EIGHT DAY REPEATED DOSE ORAL (GAVAGE) TOXICITY STUDY IN THE RAT

1. INTRODUCTION AND OBJECTIVES

This protocol details a study designed to comply with the following regulatory guidelines:

- i) Commission Directive 96/54/EC (Method B7).
- ii) The Japanese Ministry of Health and Welfare (MHW) Guidelines 1986 for a twenty-eight day repeat dose oral toxicity study as required by the Japanese Chemical Substances Control Law 1973 of the Ministry of International Trade and Industry (MITI) amended 1986.
- iii) The OECD Guidelines for Testing of Chemicals No. 407 "Repeated Dose 28 Day Oral Toxicity Study in Rodents" (Adopted 27 July 1995).

The purpose of this study is to establish the effects of repeated oral administration of the test material to rats over a period of twenty-eight consecutive days. The results of the study are believed to be of value in predicting the toxicity of the test material to man and can identify the organs and tissues which may be injured by exposure, can enable detection of possible cumulative toxicity and the estimation of the "No Observed Effect Level" (NOEL).

The work will be performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1997 (SI 1997/654)). These regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (Revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 87/18/EEC and 88/320/EEC.

2. TEST FACILITIES

Performing Laboratory	Histology Processing:
Safeparm Laboratories Ltd	Propath UK Ltd
Shardlow Business Park	Willow Court
Shardlow	Netherwood Road
Derbyshire	Rotherwas
DE72 2GD	Hereford
	HR2 6LU

3. ANIMALS

Specification: Sprague Dawley Crj:CD® BR strain rats obtained from Charles River (UK) Limited, Margate, Kent. At the start of the study animals will be aged five to eight weeks. The weight variation will not exceed $\pm 20\%$ of the mean weight for either sex.

NINOX® HCDO : TWENTY-EIGHT DAY REPEATED DOSE ORAL (GAVAGE) TOXICITY STUDY
IN THE RAT
APPENDIX XVI (continued)
PROTOCOL

- Justification: Preferred species of choice as historically used for safety evaluation studies and specified by appropriate regulatory authorities.
4. **ANIMAL HUSBANDRY**
- Environment: Target temperature: $21 \pm 2^{\circ}\text{C}$
Target humidity: $55 \pm 15\%$
Lighting: Twelve hours of continuous artificial light in each twenty-four hour period.
Ventilation: At least fifteen air changes per hour.
- Housing: Groups of five by sex in polypropylene cages with stainless steel mesh lids and grid bases, suspended over trays containing absorbent paper.
- Diet and Water: Rat and Mouse SQC Expanded Diet No. 1 (Special Diets Services Limited, Witham, Essex, UK) with batch analysis, and tap water *ad libitum*.
- The diet and drinking water are routinely analysed and are considered not to contain any contaminant that could reasonably be expected to affect the purpose or integrity of the study.
5. **PRE-TEST PROCEDURES**
- Acclimatisation Period: At least seven days.
- Allocation: Animals will be allocated to dose groups using total randomisation procedure.
- Identification: Each animal, selected at random, will be uniquely identified within the study by ear-punch. A colour-coded cage card will be prepared with details of test material, project number, dose level, sex, numbers of animals, route of administration and Study Director responsible for the study.
6. **TEST MATERIAL AND EXPERIMENTAL PREPARATION**
- Identification: Supplied by the Sponsor with details of purity, stability and hazardous properties if known.
- Storage: Room temperature in the dark unless otherwise specified by the Sponsor.

**NINOX® HCDO : TWENTY-EIGHT DAY REPEATED DOSE ORAL (GAVAGE) TOXICITY STUDY
IN THE RAT
APPENDIX XVI (continued)
PROTOCOL**

Preparation: The test material will be dissolved or suspended in a suitable vehicle weekly (subject to confirmation of stability). Wherever possible, an aqueous formulation will be used, followed by consideration of formulation in vegetable oil (eg Arachis oil), then other specified vehicles. The method of preparation will be documented in the study records.

Analysis: Details of identification of the test material will be supplied by the Sponsor. The test material formulations will be analysed for concentration, stability and, if applicable, homogeneity by Satepharm Analytical Laboratory.

7. STUDY DESIGN

Administration: Once daily, by gavage, using a stainless steel dosing cannula attached to a graduated syringe for twenty-eight consecutive days. Dosing will be performed at a similar time each day wherever possible.

Dose Groups: Six dose groups each comprising ten animals (five male and five female) will be used. Groups will be allocated as follows:

Group	Duration of Treatment	Necropsy Day
Control	28 days	29
Recovery control	28 days	43
Low dose	28 days	29
Intermediate dose	28 days	29
High dose	28 days	29
Recovery high dose	28 days	43

Control animals will be treated for twenty-eight consecutive days with vehicle alone. Recovery group animals will be maintained for a further fourteen days without treatment after completion of the twenty-eight day dosing period.

Dose levels will be based on available toxicity data following a range-finding study (Appendix I), up to a maximum dose of

NINOX® HCDO : TWENTY-EIGHT DAY REPEATED DOSE ORAL (GAVAGE) TOXICITY STUDY
IN THE RAT
APPENDIX XVI (continued)
PROTOCOL

1000 mg/kg/day. The dose levels to be used in the study will be documented as a protocol addendum together with the treatment volume. The control groups will be handled in an identical manner to the test animals, except for administration of the test material.

B. OBSERVATIONS

Morbidity/Mortality

Inspection:

Twice daily, early and late during the working period.

Clinical Observations:

Individual clinical observations will be performed immediately before dosing and one hour after dosing. An additional observation will be made five hours after dosing during the normal working week (not at weekends or on public holidays). Recovery groups will be observed twice daily during the treatment-free period (once daily at weekends and on public holidays). All observations will be recorded.

Functional

Observations:

Detailed individual clinical observations will be performed on all test and control group animals before the first exposure to the test material and once weekly thereafter. These observations will be performed outside the home cage, in a standard arena, at approximately two hours after dosing (where applicable) to ensure that any transient effects of treatment are identified. All observations will be recorded. In addition, sensory reactivity to different stimuli (auditory, visual and proprioceptive), grip strength (fore and hind limb) and motor activity (using a 44 photobeam unit) will be measured once during Week 4, at least two hours after dosing.

If specific functional changes are identified during the treatment period, functional observations will be performed once in recovery group animals during the final week of the treatment-free period, at the discretion of the Study Director.

Bodyweights:

Individual bodyweights will be recorded on Day 0 (the day before the start of dosing) and at weekly intervals thereafter. Individual bodyweights will also be recorded at terminal kill.

Food Consumption

Dietary intake will be recorded weekly for each cage group. Weekly food efficiency (bodyweight gain/food intake) will be calculated.

NINOX® HCDO : TWENTY-EIGHT DAY REPEATED DOSE ORAL (GAVAGE) TOXICITY STUDY
 IN THE RAT
 APPENDIX XVI (continued)
 PROTOCOL

ii) Blood Chemistry: Blood urea
 Total protein
 Albumin
 Albumin/Globulin ratio (by calculation)
 Sodium
 Potassium
 Chloride
 Calcium
 Inorganic phosphorus
 Creatinine
 Alkaline phosphatase
 Alanine aminotransferase
 Aspartate aminotransferase
 Glucose
 Triglycerides
 Total cholesterol
 Gamma glutamyl transpeptidase
 Total bilirubin

iii) Urinalysis: Volume Ketones
 Specific gravity Bilirubin
 pH Urobilinogen
 Protein Reducing substances
 Glucose Blood

Post Mortem Studies: Post mortem studies will be performed on animals found dead or killed in extremis during the study and on all animals killed by intravenous overdose of sodium pentobarbitone followed by exsanguination at termination.

i) Gross Examination: Full external and internal examination of all animals

ii) Organ Weights: Adrenals Brain Epididymides Heart Kidneys
 Liver Ovaries Spleen Testes Thymus

Carried out on all survivors at termination.

iii) Histopathology: Samples of the following tissues from all animals will be preserved in buffered 10% formalin:

PART II

NINOX® HCDO:
FOURTEEN DAY REPEATED DOSE ORAL (GAVAGE)
RANGE-FINDING TOXICITY STUDY
IN THE RAT

**NINOX® HCDO:
FOURTEEN DAY REPEATED DOSE ORAL (GAVAGE)
RANGE-FINDING TOXICITY STUDY
IN THE RAT**

1. INTRODUCTION

The study was performed to establish the maximum tolerated dose level (up to 1000 mg/kg/day) of the test material following repeated oral administration to the Sprague-Dawley CrI:CD®BR strain rat, and to establish a low dose level that did not produce evidence of toxicity. This information was used to determine dose levels for use in a twenty-eight day oral toxicity study.

2. TEST MATERIAL AND EXPERIMENTAL PREPARATION

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	NINOX® HCDO
Description	:	off white paste
Batch number	:	876TK
Purity	:	81.47%
Sample number	:	IX
Date received	:	10 May 1999
Storage conditions	:	room temperature in the dark

Data relating to the identity, purity and stability of the test material are the responsibility of the Sponsor.

2.2 Experimental Preparation

For the purpose of the study the test material was prepared as a solution in distilled water. A fresh formulation was made each day and the animals were dosed within three hours of preparation.

The concentration and stability of the test material formulations were not determined analytically.

3. METHODS

3.1 Animals and Animal Husbandry

Six male and six female Sprague-Dawley CrI:CD®BR strain rats were obtained from Charles River (UK) Limited, Margate, Kent. After an acclimatisation period of at least seven days, animals were selected at random and given a unique number within the study by ear punching.

At the start of treatment the males weighed 127 to 148g and the females weighed 120 to 142g. The animals were housed in groups of three by sex in polypropylene grid-floor cages suspended over trays containing absorbent paper. Free access to mains drinking water and food (Rat and Mouse SQC Expanded Diet No.1, Special Diets Services Limited, Witham, Essex, UK) was allowed throughout the study.

The animals were housed in a single air-conditioned room within the Safepharm Barrier Maintained Rodent Facility. The rate of air exchange was at least fifteen air changes per hour and the low intensity fluorescent lighting was controlled to give twelve hours continuous light and twelve hours darkness. Environmental conditions were continuously monitored by a computerised system, and print-outs of the mean temperatures and humidities were included in the study records. The temperature and relative humidity were controlled to remain within target ranges of $21 \pm 2^{\circ}\text{C}$ and $55 \pm 15\%$ respectively. Occasional deviations from the humidity targets were considered not to have affected the purpose or integrity of the study.

3.2 Procedure

Following a single dose sighting study treating one male and one female at 1000 mg/kg/day two groups, each of six rats (three males and three females) were dosed as follows:

DOSE LEVEL OF TEST MATERIAL* (mg/kg/day)	DOSE LEVEL OF ACTIVE INGREDIENT (mg/kg/day)	TREATMENT VOLUME (ml/kg)	CONCENTRATION OF TEST MATERIAL* (mg/ml)	CONCENTRATION OF ACTIVE INGREDIENT (mg/ml)
0	0 (Control)	5	0	0
1250	1000	5	250	200

* - incorporating a correction factor for 80% purity

The test material was administered daily, for fourteen consecutive days, by gavage using a stainless steel cannula attached to a disposable plastic syringe. Control animals were treated in an identical manner with 5 ml/kg/day of distilled water.

The volume of test and control material administered to each animal was based on the most recent bodyweight and was adjusted at Days 4, 8 and 11.

3.3 Observations

3.3.1 Clinical Observations

All animals were examined for overt signs of toxicity, ill health or behavioural change immediately before dosing and one hour after dosing. All observations were recorded.

3.3.2 Bodyweight

Individual bodyweights were recorded on Days 1, 4, 8, 11 and 14 of the study.

3.3.3 Necropsy

On completion of the dosing period, all animals were killed by cervical dislocation and immediately subjected to an internal and external macroscopic examination. No tissues were retained.

3.4 Evaluation of Data

Necropsy data, bodyweights and clinical observations were examined for any adverse effects resulting from treatment.

The data obtained was summarised in tabular form and used to provide the basis for selection of dose levels for the main study.

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for a period of five years. After this period, the Sponsor's instructions will be sought.

5. RESULTS

5.1 Mortality

There were no deaths during the study.

5.2 Clinical Observations

A summary incidence of daily clinical observations is given in Tables 1 and 2.

Animals of either sex treated with 1000 mg/kg/day showed increased salivation up to ten minutes after dosing from Day 3 together with incidents of increased salivation up to one hour after dosing and associated staining and wetting of the external body surface. Females were more adversely affected with clinical signs including diuresis, fur loss, hunched posture and noisy respiration from Day 4 onwards. Males showed noisy respiration one hour after dosing on Day 12 only.

5.3 Bodyweight

Individual bodyweights are given in Table 3.

Animals treated with 1000 mg/kg/day showed a slight reduction in bodyweight gain throughout the treatment period.

5.4 Necropsy

Individual necropsy findings are given in Table 4.

Animals treated with 1000 mg/kg/day showed sloughing of the non-glandular gastric epithelium at terminal kill.

6. CONCLUSION

The dose levels for the main twenty-eight day study were chosen, following consultation with the Sponsor, as:

- High dose : 1000 mg/kg/day
- Intermediate dose : 150 mg/kg/day
- Low dose : 15 mg/kg/day
- plus a control group treated with vehicle alone