REPORT No. 2500370
Regulatory Document

DSM

Document Date:	25-May-2007
Author(s):	Thiel A (NRD/CH), Köhl W (SCC-GmbH), Braun W (RCC Ltd)
Title:	Ultrazine FG-R (Food Grade Lignosulphonate): 13-Week Oral Toxicity (Feeding) Study in the Wistar Rat (study conducted at RCC Ltd., CH-4452 Itingen, Switzerland, RCC Study Number A29553)
Project No.	6309
Compound No.	Ultrazine FG-R (Food Grade Lignosulphonate), Calcium Lignosulphonate, LS FG DP-995 FGR004

Summary
In a subchronic toxicity study, ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE) was administered daily via the feed to SPF-bred Wistar rats of both sexes at target dose levels of 500, 1000 and 2000 mg/kg body weight/day, for a period of at least 90 days. A control group received untreated control feed. The groups comprised 20 animals per sex, which were sacrificed after 13 weeks of treatment (Allocation A animals). Additional 10 rats per sex and group were used at 0 and 2000 mg/kg bw/day (Allocation B animals). These animals were treated for 13 weeks and then allowed a 28-day treatment-free recovery period, after which they were sacrificed. A further six animals per sex and group were used to assess possible changes in the primary immunological response after 13 weeks of treatment (Allocation C animals).

(Summary continued)

This report consists of Pages 1-4 and Pages 1-1095

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Name	Signature	Date
Thiel A Main Author		29 May 2007
Bausch J Principal Scientist / Competence Mgr		23 May 07
Pasamontes L Research Center Head		11 Jun 2007
Lechevanton L Project Manager		30 May 2007

Summary continued

Clinical signs were recorded daily during the acclimatization, treatment and recovery periods. Food consumption and body weights were recorded weekly during the acclimatization, the treatment and recovery periods. Ophthalmoscopic examinations were performed at acclimatization, the end of the treatment and recovery periods. Functional observational battery, locomotor activity and grip strength were performed during week 13 and 17. During week 13, blood samples were taken from the Allocation C animals for immunotoxicological evaluation. Leukocyte analyses were performed on Allocation B animals after the treatment (week 14) and after the recovery (week 17) periods. Sperm count, motility and morphology were examined in all treatment and control males after 13 (Allocation A) and 17 (Allocation B) weeks. The estrus cycle was determined over a two-week period in all control and treatment group females starting week 10 (Allocation A and B) and week 15 (Allocation B). After 2 and 6 weeks and at the end of the dosing and the treatment-free recovery periods, blood samples were withdrawn for hematology and plasma chemistry analyses. Urine samples were collected for urine analyses and fecal samples were taken for fecal pH measurements. All animals were killed, necropsied and examined post mortem. Histological examinations were performed on organs and tissues from all control and high dose animals of Allocations A and B, and all gross lesions from all animals. The rectums of all animals of all dose groups were examined histopathologically. As microscopical changes were noted in the following organs of animals in the high dose group, the mesenteric lymph nodes and kidneys were examined in all Allocation A and B animals.

The target dose levels were attained. Feed samples were adjusted weekly according to body weights and food consumption of animals. Control feed samples did not contain Ultrazine FG-R. The test item content in all spiked samples was found to be within the accepted range of ± 20% nominal content. Ultrazine FG-R was homogeneously distributed in feed samples. Stability of formulations was confirmed for at least 21 days when kept at room temperature or at -20 °C.
No test item-related mortalities were ascertained in any dose group. Two male rats were killed for ethical reasons unrelated to the test item. Male no. 44 (500 mg/kg bw/day) was killed on day 32 and found to have an intestinal intussusception. Male no. 87 (1000 mg/kg bw/day) had a markedly enlarged right eye, was removed from the study on day 43 and sacrificed. All other animals survived. During daily observations, there was no indication of adverse clinical signs related to the consumption of the test item. Evaluation of the findings recorded after 13 weeks' treatment and four weeks' recovery did not reveal any indication of neurotoxicologic effects of the test item, and no test item-related changes were noted. No test item-related differences in the mean fore- and hind limb grip strength and mean locomotor activity were noted after 13 weeks of treatment or after 4 weeks of recovery. No test item-related ophthalmoscopic findings of toxicological relevance were noted at any dose level after the treatment and recovery periods. No differences of toxicological relevance were noted in the pH of feces from test item-treated rats when compared with the controls (weeks 2, 6 and 13 of treatment and during week 17 of recovery).
No toxicologically relevant differences in sperm motility, sperm morphology or sperm head count were noted at any dose level. After the recovery period, no differences of toxicological relevance were noted in sperm motility, morphology or head count. No test item-related differences in the duration of estrus, diestrus, proestrus or metestrus phases were noted during treatment or recovery at any dose level.
The mean daily food consumption of the test item-treated males and females compared favorably with those of the respective control animals. No differences of toxicological relevance were noted during the treatment period and no late effects were noted during the recovery period in the mean body weights of test item-treated and control rats.
Hematology parameters measured after 2, 6 and 13 weeks of treatment did not indicate any changes of toxicological relevance. After a 4-week recovery period, no late effects were seen in the rats previously treated with the highest dose level. Differences to the control values seen in the clinical biochemistry parameters were not considered to be of toxicological relevance. Differences in electrolyte balance were seen during the treatment period at all dose levels but all recovered to control levels after the recovery period (or in the case of phosphorus, lower than the control value after the recovery period). Therefore, these changes were considered likely to be physiological rather than of toxicological origin. No test item-related

effects on urinalysis parameters or fecal pH were noted at any time during treatment or after the recovery period.
There was no evidence for any changes in primary immune response after 13 weeks of treatment, nor were there toxicologically relevant alterations in leukocyte populations after 13 weeks of treatment or 4 weeks of recovery.
No test item-related effects upon organ weights or ratios were noted after the treatment or recovery periods. No test item-related macroscopic findings were recorded at necropsy at the end of treatment and recovery period. No microscopic findings were seen in the rectal tissue. Microscopically, focal/multifocal aggregates of foamy histiocytes were recorded in mesenteric lymph nodes of animals treated with 500 mg/kg bw/day, 1000 mg/kg bw/day or 2000 mg/kg bw/day. After the recovery period this finding was still recorded in animals treated with 2000 mg/kg bw/day, with a decrease in its severity grade. Therefore a tendency to regression was stated. This finding is of no adverse nature, because of this trend to regression and because of the absence of coexisting tissue damage or reaction. Minimal tubular vacuolation was recorded in kidneys of males treated with 2000 mg/kg bw/day, females treated with 1000 mg/kg bw/day and 2000 mg/kg bw/day, and recovery males and females treated with 2000 mg/kg bw/day. This finding, per se, with non-dose dependant severity grade, in absence of tubular damage or any other sign of renal toxicity or impairment is of no adverse nature.

Conclusion
Based upon the study findings, the no-observed-adverse-effect-level (NOAEL) was considered to be 2000 mg/kg bw/day.

Nomenclature and Structural Formula

Test Article Name:	Ultrazine GF-R (Food Grade Lignosulphonate)
Chemical Name:	Calcium Lignosulfonate
Batch No.:	FGR-004

RCC Study Number: A29553

ULTRAZINE FG-R
(FOOD GRADE LIGNOSULPHONATE)

13-Week Oral Toxicity (Feeding)
Study in the Wistar Rat

Report (Part I of IV)

Study Director:	W. H. Braun
Sponsor:	DSM Nutritional Products AG Wurmisweg 576 CH-4303 Kaiseraugst / Switzerland
Test Facility:	RCC Ltd Toxicology CH-4452 Itingen / Switzerland
Test Guidelines:	"Subchronic Toxicity Studies with Rodents", US Food and Drug Administration, USA. "Repeated Dose 90-Day Oral Toxicity Study in Rodents", OECD Guidelines for the Testing of Chemicals. Directive 96/54/EC, B.26. "Subchronic Oral Toxicity."
Date:	25 May 2007

RCC

RCC STUDY NUMBER A29553 REPORT
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

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ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

GOOD LABORATORY PRACTICE

1 STATEMENT OF COMPLIANCE / GLP GUIDELINES

RCC STUDY NUMBER:	A29553
TEST ITEM:	ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)
STUDY DIRECTOR:	W. H. Braun
TITLE:	ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE): 13-Week Oral Toxicity (Feeding) Study in the Wistar Rat

The pathology peer review was not audited by QA and was therefore excluded from this Statement of Compliance. All other aspects of this study were performed in compliance with the:

Swiss Ordinance relating to Good Laboratory Practice adopted May 18th, 2005 [RS 813.112.1]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C (97)186/Final].

These principles are compatible with Good Laboratory Practice regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA) and Japan (MHLW, MAFF and METI).

There were no circumstances that may have affected the quality or integrity of the data and the study overall.

Study Director: W. H. Braun

Date: 25 May 2007

2 QUALITY ASSURANCE GLP TOXICOLOGY

RCC Ltd, Toxicology, CH-4452 Itingen / Switzerland

STATEMENT

RCC STUDY NUMBER:	A29553
TEST ITEM:	ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)
STUDY DIRECTOR:	W. H. Braun
TITLE:	ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE): 13-Week Oral Toxicity (Feeding) Study in the Wistar Rat

The general facilities and activities are inspected periodically and the results are reported to the responsible person and the management.

Study procedures were periodically audited. The study plan and this report were audited by the Quality Assurance. The dates are given below.

Dates and Types of QA Inspections		Dates of Reports to the Study Director and Test Facility Management
15 Dec 2005	Study plan	15 Dec 2005
29 Dec 2005	Feed preparation, test item, raw data	29 Dec 2005
02 Jan 2006	Treatment, test item, raw data	02 Jan 2006
13 Feb 2006	Fecal pH, test system, raw data	13 Feb 2006
16 Feb 2006	Test item, raw data	16 Feb 2006
07 Mar 2006	Estrus cycle, test system, raw data	07 Mar 2006
04 Apr 2006	Necropsy, test system, raw data	04 Apr 2006
07 Apr 2006	Sperm analysis, raw data	07 Apr 2006
29 Mar 2006	Ophthalmoscopy (process-based)	29 Mar 2006
10 Apr 2006	Clinical laboratory investigations (process-based)	10 Apr 2006
24 Apr 2006	Histotechnique (process-based)	24 Apr 2006
11 - 19 Sept 2006	Draft report	19 Sept 2006
09 - 15 May 2007	Report	15 May 2007

This statement also confirms that this final report reflects the raw data. In addition, this final report includes a QA-Statement issued by the Test Site Quality Assurance.

Quality Assurance for U. Böhlmann

Date: 25 May 2007

3 PREFACE

3.1 GENERAL

Title	ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE): 13-Week Oral Toxicity (Feeding) Study in the Wistar Rat
Sponsor	DSM Nutritional Products AG Wurmisweg 576 CH-4303 Kaiseraugst / Switzerland
Study Monitor	SCC Scientific Consulting Company Chemisch-Wissenschaftliche Beratung GmbH Mikroforum Ring 1 D-55234 Wendelsheim / Germany Attn: Dr. W. Köhl Phone: 0049 67 34 919 0 Fax: 0049 67 34 919 191 eMail: scc@scc-gmbh.de
Test Facility	RCC Ltd Toxicology CH – 4452 Itingen / Switzerland (a) CH – 4414 Füllinsdorf / Switzerland (b)
Test Site(s)	RCC Ltd Environmental Chemistry & Pharmanalytics CH – 4452 Itingen / Switzerland (c) RCC Cytotest Cell Research GmbH In den Leppsteinswiesen 19 D–64380 Rossdorf / Germany (d)
Responsible Scientist	Dr. E. Karbe, Prof. Dr. med. vet. habil. Consultant in Toxicologic Pathology Langendorfer Strasse 17 42489 Wülfrath / Germany (e)
Lead QA	RCC Ltd Quality Assurance GLP Toxicology CH – 4452 Itingen / Switzerland
Test Site QAs	RCC Ltd Quality Assurance GLP Environmental Chemistry & Pharmanalytics CH–4452 Itingen / Switzerland Contact person: T. Fink (Responsible for test site c)

RCC Cytotest Cell Research GmbH
Quality Assurance GLP
In den Leppsteinswiesen 19
D–64380 Rossdorf / Germany
Contact person: F. Hermann
(Responsible for test site d)

3.2 RESPONSIBILITIES

Study Personnel:

Study Director	W. H. Braun (a)
Deputy Study Director	Dr. A. Eichinger-Chapelon (a)
Laboratory Coordinator	R. Sacher (a)
Clinical Laboratory Investigations	R. Draheim (b)
Necropsy/ Histotechnology	Dr. K. Weber (a)
Pathology	Dr. L. Romeo (a)

Principal Investigators:

Study Phase:	
Analytical Chemistry	Dr. D. Flade (c)
Immunotoxicity Parameters	Dr. N. Honarvar (d)

Quality Assurance:

Head of Lead QA	I. Wüthrich

Responsible Scientist:

Peer Reviewing Pathologist	Prof. Dr. med. vet. habil. E. Karbe, PhD. (e)

3.3 SCHEDULE

Experimental Starting Date	27 December 2005
Experimental Completion Date	14 February 2007
Delivery of Animals	27 December 2005
Acclimatization	27 December 2005 to 01 January 2006
Administration/Treatment	02 January 2006 to 03/04/05/06 April 2006
Recovery	04 April 2006 - 02 May 2006
Termination (Necropsy)	04 to 07 April 2006 (Allocation A) 02 May 2006 (Allocation B) 04 to 07 April 2006 (Allocation C)

3.4 ARCHIVING

RCC Ltd (CH-4452 Itingen / Switzerland) will retain the study plan (including any amendment), raw data, sample of test item(s), specimens (as long as the quality permits evaluation) and the final report of the present study for at least ten years. Wet tissue samples will be archived at RCC Ltd for a minimum of five years, after issuing the final report. Thereafter, in agreement with the Sponsor, these samples may be further archived at RCC Ltd or transferred to another GLP archive facility for the remainder of the prescribed period. No data will be discarded without the Sponsor's written consent.

All raw data generated by Principal Investigators will be archived at their own facilities for the duration specified above (10 years)[1]. Insofar as the test site is recognized as GLP-compliant, the test site management is responsible for archiving the raw data, although RCC reserves the right to conduct GLP inspections of archived data pertaining to this study.

3.5 TEST GUIDELINES

The study procedures described in this report meet or exceed with the following guidelines:

“Subchronic Toxicity Studies with Rodents”, Section IV.C.4.a., Toxicological Principles for the Safety Assessment of Food Ingredients, Office of Food Additive Safety, Redbook 2000, US Food and Drug Administration, USA.

"Repeated Dose 90-Day Oral Toxicity Study in Rodents", OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects, Number 408, 21 September 1998.

Directive 96/54/EC, B. 26. "Subchronic Oral Toxicity", 30 September 1996, including Additional Testing for Neurotoxicity.

Test guidelines pertaining to the specific requirements of work to be performed by the Principle Investigators are listed in the respective raw data and phase reports.

3.6 STUDY PLAN AMENDMENTS (SUMMARY)

Amendment 1: Methods for seminology described in detail; clarification of methods for immunology.

Amendment 2: Rescheduling for clinical laboratory investigations.

Amendment 3: Change in the planned number of evaluations for seminology.

Amendment 4: Clarification of methods for immunology.

Amendment 5: Change of study pathologist.

Amendment 6: Clarification of clinical pathologist; addition of peer reviewing pathologist; clarification of test item spelling.

[1] RCC-CCR will archive the raw data of the Immunotoxicity phase and the original phase report for 15 years.

3.7 STUDY PLAN DEVIATIONS (SUMMARY)

Samples for analysis of feed concentrations were taken from all dose levels, instead of from only the low and high dose.

Weekly detailed observations (weeks 1-12 and 14-16) were omitted due to a technical oversight. However, in view of the relatively high doses used in this study, the absence of any possible neurological changes during the functional observational battery at week 13, and the absence of any indications during the daily observations, this was not considered to have affected the evaluation of this test item in this study.

The relative humidity of the room appeared to be below the lower limit of 30% briefly on 23 January 2006 but this was considered to be due to a data communication error rather than a sudden drop and recovery of the relative humidity.

3.8 ANIMAL WELFARE

This study was performed in an AAALAC-approved (Association for the Assessment and Accreditation of Laboratory Animal Care) laboratory in accordance with the Swiss Animal Protection Law under license no. 263.

3.9 ACCREDITATION

“RCC Ltd, Toxicology” is accredited as a testing laboratory for analysis in the fields of clinical chemistry, hematology, blood-coagulation and urine diagnostics in accordance with the Standard ISO/IEC 17025 under accreditation number STS 085 by the Swiss Accreditation Service.

4 SIGNATURES

Study Director: W. H. Braun

Date: 25 May 2007

Management: Dr. H. Fankhauser

Date: 25 May 2007

The signature of Dr. L. Romeo (Study Pathologist) is included in the attached report.
The signatures of the respective PIs are included in the respective phase reports.

5 SUMMARY

GENERAL

In this subchronic toxicity study, ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE) was administered daily via the feed to SPF-bred Wistar rats of both sexes at target dose levels of 500, 1000 and 2000 mg/kg body weight/day, for a period of at least 90 days. A control group received untreated control feed.

The groups comprised 20 animals per sex, which were sacrificed after 13 weeks of treatment (Allocation A animals). Additional 10 rats per sex and group were used at 0 and 2000 mg/kg bw/day (Allocation B animals). These animals were treated for 13 weeks and then allowed a 28-day treatment-free recovery period, after which they were sacrificed. A further six animals per sex and group were used to assess possible changes in the primary immunological response after 13 weeks of treatment (Allocation C animals).

Clinical signs were recorded daily during the acclimatization, treatment and recovery periods. Food consumption and body weights were recorded weekly during the acclimatization, the treatment and recovery periods. Ophthalmoscopic examinations were performed at acclimatization, the end of the treatment and recovery periods. Functional observational battery, locomotor activity and grip strength were performed during week 13 and 17.

During week 13, blood samples were taken from the Allocation C animals for immunotoxicological evaluation. Leukocyte analyses were performed on Allocation B animals after the treatment (week 14) and after the recovery (week 17) periods.

Sperm count, motility and morphology were examined in all treatment and control males after 13 (Allocation A) and 17 (Allocation B) weeks. The estrus cycle was determined over a two-week period in all control and treatment group females starting week 10 (Allocation A and B) and week 15 (Allocation B).

After 2 and 6 weeks and at the end of the dosing and the treatment-free recovery periods, blood samples were withdrawn for hematology and plasma chemistry analyses. Urine samples were collected for urine analyses and fecal samples were taken for fecal pH measurements. All animals were killed, necropsied and examined post mortem. Histological examinations were performed on organs and tissues from all control and high dose animals of Allocations A and B, and all gross lesions from all animals. The rectums of all animals of all dose groups were examined histopathologically. As microscopical changes were noted in the following organs of animals in the high dose group, the mesenteric lymph nodes and kidneys were examined in all Allocation A and B animals to establish a no adverse effect level.

FEED ANALYSES

Feed samples were adjusted weekly according to body weights and food consumption of animals. Control feed samples did not contain Ultrazine FG-R. The test item content in all spiked samples was found to be within the accepted range of ± 20% nominal content, except in three outlier samples. Ultrazine FG-R was homogeneously distributed in feed samples at all dose levels with variations in concentration of ≤ 15.2%. Stability of formulations was confirmed for at least 21 days when kept at room temperature or at -20 °C.

MORTALITY / VIABILITY

No test item-related mortalities were ascertained in any dose group.

Two male rats were killed for ethical reasons unrelated to the test item.

- Male no. 44 (500 mg/kg bw/day) was killed on 02-Feb-2006 (day 32) and found to have an intestinal intussusception;
- Male no. 87 (1000 mg/kg bw/day) had a markedly enlarged right eye, was removed from the study on 13-Feb-2006 (day 43) and sacrificed.

CLINICAL SIGNS

During daily observations, there was no indication of adverse clinical signs related to the consumption of the test item.

FUNCTIONAL OBSERVATIONAL BATTERY

Evaluation of the findings recorded after 13 weeks' treatment and four weeks' recovery did not reveal any indication of neurotoxicologic effects of the test item, and no test item-related changes were noted.

Grip Strength and Locomotor Activity

No test item-related differences in the mean fore- and hind limb grip strength and mean locomotor activity were noted after 13 weeks of treatment or after 4 weeks of recovery.

OPHTHALMOSCOPIC FINDINGS

No test item-related ophthalmoscopic findings of toxicological relevance were noted at any dose level after the treatment and recovery periods.

FECAL PH

No differences of toxicological relevance were noted in the pH of feces from test item-treated rats when compared with the controls (weeks 2, 6 and 13 of treatment and during week 17 of recovery).

SEMINOLOGY

No toxicologically relevant differences in sperm motility, sperm morphology or sperm head count were noted at any dose level.

After the recovery period, no differences of toxicological relevance were noted in sperm motility, morphology or head count.

ESTROUS CYCLES

No test item-related differences in the duration of estrus, diestrus, proestrus or metestrus phases were noted during treatment or recovery at any dose level.

FOOD CONSUMPTION

The mean daily food consumption of the test item-treated males and females compared favorably with those of the respective control animals.

BODY WEIGHT

No differences of toxicological relevance were noted during the treatment period and no late effects were noted during the recovery period in the mean body weights of test item-treated and control rats.

TEST ITEM INTAKE

The target dose levels were attained.

Males consumed -0.8%, -0.6% and -1.13% of the target dose levels of 500, 1000 and 2000 mg/kg bw/day. Females consumed +1.3%, +1.8% and +2.0% of the target dose levels of 500, 1000 and 2000 mg/kg bw/day. The values were not corrected for analytical concentrations.

CLINICAL LABORATORY INVESTIGATIONS

Hematology parameters measured after 2, 6 and 13 weeks of treatment did not indicate any changes of toxicological relevance. After a 4-week recovery period, no late effects were seen in the rats previously treated with the highest dose level.

Differences to the control values seen in the clinical biochemistry parameters were not considered to be of toxicological relevance. Differences in electrolyte balance were seen during the treatment period at all dose levels but all recovered to control levels after the recovery period (or in the case of phosphorus, lower than the control value after the recovery period). Therefore, these changes were considered likely to be physiological rather than of toxicological origin.

No test item-related effects on urinalysis parameters or fecal pH were noted at any time during treatment or after the recovery period.

IMMUNOTOXICITY

There was no evidence for any changes in primary immune response after 13 weeks of treatment, nor were there toxicologically relevant alterations in leukocyte populations after 13 weeks of treatment or 4 weeks of recovery.

ORGAN WEIGHTS

No test item-related effects upon organ weights or ratios were noted after the treatment or recovery periods.

MACROSCOPIC / MICROSCOPIC FINDINGS

There was one premature death; one male animal (male no. 44) treated with 500 mg/kg bw/day was killed in extremis on treatment day 32 of the study. The possible cause of death of this animal was either an intestinal intussusception and/or pre-existing polycystic kidney disease. Male no. 87 (group 3), which had a markedly enlarged right eye (not test item related), was removed from the study on day 43 for ethical reasons. Neither of these deaths was test item related. All other animals survived their scheduled study period.

No test item-related macroscopic findings were recorded at necropsy at the end of treatment and recovery period.

No microscopic findings were seen in the rectal tissue.

Microscopically, focal/multifocal aggregates of foamy histiocytes were recorded in mesenteric lymph nodes of animals treated with 500 mg/kg bw/day, 1000 mg/kg bw/day or 2000 mg/kg bw/day. After the recovery period this finding was still recorded in animals treated with 2000 mg/kg bw/day, with a decrease in its severity grade. Therefore a tendency to regression was stated. This finding is of no adverse nature, because of this trend to regression and because of the absence of coexisting tissue damage or reaction.

Minimal tubular vacuolation was recorded in kidneys of males treated with 2000 mg/kg bw/day, females treated with 1000 mg/kg bw/day and 2000 mg/kg bw/day, and recovery males and females treated with 2000 mg/kg bw/day. This finding, per se, with non-dose dependant severity grade, in absence of tubular damage or any other sign of renal toxicity or impairment is of no adverse nature.

CONCLUSION

Based upon the study findings, the no-observed-adverse-effect-level (NOAEL) was considered to be 2000 mg/kg bw/day.

6 OBJECTIVE

6.1 PURPOSE AND RATIONALE

The purpose of this oral toxicity study was to assess the possible cumulative toxicity of ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE) when administered to rats in their diet for a period of at least 13 weeks (Allocation A and B).

In addition, the reversibility or progression of any test item-related changes were assessed in Allocation B animals after a 4- week treatment-free recovery period. Immunotoxicological parameters were investigated in the Allocation C animals.

This study provides a rational basis for a risk assessment in man, indicates potential target organs and permits evaluation of neurotoxic potential.

7 MATERIALS AND METHODS

7.1 TEST SYSTEM

Test system	Rat, HanRcc:WIST (SPF)
Rationale	Recognized by the international guidelines as the recommended test system.
Source	RCC Ltd Laboratory Animal Services CH-4414 Füllinsdorf / Switzerland
Group allocation	Groups 1 and 4: 72 males and 72 females Groups 2 and 3: 52 males and 52 females
Total number of animals used	124 males; 124 females
Total number of animals ordered	127 males; 127 females
Age at delivery	5 weeks
Body weight range at acclimatization	Males: 95 - 122 grams (mean 110 grams) Females: 86 - 109 grams (mean 97 grams)
Identification: Acclimatization Treatment	 Cage card and tail mark (later ear tattoo) Cage card and individual ear tattoo
Randomization	Computer-generated random algorithm
Acclimatization	Seven days under test conditions after health examination. Only animals without any visible signs of illness were used for the study.

7.2 ALLOCATION

Allocation And Target Dose Levels	Group 1* 0 mg/kg bw/day	Group 2 500 mg/kg bw/day	Group 3 1000 mg/kg bw/day	Group 4 2000 mg/kg bw/day
Males A	1 – 20	37 - 56	63 - 82	89 – 108
Males B	21 – 30			109 – 118
Males C	31 – 36	57 - 62	83 - 88	119 – 124
Females A	125 – 144	161 – 180	187 – 206	213 – 232
Females B	145 – 154			233 – 242
Females C	155 - 160	181 - 186	207 - 212	243 – 248

A – Main Study (termination after 13 weeks of treatment)
B – Recovery (termination after 13 weeks of treatment and 4 weeks of recovery)
C – Immunotoxicity parameters (termination after 13 weeks of treatment)
* – Control animals were treated with control diet only.

7.3 HUSBANDRY

Room number	139 1OG; and 135 1OG, RCC Itingen
Conditions	Standard Laboratory Conditions. Air-conditioned with 10-15 air changes per hour, and continuously monitored environmental conditions (temperature range: 22 ± 3 °C; relative humidity range: 30-70%). There was 12-hour fluorescent light/12-hour dark cycle with music during the light period.
Accommodation	Individually in Makrolon type-3 cages with wire mesh tops and standardized softwood bedding ('Lignocel' Schill AG, CH-4132 Muttenz/Switzerland, batch nos 0210551208, 0210560118 and 0210560222, analytical certificates are included in the raw data file).
Water	Community tap-water from Itingen was available *ad libitum* in water bottles. Results of bacteriological assay, chemical and contaminant analyses of representative samples are attached to this report (see Appendix II).
Diet	Pelleted standard Provimi Kliba 3433 (batch no. 2/06, 79/05, 67/05) rat maintenance diet (Provimi Kliba AG, CH-4303 Kaiseraugst/ Switzerland) was available *ad libitum*. The feed batch was analyzed for contaminants (see Appendix III). None of the contaminants analyzed in the water and diet was considered to have been present at a concentration that would have affected the validity of the results.

7.4 TEST ITEM

Test item and test item data were provided by the sponsor.

Identity	ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)
Chemical name	Calcium Lignosulphonate
CAS no.	8061-52-7
Batch number	FG-R 004
Description	Brown
Purity	95.5%
Stability of test item	Stable under storage conditions
Expiry Date	August 26, 2007
Storage	Dry at room temperature
Safety precautions	Routine hygienic procedures (gloves, goggles, face mask)
Analytical standard	ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE) (the test item served as its own analytical standard)

7.5 FEED PREPARATION

Dietary admixtures were prepared with material as supplied.

Fresh batches of the feed pellets for this study were prepared weekly, based upon the food consumption and body weights.

The test item was weighed into a tared glass beaker on a suitable precision balance, and mixed with microgranulated feed separately for each dose group. Water (ca 1:10 volume/weight/ratio) was added to aid pelleting. However, no vehicle was used. The pellets were dried with air (maximum 45 °C) for ca. 48 hours before storage.

Control feed for the animals of group 1 was prepared similarly, but without test item.

7.5.1 ANALYSIS OF FEED PREPARATIONS

Samples of the feed samples were analyzed for concentration and homogeneity of the test item in the feed before initiating the study. Stability of the lowest and highest concentrations of the test item in feed was assessed up to 21 days at room temperature and at –20 °C in RCC Study number A29992 (with target levels of 100 and 1000 mg/kg/day, respectively).

Analysis of dose concentration and homogeneity was carried out on all dose groups at the beginning, after 7 weeks and at the end of the study. Analyses of feed test item concentrations were carried out after each adjustment (once weekly for 13 weeks, based on body weight gain and feed consumption noted in the previous week).

The analyses were performed in the Analytical Laboratories of RCC Ltd, based on an analytical method supplied by the Sponsor, which was adapted and modified by RCC, and a phase report was prepared by Dr. D. Flade; see Appendix IV.

7.5.2 STORAGE OF FEED PREPARATIONS

Feed preparations were stored at room temperature (ca. 15-25 °C) in disposable paper bags.

Samples of each feed batch will be retained at –20 °C until three months after the final report is issued or until written permission for disposal is given by the sponsor, whichever comes first.

Alternatively, the deep-frozen feed samples will be sent to the sponsor upon request and a suitable notice to the raw data will be made.

7.6 TREATMENT

Method	Oral, by feeding, adjusted at least weekly.
Rationale	Oral ingestion is a route of human exposure.
Daily target dose levels	Group 1: 0 mg/kg body weight Group 2: 500 mg/kg body weight Group 3: 1000 mg/kg body weight Group 4: 2000 mg/kg body weight
Rationale for dose selection*	Doses were selected based on a previous 28-day feeding study in Wistar rats, using dose levels of 0, 500, 1500 and 4000 mg/kg bw/day, resulting in a NOEL of 1500 mg/kg bw/day. *Based upon information provided by the sponsor.
Frequency of administration	*Ad libitum*
Duration of acclimatization period	6 days
Duration of treatment	91-94 days
Duration of recovery period	29 days

7.7 OBSERVATIONS

7.7.1 MORTALITY / VIABILITY

Observations for mortality/viability were recorded twice daily.

7.7.2 GENERAL CAGESIDE OBSERVATIONS (DAILY)

The animals were observed for clinical signs once before commencement of administration, and once daily during the treatment phase, and once daily during the recovery phase

7.7.3 WEEKLY DETAILED OBSERVATIONS (WEEKS 1-12)

The weekly detailed observations were inadvertently omitted. However, in view of the relatively high doses used in this study, the absence of any possible neurological changes during the functional observational battery at week 13, and the absence of any indications during the daily observations, this was not considered to have affected the evaluation of this test item in this study.

7.7.4 FOOD CONSUMPTION

The food consumption was recorded once during the acclimatization period and weekly thereafter, using an on-line electronic recording system consisting of a Mettler balance connected to the RCC computer.

7.7.5 BODY WEIGHTS

Body weights were recorded weekly during acclimatization, treatment and recovery and before necropsy, using an on-line electronic recording system consisting of a Mettler balance connected to the RCC computer.

7.7.6 OPHTHALMOSCOPIC EXAMINATIONS

The ophthalmoscopic examinations of both eyes of all animals were performed after the application of a mydriatic solution (Ciba Vision AG, CH-3172 Niederwangen) using a Miroflex 2 Ophthalmoscope (Eisenhut Vet. AG, CH-4123 Allschwil). A description of any abnormality was recorded. For unilateral findings unless otherwise indicated in the tables, the contralateral eye was without abnormalities.

All Allocation A and B animals were examined during acclimatization, all Allocation A and B animals of the control and high dose groups were examined during week 13 of treatment and all Allocation B animals of the control and high dose groups were examined during week 17 (week 4 of recovery).

7.7.7 FECAL PH

Fecal samples were taken using metabolism cages after weeks 2, 6 and 13 in all animals (Allocation A and B), and during week 17 in control and high dose animals (Allocation B). The pH of fecal samples was measured. Fecal samples were collected overnight from rats housed in metabolism cages. After the pH of the diluent (ie bidistilled or tap water) was measured, and the fecal samples were weighed, they were mixed with the diluent at a ratio of 1:10 weight/volume. Thereafter they were homogenized with an Ultra-Turrax. The pH of the resulting slurry was measured and recorded.

7.7.8 SPERM ANALYSIS

Sperm count, motility and morphology were assessed at necropsy in all control and treatment group males after 13 weeks (Allocation A) and 17 weeks (Allocation B).

Seminology and Spermatid Count	Sperm count, motility and morphology were assessed at necropsy in all control and treatment group males after 13 weeks (Allocation A) and 17 weeks (Allocation B).
Motility	At necropsy of adult males an epididymal sperm sample was obtained from the left caudal epididymidis of each male. The sample was diluted with a prewarmed (about 35 °C) physiological medium, and within one hour after being obtained, one hundred sperm (100) were counted microscopically for determination of percentage of not motile, stationary motile and progressively motile sperm.

Morphology	An additional sperm sample from the caudal epididymidis was taken and used for morphological assessment after fixation and eosin staining. Five hundred (500) sperm per sample were evaluated microscopically and classified into the following categories:

Code	Description
A	Sperm with normal hook and tail
B	Normal hook without tail
C	Misshapen sperm hook with tail
D	Sperm with abnormally curved hook with tail
E	Sperm with reversed hook with tail
F	Abnormal hook without tail

Sperm, spermatid count	The left caudal epididymis and left testis were taken for determination of homogenization-resistant spermatids and caudal epididymal sperm reserve. These tissues were frozen at -20 ± 5 °C pending evaluation. For evaluation, the weighed tissues were placed in Triton-X-100 solution and homogenized with a blender (Ultra Turrax) and an ultrasonic water bath. Sperm or spermatid heads were counted microscopically using a Neubauer chamber.

7.7.9 ESTRUS CYCLE MEASUREMENT

The estrus cycles were determined for a two-week period from vaginal smears taken from control and treatment group females starting week 10 (Allocation A and B) and week 15 (Allocation B).

7.7.10 FUNCTIONAL OBSERVATIONAL BATTERY

During week 13, relevant parameters (presented in Appendix I) from a modified Irwin screen test were evaluated in all animals of Allocations A and B. During week 17, relevant parameters (presented in Appendix I) from a modified Irwin screen test were evaluated in all animals of Allocations B.

NB. The results of the Functional Observational Battery are presented in the summary and individual tables of the Detailed Clinical Observations (Weekly) under weeks 13 and 17. This method of data presentation permits a clear evaluation and assessment of weekly clinical signs observed during the study.

GRIP STRENGTH

Forelimb and hind limb grip strength measurements were performed using a push-pull strain gauge (Mecmesin, AFG 25N). The animals were placed with the forepaws inside a triangular grasping ring and with the hind paws outside a triangular grasping ring. Using one hand, the animals were held towards the base of the tail and steadily pulled away or towards the ring until the grip was broken. Each measurement was repeated three times, the means were calculated and recorded.

LOCOMOTOR ACTIVITY

Locomotor (decreased or increased) activity was measured quantitatively with AMS Föhr Medical Instruments GmbH (FMI) and DeMeTec GmbH Activity Monitor System. Animals were monitored during treatment week 13 for a 60-minute period and the total activity of this time period was recorded. Low beams count was reported in 10-minute intervals as well as the total activity of the measuring period.

7.8 CLINICAL LABORATORY INVESTIGATIONS

Blood and urine sampling:

After 2 weeks	17 and 18 January 2006 (males and females, respectively), Allocations A and B
After 6 weeks	13 and 14 February 2006 (Allocations A and B)
After 13 weeks	04 to 07 April 2006 (Allocations A and B)
After 17 weeks (recovery)	02 May 2006 (Allocation B)

Blood samples for hematology and clinical biochemistry were collected from all animals under light isoflurane anesthesia. The animals were fasted in metabolism cages for approximately 18 hours before blood sampling but allowed access to water *ad libitum*. Blood samples were collected early in the working day to reduce biological variation caused by circadian rhythms. Blood samples were drawn from the retro-orbital plexus using a micro-hematocrit glass capillary tube.

Urine was collected during the 18-hour fasting period into a specimen vial.

All assays were performed at RCC Ltd (Füllinsdorf).

In the summary and individual tables the names of some parameters have been abbreviated. Any abbreviation has been defined in Appendix V.

Detailed methodology, abbreviations and general remarks are described in Appendix V.

Clinical laboratory data are expressed, with a few exceptions, in general accordance with the International System of Units (SI).

7.8.1 HEMATOLOGY

The following hematology parameters were determined:

Erythrocyte count	Reticulocyte count
Hemoglobin	Reticulocyte maturity index
Hematocrit	Methemoglobin
Mean corpuscular volume	Heinz bodies
Red cell volume distribution width	Total leukocyte count
Mean corpuscular hemoglobin	Differential leukocyte count
Mean corpuscular hemoglobin concentration	Coagulation:
Hemoglobin concentration distribution width	Thromboplastin time
Platelet (thrombocyte) count	Activated partial thromboplastin time

7.8.2 CLINICAL BIOCHEMISTRY

The following clinical biochemistry parameters were determined:

Glucose	Creatine kinase
Urea	Alkaline phosphatase
Blood urea nitrogen	Gamma-glutamyl-transferase
Creatinine	Sodium
Bilirubin, total	Potassium
Cholesterol, total	Chloride
Triglycerides	Calcium
Phospholipids	Phosphorus inorganic
Aspartate aminotransferase	Protein, total
Alanine aminotransferase	Albumin
Lactate dehydrogenase	Globulin
Glutamate dehydrogenase	Albumin/Globulin ratio

7.8.3 URINALYSIS

The following urinalysis parameters were determined:

Volume (18 hours)	Glucose
Specific gravity (relative density)	Ketones
Osmolality	Urobilinogen
Color	Bilirubin
Appearance	Erythrocytes
pH	Leukocytes
Nitrite	Sediment (if present)
Protein	

7.9 IMMUNOTOXICOLOGICAL INVESTIGATIONS

The immunotoxicological investigations on serum and/or fresh blood were performed at RCC Cytotest Cell Research GmbH under the phase number 917300. A phase report containing the results of both analyses (see 7.9.1 and 7.9.2) was prepared by the PI (Dr. N. Honarvar, Test site (d), see 3.2 Responsibilities) responsible for Immunotoxicological Investigations and is attached (see Appendix VI).

7.9.1 ANALYSIS OF PRIMARY IMMUNE RESPONSE TO IMMUNOGEN (TYPE 2 BASIC)

For the determination of the primary immune response, all Allocation C animals were treated with a sheep erythrocyte suspension (approx. 4 x 10^8 cells/ml in 0.9% NaCl). This suspension was provided to RCC by the responsible Test Site (Test site (d), see 3.2 Responsibilities) and was kept refrigerated (2-8 °C) until use. Five days before necropsy, all animals of Allocation C (groups 1-4) were immunized (intravenous bolus injection into the tail vein) with 0.5 ml of the erythrocyte suspension. At terminal necropsy, blood samples were taken from the immunized animals. Serum was isolated from the blood samples and sent on dry ice to the PI (Dr. N. Honarvar, Test site (d), see 3.2 Responsibilities). The Test Site will retain any remaining sample material for three months after the completion of the final report. No test material will be discarded without the sponsor's written consent.

Further details of the methods used are described in the attached phase report

Immunization	30 March to 02 April 2006 (Allocation C)
Blood sampling after 13 weeks	04 to 07 April 2006 (Allocation C)

7.9.2 ANALYSIS OF LEUKOCYTE POPULATIONS IN BLOOD (LEVEL 1 EXTENDED)

Sampling:

After 13 weeks	04 to 07 April 2006 (Allocation B)
After 17 weeks	02 May 2006 (Allocation B)

EDTA-blood samples (approx. 0.5 ml) were taken from all Allocation B animals per group and sex at the end of treatment and at the end of the recovery period. The blood tubes were immediately sent in a container (at ambient temperature) to the Principal Investigator (Dr. N. Honarvar, Test site (d), see 3.2 Responsibilities) for analysis by flow cytometry.

All blood samples were taken for CD3+/CD4+ T-lymphocytes, CD3+/CD8+ T-lymphocytes, CD45RA+ Cells (= B-lymphocytes) and CD11b+ cells (= monocytes & granulocytes) determination.

Further details of the methods used are described in the attached phase report.

7.10 PATHOLOGY

7.10.1 NECROPSY

Sacrifice:

After 13 weeks	04-07 April 2006 (Allocations A and C)
After 17 weeks (recovery)	02 May 2006 (Allocation B)

All animals were weighed and necropsied. Descriptions of all macroscopic abnormalities were recorded. All animals surviving to scheduled necropsy and all moribund animals were anesthetized by intraperitoneal injection of sodium pentobarbitone and killed by exsanguination. Samples of the following tissues and organs were collected from all animals at necropsy and fixed in neutral phosphate buffered 4% formaldehyde solution (unless otherwise indicated):

Adrenal glands	**Nasal turbinates**
Aorta	**Ovaries**
Bone (sternum, femur including joint)	**Pancreas**
Bone marrow (sternum)	**Pituitary gland**
Brain (4 levels, incl. cerebrum, cerebellum, medulla/pons)	**Prostate gland**
	Rectum
Cecum	**Salivary glands - mandibular, sublingual, parotid**
Colon	**Sciatic nerve**
Duodenum	**Seminal vesicles**
Epididymides (fixed in Bouin's solution)	**Skeletal muscle (thigh)**
Esophagus	**Skin**
Eyes with optic nerve (fixed in Davidson's solution)	**Spinal cord - cervical, midthoracic, lumbar**
	Spleen*
Harderian glands	**Stomach**
Heart	**Testes (fixed in Bouin's solution)**
Ileum, with Peyer's patches*	**Thymus**
Jejunum with Peyer's patches*	**Thyroid (incl. parathyroid gland, if possible)**
Kidneys	Tongue**
Lacrimal gland, exorbital**	**Trachea**
Liver	**Urinary bladder, infused w/formalin at necropsy**
Lungs, infused w/formalin at necropsy	**Uterus/cervix**
Lymph nodes* – mandibular, bronchial, mesenteric	**Vagina**
	Zymbal's gland
Mammary gland area	**All gross lesions**

* with particular attention to possible immunotoxic response
** only tissues in bold typing were processed for histopathology

7.10.2 ABSOLUTE AND RELATIVE ORGAN WEIGHTS

The following organ weights were recorded on the scheduled dates of necropsy:

Brain	Thymus	Spleen	Thyroid/parathyroid
Heart	Kidneys	Testes	Ovaries
Liver	Adrenals	Epididymides	Uterus

The organ to terminal body weight ratios as well as organ to brain weight ratios were calculated.

The determination of the terminal body weight was performed immediately prior to necropsy.

The organ weights of the Allocation C animals (foreseen for immunotoxicity testing) were included in this report (pp. 164-169), but were not used for evaluation of possible toxicological effects.

7.10.3 HISTOTECHNIQUE

All organ and tissue samples, as defined under Histopathology (following), were processed, embedded and cut at an approximate thickness of 2 to 4 micrometers, and stained with hematoxylin and eosin.

7.10.4 HISTOPATHOLOGY

Slides of all organs and tissues listed in boldface type (see 7.10.1, Necropsy, above) that were collected at scheduled sacrifice from the animals of control and high-dose groups were examined by a pathologist.

Organ and tissue samples taken from animals which died spontaneously or which were killed in extremis were evaluated similarly to those organs taken from animals of the high-dose group.

The rectums of all animals of dose groups were examined histopathologically.

As test item-related morphologic changes were detected in the organs of high-dose animals, the same organs (the mesenteric lymph nodes and kidneys) from animals of the mid- and low-dose groups were examined.

7.11 DATA COMPILATION

7.11.1 GENERAL

The RCC-TOX LIMS computer was used to sort and present suitable data for inclusion in the report. All electronically recorded data are conserved on a magnetic medium.

Individual values were rounded before printing. All derived values that appear in the tables represent the rounded results of calculations that used the exact raw data value.

Locomotor activity was recorded on-line, and the results were printed and transcribed into the computer system for compilation and analysis.

Daily clinical signs as well as data from the functional observational battery and grip strength were recorded on data sheets and transcribed into the computer system for compilation and analysis.

7.12 DATA CALCULATION

7.12.1 FOOD CONSUMPTION

The food consumption was calculated per rat and per food consumption interval. It expresses the average food consumed per animal and per day over the food consumption interval.

$$FC = \frac{C}{AD}$$

where

FC is Food consumption in grams of food per animal and day;
C is measured food consumption in grams per cage over the consumption interval and
AD is total consumption days over all animals in the cage during the consumption interval.

7.12.2 RELATIVE FOOD CONSUMPTION

The relative food consumption was calculated according to the following formula:

$$RFC = \left[\frac{FC}{BW(i)} \right] \times 1000$$

where

BW(i) is the most ideal body weight in grams or the body weight (of the corresponding rats) recorded on the day most close to the middle of the food consumption interval. In cases of equal "closeness" of two body weight records the latter one was chosen;
RFC is relative food consumption in grams of food per kg body weight and day, and
FC is food consumption in grams of food per animal and day.

7.12.3 TEST ITEM INTAKE

The test item intake was calculated for each cage according to the following formula:

TAI = (C x ND) / AD * BW(i)

C measured cage food consumption over the consumption interval (in grams food)
ND Nominal dose (mg test item / gram food)
AD Total of consumption days of the animals in the cage during the consumption interval.
BW(i) Most ideal body weight (in gram) or the body weight (of the corresponding rats) recorded on the day most close to the middle of the food consumption interval. In cases of equal "closeness" of two body weight records the latter one was chosen.

7.13 STATISTICAL ANALYSIS

The following statistical methods were used to analyze the grip strength, locomotor activity, food consumption, relative food consumption, body weight, body weight gain, ophthalmoscopic findings, clinical laboratory data, seminological data and estrus cycles, macroscopical findings, organ weights and ratios:

- The Dunnett-test (many to one t-test, or T-Test as appropriate) based on a pooled variance estimate were applied if the variables could be assumed to follow a normal distribution for the comparison of the treated groups and the control groups for each sex.
- The Steel-test (many-one rank test) were applied instead of the Dunnett-test when the data can not be assumed to follow a normal distribution.
- Chi-Square, Kruskal-Wallis or Fisher's exact-test.

References :

C.W. Dunnett: A Multiple Comparison Procedure for Comparing Several Treatments with a Control, J. Amer. Stat. Assoc. 50, 1096-1121 (1955).

S.C. Gad and C.S. Weil: Statistics and Experimental Design for Toxicologists. The Telford Press, Caldwell, New Jersey, 43-45 (1986).

R.G. Miller: Simultaneous Statistical Inference, Springer Verlag, New York (1981).

R.A. Fisher: Statistical Methods for Research Workers, Oliver and Boyd, Edinburgh (1950).

8 RESULTS

8.1 FEED ANALYSES

See Appendix IV

The linearity of the analytical systems used for sample analyses was demonstrated with a good relationship between absorbance measured and working standard concentrations. All used calibration points met the acceptance limit of ± 20% variation from the calibration curve, derived by linear regression analysis. Correlation coefficients calculated were found to be better than 0.99.

A reference standard, which contained blank diet only, was used to determine the absence of test item in control samples. The reference solution obtained showed a UV absorbance of 0.145. Measured solutions derived from control samples (group 1) showed UV absorbances of 0.110 (29-Dec-05), 0.109 (10-Mar-06) and 0.123 (22-Mar-06). The control sample results are lower than that of the reference and, thus, it was confirmed that only control feed was applied within the control experiment.

All individual feed samples of dose groups 2, 3 and 4 (500, 1000 and 2000 mg/kg/day, respectively) investigated during the study were found to comprise Ultrazine FG-R in the range from 80.5% to 118.2%, except three (dose group 3 female, 78.1%, middle, prepared 29 December 2005, dose group 2 male, 79.6%, bottom, prepared 10 March 2006, dose group 2 female, 120.4%, top, prepared 22 March 2006). Considering the results obtained, the samples with low recovery were considered outliers. Thus, for individual samples and preparation means the required content limit of ± 20%, with reference to the nominal concentrations, was not exceeded.

The homogenous distribution of Ultrazine FG-R in the preparations was approved, because single samples did not deviate more than 15.2% from the corresponding mean.

Stability of formulations was determined during RCC study number 29992 and confirmed for at least 21 days when kept at room temperature or at -20 °C (deviation ≤ 16.5% from mean). The doses used in RCC study number 29992 and the stability results were considered to be representative for this test item's behavior in feed admixture.

8.2 VIABILITY/MORTALITY

See pp. 174

No test item-related mortalities were ascertained in any dose group.

Two male rats were killed for ethical reasons unrelated to the test item.

- Male no. 44 (500 mg/kg bw/day) was killed on day 32 (02-Feb-2006) and found to have an intestinal intussusception,
- Male no. 87 (1000 mg/kg bw/day) had a markedly enlarged right eye, was removed from the study on day 43 (13-Feb-2006) and sacrificed.

All other animals survived until their respective necropsy dates.

8.3 OBSERVATIONS

8.3.1 GENERAL OBSERVATIONS (DAILY)

See pp. 57 and 182

A number of typical clinical signs were recorded in animals of all dose groups. There was no indication of adverse clinical signs related to the consumption of the test item.

A subcutaneous nodule noted at week 13 of treatment in the left inguinal region of one female treated with 500 mg/kg bw/day. A similar change was not seen in any rat of the high dose group and therefore this single finding was not considered to be related to the treatment with the test item.

8.3.2 WEEKLY DETAILED OBSERVATIONS

The weekly observations were not recorded due to a planning error. However, in view of the relatively high doses used in this study, the absence of any possible neurological changes during the functional observational battery at week 13, and the absence of any indications during the daily observations, this was not considered to have affected the evaluation of this test item in this study.

8.3.3 FUNCTIONAL OBSERVATIONAL BATTERY

See Appendix I

Evaluation of the findings recorded after 13 weeks' treatment or 4 weeks' recovery did not reveal any indications of neurotoxicologic effects of the test item.

Grip Strength

See pp. 89 and 263

In males treated with 2000 mg/kg bw/day, the mean fore limb grip strength values were significantly reduced ($p<0.05$), whereas females treated with 1000 mg/kg/day had significantly reduced hind limb grip strength values ($p<0.05$). The differences between control and treated group were considered to be of no toxicological relevance, insofar as consistent reductions were not seen in the other extremities. All other animals compared favorably.

No differences were noted in animals measured after the recovery period.

Locomotor Activity

See pp. 91 and 268

No test item-related differences in the mean locomotor activity were noted after 13 weeks of treatment or after 4 weeks of recovery.

During the treatment period, statistically significant decreased locomotor activity was noted in males treated with 500 mg/kg bw/day at 0-10 minutes ($p<0.01$), 10-20 minutes ($p<0.05$), 50-60 minutes ($p<0.05$) and 0-60 minutes (overall, $p<0.01$). In females treated with 500 mg/kg bw/day, decreased mean locomotor activity attained statistical significance during 0-10 minutes ($p<0.01$), 10-20 minutes ($p<0.01$), 20-30 minutes ($p<0.05$), 30-40 minutes ($p<0.01$) and 50-60 minutes ($p<0.01$), as well as 0-60 minutes (overall, $p<0.01$). These differences were not supported by similar changes at higher doses and were therefore considered to be incidental.

At 1000 mg/kg bw/day, males had reduced locomotor activity from 40-50 minutes ($p<0.01$). Females did not differ from the control values.

At 2000 mg/kg bw/day, differences were seen in males during 50-60 minutes (reduced, $p<0.01$) and in females during 0-10 minutes (increased, $p<0.05$). These sporadic differences were considered to be incidental findings.

During the recovery period, statistically significant increases in mean locomotor activity were noted from 10-20 minutes in both sexes previously treated with 2000 mg/kg bw/day ($p<0.01$). These transient differences were not considered to be late effects of the test item.

8.3.4 OPHTHALMOSCOPIC FINDINGS

See pp. 95 and 280

No test item-related ophthalmoscopic findings of toxicological relevance were noted at any dose level after the treatment and recovery periods.

8.3.5 TEST ITEM INTAKE

See pp. 54 , 125 and 368

The target dose levels were attained.

Males consumed -0.8%, -0.6% and -1.13% of the target dose levels of 500, 1000 and 2000 mg/kg bw/day. Females consumed +1.3%, +1.8% and +2.0% of the target dose levels of 500, 1000 and 2000 mg/kg bw/day. Nominal concentrations were used for test item intake calculations (see 8.1, Dose Formulation Analyses).

8.3.6 FECAL PH

See pp. 127 and 378

No differences of toxicological relevance were noted in the pH of feces. Samples were analyzed during weeks 2, 6 and 13 of treatment and during week 17 of recovery.

8.3.7 SEMINOLOGY

Motility

See pp. 129 and 390

With the exception of a statistically significant increase in the number of progressively motile sperm in male rats treated with 1000 mg/kg bw/day ($p<0.05$), the numbers of stationary motile and progressively motile sperm were similar in control and test item-treated groups. The described difference was not dose related and therefore considered to be the result of biological variation.

After the recovery period, the numbers of progressively motile, stationary motile and non-motile sperm in control males and males previously treated with 2000 mg/kg bw/day were similar.

Morphology

See pp. 129 and 394

After the treatment period, the sperm morphology of control and test item-treated male rats showed no differences of toxicological relevance. In males treated with 1000 mg/kg bw/day, the mean number of tailed sperm with reversed hook was significantly reduced ($p<0.05$) when compared with the control males[2]. This finding was considered to be of no toxicological relevance, as no dose-response relationship was established.

No differences of toxicological relevance were noted after the recovery period.

Sperm Head Count

See pp. 129 and 386

No differences of toxicological relevance were noted between the sperm head counts of control males and test item-treated males after the treatment period or in the remaining males after the recovery period.

8.3.8 ESTROUS CYCLES

See pp. 135 and 398

No test item-related differences in the duration of estrus were noted during treatment or recovery at any dose level.

8.3.9 FOOD CONSUMPTION

See pp. 46 , 101 and 288

The mean daily food consumption of the test item-treated males and females compared favorably with those of the respective control animals.

8.3.10 BODY WEIGHTS

See pp. 50 , 113 and 328

In males treated with 2000 mg/kg bw/day, a very marginal (and statistically insignificant) difference in the mean body weight development was noted when compared with the control males. No differences of toxicological relevance were noted in the mean body weights of test item-treated and control rats.

[2] Although numerically identical to the males treated with 2000 mg/kg/day, the statistical significance resulted from the slightly higher standard deviation noted in the high dose group.

8.4 CLINICAL LABORATORY INVESTIGATIONS

8.4.1 HEMATOLOGY

See pp. 138 and 402

Hematology parameters measured after 2, 6 and 13 weeks of treatment did not indicate any changes of toxicological relevance. After a 4-week recovery period, no late effects were seen in the rats previously treated with the highest dose level.

After 2 Weeks' Treatment

Males treated with 2000 mg/kg bw/day had significantly elevated methemoglobin ($p<0.05$) when compared with controls. A similar finding was noted in the males treated with 1000 mg/kg bw/day ($p<0.05$). The findings were not dose-related and they did not exceed the ranges of the historical control values. Heinz bodies were not seen. In males treated with 500 mg/kg bw/day or 1000 mg/kg bw/day, the mean hemoglobin levels were significantly decreased (both $p<0.05$) when compared with the control males. No further differences to the control values were noted in any of the treatment groups. These changes were not considered to be test item-related.

In females treated with 2000 mg/kg bw/day, the mean hemoglobin level and the mean corpuscular hemoglobin concentration were significantly elevated ($p<0.05$ and $p<0.01$, respectively). These rats also had a significant reduction in the mean corpuscular volume ($p<0.01$) when compared with control females. The mean platelet count was also significantly elevated in the females treated with 2000 mg/kg bw/day ($p<0.05$). All differences noted after two weeks of treatment in females treated with 2000 mg/kg bw/day remained within the ranges of the historical control data and were considered to be incidental.

Females treated with 500 mg/kg bw/day or 1000 mg/kg bw/day showed a small number of toxicologically irrelevant differences to the control values, including elevated hemoglobin distribution width (500 mg/kg bw/day only, $p<0.05$), and reduced high-fluorescence reticulocyte population (500 mg/kg bw/day only, $p<0.05$), all of which remained within the ranges of the respective historical control values and were considered to be incidental.

After 6 Weeks' Treatment

Male and female rats treated with 2000 mg/kg bw/day showed several differences when compared with the controls. The mean absolute reticulocyte counts were elevated (significantly in males, $p<0.05$), and significant reductions in the population of low-fluorescence reticulocytes ($p<0.05$ in both sexes) were seen. The populations of medium fluorescence and low fluorescence reticulocytes significantly differed (middle fluorescence reticulocytes were elevated in males, $p<0.05$, whereas low fluorescence reticulocytes were reduced in both sexes, $p<0.05$) when compared with controls. Significantly reduced methemoglobin ($p<0.05$) was noted in males treated with 2000 mg/kg bw/day; this finding is not associated with any toxicological relevance. The relative prothrombin time was significantly increased ($p<0.05$) in males treated with 2000 mg/kg bw/day. None of the observed differences exceeded the ranges of the historical control data and all were considered to be incidental.

Females treated with 2000 mg/kg bw/day had an elevated white blood cell count ($p<0.05$) when compared with the control females. A slight elevation (without statistical significance) noted in mean absolute lymphocytes was considered to contribute to the difference, but all values remained within the ranges of the historical control data. The relative eosinophil count was reduced in females treated with 2000 mg/kg bw/day. The mean platelet count was also significantly elevated ($p<0.01$) in females treated with 2000 mg/kg bw/day. All findings in high dose group females were within the range of historical control data and considered incidental.
At 1000 mg/kg bw/day, male and female rats showed a significant reduction ($p<0.05$ and $p<0.01$, respectively) in the mean absolute eosinophil population, whereas females showed also a reduced mean relative eosinophil count ($p<0.05$) when compared with the controls. With the exception of a significantly prolonged activated partial thromboplastin time in females ($p<0.05$), all other parameters compared favorably with those of the controls females. Significantly reduced methemoglobin ($p<0.05$) noted in males treated with 1000 mg/kg bw/day. None of the findings for males and females of the 1000 mg/kg bw/day dose groups were dose-related and all were within the ranges of the historical control data; the findings were not associated with any toxicological relevance.

At 500 mg/kg bw/day, males showed significantly increased low-fluorescence reticulocytes and significantly reduced medium fluorescence reticulocytes (both $p<0.05$) when compared with controls. Significantly elevated mean absolute monocytes ($p<0.05$) and thrombocytes ($p<0.05$) were noted in males, but these values remained in the ranges of the historical control data and not considered toxicologically relevant.

No further differences were noted in males at this dose level and females treated with 500 mg/kg bw/day were unaffected.

After 13 Weeks' Treatment

At 2000 mg/kg bw/day, only a slight (but statistically significant) decrease in mean absolute and relative eosinophil counts (both ($p<0.05$)) were noted in males. Females at this dose level showed a significant elevation in the mean number of basophils ($p<0.05$) and a significantly elevated number of platelets ($p<0.01$). All values remained in the ranges of the historical control data.

At 1000 mg/kg bw/day, a slight, but statistically significant reduction of the white blood cell count ($p<0.05$) was noted in males only. Significantly lower counts of mean absolute neutrophils ($p<0.05$) and eosinophils ($p<0.01$) were noted in these rats, as well as a slight reduction in lymphocytes (not significant). All values remained in the ranges of the historical control data.

No differences were noted in females treated with 1000 mg/kg bw/day or in either sex treated with 500 mg/kg bw/day.

After 13 Weeks' Treatment and 4 Weeks' Recovery

After the recovery period, male rats previously treated with 2000 mg/kg bw/day had reduced white blood cell count ($p<0.01$) as a result of reductions noted in the populations of neutrophils ($p<0.05$), eosinophils ($p<0.05$), lymphocytes ($p<0.05$), monocytes ($p<0.01$) and "large unstained cells" ($p<0.05$). Statistical significance was attained only in the mean absolute values; the mean relative values were similarly affected but did not attain statistical significance. Insofar as these differences were not seen in rats after the end of the treatment period and remained within the ranges of the historical control data, they were not considered to be a test item-related change.

In female rats previously treated with 2000 mg/kg bw/day, a significant reduction of the mean corpuscular hemoglobin concentration ($p<0.05$) was noted when compared with the controls. The mean platelet count was slightly elevated ($p<0.05$). The differences were within the ranges of the historical control data.

8.4.2 CLINICAL BIOCHEMISTRY

See pp. 144 and 473

Differences to the control values seen in the clinical biochemistry parameters were not considered to be of toxicological relevance. Differences in electrolyte balance were seen during the treatment period at all dose levels, but nearly all parameters recovered to control levels after the recovery period. Therefore, these differences to control values were considered likely to be physiological rather than of toxicological origin.

After 2 Weeks' Treatment

At 2000 mg/kg bw/day, blood glucose levels were significantly elevated in males ($p<0.01$) when compared with controls, whereas mean creatinine levels were reduced ($p<0.01$). The activities of aspartate aminotransferase and alanine aminotransferase were significantly reduced ($p<0.01$), whereas glutamate dehydrogenase activity was significantly higher in males and females (both $p<0.05$).

The significant reduction in lactate dehydrogenase ($p<0.01$) noted in both sexes was not considered to be of toxicological relevance; increases in this parameter are generally considered to be indicative of possible blood cell hemolysis. Creatine kinase was significantly reduced in males ($p<0.05$) and females ($p<0.01$) at 2000 mg/kg bw/day. Several electrolytes were significantly higher than the controls: sodium (females only, $p<0.05$), potassium (both sexes, $p<0.01$), chloride (males, $p<0.05$ and females, $p<0.01$) and calcium ($p<0.01$, females only) when compared with the controls. Phosphorus was also elevated in both sexes ($p<0.01$).

The total protein was significantly reduced in males ($p<0.05$), chiefly as a result of the lower globulin ($p<0.01$) which exceeded the lower limit of the historical control data. The latter change was reflected also in the significant elevation of the albumin-globulin ratio ($p<0.05$), but was not considered to be related to the treatment with the test item since a larger reduction was noted in the males treated with 1000 mg/kg bw/day. In females, the total protein was increased ($p<0.01$) as a result of elevated albumin (not significant).

At 1000 mg/kg bw/day, elevated cholesterol was seen in males ($p<0.05$) when compared with the controls. Reduced lactate dehydrogenase ($p<0.05$) was recorded in males only, and considered to be incidental.

Differences in the electrolytes were inconsistent: sodium was reduced in males ($p<0.01$) and increased in females ($p<0.05$); potassium was increased in males only ($p<0.05$), chloride was increased in females only ($p<0.01$), calcium was reduced in males and increased in females (both $p<0.05$). The mean total protein was significantly lower ($p<0.01$) in males when compared with the controls. This value, together with the reduction in globulin ($p<0.01$), exceeded the lower limits of the respective historical control data. This finding was independent of dose and not considered to be related to the test item. The albumin-to-globulin ratio was elevated ($p<0.05$) in the males and unaffected in females.

At 500 mg/kg bw/day, increased blood glucose ($p<0.01$) was noted in males. Changes in electrolytes were also noted: sodium levels were increased in both sexes ($p<0.01$) and chloride was increased in both sexes ($p<0.01$). Total protein was elevated in females ($p<0.01$) only, primarily as a result of higher globulin (not significant).

With the exception of the significant reduction ($p<0.01$) in mean total protein level seen in males at 1000 mg/kg bw/day which was neither dose-related nor biologically significant, and considered to be incidental, all of the differences noted after 2 weeks of treatment remained within the ranges of the historical control data.

After 6 Weeks' Treatment

A significant reduction in glucose levels was noted in both sexes at 2000 mg/kg bw/day (all $p<0.05$), whereas females treated with 1000 mg/kg bw/day showed increased glucose levels ($p<0.05$).

Total bilirubin and cholesterol were significantly elevated in males at 2000 mg/kg bw/day ($p<0.05$). Cholesterol was also elevated in males treated with 1000 mg/kg bw/day. All remained within the historical control data ranges and were considered to be incidental.

Elevated phospholipids ($p<0.05$) were noted only in males treated with 1000 mg/kg bw/day, and were not seen in males treated with 2000 mg/kg bw/day. These findings were considered to be incidental.

Reduced activity of alanine aminotransferase ($p<0.05$) noted in females treated with 2000 mg/kg bw/day is a finding not generally associated with toxicity and is therefore considered to be incidental.

Lactate dehydrogenase was elevated in males treated with 2000 mg/kg bw/day ($p<0.01$), but this finding is strongly dependent upon blood sample quality and subject to wide variation. It is not considered to be an indication of toxicity. Glutamate dehydrogenase was significantly elevated in males treated with 2000 mg/kg bw/day ($p<0.01$) but remained within the ranges of the historical control data. Creatine kinase, significantly elevated in females treated with 1000 mg/kg bw/day ($p<0.05$), showed no dose-related changes at 2000 mg/kg bw/day and was considered to be incidental.

Numerous differences in electrolytes were seen in both sexes: sodium was significantly elevated at all dose levels ($p<0.01$), potassium was significantly elevated in males at all dose levels ($p<0.05$ and $p<0.01$) and in females at 1000 mg/kg bw/day ($p<0.05$), chloride was elevated in males at 1000 mg/kg bw/day and 2000 mg/kg bw/day, and in females at all dose levels (all $p<0.01$). Calcium was elevated in all treated males and in females treated with 2000 mg/kg bw/day, and phosphorus was increased in both sexes treated with 500 mg/kg bw/day ($p<0.05$), 1000 mg/kg bw/day ($p<0.01$) and 2000 mg/kg bw/day ($p<0.01$).

Total protein levels were elevated in males treated with 1000 mg/kg bw/day ($p<0.05$) and in both sexes treated with 2000 mg/kg bw/day ($p<0.01$). At 2000 mg/kg bw/day, significant elevations of albumin ($p<0.01$) and globulins ($p<0.05$) were noted in males only. The elevated sodium levels of females treated with 1000 mg/kg bw/day ($p<0.01$) and both sexes treated with 2000 mg/kg bw/day both ($p<0.01$), as well as the elevated chloride level of females treated with 2000 mg/kg bw/day exceed the upper ranges of the historical control data. However, these findings were not biologically significant and since all were reversible during the recovery period, were not considered to be toxicologically relevant.

All other differences after 6 weeks treatment remained within the range of historical control data.

After 13 Weeks' Treatment

Reduced creatinine was noted in males treated with 2000 mg/kg bw/day ($p<0.05$). Lactate dehydrogenase was increased in these males but considered to be of no toxicological significance.

Alkaline phosphatase was reduced in males treated with 1000 mg/kg bw/day ($p<0.05$) and 2000 mg/kg bw/day ($p<0.01$).

Several electrolytes in the treatment groups were different from controls: sodium was significantly elevated in males and females treated with 1000 and 2000 mg/kg bw/day ($p<0.05$ or $p<0.01$), potassium was elevated in males treated with 1000 mg/kg bw/day, chloride was elevated in females treated with 500 mg/kg bw/day ($p<0.01$) and in both sexes treated with 1000 mg/kg bw/day and 2000 mg/kg bw/day (all $p<0.01$), phosphorus was increased in males treated with 2000 mg/kg bw/day ($p<0.05$). Slightly elevated albumin ($p<0.05$) was noted in males treated with 2000 mg/kg bw/day, with a significant increased in the albumin-to-globulin ratio ($p<0.05$) in these males as well.

All differences remained within the ranges of the historical control data; all were considered incidental and not of toxicological relevance.

After 13 Weeks' Treatment and 4 Weeks' Recovery

After the recovery period, differences were noted only in males and all remained within the ranges of the historical control data.

Reductions in aspartate aminotransferase activity ($p<0.05$), alanine aminotransferase activity ($p<0.01$), alkaline phosphatase ($p<0.01$), phosphorus ($p<0.05$) and protein ($p<0.01$) were noted. The globulin fraction was also decreased ($p<0.01$), resulting in an elevated albumin-to-globulin ratio ($p<0.05$).

8.4.3 URINALYSIS

See pp. 148 and 529

No test item-related effects on urinalysis parameters were noted.

After 2 Weeks' Treatment

The urinalysis parameters of the test item-treated rats compared favorably with those of the control rats.

After 6 Weeks' Treatment

With the exception of squamous epithelial cells present in the urine of test item-treated females, all results compared favorably with those of the control rats. The presence of squamous epithelial cells was not seen in the urine after 13 weeks of treatment and therefore it was considered to be incidental.

After 13 Weeks' Treatment

The statistically significant reduction in the relative density of the urine noted in the males treated with 500 mg/kg bw/day ($p<0.05$) was not dose related and considered to be incidental. The statistically significant increase in erythrocytes noted in females at 2000 mg/kg bw/day ($p<0.01$) was considered to be unrelated to treatment and probably resulted from an unusually low control value, and did not exceed the upper limits of the historical control values.

After 13 Weeks' Treatment and 4 Weeks' Recovery

The urinalysis parameters of the test item-treated rats compared favorably with those of the control rats.

8.4.4 IMMUNOTOXICITY

See Appendix VI

There was no evidence for any changes in primary immune response nor were there toxicologically relevant alterations in leukocyte populations in all groups.

ANALYSIS OF PRIMARY IMMUNE RESPONSE TO IMMUNOGEN (TYPE 2 BASIC)

The data show that the primary immune response of the test item treated animals to sheep's red blood cells were similar to untreated immunised animals. The mean relative titers of the animals treated with 500, 1000 and 2000 mg/kg bw/day were 396.10 ± 265.88, 440.33 ± 189.33, and 333.21 ± 119.44%, respectively. The mean relative titer of the control animals was 437.03 ± 250.73%. The values are within the historical range and not statistically significantly different from controls.

ANALYSIS OF LEUKOCYTE POPULATIONS IN BLOOD (LEVEL 1 EXTENDED)

The analysis of the leukocyte populations showed that neither the control animals nor the treatment of the animals with the indicated doses of the test item affected the distribution of the $CD3^+/CD4^+$, CD45 RA^+ and the $CD11b^+$ cell populations in the blood.

The analysis of the $CD3^+/CD8^+$ population showed in Allocation B from April 04-07, 2006, and Allocation B (May 02), a slight but statistically significant decrease of the test item treated group (mean value =14.82 ± 3.09% and 15.63 ± 3.17% for Allocation B animals April 4-7 and May 2, 2006, respectively) as compared to the corresponding controls (mean value = 17.42 ± 3.19% and 17.7 ± 2.97% for Allocation B animals on April 4-7 and May 2, 2006, respectively).

These values were, however, within the historical control range for the $CD3^+/CD8^+$ population (range = 12.8 – 35.2%). Since the values are within the historical range, this slight drop is not biologically significant.

8.5 PATHOLOGY

8.5.1 ORGAN WEIGHTS

See pp. 152 and 585

After 13 Weeks

In males, no test item-related or toxicologically relevant differences were seen in the mean absolute or relative organ weights at any dose level.

A statistically significant increase in the mean thyroid-to-body weight ratio ($p<0.05$), noted in the males treated with 2000 mg/kg bw/day was considered to be toxicologically irrelevant as it was not accompanied by microscopic changes. Likewise, a significantly elevated thyroid-to-brain weight ratio in males treated with 500 mg/kg bw/day and a significantly reduced thymus-to-brain weight ratio in males treated with 1000 mg/kg bw/day (both $p<0.05$) were not dose-related. In females, no differences were noted in the mean absolute organ weights at any dose level. Significant increases in the mean liver-to-body weight ratio and liver-to-brain weight ratio (both $p<0.05$) noted in females treated with 2000 mg/kg bw/day were considered likely to be fortuitous differences, but may indicate a minor adaptive metabolic response. There was no microscopic correlation to these changes and no weight differences for animals livers were noted after the recovery period.

A significant increase in the mean kidney-to-body weight ratio ($p<0.05$) in females treated with 1000 mg/kg bw/day was unrelated to dose and therefore considered to be fortuitous.

After 17 Weeks

In males, no test item-related or toxicologically relevant differences were seen in the mean absolute or relative organ weights at any dose level.

In females, no differences were noted in the mean absolute organ weights at any dose level.

Significant increases in the mean heart-to-body weight ratio and kidney-to-body weight ratio (both $p<0.05$) of females previously treated with 2000 mg/kg bw/day were considered likely to be a fortuitous difference, since no relevant microscopical changes were seen. No changes in liver weights were seen after 4 weeks recovery.

8.5.2 MACROSCOPIC FINDINGS

See pp. 170 and 627

At the end of the treatment and recovery periods no test item-related gross lesions were observed. The macroscopic findings recorded were considered to be within the range of normal background lesions, which may be seen in rats of this strain and age in this study type and were considered incidental, reflecting the usual individual variability.

8.5.3 MICROSCOPIC FINDINGS

See Appendix VIII

After 13 Weeks

In Table 1, the finding that distinguished test item-treated animals from controls was the presence of large focal/multifocal aggregates of foamy histiocytes in mesenteric lymph node of animals treated with 500, 1000, or 2000 mg/kg bw/day. Although the incidence and mean

severity of this finding increased with dose, no co-existing tissue damage or reaction was noted. Therefore, the microscopic changes in lymph nodes were considered to be of no adverse nature.

Table 1. Incidence and mean severity of main microscopic findings in mesenteric lymph node (Allocation A, Main Study)

	Group 1 Control		Group 2 500 mg/kg bw/day		Group 3 1000 mg/kg bw/day		Group 4 2000 mg/kg bw/day	
Finding	Male	Female	Male	Female	Male	Female	Male	Female
	20	20	20	20	20	20	20	20
Foamy Histiocytosis								
Incidence/Mean Severity	-	-	4/1.0	3/1.0	17/1.3	8/1.3	20/2.3	19/2.1
Severity scale: 1: minimal; 2: slight; 3: moderate; 4: marked								

In Table 2, minimal tubular vacuolation, recorded in kidneys of females treated with 1000 mg/kg bw/day and in both sexes treated with 2000 mg/kg bw/day, is described. This finding, per se, with non-dose dependant severity grade, in absence of tubular damage or any other sign of renal toxicity or impairment is not of adverse nature.

Table 2. Incidence and mean severity of main microscopic findings in kidneys (Allocation A, Main Study)

	Group 1 Control		Group 2 500 mg/kg bw/day		Group 3 1000 mg/kg bw/day		Group 4 2000 mg/kg bw/day	
Finding	Male	Female	Male	Female	Male	Female	Male	Female
	20	20	20	20	20	20	20	20
Tubular vacuolation								
Incidence/Mean Severity	-	-	-	-	-	5/1.0	3/1.0	13/1.0
Severity scale: 1: minimal; 2: slight; 3: moderate; 4: marked								

After 17 Weeks

After a 4-week recovery period, the presence of large focal/multifocal aggregates of foamy histiocytes in mesenteric lymph nodes of animals previously treated with 2000 mg/kg bw/day was still recorded, however, the severity was reduced and thus a tendency to regression observed. No co-existing tissue damage or reaction was noted.

Table 3. Incidence and mean severity of main microscopic findings in mesenteric lymph node (Allocation B, Recovery)

	Group 1 Control		Group 4 2000 mg/kg bw/day	
Finding	Male	Female	Male	Female
	10	10	10	10
Foamy Histiocytosis				
Incidence/Mean Severity	-	-	10/2.1	10/1.8
Severity scale: 1: minimal; 2: slight; 3: moderate; 4: marked				

After a 4-week recovery period, the minimal tubular vacuolation was still recorded in animals treated previously with 2000 mg/kg bw/day. Tubular damage and other signs of renal toxicity or impairment were absent.

Table 4. Incidence and mean severity of main microscopic findings in kidneys (Allocation B, Recovery)

	Group 1 Control		Group 4 2000 mg/kg bw/day	
Finding	Male	Female	Male	Female
	10	10	10	10
Tubular vacuolation				
Incidence/Mean Severity	-	-	4/1.0	5/1.0
Severity scale: 1: minimal; 2: slight; 3: moderate; 4: marked				

9 CONCLUSION

Oral administration of ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE) to Wistar rats at target doses of 500, 1000 and 2000 mg/kg bw/day, for at least 90 days resulted in no test item-related mortalities in any dose group, no indications of adverse clinical signs during daily observations or of neurotoxicological effects during the functional observational battery (performed at week 13 and week 17), no effects upon mean grip strength or locomotor activity, no ophthalmoscopic findings of toxicological relevance at any dose level, no differences of toxicological relevance in fecal pH after weeks 2, 6 or 13 (treatment) or after week 17 (recovery). There were no effects upon sperm motility, sperm morphology or sperm head counts, and no relevant differences in the duration of estrus cycle were noted during treatment or recovery.

The mean food consumption was unaffected at all dose levels, and no toxicologically relevant differences in body weight and body weight development were noted.

The hematology, clinical biochemistry and urinalysis parameters were not considered to be affected by the treatment with the test item. Changes or differences in organ weight or ratios were not seen, and no test item-related macroscopical changes were evident.

There was no evidence for any primary immune response nor were there toxicologically relevant alterations in leukocyte populations.

The achieved dose levels ranged from -0.6% to -1.13% in males and +1.3% and +2.0% of the respective uncorrected target values.

No microscopic findings were seen in the rectal tissue.

Test item-related findings were restricted to microscopical changes in the mesenteric lymph nodes: focal/multifocal aggregates of foamy histiocytes were recorded in animals at all dose levels after 13 weeks' treatment. Further, minimal tubular vacuolation was recorded in kidneys of females treated with 1000 mg/kg bw/day and in both sexes treated 2000 mg/kg bw/day. After the 4-week treatment-free recovery period, both microscopic findings persisted in rats previously treated with 2000 mg/kg bw/day. However, neither the microscopical changes in mesenteric lymph nodes nor kidneys were accomplished by co-existing tissue damage after treatment and recovery periods.

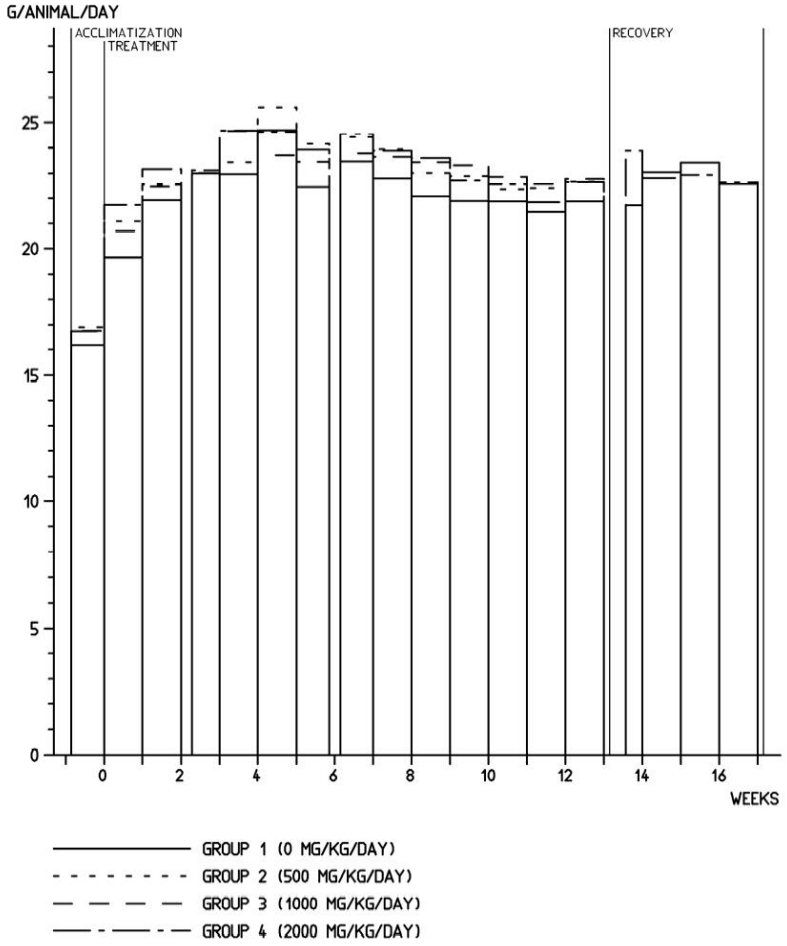
Because no co-existing tissue damage was noted and there was no dose-dependent severity grade or even a tendency to regression, the microscopic changes in lymph nodes and kidneys were considered to be of no adverse nature.

Based upon the study findings, the no-observed-adverse-effect-level (NOAEL) was considered to be 2000 mg/kg bw/day.

10 FIGURES

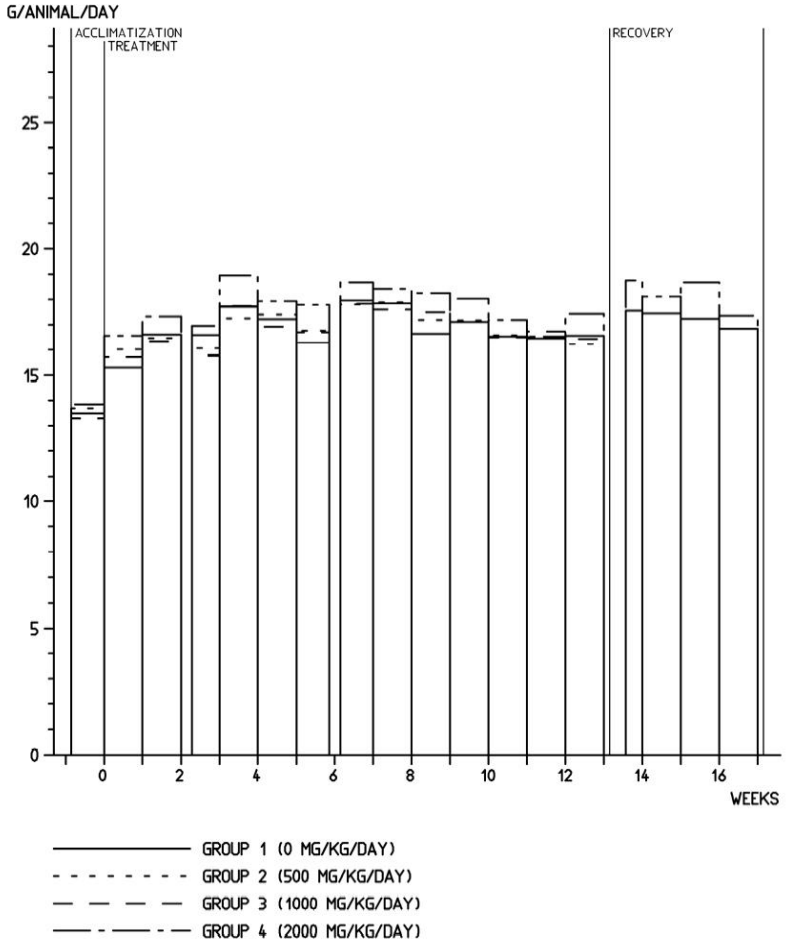
RCC STUDY NUMBER A29553 REPORT
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

FOOD CONSUMPTION
MALES



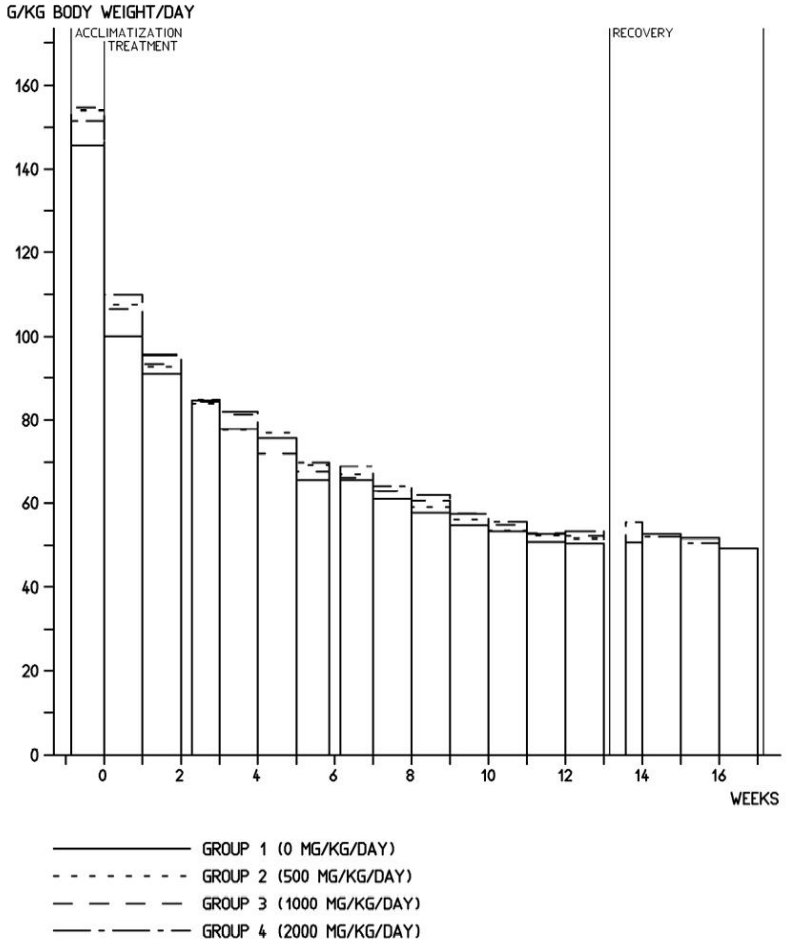
RCC STUDY NUMBER A29553 REPORT
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

FOOD CONSUMPTION
FEMALES



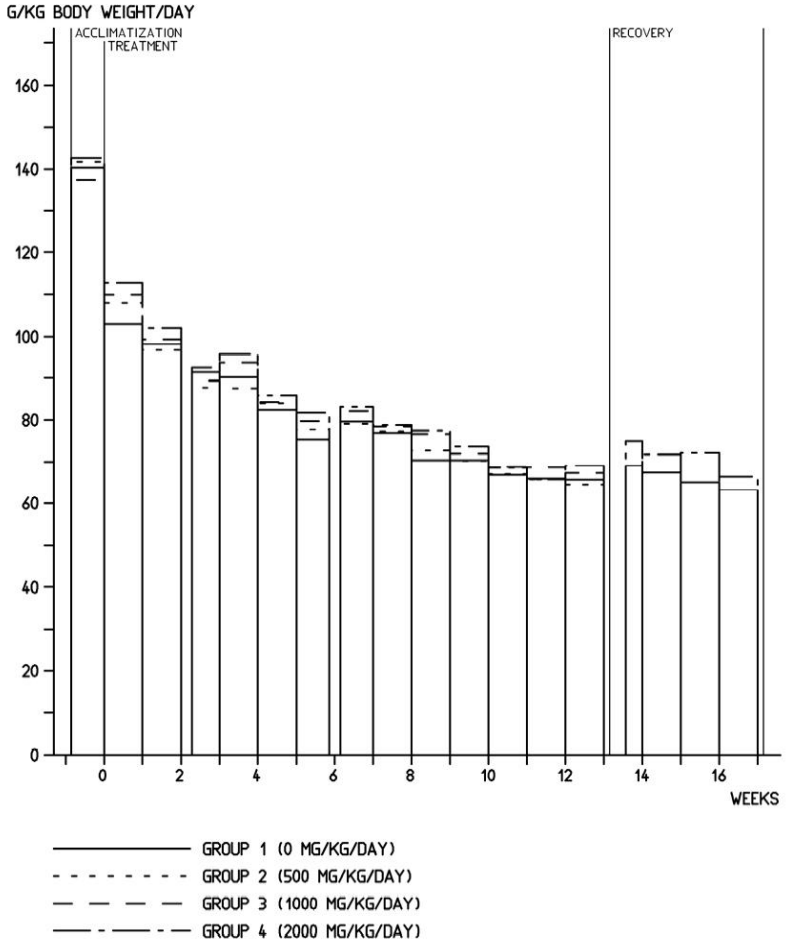
RCC STUDY NUMBER A29553 REPORT
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

RELATIVE FOOD CONSUMPTION
MALES



RCC STUDY NUMBER A29553 REPORT
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

RELATIVE FOOD CONSUMPTION
FEMALES



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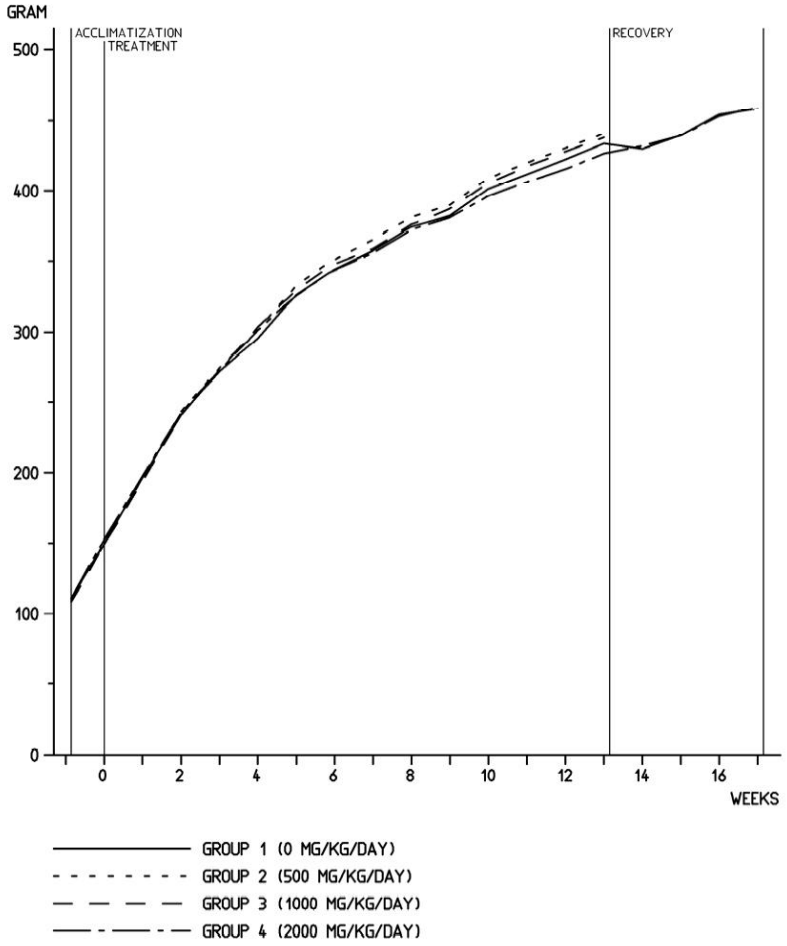
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

BODY WEIGHTS
MALES



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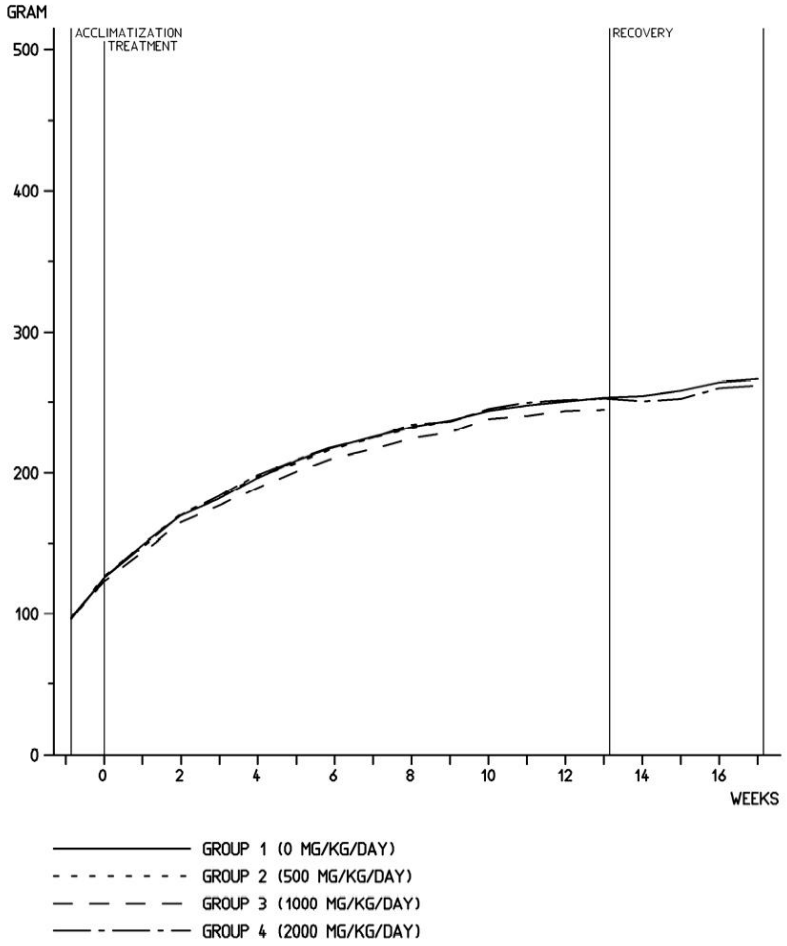
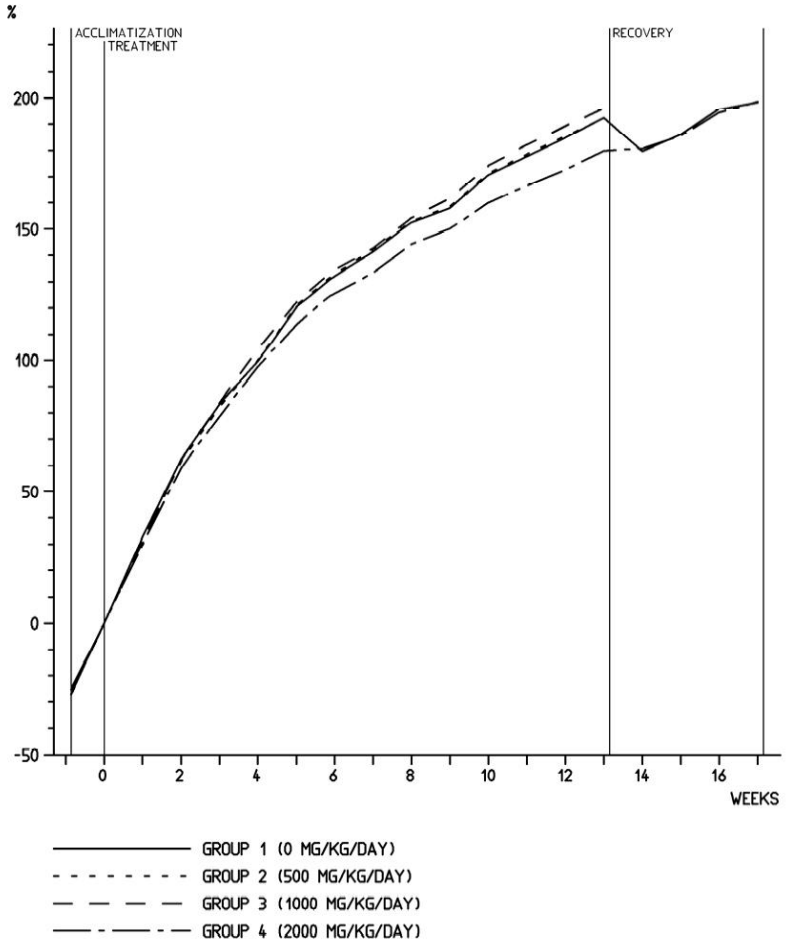
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

BODY WEIGHTS
FEMALES



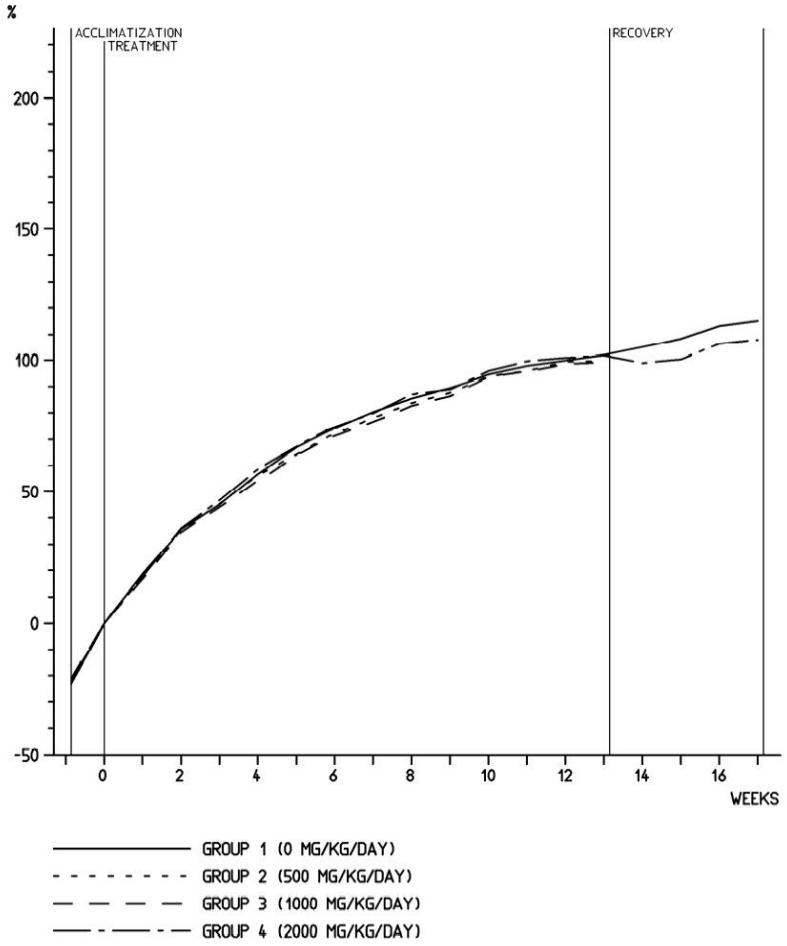
RCC STUDY NUMBER A29553 REPORT
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

BODY WEIGHT GAIN
MALES



RCC STUDY NUMBER A29553 REPORT
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

BODY WEIGHT GAIN
FEMALES



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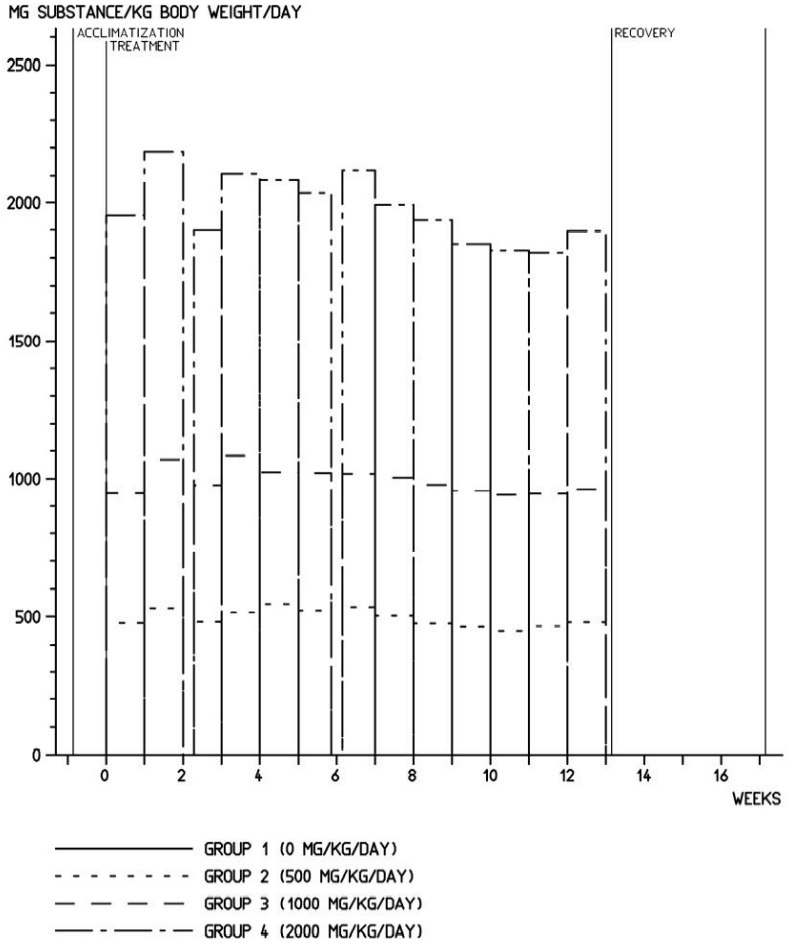
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

TEST ITEM INTAKE
MALES



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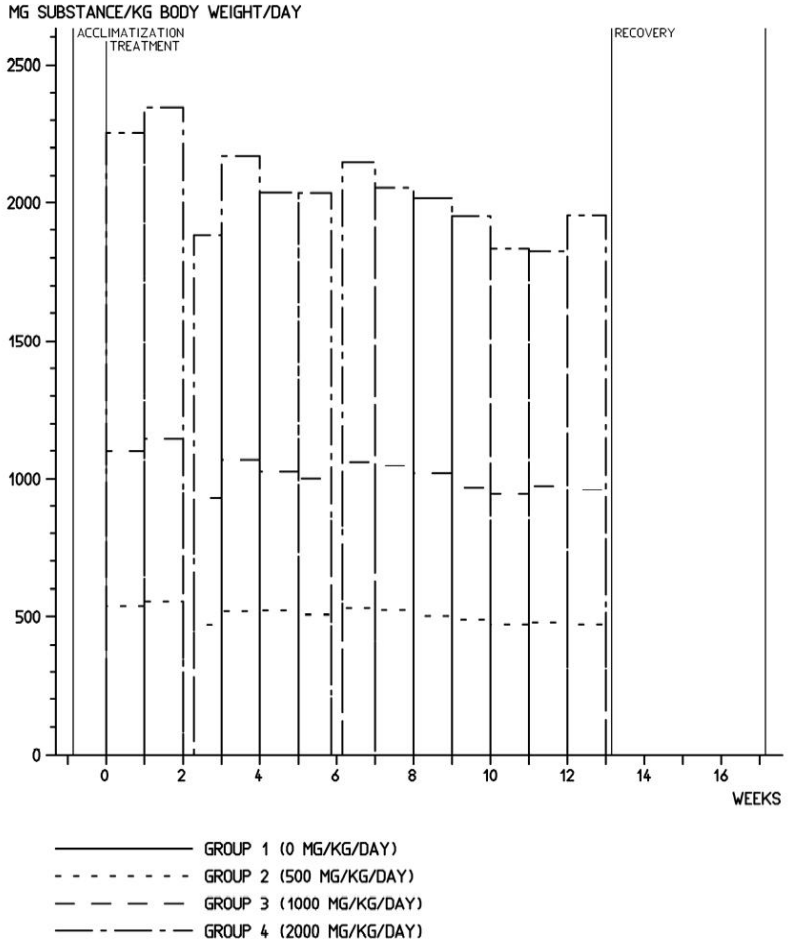
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

TEST ITEM INTAKE
FEMALES



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ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

11 SUMMARY TABLES

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ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

CLINICAL SIGNS, DAILY (SUMMARY)
MALES
GROUP 1 (0 MG/KG/DAY)

SIGN (MAX.GRADE) / LOCATION	WEEKS:	ACCLIMATIZATION 1.....	TREATMENT 1.....2.....3.....4.....5.....6.....
VARIOUS			
EMACIATED (3)	G:	.....	111.....
	%:	.....	000.....
EYE RIGHT, ENLARGED (1)	G:	.....	.....
	%:	.....	.....
EYE RIGHT, WHITE (3)	G:	.....	.....
	%:	.....	.....
EXOPHTHALMOS (1)	G:	.....	.....
	%:	.....	.....
OPACITY (1) (EYE RIGHT)	G:	.....	.....
	%:	.....	.....
KINKED (3) (TAIL APEX)	G:	.....	.....
	%:	.....	.....

G: Median value of the highest individual daily grades
%: Percent of affected animals (0 = less than 5%, 1 = between 5% and 15%,..., A = more than 95%)

RCC STUDY NUMBER A29553 REPORT
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

CLINICAL SIGNS, DAILY (SUMMARY)
MALES
GROUP 2 (500 MG/KG/DAY)

SIGN (MAX.GRADE) / LOCATION	WEEKS:	ACCLIMATIZATION 1.....	TREATMENT 1.....2.....3.....4.....5.....6.....
BEHAVIOR			
SEDATED (3)	G:	.....	.....3.....
	%:	.....	.....0.....
POSTURE			
HUNCHED POSTURE (1)	G:	.....	.....111.....
	%:	.....	.....000.....
KINKED (3)	G:	.....	.....
(TAIL APEX)	%:	.....	.....
SKIN / FUR			
ROUGH COAT (1)	G:	.....	.....1111.....
	%:	.....	.....0000.....
HAIR LOSS (3)	G:	.....	.....222222222111.....
(SHOULDER RIGHT)	%:	.....	.....000000000000.....
CRUSTS (3)	G:	.....	.....111111111111.....
(SHOULDER RIGHT)	%:	.....	.....000000000000.....
WOUND (3)	G:	.....	.....221.....
(SHOULDER RIGHT)	%:	.....	.....000.....
SECRETION / EXCRETION			
LACRIMATION (3)	G:	.....	.....2.....
	%:	.....	.....0.....
REDUCED SIZE FECALBOLI (1)	G:	.....	.....1.....
	%:	.....	.....0.....
VARIOUS			
EMACIATED (3)	G:	.....	.....1223.....
	%:	.....	.....0000.....
ENOPHTHALMOS (1)	G:	.....	.....1.....
	%:	.....	.....0.....
EXOPHTHALMOS (1)	G:	.....	.....
	%:	.....	.....
KINKED (3)	G:	.....	.....
(TAIL APEX)	%:	.....	.....

G: Median value of the highest individual daily grades
%: Percent of affected animals (0 = less than 5%, 1 = between 5% and 15%,..., A = more than 95%)

RCC STUDY NUMBER A29553 REPORT
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

CLINICAL SIGNS, DAILY (SUMMARY)
MALES
GROUP 3 (1000 MG/KG/DAY)

SIGN (MAX.GRADE) / LOCATION	WEEKS:	ACCLIMATIZATION 1.....	TREATMENT 1.....2.....3.....4.....5.....6.....
SKIN / FUR			
HAIR LOSS (3)	G:	.....	.....
(SHOULDER RIGHT)	%:	.....	.....
SCABS (3)	G:	.....	.....111111111111111.....
(SHOULDER LEFT)	%:	.....	.....000000000000000.....
SCABS (3)	G:	.....	.....
(SHOULDER RIGHT)	%:	.....	.....
SCABBED WOUND (1)	G:	.....	.....
(SHOULDER RIGHT)	%:	.....	.....
VARIOUS			
EYE RIGHT, ENLARGED (1)	G:	.....	1111111111111111111111111111111111111111111
	%:	.....	0000000000000000000000000000000000000000000
EXOPHTHALMOS (1)	G:	.....	.....
	%:	.....	.....
MYDRIASIS (1)	G:	.....	...1111111111111111111111111111111111111..
	%:	.....	...0000000000000000000000000000000000000..

G: Median value of the highest individual daily grades
%: Percent of affected animals (0 = less than 5%, 1 = between 5% and 15%,..., A = more than 95%)

RCC STUDY NUMBER A29553 REPORT
ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

CLINICAL SIGNS, DAILY (SUMMARY)
MALES
GROUP 4 (2000 MG/KG/DAY)

SIGN (MAX.GRADE) / LOCATION		ACCLIMATIZATION WEEKS: 1.....	TREATMENT 1.....2.....3.....4.....5.....6.....
SKIN / FUR			
HAIR LOSS (3)	G:	.....	.....
(SHOULDER RIGHT)	%:	.....	.....
FISSURES (3)	G:	.....	.....
(EAR LEFT)	%:	.....	.....
SCABS (3)	G:	.....	.....
(SHOULDER RIGHT)	%:	.....	.....
VARIOUS			
EXOPHTHALMOS (1)	G:	.....	.....
	%:	.....	.....

G: Median value of the highest individual daily grades
%: Percent of affected animals (0 = less than 5%, 1 = between 5% and 15%,..., A = more than 95%)