Food Grade Lignosulphonate (Ca)
Batch no 2004 163-01-1

REPORT

Study Title

ASSESSMENT OF CONTACT HYPERSENSITIVITY TO PURIFIED CALCIUM LIGNOSULFONATE IN THE MOUSE (LOCAL LYMPH NODE ASSAY)

Author

Drs. A.H.B.M. van Huygevoort

Study completion date

23 December 2004

Test Facility

NOTOX B.V.
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

Laboratory Project Identification

NOTOX Project 419535
NOTOX Substance 146844/A

PROTOCOL AMENDMENT NO : 1

Study Title	Assessment of contact hypersensitivity to purified calcium lignosulfonate in the mouse (Local Lymph Node Assay)
Sponsor	Borregaard Lignotech P.O. Box 162 N-1701 SARPSBORG Norway
Study Monitor	Mrs. C. Krogsveld
NOTOX Substance	146844/A
NOTOX Project	419535

AMENDMENT DESCRIPTION

1. Preliminary irritation study: In principle, this concentration should be well tolerated systemically by the animals and may give moderate irritation **(grade 2)** at the highest.

REASONS FOR AMENDMENT

1. Since the scoring grades were changed, moderate irritation became grade 2 (instead of grade 3)

APPROVAL
Study director

.....
Drs. A.H.B.M. van Huygevoort

23-12-04
.....
date:

1. CONTENTS

2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

NOTOX B.V.

Drs. A.H.B.M. van Huygevoort
Study Director

Drs. M.S. Teunissen
Section Head Toxicology

Date: 23 December 2004

Date: 24 December 2004

3. QUALITY ASSURANCE STATEMENT

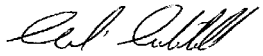
NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected

Type of inspections	Phase / Section	Start Inspection date(s)	End Inspection date(s)	Reporting date
Protocol (Study)		07-Oct-04	07-Oct-04	07-Oct-04
On-site (Process)	SPF Unit	05-Jul-04	09-Jul-04	09-Jul-04
On-site (Process)	Pathology	23-Aug-04	30-Aug-04	30-Aug-04
On-site (Process)	Environmental fate	19-Jul-04	23-Jul-04	26-Jul-04
Report (Study)		16-Dec-04	16-Dec-04	16-Dec-04

Head of Quality Assurance
C.J.Mitchell B.Sc.



Date: 24-12-04

4. SUMMARY

Assessment for Contact Hypersensitivity to Purified calcium lignosulfonate in the Mouse (Local Lymph Node Assay).

The study was carried out based on the guidelines described in: OECD, Section 4, Health Effects, No.429 (2002), Paris Cedex; EC, Council Directive 67/548/EEC, Annex V, B.42 (2004); Environmental Protection Agency (EPA): Health Effects Test Guidelines OPPTS 870.2600 "Skin Sensitisation" (2003).

Test substance concentrations selected for the main study were based on the results of a preliminary study.
In the main study, three groups of five experimental animals were epidermally exposed to a 2.5%, 10% and 25% concentration respectively on three consecutive days. Five vehicle control animals were similarly treated, but with vehicle alone (Propylene glycol).
Three days after the last exposure, all animals were injected with ^{3}H-methyl thymidine and after five hours the draining (auricular) lymph nodes were excised.
After precipitating the DNA of the lymph node cells, radioactivity measurements were done.

Mean DPM/animal values for the experimental groups treated with test substance concentrations 2.5, 10 and 25% were 151, 105 and 169 respectively.
The mean DPM/animal value for the vehicle control group was 198.

The SI values calculated for the substance concentrations 2.5, 10 and 25% were 0.8, 0.5 and 0.9 respectively.

There was no indication that the test substance could elicit an SI ≥ 3. The EC3 value was established to exceed 25%.

Based on these results and according to the recommendations made in the test guidelines (OECD No.429, EC B.42 and EPA OPPTS 870.2600), Purified calcium lignosulfonate should not be regarded as a skin sensitiser.

Based on these results and according to the:
- OECD Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances (OECD, 1998), Purified calcium lignosulfonate does not have to be classified for sensitisation by skin contact.

- EC criteria for classification and labelling requirements for dangerous substances and preparations (Council Directive 67/548/EEC), Purified calcium lignosulfonate does not have to be classified and has no obligatory labelling requirement for sensitisation by skin contact.

5. INTRODUCTION

5.1. Preface

Sponsor	Borregaard Lignotech P.O. Box 162 N-1701 SARPSBORG Norway
Study Monitor	Mrs. C. Krogsveld
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Drs. A.H.B.M. van Huygevoort
Study Plan	Start : 11 October 2004 Completion : 01 November 2004

5.2. Aims of the study

The purpose of this study was to evaluate whether the test substance induces contact hypersensitivity in mice after three epidermal exposures of the animals under the conditions described in this report. This study should provide a rational basis for risk assessment in man. Compared to sensitisation tests using Guinea pigs, the Local Lymph Node Assay (LLNA) provides certain advantages with regard to animal welfare and scientific aspects. The sponsor selected the LLNA type of sensitisation test.

5.3. Guidelines

As required by the Dutch Act on Animal Experimentation (February 1997), the study protocol was reviewed and agreed by the Article 14-functionary and the Ethical Committee of NOTOX (DEC NOTOX 01-21). The study procedures described in this report were based on the following guidelines:

Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No.429, "Skin Sensitisation: Local Lymph Node Assay", Paris Cedex, April 2002.

European Community (EC), Council Directive 67/548/EEC, Annex V, Part B, Methods for the Determination of Toxicity, as last amended by Commission Directive 2004/73/EC, B.42: "Skin sensitisation: Local Lymph Node Assay", April 2004.

Environmental Protection Agency (EPA): Health Effects Test Guidelines OPPTS 870.2600. "Skin Sensitisation", March 2003.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data, specimens (except specimens requiring refrigeration or freezing) and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

Those specimens requiring refrigeration or freezing will be retained by NOTOX for as long as the quality of the specimens permits evaluation but no longer than three months after finalization of the report.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

6. MATERIALS AND METHODS

6.1. Test Substance

6.1.1. Test Substance

The sponsor is responsible for all test substance data unless determined by NOTOX.

Identification	Purified calcium lignosulfonate
Molecular weight	46100
CAS Number	8061-52-7
Description	Brown powder
Batch	DP 1075
Composition	48 % C 40 % O 5 % S 4 % H 0,1 % Na 3 % Ca
Test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	23 August 2006
Stability in vehicle	
Water	At least 96 h
1% Aq. Carboxymethyl cellulose	Unknown
Corn oil	No
Propylene glycol	At least 96 h
Polyethylene glycol	No
Methyl ethyl ketone	No
Dimethyl sulphoxide	No
Ethanol	No
Acetone	No
Olive oil	No
Dimethyl formamide	No

6.1.2. Test substance preparation

Vehicle	Propylene glycol
Rationale	The vehicle was selected based on trial formulations performed at NOTOX.
Preparation	The test substance formulations (w/w) were prepared within 4 hours prior to each treatment. No adjustment was made for specific gravity of the vehicle. Homogeneity was obtained to visually acceptable levels.

6.2. Test System

Species	Mouse, CBA strain, inbred, SPF-Quality. Recognised by the international guidelines as the recommended test system (e.g. OECD, EC, EPA) Source: Charles River France, L'Arbresle Cedex, France
Number of animals	20 females (four groups of five females each group). (nulliparous and non-pregnant).
Age and bodyweight	Young adult animals (approx. 10 weeks old) were selected. Body weight variation was within +/- 20% of the sex mean.
Identification	Tailmark.

Reliability check — The results of a reliability test performed not more than 6 months previously or 2 months afterwards are summarised in the Appendix. Similar procedures were used in the reliability test and in this study.

6.3. Animal husbandry

Conditions
Animals were housed in a controlled environment, in which optimal conditions were considered to be approximately 15 air changes per hour, a temperature of 21.0 ± 3.0°C (actual range: 18.4 - 21.8°C), a relative humidity of 30-70% (actual range: 29 - 96%) and 12 hours artificial fluorescent light and 12 hours darkness per day.

Accommodation
Individual housing in labelled Macrolon cages (type I; height 12.5 cm) containing sterilised sawdust as bedding material (Woody-Clean type 3/4; Tecnilab-BMI BV, Someren , The Netherlands).

Acclimatisation period
The acclimatisation period was at least 5 days before the start of treatment under laboratory conditions. Accommodation was as described above except that the animals were group housed in polycarbonate cages (Macrolon II type; height 15 cm). Paper (Enviro-dri, BMI, Helmond, The Netherlands) was supplied as cage-enrichment.

Diet
Free access to standard pelleted laboratory animal diet (code VRF 1, Altromin, Lage, Germany).

Water
Free access to tap-water.

Certificates of analysis for ingredients and/or contaminants of diet, sawdust, paper and water were examined and then retained in the NOTOX archives. Results of analysis were assessed and did not reveal any findings that were considered to have affected study integrity.

6.4. Preliminary irritation study

A preliminary irritation study was conducted in order to select the highest test substance concentration to be used in the main study. In principle, this concentration should be well tolerated systemically by the animals and may give moderate irritation (grade 2) at the highest.

A series of two test substance concentrations were tested, the highest concentration being the maximum concentration that could technically be applied. The starting- and subsequent concentrations were taken from the series: 100% (undiluted), 50%, 25%, 10%, 5%, 2.5%, 1% and if needed further lower concentrations using the same steps. The test system, procedures and techniques were identical to those used during days 1 to 3 of the main study unless otherwise specified.
Two young adult animals were selected (5-14 weeks old). Each animal was treated with one concentration on three consecutive days. Approximately 4 hours after the last exposure, the ear was cleaned of residual test substance with water and the irritation was assessed. Bodyweights were determined on day 3. No necropsy was performed after termination.

6.5. Main study

Three groups of five animals were treated with three test substance concentrations respectively. One group of five animals was treated with vehicle.

ALLOCATION

GROUP*		INDUCTION
1 (1-5)	Vehicle control	Vehicle
2 (6-10)	Experimental	Lowest test substance concentration: 2.5%
3 (11-15)	Experimental	Intermediate test substance concentration: 10%
4 (16-20)	Experimental	Highest test substance concentration: 25%

*. five females each group, animal numbers between brackets

INDUCTION - Days 1, 2 and 3

Experimental animals:
The dorsum surface of both ears was epidermally treated (25 µl/ear) with the test substance concentration, at approximately the same time each day.

Vehicle control animals:
The control animals were treated the same as the experimental animals, except that, instead of the test substance, the vehicle alone was administered.

TREATMENT - Day 6:

All animals:
Each animal was injected via the tail vein with 0.25 ml of sterile phosphate buffered saline (PBS) containing 20 µCi of ^{3}H-methyl thymidine (Amersham Pharmacia Biotech, NOTOX Substance 105624).

After approximately five hours, all animals were killed by intra peritoneal injection with an overdose of pentobarbital. The draining (auricular) lymph node of each ear was excised.
The relative size of the nodes (as compared to normal) was estimated by visual examination and abnormalities of the nodes were recorded. The nodes were pooled for each animal in 3 ml PBS.

6.6. Tissue processing for radioactivity

A single cell suspension of lymph node cells (LNC) was prepared in PBS by gentle separation through stainless steel gauze (diameter 125 µm). LNC were washed twice with an excess of PBS by centrifugation at 200*g* for 10 minutes at 4° C. The DNA was precipitated with 3 ml 5% trichloroacetic acid (TCA) at 4° C for approximately 18 hours.
Precipitates were recovered by centrifugation at 200*g* for 10 minutes, resuspended in 1 ml TCA and transferred to 10 ml of Ultima Gold (Packard) as the scintillation fluid.

6.7. Radioactivity measurements

All radioactive measurements were performed using a Packard scintillation counter (1900TR). Counting time was to a statistical precision of ± 0.2% or a maximum of 5 minutes whichever comes first. The Packard 1900TR was programmed to automatically subtract background and convert CPM to DPM.

6.8. Observation

Mortality/Viability	Twice daily.
Toxicity	At least once daily.
Body weights	On days 1 (pre-treatment) and 6.
Necropsy	The animals were sacrificed by intra peritoneal injection with an overdose of pentobarbital and were subjected to necropsy for gross macroscopic examination.
Irritation	On day 3 (3-4 hours after treatment), the skin reactions were assessed. If possible, skin reactions were graded according to the following numerical scoring system. Furthermore descriptions of all other (local) effects were recorded.

Grading Irritation Reactions:

Erythema and eschar formation:
No erythema 0
Slight erythema (barely perceptible) 1
Well-defined erythema 2
Severe erythema (beet redness) to slight eschar formation (injuries in depth) 3

Oedema formation:
No oedema 0
Slight oedema (barely perceptible) 1
Moderate oedema 2
Severe oedema 3

6.9. Interpretation

DPM values are presented for each animal and for each dose group. A Stimulation Index (SI) is calculated for each group. The SI is the ratio of the DPM/group compared to DPM/vehicle control group.

If the results indicate a SI ≥ 3, the test substance may be regarded as a skin sensitiser, based on the test guideline and recommendations done by ICCVAM (NIH publication; No 99-4494, February 1999).

The results were evaluated according to the OECD Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances (OECD, 1998) and the EC criteria for classification and labelling of dangerous substances and preparations (Council Directive 67/548/EEC and all adaptations to technical progress and amendments of this Directive published in the Official Journal of the European Communities).

If possible, an EC3 value (the estimated test substance concentration that will give a SI =3) was determined, using linear interpolation (Reference 2).

6.10. List of protocol deviations

1. Deviations from the minimum and maximum level for relative humidity occurred. Evaluation: Based on laboratory historical data these deviations were considered not to have affected the study integrity.

The study integrity was not adversely affected by the deviations.

7. RESULTS

PRELIMINARY IRRITATION STUDY

The results of the epidermal exposures for the selection of highest test substance concentration to be tested in the main study are described in Table 1.

The highest test substance concentration selected for the main study was a 25% concentration, based on the technical applicability of the test substance.

MAIN STUDY

Induction phase (Table 2)
The skin effects seen after the third epidermal exposure are presented in the table. No irritation was observed in any of the animals examined.

Macroscopy (Table 2)
All nodes of the experimental and control groups were considered normal in size.

Body Weights (Table 2)
Body weights and body weight gain of experimental animals remained in the same range as controls over the study period.

Radioactivity Measurements (Table 3/4)
Mean DPM/animal values for the experimental groups treated with test substance concentrations 2.5, 10 and 25% were 151, 105 and 169 respectively.
The mean DPM/animal value for the vehicle control group was 198.

Toxicity / Mortality
No mortality occurred and no symptoms of systemic toxicity were observed in the animals of the main study.

8. CONCLUSION

The SI values calculated for the substance concentrations 2.5, 10 and 25% were 0.8, 0.5 and 0.9 respectively.

There was no indication that the test substance could elicit an SI ≥ 3. The EC3 value was established to exceed 25%.

Based on these results and according to the recommendations made in the test guidelines (OECD No.429, EC B.42 and EPA OPPTS 870.2600), Purified calcium lignosulfonate should not be regarded as a skin sensitiser.

Based on these results and according to the:
- OECD Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances (OECD, 1998), Purified calcium lignosulfonate does not have to be classified for sensitisation by skin contact.

- EC criteria for classification and labelling requirements for dangerous substances and preparations (Council Directive 67/548/EEC), Purified calcium lignosulfonate does not have to be classified and has no obligatory labelling requirement for sensitisation by skin contact.

9. REFERENCES

1. National Institute of Environmental Health Sciences, The Murine Lymph Node Assay: A test method for assessing the allergic contact dermatitis potential of chemicals/compounds. Independent peer review by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods (NICEATM), NIH publication; No 99-4494, February 1999.

2. Basketter DA, Lea LJ, Dickens A, Briggs, D, Pate I, Dearman RJ and Kimber I. A comparison of statistical approaches to the derivation of EC3 values from local lymph node assay dose responses. J Appl Toxicol 1999;19:261-266

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PRELIMINARY IRRITATION STUDY

Table 1: Grading of Skin Reactions after Epidermal Exposure

	Day 1		Day 3				
animal number	Body weight (g)	conc. %	dorsal surface left ear erythema	dorsal surface left ear oedema	dorsal surface right ear erythema	dorsal surface right ear oedema	Body weight (g)
1	20	25	1	0	0	0	19
2	25	50	1	0	0	0	24

Note: The 50% concentration was applied using a spatula, instead of using a pipette. The 50% concentration did not attach to the ear properly.

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MAIN STUDY

Table 2: Skin reactions after 3rd induction, Body Weights and Relative Size Nodes

group treatment	an no	Day1 bw (g)	Day3 skin grading dorsal surface ear left erythema	left oedema	right erythema	right oedema	Day6 bw (g)	size nodes left	size nodes right
1	1	18	0	0	0	0	19	n	n
Vehicle *	2	22	0	0	0	0	22	n	n
Control	3	22	0	0	0	0	23	n	n
	4	24	0	0	0	0	26	n	n
	5	24	0	0	0	0	25	n	n
2	6	22	0	0	0	0	22	n	n
2.5%	7	21	0	0	0	0	21	n	n
test	8	23	0	0	0	0	24	n	n
substance	9	23	0	0	0	0	24	n	n
	10	21	0	0	0	0	22	n	n
3	11	22	0	0	0	0	22	n	n
10% test	12	22	0	0	0	0	23	n	n
substance	13	21	0	0	0	0	23	n	n
	14	23	0	0	0	0	24	n	n
	15	22	0	0	0	0	23	n	n
4	16	22	0	0	0	0	22	n	n
25% test	17	20	0	0	0	0	21	n	n
substance	18	21	0	0	0	0	23	n	n
	19	23	0	0	0	0	25	n	n
	20	25	0	0	0	0	26	n	n

Legends: BW = Body weight, Relative size nodes (-: reduced, +: enlarged, n: considered to be normal)

*. Vehicle: Propylene glycol

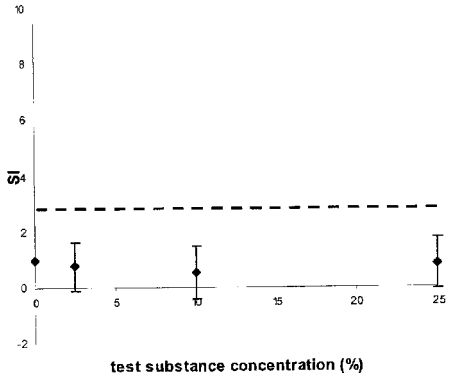
Table 3: Radioactivity measurements (individual animals)

group	animal	treatment	Induction	DPM / animal
1	1	vehicle control	Propylene glycol	109
	2			384
	3			228
	4			108
	5			159
2	6	experimental	2.5% test substance	92
	7			53
	8			251
	9			103
	10			257
3	11	experimental	10% test substance	233
	12			54
	13			97
	14			40
	15			101
4	16	experimental	25% test substance	64
	17			309
	18			256
	19			23
	20			194

Table 4: Calculation of Stimulation Index (SI)

group	treatment	induction	mean DPM ± SD	SI ± SD
2	experimental	2.5% test substance	151 ± 96	0.8 ± 0.9
3	experimental	10% test substance	105 ± 76	0.5 ± 0.9
4	experimental	25% test substance	169 ± 123	0.9 ± 0.9
1	vehicle control	Propylene glycol	198 ± 115	1.0

Figure 1: Dose-response curve



APPENDIX

ASSESSMENT OF CONTACT HYPERSENSITIVITY

TO ALPHA-HEXYLCINNAMIC ALDEHYDE, TECH., 85%

IN THE MOUSE (LOCAL LYMPH NODE ASSAY)

A RELIABILITY CHECK.

NOTOX Project 416082

Purified calcium lignosulfonate NOTOX Project 419535

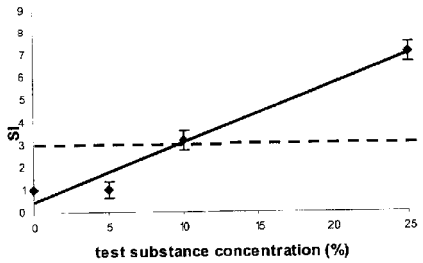
SUMMARY

A reliability check is carried out at regular intervals to check the sensitivity of the test system and the reliability of the experimental techniques as used by NOTOX. In this study, performed in July / August 2004, females of the CBA mouse strain (from Charles River France, L'Arbresle Cedex, France) were checked for the sensitivity to ALPHA-HEXYLCINNAMICALDEHYDE, TECH. 85%. The females were approx. 12 weeks old at commencement of the study. The study was based on the OECD Guideline No. 429, the EC Directive 67/548/EC, Part B.42 and on the method described by ICCVAM (NIH publication; No 99-4494, February 1999). ALPHA-HEXYLCINNAMICALDEHYDE, TECH. 85% (CAS no. 101-86-0) was fabricated under lot no. 10021HF (Aldrich Chemicals Co., Germany). Concentrations used for this study were 5, 10 and 25% in Acetone:Olive oil (4:1).

group	treatment	induction	mean DPM ± SD	SI ± SD
2	experimental	5% test substance	235 ± 53	1.0 ± 0.4
3	experimental	10% test substance	766 ± 225	3.2 ± 0.4
4	experimental	25% test substance	1708 ± 509	7.1 ± 0.4
1	vehicle control	Acetone : Olive oil (4:1)	241 ± 75	1.0

*. five females each group

Dose-response curve



CONCLUSION

The SI values calculated for the substance concentrations 5, 10 and 25% were 1.0, 3.2 and 7.1 respectively. An EC3 value of 9.5% was calculated using linear interpolation.

The calculated EC3 value was found to be in the acceptable range of 2 and 20%. The results of the 6 monthly HCA reliability checks of the recent years were 8.8, 5.5, 7.3 and 10.3%.

Based on the results, it was concluded that the Local Lymph Node Assay as performed at NOTOX is an appropriate model for testing for contact hypersensitivity.

The raw data, protocol and report from this study are kept in the NOTOX archives. The test described above was performed in accordance with NOTOX Standard Operating Procedures and the report was audited by the QA-unit.