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October 12, 2005

US Environmental Protection Agency  
OPPT Document Control Office (Mail Code 7407)  
1200 Pennsylvania Ave, NW  
EPA East Room 6428  
Washington, DC 20460  
ATTN: Section 8(e) Coordinator



CONTAIN NO CBI

RE: Notification of Substantial Risk

Dear Sir or Madam:

In accordance with the provisions of Section 8(e) of the Toxic Substances Control Act (TSCA), Rhodia Inc. (Rhodia) is submitting the following information:

Study Number 1: "Alcodet 218: Local Lymph Node Assay (LLNA) in Mice (Identification of Contact Allergens)"<sup>1</sup>

Study Number 2: "Mirataine D/40: Local Lymph Node Assay (LLNA) in Mice (Identification of Contact Allergens)"<sup>2</sup>

Although Rhodia Inc. does not believe that this substance would present an unreasonable hazard to workers or to the public when properly used in its intended applications, we believe the studies may meet the EPA's reporting criteria for TSCA Section 8(e).

In both studies, five (5) groups of female mice (4 mice per group) were treated daily with the test material at concentrations of 5%, 10%, 25%, and 50% (diluted in either acetone/olive oil (4:1), or ethanol/water (7:3)) and at a final concentration of 100% test material. The compounds were applied to the ear lobes (left and right) of the mice for 3 consecutive days. A control group of 4 mice was treated with the vehicle only. Five days after the first topical application, the mice were injected intravenously with radio-labeled thymidine (<sup>3</sup>H-methyl thymidine). Approximately five hours after the intravenous injection, the mice were sacrificed, and the draining auricular lymph nodes excised and pooled per group. Single-cell suspensions of lymph node cells were prepared from the pooled nodes, washed and incubated with trichloroacetic acid overnight. The proliferation of pooled lymph node cells was determined by the incorporation of <sup>3</sup>H-methyl thymidine, as measured by a scintillation counter.

<sup>1</sup> This test material is identified by CAS# 9004-83-5, with a CAS name of "Poly(oxy-1,2-ethanediyl), alpha-[2-(tert-dodecylthio)ethyl]-omega-hydroxy".

<sup>2</sup> This test material is identified by CAS# 683-10-3, with a CAS name of "Dodecanaminium, N-(carboxymethyl), N, N-dimethyl-, hydroxide, inner salt" (at an approximate concentration of 22%), and CAS# 2601-33-4, with a CAS name of "Tetradecanaminium, N-(carboxymethyl), N, N-dimethyl-, hydroxide, inner salt" (at an approximate concentration of 8%) as an aqueous solution

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No clinical signs were observed in any animals in the control group, or the group dosed at 5% of test material. On the second application day, slight to severe ear erythema was observed among mice in all the other treatment groups.. This persisted through the remainder of the study. The size of the draining lymph nodes of animals dosed at 50% and 100% test compound were observed to be twice as large as the corresponding nodes among mice in the control group.

Using this study design, the criterion for a test compound to be considered a potential sensitizer is if the exposure to the test compound results in a 3-fold (or greater) increase in incorporation of <sup>3</sup>H-methyl thymidine, as compared to the control animals. That concentration at which a 3- fold increase is calculated to occur is designated the EC3. In Study Number 1 as named above the test material was calculated to have an EC3 concentration of 14.0%.. In Study Number 2, the test material was calculated to have an EC3 concentration of 5.8%.

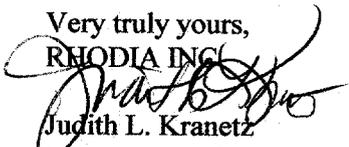
These studies were communicated to Rhodia on September 15, 2005.

Rhodia asserts that none of the information contained within this notice constitutes confidential business information.

Should you have any questions, or require any further information, please call (215) 369-9734. Thank you.

Very truly yours,

RHODIA INC



Judith L. Kranetz

Manager, Product Safety

JLK/

Atts

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## **RCC Study Number 859435**

*Study #1*

### **ALCODET 218:**

### **Local Lymph Node Assay (LLNA) in Mice (Identification of Contact Allergens)**

#### **Report**

**Author:** Dr. W. Wang-Fan  
**Sponsor:** RHODIA-HPCII  
40 rue de la Haie Coq  
F-93306 AUBERVILLIERS / FRANCE

**Study Completion Date:** 07 June 2005

Total Number of Pages: 38



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## 1 PREFACE

### 1.1 GENERAL

Title	ALCODET 218: Local Lymph Node Assay (LLNA) in Mice (Identification of Contact Allergens)
Sponsor	Mr. P-F VALENTIN RHODIA-HPCII 40 rue de la Haie Coq F-93306 AUBERVILLIERS / FRANCE
Study Monitor	Miss Annie Buard Rhodia Services Saint-Fons DELTA Boîte Postale 70026 20, rue Marcel Sembat F-69191 SAINT FONS CEDEX / France
Test Facility	a) RCC Ltd Toxicology Zelgliweg 1 CH - 4452 Itingen / Switzerland
Test Site	b) RCC Ltd Environmental Chemistry & Pharmanalytics Zelgliweg 1 CH - 4452 Itingen / Switzerland
Lead QA	RCC Ltd Quality Assurance GLP Toxicology Zelgliweg 1 CH - 4452 Itingen / Switzerland
Test Site QA	RCC Ltd Quality Assurance GLP Environmental Chemistry & Pharmanalytics Zelgliweg 1 CH - 4452 Itingen / Switzerland (Responsible for test site)

## 1.2 RESPONSIBILITIES

<b>Study Director</b>	Dr. W. Wang-Fan (a)
Deputy Study Director	L.G. Ullmann (a)
Technical Coordinator / Necropsy	E. Deperade (a)
Head of Lead Quality Assurance	I. Wüthrich

### Principal Investigator

Study Phase: <sup>3</sup>HTdR Determination Dr. R. Burri (b)

## 1.3 SCHEDULE

Experimental Starting Date	23-MAR-2005	
Experimental Completion Date	06-APR-2005	
Delivery of Animals	23-MAR-2005	
Acclimatization	23-MAR-2005	to 29-MAR-2005
Treatment (epicutaneous)	30-MAR-2005	to 01-APR-2005
Treatment (intravenous)	04-APR-2005	
Observation	23-MAR-2005	to 04-APR-2005
<sup>3</sup> HTdR Determination	05-APR-2005	

## 1.4 ARCHIVING

RCC Ltd (CH-4452 Itingen / Switzerland) will retain the study plan, raw data of the test facility and the test site, sample of test item(s) and the final report of the present study for at least ten years. No data will be discarded without the Sponsor's written consent.

## 1.5 SIGNATURES

STUDY DIRECTOR:

Dr. W. Wang-Fan

W. Wang-Fan  
date: 07 June 2005

TEST FACILITY MANAGEMENT:

Dr. H. Fankhauser

H. Fankhauser  
date: 07 June 2005

## 1.6 QUALITY ASSURANCE GLP TOXICOLOGY

RCC Ltd, Toxicology, CH-4452 Itingen / Switzerland

### STATEMENT

RCC Study Number: 859435  
Test Item: ALCODET 218  
Study Director: Dr. W. Wang-Fan  
Title: ALCODET 218:  
Local Lymph Node Assay (LLNA) in Mice  
(Identification of Contact Allergens)

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

Study procedures, with the exception of the pre-test(s), were periodically inspected. The study plan and this report were audited by the RCC Quality Assurance. The dates are given below:

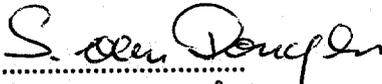
Dates and Types of QA Inspections		Dates of Reports to the Study Director and Test Facility Management
15-MAR-2005	Study Plan	15-MAR-2005
06-APR-2005	Process Based (Raw Data, Test System, Test Item, Administration, Dose Preparation)	06-APR-2005
09-MAY-2005	Report	09-MAY-2005

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:

S. van Dongen

  
date: 07-Sept-2005

GOOD LABORATORY PRACTICE

**1.7 STATEMENT OF COMPLIANCE**

RCC Study Number	859435
Test Item	ALCODET 218
Study Director	Dr. W. Wang-Fan
Title	ALCODET 218: <u>L</u> ocal <u>L</u> ymph <u>N</u> ode <u>A</u> ssay (LLNA) in Mice (Identification of Contact Allergens)

The non-GLP pretest was performed and is excluded from this Statement of Compliance.

The purity / composition of the test item are available in sponsor's file and excluded from this Statement.

This study has been performed in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted February 2<sup>nd</sup>, 2000 [RS 813.016.5]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26<sup>th</sup>, 1997 by decision of the OECD Council [C(97) 186/Final].

Study Director:

Dr. W. Wang-Fan

W. Wang-Fan  
date: 07 June 2005

## 1.8 TEST GUIDELINES

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

OECD Guideline for the Testing of Chemicals, Guideline 429: Skin Sensitization: Local Lymph Node Assay (adopted 24 April 2002).

Commission Directive 2004/73/EC, B.42: Skin Sensitization: Local Lymph Node Assay, 29 April 2004.

## 1.9 ANIMAL WELFARE

This study was performed in an AAALAC-approved laboratory in accordance with the Swiss Animal Protection Law under license no. 114.

## 1.10 REFERENCES

Kimber I., Hilton J. and Weisenberger C. (1989). The murine local lymph node assay for identification of contact allergens: a preliminary evaluation of in situ measurement of lymphocyte proliferation. *Contact Dermatitis*, 21, 215-220.

Kimber I. and Basketter D.A. (1992). The murine local lymph node assay. A commentary on collaborative studies and new directions. *Food and Chemical Toxicology*, 30, 165-169.

Basketter D.A., Gerberick G.F., Kimber I. and Loveless S.E. (1996). The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. *Food and Chemical Toxicology*, 34, 985-997.

Chamberlain M. and Basketter D.A. (1996). The local lymph node assay: status of validation. *Food and Chemical Toxicology*, 34, 999-1002.

Basketter D.A., Lea L.J., Cooper K., Stocks J., Dickens A., Pate I., Dearman R.J. and Kimber I. (1999). Threshold for Classification as a Skin Sensitizer in the Local Lymph Node Assay: A Statistical Evaluation. *Food and Chemical Toxicology*, 37, 1-8.

Steiling W., Basketter D.A., Berthold K., Butler M., Garrigue J-L., Kimber I., Lea L.J., Newsome C., Roggeband R., Stropp G., Waterman S. and Wiemann C. (2001): Skin Sensitisation Testing - New Perspectives and Recommendations. *Food and Chemical Toxicology*, 39, 293-301.

## 2 SUMMARY

In order to study a possible contact allergenic potential of ALCODET 218, five groups each of four female mice were treated daily with the test item at concentrations of 5 %, 10 %, 25 %, 50 % (w/v) in acetone/olive oil (4/1, v/v) and 100 % (undiluted) by topical application to the dorsum of each ear lobe (left and right) for three consecutive days. A control group of four mice was treated with the vehicle (acetone/olive oil (4/1, v/v)) only. Five days after the first topical application the mice were injected intravenously into a tail vein with radio-labelled thymidine (<sup>3</sup>H-methyl thymidine). Approximately five hours after intravenous injection, the mice were sacrificed, the draining auricular lymph nodes excised and pooled per group. Single cell suspensions of lymph node cells were prepared from pooled lymph nodes which were subsequently washed and incubated with trichloroacetic acid overnight. The proliferative capacity of pooled lymph node cells was determined by the incorporation of <sup>3</sup>H-methyl thymidine measured in a  $\beta$ -scintillation counter.

All treated animals survived the scheduled study period.

No clinical signs were observed in any animals of the control group or Groups 2-4. On the third application day, the touch sensitivity was observed at the range of head in all mice of Group 5 (50 %) and Group 6 (100 %, undiluted).

The results obtained (STIMULATION INDEX (S.I.)) are reported in the following table. The estimated concentration of test item required to produce a S.I. of 3 is referred to as the EC3 value.

	Test item concentration % (w/v)	S.I.
Group 2	5	2.6
Group 3	10 *	2.6 *
Group 4	25 *	4.1 *
Group 5	50	6.3
Group 6	100 (undiluted)	7.3
<b>EC3 = 14.0 % (w/v)</b>		
* This value was used in calculation of EC3.		

### **3 CONCLUSION**

A test item is regarded as a sensitizer in the LLNA if the exposure to one or more test concentrations resulted in 3-fold or greater increase in incorporation of <sup>3</sup>HTdR compared with concurrent controls, as indicated by the STIMULATION INDEX (S.I.).

In this study STIMULATION INDICES of 2.6, 2.6, 4.1, 6.3 and 7.3 were determined with the test item at concentrations of 5 %, 10 %, 25 %, 50 % (w/v) in acetone/olive oil (4/1, v/v) and 100 % (undiluted), respectively.

ALCODET 218 was therefore found to be a skin sensitizer and an EC3 value of 14.0 % (w/v) was derived.

## 4 PURPOSE

The purpose of this Local Lymph Node Assay was to identify the contact allergenic potential of ALCODET 218 when administered to the dorsum of both ear lobes of mice.

This study should provide a rational basis for risk assessment on the sensitizing potential of the test item in man.

## 5 MATERIALS AND METHODS

### 5.1 TEST SYSTEM

Test system	Mice, CBA/CaHsdRcc(SPF)
Rationale	Recognized by OECD Guideline 429 as the recommended test system.
Source	RCC Ltd CH-4414 Füllinsdorf / Switzerland
Number of animals for the pre-test (non-GLP)	2 females
Number of animals for the main study	24 females
Number of animals per group	4 females (nulliparous and non-pregnant)
Number of test groups	5
Number of negative control group	1
Age	8 - 12 weeks (beginning of acclimatization)
Body weight	16 g - 24 g (ordered)
Identification	Each cage by unique cage card.
Randomization	Randomly selected by computer algorithm at time of delivery.
Acclimatization	Under test conditions after health examination. Only animals without any visible signs of illness were used for the study.

## 5.2 ALLOCATION

The animals were distributed as follows:

GROUP	CONCENTRATION <sup>b)</sup> % (w/v)	NUMBER OF ANIMALS PER GROUP	CAGE NUMBER (Individually housed)
1 (Control Group <sup>a)</sup> )	-	4	1 - 4
2	5	4	5 - 8
3	10	4	9 - 12
4	25	4	13 - 16
5	50	4	17 - 20
6	100 (undiluted)	4	21 - 24

<sup>a)</sup> vehicle group = acetone/olive oil (4/1, v/v)

<sup>b)</sup> In a non-GLP conform pre-test in two mice, test item concentrations of 10 %, 25 %, 50 % (w/v) in acetone/olive oil (4/1, v/v) and 100 % (undiluted) were tested on one ear each. One day after a single topical application no irritation effects were observed at these concentrations.

100 % (undiluted) was the highest technically applicable concentration.

The sensitivity and reliability of the experimental technique employed was assessed by use of a substance which is known to have skin sensitization properties in CBA/CaOlaHsd mice. The validation- / positive control study was performed with ALPHA-HEXYLCINNAMALDEHYDE in acetone/olive oil (4/1, v/v) using CBA/CaOlaHsd mice (RCC Study Number 858384) between 19-JAN-2005 to 02-FEB-2005, results see Appendix C.

## 5.3 HUSBANDRY

Room no.

E24 / RCC Itingen

Conditions

Standard Laboratory Conditions. Air-conditioned with ranges for room temperature  $22 \pm 3$  °C, relative humidity 30 - 70 % and 10 - 15 air changes per hour. Room temperature and humidity were monitored continuously and values outside of these ranges occasionally occurred, usually following room cleaning. These transient variations are considered not to have any influence on the study and, therefore, these data are not reported but are retained at RCC. There was a 12 hour fluorescent light / 12 hour dark cycle with at least 8 hours music during the light period.

Accommodation

Individual in Makrolon type-2 cages with standard softwood bedding ("Lignocel", Schill AG, CH-4132 Muttenz).

Diet

Pelleted standard Kliba 3433, batch no. 94/04 mouse maintenance diet (Provimi Kliba AG, CH-4303 Kaiseraugst) available *ad libitum*. Results of

analyses for contaminants are archived at RCC. There was no contamination of the diet.

**Water**

Community tap water from Itingen, available *ad libitum*. Results of representative bacteriological, chemical and contaminant analyses are archived at RCC. There was no contamination of water.

**5.4 CHEMICALS**

**<sup>3</sup>H-methyl Thymidine**

Amersham TRA 310, aqueous solution, sterilized 74 GBq/mmol (2 Ci/mmol), 37 MBq/ml (1 mCi/ml) quantities: 9.25 MBq (250 µCi), 37 MBq (1 mCi)

Supplier

Amersham Biosciences UK Limited, Buckinghamshire England HP7 9NA, UK

Batch number

318

Storage conditions

In the original container at 5 °C ± 3 °C.

**Trichloroacetic acid**

Fluka no. 91230 (min. 99.5 %)

Supplier

Fluka Chemie AG (Industriestrasse 25, CH-9471 Buchs, Switzerland)

Batch number

422767/1 41801

Expiry date

14-DEC-2006

Storage conditions

At room temperature (20 °C ± 5 °C), away from direct sunlight.

**Phosphate buffered saline**

(1 tablet dissolved in 200 ml bi-distilled water)

Supplier

Fluka Chemie AG (Industriestrasse 25, CH-9471 Buchs, Switzerland)

Batch number

453405/1

Expiry date

OCT-2006

Storage conditions

In the original container at room temperature (20 °C ± 5 °C), away from direct sunlight.

## 5.5 VEHICLES

Acetone/olive oil (4/1, v/v)

### 1) Acetone

Supplier	Baker, P. H. Stehelin & Cie AG (Spalentorweg 62, CH-4003 Basel, Switzerland)
Batch number	0500400001
Expiry date	MAY-2006

Storage conditions	In the original container at room temperature (20 °C ± 5 °C), away from direct sunlight.
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### 2) Olive oil

Supplier	Roth AG (Chr. Merian-Ring 7, CH-4153 Reinach BL, Switzerland)
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Batch number	8873.1
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Expiry date	23-SEP-2006
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Storage conditions	In the original container at room temperature (20 °C ± 5 °C), away from direct sunlight.
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## 5.6 TEST ITEM

Identity	ALCODET 218
Description	Colourless to pale yellow liquid
Batch number	MH3HC00742
Purity / Composition	Available in sponsor's file, excluded from Statement of Compliance.
Stability of test item	Stable under storage conditions
Expiry date	04-AUG-2005
Storage conditions	At room temperature (20 °C ± 5 °C), away from direct sunlight.
Safety precautions	Routine hygienic procedures (gloves, goggles, face mask).

The test item information was supplied by the Sponsor.

## **5.7 TEST ITEM FORMULATIONS PREPARATION**

The test item was placed into a volumetric flask on a tared Mettler balance and the vehicle (acetone/olive oil (4/1, v/v)) was quantitatively added. The weight/volume (w/v) dilutions were prepared individually using a magnetic stirrer as homogenizer. The test item was soluble in the vehicle very well.

Test item formulations were made freshly before each dosing occasion and no more than 4 hours prior to application to the ears.

Homogeneity of the test item in the vehicle was maintained during treatment with the magnetic stirrer.

To determine the highest non-irritant and technically applicable test item concentration, a non-GLP pretest was performed in two mice with concentrations of 10 %, 25 %, 50 % (w/v) in acetone/olive oil (4/1, v/v) and 100 % (undiluted) (pretest excluded from Statement of Compliance).

The test item in the main study was assayed at five consecutive concentrations. The top dose is the highest technically applicable concentration while avoiding systemic toxicity and excessive local irritation. No severe irritant effects were tolerated choosing the test concentrations.

Concentrations were in terms of material as supplied.

## **5.8 RATIONALE**

The study procedure was used to detect a possible contact allergenic potential of the test item applied.

## 6 STUDY CONDUCT

### 6.1 PRE-TEST

In a non-GLP animal pre-test in two mice, the test item was tested at four different concentrations: 10 %, 25 %, 50 % (w/v) in acetone/olive oil (4/1, v/v) and 100 % (undiluted), on one ear each.

One day after a single topical application no irritation effects were observed at these concentrations. The pre-test results determined that 100 % (undiluted) was the highest technically applicable concentration.

### 6.2 TREATMENT PROCEDURES

#### 6.2.1 TOPICAL APPLICATION

Each test group of mice was treated by topical (epidermal) application to the dorsal surface of each ear lobe (left and right) with different test item concentrations of 5 %, 10 %, 25 %, 50 % (w/v) in acetone/olive oil (4/1, v/v) and 100 % (undiluted). The application volume, 25  $\mu$ l, was spread over the entire dorsal surface ( $\varnothing$  ~ 8 mm) of each ear lobe once daily for three consecutive days. A further group of mice was treated with an equivalent volume of the relevant vehicle alone (control animals). A hair dryer was passed briefly over the ear's surface to prevent the loss of any of the test item applied.

#### 6.2.2 ADMINISTRATION OF <sup>3</sup>H-METHYL THYMIDINE\*

<sup>3</sup>H-methyl thymidine (<sup>3</sup>HTdR) was purchased from Amersham International (Amersham product code no. TRA 310; specific activity, 2 Ci/mmol; concentration, 1 mCi/ml).

Five days after the first topical application, all mice were administered with 250  $\mu$ l of 81.63  $\mu$ Ci/ml <sup>3</sup>HTdR (equal to 20.4  $\mu$ Ci <sup>3</sup>HTdR) by intravenous injection via a tail vein.

#### 6.2.3 DETERMINATION OF INCORPORATED <sup>3</sup>HTDR\*

Approximately five hours after treatment with <sup>3</sup>HTdR all mice were euthanized by inhalation of CO<sub>2</sub> (dry ice).

The draining lymph nodes were rapidly excised and pooled for each experimental group (8 nodes per group). Single cell suspensions (phosphate buffered saline) of pooled lymph node cells were prepared by gentle mechanical disaggregation through stainless steel gauze (200  $\mu$ m mesh size). After washing twice with phosphate buffered saline (approx. 10 ml) the lymph node cells were resuspended in 5 % trichloroacetic acid (approx. 3 ml) and incubated at approximately +4 °C for at least 18 hours for precipitation of macromolecules. The precipitates were then resuspended in 5 % trichloroacetic acid (1 ml) and transferred to glass scintillation vials with 10 ml of 'Irga-Safe Plus' scintillation liquid and thoroughly mixed.

The level of <sup>3</sup>HTdR incorporation was then measured on a  $\beta$ -scintillation counter. Similarly, background <sup>3</sup>HTdR levels were also measured in two 1 ml-aliquots of 5 % trichloroacetic acid.

\* Preparation of <sup>3</sup>HTdR solutions and <sup>3</sup>HTdR measurements at RCC Ltd, Environmental Chemistry & Pharamalytics  
No phase report of the results of the <sup>3</sup>HTdR level analysis was provided by the Principal Investigator.

The  $\beta$ -scintillation counter expresses  $^3\text{HTdR}$  incorporation as the number of radioactive disintegrations per minute (dpm).

#### 6.2.4 INTERPRETATION OF RAW DATA

The proliferative response of lymph node cells is expressed as the number of radioactive disintegrations per minute per lymph node (dpm/node) and as the ratio of  $^3\text{HTdR}$  incorporated into lymph node cells of test group relative to that recorded for control group (STIMULATION INDEX) (S.I.). Before dpm/node values were determined, mean scintillation-background dpm was subtracted from test and control raw data.

A test item is regarded as a sensitizer in the LLNA if the following criteria are fulfilled:

- First, exposure to at least one concentration of the test item resulted in an incorporation of  $^3\text{HTdR}$  at least 3-fold or greater than that recorded in control mice, as indicated by the STIMULATION INDEX (S.I.).
- Second, the data are compatible with a conventional dose response, although allowance must be made (especially at high topical concentrations) for either local toxicity or immunological suppression.

### 6.3 OBSERVATIONS

Mortality / Viability	Twice daily from acclimatization start to the termination of in-life phase.
Body weights	On the test day 1 (prior to the 1 <sup>st</sup> application) and on the test day 6.
Clinical signs (local / systemic)	Daily from acclimatization start to the termination of in-life phase. Especially the treatment sites will be observed carefully.

### 6.4 STATISTICAL ANALYSIS

The mean values and standard deviations were calculated in the body weight tables.

A statistical analysis was conducted for assessment of the dose-response relationship, and the EC3 value was calculated according to the equation

$$\text{EC3} = (a-c) \left[ \frac{(3-d)}{(b-d)} \right] + c$$

where EC3 is the estimated concentration of the test item required to produce a 3-fold increase in draining lymph node cell proliferative activity; (a, b) and (c, d) are respectively the co-ordinates of the two pair of data lying immediately below and above the S.I. value of 3 on the local lymph node assay dose response plot.

### 6.5 DATA COMPILATION

Body weights were recorded on-line in RCC-TOX LIMS.

Clinical signs were compiled on data sheets.

## 7 RESULTS

### 7.1 CALCULATION AND RESULTS OF INDIVIDUAL DATA

The proliferative capacity of pooled lymph node cells was determined by the incorporation of <sup>3</sup>H-methyl thymidine measured on a β-scintillation counter.

	Test Item concentration % (w/v)	S.I.
Group 2	5	2.6
Group 3	10 *	2.6 *
Group 4	25 *	4.1 *
Group 5	50	6.3
Group 6	100 (undiluted)	7.3
<b>EC3 = 14.0 % (w/v)</b>		
* This value was used in calculation of EC3.		

The radioactive disintegration values for the individual treatment groups are included in Appendix A.

### 7.2 VIABILITY / MORTALITY

No deaths occurred during the study period.

### 7.3 CLINICAL SIGNS

No clinical signs were observed in any animals of the control group or Groups 2-4. On the third application day, the touch sensitivity was observed at the range of head in all mice of Group 5 (50 %) and Group 6 (100 %, undiluted).

Clinical signs were compiled on data sheets.

### 7.4 BODY WEIGHTS

The body weight of the animals, recorded prior to the first application and prior to necropsy, was within the range commonly recorded for animals of the strain and age.

The individual as well as groupwise summarized body weight values are included in Appendix B.

## 7.5 DISCUSSION AND CONCLUSION

In this study STIMULATION INDICES of 2.6, 2.6, 4.1, 6.3 and 7.3 were determined with the test item at concentrations of 5 %, 10 %, 25 %, 50 % (w/v) in acetone/olive oil (4/1, v/v) and 100 % (undiluted), respectively. A dose-response relationship was observed.

No clinical signs were observed in any animals of the control group or Groups 2-4. On the third application day, the touch sensitivity was observed at the range of head in all mice of Group 5 (50 %) and Group 6 (100 %, undiluted).

A test item is regarded as a sensitizer in the LLNA if the exposure to one or more test concentrations resulted in 3-fold or greater increase in incorporation of <sup>3</sup>HTdR compared with concurrent controls, as indicated by the STIMULATION INDEX (S.I.). ALCODET 218 was therefore found to be a skin sensitizer and an EC3 value of 14.0 % (w/v) was derived.

## **APPENDIX A**

### **CALCULATION AND RESULTS OF INDIVIDUAL DATA**

21

## CALCULATION AND RESULTS OF INDIVIDUAL DATA

The following results were obtained:

Vehicle: acetone/olive oil (4/1, v/v)

Test item concentration % (w/v)		Measurement dpm	Calculation			Result
			dpm - BG <sup>a)</sup>	number of lymph nodes	dpm per lymph node <sup>b)</sup>	S.I.
--	BG I	9	--	--	--	--
--	BG II	7	--	--	--	--
--	CG 1	6444	6436	8	805	--
5	TG 2	16601	16593	8	2074	2.6
10	TG 3	16603	16595	8	2074	2.6
25	TG 4	26596	26588	8	3324	4.1
50	TG 5	40442	40434	8 **	5054	6.3
100 *	TG 6	46859	46851	8 **	5856	7.3

\* Undiluted as delivered by the Sponsor

\*\* The size of the draining lymph nodes of this group was doubly large compared to those of the control group.

BG = Background (1 ml 5 % trichloroacetic acid) in duplicate

CG = Control Group

TG = Test Group

S.I. = Stimulation Index

a) = The mean value was taken from the figures BG I and BG II

b) = Since the lymph nodes of the animals of a dose group were pooled, dpm/node was determined by dividing the measured value by the number of lymph nodes pooled

	Test Item concentration % (w/v)	S.I.
Group 3	10 (a)	2.6 (b)
Group 4	25 (c)	4.1 (d)
<b>EC3 = (a-c) [(3-d)/(b-d)] + c = 14.0 % (w/v)</b>		

EC3 = Estimated concentration for a STIMULATION INDEX (S.I.) of 3.

a,b,c,d = Co-ordinates of the two pair of data lying immediately below and above the S.I. value of 3 on the LLNA dose response plot.

## **APPENDIX B**

### **INDIVIDUAL / SUMMARY BODY WEIGHTS**

RCC STUDY NUMBER 859435  
ALCODET 218

Report

BW-IND - 1  
27-APR-05

**BODY WEIGHTS (GRAM)  
FEMALES**

---

	Treatment	
	-----	-----
DAYS	1	6
WEEKS	1	1
ANIMAL		

---

GROUP 1 (NEG. CONTROL)

1	21.2	22.0
2	21.0	22.8
3	20.1	21.3
4	20.8	21.6

GROUP 2 (5%)

5	19.9	21.5
6	21.2	21.1
7	21.8	24.3
8	21.9	23.0

GROUP 3 (10%)

9	20.1	21.3
10	22.5	22.6
11	20.3	20.6
12	22.5	23.8

GROUP 4 (25%)

13	20.8	21.1
14	19.9	20.7
15	18.1	18.9
16	20.3	21.4

GROUP 5 (50%)

17	20.4	20.7
18	19.4	19.0
19	19.0	19.7
20	20.5	20.4

GROUP 6 (100%)

21	19.6	21.6
22	19.4	21.5
23	19.8	21.3
24	18.8	18.9

RCC STUDY NUMBER 859435  
 ALCODET 218

Report

BW-SUM - 1  
 27-APR-05

**BODY WEIGHTS (GRAM) SUMMARY  
 FEMALES**

Treatment		GROUP 1 NEG. CONTROL	GROUP 2 5%	GROUP 3 10%	GROUP 4 25%
DAY 1	MEAN	20.8	21.2	21.4	19.8
WEEK 1	ST.DEV.	0.5	0.9	1.3	1.2
	N	4	4	4	4
		GROUP 5 50%	GROUP 6 100%		
	MEAN	19.8	19.4		
	ST.DEV.	0.7	0.4		
	N	4	4		
Treatment		GROUP 1 NEG. CONTROL	GROUP 2 5%	GROUP 3 10%	GROUP 4 25%
DAY 6	MEAN	21.9	22.5	22.1	20.5
WEEK 1	ST.DEV.	0.7	1.5	1.4	1.1
	N	4	4	4	4
		GROUP 5 50%	GROUP 6 100%		
	MEAN	20.0	20.8		
	ST.DEV.	0.8	1.3		
	N	4	4		

## **APPENDIX C**

### **RESULTS OF POSITIVE CONTROL**

## **RCC Study Number 858384**

### **ALPHA-HEXYLCINNAMALDEHYDE: Local Lymph Node Assay (LLNA) in Mice (Identification of Contact Allergens) Validation- / Positive Control Study**

(performed between 19-JAN-2005 to 02-FEB-2005)

## SUMMARY

In order to study a possible contact allergenic potential of ALPHA-HEXYLCINNAMALDEHYDE, three groups each of four female mice were treated daily with the test item at concentrations of 5 %, 10 % and 25 % (w/v) in acetone:olive oil, 4:1 (v/v) by topical application to the dorsum of each ear lobe (left and right) for three consecutive days. A control group of four mice was treated with the vehicle (acetone:olive oil, 4:1 (v/v)) only. Five days after the first topical application the mice were injected intravenously into a tail vein with radio-labelled thymidine (<sup>3</sup>H-methyl thymidine). Approximately five hours after intravenous injection, the mice were sacrificed, the draining auricular lymph nodes excised and pooled per group. Single cell suspensions of lymph node cells were prepared from pooled lymph nodes which were subsequently washed and incubated with trichloroacetic acid overnight. The proliferative capacity of pooled lymph node cells was determined by the incorporation of <sup>3</sup>H-methyl thymidine measured in a  $\beta$ -scintillation counter.

All treated animals survived the scheduled study period.

No clinical signs were observed in any animals of the control group. On the second application day, a slight ear swelling was observed at both dosing sites in all mice of Group 4 (25 %), persisting for the remainder of the in-life phase of the study. One day after the third local application, a slight ear swelling was observed at both dosing sites in all mice of Group 2 (5 %) and Group 3 (10 %), persisting for the remainder of the in-life phase of the study.

The results obtained (STIMULATION INDEX (S.I.)) are reported in the following table. The estimated concentration of test item required to produce a S.I. of 3 is referred to as the EC3 value.

	Test item concentration % (w/v)	S.I.
Group 2	5 *	2.4 *
Group 3	10 *	3.6 *
Group 4	25	11.2
<b>EC3 = 7.5 % (w/v)</b>		
A clear dose-response relationship was observed.		
* This value was used in calculation of EC3.		

## CONCLUSION

A test item is regarded as a sensitizer in the LLNA if the exposure to one or more test concentrations resulted in 3-fold or greater increase in incorporation of <sup>3</sup>HTdR compared with concurrent controls, as indicated by the STIMULATION INDEX (S.I.).

In this study STIMULATION INDICES of 2.4, 3.6 and 11.2 were determined with the test item at concentrations of 5 %, 10 % and 25 % (w/v), respectively, in acetone:olive oil, 4:1 (v/v).

ALPHA-HEXYLCINNAMALDEHYDE was therefore found to be a skin sensitizer and an EC3 value of 7.5 % (w/v) was derived.

## RESULTS

### CALCULATION AND RESULTS OF INDIVIDUAL DATA

The proliferative capacity of pooled lymph node cells was determined by the incorporation of <sup>3</sup>H-methyl thymidine measured on a  $\beta$ -scintillation counter.

	Test item concentration % (w/v)	S.I.
Group 2	5 *	2.4 *
Group 3	10 *	3.6 *
Group 4	25	11.2
EC3 = 7.5 % (w/v)		
A clear dose-response relationship was observed.		
* This value was used in calculation of EC3.		

### VIABILITY / MORTALITY

No deaths occurred during the study period.

### CLINICAL SIGNS

No clinical signs were observed in any animals of the control group. On the second application day, a slight ear swelling was observed at both dosing sites in all mice of Group 4 (25 %), persisting for the remainder of the in-life phase of the study. One day after the third local application, a slight ear swelling was observed at both dosing sites in all mice of Group 2 (5 %) and Group 3 (10 %), persisting for the remainder of the in-life phase of the study.

### BODY WEIGHTS

The body weight of the animals, recorded prior to the first application and prior to necropsy, was within the range commonly recorded for animals of the strain and age.

## CALCULATION AND RESULTS OF INDIVIDUAL DATA

The following results were obtained:

Vehicle: acetone/olive oil (4/1, v/v)

Test item concentration % (w/v)		Measurement dpm	Calculation			Result
			dpm - BG <sup>a)</sup>	number of lymph nodes	dpm per lymph node <sup>b)</sup>	S.I.
--	BG I	0	--	--	--	--
--	BG II	0	--	--	--	--
--	CG 1	2257	2257	8	282	--
5	TG 2	5310	5310	8	664	2.4
10	TG 3	8055	8055	8	1007	3.6
25	TG 4	25351	25351	8	3169	11.2

BG = Background (1 ml 5 % trichloroacetic acid) in duplicate

CG = Control Group

TG = Test Group

S.I. = STIMULATION INDEX

a) = The mean value was taken from the figures BG I and BG II

b) = Since the lymph nodes of the animals of a dose group were pooled, dpm/Node was determined by dividing the measured value by the number of lymph nodes pooled

	Test item concentration % (w/v)	S.I.
Group 2	5 (a)	2.4 (b)
Group 3	10 (c)	3.6 (d)
<b>EC3 = (a-c) [(3-d)/(b-d)] + c = 7.5 % (w/v)</b>		

EC3 = Estimated concentration for a STIMULATION INDEX (S.I.) of 3.

a,b,c,d = Co-ordinates of the two pair of data lying immediately above and below the S.I. value of 3 on the LLNA dose response plot.

## **APPENDIX D**

### **GOOD LABORATORY PRACTICE**

- STATEMENT OF COMPLIANCE (PRINCIPAL INVESTIGATOR)**
- QUALITY ASSURANCE UNIT (PRINCIPAL INVESTIGATOR)**

**GOOD LABORATORY PRACTICE**

**STATEMENT OF COMPLIANCE**

RCC Study Number: 859435  
Study Director: Dr. W. Wang-Fan, Toxicology  
Test Item: ALCODET 218  
Principal Investigator  
<sup>3</sup>HTdR Determination: Dr. R. Burri, Environmental Chemistry &  
Pharmanalytics  
Phase to: ALCODET 218:  
Local Lymph Node Assay (LLNA) in Mice  
(Identification of Contact Allergens)

The preparation of the [methyl-<sup>3</sup>H]Thymidine solution and determination of radioactivity content were conducted in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted February 2<sup>nd</sup>, 2000 [RS 813.016.5]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26<sup>th</sup>, 1997 by decision of the OECD Council [C(97)186/Final].

Principal Investigator  
<sup>3</sup>HTdR Determination:

Dr. R. Burri

.....  
Date:

*R. Burri*  
April 11, 2005

## QUALITY ASSURANCE

RCC Ltd, Environmental Chemistry & Pharmanalytics, CH-4452 Itingen / Switzerland

### STATEMENT

RCC Study Number: 859435  
Study Director: Dr. W. Wang-Fan, Toxicology  
Test Item: ALCODET 218  
Principal Investigator  
<sup>3</sup>HTdR Determination: Dr. R. Burri, Environmental Chemistry & Pharmanalytics  
Phase to: ALCODET 218:  
Local Lymph Node Assay (LLNA) in Mice  
(Identification of Contact Allergens)

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

Study procedures were periodically inspected by the quality assurance. The date is given below.

Dates and Types of QA Inspections	Dates of Reports to the Principal Investigator and to the Management
January 14, 2005 Process based (Preparation of application solution)	January 14, 2005

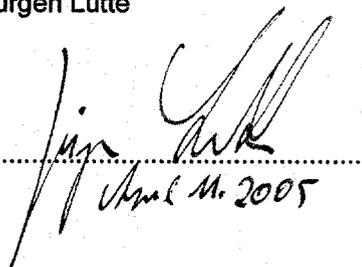
Sections of the draft study plan relating to the phase were reviewed and reported to the study director, lead QA and test facility management on March 16, 2005

Summary report(s) of study related inspection(s) (if applicable) were issued to the study director, lead QA and test facility management.

Quality Assurance:

Mr. Jürgen Lütte

Date:



April 14, 2005

## **APPENDIX E**

### **GLP - CERTIFICATION**

The Swiss GLP Monitoring Authorities



Swiss Federal  
Office of  
Public Health



Swiss Agency for the  
Environment, Forests  
and Landscape



Intercantonal Office  
for the Control of  
Medicines

## Statement of GLP Compliance

It is hereby confirmed that

during the period of

August 15 – 17, 2000  
August 28 - 29, 2001 and  
April 15, 2002

the following Test Facilities of

RCC Ltd  
4452 Itingen  
Switzerland

were inspected by the Federal Office of Public Health, the Swiss Agency for the Environment, Forests and Landscape and the Intercantonal Office for the Control of Medicines with respect to the compliance with the Swiss legislation on Good Laboratory Practice.

Test Facilities	areas of expertise*
- Toxicology Division	TOX, ACC, MUT
- Environmental Chemistry and Pharmanalytics Division	ACC, ECT, ENF, EMN, PCT, RES, OTH (Animal metabolism)
- Microbiological Diagnostics by Biotechnology & Animal Breeding Division	OTH (Microbiology)

The inspection was performed in agreement with the OECD Guidelines for National GLP Inspections and Audits. It was found that the aforementioned test facilities were operating in compliance with the Swiss Ordinance relating to Good Laboratory Practice [RS 813.016.5] at the time they were inspected.

Federal Office of Public Health  
The Director

Prof. Th. Zeltner

Bern, May 2002

\* TOX = Toxicology ; ACC = Analytical and Clinical Chemistry ; ECT = Environmental toxicity on aquatic and terrestrial organisms ; ENF = Behaviour in water, soil and air. Bioaccumulation ; EMN = Studies on effects on mesocosms and natural ecosystems ; MUT = Mutagenicity ; PCT = Physical-chemical testing ; RES = Residue studies ; OTH = Other, to be specified.

**APPENDIX F**  
**CERTIFICATE OF ANALYSIS**

Report

RHODIA INC.

CERTIFICATE OF ANALYSIS

CN 7500 Cranbury, N.J. 08512-7500  
1-888-776-7337 www.rhodia-hpcii.com

MAIL TO

THIS ANALYSIS COVERS OUR SHIPMENT TO

RHODIA PRODUCT CODE NO	RHODIA PRODUCT DESCRIPTION			
035214	ALCODET 218	460 CHS		
CUSTOMER PRODUCT CODE NO	CUSTOMER PRODUCT DESCRIPTION	CUSTOMER ORDER NO.	RHODIA	
035214	ALCODET 218	4606966	PLANT ORDER 0059298	LOT NO. [REDACTED]

FINAL PRODUCT ANALYSIS

TEST DESCRIPTION	ANALYSIS	Min - SPECIFICATION - Max	
MOISTURE (KARL FISHER), %	5.4	4.0	6.0
COLOR GARDNER	1.0		6.0
FOREIGN MATTER Free of foreign matter	PASS		
APPEARANCE @ 25 DEG C Clear liquid	PASS		
CLOUD POINT (1% SOLUTION), DEG C	53.1	50.5	54.5

CUSTOMER NO	QUALITY CONTROL ANALYST	
	T. J. BURKE	8/05/03

RECEIVED  
CPPT 2910

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## **RCC Study Number 859355**

*Study #2*

### **MIRATAINE D/40:**

**Local Lymph Node Assay (LLNA) in Mice  
(Identification of Contact Allergens)**

#### **Report**

**Author:**

**Dr. W. Wang-Fan**

**Sponsor:**

**Rhodia Asia Pacific Pte Ltd  
300 Beach Road #27-06/07  
SINGAPORE - 199555  
SINGAPORE**

**Study Completion Date: 30 August 2005**

Total Number of Pages: 53



*289672*

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# 1 PREFACE

## 1.1 GENERAL

Title	MIRATAINE D/40: Local Lymph Node Assay (LLNA) in Mice (Identification of Contact Allergens)
Sponsor	Mr Thierry SCLAPARI Rhodia Asia Pacific Pte Ltd 300 Beach Road #27-06/07 SINGAPORE - 199555 SINGAPORE
Study Monitor	Miss Annie Buard Rhodia Services Saint-Fons DELTA Boîte Postale 70026 20, rue Marcel Sembat F-69191 SAINT FONS CEDEX / France
Test Facility	a) RCC Ltd Toxicology Zelgliweg 1 CH - 4452 Itingen / Switzerland
Test Site	b) RCC Ltd Environmental Chemistry & Pharmanalytics Zelgliweg 1 CH - 4452 Itingen / Switzerland
Lead QA	RCC Ltd Quality Assurance GLP Toxicology Zelgliweg 1 CH - 4452 Itingen / Switzerland
Test Site QA	RCC Ltd Quality Assurance GLP Environmental Chemistry & Pharmanalytics Zelgliweg 1 CH - 4452 Itingen / Switzerland (Responsible for test site)

## 1.2 RESPONSIBILITIES

Study Director	Dr. W. Wang-Fan (a)
Deputy Study Director	L.G. Ullmann (a)
Technical Coordinator / Necropsy	E. Deparade (a)
Head of Lead Quality Assurance	I. Wüthrich

### Principal Investigator

Study Phase: <sup>3</sup>HTdR Determination Dr. R. Burri (b)

## 1.3 SCHEDULE

Experimental Starting Date	09-MAR-2005	
Experimental Completion Date	23-MAR-2005	
Delivery of Animals	09-MAR-2005	
Acclimatization	09-MAR-2005	to 15-MAR-2005
Treatment (epicutaneous)	16-MAR-2005	to 18-MAR-2005
Treatment (intravenous)	21-MAR-2005	
Observation	09-MAR-2005	to 21-MAR-2005
<sup>3</sup> HTdR Determination	22-MAR-2005	

## 1.4 ARCHIVING

RCC Ltd (CH-4452 Itingen / Switzerland) will retain the study plan, raw data of the test facility and the test site, sample of test item(s) and the final report of the present study for at least ten years. No data will be discarded without the Sponsor's written consent.

## 1.5 SIGNATURES

STUDY DIRECTOR:

Dr. W. Wang-Fan

*W. Wang-Fan*  
.....  
date: 30 August 2005

TEST FACILITY MANAGEMENT:

Dr. H. Fankhauser

*H. Fankhauser*  
.....  
date: 29 August 2005

## 1.6 QUALITY ASSURANCE GLP TOXICOLOGY

RCC Ltd, Toxicology, CH-4452 Itingen / Switzerland

### STATEMENT

RCC Study Number: 859355  
Test Item: MIRATAINE D/40  
Study Director: Dr. W. Wang-Fan  
Title: MIRATAINE D/40:  
Local Lymph Node Assay (LLNA) in Mice  
(Identification of Contact Allergens)

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

Study procedures, with the exception of the pre-test(s), were periodically inspected. The study plan and this report were audited by the RCC Quality Assurance. The dates are given below:

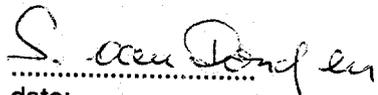
Dates and Types of QA Inspections		Dates of Reports to the Study Director and Test Facility Management
07-MAR-2005	Study Plan	07-MAR-2005
06-APR-2005	Process Based (Raw Data, Test System, Test Item, Administration, Dose Preparation)	06-APR-2005
07-JUN-2005	Report	07-JUN-2005

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:

S. van Dongen

  
date: 30-Aug-2005

GOOD LABORATORY PRACTICE

**1.7 STATEMENT OF COMPLIANCE**

RCC Study Number	859355
Test Item	MIRATAINE D/40
Study Director	Dr. W. Wang-Fan
Title	MIRATAINE D/40: Local Lymph Node Assay (LLNA) in Mice (Identification of Contact Allergens)

The non-GLP pretest was performed and is excluded from this Statement of Compliance.

The purity / composition of the test item are excluded from this Statement.

This study has been performed in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted February 2<sup>nd</sup>, 2000 [RS 813.016.5]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26<sup>th</sup>, 1997 by decision of the OECD Council [C(97) 186/Final].

Study Director:

Dr. W. Wang-Fan

*W. Wang - Fan*  
.....  
date: *30 August 2005*

## 1.8 TEST GUIDELINES

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

OECD Guideline for the Testing of Chemicals, Guideline 429: Skin Sensitization: Local Lymph Node Assay (adopted 24 April 2002).

Commission Directive 2004/73/EC, B.42: Skin Sensitization: Local Lymph Node Assay, 29 April 2004.

## 1.9 ANIMAL WELFARE

This study was performed in an AAALAC-approved laboratory in accordance with the Swiss Animal Protection Law under license no. 114.

## 1.10 REFERENCES

Kimber I., Hilton J. and Weisenberger C. (1989). The murine local lymph node assay for identification of contact allergens: a preliminary evaluation of in situ measurement of lymphocyte proliferation. *Contact Dermatitis*, 21, 215-220.

Kimber I. and Basketter D.A. (1992). The murine local lymph node assay. A commentary on collaborative studies and new directions. *Food and Chemical Toxicology*, 30, 165-169.

Basketter D.A., Gerberick G.F., Kimber I. and Loveless S.E. (1996). The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. *Food and Chemical Toxicology*, 34, 985-997.

Chamberlain M. and Basketter D.A. (1996). The local lymph node assay: status of validation. *Food and Chemical Toxicology*, 34, 999-1002.

Basketter D.A., Lea L.J., Cooper K., Stocks J., Dickens A., Pate I., Dearman R.J. and Kimber I. (1999). Threshold for Classification as a Skin Sensitizer in the Local Lymph Node Assay: A Statistical Evaluation. *Food and Chemical Toxicology*, 37, 1-8.

Steiling W., Basketter D.A., Berthold K., Butler M., Garrigue J-L., Kimber I., Lea L.J., Newsome C., Roggeband R., Stropp G., Waterman S. and Wiemann C. (2001): Skin Sensitisation Testing - New Perspectives and Recommendations. *Food and Chemical Toxicology*, 39, 293-301.

## 2 SUMMARY

In order to study a possible contact allergenic potential of MIRATAINE D/40, five groups each of four female mice were treated daily with the test item at concentrations of 5 %, 10 %, 25 %, 50 % (w/v) in ethanol/water, (7/3, v/v) and 100 % (undiluted) by topical application to the dorsum of each ear lobe (left and right) for three consecutive days. A control group of four mice was treated with the vehicle (ethanol/water, (7/3, v/v)) only. Five days after the first topical application the mice were injected intravenously into a tail vein with radio-labelled thymidine (<sup>3</sup>H-methyl thymidine). Approximately five hours after intravenous injection, the mice were sacrificed, the draining auricular lymph nodes excised and pooled per group. Single cell suspensions of lymph node cells were prepared from pooled lymph nodes which were subsequently washed and incubated with trichloroacetic acid overnight. The proliferative capacity of pooled lymph node cells was determined by the incorporation of <sup>3</sup>H-methyl thymidine measured in a  $\beta$ -scintillation counter.

All treated animals survived the scheduled study period.

No clinical signs were observed in any animals of the control group or Group 2 (5 %). On the second application day, a slight to severe ear erythema was observed at both dosing sites in all mice of Group 3 (10 %, slight), Group 4 (25 %, moderate), Group 5 (50 %, moderate) and Group 6 (100 %, undiluted, severe), persisting for the remainder of the in-life phase of the study. The size of the draining lymph nodes of the groups 5 (50 %) and 6 (100 %, undiluted) was doubly large compared to those of the control group. The local erythema increased with the doses in Groups 3-6.

The results obtained (STIMULATION INDEX (S.I.)) are reported in the following table. The estimated concentration of test item required to produce a S.I. of 3 is referred to as the EC3 value.

	Test item concentration % (w/v)	S.I.	Clinical Signs <sup>a)</sup> (on both ears)
Group 2	5 *	2.4 *	no
Group 3	10 *	6.2 *	erythema, slight
Group 4	25	14.7	erythema, moderate
Group 5	50	19.0	erythema, moderate
Group 6	100 (undiluted)	26.0	erythema, severe
<b>EC3 = 5.8 % (w/v)</b>			
A dose-response relationship was observed.			
* This value was used in calculation of EC3.			
<sup>a)</sup> The signs were observed from the second application day till to the termination of in-life phase.			

### **3 CONCLUSION**

A test item is regarded as a sensitizer in the LLNA if the exposure to one or more test concentrations resulted in 3-fold or greater increase in incorporation of <sup>3</sup>HTdR compared with concurrent controls, as indicated by the STIMULATION INDEX (S.I.).

In this study STIMULATION INDICES of 2.4, 6.2, 14.7, 19.0, and 26.0 were determined with the test item at concentrations of 5 %, 10 %, 25 %, 50 % (w/v) in ethanol/water, (7/3, v/v) and 100 % (undiluted), respectively.

MIRATAINE D/40 was therefore found to be a sensitiser and an EC3 value of 5.8 % (w/v) was derived from concentrations that presented limited or no local effects.

## 4 PURPOSE

The purpose of this Local Lymph Node Assay was to identify the contact allergenic potential of MIRATAINE D/40 when administered to the dorsum of both ear lobes of mice.

This study should provide a rational basis for risk assessment on the sensitizing potential of the test item in man.

## 5 MATERIALS AND METHODS

### 5.1 TEST SYSTEM

Test system	Mice, CBA/CaOlaHsd
Rationale	Recognized by OECD Guideline 429 as the recommended test system. Source Harlan Netherlands B.V. Postbus 6174 NL - 5960 AD Horst / The Netherlands
Number of animals for the pre-test (non-GLP)	2 females
Number of animals for the main study	24 females
Number of animals per group	4 females (nulliparous and non-pregnant)
Number of test groups	5
Number of control (vehicle) group	1
Age	8 - 12 weeks (beginning of acclimatization)
Body weight	16 g - 24 g (ordered)
Identification	Each cage by unique cage card.
Randomization	Randomly selected by computer algorithm at time of delivery.
Acclimatization	Under test conditions after health examination. Only animals without any visible signs of illness were used for the study.

## 5.2 ALLOCATION

The animals were distributed as follows:

GROUP	CONCENTRATION <sup>b)</sup> % (w/v)	NUMBER OF ANIMALS PER GROUP	CAGE NUMBER (Individually housed)
1 (Control Group <sup>a)</sup> )	-	4	1 - 4
2	5	4	5 - 8
3	10	4	9 - 12
4	25	4	13 - 16
5	50	4	17 - 20
6	100 (undiluted)	4	21 - 24

a) vehicle group = ethanol/water, (7/3, v/v)

b) In a non-GLP conform pre-test in two mice, test item concentrations of 10 %, 25 %, 50 % in ethanol/water, (7/3, v/v) and 100 % (undiluted) were tested on one ear each. One day after a single topical application no irritation effects were observed at these concentrations.

100 % (undiluted) was the highest technically applicable concentration.

The sensitivity and reliability of the experimental technique employed was assessed by use of a substance which is known to have skin sensitization properties in CBA/CaOlaHsd mice. The validation- / positive control study was performed with ALPHA-HEXYLCINNAMALDEHYDE in acetone/olive oil (4/1, v/v) using CBA/CaOlaHsd mice (RCC Study Number 858384) between 19-JAN-2005 to 02-FEB-2005, results see Appendix E.

## 5.3 HUSBANDRY

Room no.

E24 / RCC Itingen

Conditions

Standard Laboratory Conditions. Air-conditioned with ranges for room temperature  $22 \pm 3$  °C, relative humidity 30 - 70 % and 10 - 15 air changes per hour. Room temperature and humidity were monitored continuously and values outside of these ranges occasionally occurred, usually following room cleaning. These transient variations are considered not to have any influence on the study and, therefore, these data are not reported but are retained at RCC. There was a 12 hour fluorescent light / 12 hour dark cycle with at least 8 hours music during the light period.

Accommodation

Individual in Makrolon type-2 cages with standard softwood bedding ("Lignocel", Schill AG, CH-4132 Muttenz).

Diet

Pelleted standard Kliba 3433, batch no. 94/04 mouse maintenance diet (Provimi Kliba AG, CH-4303 Kaiseraugst) available *ad libitum*. Results of

Water

analyses for contaminants are archived at RCC. There was no contamination of the diet.

Community tap water from Itingen, available *ad libitum*. Results of representative bacteriological, chemical and contaminant analyses are archived at RCC. There was no contamination of water.

## 5.4 CHEMICALS

### <sup>3</sup>H-methyl Thymidine

Supplier

Amersham TRA 310, aqueous solution, sterilized 74 GBq/mmol (2 Ci/mmol), 37 MBq/ml (1 mCi/ml) quantities: 9.25 MBq (250  $\mu$ Ci), 37 MBq (1 mCi)

Batch numbers

Amersham Biosciences UK Limited, Buckinghamshire England HP7 9NA, UK

Storage conditions

316 and 318

In the original container at 5 °C  $\pm$  3 °C.

### Trichloroacetic acid

Supplier

Fluka no. 91230 (min. 99.5 %)

Fluka Chemie AG (Industriestrasse 25, CH-9471 Buchs, Switzerland)

Batch number

452262/1 32404133

Expiry date

02-SEP-2009

Storage conditions

At room temperature (20 °C  $\pm$  5 °C), away from direct sunlight.

### Phosphate buffered saline

Supplier

(1 tablet dissolved in 200 ml bi-distilled water)

Fluka Chemie AG (Industriestrasse 25, CH-9471 Buchs, Switzerland)

Batch number

453405/1

Expiry date

OCT-2006

Storage conditions

In the original container at room temperature (20 °C  $\pm$  5 °C), away from direct sunlight.

## 5.5 VEHICLES

Ethanol/water, (7/3, v/v)

Ethanol

Supplier

Baker, P. H. Stehelin & Cie AG (Spalentorweg 62, CH-4003 Basel, Switzerland)

Batch number

0307010001

Expiry date

APR-2008

Storage conditions

In the original container at room temperature (20 °C ± 5 °C), away from direct sunlight.

## 5.6 TEST ITEM

Identity

MIRATAINE D/40

Description

Colourless to pale yellow liquid

Batch number

BD050201

Purity / Composition

C<sub>12</sub> - C<sub>14</sub> DIMETHYL AMINE BETAINE 29.53 %, were excluded from Statement of Compliance.

Stability of test item

Stable under storage conditions

Expiry date

01-FEB-2006

Storage conditions

At room temperature (20 °C ± 5 °C), away from direct sunlight.

Safety precautions

Routine hygienic procedures (gloves, goggles, face mask). Irritant.

The test item information was supplied by the Sponsor.

## **5.7 TEST ITEM FORMULATIONS PREPARATION**

The test item was placed into a volumetric flask on a tared Mettler balance and the vehicle (ethanol/water, (7/3, v/v)) was quantitatively added. The weight/volume (w/v) dilutions were prepared individually. The test item was soluble in the vehicle very well.

Test item formulations were made freshly before each dosing occasion and no more than 4 hours prior to application to the ears.

Homogeneity of the test item in the vehicle was maintained during treatment using an appropriate homogenizer.

To determine the highest non-irritant and technically applicable test item concentration, a non-GLP pretest was performed in two mice with concentrations of 10 %, 25 %, 50 % in ethanol/water, (7/3, v/v) and 100 % (undiluted) (pretest excluded from Statement of Compliance).

The test item in the main study was assayed at five consecutive concentrations. The top dose is the highest technically applicable concentration while avoiding systemic toxicity and excessive local irritation.

Concentrations were in terms of material as supplied.

## **5.8 RATIONALE**

The study procedure was used to detect a possible contact allergenic potential of the test item applied.

## 6 STUDY CONDUCT

### 6.1 PRE-TEST

In a non-GLP solubility pre-test, the test item was tested in different vehicles: acetone/olive oil (4/1, v/v), ethanol/water (7/3, v/v), N,N-dimethylformamide (DMF), dimethylsulfoxide (DMSO) and propylene glycol. A suitable vehicle (ethanol/water (7/3, v/v)) was selected and used in the main test. The detailed results are included in Appendix A.

In a non-GLP animal pre-test in two mice, the test item was tested at four different concentrations: 10 %, 25 %, 50 % in ethanol/water, (7/3, v/v) and 100 % (undiluted), on one ear each.

One day after a single topical application no irritation effects were observed at these concentrations. The pre-test results determined that 100 % (undiluted) was the highest technically applicable concentration.

### 6.2 TREATMENT PROCEDURES

#### 6.2.1 TOPICAL APPLICATION

Each test group of mice was treated by topical (epidermal) application to the dorsal surface of each ear lobe (left and right) with different test item concentrations of 5 %, 10 %, 25 %, 50 % in ethanol/water, (7/3, v/v) and 100 % (undiluted). The application volume, 25  $\mu$ l, was spread over the entire dorsal surface ( $\varnothing \sim 8$  mm) of each ear lobe once daily for three consecutive days. A further group of mice was treated with an equivalent volume of the relevant vehicle alone (control animals). A hair dryer was passed briefly over the ear's surface to prevent the loss of any of the test item applied.

#### 6.2.2 ADMINISTRATION OF $^3\text{H}$ -METHYL THYMIDINE\*

$^3\text{H}$ -methyl thymidine ( $^3\text{HTdR}$ ) was purchased from Amersham International (Amersham product code no. TRA 310; specific activity, 2 Ci/mmol; concentration, 1 mCi/ml).

Five days after the first topical application, all mice were administered with 250  $\mu$ l of 77.01  $\mu\text{Ci/ml}$   $^3\text{HTdR}$  (equal to 19.3  $\mu\text{Ci}$   $^3\text{HTdR}$ ) by intravenous injection via a tail vein.

#### 6.2.3 DETERMINATION OF INCORPORATED $^3\text{HTdR}$ \*

Approximately five hours after treatment with  $^3\text{HTdR}$  all mice were euthanized by inhalation of  $\text{CO}_2$  (dry ice).

The draining lymph nodes were rapidly excised and pooled for each experimental group (8 nodes per group). Single cell suspensions (phosphate buffered saline) of pooled lymph node cells were prepared by gentle mechanical disaggregation through stainless steel gauze (200  $\mu\text{m}$  mesh size). After washing twice with phosphate buffered saline (approx. 10 ml) the lymph node cells were resuspended in 5 % trichloroacetic acid (approx. 3 ml) and incubated at approximately +4  $^{\circ}\text{C}$  for at least 18 hours for precipitation of macromolecules. The

\* Preparation of  $^3\text{HTdR}$  solutions and  $^3\text{HTdR}$  measurements at RCC Ltd, Environmental Chemistry & Pharamanalytics

No phase report of the results of the  $^3\text{HTdR}$  level analysis was provided by the Principal Investigator.

precipitates were then resuspended in 5 % trichloroacetic acid (1 ml) and transferred to glass scintillation vials with 10 ml of 'Irga-Safe Plus' scintillation liquid and thoroughly mixed.

The level of <sup>3</sup>HTdR incorporation was then measured on a β-scintillation counter. Similarly, background <sup>3</sup>HTdR levels were also measured in two 1ml-aliquots of 5 % trichloroacetic acid. The β-scintillation counter expresses <sup>3</sup>HTdR incorporation as the number of radioactive disintegrations per minute (dpm).

#### 6.2.4 INTERPRETATION OF RAW DATA

The proliferative response of lymph node cells is expressed as the number of radioactive disintegrations per minute per lymph node (dpm/node) and as the ratio of <sup>3</sup>HTdR incorporated into lymph node cells of test group relative to that recorded for control group (STIMULATION INDEX) (S.I.). Before dpm/node values were determined, mean scintillation-background dpm was subtracted from test and control raw data.

A test item is regarded as a sensitizer in the LLNA if the following criteria are fulfilled:

- First, exposure to at least one concentration of the test item resulted in an incorporation of <sup>3</sup>HTdR at least 3-fold or greater than that recorded in control mice, as indicated by the STIMULATION INDEX (S.I.).
- Second, the data are compatible with a conventional dose response, although allowance must be made (especially at high topical concentrations) for either local toxicity or immunological suppression.

#### 6.3 OBSERVATIONS

Mortality / Viability	Twice daily from acclimatization start to the termination of in-life phase.
Body weights	On the test day 1 (prior to the 1 <sup>st</sup> application) and on the test day 6.
Clinical signs (local / systemic)	Daily from acclimatization start to the termination of in-life phase. Especially the treatment sites will be observed carefully.

#### 6.4 STATISTICAL ANALYSIS

The mean values and standard deviations were calculated in the body weight tables.

A statistical analysis was conducted for assessment of the dose-response relationship, and the EC3 value was calculated according to the equation

$$EC3 = (a-c) [(3-d)/(b-d)] + c$$

where EC3 is the estimated concentration of the test item required to produce a 3-fold increase in draining lymph node cell proliferative activity; (a, b) and (c, d) are respectively the co-ordinates of the two pair of data lying immediately below and above the S.I. value of 3 on the local lymph node assay dose response plot.

#### 6.5 DATA COMPILATION

Body weights were recorded on-line in RCC-TOX LIMS.

Clinical signs were compiled directly into the RCC computer system.

## 7 RESULTS

### 7.1 CALCULATION AND RESULTS OF INDIVIDUAL DATA

The proliferative capacity of pooled lymph node cells was determined by the incorporation of <sup>3</sup>H-methyl thymidine measured on a β-scintillation counter.

	Test item concentration % (w/v)	S.I.	Clinical Signs <sup>a)</sup> (on both ears)
Group 2	5 *	2.4 *	no
Group 3	10 *	6.2 *	erythema, slight
Group 4	25	14.7	erythema, moderate
Group 5	50	19.0	erythema, moderate
Group 6	100 (undiluted)	26.0	erythema, severe
<b>EC3 = 5.8 % (w/v)</b>			
A dose-response relationship was observed.			
* This value was used in calculation of EC3.			
<sup>a)</sup> The signs were observed from the second application day till to the termination of in-life phase.			

The radioactive disintegration values for the individual treatment groups are included in Appendix B.

### 7.2 VIABILITY / MORTALITY

No deaths occurred during the study period.

### 7.3 CLINICAL SIGNS

No clinical signs were observed in any animals of the control group or Group 2 (5 %). On the second application day, a slight to severe ear erythema was observed at both dosing sites in all mice of Group 3 (10 %, slight), Group 4 (25 %, moderate), Group 5 (50 %, moderate) and Group 6 (100 %, undiluted, severe), persisting for the remainder of the in-life phase of the study.

The individual clinical signs are included in Appendix C.

(In Appendix C the numbers in brackets, e.g. (4) show that the severity of the symptoms may be classified into four grades: slight (1), moderate (2), severe (3) and very severe (4); the points indicate the application days; the numbers indicate the severity of the symptoms.)

### 7.4 BODY WEIGHTS

The body weight of the animals, recorded prior to the first application and prior to necropsy, was within the range commonly recorded for animals of the strain and age.

The individual as well as groupwise summarized body weight values are included in Appendix D.

## 7.5 DISCUSSION AND CONCLUSION

In this study STIMULATION INDICES of 2.4, 6.2, 14.7, 19.0, and 26.0 were determined with the test item at concentrations of 5 %, 10 %, 25 %, 50 % in ethanol/water, (7/3, v/v) and 100 % (undiluted), respectively. A clear dose-response relationship was observed.

No clinical signs were observed in any animals of the control group or Group 2 (5 %). On the second application day, a slight to severe ear erythema was observed at both dosing sites in all mice of Group 3 (10 %, slight), Group 4 (25 %, moderate), Group 5 (50 %, moderate) and Group 6 (100 %, undiluted, severe), persisting for the remainder of the in-life phase of the study. The size of the draining lymph nodes of the groups 5 (50 %) and 6 (100 %, undiluted) was doubly large compared to those of the control group. The local erythema increased with the doses in Groups 3-6.

A test item is regarded as a sensitizer in the LLNA if the exposure to one or more test concentrations resulted in 3-fold or greater increase in incorporation of <sup>3</sup>HTdR compared with concurrent controls, as indicated by the STIMULATION INDEX (S.I.). MIRATAINE D/40 was therefore found to be a skin sensitizer and an EC3 value of 5.8 % (w/v) was derived.

No or slight erythema was observed at the 2 lowest doses (Groups 2 and 3) which were used for calculation of an EC3 value.

**APPENDIX A**  
**SOLUBILITY PRE-TEST RESULTS**

**Table 1 Solubility test results**

Vehicle	Concentration (%)	Note
AOO	50	emulsion
EtOH70%	50	very clear solution
DMSO *	25	solution
DMF	25	somewhat turbid
PG **	25, 50	oil-like solution

\* Based on the test results, DMSO shows a significant high level of dpm/LN in CBA/CaOlaHsd mice compared to those in other vehicles (Table 2). Therefore DMSO is not often used in the LLNA studies in our laboratory, unless it stated by the Sponsor.

\*\* From experimental viewpoint, the mixtures of PG and other solvents (e.g. acetone, ethanol, etc.) are more suitable for application in the LLNA than pure PG due to its high viscosity.

**Table 2 Comparison of DPM/LN background level in the vehicle-control LLNA studies with six vehicles in CBA/CaOlaHsd mice <sup>a)</sup>**

Solvent	AOO	EtOH 70%	BN	PG	DMSO	DMF
DPM/LN (N = 8)	334	211	296	364	1055	437
DPM/LN (SD) in water control groups: 288 (109), from 44 groups, total of 176 mice						

<sup>a)</sup> The test results are based on the vehicle-validation studies performed at RCC. The detailed results have been presented at Society of Toxicology 44th Annual Meeting New Orleans, Louisiana, March 6-10, 2005: Positive- and Vehicle-Control Tests in Murine Local Lymph Node Assay (LLNA) - Comparison of the Allergenic Potency of Alpha-Hexylcinnamaldehyde (HCA) Tested with Six Vehicles in CBA/CaOlaHsd Mice in LLNA Studies and Rationalization of the Ethanol/Water (7/3, v/v) as a Vehicle in LLNA Tests.

## **APPENDIX B**

### **CALCULATION AND RESULTS OF INDIVIDUAL DATA**

### CALCULATION AND RESULTS OF INDIVIDUAL DATA

The following results were obtained:

Vehicle: ethanol/water, (7/3, v/v)

Test item concentration % (w/v)		Measurement dpm	Calculation			Result
			dpm - BG <sup>a)</sup>	number of lymph nodes	dpm per lymph node <sup>b)</sup>	S.I.
--	BG I	10	--	--	--	--
--	BG II	14	--	--	--	--
--	CG 1	2019	2007	8	251	--
5	TG 2	4827	4815	8	602	2.4
10	TG 3	12448	12436	8	1555	6.2
25	TG 4	29492	29480	8	3685	14.7
50	TG 5	38127	38115	8 **	4764	19.0
100 *	TG 6	52287	52275	8 **	6534	26.0

\* Undiluted as delivered by the Sponsor

\*\* The size of the draining lymph nodes of this group was doubly large compared to those of the control group.

BG = Background (1 ml 5 % trichloroacetic acid) in duplicate

CG = Control Group

TG = Test Group

S.I. = Stimulation Index

a) = The mean value was taken from the figures BG I and BG II

b) = Since the lymph nodes of the animals of a dose group were pooled, dpm/node was determined by dividing the measured value by the number of lymph nodes pooled

	Test item concentration % (w/v)	S.I.
Group 2	5 (a)	2.4 (b)
Group 3	10 (c)	6.2 (d)
<b>EC3 = (a-c) [(3-d)/(b-d)] + c = 5.8 % (w/v)</b>		

EC3 = Estimated concentration for a STIMULATION INDEX of 3.

a,b,c,d = Co-ordinates of the two pair of data lying immediately below and above the S.I. value of 3 on the LLNA dose response plot.

## **APPENDIX C**

### **INDIVIDUAL / SUMMARY CLINICAL SIGNS**

RCC STUDY NUMBER 859355  
MIRATAINE D/40

Report

SYM-IND - 1  
08-AUG-05

**CLINICAL SIGNS, DAILY  
FEMALES  
GROUP 1 (NEG. CONTROL)**

SIGN (MAX.GRADE) (LOCATION)	ACCLIM. WEEKS: 1.....	TREATMENT 1.....
--------------------------------	--------------------------	---------------------

---

ANIMAL 1  
-----  
NO CLINICAL SIGNS NOTED

ANIMAL 2  
-----  
NO CLINICAL SIGNS NOTED

ANIMAL 3  
-----  
NO CLINICAL SIGNS NOTED

ANIMAL 4  
-----  
NO CLINICAL SIGNS NOTED

RCC STUDY NUMBER 859355  
MIRATAINE D/40

Report

SYM-IND - 2  
08-AUG-05

**CLINICAL SIGNS, DAILY  
FEMALES  
GROUP 2 (5%)**

SIGN (MAX.GRADE) (LOCATION)	ACCLIM. WEEKS: 1.....	TREATMENT 1.....
--------------------------------	--------------------------	---------------------

---

ANIMAL 5

-----  
NO CLINICAL SIGNS NOTED

ANIMAL 6

-----  
NO CLINICAL SIGNS NOTED

ANIMAL 7

-----  
NO CLINICAL SIGNS NOTED

ANIMAL 8

-----  
NO CLINICAL SIGNS NOTED

CLINICAL SIGNS, DAILY  
FEMALES  
GROUP 3 (10%)

SIGN (MAX.GRADE) (LOCATION)	ACCLIM. WEEKS: 1.....	TREATMENT 1.....
ANIMAL 9		
-----		
SKIN / FUR		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....	.11111
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....	.11111
ANIMAL 10		
-----		
SKIN / FUR		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....	.11111
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....	.11111
ANIMAL 11		
-----		
SKIN / FUR		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....	.11111
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....	.11111
ANIMAL 12		
-----		
SKIN / FUR		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....	.11111
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....	.11111

CLINICAL SIGNS, DAILY  
FEMALES  
GROUP 4 (25%)

SIGN (MAX.GRADE) (LOCATION)	WEEKS: 1.....	ACCLIM. 1.....	TREATMENT 1.....
ANIMAL 13			
-----			
SKIN / FUR			
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....		.22222
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....		.22222
ANIMAL 14			
-----			
SKIN / FUR			
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....		.22222
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....		.22222
ANIMAL 15			
-----			
SKIN / FUR			
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....		.22222
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....		.22222
ANIMAL 16			
-----			
SKIN / FUR			
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....		.22222
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....		.22222

CLINICAL SIGNS, DAILY  
FEMALES  
GROUP 5 (50%)

SIGN (MAX.GRADE) (LOCATION)	ACCLIM. WEEKS: 1.....	TREATMENT 1.....
ANIMAL 17		
-----		
SKIN / FUR		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....	.22222
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....	.22222
ANIMAL 18		
-----		
SKIN / FUR		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....	.22222
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....	.22222
ANIMAL 19		
-----		
SKIN / FUR		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....	.22222
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....	.22222
ANIMAL 20		
-----		
SKIN / FUR		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....	.22222
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....	.22222

CLINICAL SIGNS, DAILY  
FEMALES  
GROUP 6 (100%)

SIGN (MAX.GRADE) (LOCATION)	ACCLIM. WEEKS: 1.....	TREATMENT 1.....
ANIMAL 21		
-----		
SKIN / FUR		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....	.33333
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....	.33333
ANIMAL 22		
-----		
SKIN / FUR		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....	.33333
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....	.33333
ANIMAL 23		
-----		
SKIN / FUR		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....	.33333
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....	.33333
ANIMAL 24		
-----		
SKIN / FUR		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: .....	.33333
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: .....	.33333

RCC STUDY NUMBER 859355  
MIRATAINE D/40

Report

SYM-SUM - 1  
08-AUG-05

CLINICAL SIGNS, DAILY (SUMMARY)  
FEMALES  
GROUP 1 (NEG. CONTROL)

SIGN (MAX.GRADE) LOCATION	ACCLIM. WEEKS: 1.....	TREATMENT 1.....
------------------------------	--------------------------	---------------------

---

NO CLINICAL SIGNS NOTED

RCC STUDY NUMBER 859355  
MIRATAINE D/40

Report

SYM-SUM - 2  
08-AUG-05

**CLINICAL SIGNS, DAILY (SUMMARY)**  
**FEMALES**  
**GROUP 2 (5%)**

SIGN (MAX.GRADE) LOCATION	ACCLIM. WEEKS: 1.....	TREATMENT 1.....
------------------------------	--------------------------	---------------------

---

NO CLINICAL SIGNS NOTED

CLINICAL SIGNS, DAILY (SUMMARY)  
FEMALES  
GROUP 3 (10%)

SIGN (MAX.GRADE) LOCATION	ACCLIM. WEEKS: 1.....	TREATMENT 1.....
<hr/>		
SKIN / FUR -----		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: ..... %: .....	.11111 .AAAAA
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: ..... %: .....	.11111 .AAAAA

CLINICAL SIGNS, DAILY (SUMMARY)  
FEMALES  
GROUP 4 (25%)

SIGN (MAX.GRADE) LOCATION	ACCLIM. WEEKS: 1.....	TREATMENT 1.....
<hr/>		
SKIN / FUR -----		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: ..... %: .....	.22222 .AAAAA
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: ..... %: .....	.22222 .AAAAA

CLINICAL SIGNS, DAILY (SUMMARY)  
FEMALES  
GROUP 5 (50%)

SIGN (MAX.GRADE) LOCATION	ACCLIM. WEEKS: 1.....	TREATMENT 1.....
SKIN / FUR		
-----		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: ..... %: .....	.22222 .AAAAA
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: ..... %: .....	.22222 .AAAAA

RCC STUDY NUMBER 859355  
MIRATAINE D/40

Report

SYM-SUM - 6  
08-AUG-05

CLINICAL SIGNS, DAILY (SUMMARY)  
FEMALES  
GROUP 6 (100%)

SIGN (MAX.GRADE) LOCATION	ACCLIM. WEEKS: 1.....	TREATMENT 1.....
<hr/>		
SKIN / FUR -----		
GENERAL ERYTHEMA (4) (EAR LEFT)	G: ..... %: .....	.33333 .AAAAA
GENERAL ERYTHEMA (4) (EAR RIGHT)	G: ..... %: .....	.33333 .AAAAA

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%)

## **APPENDIX D**

### **INDIVIDUAL / SUMMARY BODY WEIGHTS**

**BODY WEIGHTS (GRAM)  
FEMALES**

---

	TREATMENT	
	1	6
DAYS		
WEEKS	1	1
ANIMAL		

---

GROUP 1 (NEG. CONTROL)

1	19.1	20.5
2	23.5	24.5
3	22.0	22.8
4	22.9	24.5

GROUP 2 (5%)

5	21.3	22.2
6	21.5	24.6
7	19.9	21.2
8	19.5	20.8

GROUP 3 (10%)

9	21.8	22.5
10	22.1	22.4
11	23.5	23.9
12	19.4	19.0

GROUP 4 (25%)

13	22.4	22.3
14	20.3	20.4
15	20.5	22.0
16	21.7	22.7

GROUP 5 (50%)

17	20.2	19.9
18	18.6	18.9
19	18.5	19.6
20	20.9	20.9

GROUP 6 (100%)

21	19.9	21.7
22	20.7	21.6
23	19.0	18.7
24	20.7	20.8

**BODY WEIGHTS (GRAM) SUMMARY  
 FEMALES**

TREATMENT		GROUP 1 NEG. CONTROL	GROUP 2 5%	GROUP 3 10%	GROUP 4 25%
DAY 1	MEAN	21.9	20.6	21.7	21.2
WEEK 1	ST.DEV.	2.0	1.0	1.7	1.0
	N	4	4	4	4
		GROUP 5 50%	GROUP 6 100%		
	MEAN	19.6	20.1		
	ST.DEV.	1.2	0.8		
	N	4	4		
TREATMENT		GROUP 1 NEG. CONTROL	GROUP 2 5%	GROUP 3 10%	GROUP 4 25%
DAY 6	MEAN	23.1	22.2	22.0	21.9
WEEK 1	ST.DEV.	1.9	1.7	2.1	1.0
	N	4	4	4	4
		GROUP 5 50%	GROUP 6 100%		
	MEAN	19.8	20.7		
	ST.DEV.	0.8	1.4		
	N	4	4		

## **APPENDIX E**

### **RESULTS OF POSITIVE CONTROL**

## **RCC Study Number 858384**

### **ALPHA-HEXYLCINNAMALDEHYDE: Local Lymph Node Assay (LLNA) in Mice (Identification of Contact Allergens) Validation- / Positive Control Study**

(performed between 19-JAN-2005 to 02-FEB-2005)

## SUMMARY

In order to study a possible contact allergenic potential of ALPHA-HEXYLCINNAMALDEHYDE, three groups each of four female mice were treated daily with the test item at concentrations of 5 %, 10 % and 25 % (w/v) in acetone:olive oil, 4:1 (v/v) by topical application to the dorsum of each ear lobe (left and right) for three consecutive days. A control group of four mice was treated with the vehicle (acetone:olive oil, 4:1 (v/v)) only. Five days after the first topical application the mice were injected intravenously into a tail vein with radio-labelled thymidine (<sup>3</sup>H-methyl thymidine). Approximately five hours after intravenous injection, the mice were sacrificed, the draining auricular lymph nodes excised and pooled per group. Single cell suspensions of lymph node cells were prepared from pooled lymph nodes which were subsequently washed and incubated with trichloroacetic acid overnight. The proliferative capacity of pooled lymph node cells was determined by the incorporation of <sup>3</sup>H-methyl thymidine measured in a  $\beta$ -scintillation counter.

All treated animals survived the scheduled study period.

No clinical signs were observed in any animals of the control group. On the second application day, a slight ear swelling was observed at both dosing sites in all mice of Group 4 (25 %), persisting for the remainder of the in-life phase of the study. One day after the third local application, a slight ear swelling was observed at both dosing sites in all mice of Group 2 (5 %) and Group 3 (10 %), persisting for the remainder of the in-life phase of the study.

The results obtained (STIMULATION INDEX (S.I.)) are reported in the following table. The estimated concentration of test item required to produce a S.I. of 3 is referred to as the EC3 value.

	Test item concentration % (w/v)	S.I.
Group 2	5 *	2.4 *
Group 3	10 *	3.6 *
Group 4	25	11.2
<b>EC3 = 7.5 % (w/v)</b>		
A clear dose-response relationship was observed.		
* This value was used in calculation of EC3.		

## CONCLUSION

A test item is regarded as a sensitizer in the LLNA if the exposure to one or more test concentrations resulted in 3-fold or greater increase in incorporation of <sup>3</sup>HTdR compared with concurrent controls, as indicated by the STIMULATION INDEX (S.I.).

In this study STIMULATION INDICES of 2.4, 3.6 and 11.2 were determined with the test item at concentrations of 5 %, 10 % and 25 % (w/v), respectively, in acetone:olive oil, 4:1 (v/v).

ALPHA-HEXYLCINNAMALDEHYDE was therefore found to be a skin sensitizer and an EC3 value of 7.5 % (w/v) was derived.

## RESULTS

### CALCULATION AND RESULTS OF INDIVIDUAL DATA

The proliferative capacity of pooled lymph node cells was determined by the incorporation of <sup>3</sup>H-methyl thymidine measured on a  $\beta$ -scintillation counter.

	Test item concentration % (w/v)	S.I.
Group 2	5 *	2.4 *
Group 3	10 *	3.6 *
Group 4	25	11.2
<b>EC3 = 7.5 % (w/v)</b>		
A clear dose-response relationship was observed.		
* This value was used in calculation of EC3.		

### VIABILITY / MORTALITY

No deaths occurred during the study period.

### CLINICAL SIGNS

No clinical signs were observed in any animals of the control group. On the second application day, a slight ear swelling was observed at both dosing sites in all mice of Group 4 (25 %), persisting for the remainder of the in-life phase of the study. One day after the third local application, a slight ear swelling was observed at both dosing sites in all mice of Group 2 (5 %) and Group 3 (10 %), persisting for the remainder of the in-life phase of the study.

### BODY WEIGHTS

The body weight of the animals, recorded prior to the first application and prior to necropsy, was within the range commonly recorded for animals of the strain and age.

## CALCULATION AND RESULTS OF INDIVIDUAL DATA

The following results were obtained:

Vehicle: acetone/olive oil (4/1, v/v)

Test item concentration % (w/v)		Measurement dpm	Calculation			Result
			dpm - BG <sup>a)</sup>	number of lymph nodes	dpm per lymph node <sup>b)</sup>	S.I.
--	BG I	0	--	--	--	--
--	BG II	0	--	--	--	--
--	CG 1	2257	2257	8	282	--
5	TG 2	5310	5310	8	664	2.4
10	TG 3	8055	8055	8	1007	3.6
25	TG 4	25351	25351	8	3169	11.2

BG = Background (1 ml 5 % trichloroacetic acid) in duplicate

CG = Control Group

TG = Test Group

S.I. = STIMULATION INDEX

a) = The mean value was taken from the figures BG I and BG II

b) = Since the lymph nodes of the animals of a dose group were pooled, dpm/Node was determined by dividing the measured value by the number of lymph nodes pooled

	Test item concentration % (w/v)	S.I.
Group 2	5 (a)	2.4 (b)
Group 3	10 (c)	3.6 (d)
<b>EC3 = (a-c) [(3-d)/(b-d)] + c = 7.5 % (w/v)</b>		

EC3 = Estimated concentration for a STIMULATION INDEX (S.I.) of 3.

a,b,c,d = Co-ordinates of the two pair of data lying immediately above and below the S.I. value of 3 on the LLNA dose response plot.

## **APPENDIX F**

### **GOOD LABORATORY PRACTICE**

- STATEMENT OF COMPLIANCE (PRINCIPAL INVESTIGATOR)**
- QUALITY ASSURANCE UNIT (PRINCIPAL INVESTIGATOR)**

**GOOD LABORATORY PRACTICE**

**STATEMENT OF COMPLIANCE**

RCC Study Number: 859355  
Study Director: Dr. W. Wang-Fan, Toxicology  
Test Item: MIRATAINE D/40  
Principal Investigator  
<sup>3</sup>HTdR Determination: Dr. R. Burri, Environmental Chemistry &  
Pharmanalytics  
Phase to: MIRATAINE D/40:  
Local Lymph Node Assay (LLNA) in Mice  
(Identification of Contact Allergens)

The preparation of the [methyl-<sup>3</sup>H]Thymidine solution and determination of radioactivity content were conducted in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted February 2<sup>nd</sup>, 2000 [RS 813.016.5]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26<sup>th</sup>, 1997 by decision of the OECD Council [C(97)186/Final].

Principal Investigator  
<sup>3</sup>HTdR Determination:

Dr. R. Burri

.....  
Date:

*R. Burri*  
April 01, 2005

## QUALITY ASSURANCE

RCC Ltd, Environmental Chemistry & Pharamanalytics, CH-4452 Itingen / Switzerland

### STATEMENT

RCC Study Number: 859355  
Study Director: Dr. W. Wang-Fan, Toxicology  
Test Item: MIRATAINE D/40  
Principal Investigator  
<sup>3</sup>HTdR Determination: Dr. R. Burri, Environmental Chemistry & Pharamanalytics  
Phase to: MIRATAINE D/40:  
Local Lymph Node Assay (LLNA) in Mice  
(Identification of Contact Allergens)

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

Study procedures were periodically inspected by the quality assurance. The date is given below.

Dates and Types of QA Inspections	Dates of Reports to the Principal Investigator and to the Management
January 14, 2005 Process based (Preparation of application solution)	January 14, 2005

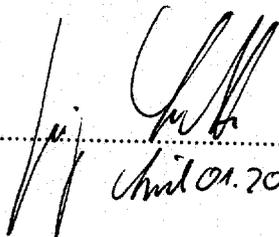
Sections of the draft study plan relating to the phase were reviewed and reported to the study director, lead QA and test facility management on March 07, 2005

Summary report(s) of study related inspection(s) (if applicable) were issued to the study director, lead QA and test facility management.

Quality Assurance:

Mr. Jürgen Lütte

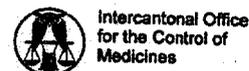
Date:

  
March 07, 2005

## **APPENDIX G**

### **GLP - CERTIFICATION**

The Swiss GLP Monitoring Authorities



## Statement of GLP Compliance

It is hereby confirmed that

during the period of

August 15 – 17, 2000  
August 28 - 29, 2001 and  
April 15, 2002

the following Test Facilities of

RCC Ltd  
4452 Itingen  
Switzerland

were inspected by the Federal Office of Public Health, the Swiss Agency for the Environment, Forests and Landscape and the Intercantonal Office for the Control of Medicines with respect to the compliance with the Swiss legislation on Good Laboratory Practice.

Test Facilities	areas of expertise*
- Toxicology Division	TOX, ACC, MUT
- Environmental Chemistry and Pharmanalytics Division	ACC, ECT, ENF, EMN, PCT, RES, OTH (Animal metabolism)
- Microbiological Diagnostics by Biotechnology & Animal Breeding Division	OTH (Microbiology)

The inspection was performed in agreement with the OECD Guidelines for National GLP Inspections and Audits. It was found that the aforementioned test facilities were operating in compliance with the Swiss Ordinance relating to Good Laboratory Practice [RS 813.016.5] at the time they were inspected.

Federal Office of Public Health  
The Director



Prof. Th. Zeltner

Bern, May 2002

\* TOX = Toxicology ; ACC = Analytical and Clinical Chemistry ; ECT = Environmental toxicity on aquatic and terrestrial organisms ; ENF = Behaviour in water, soil and air. Bioaccumulation ; EMN = Studies on effects on mesocosms and natural ecosystems ; MUT = Mutagenicity ; PCT = Physical-chemical testing ; RES = Residue studies ; OTH = Other, to be specified.

RCC STUDY NUMBER 859355  
MIRATAINE D/40

REPORT

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**APPENDIX H**  
**CERTIFICATE OF ANALYSIS**



**Asia Pacific**

Date: February 18, 2005  
To: Whom it may concern

**CERTIFICATE OF ANALYSIS**

RE: Sample submitted for Toxicology Test

Sample Name: Mirataine D/40

Item	Results / Remarks
1. Chemical Description	Alkyl Dimethyl Betaine
2. Active	29.53%
3. Batch Number	BD050201
4. Manufacturing Date	January 25, 2005
5. Appearance	Clear colourless to pale yellow liquid
6. pH (1%)	8.0
7. Solid content	38.4%
8. Free Amine	0.60%
9. SMCA, SGA, SDCA, SDGA	812ppm, 1.32%, 14ppm, 255ppm
10. NaCl	6.842%
11. Expiry Date	January 25, 2007 (2 years from manufacturing date)

Prepared by

P H Chuah  
Singapore Technical Centre  
Rhodia AP - HPCII Ag

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