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Chevron Phillips Chemical Company LP

Fred Marashi, Ph.D.
Global Regulatory Compliance Advisor

10001 Six Pines Drive
Suite 4104
The Woodlands, Texas
77380

Telephone: 832-813-4675
Fax: 832-813-4435

marasf@cpchem.com

www.cpchem.com

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Dear Document Control Officer:

Chevron Phillips Chemical Company LP submits the attached study on the substance Cyclohexylmercaptan (CAS# 1569-69-3) as an FYI. The title of the study is:

- Cyclohexyl mercaptan: Local Lymph Node Assay in the Mouse

This submission does not contain any confidential business information. Please let me if you have any question.

Sincerely yours,

Fred Marashi

FYI-10-01626

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CYCLOHEXYL MERCAPTAN:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

PROJECT NUMBER: 1635/0076

SPONSOR REFERENCE NUMBER: CPC-02-099

AUTHOR: J Bradshaw

STUDY SPONSOR:

Chevron Phillips Chemical Co. LP
10001 Six Pines Drive, Suite 4103
The Woodlands, TX 77380
USA

TEST FACILITY:

Harlan Laboratories Ltd
Shardlow Business Park
Shardlow
Derbyshire
DE72 2GD
UK

Telephone: +44 (0) 1332 792896

Facsimile: +44 (0) 1332 799018

QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress. In addition, inspection of general facilities not specifically related to this study are done monthly or annually in accordance with QA Standard Procedure.

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

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

	21 August 2009	Standard Test Method Compliance Audit
	03 February 2010	Test Material Preparation
	17 February 2010	Test System Preparation
	17 February 2010	Animal Preparation
	04 February 2010	Dosing
	09 February 2010	Assessment of Response
§	18 March 2010	Draft Report Audit
§	Date of QA Signature	Final Report Audit

..... G. Wren ..... DATE: ..... 19 APR 2010 .....
For the Quality Assurance Unit*

***Authorised QA Signatures:**
Senior Audit Staff: J G Riley BSc (Hons) MRQA, J M Crowther MIScT MRQA, G Wren ONC MRQA, S Bevan BSc (Hons) MRQA, L Blaney MRQA

GLP COMPLIANCE STATEMENT

With the exception noted below the work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

No analysis was carried out to determine the homogeneity, concentration or stability of the test material formulation. The test material was formulated within two hours of being applied to the test system; it is assumed that the formulation was stable for this duration. This exception is considered not to affect the purpose or integrity of the study.

This report fully and accurately reflects the procedures used and data generated.

..... DATE: 14 APR 2010

J Bradshaw BSc (Hons)
Study Director

This report may be presented in final form as a digital (pdf) document. Such documents are prepared by scanning the paper original, and are considered of equivalent integrity and authenticity to versions produced by optical photocopy. However, in all cases the hand-signed paper original, held in secure archives, is the definitive document.

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CYCLOHEXYL MERCAPTAN:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

SUMMARY

Introduction. A study was performed to assess the skin sensitisation potential of the test material in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to meet the requirements of the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 24 April 2002)
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Regulation (EC) No. 440/2008

Methods. Following a preliminary screening test in which no clinical signs of toxicity were noted at a concentration of 100%, this concentration was selected as the highest dose investigated in the main test of the Local Lymph Node Assay. Three groups, each of five animals, were treated with 50 µl (25 µl per ear) of the undiluted test material or the test material as a solution in acetone/olive oil 4:1 at concentrations of 50% or 25% v/v. A further group of five animals was treated with acetone/olive oil 4:1 alone.

Results. The Stimulation Index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

Concentration (% v/v) in acetone/olive oil 4:1	Stimulation Index	Result
25	13.61	Positive
50	17.65	Positive
100	18.27	Positive

Conclusion. The test material was considered to be a sensitiser under the conditions of the test.

CYCLOHEXYL MERCAPTAN:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

1. INTRODUCTION

A study was performed to assess the skin sensitisation potential of the test material in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to meet the requirements of the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 24 April 2002)
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Regulation (EC) No. 440/2008

The assay has undergone extensive inter-laboratory validation and has been shown to reliably detect test materials that are moderate to strong sensitisers.

The strain of mouse used in these laboratories has been shown to produce satisfactory responses using known sensitisers and non-sensitisers during the in-house validation. The results of routine positive control studies are shown in Appendix 1 and Appendix 2. The results of the study are believed to be of value in predicting the sensitisation potential of the test material to man.

The study was performed between 01 February 2010 and 25 February 2010.

2. TEST MATERIAL

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	CYCLOHEXYL MERCAPTAN
CAS #	:	1569-69-3
Description	:	clear colourless liquid
Batch number	:	2009-06-1550
Date received	:	02 July 2009
Expiry date	:	not supplied
Storage conditions	:	room temperature in the dark

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor.

A Certificate of Analysis supplied by the Sponsor is given in Appendix 5.

2.2 Preparation of Test Material

For the purpose of the study, the test material was used undiluted and freshly prepared as a solution in acetone/olive oil 4:1. This vehicle was chosen as it produced the most suitable formulation at the required concentration. The concentrations used are given in the procedure section. The vehicle determination record is included as Appendix 3.

The test material was formulated within two hours of being applied to the test system. It is assumed that the formulation was stable for this duration.

No analysis was conducted to determine the homogeneity, concentration or stability of the test material formulation. This is an exception with regard to GLP and has been reflected in the GLP compliance statement.

3. METHODS

3.1 Animals and Animal Husbandry

Female CBA/Ca (CBA/CaOlaHsd) strain mice were supplied by Harlan Laboratories UK Limited, Bicester, Oxon, UK. On receipt the animals were randomly allocated to cages. The animals were nulliparous and non-pregnant. After an acclimatisation period of at least five days the animals were selected at random and given a number unique within the study by indelible ink-marking on the tail and a number written on a cage card. At the start of the study the animals were in the weight range of 15 to 23 g, and were eight to twelve weeks old.

The animals were individually housed in suspended solid-floor polypropylene cages furnished with softwood woodflakes. Free access to mains tap water and food (2014 Teklad Global Rodent diet supplied by Harlan Teklad, Blackthorn, Bicester, Oxon, UK) was allowed throughout the study.

The temperature and relative humidity were controlled to remain within target ranges of 19 to 25°C and 30 to 70%, respectively. Any occasional deviations from these targets were considered not to have affected the purpose or integrity of the study. The rate of air exchange was approximately fifteen changes per hour and the lighting was controlled by a time switch to give twelve hours continuous light (06.00 to 18.00) and twelve hours darkness.

The animals were provided with environmental enrichment items which were considered not to contain any contaminant of a level that might have affected the purpose or integrity of the study.

3.2 Procedure

3.2.1 Preliminary Screening Test

Using available information regarding the systemic toxicity of the test material, a preliminary screening test was performed using two mice, one per test material concentration. The mice were treated by daily application of 25 µl of the undiluted test material or the test material at a concentration of 50% v/v in acetone/olive oil 4:1, to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The mice were observed twice daily on Days 1, 2 and 3 and once daily on Days 4, 5 and 6. Any signs of toxicity or excessive local irritation noted during this period were recorded. The bodyweight of each mouse was recorded on Day 1 (prior to dosing) and on Day 6.

3.2.2 Main Test

3.2.2.1 Test Material Administration

Groups of five mice were treated with the undiluted test material or the test material at concentrations of 50% or 25% v/v in acetone/olive oil 4:1. The preliminary screening test suggested that the test material would not produce systemic toxicity or excessive local irritation at the highest suitable concentration. The mice were treated by daily application of 25 µl of the appropriate concentration of the test material to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The test material formulation was administered using an automatic micropipette and spread over the dorsal surface of the ear using the tip of the pipette.

A further group of five mice received the vehicle alone in the same manner.

3.2.2.2 ^{3}H-Methyl Thymidine Administration

Five days following the first topical application of the test material or vehicle (Day 6) all mice were injected via the tail vein with 250 µl of phosphate buffered saline (PBS) containing ^{3}H-methyl thymidine (^{3}HTdR:80µCi/ml, specific activity 2.0 Ci/mmol, ARC UK Ltd) giving a total of 20 µCi to each mouse.

3.2.2.3 Observations

Clinical Observations: All animals were observed twice daily on Days 1, 2 and 3 and on a daily basis on Days 4, 5 and 6. Any signs of toxicity or signs of ill health during the test were recorded.

Bodyweights: The bodyweight of each mouse was recorded on Day 1 (prior to dosing) and Day 6 (prior to termination).

3.2.2.4 Terminal Procedures

Termination: Five hours following the administration of ^{3}HTdR all mice were killed by carbon dioxide asphyxiation followed by cervical separation. For each individual animal of each group the draining auricular lymph nodes were excised and processed. For each individual animal 1 ml of PBS was added to the lymph nodes.

Preparation of Single Cell Suspension: A single cell suspension of the lymph node cells for each individual animal was prepared by gentle mechanical disaggregation through a 200-mesh stainless steel gauze. The lymph node cells were rinsed through the gauze with 4 ml of PBS into a petri dish labelled with the project number and dose concentration. The lymph node cells suspension was transferred to a centrifuge tube. The petri dish was washed with an additional 5 ml of PBS to remove all remaining lymph node cells and these were added to the centrifuge tube. The lymph node cells were pelleted at 1400 rpm (approximately 190 *g*) for ten minutes. The pellet was resuspended in 10 ml of PBS and re-pelleted. To precipitate out the radioactive material, the pellet was resuspended in 3 ml of 5% Trichloroacetic acid (TCA).

Determination of ^{3}HTdR Incorporation: After approximately eighteen hours incubation at approximately 4°C, the precipitates were recovered by centrifugation at 2100 rpm (approximately 450 *g*) for ten minutes, resuspended in 1 ml of TCA and transferred to 10 ml of scintillation fluid (Optiphase 'Trisafe'). ^{3}HTdR incorporation was measured by

β-scintillation counting. The "Poly Q™" vials containing the samples and scintillation fluid were placed in the sample changer of the scintillator and left for approximately twenty minutes. The purpose of this period of time in darkness was to reduce the risk of luminescence, which has been shown to affect the reliability of the results. After approximately twenty minutes, the vials were shaken vigorously. The number of radioactive disintegrations per minute was then measured using the Beckman LS6500 scintillation system (Beckman Instruments Inc, Fullerton, CA, USA).

3.3 Statistical Analysis

Data was processed to give group mean values for disintegrations per minute and standard deviations where appropriate. Individual and group mean disintegrations per minute values were assessed for dose response relationships by analysis of homogeneity of variance followed by one way analysis of variance (ANOVA). In the event of a significant result from the ANOVA, pairwise comparisons were performed between control and treated groups. For homogenous datasets Dunnett's Multiple Comparison test was used and for non-homogenous datasets Dunnett's T3 Multiple Comparison Method was used.

Probability values (p) are presented as follows:

$P<0.001$	***
$P<0.01$	**
$P<0.05$	*
$P\geq0.05$	(not significant)

3.4 Interpretation of Results

The proliferation response of lymph node cells was expressed as the number of radioactive disintegrations per minute per lymph nodes from each individual animal and as the ratio of ^{3}HTdR incorporation into lymph node cells of test nodes relative to that recorded for the control nodes (Stimulation Index).

The test material will be regarded as a sensitiser if at least one concentration of the test material results in a threefold or greater increase in ^{3}HTdR incorporation compared to control values. Any test material failing to produce a threefold or greater increase in ^{3}HTdR incorporation will be classified as a "non-sensitiser".

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Harlan Laboratories Ltd, Shardlow, UK archives for five years, after which instructions will be sought as to further retention or disposal.

5. RESULTS

5.1 Preliminary Screening Test

Clinical observations, bodyweight and mortality data are given in Table 1.

No signs of local irritation or systemic toxicity were noted.

Based on this information the undiluted test material and the test material at concentrations of 50% and 25% v/v in acetone/olive oil 4:1 were selected for the main test.

5.2 Main Test

5.2.1 Estimation of the Proliferative Response of Lymph Node Cells

The radioactive disintegrations per minute per lymph nodes for each individual animal and the stimulation index are given in Table 2.

The Stimulation Index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

Concentration (% v/v) in acetone/olive oil 4:1	Stimulation Index	Result
25	13.61	Positive
50	17.65	Positive
100	18.27	Positive

5.2.2 Clinical Observations and Mortality Data

Individual clinical observations and mortality data for test and control animals are given in Table 3.

There were no deaths. No signs of systemic toxicity were noted in the test or control animals during the test.

5.2.3 Bodyweight

Individual bodyweights and bodyweight changes for test and control animals are given in Table 4.

Bodyweight changes of the test animals between Day 1 and Day 6 were comparable to those observed in the corresponding control group animals over the same period.

6. CONCLUSION

The test material was considered to be a sensitiser under the conditions of the test.

CYCLOHEXYL MERCAPTAN : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 1 Clinical Observations, Bodyweight and Mortality Data – Preliminary Screening Test

Concentration (% v/v) in acetone/olive oil 4:1	Animal Number	Bodyweight (g)		Day								
				1		2		3		4	5	6
		Day 1	Day 6	Pre-Dose	Post Dose	Pre-Dose	Post Dose	Pre-Dose	Post Dose			
50	S-1	18	19	0	0	0	0	0	0	0	0	0
100	S-2	21	20	0	0	0	0	0	0	0	0	0

0 = No signs of systemic toxicity

CYCLOHEXYL MERCAPTAN : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 2 Individual Disintegrations per Minute and Stimulation Indices

Concentration (% v/v) in acetone/olive oil 4:1	Animal Number	dpm/ Animal [a]	Mean dpm/Animal (Standard Deviation)	Stimulation Index [b]	Result
Vehicle	1-1	1854.64	1939.49 (±507.62)	N/A	N/A
	1-2	2752.73			
	1-3	1694.06			
	1-4	2002.40			
	1-5	1393.63			
25	2-1	25023.43	26399.75*** (±3572.26)	13.61	Positive
	2-2	23860.43			
	2-3	29737.63			
	2-4	22726.72			
	2-5	30650.53			
50	3-1	47185.48	34232.22** (±8082.46)	17.65	Positive
	3-2	31645.21			
	3-3	28177.38			
	3-4	36597.02			
	3-5	27556.00			
100	4-1	34510.93	35440.16*** (±4457.09)	18.27	Positive
	4-2	28255.55			
	4-3	38981.55			
	4-4	39138.05			
	4-5	36314.70			

dpm = Disintegrations per minute
a = Total number of lymph nodes per animal is 2
b = Stimulation Index of 3.0 or greater indicates a positive result
N/A = Not applicable
*** = Significantly different from control group $p<0.001$
** = Significantly different from control group $p<0.01$

CYCLOHEXYL MERCAPTAN : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 3 Individual Clinical Observations and Mortality Data

Concentration (% v/v) in acetone/olive oil 4:1	Animal Number	Day 1		Day 2		Day 3		Day 4	Day 5	Day 6
		Pre-Dose	Post Dose	Pre-Dose	Post Dose	Pre-Dose	Post Dose			
Vehicle	1-1	0	0	0	0	0	0	0	0	0
	1-2	0	0	0	0	0	0	0	0	0
	1-3	0	0	0	0	0	0	0	0	0
	1-4	0	0	0	0	0	0	0	0	0
	1-5	0	0	0	0	0	0	0	0	0
25	2-1	0	0	0	0	0	0	0	0	0
	2-2	0	0	0	0	0	0	0	0	0
	2-3	0	0	0	0	0	0	0	0	0
	2-4	0	0	0	0	0	0	0	0	0
	2-5	0	0	0	0	0	0	0	0	0
50	3-1	0	0	0	0	0	0	0	0	0
	3-2	0	0	0	0	0	0	0	0	0
	3-3	0	0	0	0	0	0	0	0	0
	3-4	0	0	0	0	0	0	0	0	0
	3-5	0	0	0	0	0	0	0	0	0
100	4-1	0	0	0	0	0	0	0	0	0
	4-2	0	0	0	0	0	0	0	0	0
	4-3	0	0	0	0	0	0	0	0	0
	4-4	0	0	0	0	0	0	0	0	0
	4-5	0	0	0	0	0	0	0	0	0

0 = No signs of systemic toxicity

CYCLOHEXYL MERCAPTAN : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 4 Individual Bodyweights and Bodyweight Changes

Concentration (% v/v) in acetone/olive oil 4:1	Animal Number	Bodyweight (g)		Bodyweight Change (g)
		Day 1	Day 6	
Vehicle	1-1	20	19	–1
	1-2	18	19	1
	1-3	18	18	0
	1-4	18	17	–1
	1-5	19	18	–1
25	2-1	17	17	0
	2-2	18	19	1
	2-3	21	20	–1
	2-4	18	18	0
	2-5	20	18	–2
50	3-1	19	19	0
	3-2	19	19	0
	3-3	19	19	0
	3-4	18	18	0
	3-5	19	18	–1
100	4-1	18	18	0
	4-2	18	18	0
	4-3	20	20	0
	4-4	21	20	–1
	4-5	20	20	0

Appendix 1 Current Positive Control Study for the Local Lymph Node Assay

Introduction. A study was performed to assess the sensitivity of the strain of mouse used at these laboratories to a known sensitiser. The methodology for the LLNA is detailed in the OECD Guideline for the Testing of Chemicals, No. 429, and Method B.42 of Commission Regulation (EC) No. 440/2008. The study described in this document is based on these test methods but has been refined in order to reduce the number of animals required. The reduced LLNA (rLLNA) has been endorsed by the non-Commission members of the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC) at its 26th meeting held on 26 – 27 April 2007 at ECVAM, Ispra, Italy.

Test Material: α-Hexylcinnamaldehyde, tech., 85%

Project number: 0039/1120

Study dates: 05 November 2009 to 11 November 2009

Methods. A group of five animals was treated with 50 µl (25 µl per ear) of α-Hexylcinnamaldehyde, tech., 85% as a solution in acetone/olive oil 4:1 at a concentration of 15% v/v. A further control group of five animals was treated with acetone/olive oil 4:1 alone.

Results. The Stimulation Index expressed as the mean radioactive incorporation for the treatment group divided by the mean radioactive incorporation of the vehicle control group is as follows:

Concentration (% v/v) in acetone/olive oil 4:1	Stimulation Index	Result
15	3.12	Positive

Conclusion. α-Hexylcinnamaldehyde, tech., 85% was considered to be a sensitiser under the conditions of the test.

Appendix 2 Summary of Positive Control Data for the Local Lymph Node Assay

Project Number	Start Date	Finish Date	Test Material	Concentration	Vehicle	Stimulation Index[a]	Classification[b]
0039/1083	16/04/09	22/04/09	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	butanone	4.08	Positive
0039/1084	17/04/09	23/04/09	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	dimethyl sulphoxide	4.60	Positive
0039/1091	25/06/09	01/07/09	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	ethanol/distilled water 7:3	10.68	Positive
0039/1092	25/06/09	01/07/09	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	acetone	10.72	Positive
0039/1099	08/07/09	14/07/09	α-Hexylcinnamaldehyde, tech., 85%	5%, 10% 25% v/v	1% pluronic L92 in distilled water	1.30, 2.37, 8.14	Positive
0039/1107	11/09/09	17/09/09	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	acetone/olive oil 4:1	3.70	Positive
0039/1108	01/10/09	07/10/09	α-Hexylcinnamaldehyde, tech., 85%	25% v/v	1% pluronic L92 in distilled water	5.11	Positive
0039/1115	05/11/09	11/11/09	Phenylacetaldehyde (90%)	2.5% v/v	propylene glycol	4.20	Positive
0039/1116	11/11/09	17/11/09	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	dimethyl formamide	5.16	Positive
0039/1117	11/11/09	17/11/09	α-Hexylcinnamaldehyde, tech., 85%	50% v/v	cottonseed oil	6.40	Positive
0039/1118	11/11/09	17/11/09	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	butanone	7.45	Positive
0039/1119	11/11/09	17/11/09	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	dimethyl sulphoxide	6.07	Positive
0039/1120	05/11/09	11/11/09	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	acetone/olive oil 4:1	3.12	Positive

a = Ratio of test to control lymphocyte proliferation
b = Stimulation index greater than 3.0 indicates a positive result

CYCLOHEXYL MERCAPTAN : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Appendix 3 Vehicle Determination Record

Vehicle	Concentration	Method of Preparation	Description of Formulation	Suitability*
acetone/olive oil (4:1)	50% 0.5 ml test material + 0.5 ml vehicle	Vortex mixer	solution	suitable for dosing

* = Suitable for dosing if formulation is a solution or fine homogenous suspension which can be administered via a micropipette

CYCLOHEXYL MERCAPTAN : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Appendix 4 Copy of Protocol

harlan™

PROTOCOL

TEST MATERIAL	:	CYCLOHEXYL MERCAPTAN
STUDY TYPE	:	Local Lymph Node Assay in the Mouse
TEST METHOD	:	599.08
PROJECT NUMBER	:	1635/0076
PROPOSED START DATE	:	Mid January 2010
PROPOSED COMPLETION DATE	:	Mid February 2010
TARGET (DRAFT) REPORT DATE	:	Mid March 2010
SPONSOR	:	Chevron Phillips Chemical Co. LP 10001 Six Pines Drive, Suite 4103 The Woodlands, TX 77380 USA
SPONSOR REFERENCE NUMBER	:	CPC-02-099
SPECIAL CONDITIONS	:	None

This study is compliant with the following toxicology guidelines:

- OECD Guideline for the Testing of Chemicals 429, 2002
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Regulation (EC) No. 440/2008

APPROVED FOR SPONSOR BY: ..... DATE: 06/01/2010

AUTHORISED BY: ..... DATE: 07 JAN 2010

J Bradshaw BSc (Hons)
STUDY DIRECTOR

This protocol is issued without signature by the Study Director to enable changes to be made if necessary prior to authorisation. Sponsors should sign and return the document to indicate approval and GLP authorisation will be confirmed by the Study Director's signature prior to the start of the study.

Appendix 4 (continued) Copy of Protocol

LOCAL LYMPH NODE ASSAY IN THE MOUSE

1. INTRODUCTION AND OBJECTIVES

The test is designed to assess the skin sensitising potential (delayed type hypersensitivity) of the test material in the mouse following topical application to the dorsal surface of the ear. Primary lymphocyte proliferation is assessed during the sensitising (induction) phase of the response. Lymphocyte proliferation in lymph nodes, from individual animals, draining the application site of the test material is quantified by measuring the incorporation of radiolabelled thymidine by β-Scintillation Counting.

The Local Lymph Node Assay (LLNA) can be used in the assessment of skin sensitisation potential as indicated in the OECD Guideline for the Testing of Chemicals, No 429, 2002 "Skin Sensitisation· Local Lymph Node Assay", Method B.42 of Commission Regulation (EC) No 440/2008 and the EPA OPPTS Guideline, No 870.2600, "Skin Sensitisation", 2003.

The work will be performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106) as amended by SI 2004/0994). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM (98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

2. TEST FACILITY

Harlan Laboratories Ltd
Shardlow Business Park
Shardlow
Derbyshire
DE72 2GD
UK

Appendix 4 (continued) Copy of Protocol

3. ANIMALS

Specification

Female CBA/Ca strain mice obtained from an accredited breeder which will be detailed in the study data. At the start of the study the mice will weigh 15 to 23g and will be 8 to 12 weeks old.

Number

A minimum of five animals per dose group are used.

Justification

Preferred species of choice since quantitative methods have been developed for the measurement of skin sensitisation responses in the mouse.

4. ANIMAL HUSBANDRY

Environment

Target temperature:	19 to 25°C
Target humidity:	30 to 70%
Lighting:	Twelve hours of continuous artificial light in each twenty-four hour period
Ventilation:	at least fifteen air changes per hour

Housing

Individually housed, in suspended polypropylene cages fitted with stainless steel mesh lids and furnished with softwood woodflakes. Results of routine analysis of the woodflakes are made available to Harlan Laboratories Ltd.

Diet and Water

Certified rat and mouse diet obtained from an accredited supplier and detailed in the report and mains tap water *ad libitum*.

Appendix 4 (continued) Copy of Protocol

The diet and water are routinely analysed and are considered not to contain any contaminants that could reasonably be expected to affect the purpose or integrity of the study.

5. ANIMAL WELFARE

Environmental Enrichment

Animals will be provided with suitable environmental enrichment items.

Study Conduct

The study was designed and will be conducted to cause the minimum suffering or distress to the animals consistent with the scientific objectives and in accordance with the Harlan Laboratories Ltd, Shardlow, UK policy on animal welfare and the requirements of the United Kingdom's Animals (Scientific Procedures) Act 1986. This standard test method is subject to review and the conduct of the study may be retrospectively reviewed, as part of the Harlan Laboratories Ltd, Shardlow, UK Ethical Review Process.

6. INITIAL CONSIDERATIONS

Preliminary Screening Test

The maximum concentration of test material to be investigated in the LLNA will be that which can be uniformly applied to the dorsal surface of the ears of the mice and which causes no more than mild skin irritation and no clinical signs of toxicity after three consecutive daily applications.

In the absence of information regarding the potential of the test material to cause skin irritation and/or systemic toxicity, or if information suggests that skin irritation and/or toxicity is possible, then a preliminary screening test will be conducted.

A single animal will be treated with the test material at the maximum concentration that is suitable for application to the dorsal surface of the ears (unless information is available to suggest that this concentration will be irritant or toxic, in which case a lower concentration may be investigated). The animal will be treated, as detailed in the procedures section, for three consecutive days (Days 1, 2 and 3) and will be observed for five days from initiation of treatment. Any clinical signs of toxicity or irritation at the treatment sites will be recorded at least

Appendix 4 (continued) Copy of Protocol

once per day. In the event of clinical signs of toxicity or excessive skin irritation being noted, lower concentrations of test material will be investigated in a stepwise manner, using one animal per test concentration until the maximum concentration is identified that produces no more than mild skin irritation and no clinical signs of toxicity.

7. PRE-TEST PROCEDURES

Acclimatisation

At least five days.

Allocation

Animals will be allocated to studies using a total randomisation procedure and caged individually.

Identification

Each animal will be identified using indelible tail markings. A cage card will be prepared with details of test material, project number, dose level, sex, number of animal, study dates and initials of the Study Director.

8. TEST MATERIAL AND EXPERIMENTAL PREPARATION

Supply

Supplied by the Sponsor with details of hazardous properties if known. Data relating to the identification, purity and stability of the test material will be the responsibility of the Sponsor.

Storage

Room temperature in the dark unless otherwise specified by Sponsor.

Appendix 4 (continued) Copy of Protocol

Preparation

The test material will be prepared in a suitable vehicle at the required concentrations. The vehicle should be selected on the basis of maximising the concentration and solubility whilst producing a solution/suspension suitable for application of the test material. Suitable vehicles for use (in order of preference) are; acetone:olive oil (4:1 v/v), dimethyl formamide, methyl ethyl ketone (2-butanone), and dimethyl sulphoxide. Other vehicles such as ethanol:water (7:3 v/v), ethanol:diethyl phthalate (1:3 v/v) 1% v/v pluronic L92 in distilled water, tetrahydrofuran, absolute ethanol or acetone may be used if sufficient scientific rationale is provided. In certain situations it may be necessary to use a clinically relevant solvent or the commercial formulation in which the test material is marketed as an additional control. Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system, which wets the skin and does not immediately run off. Thus, wholly aqueous vehicles are to be avoided.

Analysis

All formulations will be used within two hours of preparation and will be assumed to be stable for this period unless specified otherwise by the Sponsor. The concentration and homogeneity of the formulations will not be determined by analysis.

Absorption

The absorption of the test material will not be determined.

9. SELECTION OF CONCENTRATIONS OF TEST MATERIAL

At least three consecutive concentrations will be selected from the following concentration series: 100, 50, 25, 10, 5, 2.5, 1, 0.5, 0.25 and 0.1%.

Lower concentrations or intermediate concentrations may be investigated if considered necessary.

The concentrations used will be based upon previous experience, (ie, preliminary screening test data) expected toxicity of the test material and/or the physical properties of the test material eg solubility, viscosity.

Appendix 4 (continued) Copy of Protocol

10. PROCEDURE

Test Material Administration

Groups of five mice will be treated at each of the concentrations of the test material. The mice will be treated by daily application of 25 µl of the appropriate concentration of the test material to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The test material will be administered using an automatic micropipette. The test substance will be spread over the entire dorsal surface of the ear using the tip of the pipette.

A further group of five mice will receive the vehicle alone in the same manner.

^{3}H-methyl thymidine Administration

Five days following the first topical application of the test material (Day 6) all mice will be injected via the tail vein with 250 µl of phosphate buffered saline (PBS) containing ^{3}H-methyl thymidine (^{3}HTdR:80µCi/ml) giving a total of 20 µCi to each mouse. If intravenous injection of the required amount of ^{3}H-methyl thymidine is unsuccessful, then the mouse/mice will be removed from the group and the assay continued using the remaining mice. Rejection of the animal(s) will be documented and at the end of the study a decision will be taken by the Study Director whether the results from the reduced number of animals are considered to be valid.

11. OBSERVATIONS

Clinical Observations

All animals will be observed on a daily basis, including pre and post-dose observations on Days 1, 2 and 3. Any clinical signs of toxicity, skin irritation or signs of ill health during the study will be recorded.

Bodyweights

Recorded on Day 1 (prior to dosing) and prior to termination.

Appendix 4 (continued) Copy of Protocol

12. TERMINAL PROCEDURE

Termination

Five hours following the administration of ^{3}HTdR the mice will be killed by carbon dioxide asphyxiation followed by cervical separation. For each individual animal of each group the draining auricular lymph nodes will be excised and processed (maximum of 2 nodes per animal). For each individual animal 1 ml of PBS will be added to the lymph nodes. If it is not possible to process all of the lymph nodes this will be documented. At the conclusion of the study, the Study Director will decide whether the results from the reduced number of lymph nodes are considered to be valid.

Preparation of Single Cell Suspensions

A single cell suspension of the lymph node cells for each individual animal will be prepared by gentle mechanical disaggregation through a 200-mesh stainless steel gauze. The lymph node cells (LNC) will be rinsed through the gauze with 4 ml of PBS into a petri dish. The LNC suspension will be transferred to an appropriately sized centrifuge tube. The petri dish will be washed with an additional 5 ml of PBS to remove all remaining cells, this will be added to the centrifuge tube. The lymph node cells will be pelleted at 1400 rpm (approximately 190*g*) for 10 minutes. The pellet will be re-suspended in 10 ml of PBS and re-pelleted. To precipitate out the radioactive material, the pellet will be re-suspended in 3 ml of 5% Trichloroacetic acid (TCA).

Determination of ^{3}HTdR Incorporation

After an incubation period of approximately 18 hours at approximately 4°C, the precipitates will be recovered by centrifugation at 2100 rpm (approximately 450*g*) for 10 minutes, followed by resuspension in 1 ml of TCA. The cell suspension of each individual animal will be transferred to a "Poly Q™" vial containing 10 ml of scintillation fluid. The vials will be placed in the sample changer of the scintillator and allowed to stand in darkness for approximately 20 minutes. This period of time in the dark will reduce the risk of luminescence, which can affect the reliability of the results. On completion of this period the vials will be shaken vigorously, preferably in darkness. ^{3}HTdR incorporation will be measured by β-scintillation counting.

Appendix 4 (continued) Copy of Protocol

13. INTERPRETATION OF RESULTS

For each individual animal, the proliferative response of the lymph node cells will be expressed as the number of radioactive disintegrations per minute per animal (DPM/animal). The mean DPM/animal will be determined for each test group and also for the vehicle control group. The ratio of ^{3}HTdR incorporation into the lymph node cells of test nodes relative to that recorded for the control nodes (the Stimulation Index; SI) will be calculated for each test concentration by dividing the mean DPM/animal of each test group by the DPM/animal of the vehicle control group.

The decision process with regard to identification of a positive response will include a SI of ≥ 3, together with consideration of dose-response, and where appropriate, statistical significance. Any test material failing to produce a SI of ≥ 3 at all test concentrations will not be regarded as a skin sensitiser.

When a positive response is obtained, it may be possible to calculate an EC3 value. The EC3 represents the concentration of test item expected to cause a 3-fold increase in ^{3}HTdR incorporation (increase in draining lymph node cell proliferative activity) compared with the concurrent vehicle treated controls. It is therefore the effective concentration of test item required to produce a SI of 3. Where appropriate, the EC3 will be determined by linear interpolation of points on the dose-response curve, immediately above and below the 3-fold threshold using the following equation:

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

where:

a = the lowest concentration giving stimulation >3

b = the actual SI caused by 'a'

c = the highest concentration failing to produce a SI of 3

d = the actual SI caused by 'c'

Appendix 4 (continued) Copy of Protocol

14. STATISTICAL ANALYSIS

Appropriate statistical analysis of the data may be performed using commercially available software.

15. POSITIVE CONTROL DATA

Studies conducted to meet the requirements of EPA OPPTS Guidelines 870.2600 may require inclusion of a concurrent positive control group to demonstrate the sensitivity and reliability of the test system. A concurrent positive control group will be included only after prior agreement with, and at extra cost to the Study Sponsor. The positive control material must produce a positive LLNA response at a concentration level expected to give an increase in the stimulation index (SI) of three or greater over the vehicle control group. The positive control dose is to be such that the induction is clear but not excessive. Preferred positive control substances are α-hexyl cinnamicaldehyde, (CAS No. 101-86-0) and mercaptobenzothiazole (CAS No. 149-30-4). There may be circumstances where, given adequate justifications, other positive control substances may be used.

The positive control material will be diluted using the vehicle used to dilute the test material.

If a concurrent positive control group is not included in the study, satisfactory positive control data must be available which has been generated within 6 months of the initiation of the study.

16. QUALITY ASSURANCE

This standard test method will be reviewed for GLP compliance and the final report will be audited by Harlan Laboratories Ltd, Shardlow, UK Quality Assurance Unit. This type of study is subject to process-based QA inspection designed to encompass the major phases once per month.

17. PROTOCOL AMENDMENTS

Amendments to protocol will be made only by completion of an Amendment to Protocol form authorised by the Study Director.

Appendix 4 (continued) Copy of Protocol

18. FINAL REPORT

The final report will include, as a minimum, the following information:

Species, strain of animals
Experimental design
The DPM/animal for each animal in the study. The mean DPM/animal for each group and the Stimulation Index for each test group in tabular form

The EC3, where appropriate

Classification of sensitisation potential

Concurrent positive control data or positive control data generated within 6 months of initiation of the study.

19. ARCHIVE

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Harlan Laboratories Ltd, Shardlow, UK archives for five years after which instructions will be sought as to further retention or disposal. Further retention or return will be chargeable to the Sponsor.

Appendix 5 Certificate of Analysis

CHEVRON PHILLIPS CHEMICALS INTERNATIONAL N.V.

CPChem
QUALITY FIRST

Fabrieksstraat 5
Industriepark Schoonhees 2166
Tel. (013) 61.04.11 Fax (013) 66.30.06

B-3980 TESSENDERLO
Belgium

EN ISO 9001 : 2000 CERTIFIED

Certificate Of Analysis / Quality

Customer References

Product : CYCLO HEXYL MERCAPTAN

Your Order : e-mail
Our Order : 6809860
Order Date : 26/06/09
Certificate Date : 29/06/09

Product Code : CHM
Customer Number : 10032662
Quantity : 1 kg
Packaging :

Ship To :
HARLAN LABORATORIES
ATTN. CHRIS PIDDUCK
LONDON ROAD, SHARDLOW
DE72 2GD DERBY
GB

Sold To :
CHEVRON PHILLIPS CHEMICALS
INTERNATIONAL NV
BRUSSELSESTEENWEG 355
3090 OVERUSE
BE

Lab Specifications

Lot : 2009-06-1550
Sample : 2009-06-1550

Specification code : DS10CHM.5
Tank : E_108

Analysed Property	Method Of Analysis	Technical Specifications	Analysis Results	Unit
Cyclohexyl mercaptan	Gc	99.4 Min.	99.85	wt %
Cyclohexene	Gc	0.2 Max.	<0.01	wt %
Heavies	Gc	0.4 Max.	0.10	wt %
Distillation 5 %	ASTM D 1078	157 Min.	158.7	° C
Distillation 95 %	ASTM D 1078	160 Max.	158.9	° C
Mercaptan sulfur	CPChem	27.0 Min.	27.39	wt %
Cloudpoint	ASTM D 2500	-18 Max.	<-36	° C
Dissolved gasses	Gc	0.05 Max.	<0.0500	wt %
Color	ASTM E 308	0.5 Max.	0.30	delta E
Appearance	Visual	Clear with no particulate matter	with no particulate matter	

Comment :

Shelflife : Undetermined
The data set forth herein have been carefully compiled by Chevron Phillips Chemicals Company LP.
However, there is no warranty of any kind, either expressed or implied, applicable to its use, and the user assumes all risk and liability in connection therewith.

Process & Q.C. Manager
Van Kerckhoven Dany

Appendix 6 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY	TEST TYPE
Harlan Laboratories Ltd. **Shardlow Business Park** **London Road** **Shardlow** **Derby** **DE72 2GD**	**Analytical/Clinical Chemistry** **Environmental Fate** **Environmental Toxicology** **Mutagenicity** **Phys/Chem** **Toxicology**

DATE OF INSPECTION

15th September 2009

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

26/11/09

Dr. Andrew J. Gray
Head, UK GLP Monitoring Authority

MHRA

RACHEL VILLARREAL
(832) 813-4577
CHEVRON PHILLIPS
10001 SIX PINES RD
SPRING TX 77380-1498

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WASHINGTON DC 20004-3302

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WS 12.0.17 LP2844 03.0A 04/2010

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