Section A6.1.5 Skin sensitisation -

Annex Point IIA6.1.5 **Mouse Local Lymph Node Assay (LLNA)**

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted]: Cypermethrin cis:trans/40:60: local lymph node assay in the mouse (individual method); [redacted].	
		Dates of experimental work: 3 January 2006 – 31 January 2006	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Chimac-Agriphar s.a.	
1.2.2			
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes,	
		OECD guideline 429 (April 2002), method B42 of Directive 2004/73/EC and EPA OPPTS 870.2600 (2003)	
2.2	**GLP**	Yes	
2.3	**Deviations**	None	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	SL25163S63	
3.1.2	Specification	As given in section 2	
3.1.2.1	Description	Viscous liquid	
3.1.2.2	Purity	93.05%	
3.1.2.3	Stability	Stable	X
3.1.2.4	Preparation of test substance for application	Vehicle for the test article was 80% v/v acetone in olive oil. Formulations were freshly prepared on days 1, 2 and 3 and were mixed by multiple inversion of the containers prior to use.	
3.1.2.5	Pretest performed on irritant effects	Yes. Concentrations of 50%, 25%, 10%, 5%, 2.5% and 1% v/v cypermethrin in acetone/olive oil (4:1 v/v) were tested in a preliminary screening test.	

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3.2	**Test Animals**	
3.2.1	Species	Mouse
3.2.2	Strain	CBA/CaCrl
3.2.3	Source	[redacted]
3.2.4	Sex	Female (non-pregnant, nulliparous)
3.2.5	Age/weight at study initiation	Approximately 8 to 10 weeks old, weighing between 17 and 22g on day before dosing.
3.2.6	Number of animals per group	5
3.2.7	Control animals	Yes (vehicle control and positive control)
3.3	**Administration/ Exposure**	
3.3.1	Induction schedule	Epidermal induction was carried out on three occasions (days 1, 2 and 3). On day 6 all animals were injected (intravenous) with 20 µCi ^{3}H-methyl thymidine in PBS. After approximately 5 hours all animals were killed (CO_2) and the auricular lymph nodes removed from each ear for examination.
3.3.2	Way of Induction	Epidermal (no dressing applied). The outer part of both pinnae of each mouse was treated by direct application of the test or control formulation (0.025 ml/pinna) dispensed from an automatic micro pipette
3.3.3	Concentrations used for induction	Three test substance concentrations were used in the main study. Three groups of 5 animals were treated with either 2.5%, 1% or 0.5% test substance in 80% acetone in olive oil. A vehicle control group was also treated.
3.3.4	Positive control substance	Yes. A positive control group was treated wit 25% α—hexylcinnamaldehyde formulated in acetone/olive oil (4:1 v/v).
3.4	**Examinations**	
3.4.1	Pilot study	Yes. A preliminary irritation study was performed in order to select the highest concentration in the main study. Six mice were used, 1 mouse for each dose level tested (50%, 25%, 10%, 5%, 2.5%, 1.0% and 0.5% v/v respectively)
3.4.2	Recovery of Lymph nodes	The lymph nodes collected into each petri dish were cut open and disaggregated by squashing the fragments with a sharp blade. The resultant liquor was transferred into code-identified conical tubes. The petri dishes were rinsed with an additional 5 mL phosphate buffered saline and the second liquor was added to the first liquor. At each transfer, debris such as fragments of capsule were retained in the petri dish wherever possible. After 5 minutes the pooled liquor was filtered (200µm) and centrifuged at 190 G for 10 minutes. Following centrifugation, the supernatant was discarded and the pellet resuspended in 5 mL phosphate buffered saline. This was centrifuged again and the pellet resuspended in 3 mL of 5% w/v aqueous trichloroacetic acid. The suspension was refrigerated for 18 hours at nominally 4ºC. On the following day the suspension was re-centrifuged and the pellet resuspended in 1 mL 5% w/v aqueous trichloroacetic acid, which was

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then subjected to ultrasonic dispersion for 25 minutes to ensure a homogenous suspension. The suspension (1 mL) was transferred to a scintillation vial and scintillation fluid (*ca* 10 mL) was added.

3.4.3 Scintillation counting

All vials, including the background samples, were submitted for liquid scintillation counting for 10 minutes, using a ^{3}H quench curve. Incorporation of ^{3}HTdR is measured by ß-scintillation counting as disintegrations per minute (DPM) over a ten-minute period. This value was corrected to account for the background containing 5% w/v aqueous trichloroacetic acid and scintillation fluid. The DPM value was transformed into a mean DPM value for each group. The mean DPM value for each test group was divided by the mean DPM for the control group to provide the Stimulation Index (SI) value for each test group.

3.5 Further remarks

The test result is not valid for those groups producing an SI value of 3.0 or more when the sites of application have shown excessive irritation and for those groups that have shown indications of systemic toxicosis. The test article is regarded as a sensitiser when the maximum value of the SI is 3.0 or above.

The test article is classified as a non-sensitiser when the maximum value of the SI is less than 3.0. (This result is unchanged by observations of irritation at sites of application of the test formulation).

4 RESULTS AND DISCUSSION

4.1 Results of pilot studies

Signs of systemic toxicity were noted in the mice treated with the 50%, 25%, 10% and 5% v/v concentrations. The mouse treated with the 50% v/v concentration and the mouse treated with the 25% v/v concentration appeared to be very agitated and were writhing approximately five hours after dose administration on Day 1. The mouse treated with the 10% v/v concentration and the mouse treated with the 5% v/v concentration appeared to be slightly agitated approximately five hours after the dose administration on Day 2. The degree of agitation increased and was accompanied by twitching, involuntary spasms and sudden movements within approximately 1 hour. These four animals were killed for humane reasons.

Clinical signs in the two mice treated with, respectively, the 2.5% v/v and 1% v/v concentrations were restricted to greasiness to the head, neck and ears. Based on this information the dose levels selected for the main test were 2.5%, 1% and 0.5% v/v in 80% v/v acetone in olive oil.

4.2 Results of test

4.2.1 Clinical signs

There were no clinical signs indicative of a systemic effect of treatment among mice treated with the vehicle or with 0.5, 1.0 or 2.5% v/v formulations of Cypermethrin *cis:trans* 40:60. However, all groups displayed greasiness on the head and neck on all six days of the observation period and all animals treated with the 2.5% v/v concentration showed slight reddening of the head, ears and neck on Days 2, 3 and 4. This sign was also noted in the positive controls from Day 2 until the end of the observation period.

All animals survived treatment with the test article.

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4.2.2	Stimulation indices	See Table A6_1_5-01
4.2.3	Other findings	No indication of a treatment related effect on bodyweight.
4.3	**Overall result**	The Local Lymph Node Assay demonstrated that Cypermethrin cis:trans/40:60 does not have the potential to cause skin sensitisation at concentrations of up to 2.5% v/v in 80% v/v acetone in olive oil.
		5 APPLICANT'S SUMMARY AND CONCLUSION
5.1	**Materials and methods**	The Local Lymph Node Assay (LLNA) was used to assess the potential of cypermethrin cis:trans/40:60 to cause skin sensitisation in the mouse according to OECD guideline 429 (2002). Following a preliminary study, the test article was prepared for administration at 0.5%, 1.0% and 2.5% v/v in 80% v/v acetone in olive oil. Groups of five female CBA / Ca mice were subjected to topical applications of vehicle or of one of the test formulations to the outer aspect of the auditory pinnae once daily on Days 1, 2 and 3. In addition, a concentration of 25% v/v α-Hexylcinnamaldehyde in acetone / olive oil (4:1 v/v) was administered to a positive control group of five mice. On Day 6 a 20 µCi dose of tritiated ^{3}H-methyl thymidine was injected intravenously into each mouse. Five hours later the auricular lymph nodes were recovered from each animal. The pairs of nodes from each animal were pooled and suspensions of the cellular components of the lymph nodes were prepared in 5% w/v trichloroacetic acid and processed through a scintillation counter.
5.2	**Results and discussion**	Test results are expressed in terms of Stimulation Indices, the ratios of the mean scintillation counts obtained from the test groups relative to the corresponding mean scintillation count obtained from controls. The threshold level for the Stimulation Index to be considered a positive indicator of the potential to cause skin sensitisation is 3.0. The Local Lymph Node Assay demonstrated that Cypermethrin *cis:trans* 40:60 does not have the potential to cause skin sensitisation at concentrations of up to 2.5% v/v in 80% v/v acetone in olive oil.
5.3	**Conclusion**	The test article did not meet the criteria for classification as a sensitiser according to EU labelling regulations Commission Directive 2001/59/EC. No symbol and risk phrase are required.
5.3.1	Reliability	1
5.3.2	Deficiencies	None

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	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	March, 2007.
Materials and Methods	The applicant's version is acceptable with the following amendment: 3.1.2.3 Stability: Stability of test substance in vehicle: data not shown.
Results and discussion	The applicant's version is adopted.
Conclusion	Cypemethrin is not found a dermal sensitizer as tested with the LLNA (conc. 2.5%, 1%, 0.5%). According to these test results, cypermethrin does not require classification.
Reliability	1
Acceptability	acceptable
Remarks	-
	COMMENTS FROM ...
Date	Give date of comments submitted
Materials and Methods	Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion. Discuss if deviating from view of rapporteur member state
Results and discussion	Discuss if deviating from view of rapporteur member state
Conclusion	Discuss if deviating from view of rapporteur member state
Reliability	Discuss if deviating from view of rapporteur member state
Acceptability	Discuss if deviating from view of rapporteur member state
Remarks	

Table A6_1_5-01: Individual DPM's and Stimulation Index (SI)

Concentration (% v/v)	Group number	Animal number	DPM/ animal	Mean DPM/animal (Standard Deviation)	Stimulation Index (SI) [a]
Vehicle	1	293 294 295 296 297	704 943 1212 470 963	858 (± 282)	NA
0.5	2	298 299 300 301 302	520 57 208 413 311	302 (± 179)	0.35
1	3	303 304 305 306 307	85 280 60 561 988	395 (± 388)	0.46
2.5	4	308 325 326 327 328	447 557 860 457 400	544 (± 186)	0.63
Positive control	5	329 330 331 332 333	5792 3025 2050 4551 3594	3802 (± 1435)	4.43

[a] = Stimulation Index of 3.0 or greater indicates a positive result

NA = Not applicable