Report author:	N.H.
Report year:	2005
Report title:	Local lymph node assay in mice (LLNA) in mice with Pendimethalin technical
Report No.:	RCC-CCR 893601
Document No.:	000010488
Guideline(s) followed in study:	OECD 429 (2002), EU method B.42 (2004/73/EC)
Deviations from current test guidelines:	Yes The following deviation(s) from the OECD-Guideline 429 (2010) occurred: - The mice were individually housed, guideline requires group-housing, unless adequate scientific rationale for housing mice individually is provided. There were no deviations from the guidelines in force at the time the study was conducted.
GLP/Officially recognized testing facilities[1,2]	Yes, conducted under GLP/Officially recognized testing facilities. Laboratory certified by Hessisches Ministerium für Umwelt, ländlichen Raum und Verbraucherschutz, Wiesbaden, Germany, 06 January 2005)
Acceptability / reliability:	The study is acceptable.

[1] See Art.3 of Annex of Regulation No 283/2013 and 284/2013

[2] RMS shall check that the GLP statement has been properly signed in the study report, that the study results are properly reported in accordance with GLP standards and following the relevant guidance by OECD on the review of the GLP status of non-clinical safety data (currently under development).

Executive Summary

Following a preliminary test to determine the highest tolerated and non-irritant concentration of Pendimethalin technical, a local lymph node assay (LLNA) was carried out. Groups of four female mice (CBA/CaOlaHsd) were treated with Pendimethalin technical at concentrations of 10, 25 or 50% w/v in acetone/olive oil 4:1 (v/v). 25 µL of the appropriate test solutions were applied to the dorsal surface of each ear lobe for three consecutive days (topical application, days 1 to 3). Five days following the first topical application (day 6) all mice were injected via the tail vein with ^{3}H-methyl thymidine and the mice were killed 5 hours thereafter. The draining auricular lymph nodes were excised and pooled for each experimental group. A vehicle control group was run concurrently. ^{3}HTdR incorporation was measured in single cell suspensions by β-scintillation counting and the stimulation indices calculated. A test item is regarded as a sensitiser in the LLNA if the exposure to one or more test concentrations results in ≥3-fold increase in incorporation of ^{3}HTdR compared to concurrent controls, as indicated by the stimulation index (S.I.).

No mortality or clinical signs were observed during the study. Body weights recorded prior to the first application and prior to treatment with ^{3}HTdR were within the expected range. The calculated stimulation indices were 2.42, 1.43 and 1.71 at concentrations of 10, 25 and 50% (w/v) Pendimthalin technical in acetone:olive oil 4:1 (v/v), respectively.

A validation/positive control study was performed with α-hexylcinnamaldehyde in March 2005 which confirmed the validity of the test method.

In conclusion, Pendimethalin technical was not a skin sensitiser in this assay.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Pendimethalin technical
Chemical name:	N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine
CAS:	40487-42-1
Description:	Brown/orange solid
Batch No.:	D-TR00389
Purity:	96.5%
Stability:	Expiry date 30 June 2007 (stored at room temperature, light and moisture protected)
2. Vehicle:	Acetone:olive oil (4:1 v/v)
3. Test animals:	
Species:	Mouse
Strain:	CBA/CaOlaHsd
Sex:	Female
Weight at start of study:	17.6-22.4 g (8-12 weeks)
Source:	Harlan Netherlands, B.V. Postbus 6174, NL-5960 AD Horst, The Netherlands
Acclimation period:	At least 5 days
Diet:	Pelleted standard diet (supplied by Harlan Winkelmann GmbH, D-33178 Borchen, Germany) *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Individually housed in type I Makrolon® cages with wire mesh tops and granulated soft wood bedding (supplied by Harlan Winkelmann GmbH, D-33178 Borchen, Germany)
Environmental conditions:	
Temperature:	22 ± 3°C
Humidity:	30-70%
Air changes:	Not reported
Photo period:	Alternating 12 hour light/dark cycle

B. STUDY DESIGN

1. Dates of work: 03 August 2005 – 09 August 2005.

2. Test substance preparation and analysis

Prior to each dose, the highest concentration dosing preparation was freshly prepared by adding the appropriate quantity of test item into a volumetric flask glass beaker on a tared balance, the vehicle was then quantitatively added to achieve a 50% concentration (high dose). The mid (25%) and low (10%) doses were prepared by dilution of the 50% concentration with vehicle. All preparations were stirred with a magnetic stirrer.

To determine the highest non-irritant and technically applicable test item concentration, a non-GLP pre-test was performed in two mice with concentrations of 5, 10, 25 and 50% (w/v). The treated animals did not show any signs of toxicity or irritation and, therefore, concentrations of 10, 25 and 50% w/v were chosen for the main study.

The dosing preparations were not analysed.

3. Animal assignment and treatment

The animals were randomly distributed as follows:

Table 1: Study design

Group	Test substance	Concentration (%)	Number of animals
1 (control)	Acetone:olive oil 4:1 (v/v)	not applicable	4
2	Pendimethalin technical	10	4
3	Pendimethalin technical	25	4
4	Pendimethalin technical	50	4

Each test group of mice was treated by topical (epidermal) application to the dorsal surface of each ear lobe (left and right) with test item concentrations of 10, 25 or 50% (w/v) in vehicle. 25 µL of the dosing preparation was spread over the entire dorsal surface (0-8 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 used to dry the ear's surface as quickly as possible to avoid loss of test item applied.

Five days after the first topical application, the mice were dosed intravenously with 250 µL of 80.6 µCi/mL ^{3}H-methyl thymidine (^{3}HTdR), corresponding to 20.15 µCi ^{3}HTdR per mouse. The specific activity of ^{3}HTdR was 2 Ci/mmol; concentration 1 mCi/mL).

Approximately five hours after treatment with ^{3}HTdR all mice were killed by intraperitoneal injection of Na-thiopental. The draining auricular lymph nodes were excised and pooled for each experimental group (8 lymph nodes/group).

4. Observations / body weight

Mortality/viability were checked once daily from experimental start to necropsy. Body weights were recorded prior to the first application and prior to treatment with ^{3}HTdR. Clinical signs (local and systemic) were assessed 1-2 hours after each application.

5. Determination of Incorporated ^{3}HTdR

Single cell suspensions (in phosphate buffered saline) of pooled lymph node cells were prepared by gentle mechanical disaggregation through stainless steel gauze (200 µm mesh size). After washing twice with PBS, the lymph node cells were resuspended in approximately 3 mL 5% trichloroacetic acid (TCA) at approximately 4°C for at least 18 hours for precipitation of macromolecules. The precipitates were then resuspended in 1 mL 5% TCA, transferred into glass scintillation vials with 10 mL "Ultime Gold" scintillation liquid and thoroughly mixed.

The level of ^{3}HTdR incorporation was then measured on a β-scintillation counter. Similarly, background ^{3}HTdR levels were also measured in two 1 mL aliquots of 5% trichloroacetic acid. The β-scintillation counter expresses ^{3}HTdR incorporation as the number of radioactive disintegrations per minute (DPM).

The proliferative response of lymph node cells is expressed as the number of DPM/node and as the ratio of ^{3}HTdR incorporated into lymph node cells of test lymph nodes relative to that recorded for control lymph nodes (stimulation index). Before DPM/node values were determined, mean scintillation-background DPM was subtracted from test and control raw data. EC3 values, calculated concentrations which induce stimulation indices of three, are determined by linear interpolation between two points on the stimulation indices axis. Since all measured points were below the stimulation index of 3, the EC3 value could not be determined.

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

- First, that exposure to at least one concentration of the test item resulted in an incorporation of ^{3}HTdR at least 3-fold or greater than that recorded in control mice, as indicated by the stimulation index.
- Second, that 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. Positive control / validity study

A study (Study no. 881400) was performed in March 2005 in an identical manner as described above except that the test item was α-hexylcinnamaldehyde, tested at concentrations of 5, 10 and 25%.

7. Statistics

Not applicable.

II. RESULTS AND DISCUSSION

1. Viability / mortality / clinical signs / body weight

No mortality or clinical signs were observed during the study. Body weights recorded prior to the first application and prior to treatment with ^{3}HTdR were within the expected range.

2. Stimulation index

The calculated stimulation indices were 2.42, 1.43 and 1.71 at concentrations of Pendimethalin of 10, 25 and 50% w/v in acetone:olive oil v/v, respectively.

Table 2: Summary of results

Group	Pendimethalin concentration (%)	Measured DPM	Calculations			Stimulation index (SI)
			DPM-BG[a]	Number of lymph nodes	DPM per lymph node	
1 (control)	0	4187.8	4187.8	8	523.5	-
2	10	10131.3	10131.3	8	1266.4	2.42
3	25	5988.0	5988.0	8	748.5	1.43
4	50	7181.0	7181.0	8	897.6	1.71
BGI	n/a	0.0	n/a	n/a	n/a	n/a
BGII	n/a	0.0	n/a	n/a	n/a	n/a

BGI and BGII = background (1 mL 5% trichloroacetic acid)
[a] Mean of BGI and BGII

The EC3 value (derived by linear extrapolation) could not be calculated as the stimulation indices of all concentrations were less than 3.

3. Positive control / validity study

The validity of the test method was confirmed. The stimulation indices were 2.29, 3.21 and 8.44 for test item concentrations of 5, 10 and 25% (w/v) α-hexylcinnamaldehyde, respectively, and the EC3 was calculated as 8.9% w/v.

III. CONCLUSION

The test item, Pendimethalin technical, was not a skin sensitiser in the LLNA assay.