Report author:	K.L.
Report year:	2011
Report title:	Test for skin sensitization (local lymph node assay - LLNA) with Pendimethalin technical
Report No.:	114632
Document No.:	90014935 (000072673)
Guideline(s) followed in study:	OECD 429 (2010), EU method B.42 440/2008 (2008), EPA OPPTS 870.2600 (2003), EPA OPPTS 870.1000 (2002)
Deviations from current test guidelines:	Yes The following deviation(s) from the OECD-Guideline 429 (2010) occurred: - P-phenylenediamine was used as the positive control substance and justification for its selection was not reported; the guideline recommends 25% hexyl cinnamic aldehyde or 5% mercaptobenzothiazole.
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 five female mice (CBA/CaOlaHsd) were treated with Pendimethalin technical at concentrations of 12.5, 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 250 µL ^{3}H-methyl thymidine and the mice were killed 5 hours thereafter. The thickness of both ears was measured immediately before the first application, 48 hours after the first application and shortly before termination. The draining auricular lymph nodes were excised, individually pooled for each animal (2 lymph nodes/animal) and collected in phosphate buffered saline. 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. There was no treatment-related effect on body weight. The mean ear thickness of the test groups was not affected. The calculated stimulation indices were 1.0, 2.1, 1.9 and 2.2 at concentrations of Pendimethalin of 0, 12.5, 25 and 50% w/v in acetone:olive oil v/v, respectively.

A validation/positive control study was performed with P-phenylenediamine in October 2011 which confirmed the validity of the test method.

In conclusion, Pendimethalin technical was not a skin sensitiser in this assay. According to Commission Regulation (EU) No 286/2011 and the GHS (Globally Harmonized Classification System), Pendimethalin technical has no obligatory labelling requirement for skin sensitisation and is unclassified. According to the EPA Health Effects Guidelines, Pendimethalin technical at the tested concentration is negative for sensitisation.

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:	Orange/brown solid
Batch No.:	20101207
Purity:	97.67% (w/w)
Stability:	Expiry date December 2015 (stored at room temperature, protected from light)
2. Vehicle:	Acetone:olive oil (4:1 v/v)
3. Test animals:	
Species:	Mouse
Strain:	CBA/CaOlaHsd
Sex:	Female
Weight at start of study:	17-22 g (8-9 weeks)
Source:	Harlan Winkelmann, 33178 Borchen, Germany
Acclimation period:	At least 5 days
Diet:	Altromin 1324 maintenance diet for rats and mice *ad libitum*
Water:	Tap water, sulphur acidified to approximately pH 2.8, *ad libitum*
Housing:	Group housed (5/cage) in IVC cages, type IIL polysulphone on Altromin saw fibre bedding
Environmental conditions:	
Temperature:	22 ± 3°C
Humidity:	55±10%
Air changes:	At least 10/hour
Photo period:	Alternating 12 hour light/dark cycle

B. STUDY DESIGN

1. Dates of work: 26 October 2011 – 14 November 2011.

2. Test substance preparation and analysis

Prior to each dose, the dosing preparations were freshly prepared by mixing the appropriate quantity of test substance with vehicle. The dosing preparations were not analysed.

3. Preliminary test

In order to determine the highest tolerated and non-irritant test concentration, a preliminary test was performed. Two animals were treated by topical application with the test item on three consecutive days at a concentration of 50% (suspended in vehicle) to the entire dorsal surface of each ear. One further animal was treated with vehicle alone and served as a negative control. Immediately before the first application, approximately 48 hours after the first application and shortly before termination, the thickness of both ears of all animals was measured. Clinical signs were recorded daily and body weights were recorded at the start and end of the study.

4. Animal assignment and treatment

The animals, in the main test, 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	5
2	Pendimethalin technical	12.5	5
3	Pendimethalin technical	25	5
4	Pendimethalin technical	50	5

Immediately before the first application the thickness of both ears of all animals was measured. Each mouse was treated by topical (epidermal) application of the appropriate test item concentration (12.5, 25 or 50% (w/v) in vehicle) to the dorsal surface of each ear lobe (left and right). 25 µL of the dosing preparation was spread over the entire dorsal surface of each ear lobe once daily for three consecutive days. A second measurement of the ear thickness of all animals was carried out approximately 48 hours after the first application. A further group of mice was treated with an equivalent volume of the relevant vehicle alone (control animals).

Five days after the first topical application, the mice were dosed with 250 µL of 80 µCi/mL ^{3}H-methyl thymidine (^{3}HTdR) corresponding to 20 µCi ^{3}HTdR per mouse.

Approximately five hours after treatment with ^{3}HTdR, all mice were killed by cervical dislocation. Shortly before sacrifice, the thickness of the ears of all animals was measured for a third time. The draining auricular lymph nodes were excised and individually pooled for each animal (2 lymph nodes/animal) and collected in phosphate buffered saline (PBS).

5. Observations

Prior to application and once a day thereafter, all animals were observed in order to detect signs of toxicity, including dermal irritation at the application site.

6. Body weight

All animals were weighed prior to application and at the end of the treatment period (prior to treatment with ^{3}HTdR).

7. Determination of Incorporated ^{3}HTdR

A single cell suspension of pooled lymph node cells was prepared by gentle mechanical disaggregation through polyamide gauze (200 mesh size). After washing the gauze with PBS, the cell suspension was pelleted in a centrifuge. The supernatant was discarded and the pellets were resuspended with PBS. This washing procedure was repeated. After the final wash each pellet was resuspended in approximately 1 mL 5% trichloroacetic acid (TCA) at approximately 4°C for approximately 18 hours for precipitation of macromolecules. Each precipitate was washed again, resuspended in 1 mL 5% TCA and 7 mL scintillation fluid was added, then transferred into scintillation vials and stored at room temperature overnight.

The level of ^{3}HTdR incorporation was measured on a β-scintillation counter and expressed as the number of disintegrations per minute (DPM). Similarly, background ^{3}H-methyl thymidine levels were also measured (5% TCA). Determination of radioactivity was performed individually for each animal.

The proliferative response of lymph node cells was 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 at least one concentration of the test item results

in a 3-fold or greater increase in ^{3}H-methyl thymidine incorporation into lymph node cells of the test group animals, relative to that recorded for the lymph nodes of control group animals (stimulation index equal to or greater than 3.0).

8. Positive control / validity study

A study (BSL Project ID 1146I2B) was performed in October 2011 in an identical manner as described above except that the test item was P-phenylenediamine tested at concentrations of 1% over three consecutive days.

9. Statistics

Not applicable.

II. RESULTS AND DISCUSSION

1. Preliminary test

No signs of systemic toxicity or signs of irritation at the application site were seen in any animal. All animals showed expected weight development, which included a weight loss of up to 2 g during the duration of the test. Ear thickness measurements are shown below:

Table 2: Ear thickness – preliminary test (mm)

Concentration Pendimethalin technical (%)	Measurement of ear thickness (mm)					
	Day 1		Day 3		Day 4	
	left	right	left	right	left	right
50	0.18	0.20	0.20	0.21	0.19	0.19
50	0.20	0.20	0.20	0.19	0.20	0.19
0 (control)	0.18	0.19	0.19	0.19	0.18	0.18

2. Viability / mortality / clinical signs / body weight

No mortality or clinical signs were observed during the study. All animals showed expected weight development, which included a weight loss of up to 2 g during the duration of the test.

3. Ear thickness

There was no treatment-related effect on mean ear thickness.

Table 3: Mean ear thickness – main test (mm)

Concentration Pendimethalin technical (%)	Measurement of ear thickness (mm)		
	Day 1	Day 3	Day 4
12.5	0.19	0.19	0.20
25	0.19	0.20	0.20
50	0.20	0.20	0.21
0 (control)	0.19	0.19	0.20

4. Stimulation index

The calculated stimulation indices were 2.1, 1.9 and 2.2 at concentrations of Pendimethalin of 12.5, 25 and 50% w/v in acetone:olive oil v/v, respectively.

Table 4: Summary of results

Pendimethalin concentration (%)	Measured mean DPM	Mean DPM-BG	DPM per lymph node	Stimulation index (SI)
0 (control)	1040.8±40.1	1026.0±40.1	513.0±20.0	1.0
12.5	2191.8±305.1	2177.0±305.1	1088.5±152.6	2.1±0.3
25	1919.4±477.1	1904.6±477.1	952.3±238.5	1.9±0.5
50	2250.8±228.4	2236.0±228.4	1118.0±114.2	2.2±0.2
BG	14.8±1.9	0.0	0.0	0.0

BG = background

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

5. Positive control / validity study

The validity of the test method was confirmed; the stimulation index was 10.1.

III. CONCLUSION

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