CHD-FA 5000:

MICRONUCLEUS TEST IN THE MOUSE

PROJECT NUMBER: 2814/0006

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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:

	17 November 2008	Standard Test Method Compliance Audit
§	17 March 2010	Test Material Preparation
§	17 March 2010	Animal Preparation
§	17 March 2010	Dosing
§	19 March 2010	Assessment of Response
§	19 March 2010	Harvest
§	19 March 2010	Staining/Slide Preparation
§	16 April 2010	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: .....
For the Quality Assurance Unit*

***Authorised QA Signatures:**
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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 it 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: .....

L Flanders
Study Director

CONTENTS

CHD-FA 5000:

MICRONUCLEUS TEST IN THE MOUSE

SUMMARY

Introduction. The study was performed to assess the potential of the test material to produce damage to chromosomes or aneuploidy when administered to mice. The method was designed to comply with the 1997 OECD Guidelines for Testing of Chemicals No.474 "Micronucleus Test", Method B12 of Commission Regulation (EC) No. 440/2008 of 30 May 2008, the USA EPA, TSCA and FIFRA guidelines and the Japanese METI/MHLW guidelines for testing of new chemical substances.

Methods. A range-finding test was performed to find suitable dose levels of the test material and route of administration. The micronucleus test was conducted using the intraperitoneal route in groups of seven mice (males) at the maximum tolerated dose (MTD) 100 mg/kg and with 50 and 25 mg/kg as the two lower dose levels. Animals were killed 24 or 48 hours later, the bone marrow was extracted, and smears preparations made and stained. Polychromatic (PCE) and normochromatic (NCE) erythrocytes were scored for the presence of micronuclei.

Further groups of mice were given a single intraperitoneal dose of phosphate buffered saline (2 groups each of 7 mice) or dosed orally with cyclophosphamide (5 mice), to serve as vehicle and positive controls respectively. Vehicle control animals were killed 24 or 48 hours later, and positive control animals were killed after 24 hours.

Results. In the main test there were no premature deaths seen in any of the dose groups. Clinical signs were observed in animals dosed with the test material at and above 50 mg/kg in both the 24 and 48-hour groups where appropriate, these were as follows: Hunched posture, ptosis and ataxia.

A modest statistically significant decrease in the PCE/NCE ratio was observed in the 48-hour 100 mg/kg dose group and, whilst not statistically significant, a modest decrease was also observed in the 24-hour test 100 mg/kg dose group when compared to their concurrent vehicle control groups. On review of the slide for animal number 20 of the 48-hour 100 mg/kg test material dose group, it was noted that there was a vast majority

of NCE over PCE. It was considered that test material induced toxicity had halted the formation of PCE. Therefore, the decreases in PCE/NCE ratio, together with the high level of toxicity observed in animal number 20 and the observation of clinical signs at and above 50 mg/kg, were taken to indicate that systemic absorption had occurred and exposure to the target tissue had been achieved.

There was no evidence of a significant increase in the incidence of micronucleated polychromatic erythrocytes in animals dosed with the test material when compared to the concurrent vehicle control groups.

The positive control group showed a marked increase in the incidence of micronucleated polychromatic erythrocytes hence confirming the sensitivity of the system to the known mutagenic activity of cyclophosphamide under the conditions of the test.

Conclusion. The test material was considered to be non-genotoxic under the conditions of the test.

CHD-FA 5000:

MICRONUCLEUS TEST IN THE MOUSE

1. INTRODUCTION

The micronucleus test is a mammalian *in vivo* test that detects damage to the chromosomes induced by chemicals. In addition, numerical changes due to chromosome loss during cell division can be detected in this test.

The study was performed according to a method that was designed to comply with the 1997 OECD Guidelines for Testing of Chemicals No.474 "Micronucleus Test", Method B12 of Commission Regulation (EC) No. 440/2008 of 30 May 2008, the USA EPA, TSCA and FIFRA guidelines and the Japanese METI/MHLW guidelines for testing of new chemical substances. The results of the test are believed to be of value in predicting the mutagenic potential of the test material to man. The test system was chosen because the mouse has been shown to be a suitable model for this type of study and is recommended in the test method.

The experimental phases of the study were performed between 23 February 2010 and 30 March 2010.

2. PRINCIPLES OF INVESTIGATION

In mitotic cells in which chromosome damage has been caused by the test material or its metabolites, fragments (centric or acentric) or whole chromosomes tend to lag behind in the anaphase stage of cell division. After telophase a large proportion of the fragments are not included in the nuclei of the daughter cells and hence form a single or multiple micronuclei (Howell-Jolly bodies) in the cytoplasm of these cells. These micronuclei are seen in a wide variety of cell types, but erythrocytes are chosen since micronuclei are easily detected in these cells.

A few hours after the last mitosis is completed, erythrocytes expel their nuclei. Immature erythrocytes, less than 24 hours old, stain blue with May-Grünwald/Giemsa due to the presence of minute fragments of nuclear material in the cytoplasm. This material is mainly ribonucleic acid (RNA), which gradually disappears so that more mature erythrocytes (normochromatic erythrocytes) stain pink with May-Grünwald/Giemsa. The

immature blue staining cells are known as polychromatic erythrocytes and mauve stained micronuclei are easily detected in this cell type. If scoring is restricted to polychromatic erythrocytes, all the chromosomal damage detected will have been caused during the final cell cycle of the nucleated precursor cells. Thus by examining polychromatic cells at various periods after administration, the effect of the test material over the previous 30 hours can be monitored.

Any toxic effects of the test material on the immature nucleated cells may lead to a reduction in cell division and cell death. This in turn leads to a reduction in cell volume and to compensate for this, peripheral blood is shunted into the bone marrow. If the ratio of polychromatic to normochromatic erythrocytes is scored and found to be significantly lower than the control value, this is taken as being indicative of cytotoxicity.

3. TEST AND CONTROL MATERIALS AND EXPERIMENTAL PREPARATION

3.1 Test Material

Sponsor's identification	:	CHD-FA 5000
Description	:	Light brown liquid
Batch number	:	12
Date received	:	20 May 2009
Expiry date	:	26 February 2011
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.

For the purpose of this study the test material was freshly prepared as required as a solution at the appropriate concentration in phosphate buffered saline. Initially the test material was dosed undiluted orally at 20 ml/kg to achieve the maximum practical dose level of 942 mg/kg.

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 it being applied to the test system; it is assumed that the formulation was stable for this duration. This is an exception with regard to GLP and has been reflected in the GLP compliance statement.

3.2 Positive Control Material

The positive control material was supplied by Acros Organics, as follows:

Supplier's identification	:	Cyclophosphamide
Supplier's lot number	:	A0164185
In-house serial number	:	R-4447
Date received	:	27 October 2008
Expiry date	:	27 October 2010
Storage conditions	:	Approximately 4ºC in the dark

For the purpose of this study the positive control material was freshly prepared as required as a solution at the appropriate concentration in distilled water (Aguettant batch no. 300522801).

The concentration, homogeneity and stability of the positive control material and its preparation were not determined by analysis.

3.3 Vehicle Control

The vehicle was supplied by Gibco, as follows:

Supplier's identification	:	Phosphate buffered saline
Supplier's lot number	:	710923 and 722040A
Description	:	Clear colourless liquid
Storage conditions	:	Room temperature

4. METHODS

4.1 Animals and Animal Husbandry

Sufficient albino Hsd: ICR (CD-1®) strain mice were obtained from Harlan UK. At the start of the main test the male mice weighed 24 to 30g and were approximately five to eight weeks old. After a minimum acclimatisation period of five days the animals were selected at random and given a number unique within the study by tail marking and a number written on a colour coded cage card.

The animals were housed in groups of up to seven, by sex, in solid-floor polypropylene cages with wood-flake bedding. Free access to mains drinking water and food (Harlan Teklad 2014 Rodent Pelleted Diet) was allowed throughout the study.

The temperature and relative humidity were set to achieve limits of 19 to 25ºC 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 light and twelve hours darkness.

4.2 Procedure

4.2.1 Range-finding Toxicity Test

A range-finding toxicity test was performed to determine a suitable dose level and route of administration for the micronucleus test. The dose level selected should ideally be the maximum tolerated dose level or that which produces some evidence of toxicity up to a maximum recommended dose of 2000 mg/kg. With no evidence of toxicity via the oral route it was considered necessary to investigate the intraperitoneal route of administration.

Groups of mice were dosed as follows:

Dose Level (mg/kg)	Concentration (mg/ml)	Dose Volume (ml/kg)	Number of Male Mice
942 (oral)	47.1	20	1
942 (ip)	47.1	20	1
471 (ip)	47.1	10	1
400 (ip)	20	20	2
200 (ip)	20	10	2
100 (ip)	10	10	1
100 (ip)	10	10	1

All animals were dosed once only at the appropriate dose level by gavage using a metal cannula or with a hypodermic needle attached to a graduated syringe. The volume

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ip = Intraperitoneal

administered to each animal was calculated according to its bodyweight at the time of dosing.

Animals were observed within the time periods of 0.5, 1, 2, or 4 hours after dosing and subsequently once daily for two days. Any deaths and evidence of overt toxicity were recorded at each observation. No necropsies were performed.

4.2.2 Micronucleus Test

Groups, each of seven male mice, were dosed once only via the intraperitoneal route with the test material at 100, 50 or 25 mg/kg. One group of mice from each dose level was killed by cervical dislocation 24 hours following treatment and a second group dosed with test material at 100 mg/kg was killed after 48 hours. In addition, three further groups of male mice were included in the study; two groups (each of seven mice) were dosed via the intraperitoneal route with the vehicle alone (phosphate buffered saline) and a third group (five mice) was dosed orally with cyclophosphamide. Cyclophosphamide is a positive control material known to produce micronuclei under the conditions of the test. The vehicle controls were killed 24 or 48 hours following dosing and positive control group animals were killed 24 hours following dosing. The experimental design is summarised as follows:

Dose Group	Dose Level (mg/kg)	Concentration (mg/ml)	Dose Volume (ml/kg)	Kill Time (Hours After Dosing)	Animal Numbers
1. Vehicle Control (Phosphate buffered saline)	0	0	10	48	1 - 7
2. Vehicle Control (Phosphate buffered saline)	0	0	10	24	8 -14
3. Positive Control (Cyclophosphamide)	50	5	10	24	15 - 19
4. CHD-FA 5000	100	10	10	48	20 - 26
5. CHD-FA 5000	100	10	10	24	27 - 33
6. CHD-FA 5000	50	5	10	24	34 - 40
7. CHD-FA 5000	25	2.5	10	24	41 - 47

All animals were observed for signs of overt toxicity and death one hour after dosing and then once daily as applicable and immediately prior to termination.

4.2.3 Slide Preparation

Immediately following termination (*i.e.* 24 or 48 hours following dosing), both femurs were dissected from each animal, aspirated with foetal calf serum and bone marrow smears prepared following centrifugation and re-suspension. The smears were air-dried, fixed in absolute methanol, stained in May-Grünwald/Giemsa, allowed to air-dry and a cover slip applied using mounting medium.

4.2.4 Slide Evaluation

Stained bone marrow smears were coded and examined blind using light microscopy at x1000 magnification. The incidence of micronucleated cells per 2000 polychromatic erythrocytes (PCE-blue stained immature cells) per animal was scored. Micronuclei are normally circular in shape, although occasionally they may be oval or half-moon shaped, and have a sharp contour with even staining. In addition, the number of normochromatic erythrocytes (NCE-pink stained mature cells) associated with 1000 erythrocytes was counted; these cells were also scored for incidence of micronuclei.

The ratio of polychromatic to normochromatic erythrocytes was calculated together with appropriate group mean values and standard deviations.

4.2.5 Interpretation of Results

A comparison was made between the number of micronucleated polychromatic erythrocytes occurring in each of the test material groups and the number occurring in the corresponding vehicle control group.

A positive mutagenic response would be demonstrated when a statistically significant, dose-responsive, toxicologically relevant increase in the number of micronucleated polychromatic erythrocytes was observed for either the 24 or 48-hour kill times when compared to their corresponding control group.

If these criteria were not fulfilled, then the test material was considered to be non-genotoxic under the conditions of the test.

A positive response for bone marrow toxicity would be demonstrated when the dose group mean polychromatic to normochromatic ratio was shown to be statistically significantly lower than the concurrent vehicle control group.

All data were statistically analysed using appropriate statistical methods as recommended by the UKEMS Sub-committee on Guidelines for Mutagenicity Testing Report, Part III (1989). The data was analysed following a $\sqrt{(x+1)}$ transformation using Student's t-test (two tailed) and any significant results were confirmed using the one way analysis of variance.

5. 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.

6. RESULTS

6.1 Range-finding Toxicity Test

The mortality data are summarised as follows:

Dose Level (mg/kg)	Sex	Number of Animals Treated	Route	Deaths on Day			Total Deaths
				0	1	2	
942	Male	1	oral	0	0	0	0/1
942	Male	1	ip	1	-	-	1/1
471	Male	1	ip	0	0	0	0/1
400	Male	2	ip	2[e]	-	-	2/2
200	Male	2	ip	0	2[e]	-	2/2
100	Male	1	ip	0	0	0	0/1
100	Male	1	ip	0	0	0	0/1

No evidence of toxicity was observed in animals dosed with test material via the oral route and, therefore systemic absorption could not be confirmed using this dose route

Clear evidence of toxicity was observed in animals dosed with test material via the intraperitoneal route with a premature death at 942 mg/kg and animals killed *in extremis* at 200 and 400 mg/kg. Clinical signs were observed in animals dosed with test material at and above 200 mg/kg as follows: Hunched posture, ptosis, pilo-erection, ataxia, lethargy, dehydration, tiptoe gait, decreased respiratory rate and splayed gait. In animals dosed at 100 mg/kg the clinical signs within acceptable severity limits were observed as follows: Ptosis, hunched posture and pilo-erection. These observations were taken as adequate evidence of systemic absorption.

Adequate evidence of test material toxicity was demonstrated via the intraperitoneal route of administration; therefore, this was selected for use in the main test. The maximum tolerated dose (MTD) of the test material, 100 mg/kg, was selected for use in the main test, with 50 and 25 mg/kg as the lower dose levels.

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e = killed *in extremis*
ip = Intraperitoneal
- = No data

6.2 Micronucleus Test

6.2.1 Mortality Data and Clinical Observations

There were no premature deaths seen in any of the dose groups. Clinical signs were observed in animals dosed with the test material at and above 50 mg/kg in both the 24 and 48-hour groups where appropriate, these were as follows: Hunched posture, ptosis and ataxia.

6.2.2 Evaluation of Bone Marrow Slides

A summary of the results of the micronucleus test is given in Table 1. Individual and group mean data are presented in Tables 2 to 8.

A modest statistically significant decrease in the PCE/NCE ratio was observed in the 48-hour 100 mg/kg dose group and, whilst not statistically significant, a modest decrease was also observed in the 24-hour test 100 mg/kg dose group when compared to their concurrent vehicle control groups. On review of the slide for animal number 20 of the 48-hour 100 mg/kg test material dose group, it was noted that there was a vast majority of NCE over PCE. It was considered that test material induced toxicity had halted the formation of PCE. Therefore, the decreases in PCE/NCE ratio, together with the high level of toxicity observed in animal number 20 and the observation of clinical signs at and above 50 mg/kg, were taken to indicate that systemic absorption had occurred and exposure to the target tissue had been achieved.

There were no statistically significant increases in the frequency of micronucleated PCE in any of the test material dose groups when compared to their concurrent vehicle control groups.

The positive control group showed a marked increase in the incidence of micronucleated polychromatic erythrocytes hence confirming the sensitivity of the system to the known mutagenic activity of cyclophosphamide under the conditions of the test.

The test material was found not to produce a significant increase in the frequency of micronuclei in polychromatic erythrocytes of mice under the conditions of the test.

7. CONCLUSION

The test material was considered to be non-genotoxic under the conditions of the test.

CHD-FA 5000: MICRONUCLEUS TEST IN THE MOUSE

Table 1 Micronucleus Test - Summary of Group Mean Data

Treatment Group	Number of PCE with Micronuclei per 2000 PCE		PCE/NCE Ratio	
	Group Mean	SD	Group Mean	SD
1. Vehicle Control (Phosphate buffered saline) 10 ml/kg 48-hour Sampling Time	0.6	0.5	0.76	0.25
2. Vehicle Control (Phosphate buffered saline) 10 ml/kg 24-hour Sampling Time	1.1	1.2	0.65	0.17
3. Positive Control (Cyclophosphamide) 50 mg/kg 24-hour Sampling Time	28.8***	13.7	0.69	0.20
4. CHD-FA 5000 100 mg/kg 48-hour Sampling Time	1.4	1.6	0.48*	0.22
5. CHD-FA 5000 100 mg/kg 24-hour Sampling Time	0.4	0.5	0.49	0.24
6. CHD-FA 5000 50 mg/kg 24-hour Sampling Time	0.6	1.1	0.64	0.22
7. CHD-FA 5000 25 mg/kg 24-hour Sampling Time	0.3	0.5	0.69	0.42

PCE = Polychromatic erythrocytes
NCE = Normochromatic erythrocytes
SD = Standard deviation
* =P < 0.05
*** = P < 0.001

Table 2 Micronucleus Test - Individual Data and Group Means and Standard Deviations: Vehicle Control (10 ml/kg) 48-Hour Sampling Time

TREATMENT GROUP	ANIMAL NUMBER	BODYWEIGHT (g) WHEN DOSED	POLYCHROMATIC ERYTHROCYTES (PCE)			NORMOCHROMATIC ERYTHROCYTES (NCE)		PCE/NCE RATIO
			NUMBER SCORED	PCE + MN	%PCE + MN	NUMBER SCORED	NCE + MN	
1. VEHICLE CONTROL 10 ml/kg 48-hour sampling time	1	28	2000	1	0.05	631	1	0.58
	2	28	2000	0	0.00	565	0	0.77
	3	26	2000	0	0.00	643	0	0.56
	4	28	2000	0	0.00	513	0	0.95
	5	28	2000	1	0.05	703	0	0.42
	6	29	2000	1	0.05	520	0	0.92
	7	28	2000	1	0.05	475	0	1.11
	Group Mean	27.9	2000	0.6	0.03	579	0.1	0.76
	SD	0.9	0	0.5	0.03	83	0.4	0.25

Table 3 Micronucleus Test - Individual Data and Group Means and Standard Deviations: Vehicle Control (10 ml/kg) 24-Hour Sampling Time

TREATMENT GROUP	ANIMAL NUMBER	BODYWEIGHT (g) WHEN DOSED	POLYCHROMATIC ERYTHROCYTES (PCE)			NORMOCHROMATIC ERYTHROCYTES (NCE)		PCE/NCE RATIO
			NUMBER SCORED	PCE + MN	%PCE + MN	NUMBER SCORED	NCE + MN	
2. VEHICLE CONTROL 10 ml/kg 24-hour sampling time	8	25	2000	1	0.05	517	0	0.93
	9	24	2000	0	0.00	593	0	0.69
	10	25	2000	2	0.10	576	0	0.74
	11	24	2000	0	0.00	647	0	0.55
	12	24	2000	3	0.15	631	0	0.58
	13	26	2000	2	0.10	594	0	0.68
	14	27	2000	0	0.00	726	0	0.38
	Group Mean	25.0	2000	1.1	0.06	612	0.0	0.65
	SD	1.2	0	1.2	0.06	65	0.0	0.17

Table 4 Micronucleus Test - Individual Data and Group Means and Standard Deviations: Cyclophosphamide (50 mg/kg) 24-Hour Sampling Time

TREATMENT GROUP	ANIMAL NUMBER	BODYWEIGHT (g) WHEN DOSED	POLYCHROMATIC ERYTHROCYTES (PCE)			NORMOCHROMATIC ERYTHROCYTES (NCE)		PCE/NCE RATIO
			NUMBER SCORED	PCE + MN	%PCE + MN	NUMBER SCORED	NCE + MN	
3. CYCLOPHOSPHAMIDE 50 mg/kg 24-hour sampling time	15	28	2000	11	0.55	622	0	0.61
	16	26	2000	31	1.55	655	0	0.53
	17	26	2000	49	2.45	499	0	1.00
	18	29	2000	24	1.20	641	0	0.56
	19	27	2000	29	1.45	569	0	0.76
	Group Mean	27.2	2000	28.8	1.44	597	0.0	0.69
	SD	1.3	0	13.7	0.69	64	0.0	0.20

Table 5 Micronucleus Test - Individual Data and Group Means and Standard Deviations: Test Material (100 mg/kg) 48-Hour Sampling Time

TREATMENT GROUP	ANIMAL NUMBER	BODYWEIGHT (g) WHEN DOSED	POLYCHROMATIC ERYTHROCYTES (PCE)			NORMOCHROMATIC ERYTHROCYTES (NCE)		PCE/NCE RATIO
			NUMBER SCORED	PCE + MN	%PCE + MN	NUMBER SCORED	NCE + MN	
4. CHD-FA 5000 100 mg/kg 48-hour sampling time	20	25	186	0	0.00	814	0	0.23
	21	25	2000	4	0.20	794	0	0.26
	22	29	2000	2	0.10	726	0	0.38
	23	26	2000	0	0.00	608	0	0.64
	24	24	2000	1	0.05	542	0	0.85
	25	24	2000	0	0.00	649	0	0.54
	26	29	2000	3	0.15	683	0	0.46
	Group Mean	26.0	1741	1.4	0.07	688	0.0	0.48
	SD	2.2	686	1.6	0.08	98	0.0	0.22

Table 6 Micronucleus Test - Individual Data and Group Means and Standard Deviations: Test Material (100 mg/kg) 24-Hour Sampling Time

TREATMENT GROUP	ANIMAL NUMBER	BODYWEIGHT (g) WHEN DOSED	POLYCHROMATIC ERYTHROCYTES (PCE)			NORMOCHROMATIC ERYTHROCYTES (NCE)		PCE/NCE RATIO
			NUMBER SCORED	PCE + MN	%PCE + MN	NUMBER SCORED	NCE + MN	
5. CHD-FA 5000 100 mg/kg 24-hour sampling time	27	26	2000	1	0.05	533	0	0.88
	28	24	2000	0	0.00	589	0	0.70
	29	24	2000	0	0.00	802	0	0.25
	30	27	2000	1	0.05	737	0	0.36
	31	25	2000	0	0.00	624	0	0.60
	32	27	2000	0	0.00	725	0	0.38
	33	29	2000	1	0.05	804	0	0.24
	Group Mean	26.0	2000	0.4	0.02	688	0.0	0.49
	SD	1.8	0	0.5	0.03	107	0.0	0.24

Table 7 Micronucleus Test - Individual Data and Group Means and Standard Deviations: Test Material (50 mg/kg) 24-Hour Sampling Time

TREATMENT GROUP	ANIMAL NUMBER	BODYWEIGHT (g) WHEN DOSED	POLYCHROMATIC ERYTHROCYTES (PCE)			NORMOCHROMATIC ERYTHROCYTES (NCE)		PCE/NCE RATIO
			NUMBER SCORED	PCE + MN	%PCE + MN	NUMBER SCORED	NCE + MN	
6. CHD-FA 5000 50 mg/kg 24-hour sampling time	34	25	2000	0	0.00	598	0	0.67
	35	25	2000	0	0.00	555	0	0.80
	36	26	2000	0	0.00	599	0	0.67
	37	28	2000	3	0.15	712	0	0.40
	38	27	2000	0	0.00	642	0	0.56
	39	28	2000	0	0.00	503	0	0.99
	40	28	2000	1	0.05	733	0	0.36
	Group Mean	26.7	2000	0.6	0.03	620	0.0	0.64
	SD	1.4	0	1.1	0.06	82	0.0	0.22

Table 8 Micronucleus Test - Individual Data and Group Means and Standard Deviations: Test Material (25 mg/kg) 24-Hour Sampling Time

TREATMENT GROUP	ANIMAL NUMBER	BODYWEIGHT (g) WHEN DOSED	POLYCHROMATIC ERYTHROCYTES (PCE)			NORMOCHROMATIC ERYTHROCYTES (NCE)		PCE/NCE RATIO
			NUMBER SCORED	PCE + MN	%PCE + MN	NUMBER SCORED	NCE + MN	
7. CHD-FA 5000 25 mg/kg 24-hour sampling time	41	29	2000	0	0.00	612	0	0.63
	42	30	2000	0	0.00	456	0	1.19
	43	27	2000	1	0.05	742	0	0.35
	44	28	2000	1	0.05	698	0	0.43
	45	26	2000	0	0.00	790	0	0.27
	46	29	2000	0	0.00	609	0	0.64
	47	30	2000	0	0.00	426	1	1.35
	Group Mean	28.4	2000	0.3	0.01	619	0.1	0.69
	SD	1.5	0	0.5	0.02	138	0.4	0.42

CHD-FA 5000
MICRONUCLEUS TEST IN THE MOUSE

Appendix 1 Historical Control Data from 45 Studies (2007 and 2008)

48-Hour Vehicle Control Group Mean Data		
	PCE + MN Per 2000 PCE	**PCE/NCE 1000 Erythrocytes**
Mean	0.90	1.09
SD	0.54	0.37
Minimum	0.00	0.62
Maximum	2.60	2.54
24-Hour Vehicle Control Group Mean Data		
	PCE + MN Per 2000 PCE	**PCE/NCE 1000 Erythrocytes**
Mean	0.99	1.05
SD	0.60	0.32
Minimum	0.10	0.60
Maximum	3.20	2.32
24-Hour Positive Control Group Mean Data		
	PCE + MN Per 2000 PCE	**PCE/NCE 1000 Erythrocytes**
Mean	42.76	1.17
SD	13.55	0.31
Minimum	17.60	0.72
Maximum	66.40	2.03

PCE = Polychromatic erythrocytes
NCE = Normochromatic erythrocytes
SD = Standard deviation

Appendix 2 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

DH 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