SafePharm Laboratories

Dibromomethane:

CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO* OECD METHOD 473, US 40 CFR 799.9537 AND OPPTS 870.5375 GUIDELINES

SPL PROJECT NUMBER: 0466/0262

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QUALITY ASSURANCE REPORT

This study type is classed as short-term. 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.

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

In each case, unless otherwise indicated, 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:

§	30 August 2006	Protocol Compliance Audit
	14 November 2006	Test Material Preparation
	07 November 2006	Test System Preparation
	14 November 2006	Exposure
	15 November 2006	Assessment of Response
	10 November 2006	Cell Harvest/Staining/Slide Preparation
§	05 March 2007	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: 15 MAY 2007

For Safepharm Quality Assurance Unit*

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GLP COMPLIANCE STATEMENT

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.

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, South Africa, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America. Specifically, these GLP standards are acceptable to the United States Environmental Protection Agency, fulfilling the requirements of 40 CFR Part 792 as amended.

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

..... DATE: 14 MAY 2007

R Durward HNC
Study Director

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CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

SUMMARY

Introduction. This report describes the results of an *in vitro* study for the detection of structural chromosomal aberrations in cultured mammalian cells. It supplements microbial systems insofar as it identifies potential mutagens that produce chromosomal aberrations rather than gene mutations (Scott *et al*, 1990).

The method used followed that described in the OECD Guidelines for Testing of Chemicals (1997) No. 473 "Genetic Toxicology: Chromosome Aberration Test" and Method B10 of Commission Directive 2000/32/EC. The study design also meets the requirements of the UK Department of Health Guidelines for Testing of Chemicals for Mutagenicity and US OPPTS 870.5375 and 40 CFR 799.9537 guidelines.

Methods. Duplicate cultures of human lymphocytes, treated with the test material, were evaluated for chromosome aberrations at three dose levels, together with vehicle and positive controls. Two treatment conditions were used for the study, ie. In Experiment 1, 4 hours in the presence of an induced rat liver homogenate metabolising system (S9), at a 2% final concentration with cell harvest after a 20-hour expression period and a 4 hours exposure in the absence of metabolic activation (S9) with a 20-hour expression period. Experiment 2 was performed but not scored because a clear positive result was obtained in Experiment 1.

The dose levels used in Experiment 1 were selected on the basis of the results of a preliminary toxicity test and were 27.19, 54.38, 108.75, 217.5, 435 and 870µg/ml for the 4(20)-hour exposures both with and without S9. The dose levels selected for metaphase analysis were 54.38, 108.75 and 217.5 µg/ml in both the absence and presence of S9. The dose levels used in Experiment 2 were 54.38, 108.75, 217.5, 435, 652.5 and 870µg/ml in the absence of S9, and 13.60, 27.19, 54.38, 108.75, 217.5 and 435 µg/ml in the presence of S9.

Results. All vehicle (solvent) controls gave frequencies of cells with aberrations within the range expected for normal human lymphocytes. All the positive control materials induced statistically significant increases in the frequency of cells with aberrations indicating the satisfactory performance of the test and of the activity of the metabolising system.

The test material induced a statistically significant increase in the frequency of cells with aberrations at all dose levels analysed, in both exposure groups, using a dose range that included a dose level that induced greater than 50% mitotic inhibition. No increase in the incidence of polyploid cells was observed in either the absence or presence of S9.

Conclusion. The test material was considered to be clastogenic to human lymphocytes *in vitro*.

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1. INTRODUCTION

This report describes the results of an *in vitro* study for the detection of structural chromosomal aberrations in cultured mammalian cells. It supplements microbial systems insofar as it identifies potential mutagens that produce chromosomal aberrations rather than gene mutations (Scott *et al*, 1990).

The method used followed that described in the OECD Guidelines for Testing of Chemicals (1997) No. 473 "Genetic Toxicology: Chromosome Aberration Test" and Method B10 of Commission Directive 2000/32/EC. The study design also meets the requirements of the UK Department of Health Guidelines for Testing of Chemicals for Mutagenicity and US OPPTS 870.5375 and 40 CFR 799.9537 guidelines.

The experimental phases of the study were performed between 16 October 2006 and 02 February 2007.

2. TEST MATERIAL

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	Dibromomethane
CAS Number	:	74-95-3
Description	:	Clear colourless liquid
Purity	:	99.4%
Date received	:	14 September 2006
Storage conditions	:	Room temperature in the dark

Individual samples of the test material were supplied by both Co-Sponsors. These two test materials were then mixed in equal proportions and the resulting mixture analysed to determine its purity under Safepharm Laboratories Ltd. Project Number 0466/0267, including the original sample details provided by each Co-Sponsor, the analytical parameters and resulting test material purity is presented as Appendix 2. This mixed test material was used for all experimental testing detailed in this report.

3. METHODS

3.1 Cells

For each experiment, sufficient whole blood was drawn from the peripheral circulation of a volunteer (male) who had been previously screened for suitability. The volunteer had not been exposed to high levels of radiation or hazardous chemicals and had not knowingly recently suffered from a viral infection. The cell-cycle time for the lymphocytes from the donors used in this study was determined using BrdU (bromodeoxyuridine) incorporation to assess the number of first, second and third division metaphase cells and so calculate the average generation time (AGT). The average AGT for the regular donors used in this laboratory has been determined to be approximately 17 hours under typical experimental exposure conditions.

3.2 Cell Culture

Cells were grown in Eagle's minimal essential medium with HEPES buffer (MEM), supplemented "in-house" with L-glutamine, penicillin/streptomycin, amphotericin B and 15% foetal calf serum, at 37°C with 5% CO_2 in air. The lymphocytes of fresh heparinised whole blood were stimulated to divide by the addition of phytohaemagglutinin (PHA) at 90 µg/ml final concentration.

3.3 Preparation of Test and Control Materials

The test material was accurately weighed, dissolved in dimethyl sulphoxide (DMSO) and serial dilutions prepared. The molecular weight of the test material was 174 therefore the maximum dose level was 1740 µg/ml, the maximum recommended dose level. The purity of the test material was 99.4% and therefore was not required to be accounted for in the formulations. There was no significant change in pH when the test material was dosed into media and the osmolality did not increase by more than 50 mOsm. Chemical analysis of the test material formulations was not performed because it is not a requirement of the test method.

Vehicle and positive controls were used in parallel with the test material. The positive control materials were as follows:

In the absence of S9, mitomycin C (MMC) (Sigma, Batch No. 103K0498) at 0.4 µg/ml for cultures in Experiment 1. It was dissolved in Minimal Essential Medium.

In the presence of S9, cyclophosphamide (CP) (Acros, Batch No. A0164185) at 5 µg/ml. It was dissolved in dimethyl sulphoxide.

3.4 Microsomal Enzyme Fraction

Lot No. PB/βNF S9 03/09/06 was prepared in-house at Safepharm Laboratories from the livers of male Sprague-Dawley rats weighing ~250g. These had received three daily oral doses of a mixture of phenobarbitone (80 mg/kg) and β-naphthoflavone (100 mg/kg), prior to S9 preparation on the fourth day. The S9 was stored at -196°C in a liquid nitrogen freezer.

3.5 Culture Conditions

Duplicate lymphocyte cultures (A and B) were established for each dose level by mixing the following components, giving, when dispensed into sterile plastic flasks for each culture:

9.05 ml MEM, 15% (FCS)
0.1 ml Li-heparin
0.1 ml phytohaemagglutinin
0.75 ml heparinised whole blood

3.5.1 With Metabolic Activation (S9) Treatment

After approximately 48 hours incubation at 37°C, 5% CO_2 in humidified air, the cultures were transferred to tubes and centrifuged. Approximately 9 ml of the culture medium was removed, reserved, and replaced with the required volume of MEM (including serum) and 0.1 ml of the appropriate solution of vehicle control or test material was added to each culture. For the positive control, 0.1 ml of the appropriate solution was added to the cultures. 1 ml of 20% S9-mix (ie 2% final concentration of S9 in standard co-factors) was added to the cultures of the Preliminary Toxicity Test and of Experiment 1.

After 4 hours at 37°C, the cultures were centrifuged, the treatment medium removed by suction and replaced with an 8 ml wash of MEM culture medium. After a further centrifugation the wash medium was removed by suction and replaced with the original culture medium. The cells were then re-incubated for a further 20 hours at 37°C in 5% CO_2 in humidified air.

3.5.2 Without Metabolic Activation (S9) Treatment

In Experiment 1, after approximately 48 hours incubation at 37°C with 5% CO_2 in humidified air the cultures were decanted into tubes and centrifuged. Approximately 9 ml of the culture medium was removed and reserved. The cells were then re-suspended in the required volume of fresh MEM (including serum) and dosed with 0.1 ml of the appropriate vehicle control, test material

solution or 0.1 ml of positive control solution. The total volume for each culture was a nominal 10 ml.

After 4 hours at 37°C, the cultures were centrifuged the treatment medium was removed by suction and replaced with an 8 ml wash of MEM culture medium. After a further centrifugation the wash medium was removed by suction and replaced with the reserved original culture medium. The cells were then returned to the incubator for a further 20 hours.

3.6 Preliminary Toxicity Test

A preliminary toxicity test was performed on cell cultures using a 4-hour exposure time with and without metabolic activation followed by a 20-hour recovery period, and a continuous exposure of 24 hours without metabolic activation. The dose range of test material used was 6.80, 13.59, 27.19, 54.38, 108.75, 217.5, 435, 870 and 1740 µg/ml. Parallel flasks, containing culture medium without whole blood, were established for the three exposure conditions so that test material precipitate observations could be made. Precipitate observations were recorded at the beginning and end of the exposure periods.

Using a qualitative microscopic evaluation of the microscope slide preparations from each treatment culture, appropriate dose levels were selected for mitotic index evaluation. Mitotic index data was used to estimate test material toxicity and for selection of the dose levels for the main study.

3.7 Experiment 1

i) 4-hour exposure to the test material without S9-mix followed by 20-hour culture in treatment-free media prior to cell harvest.

ii) 4-hour exposure to the test material with S9-mix followed by 20-hour culture in treatment-free media prior to cell harvest.

3.8 Cell Harvest

Mitosis was arrested by addition of demecolcine (Colcemid 0.1 µg/ml) two hours before the required harvest time. After incubation with demecolcine, the cells were centrifuged, the culture medium was drawn off and discarded, and the cells resuspended in 0.075M hypotonic KCl. After approximately fourteen minutes (including centrifugation), most of the hypotonic solution was drawn off and discarded. The cells were resuspended and then fixed by dropping the KCl cell

suspension into fresh methanol/glacial acetic acid (3:1 v/v). The fixative was changed at least three times and the cells stored at 4°C for at least four hours to ensure complete fixation.

3.9 Preparation of Metaphase Spreads

The lymphocytes were re-suspended in several ml of fresh fixative before centrifugation and re-suspension in a small amount of fixative. Several drops of this suspension were dropped onto clean, wet microscope slides and left to air dry. Each slide was permanently labelled with the appropriate identification data.

3.10 Staining

When the slides were dry they were stained in 5% Gurrs Giemsa for 5 minutes, rinsed, dried and coverslipped using mounting medium.

3.11 Qualitative Slide Assessment

The slides were checked microscopically to determine the quality of the metaphases and also the toxicity and extent of precipitation, if any, of the test material. These observations were used to select the dose levels for mitotic index evaluation.

3.12 Coding

The slides were coded using a computerised random number generator.

3.13 Mitotic Index

A total of 2000 lymphocyte cell nuclei were counted and the number of cells in metaphase recorded and expressed as the mitotic index and as a percentage of the vehicle control value.

3.14 Scoring of Chromosome Damage

Where possible the first 100 consecutive well-spread metaphases from each culture were counted except where there were approximately 50% cells with aberrations then slide evaluation was terminated at approximately 50 cells. If the cell had 44-48 chromosomes, any gaps, breaks or rearrangements were noted according to the simplified system of Savage (1976) recommended in the 1983 UKEMS guidelines for mutagenicity testing (Appendix I). Cells with chromosome aberrations were reviewed as necessary by a senior cytogeneticist prior to decoding the slides.

3.15 Statistical Analysis

The frequency of cells with aberrations excluding gaps and the frequency of polyploid cells were compared, where necessary, with the concurrent vehicle control value using Fisher's Exact test.

4. ARCHIVES

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

5. RESULTS

5.1 Preliminary Toxicity Test

The dose range for the Preliminary Toxicity Test was 6.80, 13.59, 27.19, 54.38, 108.75, 217.5, 435, 870 and 1740 µg/ml. The maximum dose was the 10 mM concentration. Microscopic assessment of the slides prepared from the cultures showed that metaphase cells were present up to 1740 µg/ml in all exposure groups. There was no precipitate of the test material observed in the parallel blood-free cultures at the end of the exposure up to 1740 µg/ml. However, there was haemolysis observed in the blood cultures at and above 870 µg/ml in both of the 4(20)-hour exposure groups, and at 1740 µg/ml only in the 24 hours continuous exposure group. Following qualitative analysis of the slides, and metaphases being observed up to the 10 mM limit dose level, the upper three dose levels and concurrent vehicle control for each exposure group were selected for mitotic index estimation. The mitotic index data are presented in Table 1. It can be seen that the test material showed evidence of toxicity and dose related mitotic inhibition in all three exposure groups.

Dose selection for Experiment 1 was based on toxicity and the maximum dose level tested was 870 µg/ml for both 4(20) hour exposure groups.

5.2 Chromosome Aberration Test – Experiment 1

The dose levels of the controls and the test material are given in the table below:

Group	Final concentration of Dibromomethane (µg/ml)
4(20)-hour without S9	0*, 27.19, 54.38*, 108.75*, 217.5*, 435, 870, MMC 0.4*
4(20)-hour with S9	0*, 27.19, 54.38*, 108.75*, 217.5*, 435, 870, CP 5*

The qualitative assessment of the slides determined that the toxicity was similar to that observed in the Preliminary Toxicity Test and that there were metaphases suitable for scoring present at the maximum dose level tested of 870 µg/ml in both the absence and the presence of metabolic activation (S9).

* Dose levels selected for metaphase analysis
MMC = Mitomycin C
CP = Cyclophosphamide

The mitotic index data are given in Table 2. They confirm the qualitative observations in that a dose-related inhibition of mitotic index was observed, and that there was an approximate 80% mitotic inhibition observed at and above 435 µg/ml in both the absence and presence of S9. In both exposure groups there was an approximate 65% mitotic inhibition at 217.5 µg/ml. The maximum dose level selected for metaphase analysis was therefore based on toxicity and was 217.5 µg/ml.

The chromosome aberration data are given in Table 3 and Table 4. All of the vehicle control cultures had frequencies of cells with chromosome aberrations within the expected range. The positive control treatments gave statistically significant increases in the frequency of cells with aberrations. The metabolic activation system was therefore shown to be functional and the test method itself was operating as expected.

The test material induced statistically significant increases in the frequency of cells with aberrations in the absence and presence of metabolic activation (S9).

The polyploid cell frequency data are given in Table 5. The test material did not induce a statistically significant increase in the numbers of polyploid cells at any dose level in either of the exposure groups.

The study was terminated after Experiment 1 because a statistically significant increase in structural aberrations was observed in the 4(20)-hour exposures for Experiment 1. The response was considered to be clear evidence of clastogenic activity and in such cases a second experiment is not necessary and not required by the test guidelines.

6. CONCLUSION

The test material induced a statistically significant dose-related increase in the frequency of cells with chromosome aberrations in both the absence and presence of a liver enzyme metabolising system. The test material was therefore considered to be clastogenic to human lymphocytes *in vitro*.

7. REFERENCES

Savage J R K (1976). Annotation: Classification and relationships of induced chromosomal structural changes. *J. Med. Genet.*, **13**, 103 - 122.

Scott D, Dean B J, Danford N D and Kirkland D J (1990). Metaphase chromosome aberration assays *in vitro*. In: Basic mutagenicity tests: UKEMS recommended procedures (Kirkland D J Ed), *Cambridge University Press Report*. Part 1 revised, 62 - 84.

Richardson C, Williams D A, Amphlett G, Phillips B, Allen J A, Chanter D O (1989). Analysis of data from *in vitro* cytogenetics assays: Statistical evaluation of mutagenicity test data: UKEMS sub-committee on guidelines for mutagenicity testing (Kirkland D J Ed) *Cambridge University Press*.

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Table 1 Mitotic Index - Preliminary Toxicity Test

4-HOUR TREATMENT, 20-HOUR RECOVERY -S9

4-HOUR TREATMENT, 20-HOUR RECOVERY +S9

24-HOUR TREATMENT -S9

CONCENTRATION (µg/ml)	4(20)h WITHOUT S9		4(20)h WITH S9		24h WITHOUT S9	
	MITOTIC INDEX	% OF CONTROL	MITOTIC INDEX	% OF CONTROL	MITOTIC INDEX	% OF CONTROL
0	5.05	100	4.35	100	3.90	100
6.80	-	-	-	-	-	-
13.59	-	-	-	-	-	-
27.19	-	-	-	-	-	-
54.38	-	-	-	-	-	-
108.75	-	-	-	-	-	-
217.5	-	-	-	-	-	-
435	1.80	36	1.20	28	2.20	56
870	0.70H	14	0.85H	20	1.15	29
1740	0.95H	19	0.80H	18	0.60H	15

- = Not assessed for mitotic index
H = Haemolysis

Table 2 **Mitotic Index – Experiment 1**

DOSE LEVEL µg/ml	4 HOURS TREATMENT WITHOUT S9				4 HOURS TREATMENT WITH S9			
	A	B	MEAN	% OF CONTROL	A	B	MEAN	% OF CONTROL
0	7.25	7.30	7.28	100	5.65	5.00	5.33	100
27.19	-	-	-	-	-	-	-	-
54.38	6.60	6.40	6.50	89	4.25	5.45	4.85	91
108.75	3.65	4.55	4.10	56	2.90	2.60	2.75	52
217.5	2.40	3.00	2.70	37	1.85	1.35	1.60	30
435	1.35	1.65	1.50	21	1.00	1.65	1.33	25
870	1.60	1.65	1.63	22	0.75	1.35	1.05	20
MMC 0.4	4.40	3.05	3.73	51	NA	NA	NA	NA
CP 5	NA	NA	NA	NA	1.40	1.75	1.58	30

MMC = Mitomycin C
CP = Cyclophosphamide
NA = Not applicable
\- = Not assessed for mitotic index

Table 3 Results of Chromosome Aberration Test – Experiment 1 Without Metabolic Activation (S9)

Treatment Group	Replicate	Mitotic Index (%)	Number of Cells Scored	Number of Aberrations						Total Number of Aberrations		Frequency of Aberrant Cells (%)	
				Gaps	Chromatid		Chromosome		Others				
					Breaks	Exchanges	Breaks	Exchanges	X	(+ Gaps)	(-Gaps)	(+Gaps)	(-Gaps)
Vehicle Control	A	7.25	100	1	0	0	0	0	0	1	0	1	0
	B	7.30	100	0	2	0	0	0	0	2	2	2	2
	Total		200	1	2	0	0	0	0	3	2	3	2
		(100)										(1.5)	(1.0)
54.38 µg/ml	A	6.60	100	18	12	0	0	1	0	31	13	23	12
	B	6.40	100	11	17	2	2	1	0	33	22	24	16
	Total		200	29	29	2	2	2	0	64	35	47	28***
		(89)										(23.5)	(14.0)
108.75 µg/ml	A	3.65	53[a]	8	20	6	3	0	0	37	29	21	18
	B	4.55	50[a]	15	15	9	0	0	0	39	24	28	21
	Total		103	23	35	15	3	0	0	76	53	49	39***
		(56)										(47.6)	(37.9)
217.5 µg/ml	A	2.40	50[a]	13	25	13	0	0	0	51	38	24	20
	B	3.00	50[a]	20	16	10	1	0	0	47	27	22	13
	Total		100	33	41	23	1	0	0	98	65	46	33***
		(37)										(46.0)	(33.0)
Positive Control MMC 0.4 µg/ml	A	4.40	50[a]	3	17	23	3	0	0	46	43	27	26
	B	3.05	50[a]	5	22	30	2	0	0	59	54	28	28
	Total		100	8	39	53	5	0	0	105	97	55	54***
		(51)										(55.0)	(54.0)

MMC = Mitomycin C
a = Slide evaluation terminated as approximately 50% cells observed have aberrations
*** = P < 0.001(Less than1/1000 chance that the result occurred by-chance, or very highly significant)

Table 4 Results of Chromosome Aberration Test – Experiment 1 With Metabolic Activation (S9)

Treatment Group	Replicate	Mitotic Index (%)	Number of Cells Scored	Number of Aberrations						Total Number of Aberrations		Frequency of Aberrant Cells (%)	
					Chromatid		Chromosome		Others				
				Gaps	Breaks	Exchanges	Breaks	Exchanges	X	(+ Gaps)	(-Gaps)	(+Gaps)	(-Gaps)
Vehicle Control	A	5.65	100	1	1	0	0	0	0	2	1	2	1
	B	5.00	100	0	0	0	0	0	0	0	0	0	0
	Total	(100)	200	1	1	0	0	0	0	2	1	2 (1.0)	1 (0.5)
54.38 µg/ml	A	4.25	100	12	22	0	1	0	0	35	23	29	18
	B	5.45	100	11	23	1	0	0	0	35	24	23	16
	Total	(91)	200	23	45	1	1	0	0	70	47	52 (26.0)	34*** (17.0)
108.75 µg/ml	A	2.90	100	7	12	2	1	0	0	22	15	15	12
	B	2.60	100	10	11	5	2	1	0	29	19	21	16
	Total	(52)	200	17	23	7	3	1	0	51	34	36 (18.0)	28*** (14.0)
217.5 µg/ml	A	1.85	50[a]	16	21	15	2	1	1	56	40	25	20
	B	1.35	50[a]	6	33	7	1	0	0	47	41	21	18
	Total	(30)	100	22	54	22	3	1	1	103	81	46 (46.0)	38*** (38.0)
Positive Control CP 5 µg/ml	A	1.40	100	3	14	3	2	0	0	22	19	15	14
	B	1.75	50[a]	8	14	12	4	0	0	38	30	23	18
	Total	(30)	150	11	28	15	6	0	0	60	49	38 (25.3)	32*** (21.3)

CP = Cyclophosphamide
a = Slide evaluation terminated as approximately 50% cells observed have aberrations
*** = P < 0.001 (Less than1/1000 chance that the result occurred by-chance, or very highly significant)

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Table 5 Mean Frequency of Polyploid Cells (%)

EXPERIMENT 1

DOSE LEVEL µg/ml	HARVEST TIME 24 HOURS	
	4 HOURS WITHOUT S9	4 HOURS WITH S9
0	0.0	0.0
54.38	0.0	0.0
108.75	0.0	0.0
217.5	0.0	0.0
MMC 0.4	0.0	NA
CP 5	NA	0.0

MMC = Mitomycin C
CP = Cyclophosphamide
NA = Not applicable

Appendix 1 Chromosome Structural Aberrations: Classification, Evaluation Criteria and Historical Control Data

1. CLASSIFICATION

1.1 Gaps (g)

Gaps are small areas of the chromosome that are unstained. The chromatids remain aligned as normal and the gap does not extend along the chromatid for a distance greater than the width of a chromatid. If the gap occurs on one chromatid only it is a chromatid gap (g). If a gap appears in both chromatids at the same position it is a chromosome gap (G).

1.2 Chromatid Breaks (ctb)

Chromatid breaks (ct) vary in appearance. The chromatid may remain aligned but show a gap which is too large to classify as a gap. Alternatively, the chromatid may be broken so that the broken fragment is displaced. In some cases, the fragment is not seen at all. A chromatid fragment (f) should be scored if the chromosome of origin cannot be identified. Very small fragments are scored as minutes (m).

1.3 Chromosome Breaks (csb)

Chromosome breaks (CS) are breaks in both chromatids of the chromosome. A fragment with two chromatids is formed and may be displaced by varying degrees. Breaks are distinguished from gaps by the size of the unstained region. A chromosome break is scored if the fragment is associated with a chromosome from which it was probably derived. However, fragments are often seen in isolation and are then scored as chromosome fragments (F). Very small fragments are scored as minutes (M).

1.4 Exchanges (cte and cse)

Exchanges are formed by faulty rejoining of broken chromosomes and may be of the chromosome or chromatid type. Chromatid exchanges (c/c,r) have numerous different forms but are generally not further classified. Where multiple exchanges have occurred each exchange point is counted as one chromatid exchange. Chromosome exchanges generally appear as either a dicentric (D) or a ring (R) form, either of which can be associated with a fragment, which if possible should be scored as part of the exchange.

Appendix 1 (continued) Chromosome Structural Aberrations: Classification, Evaluation Criteria and Historical Control Data

1.5 Multiple Aberrations

If many aberrations are present in one metaphase, the exact details may not be scorable. This is particularly the case when chromosome pulverisation occurs. If the number of aberrations is 10 or more then the cell is classified as X.

1.6 Chromosome Number

If the chromosome (centromere) number is 44-48 then it is classified as a diploid cell and scored for aberrations. If less than 44 chromosomes are counted then the cell is ignored under the assumption that some chromosomes may have been lost for technical reasons. If greater than 44 chromosomes are scored and the number is a multiple of the haploid count then the cell is classified as a polyploid cell. If the chromosomes are arranged in closely apposed pairs, ie. 4 chromatids instead of 2, the cell is scored as endoreduplicated (E).

2. HISTORICAL CONTROL DATA AND EVALUATION CRITERIA

2.1 Historical Aberration Ranges for Vehicle Control Cultures

Many experiments with human lymphocytes have established a range of aberration frequencies acceptable for control cultures. The current in-house historical aberration ranges are presented below:

	Pulse exposure –S9		Pulse exposure +S9 (1% S9)		24-Hour –S9		Pulse exposure +S9 (2% S9)	
	% cells with aberrations (-gaps)	% cells with polyploids	% cells with aberrations (-gaps)	% cells with polyploids	% cells with aberrations (-gaps)	% cells with polyploids	% cells with aberrations (-gaps)	% cells with polyploids
Minimum	0	0	0	0	0	0	0	0
Maximum	3	1	4	1.5	4.5	1	2.5	1.5
Mean	0.95	0.13	1.09	0.13	1.18	0.12	0.86	0.19
Standard Deviation	0.74	0.28	1.02	0.28	0.99	0.24	0.68	0.37
Number	47	47	47	47	43	43	40	40

Appendix 1 (continued) Chromosome Structural Aberrations: Classification, Evaluation Criteria and Historical Control Data

2.2 Historical Aberration Range for Positive Control Cultures

	Pulse exposure –S9 MMC 0.4 µg/ml		Pulse exposure +S9 (1% S9) CP 10 - 12.5 µg/ml		24-Hour –S9 MMC 0.2 - 0.25 µg/ml		Pulse exposure +S9 (2% S9) CP 10 - 12.5 µg/ml	
	% cells with aberrations (-gaps)	% cells with polyploids	% cells with aberrations (-gaps)	% cells with polyploids	% cells with aberrations (-gaps)	% cells with polyploids	% cells with aberrations (-gaps)	% cells with polyploids
Minimum	5.5	0	7.5	0	11	0	10.8	0
Maximum	68	0.5	42	1	71	0.5	59	0.5
Mean	38.12	0.02	19.71	0.07	37.84	0.01	28.84	0.03
Standard Deviation	14.70	0.10	8.14	0.21	14.94	0.08	12.54	0.11
Number	47	47	47	47	43	43	40	40

2.3 Evaluation Criteria

A positive response was recorded for a particular treatment if the % cells with aberrations, excluding gaps, markedly exceeded that seen in the concurrent control, either with or without a clear dose-relationship. For modest increases in aberration frequency a dose response relationship is generally required and appropriate statistical tests may be applied in order to record a positive response.

Dibromomethane: CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

Appendix 2 Certificate of Analysis

SafePharm Laboratories

SafePharm Laboratories Ltd
Shardlow Business Park
London Road, Shardlow
Derbyshire DE72 2GD
Tel: 01332 792896 Fax: 01332 799018
Website: www.safepharm.co.uk

MIXING AND ANALYSIS OF TEST MATERIAL SAMPLES

CERTIFICATE OF ANALYSIS

TEST MATERIAL	:	Dibromomethane	
PROJECT NUMBER	:	0466/0267	
CO-SPONSORS	:	Bromine Compounds Ltd. Makleff House P.O.B. 180 BEER-SHEVA 84101 ISRAEL	Albemarle Corporation Health, Safety and Environment 451 Florida Street Baton Rouge LA 70801 UNITED STATES OF AMERICA
TEST DATES	:	14 September 2006 to 28 September 2006	

Test Material Details:

Supplier	:	Bromine Compounds Ltd	Albemarle Corporation
Name	:	Dibromomethane	Dibromomethane
Description	:	clear, colourless liquid	clear, colourless liquid
Lot number	:	20050298	DBM082506
Date Received	:	12 September 2006	01 September 2006
Storage Conditions	:	room temperature, in the dark	room temperature, in the dark

Testing Details:

The above two test materials were mixed in equal proportions and the purity of the resulting mixture determined by gas chromatography (GC) area normalisation. The purity was determined to be 99.4%.

Analytical Conditions:

Column	:	ZB-624 (30 m x 0.25 mm id, 1.40 µm film)
Oven temperature program	:	initial temperature : 40°C rate: 5°C/minute final temperature: 260°C for 5 minutes
Injector temperature	:	250°C
Flame ionisation detector temperature	:	300°C
Injection volume	:	1 µl
Retention time	:	approximately 9.5 minutes for Dibromomethane

Registered in England No. 3009027

Dibromomethane: CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

Appendix 2 (continued) Certificate of Analysis

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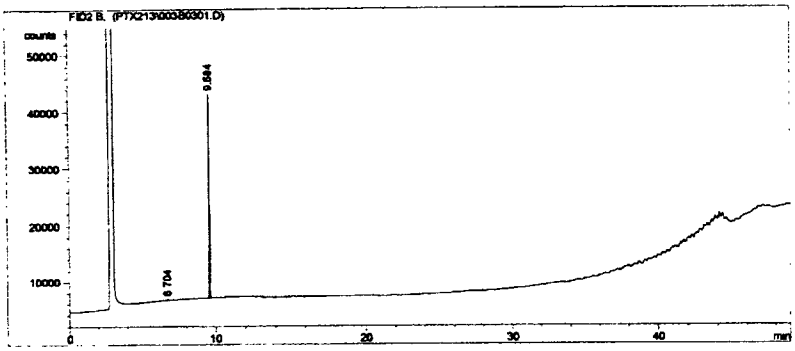
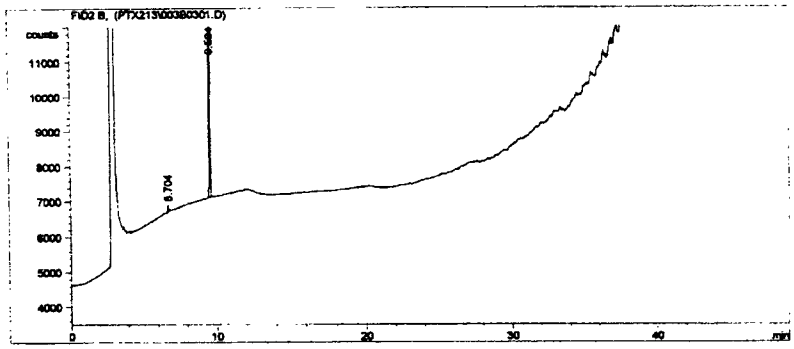
Tel: 01332 792896 Fax: 01332 799018
Website: www.safepharm.co.uk

Typical Chromatography

Sample Solution, 6.14 x 10^3 mg/l in Methanol



Sample Solution (Expanded Scale)



Registered in England No. 3009027

Dibromomethane: CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES *IN VITRO*

Appendix 2 (continued) Certificate of Analysis

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Shardlow Business Park
London Road, Shardlow
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Tel: 01332 792896 Fax: 01332 799018
Website: www.safepharm.co.uk

I the undersigned, hereby declare that this certificate of analysis accurately reflects the original data generated in SPL Project Number 0466/0267.

This study was performed in compliance with the UK GLP Regulations 1999 (as amended in 2004) and this certificate accurately reflects the results obtained. The original data and the Certificate of Analysis will be kept for five years in the archives of Safepharm Laboratories Ltd.

Signed: ..... Date: 02 NOV 2006
B J O'Connor BSc (Hons) MRSC
Study Director

Studies of this type are monitored and reported by Safepharm Laboratories Quality Assurance Unit as part of a routine sampling programme: 14, 26 September 2006.

The certificate was audited on 02 November 2006 by Safepharm Laboratories Quality Assurance Unit. It is considered to be an accurate account of the data generated and of the results produced.

Signed: ..... Date: 02 NOV 2006
For Safepharm Quality Assurance Unit

Registered in England No. 3009027

Appendix 3 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

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

LABORATORY	TEST TYPE
SafePharm Laboratories Ltd. **Shardlow Business Park** **London Road** **Shardlow** **Derby** **DE72 2GD**	**Analytical Chemistry** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION

30th August 2005

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory 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.

Bryan J Wright 21/11/05.

Mr. Bryan J. Wright
Head, UK GLP Monitoring Authority

CONTAINS NO CBI