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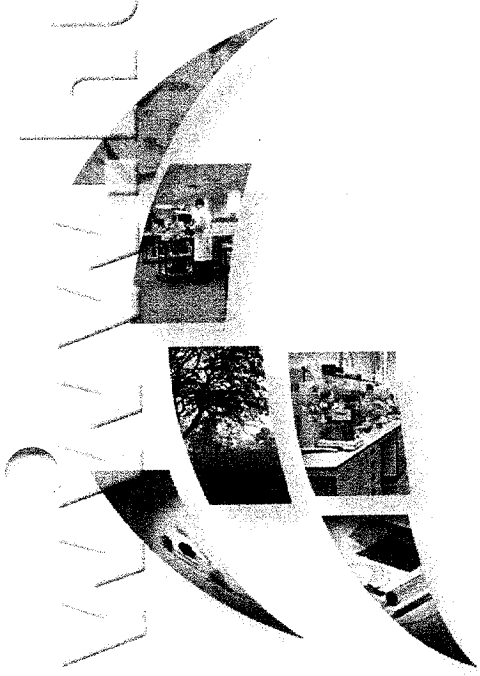
ABM-FD (EXTRACT OF HIMEMATSUTAKE)

IN VITRO MUTATION TEST USING MOUSE LYMPHOMA L5178Y CELLS



Report

ABM-FD (EXTRACT OF HIMEMATSUTAKE)

IN VITRO MUTATION TEST USING MOUSE LYMPHOMA L5178Y CELLS

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards, and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM (98) 17.
EC Commission Directive 2004/10/EC of 11 February 2004 (Official Journal No. L 50/44).

These principles of Good Laboratory Practice are accepted by the regulatory authorities of the United States of America and Japan on the basis of intergovernmental agreements.

K. May ..... 11 October 2005

Kenneth May, B.Sc., C.Biol., M.I.Biol.,
Study Director,
Huntingdon Life Sciences Ltd.

Date

QUALITY ASSURANCE STATEMENT

The following inspections and audits have been carried out in relation to this study:

Study Phase	Date(s) of Inspection	Date of Reporting to Study Director and Management
Protocol Audit	14 December 2004	14 December 2004
Report Audit	9 – 11 May 2005	11 May 2005

Process based inspections: At or about the time this study was in progress inspections of procedures employed on this type of study were carried out. These were conducted and reported to appropriate Company Management as indicated below:

Process Based Inspections	Date(s) of Inspection	Date of Reporting to Management
Formulation	12 April 2005	22 April 2005
Treatment	2 March 2005	2 March 2005
Cell culture establishment	27 January 2005	27 January 2005
Cell count, adjustment and plating out	4 March 2005	4 March 2005
Plate scoring	7 February 2005	7 February 2005
Clinical chemistry	10 January 2005	10 January 2005

In addition, an inspection of the facility where this study was conducted was carried out on an annual basis. These inspections were promptly reported to Company Management.

N Jolly ..... 11 OCTOBER 2005 .....

Neal Jolly, B.Sc., M.R.Q.A., Date
Group Manager,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

RESPONSIBLE PERSONNEL

STUDY MANAGEMENT

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SUMMARY

ABM-FD (Extract of Himematsutake) was tested for mutagenic potential in an *in vitro* mammalian cell mutation assay. This test system is based on detection and quantitation of forward mutation in the subline 3.7.2c of mouse lymphoma L5178Y cells, from the heterozygous condition at the thymidine kinase locus ($TK^{+/-}$) to the thymidine kinase deficient genotype ($TK^{-/-}$). The study consisted of a preliminary toxicity test and two main tests comprising three independent mutagenicity assays. The cells were exposed for either 3 hours or 24 hours in the absence of exogenous metabolic activation (S9 mix) or 3 hours in the presence of S9 mix.

ABM-FD was found to be soluble at approximately 53.5 mg/mL in sterile water. A solution of 53.5 mg/mL dosed at 10% (final concentration in medium of 5350 µg/mL), did not form a precipitate in the culture medium or cause any change in pH of more than 1.0 unit. Fluctuation in osmolality was found to be less than 50 mOsm/kg compared with the osmolality of the culture medium. A solution of 50 mg/mL, dosed at 10% in culture medium (final concentration 5000 µg/mL) was used as the maximum concentration in the preliminary toxicity test.

In the preliminary toxicity test, no toxicity was observed after treatment with ABM-FD in both the absence and presence of S9 mix with a 3-hour exposure at concentrations from 9.77 to 5000 µg/mL. Following a 24-hour exposure in the absence of S9 mix, relative suspension growth (RSG) was reduced from 114 to 8%. The concentrations assessed for determination of mutant frequency in the 24 hour main test were based upon these data, the objective being to assess concentrations which span the complete toxicity range of approximately 10-100% relative total growth (RTG). As no toxicity was observed following a 3-hour exposure in either the absence or presence of S9 mix, a final maximum concentration of 5000 µg/mL was used.

In the first main test (3 hour exposure) in the absence and presence of S9 mix, the maximum concentration ABM-FD plated for determination of mutant frequency was 5000 µg/mL, where RTG was reduced to 80% relative to the concurrent solvent control. There were no increases in induced mutation frequency that exceeded the Global Evaluation Factor, at any of the concentrations tested within acceptable levels of toxicity. In all tests the concurrent solvent and positive control were within acceptable ranges. In the second main test (24 hour exposure) in the absence of S9 mix, the maximum concentration of ABM-FD assessed for determination of mutant frequency was 4000 µg/mL, where RTG was reduced to 20% relative to the concurrent solvent control. Exposure to ABM-FD at a concentration of 4000 µg/mL was associated with an increased induced mutation frequency exceeding the Global Evaluation Factor. There was evidence of a concentration-response relationship, which gave a significant positive linear trend ($P \leq 0.01$). In all tests the concurrent solvent and positive control were within acceptable ranges.

ABM-FD (Extract of Himematsutake) demonstrated mutagenic potential, in the absence of S9 mix when exposed for 24 hours, in this *in vitro* cell mutation assay, under the experimental conditions described. Increased mutant frequency was obtained only after 24 hours continuous exposure to ABM-FD, and it was noted that the mutant colonies obtained were almost exclusively small. Small mutant colonies may indicate the loss of genes concerned with cell growth resulting from large deletions, clastogenicity or the loss of a chromosome (aneuploidy).

INTRODUCTION

The test substance, ABM-FD (Extract of Himematsutake), was tested for potential mutagenicity in the mouse lymphoma L5178Y cell mutation test. The test involves detection of mutation of mouse lymphoma L5178Y cells using the microtitre® fluctuation technique in 96-well microtitre plates. The cells are heterozygous at the thymidine kinase (tk) locus ($TK^{+/-}$), and forward mutation ($TK^{-/-}$) is detected by the ability of these cells to divide and form colonies in the presence of trifluorothymidine (TFT), a toxic analogue of thymidine.

This test system detects a wide range of genetic damage in viable cells capable of forming colonies. The test methodology was based on established procedures (Amacher, Paillet and Ray, 1979; Amacher *et al.,* 1980; Clive *et al.,*1979; Cole *et al.,*1990, Moore *et al.*, 2000, Moore *et al.*, April 2000, Moore *et al.*, 2003 and Moore *et al.*, 2003). Toxicity was measured in terms of relative suspension growth (RSG) and relative total growth (RTG).

Some substances do not exert their mutagenic effect until they have been metabolised by enzyme systems. Therefore, the test substance and the cells were incubated in the absence and the presence of a supplemented rat liver homogenate fraction (S9 mix).

This report describes a study designed to comply with the following guidelines:

> OECD Guidelines for Testing of Chemicals No. 476: "Genetic toxicology: *In vitro* mammalian cell gene mutation tests", 1997.
>
> US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5300 *In vitro* mammalian cell gene mutation test. EPA 712-C-98-221.
>
> Commission Directive 2000/32/EC Annex 4E – B.17. Mutagenicity – *In vitro* mammalian cell gene mutation test. No. L136/65.
>
> ICH (1996) Guideline S2A: Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. PAB/PCD Notification No. 444.
>
> ICH (1998) Guideline S2B: Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. PMSB/ELD Notification No. 554.
>
> The procedures also met the requirements of the FDA (United States Food and Drug Administration) "Redbook 2000" Toxicological Principles for the Safety Assessment of Food Ingredients.

The protocol was approved by Huntingdon Life Sciences Management on 25 November 2004, by the Sponsor on 8 December 2004 and by the Study Director on 9 December 2004.

The study was conducted at Huntingdon Life Sciences Ltd., Eye Research Centre, Eye, Suffolk, IP23 7PX, England, except for formulation chemistry and osmolality testing which was conducted at Huntingdon Life Sciences Ltd., Huntingdon Research Centre, Huntingdon, Cambridgeshire, PE28 4HS.

Experimental start date: 13 December 2004.
Experimental completion date: 7 February 2005.

TEST SUBSTANCE

Identity:	ABM-FD (Extract of Himematsutake)
Appearance:	Brown solid
Storage conditions:	Room temperature, in the dark
Batch number:	EX200408
Expiry date:	End of July 2006
Purity:	2.9 g / 100 g as beta-glucan
Date received:	31 August 2004

EXPERIMENTAL PROCEDURE

CELLS

L5178Y mouse lymphoma (3.7.2c) cells (Clive and Spector, 1975), were obtained from the MRC Cell Mutation Unit, University of Sussex, Brighton. These cells are heterozygous at the thymidine kinase locus, $TK^{+/-}$. Spontaneous thymidine kinase deficient mutants, $TK^{-/-}$, were eliminated from the cultures by a 24 hour incubation in the presence of methotrexate, thymidine, hypoxanthine and glycine two days prior to storage at -196°C, in heat-inactivated donor horse serum (HiDHS) containing 10% DMSO. Cultures were used within ten days of recovery from frozen stock. Cell stocks are periodically checked for freedom from mycoplasma contamination.

MEDIA

The following media, obtained from a suitable supplier, were used:

R0 RPMI 1640, buffered with 2 mg/mL sodium bicarbonate, supplemented with 2.0 mM L-glutamine and 50 μg/mL gentamicin.

R10p R0, supplemented with 0.1% v/v Synperonic F68, 1.0 mM sodium pyruvate and HiDHS at 10% v/v.

R30p R0, supplemented with 0.02% v/v Synperonic F68, 1.0 mM sodium pyruvate and HiDHS at 30% v/v.

R10p medium was used for cell culture unless otherwise specified.

R20p medium was used for the cloning efficiency plating. This was prepared by mixing equal volumes of R10p and R30p.

Selective medium consisted of R10p containing 4 μg/mL trifluorothymidine (TFT).

POSITIVE CONTROL COMPOUNDS

In the absence of S9 mix

Identity:	Methyl methanesulphonate (MMS)
CAS No.:	66-27-3
Molecular weight	110.1
Solvent:	DMSO
Exposure concentration:	10 µg/mL (3 hour exposure) 5 µg/mL (24 hour exposure)

In the presence of S9 mix

Identity:	3-methylcholanthrene (3MC)
CAS No.:	56-49-5
Molecular weight:	268.4
Solvent:	DMSO
Exposure concentration:	2.5 µg/mL

PREPARATION OF S9 FRACTION

The S9 fraction was from male Sprague-Dawley derived rats, dosed with Aroclor 1254 to stimulate mixed-function oxidases in the liver. The S9 fraction was purchased from Molecular Toxicology Incorporated, USA and stored at *ca* -80°C or below. The quality control statement relating to the batch of S9 preparation used is included in the raw data and a copy is included in this report as Appendix 3.

Lot No.: 1759 (Date of preparation: 9 December 2004)

PREPARATION OF S9 MIX

S9 mix contains: S9 fraction (5% v/v), isocitric acid (8.7 mM), NADP (1.6 mM) in R0. The co-factors were neutralised with 1N NaOH and filter sterilised before adding to S9 fraction and R0.

SELECTION OF VEHICLE AND FORMULATION OF TEST SUBSTANCE

Prior to commencing testing, the solubility of the test substance in solvents compatible with this test system was assessed. ABM-FD (Extract of Himematsutake) was found to be soluble at approximately 53.5 mg/mL in sterile water (purified by reverse osmosis). A solution of 53.5 mg/mL, dosed in culture medium at 10% (final concentration 5350 µg/mL), did not form a precipitate in the culture medium. The osmolality of the test substance in medium was tested at concentration of 5000 µg/mL. No fluctuations in osmolality of the medium of more than 50 mOsm/kg were observed compared with the solvent (vehicle) control. When dosed at concentrations from approximately 668 to 5350 µg/mL, ABM-FD caused a colour change in the medium. The pH was measured, but no changes in pH were observed of more than 1.0 unit at these concentrations. The maximum concentration tested in the preliminary toxicity test was, therefore, 5000 µg/mL.

ABM-FD (Extract of Himematsutake) was dissolved and diluted in sterile water, shortly before dosing. The final concentration of sterile water added to the cultures was 10% v/v.

The final concentrations (µg/mL) to which cells were exposed initially are given below. Those cultures that were assessed for determination of the mutant phenotype are underlined.

Preliminary toxicity test:			9.77, 19.53, 39.06, 78.13, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/mL
Mutation tests:	-S9 mix	Test 1 (3 hours)	<u>156.25, 312.5, 625, 1250, 2500</u> and <u>5000</u> µg/mL
	+S9 mix	Test 1 (3 hours)	<u>156.25, 312.5, 625, 1250, 2500</u> and <u>5000</u> µg/mL
	-S9 mix	Test 2 (24 hours)	<u>500, 1000, 2000, 3000, 4000</u> and 5000 µg/mL

TEST PROCEDURE

Preliminary toxicity test

Cells were exposed to the test substance for 3 hours in the absence and presence of S9 mix and for 24 hours in the absence of S9 mix.

For 3 hour exposures, a cell suspension of 2.4 x 10^6 cells/mL in R10p was incubated for approximately 30 minutes at 37°C, then dispensed in 2.5 mL aliquots before adding either 2 mL of R0 or S9 mix as appropriate. For 24 hour exposures, a cell suspension of 0.333 x 10^6 cells/mL in R10p was incubated for approximately 30 minutes at 37°C, then dispensed in 4.5 mL aliquots. One culture was prepared for each concentration of the test substance for each test condition. Solvent controls were tested in duplicate for each test condition.

The test substance was formulated and serially diluted in the solvent. Aliquots of 500 µL of test substance dilution (at 10 times the desired final concentration) or solvent were added to each culture prior to incubation for 3 or 24 hours at 37°C with continuous shaking, as appropriate. At the end of the 3-hour exposure period, the cells were washed once, resuspended in R10p to nominally 2 x 10^5 cells/mL (assuming no cell loss) or as appropriate, incubated and sampled after 24 and 48 hours to assess growth in suspension. After sampling at 24 hours the cell density was readjusted to 2 x 10^5 cells/mL with R10p, the intention being to retain at least 1 x 10^7 cells. At the end of the 24-hour exposure period, the cells were washed once, resuspended in 20 mL R10p and counted, to ascertain treatment growth. The cultures were then diluted to 2 x 10^5 cells/mL with R10p as appropriate, incubated and sampled after 24 and 48 hours to assess growth in suspension. After sampling at 24 hours the cell density was readjusted to 2 x 10^5 cells/mL with R10p, the intention being to retain at least 1 x 10^7 cells.

The RSG was used to determine the concentrations of test substance used in the main test; ideally the maximum concentration should reduce RTG to approximately 10-20% of the concurrent solvent control value. There was evidence of toxicity in the preliminary toxicity test, so the maximum concentration tested in the first main test was 5000 µg/mL, the formulations being added at 10% final volume in medium.

Mutagenicity assay – 3 hour exposure

Duplicate 5 mL aliquots of a suspension of 2.4 x 10^6 cells/mL had 4 mL of R0 or S9 mix added as appropriate. Quadruplicate cultures were prepared for solvent controls. Aliquots of 1000 µL of test substance dilution (at 10 times the desired final concentration) or solvent were added, positive control cultures contained 100 µL of positive control and 900 µL of medium. All cultures were then incubated, with continuous shaking, for 3 hours at 37°C. Six serial dilutions of the test substance were tested. Toxicity was measured in terms of RTG.

Following the 3 hour exposure, the cells were washed once, resuspended in R10p to nominally 2 x 10^5 cells/mL (assuming no cell loss) and incubated for a further 48 hours to allow for expression of mutant phenotype. The cultures were sampled after 24 and 48 hours to assess growth in suspension. After sampling at 24 hours the cell density was readjusted to 2 x 10^5 cells/mL with

R10p, the intention being to retain 1 x 10^7 cells. After 48 hours cultures with a density of more than 1 x 10^5 cells/mL were assessed for cloning efficiency (viability) and mutant potential by plating in 96 well plates. Cloning efficiency was assessed by plating 1.6 cells/well in R20p, two plates being prepared per culture. Mutant potential was assessed by plating 2 x 10^3 cells/well in selective medium, two plates being prepared per culture. The plates were placed in a humidified incubator at 37°C in an atmosphere of 5% CO_2 in air.

After the plates had been incubated for at least 7 days for viability plates and approximately 10-14 days for mutant plates, the number of empty wells was assessed for each 96-well plate (P0). P0 was used to calculate the cloning efficiency (CE) and mutant frequency (MF). The colony size distribution in the solvent, positive controls and the highest mutation frequency inducing test substance concentration was examined to ensure that there was an adequate recovery of small colony mutants.

Mutagenicity assay – 24 hour exposure in the absence of S9 mix

A second test was carried out, with a 24 hour exposure in the absence of S9 mix. In the absence of S9 mix, duplicate 9 mL cultures at 0.333 x 10^6 cells/mL were treated for 24 hours with 1000 μL of test substance, or solvent. Positive control cultures were dosed with 100 μL of positive control and 900 μL of medium. . At the end of the exposure period, the cells were washed once, resuspended in 20 mL R10p and counted to ascertain treatment growth. The cultures were then diluted to 2 x 10^5 cells/mL with R10p as appropriate, incubated and sampled after 24 and 48 hours to assess growth in suspension. After sampling at 24 hours the cell density was readjusted to 2 x 10^5 cells/mL with R10p, the intention being to retain at least 1 x 10^7 cells. Following this, the procedure was the same as in the 3 hour treatment. The maximum concentration in the second main test was 5000 μg/mL.

STABILITY AND FORMULATION ANALYSIS

The stability of ABM-FD (Extract of Himematsutake) in water was determined as part of Huntingdon Life Sciences study no. IKI/001. Analysis of achieved concentration was performed as part of this study. Results of this analysis are presented in Appendix 3. All results are considered acceptable, within the limitations of the assay.

ASSESSMENT OF RESULTS

Acceptance criteria

On completion of each main mutagenicity test, data were examined for cell growth parameters, cytotoxicity, plating efficiencies, spontaneous and positive control MF, and percent small colonies in positive control cultures.

The criteria used to assess whether an assay was valid are based on the recommendations of the Plymouth and draft Aberdeen papers (Robinson *et al.*, 1989, Moore *et al.*, 2000 and Moore *et al.*, April 2000, Moore *et al.*, 2003 and Moore *et al.*, 2003).

Tests were accepted on the basis of the following criteria:

Acceptance criteria for test substance:

The highest concentration tested was one that allowed the maximum exposure up to 5000µg/mL or 10mM for freely soluble compounds, or the limit of toxicity (ie. relative total growth reduced to approx 10-20% of the concurrent vehicle control) or the limit of solubility. For a toxic substance, at least 4 analysable concentrations should have been achieved which ideally spanned the toxicity range of 100-10% RTG.

Acceptance criteria for vehicle controls:

The mean vehicle control value for mutant frequency was between 50-170 x 10^{-6}.
The mean cloning efficiency was between 65–120%.
The mean suspension growth was between 8–32 on Day 2 following 3 hour treatments.
Obvious outliers were excluded. However, there were at least 2 vehicle control cultures remaining.

Acceptance criteria for positive controls:

Positive controls showed an absolute increase in mean total MF above the mean concurrent vehicle control MF of at least 300 x 10^{-6}. At least 40% of this was due to the number of small mutant colonies.

Mean RTG's for the positive controls were greater than 10%.

There was an absence of confounding technical problems such as contamination, excessive numbers of outliers and excessive toxicity.

There was not excessive heterogeneity between replicate cultures.

Assays that did not fulfil the required criteria were rejected and therefore are not reported. This decision was at the discretion of the Study Director.

Analysis of data

The data for acceptable tests were analysed as follows

Growth in suspension (SG) was calculated as:

$$SG = \frac{Cell\ count\ 24\ hours\ post\ treatment}{2x10^5\ *} x \frac{Cell\ count\ 48\ hours\ post\ treatment}{2x10^5\ *}$$

For a 24 hour exposure:

$$SG = \frac{Post\ treatment\ cell\ count}{Pre\ treatment\ cell\ count} x \frac{Cell\ count\ 24\ hours\ post\ treatment}{2x10^5\ *} x \frac{Cell\ count\ 48\ hours\ post\ treatment}{2x10^5\ *}$$

*or previous day's cell count if not 2 x 10^5 cells/mL

Relative suspension growth (RSG) was calculated as:

$$\frac{Individual\ SG\ x\ 100}{Mean\ solvent\ control\ SG}$$

Cloning efficiency (CE) was calculated as:

$$P(0) = \frac{Number\ of\ empty\ wells}{Total\ number\ of\ wells}$$

$$CE = \frac{-\ lnP(0)}{Number\ of\ cells\ per\ well}$$

Relative cloning efficiency (RCE) was calculated as:

$$\frac{Individual\ CE\ x\ 100}{Mean\ solvent\ control\ CE}$$

Relative total growth (RTG) for each culture was calculated as:

$$\frac{RSG\ x\ Day_2\ RCE}{100}$$

The mutant frequency per 10^6 survivors (MF) was calculated as:

$$MF = \frac{CE\ selective\ medium}{CE\ non\ selective\ medium}$$

The data were analysed using Fluctuation application SAFEStat (SAS statistical applications for end users) version 1.1, which follows the methods described by Robinson *et al.,* (1989).

Criteria for assessing mutagenic potential

The following criteria were applied for assessment of individual assay results using data for MF where the RTG normally exceeded 10%:

Definitions:

GEF = Global Evaluation Factor. For microwell assays this is 126×10^{-6}.

IMF = Induced mutation frequency (test concentration MF minus mean control MF).

The assay was considered valid in accordance with the assay acceptance criteria.

The test agent was regarded as negative if:

The IMF for any test concentration was less than the GEF.

If the IMF of any test concentration exceeded the GEF, a linear trend test was applied:

If the linear trend test was negative, the result was regarded as negative.

If the linear trend test was positive, this indicated a positive, biologically relevant response.

Where appropriate, other factors were considered in the interpretation of the results, for example, the reproducibility within and between tests, the overall number of mutant colonies (as opposed to mutation frequency) and the nature of any concentration-related effect(s).

Results that only partially satisfied the assessment criteria described above were considered on a case-by-case basis. In cases where the results were inconclusive, further testing and/or a test modification may have been required to better define the assay response.

DEVIATIONS FROM THE PROTOCOL

There were no deviations from the protocol.

MAINTENANCE OF RECORDS

All raw data, samples and specimens (if appropriate) arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

RESULTS

Preliminary toxicity test (Table 1)

In the preliminary toxicity test, a 3 hour exposure to ABM-FD at concentrations from 9.77 to 5000 µg/mL in the absence and presence of S9 mix resulted in RSG values from 123 to 88% and from 126 to 98% respectively. A continuous exposure for 24 hours to ABM-FD at concentrations of 9.77 to 5000 µg/mL in the absence of S9 mix resulted in RSG values from 114 to 8%. Concentrations used in the main tests were based upon these data.

Test 1 in the absence of S9 mix (Tables 2, 3, 4 and 5)

A 3 hour exposure to ABM-FD at concentrations from 156.25 to 5000 µg/mL resulted in RSG values of 111 to 89%. Cultures exposed to ABM-FD at concentrations from 156.25 to 5000 µg/mL were assessed for determination of mutation frequency. RTG values from 136 to 105%, and cloning efficiencies from 134 to 110%, were obtained relative to the solvent control. There were no increases in induced mutation frequency that exceeded the Global Evaluation Factor following exposure to ABM-FD at concentrations within acceptable levels of toxicity. The positive control, methyl methanesulphonate, induced an acceptable increase in mutation frequency and an acceptable increase in the number of small colony mutants.

Test 1 in the presence of S9 mix (Tables 6, 7, 8 and 9)

A 3 hour exposure to ABM-FD at concentrations of 156.25 to 5000 µg/mL resulted in RSG values from 109 to 92%. Cultures exposed to ABM-FD at concentrations of 156.25 to 5000 µg/mL were assessed for determination of mutation frequency. RTG values of 97 to 80%, and cloning efficiencies of 98 to 82%, were obtained, relative to the solvent control. There were no increases in induced mutation frequency that exceeded the Global Evaluation Factor following exposure to ABM-FD at concentrations within acceptable levels of toxicity. The positive control, 3-methylcholanthrene, induced an acceptable increase in mutation frequency and an acceptable increase in the number of small colony mutants.

Test 2 in the absence of S9 mix (Tables 10, 11, 12 and 13)

A 24 hour exposure to ABM-FD at concentrations of 500 to 5000 µg/mL resulted in RSG values from 88 to 7%. Cultures exposed to ABM-FD at concentrations from 500 to 4000 µg/mL were assessed for determination of mutation frequency. RTG values from 111 to 20%, and cloning efficiencies from 126 to 99%, were obtained relative to the solvent control. Data are presented for concentrations tested which span the toxicity range of approximately 20-100% RTG. Exposure to ABM-FD at a concentration of 4000 µg/mL was associated with an increase in induced mutation frequency that exceeded the Global Evaluation Factor. This increase represented a 3.8-fold increase

in mutation frequency compared to the mutation frequency for the concurrent solvent control. There was evidence of a concentration-response relationship, which gave a significant positive linear trend ($P \leq 0.01$) and an increase in the number of small colony mutants. The positive control, methyl methanesulphonate, induced an acceptable increase in mutation frequency and an acceptable increase in the number of small colony mutants.

The historical solvent control data are presented in Table 14.

CONCLUSION

ABM-FD (Extract of Himematsutake) demonstrated mutagenic potential, in the absence of S9 mix when exposed for 24 hours, in this *in vitro* cell mutation assay, under the experimental conditions described. Increased mutant frequency was obtained only after 24 hours continuous exposure to the test substance, and it was noted that the mutant colonies obtained were almost exclusively small. Small mutant colonies may indicate the loss of genes concerned with cell growth resulting from large deletions, clastogenicity or the loss of a chromosome (aneuploidy).

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Table 1: Preliminary toxicity test - Relative Suspension Growth

i) In the absence of S9 mix - 3 hour exposure

Concentration of ABM-FD (µg/mL)	Day_1 Cell Growth	Day_2 Cell Growth	Suspension Growth (SG)	Relative Suspension Growth (RSG)
0	4.25	4.98	21.16	100
0	3.65	4.60	16.82	
9.77	4.04	5.04	20.35	107
19.53	3.03	5.61	17.00	89
39.06	3.29	5.23	17.21	91
78.13	3.32	5.49	18.22	96
156.25	3.35	4.97	16.63	88
312.5	4.44	5.26	23.34	123
625	3.63	5.12	18.60	98
1250	3.35	5.05	16.90	89
2500	3.85	5.03	19.38	102
5000	3.73	4.61	17.19	91

ii) In the presence of S9 mix - 3 hour exposure

Concentration of ABM-FD (µg/mL)	Day_1 Cell Growth	Day_2 Cell Growth	Suspension Growth (SG)	Relative Suspension Growth (RSG)
0	3.67	4.44	16.27	100
0	4.16	4.52	18.80	
9.77	3.94	4.46	17.58	100
19.53	4.70	4.40	20.68	118
39.06	4.25	4.61	19.60	112
78.13	4.22	4.44	18.73	107
156.25	4.45	4.38	19.50	111
312.5	3.75	4.60	17.25	98
625	4.13	4.50	18.56	106
1250	4.41	4.44	19.59	112
2500	4.65	4.76	22.13	126
5000	3.86	4.66	17.96	102

Table 1: Preliminary toxicity test - Relative Suspension Growth (continued)

iii) In the absence of S9 mix - 24 hour exposure

Concentration of ABM-FD (µg/mL)	Treatment Growth	Day_1 Cell Growth	Day_2 Cell Growth	Suspension Growth (SG)	Relative Suspension Growth (RSG)
0	3.11	5.83	3.08	55.86	100
0	2.97	6.00	3.71	66.20	
9.77	3.34	4.79	3.50	55.85	92
19.53	3.34	4.48	3.12	46.64	76
39.06	2.30	6.21	3.78	53.93	88
78.13	3.16	5.22	4.23	69.63	114
156.25	3.20	4.84	3.97	61.59	101
312.5	3.00	4.56	4.52	61.89	101
625	3.02	6.22	3.64	68.40	112
1250	2.54	4.36	4.25	46.95	77
2500	1.72	3.43	3.93	23.15	38
5000	1.03	1.71	2.61	4.59	8

Table 2: Test 1 in the absence of S9 mix, Relative Suspension Growth 3 hour exposure

Concentration of ABM-FD (µg/mL)	Day_1 Cell Growth	Day_2 Cell Growth	Suspension Growth (SG)	Relative Suspension Growth (RSG)	Mean Relative Suspension Growth (% RSG)
0	2.50	6.24	15.61	100	100
0	2.62	6.18	16.19		
0	2.59	5.99	15.52		
0	2.18	6.06	13.23		
156.25	2.14	6.50	13.93	92	92
156.25	2.18	6.42	13.98	92	
312.5	2.78	5.84	16.20	107	102
312.5	2.23	6.54	14.56	96	
625	2.76	6.29	17.40	115	111
625	2.57	6.27	16.13	107	
1250	2.75	6.44	17.71	117	109
1250	2.45	6.24	15.30	101	
2500	2.54	6.12	15.57	103	103
2500	2.40	6.45	15.47	102	
5000	2.47	5.54	13.71	91	89
5000	2.35	5.69	13.36	88	
MMS - positive control					
10	2.23	5.33	11.88	78	77
10	2.06	5.60	11.53	76	

Table 3: Test 1 in the absence of S9 mix, Day_2 Cloning Efficiency
3 hour exposure

Concentration of ABM-FD (µg/mL)	Empty Wells	Total Wells	P(0)	Cloning Efficiency	Relative % Cloning Efficiency	Mean Relative % CE	Relative Total Growth (%)	Mean Relative Total Growth (%)
0	75	192	0.391	0.59	100	100	100	100
0	65	192	0.339	0.68				
0	77	192	0.401	0.57				
0	59	192	0.307	0.74				
156.25	49	192	0.255	0.85	133	125	123	115
156.25	58	192	0.302	0.75	117		108	
312.5	46	192	0.240	0.89	140	134	149	136
312.5	52	192	0.271	0.82	128		123	
625	51	192	0.266	0.83	130	118	149	132
625	64	192	0.333	0.69	107		114	
1250	56	192	0.292	0.77	120	110	141	121
1250	69	192	0.359	0.64	100		101	
2500	50	192	0.260	0.84	131	126	135	129
2500	56	192	0.292	0.77	120		123	
5000	67	192	0.349	0.66	103	118	93	105
5000	49	192	0.255	0.85	133		118	
MMS - positive control								
10	77	192	0.401	0.57	89	92	70	71
10	73	192	0.380	0.60	94		72	

Table 4: Test 1 in the absence of S9 mix, Mutant Frequency 3 hour exposure

Concentration of ABM-FD (µg/mL)	Empty Wells	Total Wells	P(0)	Cloning Efficiency	Mutant Frequency	Mean Mutant Frequency
0	161	192	0.839	0.000088	0.000150	0.000169
0	149	192	0.776	0.000127	0.000187	
0	154	192	0.802	0.000110	0.000193	
0	155	192	0.807	0.000107	0.000145	
156.25	156	192	0.813	0.000104	0.000122	0.000132
156.25	155	192	0.807	0.000107	0.000143	
312.5	161	192	0.839	0.000088	0.000099	0.000101
312.5	162	192	0.844	0.000085	0.000104	
625	154	192	0.802	0.000110	0.000133	0.000140
625	157	192	0.818	0.000101	0.000147	
1250	152	192	0.792	0.000117	0.000152	0.000155
1250	157	192	0.818	0.000101	0.000157	
2500	159	192	0.828	0.000094	0.000112	0.000131
2500	152	192	0.792	0.000117	0.000152	
5000	145	192	0.755	0.000140	0.000213	0.000202
5000	139	192	0.724	0.000162	0.000189	
MMS - positive control						
10	46	192	0.240	0.000714	0.001251	0.001080
10	62	192	0.323	0.000565	0.000935	

Table 5: Test 1 in the absence of S9 mix, Colony Size Analysis
3 hour exposure

Concentration of ABM-FD (µg/mL)	Empty Wells	Total Wells	Total Mutant Colonies	Large Mutant Colonies	Small Mutant Colonies	% Small Colony Mutants	Mean % Small Colony Mutants
0	161	192	33	10	23	70	71
0	149	192	43	7	36	84	
0	154	192	39	13	26	67	
0	155	192	38	14	24	63	
MMS - positive control							
10	46	192	212	37	175	83	84
10	62	192	153	21	132	86	

Table 6: Test 1 in the presence of S9 mix, Relative Suspension Growth 3 hour exposure

Concentration of ABM-FD (µg/mL)	Day_1 Cell Growth	Day_2 Cell Growth	Suspension Growth (SG)	Relative Suspension Growth (RSG)	Mean Relative Suspension Growth (% RSG)
0	3.07	5.67	17.40	100	100
0	3.50	5.48	19.16		
0	3.06	5.54	16.97		
0	3.43	4.58	15.70		
156.25	2.92	5.85	17.07	99	102
156.25	3.27	5.54	18.09	104	
312.5	2.62	5.72	14.98	87	92
312.5	2.97	5.70	16.93	98	
625	3.32	5.89	19.56	113	109
625	3.19	5.74	18.29	106	
1250	3.14	5.66	17.74	103	99
1250	2.73	6.01	16.42	95	
2500	2.85	5.39	15.39	89	93
2500	2.95	5.71	16.82	97	
5000	3.16	5.40	17.03	98	104
5000	3.35	5.69	19.08	110	
3MC - positive control					
2.5	2.09	4.47	9.33	54	55
2.5	2.16	4.55	9.83	57	

Table 7: Test 1 in the presence of S9 mix, Day$_2$ Cloning Efficiency
3 hour exposure

Concentration of ABM-FD (µg/mL)	Empty Wells	Total Wells	P(0)	Cloning Efficiency	Relative % Cloning Efficiency	Mean Relative % CE	Relative Total Growth (%)	Mean Relative Total Growth (%)
0	32	192	0.167	1.12	100	100	100	100
0	57	192	0.297	0.76				
0	37	192	0.193	1.03				
0	31	192	0.161	1.14				
156.25	42	192	0.219	0.95	96	89	94	90
156.25	52	192	0.271	0.82	82		86	
312.5	46	192	0.240	0.89	90	98	78	91
312.5	36	192	0.188	1.05	105		103	
625	52	192	0.271	0.82	82	83	93	91
625	51	192	0.266	0.83	84		88	
1250	48	192	0.250	0.87	87	82	90	81
1250	57	192	0.297	0.76	76		73	
2500	56	192	0.292	0.77	78	85	69	80
2500	44	192	0.229	0.92	93		90	
5000	47	192	0.245	0.88	89	93	87	97
5000	41	192	0.214	0.96	97		107	
3MC - positive control								
2.5	67	192	0.349	0.66	66	63	36	35
2.5	75	192	0.391	0.59	59		34	

Table 8: Test 1 in the presence of S9 mix, Mutant Frequency 3 hour exposure

Concentration of ABM-FD (µg/mL)	Empty Wells	Total Wells	P(0)	Cloning Efficiency	Mutant Frequency	Mean Mutant Frequency
0	159	192	0.828	0.000094	0.000084	0.000117
0	156	192	0.813	0.000104	0.000137	
0	146	192	0.760	0.000137	0.000133	
0	148	192	0.771	0.000130	0.000114	
156.25	149	192	0.776	0.000127	0.000133	0.000131
156.25	156	192	0.813	0.000104	0.000127	
312.5	142	192	0.740	0.000151	0.000169	0.000128
312.5	158	192	0.823	0.000097	0.000093	
625	151	192	0.786	0.000120	0.000147	0.000111
625	169	192	0.880	0.000064	0.000077	
1250	159	192	0.828	0.000094	0.000109	0.000111
1250	162	192	0.844	0.000085	0.000112	
2500	145	192	0.755	0.000140	0.000182	0.000159
2500	149	192	0.776	0.000127	0.000138	
5000	144	192	0.750	0.000144	0.000164	0.000158
5000	143	192	0.745	0.000147	0.000153	
3MC - positive control						
2.5	34	192	0.177	0.000866	0.001315	0.001454
2.5	29	192	0.151	0.000945	0.001609	

Table 9: Test 1 in the presence of S9 mix, Colony Size Analysis
3 hour exposure

Concentration of ABM-FD (µg/mL)	Empty Wells	Total Wells	Total Mutant Colonies	Large Mutant Colonies	Small Mutant Colonies	% Small Colony Mutants	Mean % Small Colony Mutants
0	159	192	34	13	21	62	59
0	156	192	36	20	16	44	
0	146	192	48	20	28	58	
0	148	192	44	13	31	70	
3MC - positive control							
2.5	34	192	256	44	212	83	82
2.5	29	192	245	47	198	81	

Table 10: Test 2 in the absence of S9 mix, Relative Suspension Growth 24 hour exposure

Concentration of ABM-FD (µg/mL)	Treatment Growth	Day_1 Cell Growth	Day_2 Cell Growth	Suspension Growth (SG)	Relative Suspension Growth (RSG)	Mean Relative Suspension Growth (% RSG)
0	3.40	5.49	4.15	77.47	100	100
0	3.56	5.35	4.27	81.36		
0	3.44	4.33	4.54	67.55		
0	3.52	5.27	4.56	84.68		
500	3.10	4.91	4.48	68.22	88	88
500	2.93	5.19	4.52	68.66	88	
1000	2.91	5.47	4.38	69.78	90	86
1000	2.60	5.03	4.88	63.63	82	
2000	2.19	4.51	4.91	48.61	63	60
2000	1.74	5.23	4.93	44.86	58	
3000	1.68	3.74	4.61	28.96	37	34
3000	1.51	3.78	4.30	24.62	32	
4000	1.16	2.65	4.33	13.37	17	18
4000	1.33	2.50	4.60	15.28	20	
5000	0.93	2.13	3.44	6.83	9	7
5000	0.95	1.29	3.46	4.24	5	
MMS - positive control						
5	2.41	3.88	5.03	46.90	60	60
5	2.73	4.16	4.13	46.87	60	

Table 11: Test 2 in the absence of S9 mix, Day_2 Cloning Efficiency 24 hour exposure

Concentration of ABM-FD (µg/mL)	Empty Wells	Total Wells	P(0)	Cloning Efficiency	Relative % Cloning Efficiency	Mean Relative % CE	Relative Total Growth (%)	Mean Relative Total Growth (%)
0	48	192	0.250	0.87	100	100	100	100
0	55	192	0.286	0.78				
0	60	192	0.313	0.73				
0	51	192	0.266	0.83				
500	45	192	0.234	0.91	114	126	100	111
500	33	192	0.172	1.10	138		122	
1000	58	192	0.302	0.75	94	111	84	95
1000	37	192	0.193	1.03	129		105	
2000	47	192	0.245	0.88	110	99	69	60
2000	62	192	0.323	0.71	88		51	
3000	56	192	0.292	0.77	96	111	36	38
3000	39	192	0.203	1.00	125		39	
4000	53	192	0.276	0.80	101	106	17	20
4000	46	192	0.240	0.89	112		22	
MMS - positive control								
5	75	192	0.391	0.59	74	76	44	46
5	70	192	0.365	0.63	79		48	

Table 12: Test 2 in the absence of S9 mix, Mutant Frequency 24 hour exposure

Concentration of ABM-FD (µg/mL)	Empty Wells	Total Wells	P(0)	Cloning Efficiency	Mutant Frequency	Mean Mutant Frequency
0	167	192	0.870	0.000070	0.000081	0.000102
0	158	192	0.823	0.000097	0.000125	
0	162	192	0.844	0.000085	0.000117	
0	166	192	0.865	0.000073	0.000088	
500	159	192	0.828	0.000094	0.000104	0.000082
500	167	192	0.870	0.000070	0.000063	
1000	158	192	0.823	0.000097	0.000130	0.000132
1000	147	192	0.766	0.000134	0.000130	
2000	136	192	0.708	0.000172	0.000196	0.000212
2000	139	192	0.724	0.000162	0.000229	
3000	132	192	0.688	0.000187	0.000243	0.000219
3000	130	192	0.677	0.000195	0.000196	
4000	105	192	0.547	0.000302	0.000375	0.000388
4000	94	192	0.490	0.000357	0.000400	
MMS - positive control						
5	36	192	0.188	0.000837	0.001425	0.001422
5	32	192	0.167	0.000896	0.001421	

Table 13: Test 2 in the absence of S9 mix, Colony Size Analysis 24 hour exposure

Concentration of ABM-FD (µg/mL)	Empty Wells	Total Wells	Total Mutant Colonies	Large Mutant Colonies	Small Mutant Colonies	% Small Colony Mutants	Mean % Small Colony Mutants
0	167	192	27	11	16	59	60
0	158	192	34	17	17	50	
0	162	192	33	12	21	64	
0	166	192	28	9	19	68	
4000	105	192	149	14	135	91	84
4000	94	192	128	29	99	77	
MMS - positive control							
5	36	192	214	33	181	85	84
5	32	192	209	33	176	84	

Table 14: Historical control data (relative total growth – flat bottom wells)

i) in the absence of S9 mix - 3 hour exposure

		Mean TG*	Mean Suspension Growth	Mean Day_2 CE	Mean Mutant Frequency
Solvent Controls	mean	12.07	14.78	81.82	0.000120
	maximum	17.46	21.51	116.02	0.000169
	sd	2.60	2.83	9.83	0.000023
Positive Controls	mean	55.73	11.60	57.02	0.001075
MMS 10 µg/ml	minimum	42.81	7.90	43.65	0.000467
	sd	8.52	2.10	7.54	0.000272

95% confidence limit for solvent controls 0.000157
sd = standard deviation
Number of tests 40
Data collection period 24-Mar-04 20-Dec-04
*- For positive control values Relative Total Growth

ii) in the presence of S9 mix - 3 hour exposure

		Mean TG*	Mean Suspension Growth	Mean Day_2 CE	Mean Mutant Frequency
Solvent Controls	mean	12.82	14.86	86.43	0.000126
	maximum	18.99	21.51	102.49	0.000190
	sd	2.42	2.48	8.52	0.000024
Positive Controls	mean	38.04	7.39	65.31	0.001001
3MC 2.5 µg/ml	minimum	24.48	4.28	49.91	0.000454
	sd	8.19	1.65	7.78	0.000238

95% confidence limit for solvent controls 0.000170
sd = standard deviation
Number of tests 40
Data collection period 24-Mar-04 04-Jan-05
*- For positive control values Relative Total Growth

iii) in the absence of S9 mix - 24 hour exposure

		Mean TG*	Mean Suspension Growth	Mean Day_2 CE	Mean Mutant Frequency
Solvent Controls	mean	37.50	44.84	83.54	0.000131
	maximum	47.89	59.10	99.22	0.000198
	sd	7.00	7.12	7.45	0.000030
Positive Controls	mean	40.52	28.65	52.02	0.001610
MMS 5 µg/ml	minimum	22.71	21.37	40.46	0.001071
	sd	10.96	6.13	8.17	0.000393

95% confidence limit for solvent controls 0.000164
sd = standard deviation
Number of tests 22
Data collection period 24-Mar-04 24-Jan-05
*- For positive control values Relative Total Growth

APPENDIX 1: GLP Compliance Statements

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Eye Research Centre** **Occold** **Eye** **Suffolk** **IP23 7PX**	**Analytical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Toxicology** **Phys/Chem Tests**

DATE OF INSPECTION
22nd April 2003

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

25/7/03

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Huntingdon Research Centre** **Woolley Road** **Alconbury** **Huntingdon** **Cambs.** **PE28 4HS**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Toxicology** **Phys/Chem Tests**

DATE OF INSPECTION

07th April 2003

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander
1/8/03

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

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

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Eye Research Centre** **Occold** **Eye** **Suffolk** **IP23 7PX**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Toxicology** **Phys/Chem Testing**

DATE OF INSPECTION

12th April 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 9/6/05

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

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
Huntingdon Life Sciences **Huntingdon Research Centre** **Woolley Road** **Alconbury** **Cambridgeshire** **PE28 4HS**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Toxicology** **Phys/Chem Testing**

DATE OF INSPECTION

7th March 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
9/6/05

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

APPENDIX 2: MolTox™ S9 Quality Control Certificate

MOLTOX™ POST MITOCHONDRIAL SUPERNATANT (S-9) PRODUCTION & QUALITY CONTROL CERTIFICATE

LOT NO.: 1759
PART NO.: 11-111
VOLUME: 1 ml & 2 ml

SPECIES: Rat
STRAIN: Sprague Dawley
SEX: Male
TISSUE: Liver

PREPARATION DATE: September 9, 2004
EXPIRATION DATE: September 9, 2006
BUFFER: 0.25 M Sucrose
INDUCING AGENT(s): Aroclor 1254 Monsanto Lot No. KL615 - 500mg/kg

REFERENCE: Maron, D & Ames, B, *Mutat. Res.* 113:173, 1983

BIOCHEMISTRY:

- PROTEIN

36.7 mg/ml

Assayed according to the method of Lowry et al., JBC 193:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	121.5
PROD	2B1, 3B2	24.1
BROD	3A, 2B	47.3
MROD	1A2	58.4

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-and benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, MROD) were conducted using a modification of the methods of Burke et al., *Biochem Pharm* 34:3337, 1985. Fold-inductions calculated as the ratio of the sample vs. uninduced control specific activities (SA). Control SA's (pmoles/min/mg protein) were 41.3, 18.3, 40.0, & 14.3 for EROD, PROD, BROD, & MROD respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 24 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
EtBr/ TA98	CPA/ TA1535
197.6	1326

The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* 129:299, 1984. Data were expressed as revertants per μg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames (*Mutat. Res.*113:173, 1983).

	μl S9 per plate/number his$^+$ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5.0 μg)	97	265	409	669	809	924
2-AA (2.5 μg)	106	764	1697	2281	2171	1905

MOLECULAR TOXICOLOGY, INC.
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APPENDIX 3: Formulation Chemistry

ABM-FD (EXTRACT OF HIMEMATSUTAKE)

FORMULATION CHEMISTRY

Authors:

Paul Mann,
David Fleet.

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INTRODUCTION

This report describes the analytical and sampling procedures used, and details the results obtained for the determination of concentrations of ABM-FD (Extract of Himematsutake) in test formulations analysed during the study.

The formulations for this study were prepared as solutions of ABM-FD (Extract of Himematsutake) (batch EX200408) in water by Genetic Toxicology personnel at Huntingdon Life Sciences Ltd.

The analytical work was undertaken by Formulation Chemistry and Central Laboratory Services (CLS) personnel at the Huntingdon Research Centre, Huntingdon Life Sciences between 10 January 2005 and 03 February 2005.

EXPERIMENTAL PROCEDURE

ANALYTICAL PROCEDURE

The analytical method[Ref.2] was based on methodology supplied by the Sponsor[Ref.1]. The method involved enzyme and acid hydrolysis of the test sample and determination of the resulting glucose content by CLS personnel at the Huntingdon Research Centre.

The analytical procedure was validated for ABM-FD (Extract of Himematsutake) in water formulations as part of an earlier study with respect to the specificity of the analysis, and method accuracy and precision at nominal concentrations of 50 mg/mL and 200 mg/mL. The experimental details and the analytical results obtained were presented in Huntingdon Life Sciences report IKI 001/052114.

CONCENTRATION IN TEST FORMULATIONS

Freshly prepared test formulations were sampled (25 ml) by Genetic Toxicology personnel at the Eye Research Centre and the samples submitted for analysis. On receipt, each treated sample was sub-sampled in duplicate (2 × 5 ml) and analysed in accordance with the analytical procedure.

The concentration of ABM-FD (Extract of Himematsutake) in aqueous formulations, analysed with respect to the glucose content, was conducted as part of an earlier study and confirmed at nominal concentrations of 50 mg/mL and 200 mg/mL during ambient temperature storage for 1 day and refrigerated storage for 8 days. The experimental details and analytical results obtained were presented in Huntingdon Life Sciences report IKI 001/052114.

Shared reagents were used in the analysis of dose formulations of this study and of Huntingdon Life Sciences study no. IKI/002.

REFERENCES

1. ß-glucan analytical method, client e-mail of 25 June 2004.

2. Fleet D., Johnson, S.F., Mann, P., (2004). Analysis of ABM-FD (Extract of Himematsutake) in aqueous formulations. Huntingdon Life Sciences Analytical Method HRC/FCH/M80/04 Issue 01/011104.

RESULTS AND DISCUSSION

The mean concentrations of ABM-FD (Extract of Himematsutake) in test formulations, analysed with respect to the glucose content, and the deviation of mean results from nominal values are summarised in Table 1. The mean analysed concentrations were between 9.8% below and 10.2% above the nominal values. The precision of duplicate analyses was <10%.

CONCLUSION

The mean concentration of ABM-FD (Extract of Himematsutake) in the test formulation, analysed with respect to the glucose content, was within ±10.5% of the nominal concentration.

TABLE

1. CONCENTRATIONS OF ABM-FD (EXTRACT OF HIMEMATSUTAKE) IN TEST FORMULATIONS

Date of formulation	Nominal inclusion (mg/ml)	Analysed concentration (mg/ml)			RME (%)
		Analysis 1	Analysis 2	Mean	
10-Jan-05	0	ND	-	ND	-
	50	49.2	41.0	45.1	-9.8
24-Jan-05	0	ND	-	ND	-
	50	50.4	59.9	55.1	+10.2

RME Relative mean error, representing the deviation from nominal
ND None detected