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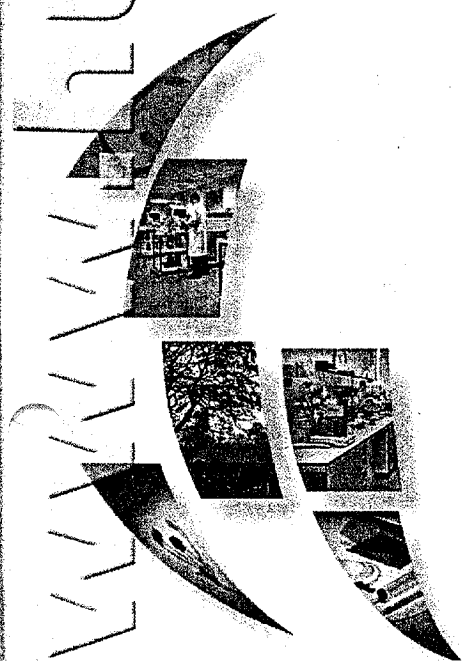

# ABM-FD (EXTRACT OF HIMEMATSUTAKE)

# RAT MICRONUCLEUS TEST

Report

IKI 004/052643

# ABM-FD (EXTRACT OF HIMEMATSUTAKE)

# RAT MICRONUCLEUS TEST

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## COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

## ABM-FD (EXTRACT OF HIMEMATSUTAKE)

## RAT MICRONUCLEUS TEST

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 on the basis of intergovernmental agreements.

..... ...16 November 2005.....

Lincoln Pritchard, B.Sc., Date
Study Director,
Department of Genetic Toxicology

# 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** | 22 April 2005 | 22 April 2005 |
| **Report Audit** | 24 – 30 June 2005 | 30 June 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 Procedures | 11 May 2005 | 11 May 2005 |
| Slide Scoring | 2 March 2005 | 2 March 2005 |
| Dosing and post dosing observations | 15 March 2005 | 15 March 2005 |
| Bone marrow extraction and slide preparation | 10 June 2005 | 13 June 2005 |
| Bodyweights | 6 April 2005 | 6 April 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
Neal Jolly, B.Sc., M.R.Q.A.,
Group Manager,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

15 NOVEMBER 2005
Date

## RESPONSIBLE PERSONNEL

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## SUMMARY

This study was designed to assess the potential induction of micronuclei by ABM-FD (Extract of Himematsutake) in bone marrow cells of Sprague-Dawley, (CD) rats. Rats were treated with ABM-FD (Extract of Himematsutake) on two consecutive occasions approximately 24 hours apart.

Data from a previous Huntingdon Life Sciences study, Study number IKI/001, indicated that a dose of 2000 mg/kg/day (the standard limit dose for the micronucleus test) was tolerated and no substantial difference in toxicity were observed between the sexes. Therefore no preliminary toxicity test was performed and in line with current guidelines male animals only were used in the main micronucleus test.

Dose levels of 500, 1000 and 2000 mg/kg/day were selected for the micronucleus test.

All animals were dosed orally by gavage. The negative control group received the vehicle, water (purified in-house by reverse osmosis) and the positive control group received Cyclophosphamide at 20 mg/kg bodyweight.

Bone marrow smears were obtained from seven male animals in the negative control and in each of the test substance groups 24 hours after administration of the second dose. In addition, bone marrow smears were also obtained from five male animals in the positive control group 24 hours after a single dose. One smear from each animal was examined for the presence of micronuclei in 2000 immature erythrocytes. The proportion of immature erythrocytes was assessed by examination of at least 1000 erythrocytes from each animal. A record of the incidence of micronucleated mature erythrocytes was also kept.

The mean concentration of ABM-FD (Extract of Himematsutake) in test formulations, analysed with respect to the glucose content, were within ±10% of the nominal concentration.

No statistically significant increases in the frequency of micronucleated immature erythrocytes and no substantial decreases in the proportion of immature erythrocytes were observed in rats treated with ABM-FD (Extract of Himematsutake) at any treatment level, compared to vehicle control values.

The positive control compound, Cyclophosphamide, produced significant increases in the frequency of micronucleated immature erythrocytes ($P<0.01$).

It is concluded that ABM-FD (Extract of Himematsutake) did not show any evidence of causing an increase in the induction of micronucleated immature erythrocytes or bone marrow cell toxicity in CD rats, when administered orally by gavage in this *in vivo* test procedure.

## INTRODUCTION

The purpose of this study was to assess the potential of ABM-FD (Extract of Himematsutake) to induce an increase in micronucleated erythrocytes in rats following acute oral administration using an *in vivo* cytogenetic system (Boller and Schmid 1970, MacGregor *et al* 1987, Mavournin *et al* 1990). The oral route was selected for use in this test as the most likely route of human exposure.

The performance of this study was in compliance with the following Guidelines:

OECD Guideline for the Testing of Chemicals. (1997) Genetic Toxicology: Mammalian Erythrocyte Micronucleus Test, Guideline 474.

EC Commission Directive 2000/32/EC Annex 4C – B.12. Mutagenicity – *In vivo* Mammalian Erythrocyte Micronucleus Test. No. L 136/50.

US EPA (1998) Health Effects Test Guidelines; OPPTS 870.5395 Mammalian Erythrocyte Micronucleus Test. EPA 712-C-98-226.

Japanese Ministry of Agriculture, Forestry and Fisheries. Test Data for Registration of Agricultural Chemicals, 12 Nohsan No. 8147, Agricultural Production Bureau, November 24, 2000.

JMHW Genotoxicity Testing Guideline, PAB Notification No. 1604 (1 November 1999).

Joint Directive of J MHLW/METI and ME (21 November 2001)

IYAKUHATSU No. 1271
SEIKYOKU No. 1 (1 November 2001)
KANPOKI No. 946

ICH (1996) Guideline S2A: 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 bone marrow micronucleus test is a short-term assay for identification of genotoxic effects associated with mutagens and carcinogens (Mavournin *et al* 1990). Young adult rats are chosen for use because of the high rate of cell division in the bone marrow, because of the wealth of background data on this species, and their general suitability for toxicological investigations.

In mitotic cells in which chromosomal breakage has been caused by the test substance or its metabolites, acentric fragments of the chromosomes do not separate at the anaphase stage of cell division. After telophase these fragments may not be included in the nuclei of the

daughter cells and hence will form single or multiple micronuclei in the cytoplasm of these cells.

A few hours after the last mitosis is completed, erythroblasts expel their nucleus. Young erythrocytes less than 24 hours old are termed immature erythrocytes (polychromatic erythrocytes). More mature erythrocytes are termed mature (normochromatic erythrocytes).

Substances which interfere with the mitotic spindle apparatus will cause non-disjunction or lagging chromosomes at anaphase which may not be incorporated into the daughter nuclei. These lagging chromosomes are not excluded from the erythroblast with the main nucleus and hence also give rise to micronuclei.

Any toxic effects of the test substance on the nucleated cells may lead either to a reduction in cell division or to cell death. These effects in turn lead to a reduction in the number of nucleated cells and immature erythrocytes. If the proportion of immature erythrocytes is found to be significantly less than the control value, this is taken as being indicative of toxicity.

The protocol was approved by Huntingdon Life Sciences Management and by the Study Director on 20 April 2005 and by the Sponsor on 26 April 2005.

The study was conducted at the Department of Genetic Toxicology, Huntingdon Life Sciences, Eye, Suffolk, IP23 7PX, England. The statistical analysis and formulation chemistry were performed at Huntingdon Life Sciences Ltd., Huntingdon Research Centre, Huntingdon, Cambridgeshire, PE28 4HS, England.

The experimental start and completion dates of the study were 20 April 2005 and 24 May 2005, respectively.

## 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: | 2.9g/100g as beta-glucan |
| Date received: | 31 August 2004 |

The above information with regard to the physical characterisation of the test substance is the responsibility of the Sponsor.

# EXPERIMENTAL PROCEDURE

## ANIMALS

All animals used on this study were Sprague Dawley (CD) rats. Males weighed between 175 and 200 grams on despatch from Charles River UK Limited, Margate, Kent, England.

On arrival the weight of the animals was checked and found to be acceptable. The animals were randomly assigned to groups and tail marked. Each group was kept in cages and maintained in a controlled environment, with the thermostat and relative humidity target ranges set at 22±3°C and 55±15% respectively. Temperature and humidity were within range throughout the study. The room was illuminated by artificial light for 12 hours per day. All animals were allowed free access to pelleted expanded rat and mouse No.1 maintenance diet (SQC grade obtained from Special Diets Services Ltd, Witham, Essex, UK) and tap water *ad libitum*. Food and tap water are routinely analysed for quality at source. All animals were given small soft white untreated wood (ASPEN) chew blocks for environmental enrichment, were acclimatised for a minimum of 6 days, examined daily and weighed prior to dosing.

## STABILITY AND HOMOGENEITY

The stability of ABM-FD (Extract of Himematsutake) was not determined as part of this study. The stability and homogeneity of ABM-FD (Extract of Himematsutake) in the vehicle were determined as part of Huntingdon Life Sciences Study No. IKI/001. Analysis of the achieved concentration of test formulations was performed as part of this study. Results of this analysis are presented in Appendix 5.

## TEST SUBSTANCE FORMULATION AND ADMINISTRATION

Solutions of ABM-FD (Extract of Himematsutake) were prepared in water (purified in-house by reverse osmosis). All concentrations are expressed in terms of the ABM-FD (Extract of Himematsutake) sample as received.

Cyclophosphamide, obtained from Asta Medica, batch number 1H494A, was used as the positive control compound. A solution was prepared using purified water at a concentration of 2 mg/ml just prior to administration.

All animals in all groups were dosed orally by gavage with the standard volume of 10 ml/kg bodyweight.

## DATES OF DOSING

Micronucleus test: 26 and 27 April 2005

## MICRONUCLEUS TEST

Data from a previous Huntingdon Life Sciences study, Study number IKI/001, indicated that a dose of 2000 mg/kg/day (the standard limit dose for the micronucleus test) was tolerated and no substantial difference in toxicity was observed between the sexes, therefore male animals only were used in the main micronucleus test. Dose levels of 500, 1000 and 2000 mg/kg bodyweight were used for the micronucleus test.

The experimental design is shown below:

| Group | Treatment | Concentration (mg/ml) | Dosage (mg/kg/day) | Number of animals Male |
|---|---|---|---|---|
| 1 | Vehicle | - | - | 7 |
| 2 | ABM-FD (Extract of Himematsutake) | 50 | 500 | 7 |
| 3 | ABM-FD (Extract of Himematsutake) | 100 | 1000 | 7 |
| 4 | ABM-FD (Extract of Himematsutake) | 200 | 2000 | 7 |
| 5 | Cyclophosphamide [a] | 2 | 20 | 5 |

Vehicle: Water (purified in-house by reverse osmosis)
[a] Positive control, dosed once only 24 hours prior to termination

Following dosing, the animals were examined regularly and any mortalities or clinical signs of reaction were recorded. Seven males from the negative control and test substance groups were sacrificed 24 hours after administration of the second dose. In addition five male animals in the positive control group were sacrificed 24 hours after a single dose.

The animals were killed by exposure to rising levels of carbon dioxide and both femurs dissected out from each animal. The femurs were cleared of tissue and the proximal epiphysis removed. The bone marrow of both femurs from each animal was flushed out and pooled in a total volume of 2 ml of filtered foetal calf serum by aspiration. The resulting cell suspensions were centrifuged at 1000 rpm (150 × *g*) for 5 minutes and the supernatant discarded. The final cell pellet was resuspended in a small volume of foetal calf serum to facilitate smearing in the conventional manner on glass microscope slides (Schmid 1976). Four smears were prepared from each animal.

**Fixation and staining of slides**

1. Fixed for a minimum of 10 minutes in methanol and allowed to air-dry.
2. Rinsed in purified water.
3. Stained in acridine orange solution (0.01 mg/ml using purified water) for 3 minutes.
4. Washed in purified water for 5 minutes.
5. Rinsed in cold tap water for 2 minutes.
6. Stored in the dark at *ca* 4°C for a minimum of 1 hour until required.
7. Immediately prior to scoring, slides were wet mounted with 50 mm glass coverslips using purified water.

**Microscopic examination**

Coded slides were examined by fluorescence microscopy and 2000 immature erythrocytes per animal were examined for the presence of micronuclei. One smear was examined per animal, any remaining smears being held temporarily in reserve in case of technical problems with the first smear.

The proportion of immature erythrocytes was assessed by examination of a total of at least 1000 erythrocytes per animal and the number of micronucleated mature erythrocytes was recorded.

## ASSESSMENT OF RESULTS

**Acceptance criteria**

The following criteria was applied for assessment of assay acceptability:

1. Each treated and control group should include at least 5 analysable animals.
2. Vehicle control values for micronucleated polychromatic erythrocytes must be consistent with the laboratory historical negative control data.
3. Positive controls must show clear unequivocal positive responses.
4. The proportion of polychromatic erythrocytes among total erythrocytes in treated groups is not less than 20% of the control value.

**Analysis of data**

The results for each treatment group were compared with the results for the vehicle control group using non-parametric statistics.

For incidences of micronucleated immature erythrocytes, exact one-sided P-values are calculated by permutation (StatXact, CYTEL Software Corporation, NC, USA). Comparison of several dose levels are made with the vehicle control using the Linear by Linear Association test for trend in a step-down fashion if significance is detected (StatXact, CYTEL Software Corporation, NC, USA). For assessment of effects on the proportion of immature erythrocytes, equivalent permutation tests based on rank scores are used, *ie* exact versions of Wilcoxon's sum of ranks test (Wilcoxon 1945) and Jonckheere's test for trend (Jonckheere 1954).

**Criteria for assessing clastogenic/aneugenic potential**

A positive response is normally indicated by a statistically significant increase in the incidence of micronucleated immature erythrocytes for the treatment group compared with the vehicle control group ($P<0.01$); individual and/or group mean values should exceed the laboratory historical control range (Morrison and Ashby 1995).

A negative result is indicated where individual and group mean incidences of micronucleated immature erythrocytes for the group treated with the test substance are not significantly greater than incidences for the vehicle control group and where these values fall within the historical control range.

An equivocal response is obtained when the results do not meet the criteria specified for a positive or negative response.

Bone marrow cell toxicity (or depression) is normally indicated by a substantial and statistically significant decrease in the proportion of immature erythrocytes ($P<0.01$).

## MAINTENANCE OF RECORDS

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

Types of sample and specimen that 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

## MICRONUCLEUS TEST

Animals were treated with ABM-FD (Extract of Himematsutake) at dose levels of 500, 1000 and 2000 mg/kg/day.

Table 1 gives a summary of the results of the micronucleus test and the results of statistical analysis. The results for individual animals are presented in Table 2.

### Clinical signs

The details of clinical signs are shown in Appendix 1. No clinical signs were noted for the vehicle and positive control group animals over the duration of the test.

At 500 mg/kg/day clinical signs of toxicity included underactive and nervous behaviour, flattened posture, fast and irregular respiration, prominent eyes, partially closed eyelids and unsteady gait. At 1000 mg/kg/day clinical signs included piloerection, underactive and nervous behaviour, hunched and flattened posture, fast and irregular respiration, prominent eyes, partially closed eyelids, flattened and unsteady gait.

At 2000 mg/kg/day clinical signs included piloerection, underactive and nervous behaviour, hunched and flattened posture, fast and irregular respiration, prominent eyes, partially closed eyelids and unsteady gait. On Day 3 only, animals treated at 1000 and 2000 mg/kg/day showed occasional extension and stretching of the hind limbs. All animals survived until scheduled termination.

No mortalities or significant incidences of body weight reduction were observed throughout the duration of this test. Animal bodyweight data is presented in Appendix 2.

### Micronucleated immature erythrocyte counts (mie)

The test substance did not cause any statistically significant increases in the number of micronucleated immature erythrocytes. An increased incidence of micronuclei was observed in animal number 221 at the intermediate dose level of 1000 mg/kg/day. Even though both the individual and group values exceed the laboratory historical control range no statistical significance was observed. This individual result was therefore considered artifactual and of no biological relevance.

Cyclophosphamide caused a significant increase in the frequency of micronucleated immature erythrocytes ($P<0.01$).

Appendix 3 summarises the historical vehicle and positive control values for micronucleated polychromatic erythrocytes obtained in previous, unrelated experiments.

**Micronucleated mature erythrocytes (mme)**

The test substance did not cause any substantial increases in the incidence of micronucleated mature erythrocytes.

**Proportion of immature erythrocytes (% ie/(ie + me))**

The test substance failed to cause any significant decreases in the proportion of immature erythrocytes.

Cyclophosphamide did not cause a statistically significant decrease in the proportion of immature erythrocytes.

**Concentrations in test formulations**

The mean concentrations of ABM-FD in test formulations, analysed with respect to the glucose content, and the deviation of mean results from nominal values are summarised in Appendix 5. The mean analysed concentrations were between 9.6 below and 1.5% above the nominal values. Results are summarised in Appendix 5.

## CONCLUSION

It is concluded that ABM-FD (Extract of Himematsutake) did not show evidence of causing an increase in the induction of micronucleated immature erythrocytes or bone marrow cell toxicity in rats, when administered orally by gavage in this *in vivo* test procedure.

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**TABLE 1**

**Summary of results and statistical analysis**

| Sampling time after 2nd dose | Treatment | Dose (mg/kg/day) | Proportion of ie (%) | Incidence mie (mean) |
|---|---|---|---|---|
| 24 Hours | Vehicle | - | 43 | 3.3 |
| | ABM-FD (Extract of Himematsutake) | 500 | 40 | 2.4 |
| | ABM-FD (Extract of Himematsutake) | 1000 | 48 | 5.9 |
| | ABM-FD (Extract of Himematsutake) | 2000 | 43 | 3.6 |
| | Cyclophosphamide [1] | 20 | 36 | 44.8** |

Vehicle Water (purified in-house by reverse osmosis)
ie Immature erythrocytes
mie Number of micronucleated cells observed per 2000 immature erythrocytes examined
[1] Positive control, dosed once only 24 hours prior to termination

Results of statistical analysis using the appropriate nonparametric method of analysis based on permutation (one-sided probabilities):

** $P < 0.01$ (significant)
otherwise $P > 0.01$ (not significant)

† Occasional apparent errors of ± 1% may occur due to rounding of values for presentation in the table

**TABLE 2**

**Results for individual animals - 24 hour sampling time**

| Treatment (mg/kg/day) | Animal number | Proportion of ie % | Incidence mie | ie | me | Incidence mme |
|---|---|---|---|---|---|---|
| Vehicle | M 201 | 51 | 5 | 520 | 505 | 0 |
| | M 202 | 45 | 1 | 512 | 632 | 0 |
| | M 203 | 40 | 4 | 419 | 632 | 0 |
| | M 204 | 50 | 4 | 535 | 529 | 0 |
| | M 205 | 35 | 3 | 418 | 763 | 0 |
| | M 206 | 41 | 4 | 422 | 617 | 0 |
| | M 207 | 39 | 2 | 404 | 642 | 0 |
| ABM-FD (Extract of Himematsutake) (500) | M 211 | 43 | 0 | 525 | 701 | 0 |
| | M 212 | 43 | 2 | 451 | 610 | 0 |
| | M 213 | 40 | 0 | 416 | 628 | 0 |
| | M 214 | 50 | 2 | 529 | 531 | 0 |
| | M 215 | 40 | 6 | 403 | 609 | 0 |
| | M 216 | 28 | 4 | 302 | 764 | 0 |
| | M 217 | 37 | 3 | 367 | 634 | 0 |
| ABM-FD (Extract of Himematsutake) (1000) | M 221 | 52 | 16 | 606 | 554 | 0 |
| | M 222 | 40 | 7 | 410 | 604 | 0 |
| | M 223 | 43 | 5 | 435 | 587 | 0 |
| | M 224 | 43 | 0 | 445 | 601 | 0 |
| | M 225 | 50 | 7 | 555 | 551 | 0 |
| | M 226 | 54 | 2 | 571 | 485 | 0 |
| | M 227 | 52 | 4 | 548 | 500 | 0 |
| ABM-FD (Extract of Himematsutake) (2000) | M 231 | 37 | 4 | 380 | 642 | 0 |
| | M 232 | 45 | 8 | 451 | 556 | 0 |
| | M 233 | 50 | 2 | 525 | 517 | 0 |
| | M 234 | 43 | 5 | 439 | 592 | 0 |
| | M 235 | 49 | 1 | 566 | 599 | 0 |
| | M 236 | 38 | 2 | 410 | 658 | 0 |
| | M 237 | 39 | 3 | 411 | 644 | 0 |
| Cyclophosphamide [1] (20) | M 241 | 39 | 30 | 400 | 628 | 0 |
| | M 242 | 32 | 52 | 364 | 762 | 0 |
| | M 243 | 36 | 48 | 427 | 773 | 0 |
| | M 244 | 39 | 49 | 422 | 653 | 0 |
| | M 245 | 33 | 45 | 356 | 731 | 0 |

Vehicle Water (purified in-house by reverse osmosis)
ie Total number of immature erythrocytes examined for micronuclei
mie Number of micronucleated cells observed per 2000 immature erythrocytes
me Total number of mature erythrocytes examined for micronuclei
mme Number of micronucleated mature erythrocytes observed
[1] Positive control, dosed once only 24 hours prior to termination

## APPENDIX 1

### Micronucleus Test - Clinical signs

| Treatment/dosage | ABM-FD(Extract of Himematsutake) – (500 mg/kg/day) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group/sex | 2M | | | | | | | | | |
| Day number | 1 | | | | 2 | | | | | 3 |
| Observation number | 1 | 2 | 3 | 4 | Pre-dose | 1 | 2 | 3 | 4 | 1 |
| No abnormalities detected | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 4 |
| Behaviour – Underactive | 6 | 6 | 7 | 6 | 1 | 7 | 7 | 4 | 6 | 3 |
| Behaviour – Nervous | 0 | 1 | 2 | 3 | 0 | 2 | 0 | 2 | 2 | 0 |
| Posture – Flattened | 0 | 5 | 3 | 4 | 0 | 2 | 3 | 3 | 1 | 0 |
| Eyes – Prominent | 2 | 7 | 0 | 3 | 0 | 2 | 2 | 2 | 3 | 0 |
| Respiration – Fast | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| Respiration – Irregular | 7 | 7 | 7 | 7 | 1 | 5 | 7 | 7 | 7 | 1 |
| Eyelids – Partially closed | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 1 |
| Abnormal gait – Unsteady | 0 | 0 | 5 | 5 | 0 | 0 | 5 | 6 | 2 | 0 |
| Mortalities | 0/7 | | | | | | | | | |

The number of animals displaying the clinical sign is indicated within each box.
No clinical signs were observed in the vehicle or positive control animals throughout the duration of the study.

## APPENDIX 1

### Micronucleus Test - Clinical signs - continued

| Treatment/dosage | ABM-FD(Extract of Himematsutake) – (1000 mg/kg/day) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group/sex | 3M | | | | | | | | | |
| Day number | 1 | | | | 2 | | | | | 3 |
| Observation number | 1 | 2 | 3 | 4 | Pre-dose | 1 | 2 | 3 | 4 | 1 |
| No abnormalities detected | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| Behaviour – Underactive | 7 | 7 | 7 | 7 | 5 | 6 | 7 | 7 | 7 | 4 |
| Behaviour – Nervous | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| Posture – Flattened | 1 | 3 | 3 | 5 | 0 | 0 | 4 | 5 | 5 | 0 |
| Posture – Hunched | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Coat - Piloerection | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Eyes – Prominent | 3 | 5 | 0 | 1 | 0 | 3 | 2 | 2 | 3 | 0 |
| Respiration – Irregular | 7 | 7 | 7 | 7 | 4 | 3 | 7 | 7 | 7 | 7 |
| Respiration – Fast | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| Eyelids – Partially closed | 0 | 0 | 2 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| Abnormal gait [a] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| Abnormal gait – Flattened | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Abnormal gait – Unsteady | 0 | 0 | 7 | 7 | 0 | 0 | 7 | 7 | 7 | 0 |
| Mortalities | 0/7 | | | | | | | | | |

The number of animals displaying the clinical sign is indicated within each box.
[a] Animals occasionally extending and stretching out hind limbs (Day 3 only).

## APPENDIX 1

### Micronucleus Test - Clinical signs - continued

| Treatment/dosage | ABM-FD(Extract of Himematsutake) – (2000 mg/kg/day) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group/sex | 4M | | | | | | | | | |
| Day number | 1 | | | | 2 | | | | | 3 |
| Observation number | 1 | 2 | 3 | 4 | Pre-dose | 1 | 2 | 3 | 4 | 1 |
| No abnormalities detected | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Behaviour – Underactive | 4 | 7 | 7 | 7 | 6 | 6 | 7 | 7 | 7 | 6 |
| Behaviour – Nervous | 2 | 1 | 2 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| Posture – Flattened | 0 | 0 | 2 | 4 | 0 | 1 | 0 | 4 | 5 | 0 |
| Posture – Hunched | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| Coat - Piloerection | 0 | 0 | 2 | 7 | 0 | 0 | 0 | 0 | 0 | 0 |
| Eyes – Prominent | 7 | 7 | 2 | 2 | 0 | 5 | 5 | 1 | 0 | 0 |
| Respiration – Irregular | 7 | 7 | 7 | 7 | 6 | 6 | 4 | 7 | 7 | 7 |
| Respiration – Fast | 0 | 0 | 0 | 0 | 0 | 1 | 3 | 0 | 0 | 0 |
| Eyelids – Partially closed | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 2 |
| Abnormal gait [a] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| Abnormal gait – Unsteady | 0 | 2 | 7 | 7 | 0 | 0 | 7 | 7 | 7 | 0 |
| Mortalities | 0/7 | | | | | | | | | |

The number of animals displaying the clinical sign is indicated within each box.
[a] Animals occasionally extending and stretching out hind limbs (Day 3 only)

# APPENDIX 2

## Animal bodyweights

| Treatment (mg/kg/day) | Animal number | Bodyweight (g) Arrival Individual | Arrival Mean | At treatment Day 1 Individual | At treatment Day 1 Mean | At treatment Day 2 Individual | At treatment Day 2 Mean | At termination Day 3 Individual | At termination Day 3 Mean |
|---|---|---|---|---|---|---|---|---|---|
| Vehicle | M 201 | 181 | 186 | 234 | 248 | 238 | 254 | 249 | 263 |
| | M 202 | 183 | | 242 | | 253 | | 260 | |
| | M 203 | 186 | | 239 | | 248 | | 255 | |
| | M 204 | 194 | | 266 | | 271 | | 277 | |
| | M 205 | 182 | | 249 | | 256 | | 266 | |
| | M 206 | 188 | | 250 | | 254 | | 268 | |
| | M 207 | 190 | | 253 | | 260 | | 267 | |
| ABM-FD Extract of Himematsutake (500) | M 211 | 179 | 186 | 232 | 243 | 239 | 249 | 247 | 256 |
| | M 212 | 180 | | 241 | | 250 | | 258 | |
| | M 213 | 168 | | 217 | | 220 | | 223 | |
| | M 214 | 196 | | 258 | | 267 | | 272 | |
| | M 215 | 190 | | 254 | | 260 | | 270 | |
| | M 216 | 204 | | 262 | | 261# | | 277 | |
| | M 217 | 182 | | 238 | | 245 | | 248 | |
| ABM-FD Extract of Himematsutake (1000) | M 221 | 163 | 188 | 218 | 246 | 223 | 251 | 233 | 258 |
| | M 222 | 200 | | 257 | | 262 | | 272 | |
| | M 223 | 185 | | 240 | | 247 | | 254 | |
| | M 224 | 186 | | 240 | | 244 | | 252 | |
| | M 225 | 195 | | 255 | | 260 | | 264 | |
| | M 226 | 195 | | 259 | | 264 | | 271 | |
| | M 227 | 193 | | 254 | | 259 | | 260 | |
| ABM-FD Extract of Himematsutake (2000) | M 231 | 182 | 182 | 225 | 238 | 232 | 243 | 242 | 253 |
| | M 232 | 184 | | 233 | | 234 | | 243 | |
| | M 233 | 172 | | 228 | | 236 | | 244 | |
| | M 234 | 191 | | 245 | | 254 | | 262 | |
| | M 235 | 171 | | 236 | | 237 | | 251 | |
| | M 236 | 185 | | 248 | | 255 | | 260 | |
| | M 237 | 188 | | 251 | | 256 | | 267 | |
| CPA (20) | M 241 | 178 | 178 | - | | 222 | 241 | 229 | 247 |
| | M 242 | 177 | | - | | 252 | | 252 | |
| | M 243 | 175 | | - | | 237 | | 245 | |
| | M 244 | 172 | | - | | 244 | | 250 | |
| | M 245 | 189 | | - | | 251 | | 259 | |

Vehicle: Water (purified in-house by reverse osmosis)
CPA - Cyclophosphamide
# Denotes weight loss from previous weighing

- Animals not weighed

## APPENDIX 3

### Historical vehicle control values

### November 2003 – March 2005 (130 Animals)

### CD Rat - Males

| Micronucleated Immature (Polychromatic) cells | | |
|---|---|---|
| | Individual | Group |
| Minimum | 0 | 0.75 |
| Maximum | 9.0 | 5.0 |
| Mean | 1.91 | 1.88 |
| Standard Deviation | 1.61 | 0.96 |

### Historical positive control values

### November 2003 – March 2005 (78 Animals)

### CD Rat - Males

| Micronucleated Immature (Polychromatic) cells | | |
|---|---|---|
| | Individual | Group |
| Minimum | 10.0 | 15.0 |
| Maximum | 72.0 | 61.8 |
| Mean | 39.4 | 38.8 |
| Standard Deviation | 16.0 | 12.9 |

# APPENDIX 4

## Eye Research Centre
## GLP Compliance Statement 2003

# 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**<br>**Eye Research Centre**<br>**Occold**<br>**Eye**<br>**Suffolk**<br>**IP23 7PX** | **Analytical Chemistry**<br>**Ecosystems**<br>**Environmental Fate**<br>**Environmental Toxicity**<br>**Mutagenicity**<br>**Toxicology**<br>**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.

Roger G. Alexander
25/7/03

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

## APPENDIX 4 – continued

## Huntingdon Research Centre
## GLP Compliance Statement 2003

**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**

**Huntingdon Life Sciences**
**Huntingdon Research Centre**
**Woolley Road**
**Alconbury**
**Huntingdon**
**Cambs.**
**PE28 4HS**

**TEST TYPE**

**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

# APPENDIX 5

## ABM-FD

## FORMULATION CHEMISTRY

**Authors:**

P. Mann,
A. Papé,
D. 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 in test formulations analysed during the study.

The formulations for this study were prepared as solutions of ABM-FD in water by Genetic Toxicology personnel at Huntingdon Life Sciences Ltd. The analytical work was undertaken by Formulation Chemistry and Central Laboratory Services personnel between 26 April 2005 and 28 April 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 in water formulations as part of an earlier study with respect to the 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 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.

## 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 in aqueous formulations. Huntingdon Life Sciences Analytical Method HRC/FCH/M80/04 Issue 01/011104.

## RESULTS AND DISCUSSION

The concentrations of ABM-FD in test formulations, analysed with respect to the glucose content, and the deviation of mean results from nominal values are presented in Table 1. The mean analysed concentrations were between 9.6% below and 1.5% above the nominal values. The individual results were within 3% of the respective mean value.

## CONCLUSION

The mean concentrations of ABM-FD in the test formulations, analysed with respect to the glucose content, were within -10% and +2% of the nominal concentrations.

# TABLES

## 1. CONCENTRATIONS OF ABM-FD IN TEST FORMULATIONS

| Date of formulation | Nominal inclusion (mg/ml) | Analysed concentration (mg/ml) | | | RME (%) |
|---|---|---|---|---|---|
| | | Analysis 1 | Analysis 2 | Mean | |
| 22-Apr-05 | 0 | ND | - | ND | |
| | 50 | 45.8 | 44.7 | 45.2 | -9.6 |
| | 100 | 92.1 | 90.3 | 91.2 | -8.8 |
| | 200 | 208 | 197 | 203 | +1.5 |

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

Analysed concentrations were calculated using unrounded figures.