STUDY TITLE
MICRONUCLEUS TEST OF ACETAMIDE IN MICE

DATA REQUIREMENT
GUIDELINES: OECD 474

STUDY DIRECTOR/REPORT AUTHOR: AVANI K. SOLANKI
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STUDY COMPLETION: NOVEMBER 18, 2017

SPONSOR
MICHIGAN STATE UNIVERSITY,
220 TROWBRIDGE RD, EAST LANSING MI,
48824, UNITED STATES

TEST FACILITY
JAI RESEARCH FOUNDATION
DEPARTMENT OF TOXICOLOGY
VALVADA - 396 105
DIST. VALSAD
GUJARAT
INDIA
STATEMENT OF GOOD LABORATORY PRACTICE COMPLIANCE

Test item : Acetamide

Study Title : Micronucleus Test of Acetamide in Mice

Except as noted below, the study described in this report was conducted in compliance with the following Good Laboratory Practice Standard:

Organisation for Economic Co-operation and Development (OECD)
ENV/MC/CHEM (98)17 and all subsequent OECD consensus documents

Exception: Test item characterisation (composition), stability, method of synthesis and location of documents for the synthesis is the responsibility of the Sponsor.

There were two amendments to the study plan generated (APPENDIX 6). There was no deviation from the study plan.

Avani K. Solanki, M.Sc. Date
Study Director

Manish V. Patel, Ph.D. Date
Test Facility Management

Sponsored and Submitted By:

Name Date
STATEMENT OF QUALITY ASSURANCE

Test item : Acetamide

Study Title : Micronucleus Test of Acetamide in Mice

This study was audited and the final report examined with respect to the study plan, standard operating procedures and raw data for conformance with the OECD Principles of Good Laboratory Practice. The report was determined to be a full and accurate reflection of the procedures adopted and the raw data generated during the study.

The audits were carried out according to the standard operating procedures of the Quality Assurance Unit of Jai Research Foundation (JRF) and in compliance with the OECD monograph N° 4, ENV/JM/MONO(99)20 (1999).

Findings resulting from the audits were reported to the Study Director and the Management on the dates specified below. These reports are kept in the GLP Archives at JRF.

| Nº    | Details                                                                 | Date               | Study Director   | Facility Management |
|-------|-------------------------------------------------------------------------|--------------------|------------------|--------------------|
| 94462 | Study plan                                                             | August 12, 2017    | August 12, 2017  | August 12, 2017    |
| 95730 | Dose formulation preparation and dosing (day 1), blood collection, sacrifice and bone marrow harvesting (day 3) | September 18, 2017 to September 20, 2017 | September 20, 2017 | September 20, 2017 |
| 95922 | Plasma sample analysis                                                 | September 25, 2017 to September 26, 2017 | September 26, 2017 | September 26, 2017 |
| 96785 | Raw data and report                                                    | October 27, 2017   | October 27, 2017  | October 27, 2017   |
| 97397 | Final report                                                           | November 16, 2017  | November 16, 2017 | November 16, 2017  |

Number of study plan amendment(s) reviewed: 02

In addition, other processes related to this type of study were inspected periodically by the Quality Assurance. The most recent process inspected is identified below:
### Statement of Quality Assurance (Continued)

| No  | Inspection                        | Details                        | Reporting Dates to |
|-----|-----------------------------------|--------------------------------|--------------------|
| 94871 | Processes of micronucleus test    | August 01, 2017 to August 25, 2017 | August 25, 2017     | August 25, 2017     |

Associated laboratory and support functions are subject to regular facility inspections in accordance with the Quality Assurance procedures.

HEMANGINI PATEL, M. Pharm.
QUALITY ASSURANCE OFFICER, JRF
DATE: November 21, 2017
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**SUMMARY**

**STUDY TYPE**: Micronucleus Test - Mice (CD1); OECD 474 (July 2016)

**TEST ITEM**: Acetamide [99.2% w/w – Provided by Supplier; 99.198% w/w – Generated at JRF]

**CITATION**: Avani K. Solanki. Micronucleus Test of Acetamide in Mice. Jai Research Foundation, India. Laboratory report number: 485-1-06-17727; November 18, 2017.

**SPONSOR**: Michigan State University, U.S.A.

**EXECUTIVE SUMMARY**: This study was performed to evaluate the micronucleus induction potential of acetamide in mice. Sixty CD1 mice were divided into 5 groups, each group comprising 6 animals/sex. The main study was conducted at the dose levels of 250, 1000 and 2000 mg acetamide/kg body weight (Groups II, III and IV, respectively). A concurrent vehicle (distilled water) control group (Group I) was maintained along with the acetamide treated animals. Acetamide was dissolved in distilled water and administered orally for two consecutive days. Animals were sacrificed approximately between 18-24 hours after the final treatment. Before sacrifice blood samples were collected from each treatment group and vehicle control group to demonstrate the target organ exposure. A concurrent positive control group (Group V) was treated with a single intraperitoneal injection of Mitomycin-C at the dose level of 1 mg/kg body weight.

No toxicity to bone marrow [decrease in polychromatic to total erythrocytes ratio (P/E)] was observed in all animals treated at the dose levels of 250, 1000 and 2000 mg/kg body weight, when compared with the concurrent vehicle control group. All animals exhibited normal behavior and there were no mortalities. The number and percentage of micronucleated polychromatic erythrocyte (MNPCE) were not increased in animals treated with acetamide up to the dose level of 2000 mg/kg body weight when compared with the vehicle control group. No statistically significant effects on body weight were observed in any of the animals from positive control or treatment groups, when compared with the concurrent vehicle control group. The positive control group yielded a statistically significant increase in the number of micronucleated polychromatic erythrocytes (MNPCE) in comparison to the vehicle control group.

The dose formulation analysis revealed that the doses complied for the presence of test item for its nominal concentration (± 10%) of active ingredient (% CV < 10%). Plasma concentration of acetamide in different groups revealed that the test item reached the target tissue, i.e. bone marrow. Negative and positive control data were consistent with historical control distributions.

From the results of the present study, it is concluded that acetamide does not have micronucleus induction potential.

**COMPLIANCE**: Signed and dated GLP and Quality Assurance statements are provided. There was no deviation from regulatory requirements.
1. INTRODUCTION

1.1 Study Objective

This study was performed to evaluate the micronucleus induction potential of acetamide in mice. The study was conducted in compliance with Principles of GLP (OECD 1998).

1.2 Study Guidelines

The present study was conducted according to:

OECD, 2016: The Organisation for Economic Co-operation and Development (OECD), Guidelines for Testing of Chemicals, Volume II, OECD 474, Mammalian Erythrocyte Micronucleus Test, adopted by the Council on July 29, 2016.

1.3 Justification for Selection of the Test System

The mouse was selected as the test system of choice because it is a readily available rodent species. It has been historically shown to be a suitable model for assessing the micronucleus induction potential and is recommended by the OECD and other regulatory authorities. The results of the study are believed to be of value in predicting the micronucleus induction potential of the test item in humans.

1.4 Test Facility and Study Period

This study was performed at the Department of Toxicology, Jai Research Foundation, Valvada - 396 105, Dist. Valsad, Gujarat, India.

Study Initiation : August 30, 2017
Experiment Start : September 04, 2017
Experiment Completion : October 11, 2017
Study Completion : November 18, 2017

1.5 Personnel Involved in the Study

Study Director : Avani K. Solanki, M.Sc.
Deputy Study Director : Dr. Rajendra M. Nagane, M.V.Sc.
Study Personnel : Pradeep D. Tekale, M.Sc.
Dibya Ranjan Panda, M.Sc.
Durga N. Chejara, M.Pharm.
Jainisha D. Rathod, M.Sc.
Bindi S. Patel, M.Sc.
Deval S. Mehta, Ph.D.
1.6 Archives

All original raw data including any storage medium for electronically recorded data, documentation, the signed study plan, the study plan amendments, the draft report, one original final report, slides and the representative sample of the test item will be retained in the GLP Archives at Jai Research Foundation for a period of ten years. At the end of this period, the Sponsor’s instructions will be sought to either extend the archiving period or return the archived material to the Sponsor or dispose of the material.
### 2. EXPERIMENTAL PROCEDURE

#### 2.1 Test Item

Details of the test item provided by the Supplier:

| Test Item Name     | Acetamide          |
|-------------------|--------------------|
| IUPAC Name        | Acetamide          |
| CAS Number        | 60-35-5            |
| Molecular Formula | C₂H₅NO             |
| Molecular Weight  | 59.07 g/mol        |
| Molecular Structure | ![Molecular Structure](image) |
| Batch/Lot Number  | QYD4G              |
| Analysed Purity (Provided by Supplier) | 99.2% w/w (Refer CoA in APPENDIX 12) |
| Analysed Purity (Generated at JRF) | 99.198% w/w (Refer CoA in APPENDIX 13) |
| Manufactured by   | Tokyo Chemical Industry Co. Ltd |
| Supplied to JRF by | Procured by JRF from Tokyo Chemical Industry Co. Ltd on behalf of sponsor |
| Date of Receipt   | July 29, 2017      |
| Retest Date       | December 03, 2017  |
| Appearance        | White Solid       |
| Storage Condition (at JRF) | As per the instruction received from the Sponsor on storage of the test item, the test item was stored: |
| Storage Temperature | Room temperature |
| Storage Container  | In original container as supplied by the Sponsor |
| Storage Condition  | Stored in its original container in isolated, dry, cool and well-ventilated area. |
| Storage Location   | Test Item Control Office, JRF |

**Source of Molecular Weight, Molecular Formula and Molecular Structure:**

www.sigmaaldrich.com
2.2 Positive Control

Name: Mitomycin-C  
Lot No: SLBP4042V  
CAS Number: 50-07-7  
Route of Administration: Intraperitoneal  
Dose: 1 mg/kg body weight (formulated at 0.1 mg/mL using distilled water as vehicle)  
Appearance: Light grey powder  
Manufactured by: Sigma  
Storage: 2 - 8 °C (Amber vial)  
expiry date: April 2020

2.3 Solvent and Chemicals

Methanol: Qualigens (Lot # 1655050117)  
Foetal Bovine Serum: Himedia (Lot # 0000296898)  
Giemsa Powder: Merck (Lot # DC6D660652)  
Potassium Dihydrogen Orthophosphate: Qualigens (Lot #2301790714)  
Sodium Hydroxide: Qualigens (Lot # 27287109-1)  
Glycerol: Qualigens (Lot # 14687201-2)  
NaH₂PO₄: Merck (Lot # QH3Q631840)  
Na₂HPO₄: Sigma (Lot # BCBN1164V)  
DPX Mountant: Qualigens (Lot # 1097020616)  
Immersion Oil: Himedia (Lot # 0000248321)  
Disinfectant: Dettol 2.5% v/v (Lot #D9354)  
Heparin: Biological E. Ltd. (Lot #AK040)

2.4 Instruments and Equipment

Digital Balance: Adventurer/AR 2140, OHAUS  
Electronic Balance: Electronic Weighing Scale - SMART  
Metal Cannula: CW12 ILA, England, size: 18 G x 15 cm.  
Syringe: BD 1 mL disposable syringe  
Needles: 1. 26 G ½ (0.45 x 13 mm), BD Precision Glide  
2. 24 G x 1" (0.6 x 25 mm), BD Precision Glide  
Vacuum Desiccator: Tarsons (CO₂ Chamber)  
Centrifuges: Thermo scientific
2.5 Principle

The mammalian micronucleus test is used to detect cytogenetic damage (which results in a chromosomal break, fragment or lagging whole chromosome) caused by the test item. The damaged chromosomal fragments remain in the anucleated cytoplasm of the erythrocyte and are visible, when stained, as a small round or oblong structure called micronuclei. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage.

2.6 Animal Welfare

The study was undertaken in compliance with the ‘Guidelines for Laboratory Animals Facility’ issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. These guidelines promote the humane care of animals used in research by providing specifications that will enhance animal well-being and experimental quality for the advancement of biological knowledge that is relevant to humans and animals.

Project proposal for the experimentation was approved by Institutional Animal Ethics Committee (IAEC), Jai Research Foundation.

JRF is also accredited with Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) that promotes the humane treatment of animals in science.
2.7 Test Animals

For the main study, Hsd: ICR (CD1) mice (*Mus musculus*) were received from the Animal Breeding Facility, Jai Research Foundation. The animals were 8-10 weeks old on day 1 of dosing. The females used were nulliparous and non-pregnant. The male mice weighed between 33 and 43 g and the female mice weighed between 27 and 33 g on day 1 of the experiment (main study).

2.8 Acclimatisation

The animals were received into the experimental room and acclimatised for a period of six days (maximum 3 animals/cage). The animals were randomised into 5 groups using validated in-house developed software. The method of randomisation used was censored randomisation method (Gad S.C. and Weil, C.S., 1994).

2.9 Identification

Before randomisation, animals were marked with nontoxic marker pen. After randomisation, each mouse was assigned a number, which was tattooed on its tail using a tattoo machine and appropriate labels were attached to the cages indicating the study number, test item code, group number and sex, dose, type of study, cage number and animal number.

2.10 Environmental Conditions

|                              | BMR Facility Room No. 31, Department of Toxicology |
|------------------------------|-----------------------------------------------------|
| Animal Room                  |                                                     |
| Temperature Range            | 19- 23 °C                                           |
| Relative Humidity Range      | 57 - 66%                                            |
| Photoperiod                  | The photoperiod was 12 h artificial light and 12 h darkness, light hours being 06:00 h - 18:00 h. |
| Air Changes                  | Minimum 15 volumes/hour.                            |

2.11 Husbandry Practices

|                              | Polypropylene mouse cages (size: 29 x 22 x 14 cm) with stainless steel grid top. Autoclaved clean rice husk was used as the bedding material. |
|------------------------------|-------------------------------------------------------------------------------------------------------------------------------------|
| Caging                       | Each cage was supplied with a polypropylene water bottle (capacity 300 mL) with a stainless steel nozzle.                               |
| Water Bottle                 | 3 animals per cage.                                                                                                                  |
| Housing                      | Each day the floor and all work tops were mopped with a disinfectant solution (Dettol 2.5% v/v).                                     |
2.12 **Feed and Water**

The quality of feed and water is regularly monitored at Jai Research Foundation. There were no known contaminants in the feed or water at levels that would have interfered with the experimental results obtained.

**Feed**: Mice pellet feed (Teklad, Certified Global 16% Protein Rodent Diet Sterilizable, USA) was provided *ad libitum* (except fasting for 2-3 h before dosing and 1 h after dosing) (APPENDIX 10).

**Water**: UV sterilized drinking water filtered through Hi-Tech reverse osmosis water filtration system was provided *ad libitum* (APPENDIX 9).

2.13 **Selection of Vehicle**

Acetamide was found soluble in distilled water (stock A, 200 mg/mL). Hence distilled water was selected as the vehicle for oral gavage for the animals in the main study.

2.14 **Rationale for Selection of Route of Administration**

A potential route of human exposure is via the oral route. Therefore, the oral route of administration was selected for this study.

2.15 **Main Study**

Based on sponsor’s suggestions and the published data from earlier studies (Michael R. et al., 2014, Chieli et al., 1987, Mirkova, 1996 and Dybing et al., 1987), the main study was conducted with dose levels of 250, 1000 and 2000 mg/kg body weight. Five groups (comprising 6 animals/sex) were used for this study. Group I served as the vehicle (distilled water) control, Group II, III and IV were low, mid and high dose groups, respectively. Group V was the positive control group and received Mitomycin-C (1.0 mg/kg body weight on day 2 of treatment) in distilled water by the intraperitoneal route on a single occasion.

A quantity of 625, 2500 and 5000 mg of acetamide were weighed and dissolved in distilled water on day 1 of dosing (Gad and Cassidy, 2006). The volume was made up to 25 mL to obtain a concentration of 25, 100 and 200 mg/mL for male and female animals for groups II, III, and IV, respectively. A quantity of 250, 1000 and 2000 mg of acetamide were weighed and dissolved in distilled water on day 2 of dosing (Gad and Cassidy, 2006). The volume was made up to 10 mL to obtain a concentration of 25, 100 and 200 mg/mL for male and female animals for groups II, III, and IV, respectively. The dose volume was 10 mL/kg body weight for all the treatment groups including vehicle and positive control groups. The acetamide was administered orally to mice using a metal cannula attached to a BD 1 mL disposable syringe. Mice from the vehicle control group (Group I) received only distilled water orally on both the days.
The mice from the positive control group (Group V) received a single injection of Mitomycin-C intraperitoneally at the dose level of 1.0 mg/kg body weight on day 2 of treatment. Each day the dose solutions were freshly prepared prior to dosing.

Body weight was recorded before dosing on day 1, day 2 and before sacrifice. The clinical signs of toxicity were recorded before dosing, post dosing (up to four hours) and before sacrifice. The body temperatures of all the animals were measured before dosing and then approximately 2 and 5 hours after each dosing and before sacrifice using microprobe thermometer (Asanami and Shimono, 1997; Asanami et al., 1998).

2.16 Dose Formulation Preparation, Sampling and Analysis

For active ingredient concentration analysis, samples were collected from each prepared dose formulations along with vehicle (distilled water) during the main study following the detailed procedures below.

Two sets of three replicates of 2 mL each concentration (25, 100 and 200 mg/mL for male and female animals) were taken from middle portion along with vehicle (distilled water). First set of replicates (three replicates of 2 mL each) were sent to Department of Chemistry (JRF) for analysis and second set of replicates were stored in deep freezer (-70 ± 10 ºC) as backup. The unused aliquots will be discarded after receiving approval for finalisation of the report from the sponsor.

Samples were analysed using following analytical parameters: (JRF Study N° 228-2-14-17729)

Instrumental Parameters

| Parameter                | Description                                                                 |
|--------------------------|----------------------------------------------------------------------------|
| Instrument               | GC-MS                                                                      |
| Column                   | Agilent VF-5MS, 0.25 mm (i.d.), 30m length, 0.25 μm film thickness          |
| Carrier Gas              | Helium                                                                     |
| Injection Volume         | 2.0 μL                                                                     |
| Injection Temperature    | 250 °C                                                                     |
| Flow Rate                | 1.2 mL/minute                                                              |
| Split Ratio              | 1:8                                                                        |
| Oven Temperature         | 40 °C (Hold 2.0 min.) to 20.0 °C to 300 °C, (hold for 10 minutes) – Total of 25 minutes |
| Mass Spectrometry        | Electron Ionization mode with 70 eV SIM Mode                              |
| Solvent Delay Time       | 4.0 minutes                                                                |
| Quadruple Temperature    | 150 °C                                                                     |
| Data Acquisition         | Selected Ion Monitoring (SIM) for masses 239 (Xanthyl-acetamide) and 253 (Xanthyl- Propionamide) |
2.16.1 Analytical Acceptance Criteria

The following criteria for acceptable specification for the concentration of the test item in the vehicle were used to determine a valid assay:

90 to 110% of nominal concentration with <10% coefficient of variance (%CV) of each concentration (Whitmire et al., 2010).

2.17 Evidence of Tissue Exposure

Blood samples were withdrawn from each animal in each treatment group and vehicle control group at the time of sacrifice before bone marrow collection. Blood samples were collected in heparinised (20 IU/mL) micro-centrifuge tubes. Blood samples were collected from orbital plexus under very light isoflurane anesthesia. To separate out the plasma, blood samples were centrifuged at 3000 rpm for 15 minutes at 4 °C. The plasma samples were stored at -70 ± 10 °C until analysis. The plasma samples were analysed for determination of test item concentration at Department of Chemistry, JRF.

Samples were analysed using following analytical parameters: (JRF Study N° 228-2-14-18476)

**Instrumental Parameters**

- **Instrument**: GC-MS
- **Column**: Agilent VF-5MS, 0.25 mm (i.d.), 30m length, 0.25 μm film thickness
- **Carrier Gas**: Helium
- **Injection Volume**: 2.0 μL
- **Injection Temperature**: 250 °C
- **Flow Rate**: 1.2 mL/minute
- **Split Ratio**: 1:8
- **Oven Temperature**: 40 °C (Hold 2.0 min.) to 20.0 °C to 300 °C, (hold for 10 minutes) – Total of 25 minutes
- **Mass Spectrometry**: Electron Ionization mode with 70 eV
- **Data Acquisition**: Selected Ion Monitoring (SIM) for masses 239 (Xanthyl-acetamide), 242 (Xanthyl-3d- acetamide) and 253 (Xanthyl- Propionamide)

2.18 Slide Preparation

Within 18 - 24 h following the last treatment, mice from the vehicle control and the treatment groups (group I - group IV) were sacrificed by CO₂ asphyxiation (MacGregor et al. 1987) and the positive control group (group V) was sacrificed 24 hour after the last treatment by CO₂ asphyxiation (Krishna and Hayashi, 2000). Femur bones from the sacrificed animals were excised and the epicondyle tips were removed. The bone marrow content was expelled by flushing and aspirating approximately 3 mL of foetal bovine serum using a 1 mL syringe and 24 gauge needle into centrifuge tubes. The aspirated bone marrow content was mixed using the syringe to dissociate the cells in order to avoid cell clump formation.
The tubes were centrifuged at around 1500 rpm for 10 minutes and the supernatant was discarded leaving about 0.2 - 0.3 mL of medium with the cell pellet. The cell pellet was dissociated thoroughly using a Pasteur pipette and a drop of suspension was placed on a clean slide. A smear was prepared and allowed to air dry.

The slides were marked with study number, animal number and slide number. Two slides were prepared per animal and the cells were fixed with methanol and allowed to air dry for 20 minutes. Slides were stained using 5% Giemsa in phosphate buffer for 25 minutes. Subsequently the slides were rinsed in distilled water, air-dried and mounted. In order to prevent bias in the scoring, the slide numbers were masked with code numbers provided by the Department of Bio-statistics and Systems Information, Jai Research Foundation.

2.19 Scoring of Bone Marrow Micronucleus

One out of two slides from each animal was used for screening of micronucleated erythrocytes whereas the other slide was kept as back up, to be used for scoring when required. The slides were examined for the presence of micronuclei in polychromatic and normochromatic erythrocytes under microscope [Nikon Eclipse E600, Nikon Eclipse 80i, Nikon Eclipse Ni-U (Fluorescence) and Nikon Eclipse Ci]. A minimum of 4000 polychromatic erythrocytes were screened per animal to evaluate the incidence of micronuclei. A minimum of 500 normochromatic erythrocytes to its corresponding polychromatic erythrocytes were recorded to determine the P/E ratio. The masked labels were removed and all the slides were decoded after scoring.

2.20 Calculation

The P/E ratios were calculated from polychromatic to total (polychromatic + normochromatic) erythrocytes. The percentage of micronucleated polychromatic erythrocytes was also calculated.

2.21 Statistical Evaluation of Results

The data of percent micronucleated polychromatic erythrocytes (% MNPCE), P/E ratio and body weight of both the sexes were statistically analysed for normality using Shapiro-Wilk’s test. Where results of normality test were significant, non-parametric test (Kruskal-Walis test) was performed. Where results of normality test were non-significant then Bartlett test was performed to meet the homogeneity of variance before conducting ANOVA test followed by Dunnett’s t-test. T-test was also performed to determine the level of significant difference between the vehicle control and the treated groups and positive control group.
2.22 Historical control data

Jai Research Foundation (JRF) has conducted more than 500 GLP studies for regulatory submission as per OECD TG 474 and established a strong historical control data base. JRF used quality control methods, such as control charts to identify data variability and to show that the methodology was 'under control'. Quality control charts (QC charts) have been added in APPENDIX 8 demonstrating the JRFs established historical positive control ranges and distribution, and a historical negative control ranges and distribution. Results of negative and positive control were within historical distribution limits. Overall results of treatment group was also within historical control limits.

2.23 Assay Acceptance and Evaluation Criteria

Before assay data were evaluated, criteria for a valid assay had to be met. The following criteria were used to determine a valid assay:

2.23.1 Acceptance Criteria

i. The vehicle (or negative) controls values were in the range of historical control data.

ii. The positive controls has produced responses that were compatible with that of the historical data and has produce statistically significant responses compared with the concurrent negative control.

iii. Mortality was not observed in control or treatment group and six animals per sex per group (group I to V) were evaluated for micronucleus induction potential of the test item in all the groups.

iv. The highest dose was a limit dose, maximum tolerable dose (MTD) which did not cause distress or death to the animal or produce toxicity to bone marrow.

v. PCE to erythrocyte ratio was more than the 20% of the vehicle control.

2.23.2 Evaluation and Interpretation Criteria

Once criteria for a valid assay had been met, responses observed in the assay were evaluated. The conditions necessary for determining a positive result were,

i. At least one of the treatment groups exhibits statistically significant increase in the frequency of micronucleated polychromatic erythrocytes compared to concurrent negative control.

ii. A positive result was defined as a dose-dependent, significant increase in the incidence of micronuclei when evaluated with an appropriate trend test e.g. Chi-square trend analysis.

iii. Statistical and biological relevance was considered in data interpretation.

iv. Any of the results falling outside the distribution of the historical negative control data i.e. Poisson based 95% control limits.
The test item was considered clearly negative, if, in all experimental conditions examined:

i. None of the treatment groups exhibits a statistically significant increase in the frequency of micronucleated immature erythrocytes compared with the concurrent negative control.

ii. There was no dose-related increase at any sampling time when evaluated by an appropriate trend test.

iii. All results were inside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits), and

iv. Bone marrow exposure to the test item(s) occurred

v. There is no requirement for verification of a clear positive or clear negative response.
3. RESULTS

3.1 Main Study

3.1.1 Clinical Observations, Body Temperature and Body Weight

All animals were normal in the vehicle control group (Group I) and treatment groups II, III and IV (250, 1000, 2000 mg/kg body weight, respectively) and positive control group (Group V), both post-treatment and pre-sacrifice.

Significant decrease or increase in body temperature was not observed after day 1 and day 2 of dosing in both male and female animals from treatment groups, when compared with the concurrent vehicle control group.

No statistically significant effect on mean body weight was observed in positive control or treatment groups, when compared with the concurrent vehicle control group. No mortalities were observed.

Individual clinical observations are provided in APPENDIX 1. The summary of mean body temperature and individual body temperature are provided in TABLE 1 and APPENDIX 2, respectively. The summary of mean body weight and individual body weight are provided in TABLE 2 and APPENDIX 3, respectively.

3.1.2 Micronucleated Polychromatic Erythrocytes

Values of % MNPCE and P/E ratio for vehicle and positive controls were within the range of historical control data limits (APPENDIX 8).

No toxicity to bone marrow [decrease in polychromatic to total erythrocytes ratio (P/E)] was observed in both male and female animals treated at the dose levels of 250, 1000 and 2000 mg/kg body weight, when compared with the concurrent vehicle control group. Percent reduction P/E ratio observed was -1.02, -1.43 and -0.41 in male animals treated at dose levels of 250, 1000, 2000 mg/kg body weight, respectively. Percent reduction P/E ratio observed was 1.15, -0.19 and 2.11 in female animals treated at dose levels of 250, 1000, 2000 mg/kg body weight, respectively.

The ratio of polychromatic erythrocytes (PCE) to total erythrocytes (P/E ratio) in treated groups at the dose levels of 250, 1000 and 2000 mg/kg body weight was comparable to the vehicle control group.
The mean P/E ratios observed in the male animals were 0.491, 0.496, 0.498 and 0.493 at the dose levels of 0.0 (vehicle control group), 250, 1000 and 2000 mg acetamide/kg body weight, respectively. The mean P/E ratios observed in the female animals were 0.521, 0.515, 0.522 and 0.510 at the dose levels of 0.0 (vehicle control group), 250, 1000 and 2000 mg acetamide/kg body weight, respectively. The mean polychromatic to total erythrocytes ratios (P/E) observed in the male and female animals treated with Mitomycin-C (1.0 mg/kg body weight) were 0.511 and 0.499, respectively.

The mean percent micronucleated polychromatic erythrocytes (% MNPCE) observed in male animals was 0.017, 0.017, 0.013 and 0.017 at the dose levels of 0.0 (vehicle control group), 250, 1000 and 2000 mg acetamide/kg body weight, respectively. The mean percent micronucleated polychromatic erythrocytes (% MNPCE) observed in female animals was 0.013, 0.025, 0.020 and 0.017 at the dose levels of 0.0 (vehicle control group), 250, 1000 and 2000 mg of acetamide/kg body weight, respectively. The mean percent micronucleated polychromatic erythrocytes (% MNPCE) observed in male and female animals treated with Mitomycin-C (1.0 mg/kg body weight) were 1.308 and 1.340, respectively.

Statistical analysis of the results did not reveal any significant difference in percent micronucleated polychromatic erythrocytes (% MNPCE) in animals belonging to any treatment groups, when compared with the vehicle control group.

A statistically significant increase in mean % MNPCE observed in the male and female animals treated with Mitomycin-C (1.0 mg/kg body weight) demonstrated the sensitivity of the test system, suitability of the procedures and efficiency of the test conditions employed in the test (TABLE 3, APPENDIX 4 and APPENDIX 5).

Group-wise total polychromatic erythrocytes (PCE), micronucleated polychromatic erythrocytes (MNPCE), percent MNPCE and mean P/E ratio in bone marrow cells are given in TABLE 3 with individual data presented in APPENDIX 4 and APPENDIX 5.

3.1.3 Dose Formulation Analysis

The dose formulations complied with the presence of test item for its nominal concentration of (±10) active ingredient (% CV < 10%). Mean recoveries were 99.69, 100.57 and 105.41% at the prepared concentrations of 25, 100 and 200 mg/mL, respectively for both male and female animals (APPENDIX 7).
3.1.4 Evidence of Tissue Exposure

The plasma samples were analysed to demonstrate the target organ exposure, i.e., for test item concentration in blood. Dose dependent increase in concentration was observed in plasma samples (APPENDIX 7). Concentration of acetamide observed in GI (Negative control) may be endogenous level. Mean concentration observed at dose levels of 250, 1000 and 2000 mg/kg body weight has been presented in below table:

| Sex   | Group and Dose (mg/kg body weight) | Mean Concentration in Plasma Samples (ppm) | Sex        | Group and Dose (mg/kg body weight) | Mean Concentration in Plasma Samples (ppm) |
|-------|-----------------------------------|-------------------------------------------|------------|-----------------------------------|-------------------------------------------|
| Male  | GI and 0.0                         | 0.394                                     | Female     | GI and 0.0                         | 0.442                                     |
|       | GII and 250                        | 35.720                                    |            | GII and 250                        | 10.950                                    |
|       | GIII and 1000                      | 171.341                                   |            | GIII and 1000                      | 68.193                                    |
|       | GIV and 2000                       | 183.465                                   |            | GIV and 2000                       | 114.759                                   |
4. CONCLUSION

From the results of the present study, it is concluded that acetamide does not have micronucleus induction potential in male and female mice up to the dose level of 2000 mg/kg body weight, following oral administration for two consecutive days.
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### Micronucleus Test of Acetamide in Mice

**TABLE 1: Summary of Mean Body Temperature - Main Study**

Number of Animals = 6 Animals/Sex/Group  
Refer: APPENDIX 2

| Group and Dose of Acetamide | Body Temperature (°C) After Dosing – Male |
|-----------------------------|------------------------------------------|
|                             | Before Dosing (Day 1) | After Dosing - Day 1 (hours) | Before Dosing (Day 2) (24 h after initial dose) | After Dosing - Day 2 (hours) | Before Sacrifice |
|                             | 2 h | 5 h | 2 h | 5 h | 2 h | 5 h |
| G I Vehicle control (Distilled water) | Mean | 37.2 | 37.0 | 37.2 | 37.1 | 37.2 | 37.0 | 37.4 |
|                             | SD | 0.2 | 0.2 | 0.3 | 0.2 | 0.2 | 0.2 |
| G II (250 mg/kg body weight) | Mean | 37.2 | 37.0 | 37.2 | 37.0 | 37.0 | 37.1 | 37.3 |
|                             | SD | 0.2 | 0.4 | 0.2 | 0.2 | 0.1 | 0.2 | 0.4 |
| G III (1000 mg/kg body weight) | Mean | 37.2 | 37.0 | 37.1 | 37.2 | 37.1 | 37.0 | 37.2 |
|                             | SD | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 |
| G IV (2000 mg/kg body weight) | Mean | 37.1 | 36.4 | 37.0 | 37.2 | 36.6 | 37.0 | 37.3 |
|                             | SD | 0.2 | 0.1 | 0.5 | 0.1 | 0.4 | 0.2 | 0.1 |
| G V Positive control Mitomycin C (1.0 mg/kg body weight) | Mean | NA | NA | NA | 36.9 | 37.2 | 37.2 | 37.2 |
|                             | SD | NA | NA | NA | 0.2 | 0.1 | 0.2 | 0.2 |

**Keys**: SD = Standard deviation, °C = Degree centigrade, h = Hour, NA = Not applicable.

**Note**: Temperature of positive control animals was not recorded on day one since positive control animals were not treated on day one.
TABLE 1 (Continued)

| Group and Dose of Acetamide | Body Temperature (°C) After Dosing – Female |
|-----------------------------|-------------------------------------------|
|                             | Before Dosing (Day 1) | After Dosing - Day 1 (hours) | Before Dosing (Day 2) (24 h after initial dose) | After Dosing - Day 2 (hours) | Before Sacrifice |
|                             | 2 h | 5 h | 2 h | 5 h | 2 h | 5 h | |
| GI  Vehicle control (Distilled water) | Mean | 37.2 | 37.2 | 37.3 | 37.3 | 37.2 | 37.2 | 37.3 |
|                             | SD  | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.1 | 0.2 |
| G II (250 mg/kg body weight) | Mean | 37.2 | 37.1 | 37.3 | 37.2 | 37.1 | 37.1 | 37.2 |
|                             | SD  | 0.2 | 0.3 | 0.1 | 0.1 | 0.2 | 0.3 | 0.3 |
| G III (1000 mg/kg body weight) | Mean | 37.2 | 37.1 | 37.3 | 37.1 | 37.2 | 37.3 |
|                             | SD  | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 |
| G IV (2000 mg/kg body weight) | Mean | 37.1 | 37.2 | 37.3 | 37.1 | 37.1 | 37.2 |
|                             | SD  | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.3 | 0.3 |
| G V  Positive control Mitomycin C (1.0 mg/kg body weight) | Mean | NA | NA | NA | 37.0 | 36.9 | 37.2 | 37.4 |
|                             | SD  | NA | NA | NA | 0.2 | 0.1 | 0.1 | 0.2 |

Keys : SD = Standard deviation, °C = Degree centigrade, h = Hour, NA = Not applicable.

Note : Temperature of positive control animals was not recorded on day one since positive control animals were not treated on day one.
Micronucleus Test of Acetamide in Mice

TABLE 2: Summary of Mean Body Weight

Number of Animals = 6 Animals/Sex/Group

| Group and Dose of Acetamide | Body Weight (g) | MALE | FEMALE |
|-----------------------------|-----------------|------|--------|
|                             | Day 1 | Day 2 | Before Sacrifice | Day 1 | Day 2 | Before Sacrifice |
| G I Vehicle control         | Mean  | 38.67 | 39.00 | 39.33 | 28.83 | 29.00 | 28.83 |
| (Distilled water)           | SD    | 2.88 | 2.61 | 2.88 | 1.47 | 1.41 | 0.75 |
| G II (250 mg/kg body weight)| Mean  | 38.50 | 38.67 | 38.33 | 28.83 | 29.17 | 29.17 |
|                             | SD    | 3.02 | 2.34 | 2.73 | 1.17 | 1.17 | 1.17 |
| G III (1000 mg/kg body weight)| Mean  | 38.67 | 38.33 | 38.67 | 29.33 | 29.00 | 29.00 |
|                             | SD    | 2.58 | 2.88 | 2.80 | 1.97 | 1.67 | 1.67 |
| G IV (2000 mg/kg body weight)| Mean  | 38.17 | 38.33 | 38.50 | 30.17 | 29.67 | 30.00 |
|                             | SD    | 3.66 | 3.67 | 3.39 | 1.72 | 2.07 | 2.10 |
| G V (Mitomycin-C,1.0 mg/kg body weight)| Mean  | NA  | 39.00 | 39.00 | NA | 28.67 | 28.67 |
|                             | SD    | NA  | 2.76 | 2.53 | NA | 1.63 | 1.63 |

Keys: SD = Standard deviation, NA = Not Applicable

Note: Body weight of positive control animals was not recorded on day one since positive control animals were not treated on day one.
Micronucleus Test of Acetamide in Mice

TABLE 3: Summary of Micronucleated Polychromatic Erythrocytes in Bone Marrow Cells

Number of Animals = 6 Animals/Sex/Group

Refer: APPENDIX 4 and APPENDIX 5

| Group and Dose of Acetamide | Total PCE | Male | | | Total PCE | Female | | |
|-----------------------------|-----------|------|-------|-------|-----------------|---------|-------|-------|
|                             |           | MNPCE | %MNPCE | P/E Ratio | MNPCE | %MNPCE | P/E Ratio |
|                             |           | Total | Mean ± SD | (Mean ± SD) | Total | Mean ± SD | (Mean ± SD) |
| GI (Vehicle control)         | 27063     | 5     | 0.833 ± 0.753 | 0.017 ± 0.015 | 0.491 ± 0.024 | 27077 | 4     | 0.667 ± 0.516 | 0.013 ± 0.010 | 0.521 ± 0.025 |
| GII (250 mg/kg body weight)  | 27060     | 5     | 0.833 ± 0.753 | 0.017 ± 0.015 | 0.496 ± 0.025 | 27041 | 7     | 1.167 ± 1.169 | 0.025 ± 0.027 | 0.515 ± 0.022 |
| GIII (1000 mg/kg body weight) | 27043 | 4     | 0.667 ± 0.816 | 0.013 ± 0.015 | 0.498 ± 0.018 | 27095 | 6     | 1.000 ± 0.894 | 0.020 ± 0.018 | 0.522 ± 0.031 |
| GIV (2000 mg/kg body weight) | 27086 | 5     | 0.833 ± 0.753 | 0.017 ± 0.015 | 0.493 ± 0.024 | 27054 | 5     | 0.833 ± 0.753 | 0.017 ± 0.015 | 0.510 ± 0.025 |
| GV (Mitomycin-C, 1.0 mg/kg body weight) | 27127 | 355  | 59.167↑↑ | ± 16.364 | 1.308↑↑ | ± 0.363 | 27066 | 363 | 60.500↑↑ | ± 18.960 | 1.340↑↑ | ± 0.422 |

Note:

\[
\text{% MNPCE} = \frac{\text{MNPCE} \times 100}{\text{Total PCE}}
\]

Keys:
PCE = Polychromatic Erythrocytes
MNPCE = Micronucleated Polychromatic Erythrocytes
P/E = Polychromatic Erythrocytes corresponding to Normochromatic Erythrocytes/Total Erythrocyte
↑↑ = Significantly higher than the control at 1% level (p≤0.01)
### Micronucleus Test of Acetamide in Mice

#### APPENDIX 1: Individual Clinical Observations – Main Study

| Group and Dose of Acetamide | Sex | Animal No. | Individual Animal Observations on Experimental Days |
|-----------------------------|-----|------------|------------------------------------------------------|
|                             |     |            | Before Dosing (Day 1) | After Dosing Day 1 (hours) | Before Dosing (Day 2) | After Dosing Day 2 (hours) | Before Sacrifice |
|                             | M   | T1         | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T2         | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T3         | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T4         | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T5         | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T6         | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
| G I                         | F   | T7         | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
| Vehicle control             |     | T8         | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
| (Distilled water)           |     | T9         | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T10        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T11        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T12        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             | M   | T13        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T14        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T15        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T16        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T17        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T18        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             | F   | T19        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
| G II                        |     | T20        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
| (250 mg/kg body weight)     |     | T21        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T22        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T23        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |
|                             |     | T24        | 1 1 1 1 1 1 1 | 1 1 1 1 1 | 1 1 1 1 1 1 | 1 | 1 |

Keys: M = Male, F = Female, 1 = Normal.
## APPENDIX 1 (Continued)

| Group and Dose of Acetamide | Sex | Animal N° | Individual Animal Observations on Experimental Days | Before Dosing (Day 1) | After Dosing Day 1 (hours) | Before Dosing (Day 2) | After Dosing Day 2 (hours) | Before Sacrifice |
|-----------------------------|-----|-----------|----------------------------------------------------|-----------------------|---------------------------|-----------------------|---------------------------|-----------------|
|                             |     |           | Before Dosing (Day 1)                              | After Dosing (Day 1)  | Before Dosing (Day 2)     | After Dosing (Day 2)  | Before Sacrifice          |                 |
|                             |     |           | 1 2 3 4                                            | 1 2 3 4               | 1 2 3 4                   | 1 2 3 4               |                           |                 |
| G III (1000 mg/kg body weight) | M   | T25       |                                                      |                       |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             | F   | T31       |                                                      |                       |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
| G IV (2000 mg/kg body weight) | M   | T37       |                                                      |                       |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             | F   | T43       |                                                      |                       |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | 1 1 1 1                                            | 1 1 1 1               |                           |                       |                           |                 |
| G V Positive control Mitomycin-C (1.0 mg/kg body weight) | M   | T49       |                                                      |                       |                           |                       |                           |                 |
|                             |     |           | - - - -                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | - - - -                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | - - - -                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | - - - -                                            | 1 1 1 1               |                           |                       |                           |                 |
|                             |     |           | - - - -                                            | 1 1 1 1               |                           |                       |                           |                 |
| Keys: M = Male, F = Female, 1 = Normal, - = Not applicable (Animals of positive control group were not treated on day one).
### APPENDIX 1 (Continued)

| Group and Dose of Acetamide | Animal No | Sex | Clinical Signs Observed after Dosing on Before | After Dosing Day 1 (hours) | Before Dosing | After Dosing Day 2 (hours) | Before Sacrifice |
|-----------------------------|-----------|-----|---------------------------------|--------------------------|-------------|-----------------------------|-----------------|
|                             |           |     | Before Dosing (Day 1) | 1 2 3 4 | 1 2 3 4 | 1 2 3 4 | 1 2 3 4 |
| G V (Mitomycin-C, 1.0 mg/kg body weight) | T55       | F   | 1 | - - - - | 1 | 1 | 1 | 1 |
|                             | T56       | F   | 1 | - - - - | 1 | 1 | 1 | 1 |
|                             | T57       | F   | 1 | - - - - | 1 | 1 | 1 | 1 |
|                             | T58       | F   | 1 | - - - - | 1 | 1 | 1 | 1 |
|                             | T59       | F   | 1 | - - - - | 1 | 1 | 1 | 1 |
|                             | T60       | F   | 1 | - - - - | 1 | 1 | 1 | 1 |

Keys: F = Female, 1 = Normal, - = Not applicable (Animals of positive control group were not treated on day one).
# Micronucleus Test of Acetamide in Mice

## APPENDIX 2: Individual Body Temperature - Main Study

### Temperature Data – Male

| Group and Dose of Acetamide | Animal No. | Before Dosing (Day 1) | Day of Dosing | After Dosing Day - 1 (hours) | After Dosing (Day 2) | Before Sacrifice |
|----------------------------|------------|-----------------------|---------------|------------------------------|----------------------|-----------------|
|                            |            |                       |               | 2 h  | 5 h  | 2 h  | 5 h  |                       |
|                            |            |                       |               | °C   | °C   | °C   | °C   | °C                      |
| G I Vehicle control (Distilled water) | T1         | 37.4                  | 37.2          | 37.5 | 36.9 | 37.2 | 37.3 | 37.5                   |
|                             | T2         | 37.2                  | 36.7          | 37.3 | 37.0 | 37.3 | 37.0 | 37.6                   |
|                             | T3         | 37.0                  | 36.9          | 37.3 | 36.8 | 36.9 | 37.1 | 37.2                   |
|                             | T4         | 37.1                  | 37.3          | 37.4 | 37.3 | 37.3 | 36.8 | 37.1                   |
|                             | T5         | 37.3                  | 36.9          | 36.8 | 37.2 | 37.0 | 37.0 | 37.3                   |
|                             | T6         | 36.9                  | 36.8          | 37.1 | 37.3 | 37.2 | 36.9 | 37.5                   |
| G II (250 mg/kg body weight) | T13        | 37.4                  | 36.9          | 37.0 | 36.9 | 36.8 | 37.1 | 38.0                   |
|                             | T14        | 37.1                  | 36.7          | 36.9 | 36.8 | 36.9 | 37.2 | 37.1                   |
|                             | T15        | 37.2                  | 37.6          | 37.4 | 36.8 | 37.0 | 36.9 | 36.9                   |
|                             | T16        | 36.9                  | 37.2          | 37.3 | 37.2 | 37.2 | 37.0 | 37.4                   |
|                             | T17        | 37.3                  | 36.7          | 37.4 | 37.1 | 37.0 | 37.1 | 36.9                   |
|                             | T18        | 37.2                  | 36.6          | 37.3 | 37.2 | 37.0 | 37.4 | 37.5                   |
| G III (1000 mg/kg body weight) | T25        | 37.6                  | 37.2          | 37.4 | 37.4 | 37.4 | 37.3 | 37.3                   |
|                              | T26        | 37.0                  | 37.4          | 37.2 | 37.2 | 37.1 | 37.3 | 37.1                   |
|                              | T27        | 36.9                  | 37.3          | 37.0 | 37.1 | 36.7 | 36.8 | 37.5                   |
|                              | T28        | 37.3                  | 36.9          | 36.8 | 37.0 | 37.1 | 36.7 | 36.9                   |
|                              | T29        | 37.1                  | 36.5          | 37.2 | 37.2 | 37.2 | 36.8 | 37.1                   |
|                              | T30        | 37.4                  | 36.9          | 37.0 | 37.3 | 37.3 | 37.0 | 37.2                   |
| G IV (2000 mg/kg body weight) | T37        | 37.2                  | 36.4          | 36.5 | 37.2 | 37.3 | 36.8 | 37.2                   |
|                              | T38        | 36.9                  | 36.3          | 36.3 | 37.1 | 36.3 | 37.1 | 37.5                   |
|                              | T39        | 37.3                  | 36.6          | 37.4 | 37.1 | 37.1 | 36.9 | 37.3                   |
|                              | T40        | 37.1                  | 36.4          | 37.3 | 37.2 | 36.4 | 37.3 | 37.5                   |
|                              | T41        | 37.3                  | 36.5          | 37.2 | 37.1 | 36.4 | 37.1 | 37.2                   |
|                              | T42        | 37.0                  | 36.3          | 37.3 | 37.3 | 36.2 | 36.9 | 37.3                   |
| G V Positive control Mitomycin-C (1.0 mg/kg body weight) | T49        | -                     | -             | -    | 36.6 | 37.0 | 37.2 | 37.2                   |
|                              | T50        | -                     | -             | -    | 37.2 | 37.3 | 37.4 | 37.3                   |
|                              | T51        | -                     | -             | -    | 36.8 | 37.1 | 37.2 | 37.5                   |
|                              | T52        | -                     | -             | -    | 37.1 | 37.2 | 37.3 | 37.3                   |
|                              | T53        | -                     | -             | -    | 37.0 | 37.0 | 36.9 | 36.8                   |
|                              | T54        | -                     | -             | -    | 36.8 | 37.3 | 37.0 | 37.2                   |

Note: Range of microprobe thermometer is -100 °C to +200 °C.

Keys: °C = Degree Centigrade, h = Hour, - = Not applicable (Positive control animals were not treated on day one).
## APPENDIX 2 (Continued)

### Temperature Data – Female

| Group and Dose of Acetamide | Animal N° | Before Dosing (Day1) | After Dosing Day – 1 (hours) | Before Dosing (Day2) | After Dosing Day – 2 (hours) | Before Sacrifice |
|-----------------------------|-----------|----------------------|-------------------------------|----------------------|-------------------------------|-----------------|
|                             |           | °C                  | °C                           | °C                  | °C                           | °C              |
|                             |           | 2 h                 | 5 h                          | 2 h                 | 5 h                          |                 |
| G I Vehicle control (Distilled water) | T7 | 37.3 | 37.1 | 37.3 | 37.4 | 37.0 | 37.3 |
|                             | T8 | 37.5 | 37.2 | 37.5 | 37.0 | 37.2 | 37.3 |
|                             | T9 | 37.1 | 37.0 | 37.3 | 37.4 | 37.3 | 37.2 |
|                             | T10 | 37.3 | 37.1 | 37.1 | 37.4 | 36.9 | 37.3 |
|                             | T11 | 37.0 | 37.4 | 37.3 | 37.2 | 37.3 | 37.2 |
|                             | T12 | 37.2 | 37.3 | 37.3 | 37.1 | 37.1 | 37.4 |
| G II (250 mg/kg body weight) | T19 | 37.4 | 37.3 | 37.4 | 37.3 | 37.2 | 37.4 |
|                             | T20 | 37.2 | 37.2 | 37.1 | 37.4 | 37.0 | 37.2 |
|                             | T21 | 37.1 | 37.5 | 37.4 | 37.2 | 36.8 | 37.1 |
|                             | T22 | 37.0 | 36.8 | 37.3 | 37.2 | 36.8 | 37.2 |
|                             | T23 | 37.3 | 36.9 | 37.1 | 37.1 | 36.8 | 36.6 |
|                             | T24 | 37.1 | 37.1 | 37.3 | 37.2 | 37.3 | 37.4 |
| G III (1000 mg/kg body weight) | T31 | 37.3 | 37.1 | 37.3 | 37.3 | 37.0 | 37.3 |
|                             | T32 | 37.3 | 37.0 | 37.1 | 37.1 | 37.0 | 37.2 |
|                             | T33 | 37.0 | 36.9 | 37.3 | 37.0 | 37.2 | 37.1 |
|                             | T34 | 37.4 | 37.1 | 37.4 | 37.3 | 37.1 | 37.2 |
|                             | T35 | 37.1 | 37.4 | 37.3 | 37.0 | 36.9 | 37.3 |
|                             | T36 | 36.8 | 37.1 | 37.0 | 37.1 | 37.0 | 37.2 |
| G IV (2000 mg/kg body weight) | T43 | 37.3 | 37.3 | 37.4 | 37.1 | 37.0 | 37.2 |
|                             | T44 | 36.8 | 36.9 | 37.2 | 37.2 | 36.9 | 37.2 |
|                             | T45 | 37.1 | 37.5 | 37.3 | 37.3 | 37.3 | 37.2 |
|                             | T46 | 37.2 | 37.1 | 37.2 | 37.0 | 37.0 | 36.9 |
|                             | T47 | 37.0 | 37.4 | 37.4 | 36.9 | 37.2 | 36.8 |
|                             | T48 | 37.4 | 37.2 | 37.5 | 37.3 | 36.9 | 36.8 |
| G V Positive control Mitomycin-C (1.0 mg/kg body weight) | T55 | - | - | - | 36.9 | 36.7 | 37.1 |
|                             | T56 | - | - | - | 37.2 | 36.9 | 37.2 |
|                             | T57 | - | - | - | 37.0 | 37.1 | 37.2 |
|                             | T58 | - | - | - | 36.8 | 37.0 | 37.0 |
|                             | T59 | - | - | - | 37.2 | 37.0 | 37.3 |
|                             | T60 | - | - | - | 37.1 | 36.9 | 37.3 |

Note: Range of microprobe thermometer is -100 °C to +200 °C.

Keys: °C = Degree Centigrade, h = Hour, - = Not applicable (Animals of positive control group were not treated on day one)
## Micronucleus Test of Acetamide in Mice

### APPENDIX 3: Individual Body Weight (g) - Main Study

| Group and Dose of Acetamide | Sex                | Animal N° | Body Weight (g) | Before Sacrifice |
|-----------------------------|--------------------|-----------|-----------------|-----------------|
|                             |                    |           | Day 1 | Day 2 |                  |
|                             | Male               | T1        | 43    | 43    | 44               |
|                             |                    | T2        | 40    | 41    | 41               |
|                             |                    | T3        | 39    | 39    | 39               |
|                             |                    | T4        | 39    | 38    | 39               |
|                             |                    | T5        | 36    | 37    | 37               |
|                             |                    | T6        | 35    | 36    | 36               |
| G I                         | Female             | T7        | 31    | 31    | 29               |
| Vehicle control             |                    | T8        | 29    | 29    | 30               |
| (Distilled water)           |                    | T9        | 30    | 30    | 29               |
|                             |                    | T10       | 27    | 27    | 28               |
|                             |                    | T11       | 28    | 28    | 29               |
|                             |                    | T12       | 28    | 29    | 28               |
| G II                        | Male               | T13       | 43    | 42    | 42               |
| (250 mg/kg body weight)     |                    | T14       | 41    | 41    | 41               |
|                             |                    | T15       | 38    | 38    | 38               |
|                             |                    | T16       | 38    | 38    | 38               |
|                             |                    | T17       | 36    | 37    | 36               |
|                             |                    | T18       | 35    | 36    | 35               |
|                             | Female             | T19       | 30    | 30    | 29               |
|                             |                    | T20       | 30    | 30    | 28               |
|                             |                    | T21       | 29    | 29    | 30               |
|                             |                    | T22       | 28    | 29    | 29               |
|                             |                    | T23       | 29    | 30    | 31               |
|                             |                    | T24       | 27    | 27    | 28               |
| G III                       | Male               | T25       | 41    | 41    | 40               |
| (1000 mg/kg body weight)    |                    | T26       | 41    | 41    | 41               |
|                             |                    | T27       | 40    | 40    | 42               |
|                             |                    | T28       | 39    | 38    | 38               |
|                             |                    | T29       | 36    | 36    | 36               |
|                             |                    | T30       | 35    | 34    | 35               |
### APPENDIX 3 (Continued)

| Group and Dose of Acetamide | Sex   | Animal N° | Body Weight (g) |
|-----------------------------|-------|-----------|-----------------|
|                             |       |           | Before Sacrifice |
|                             |       | Day 1     | Day 2           |
| G III (1000 mg/kg body weight) | Female | T31 31 | 31 30            |
|                             |       | T32 32 | 31 31            |
|                             |       | T33 30 | 29 29            |
|                             |       | T34 28 | 28 29            |
|                             |       | T35 27 | 27 26            |
|                             |       | T36 28 | 28 29            |
| G IV (2000 mg/kg body weight) | Male   | T37 42 | 42 42            |
|                             |       | T38 42 | 42 42            |
|                             |       | T39 39 | 39 39            |
|                             |       | T40 38 | 39 39            |
|                             |       | T41 35 | 35 35            |
|                             |       | T42 33 | 33 34            |
|                             | Female | T43 - 42 | 41 41        |
|                             |       | T44 31 | 31 29            |
|                             |       | T45 30 | 29 30            |
|                             |       | T46 30 | 29 30            |
|                             |       | T47 28 | 27 28            |
|                             |       | T48 29 | 29 29            |
| G V (Mitomycin-C, 1.0 mg/kg body weight) | Male   | T49 - 42 | 41 41        |
|                             |       | T50 - 41 | 40 40        |
|                             |       | T51 - 39 | 40 40        |
|                             |       | T52 - 39 | 40 40        |
|                             |       | T53 - 39 | 39 39        |
|                             |       | T54 - 34 | 34 34        |
|                             | Female | T55 - 29 | 29 29        |
|                             |       | T56 - 31 | 31 31        |
|                             |       | T57 - 28 | 28 28        |
|                             |       | T58 - 29 | 29 29        |
|                             |       | T59 - 29 | 29 29        |
|                             |       | T60 - 26 | 26 26        |

Note: Body weight of positive control animals was not recorded on day one since positive control animals were not treated on day one.
Micronucleus Test of Acetamide in Mice

APPENDIX 4: Total Erythrocytes and P/E Ratio

| Group and Dose of Acetamide | Sex   | Animal No | Total PCE Scored | PCE Corr. to NCE | NCE Scored | Total Erythrocytes | P/E Ratio |
|-----------------------------|-------|-----------|------------------|------------------|------------|--------------------|-----------|
| G I                         | Male  | T1        | 4517             | 244              | 257        | 501                | 0.487     |
|                             |       | T2        | 4505             | 242              | 263        | 505                | 0.479     |
|                             |       | T3        | 4507             | 252              | 305        | 557                | 0.452     |
|                             |       | T4        | 4517             | 255              | 250        | 505                | 0.505     |
|                             |       | T5        | 4512             | 265              | 259        | 524                | 0.506     |
|                             |       | T6        | 4505             | 277              | 257        | 534                | 0.519     |
|                             | Female| T7        | 4510             | 264              | 267        | 531                | 0.497     |
|                             |       | T8        | 4520             | 294              | 257        | 551                | 0.534     |
|                             |       | T9        | 4505             | 271              | 254        | 525                | 0.516     |
|                             |       | T10       | 4519             | 258              | 257        | 515                | 0.501     |
|                             |       | T11       | 4515             | 263              | 251        | 514                | 0.512     |
|                             |       | T12       | 4508             | 306              | 236        | 542                | 0.565     |
| G II                        | Male  | T13       | 4513             | 260              | 273        | 533                | 0.488     |
|                             |       | T14       | 4511             | 262              | 256        | 518                | 0.506     |
|                             |       | T15       | 4508             | 244              | 262        | 506                | 0.482     |
|                             |       | T16       | 4514             | 255              | 293        | 548                | 0.465     |
|                             |       | T17       | 4506             | 292              | 250        | 542                | 0.539     |
|                             |       | T18       | 4508             | 249              | 253        | 502                | 0.496     |
|                             | Female| T19       | 4512             | 263              | 241        | 504                | 0.522     |
|                             |       | T20       | 4504             | 252              | 251        | 503                | 0.501     |
|                             |       | T21       | 4504             | 274              | 264        | 538                | 0.509     |
|                             |       | T22       | 4501             | 261              | 252        | 513                | 0.509     |
|                             |       | T23       | 4519             | 261              | 267        | 528                | 0.494     |
| G III                       | Male  | T24       | 4501             | 296              | 236        | 532                | 0.556     |
|                             |       | T25       | 4506             | 263              | 264        | 527                | 0.499     |
|                             |       | T26       | 4502             | 254              | 271        | 525                | 0.484     |
|                             |       | T27       | 4505             | 250              | 280        | 530                | 0.472     |
|                             |       | T28       | 4505             | 257              | 251        | 508                | 0.506     |
|                             |       | T29       | 4502             | 256              | 257        | 513                | 0.499     |
|                             |       | T30       | 4523             | 267              | 242        | 509                | 0.525     |

Note: Polychromatic erythrocytes corresponding to normochromatic erythrocytes were recorded (minimum 500 erythrocytes) for calculating the (P/E) ratio.

Keys: PCE = Polychromatic Erythrocytes, NCE = Normochromatic Erythrocytes.

P/E = Polychromatic Erythrocytes corresponding to Normochromatic Erythrocytes/Total Erythrocytes.
### APPENDIX 4 (Continued)

| Group and Dose of Acetamide | Sex | Animal № | Total PCE Scored | PCE Corr. to NCE | NCE Scored | Total Erythrocytes | P/E Ratio |
|-----------------------------|-----|----------|------------------|------------------|------------|-------------------|-----------|
| G III (1000 mg/kg body weight) | Female | T31 | 4511 | 277 | 277 | 554 | 0.500 |
| | | T32 | 4519 | 287 | 274 | 561 | 0.512 |
| | | T33 | 4516 | 272 | 246 | 518 | 0.525 |
| | | T34 | 4510 | 285 | 271 | 556 | 0.513 |
| | | T35 | 4511 | 303 | 217 | 520 | 0.583 |
| | | T36 | 4528 | 261 | 260 | 521 | 0.501 |
| | Male | T37 | 4527 | 280 | 254 | 534 | 0.524 |
| | | T38 | 4506 | 274 | 305 | 579 | 0.473 |
| | | T39 | 4513 | 268 | 288 | 556 | 0.482 |
| | | T40 | 4508 | 266 | 289 | 555 | 0.479 |
| | | T41 | 4519 | 272 | 300 | 572 | 0.476 |
| | | T42 | 4513 | 287 | 263 | 550 | 0.522 |
| | Female | T43 | 4521 | 303 | 259 | 562 | 0.539 |
| | | T44 | 4506 | 290 | 243 | 533 | 0.544 |
| | | T45 | 4505 | 262 | 256 | 518 | 0.506 |
| | | T46 | 4507 | 263 | 266 | 529 | 0.497 |
| | | T47 | 4506 | 247 | 261 | 508 | 0.486 |
| | | T48 | 4509 | 249 | 261 | 510 | 0.488 |
| | Male | T49 | 4525 | 304 | 225 | 529 | 0.575 |
| | | T50 | 4505 | 262 | 273 | 535 | 0.490 |
| | | T51 | 4519 | 283 | 275 | 558 | 0.507 |
| | | T52 | 4508 | 257 | 274 | 531 | 0.484 |
| | | T53 | 4562 | 279 | 240 | 519 | 0.538 |
| | | T54 | 4508 | 262 | 296 | 558 | 0.470 |
| | | T55 | 4507 | 278 | 284 | 562 | 0.495 |
| | | T56 | 4515 | 293 | 266 | 559 | 0.524 |
| | | T57 | 4509 | 250 | 273 | 523 | 0.478 |
| | | T58 | 4516 | 276 | 259 | 535 | 0.516 |
| | | T59 | 4509 | 259 | 279 | 538 | 0.481 |
| | | T60 | 4510 | 252 | 255 | 507 | 0.497 |

Note: Polychromatic erythrocytes corresponding to normochromatic erythrocytes were recorded (minimum 500 erythrocytes) for calculating the (P/E) ratio.

Keys: PCE = Polychromatic Erythrocytes, NCE = Normochromatic Erythrocytes, P/E = Polychromatic Erythrocytes corresponding to Normochromatic Erythrocytes/Total Erythrocyte.
## Micronucleus Test of Acetamide in Mice

### APPENDIX 5: Frequency of Micronucleated Polychromatic Erythrocytes

| Group and Dose of Acetamide | Sex | Animal No | Total Number of PCE Scored | Number of MNPCE | Percent MNPCE |
|-----------------------------|-----|-----------|-----------------------------|----------------|---------------|
|                             | Male|           |                             |                |               |
| G I (Vehicle control)       |     |           |                             |                |               |
|                             |     | T1        | 4517                        | 2              | 0.04          |
|                             |     | T2        | 4505                        | 0              | 0.00          |
|                             |     | T3        | 4507                        | 1              | 0.02          |
|                             |     | T4        | 4517                        | 1              | 0.02          |
|                             |     | T5        | 4512                        | 0              | 0.00          |
|                             |     | T6        | 4505                        | 1              | 0.02          |
|                             | Female| T7        | 4510                        | 1              | 0.02          |
|                             |     | T8        | 4520                        | 1              | 0.02          |
|                             |     | T9        | 4505                        | 1              | 0.02          |
|                             |     | T10       | 4519                        | 0              | 0.00          |
|                             |     | T11       | 4515                        | 0              | 0.00          |
|                             |     | T12       | 4508                        | 1              | 0.02          |
| G II (250 mg/kg body weight)|     | T13       | 4513                        | 1              | 0.02          |
|                             |     | T14       | 4511                        | 2              | 0.04          |
|                             |     | T15       | 4508                        | 1              | 0.02          |
|                             |     | T16       | 4514                        | 0              | 0.00          |
|                             |     | T17       | 4506                        | 0              | 0.00          |
|                             |     | T18       | 4508                        | 1              | 0.02          |
|                             | Female| T19       | 4512                        | 1              | 0.02          |
|                             |     | T20       | 4504                        | 0              | 0.00          |
|                             |     | T21       | 4504                        | 1              | 0.02          |
|                             |     | T22       | 4501                        | 2              | 0.04          |
|                             |     | T23       | 4519                        | 0              | 0.00          |
|                             |     | T24       | 4501                        | 3              | 0.07          |
| G III (1000 mg/kg body weight)|     | T25       | 4506                        | 0              | 0.00          |
|                             |     | T26       | 4502                        | 1              | 0.02          |
|                             |     | T27       | 4505                        | 0              | 0.00          |
|                             |     | T28       | 4505                        | 0              | 0.00          |
|                             |     | T29       | 4502                        | 1              | 0.02          |
|                             |     | T30       | 4523                        | 2              | 0.04          |

Keys: PCE = Polychromatic Erythrocytes, MNPCE = Micronucleated Polychromatic Erythrocytes, Percent MNPCE = MNPCE x 100/Total PCE.
### APPENDIX 5 (Continued)

| Group and Dose of Acetamide | Sex    | Animal No | Total Number of PCE Scored | Number of MNPCE | Percent MNPCE |
|-----------------------------|--------|-----------|----------------------------|----------------|---------------|
| G III (1000 mg/kg body weight) | Female | T31       | 4511                       | 0              | 0.00          |
|                             |        | T32       | 4519                       | 2              | 0.04          |
|                             |        | T33       | 4516                       | 1              | 0.02          |
|                             |        | T34       | 4510                       | 0              | 0.00          |
|                             |        | T35       | 4511                       | 2              | 0.04          |
|                             |        | T36       | 4528                       | 1              | 0.02          |
| G IV (2000 mg/kg body weight) | Male   | T37       | 4527                       | 2              | 0.04          |
|                             |        | T38       | 4506                       | 1              | 0.02          |
|                             |        | T39       | 4513                       | 1              | 0.02          |
|                             |        | T40       | 4508                       | 0              | 0.00          |
|                             | Female | T41       | 4519                       | 1              | 0.02          |
|                             |        | T42       | 4513                       | 0              | 0.00          |
|                             |        | T43       | 4521                       | 0              | 0.00          |
|                             |        | T44       | 4506                       | 1              | 0.02          |
|                             |        | T45       | 4505                       | 2              | 0.04          |
|                             |        | T46       | 4507                       | 1              | 0.02          |
|                             |        | T47       | 4506                       | 0              | 0.00          |
|                             |        | T48       | 4509                       | 1              | 0.02          |
| G V (Mitomycin-C, 1.0 mg/kg body weight) | Male   | T49       | 4525                       | 47             | 1.04          |
|                             |        | T50       | 4505                       | 40             | 0.89          |
|                             |        | T51       | 4519                       | 49             | 1.08          |
|                             |        | T52       | 4508                       | 82             | 1.82          |
|                             |        | T53       | 4562                       | 65             | 1.42          |
|                             |        | T54       | 4508                       | 72             | 1.60          |
|                             | Female | T55       | 4507                       | 65             | 1.44          |
|                             |        | T56       | 4515                       | 43             | 0.95          |
|                             |        | T57       | 4509                       | 57             | 1.26          |
|                             |        | T58       | 4516                       | 54             | 1.20          |
|                             |        | T59       | 4509                       | 48             | 1.06          |
|                             |        | T60       | 4510                       | 96             | 2.13          |

Keys: PCE = Polychromatic Erythrocytes, MNPCE = Micronucleated Polychromatic Erythrocytes and Percent MNPCE = MNPCE x 100/Total PCE
Micronucleus Test of Acetamide in Mice

APPENDIX 6: Signed Study Plan and Study Plan Amendment

STUDY PLAN

MICRONUCLEUS TEST OF ACETAMIDE IN MICE

GUIDELINES: OECD 474

SPONSOR

MICHIGAN STATE UNIVERSITY,
220 TROWBRIGE RD, EAST LANSING MI,
48824, UNITED STATES

STUDY DIRECTOR: AVANI K. SOLANKI

TEST FACILITY

JAI RESEARCH FOUNDATION
DEPARTMENT OF TOXICOLOGY
VALVADA - 396 105
DIST. VALSAD
GUJARAT
INDIA

AUGUST- 2017
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1. GENERAL INFORMATION

1.1 Study Director
Avani K. Solanki, M.Sc.

Deputy Study Director
Dr. Rajendra M. Nagane, M.V.Sc.

1.2 Test Facility Management
Dr. Manish V. Patel

1.3 Study Schedule
Study Initiation Date : August 30, 2017
Experiment Start Date : September 04, 2017
Experiment Completion : Latest by November 2017
Draft Report Submission : Latest by November 2017
Study Completion : Within two weeks from the date of receipt of comments on
the final draft report from the Sponsor.

1.4 Study Plan and Amendment (if any) Distribution
a. Original copy in Archive and study Sponsor; b. Photocopy to Study Director, QAU, and Residue
Chemistry.

2. INTRODUCTION

2.1 Objective
The objective of this study is to evaluate the micronucleus induction potential of acetamide in mice.

2.2 Regulatory Guidelines
This study is intended for regulatory submission and will be conducted in accordance with the known
requirement of international guidelines:

OECD, 2016: The Organisation for Economic Co-operation and Development (OECD) Guidelines for the
Testing of Chemicals, Volume II, OECD 474, Mammalian Erythrocyte Micronucleus Test, adopted by the
Council on July 29, 2016.
2.3 Principle of the Test Method

The mammalian micronucleus test is used to detect cytogenetic damage (which results in a chromosomal break, fragment or lagging whole chromosome) caused by the test item. The damaged chromosomal fragments remain in the anucleated cytoplasm of the erythrocyte and are visible, when stained, as a small round or oblong structure called a micronucleus during the maturation of erythrocytes. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage.

2.4 Test Item

The Test Item Data Sheet has been completed by the Sponsor. The representative sample of the test item will be retained for Archiving. Any residual test item will be disposed of at JRF after the expiry date unless otherwise instructed by the Sponsor. The test item procured from Tokyo Chemical Industry Co. Ltd on behalf of study sponsor. The details provided by the supplier are as below:

| Test Item Name       | Acetamide                  |
|----------------------|----------------------------|
| IUPAC Name           | Acetamide                  |
| CAS Number           | 60-35-5                    |
| Molecular Formula    | C$_2$H$_5$NO               |
| Molecular Weight     | 59.07 g/mol                |
| Molecular Structure  | ![](molecular_structure.png) |
| Batch/Lot Number     | QYD4G                      |
| Analyzed Purity/Concentration | 99.2% (Information provided by the Supplier, Tokyo Chemical Industry Co., Ltd. (TCI) via Certificate of Analysis) |
| Manufactured by      | Tokyo Chemical Industry Co. Ltd |
| Supplied to JRF by   | Procured by JRF from Tokyo Chemical Industry Co. Ltd on behalf of sponsor |
| Date of Receipt      | July 29, 2017              |
| Date of Expiry       | July 28, 2019*             |
| Appearance           | White solid                |
| Test Item Characterization under GLP | Yes, by Jai Research Foundation |
APPENDIX 6 (Continued)

| Storage Condition (at JRF) | As per the instruction received from the Supplier, TCI on storage of the test item, the test item will be stored:
| Storage Temperature | Room temperature |
| Storage Container | In original container as supplied by the Supplier |
| Storage condition | Store in its original container in isolated, dry, cool and well-ventilated area. |
| Storage Location | Test Item Control Office, JRF |

| JRF Test Item Code | ATM 700 |

*Note: Test item expiry date was not provided by the supplier. Hence expiry date was mentioned as per the JRF SOP No JRF/ARC/SOP-853, Issue No. Q.

**Source of Molecular Weight, Molecular Formula and Molecular Structure:** www.sigmaaldrich.com.

3. GOOD LABORATORY PRACTICE (GLP)

3.1 GLP Compliance

This study will be conducted in compliance with the OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17, N° 1, Environment Directorate, the Organisation for Economic Co-operation and Development, Paris (1998) and all subsequent OECD consensus documents.

3.2 Standard Operating Procedures (SOP)

Unless otherwise specified all procedures mentioned in the study plan are subject to detailed Standard Operating Procedures of Jai Research Foundation.

3.3 Amendment to Study Plan

This study plan may be subjected to amendment. Amendment to study plan, whether initiated by the Sponsor or the Study Director will be generated, authorized by the Study Director and will be sent to the Sponsor for approval.

In the event that circumstances dictate immediate action, the nature of these circumstances will be communicated to the Sponsor as soon as practicable (by telephone, facsimile transmission or e-mail) and will be confirmed as soon as possible by way of formal study plan amendment.

3.4 Deviation(s)

Any deviation(s) will be documented in the study file and reported in the study report.
3.5 Quality Assurance
This study plan has been verified by JRF Quality Assurance Unit (QAU) and documented (Number 94462). The QAU JRF will inspect the critical phase(s) of the study by study based inspection and/or process based inspection. The raw data, draft and final reports will be audited to ensure that the final report accurately reflects the raw data. The audit/inspection reports will be provided to the Study Director and the Test Facility Management. The date of audits/inspections and reporting of findings to the Study Director and the Test Facility Management will be incorporated in the study report.

4. ANIMAL WELFARE
The study will be undertaken in compliance with the ‘Guidelines for Laboratory Animals Facility’ issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. These guidelines promote the humane care of animals used in research by providing specifications that will enhance animal well-being and experimental quality for the advancement of biological knowledge that is relevant to humans and animals.

Jai Research Foundation is committed to enhancing animal welfare and ensures that studies are designed and conducted to cause the minimum suffering or distress to animals, consistent with the scientific objectives and in accordance with Jai Research Foundation’s policy on animal welfare.

Project proposal for the experimentation is subject to the approval by the Institutional Animal Ethics Committee (IAEC), Jai Research Foundation.

JRF is accredited with Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) that promotes the human treatment of animals in science.

4.1 Humane Endpoint
Moribund animals or animals obviously in pain or showing signs of severe and enduring distress shall be humanely killed. Depending on the time, since dose administration, and the circumstances of death, the bone marrow may be removed and used as part of the interpretation of the results, (at the discretion of the study director).

5. EXPERIMENTAL PROCEDURE
5.1 Initial Considerations
Test item, at doses, that causes marked pain and distress due to corrosive or severely irritant actions, will not be administered. If required, study will be terminated.
5.2 **Reason for Selection of the Test System**

The mouse is selected as a test system because it is readily available laboratory rodent species. It has been shown to be a suitable model for mutagenicity studies and is also recommended by the OECD and other regulatory authorities. The results of this study are believed to be of value in predicting the potential of the test item to cause cytogenetic damage in humans.

5.3 **Animals**

Healthy and young Swiss albino mice or Hsd:ICR(CD1) (*Mus musculus*) will be obtained from the Animal Breeding Facility, JRF or any other CPCSEA approved source. The animals, 6 – 10 weeks old on the first day of dosing will be used in the study. The female mice used will be nulliparous and non-pregnant. Body weight variation among the animals should not exceed ±20% of the mean body weight for each sex at the time of initiation of dosing. The animals will be identified with unique numbers by tattooing.

5.4 **Acclimatisation**

The animals, after veterinary examination for good health, will be acclimatised to the laboratory conditions for a minimum period of 5 days prior to commencement of treatment and they will be observed for clinical symptoms daily. After acclimatisation, the animals will be randomized using Censored Randomization Method (Gad S.C. and Weil C.S., 1994) using validated in-house developed software.

5.5 **Housing and Animal Identification**

The mice will be housed (no more than four per cage) in polypropylene mice cages provided with rice husk as the bedding material. Each day cages will be supplied with a polypropylene water bottle fitted with a stainless steel nozzle.

Individual mouse will be identified with a unique number tattooed on the tail using a tattoo machine. The cages will be labeled with details of the study number, test item code, group number, sex, dose, type of study, cage number, and animal numbers. The labels used will be of different colours for different dose groups.

5.6 **Animal Room Sanitation**

Each day, the floor of the experimental procedure room will be swept and all worktops and the floor will be mopped with disinfectant solution.
5.7 Feed and Water
The mice will be provided with laboratory mice pellet feed (mice standard feed) and reverse osmosis water, filtered through reverse osmosis water purification system, *ad libitum*.

5.8 Environmental Conditions
The temperature of the experimental procedure room will be maintained at 22 ± 3 °C and the relative humidity between 40 and 70%. The photoperiod will be 12 h light and 12 h darkness, light hours being 06:00 – 18:00 h approximately, and air exchanges will be a minimum of 15 volumes/hour.

5.9 Selection of Vehicle
Acetamide is highly water soluble, so solubility will be first tested with distilled water first. In case of insolubility, test item will be suspended in vegetable oil or 0.5% carboxymethyl cellulose (CMC), unless otherwise recommended by sponsor. Fresh dose formulations will be prepared daily and administered within 2 hours of preparation. The concentration of the test item will be adjusted so as to permit constant dosing volume. All animals will receive a single standard volume of 10 mL/kg body weight by oral gavage administration. Vehicle control animals will receive the vehicle alone.

5.10 Dose Formulation Preparation, Sampling and Analysis
Since test item will be prepared freshly and will be used within 2 hours of preparation, stability of the test item in the selected vehicle will not be tested separately.

Dose formulation will be prepared as per JRF/TOX/SOP-260 and JRF/TOX/SOP-266. For active ingredient concentration and homogeneity (in case of suspension) analysis, required samples will be collected from the prepared dose formulations (high, mid, and low dose) along with vehicle during the main study following the detailed procedures below. The size of samples will be determined by the study director/study person for feasibility considerations and to allow sufficient amount for analysis.

If dose formulations are solutions, required aliquots of the vehicle and all dose formulations will be collected from the middle portion. If dosing formulations are suspensions, aliquots from the top (T), middle (M), and bottom (B) of required dose formulation will be collected for homogeneity and concentration verification immediately following the preparation of the dose formulation during main study. The vehicle control will be sampled from the middle portion only.

In all cases, 2 sets of samples per dose formulation will be collected: the 1st set of aliquots of selected dose formulations will be analyzed for homogeneity (in case of suspension) and active ingredient concentration. The 2nd set of aliquots of selected dose formulation will be stored in the deep freezer (-70 ± 10 °C) at JRF as backup and will be analyzed only if needed.
Unless otherwise requested by the Sponsor, required samples will be collected from any partial retest of main study. These samples will be held at JRF as backup and only analyzed as would be required in the amendment. Any repetition of the affected portion of the study will be specified by study plan amendment. In all cases, any unused aliquots will be discarded after receiving approval for finalization of the report from the sponsor.

All analytical work will be conducted by the Department of Chemistry, JRF, under GLP compliance. The detailed method together with the sample preparation procedure will be fully documented in the study records and described in the final report. Analytical parameters used for analysis of prepared dose formulations for the active ingredient will be added through study plan amendment. All unused samples will be handled as per the relevant Standard Operating Procedures.

5.10.1 Analytical Acceptance Criteria

The acceptable specification for the concentration of the test item in the dose formulation will be as described/mentioned below:

Solutions: 90 to 110% of nominal with <10% coefficient of variance (% CV) of each concentration.

Suspensions: 85 to 115% of nominal with <10% coefficient of variance (% CV) of each concentration.

In the event of a sample being outside the acceptable specification range, the study director will:

a) Justify the acceptability of the results,

b) Suggest re-analysis of the backup samples, or

c) Retest the affected portion of the study.

5.11 Main Study

Five groups (comprising 6 animals/sex/group) will be used for this study. Group I will serve as the vehicle control, Group II (250 mg/kg), III (1000 mg/kg) and IV (2000 mg/kg) will be low, mid and high dose groups, respectively. Group V will be the positive control and will receive mitomycin-C (1.0 mg/kg body weight on Day 2 of treatment) in distilled water by the intraperitoneal route on a single occasion. The rectal temperature of the treated animals will be monitored during the main study using digital laboratory thermometer. The temperatures will generally be measured before dosing (Day 1), approximately 2, 5 and 24 hours after each dosing. Dose levels are selected based on sponsor’s suggestions and the published data from earlier studies (Michael R. et al., 2014, Chieli et al., 1987, Mirkova, 1996 and Dybing et al., 1987).
Mortality, severity of clinical symptoms, change in body temperature for up to 48 h after the initial dose and reduction in the immature to total (immature + mature) erythrocyte ratio will be considered for the evaluation of toxicity to bone marrow. For changes in thermal regulation, the body temperature rise by, at least, 1 °C or fall by, at least, 3 °C for five or more hours will be declared as having exceeded MTD. Body temperature changes, outside this range, have been previously reported to cause an increase in micronucleus formation in absence of chemical treatment (Asanami and Shimono, 1997; Asanami et al., 1998).

5.12 Evidence of Tissue Exposure

To demonstrate target organ exposure, plasma analysis will be performed along with main study to demonstrate the absorption of test item after oral dosing as well as to demonstrate the target organ exposure i.e. test item concentration in the blood samples.

It may be inferred from the published literature (Zhao et al., 2007; Putcha, Griffith and Feldman (1984)), that (i) acetamide fed orally to rats and mice is likely to rapidly reach the bloodstream and be transported throughout the body, (ii) at the proposed regimen of two daily back-to-back doses followed by bone marrow harvest around 24 hours after last dose, significant exposure of acetamide is expected to occur, and (iii) determination of acetamide levels in blood plasma should be a suitable method to provide evidence of exposure for the micronucleus assays.

Therefore in this study, blood samples will be withdrawn from each animal in each treatment group and vehicle control group at the time of sacrifice before bone marrow collection. Blood samples will be collected in heparinised (20 IU/mL) micro-centrifuge tubes. Blood samples will be collected from orbital plexus under very light isoflurane anesthesia. To separate out the plasma, blood samples will be centrifuged at 3000 rpm for 15 minutes at 4 °C. The plasma samples will be stored at -70 ± 10 °C until analysis. The plasma samples will be analysed for determination of test item concentration at Department of Chemistry, JRF. The details of bioanalytical method and results will be presented in the report. Bioanalytical parameters used for analysis of plasma samples will be added through study plan amendment.
5.13 **Study Performance**

The test item will be dissolved or suspended in a selected vehicle (Gad and Cassidy, 2006). Fresh dose formulation will be prepared, on the day of dose administration. Animals will be fasted prior to dosing (feed, but not water, will be withheld for approximately 2-3 hours). Animals will be dosed (10 mL/kg body weight) by oral intubation for 2 consecutive days, approximately 24 hours (±1 hour) apart. The body weight will be recorded prior to the dosing on each day and also before sacrifice. Clinical signs will be recorded after dosing, each day, and before sacrifice. The animals will be sacrificed by CO₂ asphyxiation between 18 and 24 h following the last treatment of the test item (MacGregor *et al.*, 1987). Animals in the positive control group will be sacrificed by CO₂ asphyxiation approximately 24 hours after the last treatment (Krishna and Hayashi, 2000). Femur bones from the sacrificed animals will be excised and the epicondyle tips will be removed. The bone marrow content will be expelled by flushing with foetal bovine serum. The aspirated bone marrow content will be mixed using a syringe to dissociate the cells. Cell clump formation will be avoided, and the content will be centrifuged. The supernatant will be discarded. A minimum number of two slides will be prepared, per animal, with the cell pellet, fixed with methanol. Slides will be stained using 5% Giemsa (Heddle *et al.*, 1984). In order to prevent bias in the scoring procedure, the slide numbers will be masked with code numbers provided by the Department of Biostatistics and Systems Information, Jai Research Foundation.

5.14 **Historical control data**

Jai Research Foundation (JRF) has conducted more than 500 GLP studies for regulatory submission as per OECD TG 474 and established a strong historical control data base. JRF uses quality control methods, such as control charts to identify data variability and to show that the methodology is 'under control'. Quality control charts (QC charts) will be added in the report demonstrating the JRF's established historical positive control ranges and distribution, and a historical negative control ranges and distribution.

5.15 **Microscopic Observation**

Slides will be observed under a light microscope. The proportion of immature erythrocytes among the total (immature + mature), i.e., P/E ratio will be determined for each animal by counting a minimum of 500 erythrocytes. A minimum of 4500 polychromatic (immature) erythrocytes, per animal, will be scored for the incidence of micronuclei. Additional information may be obtained by scoring mature erythrocytes for micronuclei.
5.16 Statistical Analysis

The data of percent micronucleated polychromatic erythrocytes (% MNPCE), P/E ratio and body weight for both the sexes will be subjected to normality test using Shapiro-Wilk’s test and Bartlett’s test to assess homogeneity of variance. The data will be analyzed by Chi-square and Fisher’s exact test or Student's t-test depending on the nature of the data (Richardson, C. et al., 1989). If the data do show suitable homogeneity of variance, the data will be subjected to Analysis of Variance (ANOVA) followed by Dunnett’s t-test (Gad and Weil, 1994). Depending upon the nature of data non-parametric tests will be performed if applicable. If increase in % micronucleated cells are statistically significant, then dose response will be evaluated with an appropriate trend test i.e. Chi-square trend analysis.

5.17 Assay Acceptance and Evaluation Criteria

5.17.1 Acceptance Criteria

The study will be considered valid as the following criteria are met:

i. The vehicle (or negative) controls should be in the range of historical control data.

ii. The positive controls should produce responses that are compatible with that of the historical data and should produce statistically significant responses compared with the concurrent negative control.

iii. Appropriate number of animals, doses and cells has been analysed.

iv. A minimum of three treatment groups including controls are analysed if the test item produces toxicity.

v. The highest dose should be a limit dose, maximum tolerable dose (MTD) without causing distress or death to the animal or produce toxicity to bone marrow.

vi. The PCE to total erythrocyte ratio should not be less than 20 % of the negative control.

5.17.2 Evaluation and Interpretation Criteria

After fulfilling the acceptability criteria, the test item will be considered clearly positive if:

i. At least one of the treatment groups exhibits statistically significant increase in the frequency of micronucleated polychromatic erythrocytes compared to concurrent negative control.

ii. A positive result is defined as a dose-dependent, significant increase in the incidence of micronuclei when evaluated with an appropriate trend test e.g. Chi-square trend analysis.

iii. Statistical and biological relevance will be considered in data interpretation.

iv. Any of the results falling outside the distribution of the historical negative control data i.e. Poisson based 95% control limits.
The test item will be considered clearly negative, if, in all experimental conditions examined:

i. None of the treatment groups exhibits a statistically significant increase in the frequency of micronucleated immature erythrocytes compared with the concurrent negative control.

ii. There is no dose-related increase at any sampling time when evaluated by an appropriate trend test.

iii. All results are inside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits), and

iv. Bone marrow exposure to the test item(s) occurred

v. There is no requirement for verification of a clear positive or clear negative response.

6. REPORT

Unless otherwise instructed by the Sponsor, one copy of the final report will be issued along with one soft copy in PDF. The report will include the following information:

Summary

Test item:
- Identification and CAS number, if known
- Physical nature and purity
- Phys-chem. properties relevant to the conduct of the study
- Stability of the test item, if known
- Source, lot number, limit date for use, if known

Vehicle:
- Justification for choice of vehicle
- Solubility of the test item in vehicle

Test Animals:
- Species and strain of animals used
- Number, age and sex of animals
- Source and housing conditions, diet, etc.
- Method for uniquely identifying animals
- Individual weight of the animals at the start of the experiment, including body weight range, mean and standard deviation for each group

Test conditions:
- Positive and negative (vehicle/solvent) control data
- Data from range-finding study, if conducted
- Rationale for dose level selection
- Details of dose preparation
- Details of the administration of the test item
- Rationale for route and duration of administration
- Methods for verifying that the test substance(s) reached the general circulation or target tissue;
- Detailed description of treatment and sampling schedules
- Method of euthanasia
- Methods of slide preparation
- Methods for measurement of toxicity
- Criteria for scoring micronucleated immature erythrocytes
- Number of cells analysed per animal
- Criteria for acceptability of the study
- Criteria for considering studies as positive, negative or equivocal

Results

- Animal conditions, prior to and throughout test period
- Signs of toxicity
- Proportion of immature erythrocytes among total erythrocytes
- Number of micronucleated immature erythrocytes, given separately for each animal
- Mean ± standard deviation of micronucleated immature erythrocytes per group
- Dose-response relationship, where possible
- Statistical analyses and method applied
- Concurrent negative and positive control data
- Historical control data ranges of P/E ratio and % MNPCE for male and female, with ranges, means, standard deviations, and 95% control limits for the distribution, as well as the time period covered and the number of data points
- Data supporting exposure of the bone marrow occurred
- Criteria for positive or negative responses that are met

Discussion of the results

Conclusion

Dose formulation analysis report

Signed study plan and study plan amendment(s) (if any)

Record of deviation(s) (if any)

References
7. ARCHIVES

On completion of the study all original raw data including any storage medium for electronically recorded data, documentation, the signed study plan, the study plan amendment, slides, the draft report, one original final report, and the representative sample of the test item will be retained in the GLP Archives at Jai Research Foundation for a period of ten years. At the end of this period, the Sponsor’s instructions will be sought to either extend the archiving period or return the archived material to the Sponsor or dispose of the material.

8. REFERENCES

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Whitmire, M.L., Bryan, P., Henry, T.R., Holbrook, J., Lehman, P., Mollitor, T., Ohorodnik, S., Reed, D., and Wietgrefe, H.D., 2010: “Nonclinical dose formulation analysis method validation and sample analysis”. The AAPS Journal, 12(4), pp. 628-34.

Zhao, D et al., 2007: Comparative transport efficiencies of urea analogues through urea transporter UT-B, Biochimica et Biophysica Acta 1768 (2007), ScienceDirect, pp. 1815-1821.
9. STUDY PLAN APPROVAL

We, the undersigned have read the whole study plan for, “Micronucleus Test of Acetamide in Mice” and confirm that the study will be performed as per this study plan.

Study Director : AVANI K. SOLANKI

Test Facility Management : DR. MANISH V. PATEL

For Study Sponsor : MICHIGAN STATE UNIVERSITY, UNITED STATES

Name of the Sponsor’s Representative : DR. V. BRINGAL

Signature and Date : W.R. 30th, August 2017
## APPENDIX 6 (Continued)

### STUDY PLAN / PROTOCOL AMENDMENT RECORD

| STUDY N°   | AMENDMENT N° | EFFECTIVE DATE |
|------------|--------------|----------------|
| 485-1-06-17727 | 1            | September 18, 2017 |

| STUDY TITLE | Micronucleus Test of Acetamide in Mice |
|-------------|----------------------------------------|

| ORIGINAL DETAILS* | DETAILS AMENDED                                                                 | REASON FOR AMENDMENT |
|------------------|---------------------------------------------------------------------------------|----------------------|
| **5.10 Dose Formulation Preparation, Sampling and Analysis** |                                                                                       |                      |
| Study Plan Page 9 of 18 | Addition of analytical method parameters for dose formulation analysis. |
|                     | Analytical method parameters (JRF Study N°: 228-2-14-17729)                     |                      |
|                     | Instrument : GC-MS                                                              |                      |
|                     | Column : Agilent VF-5MS, 0.25 mm (i.d.), 30m length, 0.25 μm film thickness     |                      |
|                     | Carrier Gas : Helium                                                           |                      |
|                     | Injection Volume : 2.0 μL                                                      |                      |
|                     | Injection Temperature : 250 °C                                                 |                      |
|                     | Flow Rate : 1.2 mL/minute                                                      |                      |
|                     | Split Ratio : 1:8                                                              |                      |
|                     | Oven Temperature : 40 °C (Hold 2.0 min.) to 20.0 °C to 300 °C, (hold for 10 minutes) – Total of 25 minutes |                      |
|                     | Mass Spectrometry : Electron Ionization mode with 70 eV SIM Mode             |                      |
|                     | Solvent Delay Time : 4.0 minutes                                               |                      |
|                     | Quadrupole Temperature : 150 °C                                                |                      |
|                     | Data Acquisition : Selected Ion Monitoring (SIM) for masses 239 (Xanthyl-acetamide) and 253 (Xanthyl-Propionamide) |                      |

| **5.12 Evidence of Tissue Exposure** |                                                                                       |                      |
| Study Plan Page 11 of 18 | Addition of analytical method parameters for plasma sample analysis. |
| | Analytical method parameters (JRF Study N°: 228-2-14-18476)                     |                      |
| | Instrument : GC-MS                                                              |                      |
| | Column : Agilent VF-5MS, 0.25 mm (i.d.), 30m length, 0.25 μm film thickness     |                      |
| | Carrier Gas : Helium                                                           |                      |
| | Injection Volume : 2.0 μL                                                      |                      |
| | Injection Temperature : 250 °C                                                 |                      |
| | Flow Rate : 1.2 mL/minute                                                      |                      |
| | Split Ratio : 1:8                                                              |                      |
| | Oven Temperature : 40 °C (Hold 2.0 minute) to 20.0 °C to 300 °C, (hold for 10 minutes) – Total of 25 minutes |                      |
| | Mass Spectrometry : Electron Ionization mode with 70 eV                       |                      |
| | Data Acquisition : Selected Ion Monitoring (SIM) for masses 239 (Xanthyl-acetamide), 242 (Xanthyl-3d-acetamide) and 253 (Xanthyl-Propionamide) |                      |

* Reference of page N°, paragraph number etc.
### STUDY PLAN / PROTOCOL AMENDMENT RECORD (Continued)

| STUDY Nº | 485-1-06-17727 | AMENDMENT Nº | 1 | EFFECTIVE DATE | September 18, 2017 |
|----------|----------------|-------------|---|----------------|-------------------|
| STUDY TITLE | Micronucleus Test of Acetamide in Mice |
| REVIEWED BY (QAU) | Hemaangi Patel | [Signature & Date: September 18, 2013] |
| AUTHORISED BY | For JRF | For SPONSOR (S) |
| Study Director | [Signature & Date: September 18, 2013] Name: Avani K. Solanki | MICHIGAN STATE UNIVERSITY, 220 TROWBRIGE RD, EAST LANSING MI, 48824, UNITED STATES |
| Facility Management | [Signature & Date: September 18, 2017] Name: Dr. Manish V. Patel | [Signature & Date: September 19, 2017] |

The sponsor is requested to send one original, signed copy of the amendment to JRF.

Amendment Distribution: Archives (original) and photocopy to all the copy holders of study plan/protocol

JRF/GEN/F 376
### STUDY PLAN / PROTOCOL AMENDMENT RECORD

| STUDY N° | AMENDMENT N° | EFFECTIVE DATE | STUDY TITLE |
|----------|--------------|----------------|-------------|
| 485-1-06-17727 | 2 | November 09, 2017 | Micronucleus Test of Acetamide in Mice |

**ORIGINAL DETAILS***

- Study Plan Page 5 of 18
- 2.4 Test Item
- Date of Expiry: July 28, 2019

**DETAILS AMENDED**

- Retest Date: December 03, 2017
- Addition
- Analysed Purity (Generated at JRF): 99.198% w/w

**REASON FOR AMENDMENT**

- Retest date was assigned and analysed purity have been added based on results of Test item characterisation at JRF (JRF study N° 228-2-14-17729).

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* Reference of page N°, paragraph number etc.

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**REVIEWED BY (QAU)**

- Smf J. Patel
  - Signature & Date: November 07, 2017

**AUTHORISED BY**

| For JRF | For SPONSOR (S) |
|---------|-----------------|
| Study Director | MICHIGAN STATE UNIVERSITY, 220 TROWBRIDGE RD, EAST LANSING MI, 48824, UNITED STATES |
  - Signature & Date: November 09, 2017
  - Name: Avani K. Solanki |
| Facility Management | Signature & Date: November 10, 2017 |
  - Name: Dr. Nadeem Ahmad Khan |

The sponsor is requested to send one original, signed copy of the amendment to JRF.
Amendment Distribution: Archives (original) and photocopy to all the copy holders of study plan/protocol JRF/GEN/F 376
Micronucleus Test of Acetamide in Mice

APPENDIX 7: Dose Formulation Analysis and Plasma Sample Analysis Report

ABHISHEK TATER
ANALYST
APPENDIX 7 (Continued)

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ABBREVIATIONS

% - Percent
°C - Degree Centigrade
CC - Calibration Curve
µL - Microlitre(s)
AR - Analytical Reagent
C\text{max} - Maximum observed/peak plasma concentration
Conc. - Concentration
DQC - Dilution Quality Control
g - Gram(s)
GC-MS - Gas Chromatography-Mass Spectrometer
HQC - High Quality Control
i.d. - Internal diameter
ID - Identification
IS - Internal Standard
kg - Kilogram
L - Litre(s)
LLOQ - Lower Limit of Quantification
LQC - Low Quality Control
Mg - Milligram(s)
min. - Minute
mL - Millilitre(s)
mm - Millimeter
MQC - Mid Quality Control
N° - Number
RE - Relative Error
RSD - Relative Standard Deviation
SD - Standard Deviation
SS - Spiking Solution
T\text{max} - Time of maximum observed peak plasma concentration
ULOQ - Upper Limit of Quantification
SUMMARY

A. Objective
The objective of the analysis was to estimate the concentration of acetamide in dose formulation and plasma of different groups of healthy, young adult mice Hsd:ICR(CD1) (Mus musculus) strain treated with acetamide by GC-MS. Plasma analysis were performed to demonstrate the absorption of test item after oral dosing as well as to demonstrate the target organ exposure i.e. test item concentration in the blood samples.

B. Dose formulation analysis
Dose formulation was prepared as per JRF/TOX/SOP-260 and JRF/TOX/SOP-266. For active ingredient concentration and homogeneity analysis, samples were collected from the prepared dose formulations (high, mid, and low dose) along with vehicle. Dose formulations were aliquoted from the upper (T), middle (M), and bottom (B) portion for homogeneity and concentration verification immediately following the preparation of the dose formulation during study. The vehicle control was sampled from the middle portion only. Mean recovery (%) was obtained as per below table:

| Dose level and concentration (mg/mL) | Replication | Fortification level (mg/mL) | Mean Recovery (%) | %CV |
|-------------------------------------|-------------|-----------------------------|-------------------|-----|
| Vehicle Control G1 (0.0)            |             | 0.0                         | -                 | -   |
| Low dose G2 (25)                    | MR1         | 25.00                       | 99.69             | 7.56|
|                                     | MR2         |                             |                   |     |
|                                     | MR3         |                             |                   |     |
| Middle dose G3 (100)                | MR1         | 100.00                      | 100.57            | 3.61|
|                                     | MR2         |                             |                   |     |
|                                     | MR3         |                             |                   |     |
| High dose G4 (200)                  | MR1         | 200.00                      | 105.41            | 6.73|
|                                     | MR2         |                             |                   |     |
|                                     | MR3         |                             |                   |     |

A calibration curve of acetamide considered as reference standard concentration ranging from 1.01 to 50.59 ppm was prepared for dose formulation analysis. The coefficient of determination ($r^2$) was 0.99968339 (acceptance criteria: $r^2 \geq 0.98$).
C. Plasma sample analysis

Five groups (comprising 6 animals/sex/group) were used for this study. Group I served as the vehicle control, Group II (250 mg/kg), III (1000 mg/kg) and IV (2000 mg/kg) were low, mid and high dose groups, respectively. Group V was the positive control and received mitomycin-C (1.0 mg/kg body weight on Day 2 of treatment) in distilled water by the intraperitoneal route on a single occasion. Blood samples were withdrawn from each animal in each treatment group and vehicle control group at the time of sacrifice before bone marrow collection. Blood samples were collected in heparinised (20 IU/mL) micro-centrifuge tubes. Blood samples were collected from orbital plexus under very light isoflurane anesthesia. To separate out the plasma, blood samples were centrifuged at 3000 rpm for 15 minutes at 4 °C. The plasma samples were stored at -70 ± 10 °C until analysis. The experimental outline and samples details are as below:

| Group N° | Dose (mg/kg b.wt.) | Animal N° | Group N° | Dose (mg/kg b.wt.) | Animal N° |
|----------|--------------------|-----------|----------|--------------------|-----------|
|          | Vehicle control    | Male      | Female   |                    | Male      | Female   |
| GI       |                    | 1         | 7        | GIII               | 1000.0    |
|          |                    | 2         | 8        |                    | 25        | 31       |
|          |                    | 3         | 9        |                    | 26        | 32       |
|          |                    | 4         | 10       |                    | 27        | 33       |
|          |                    | 5         | 11       |                    | 28        | 34       |
|          |                    | 6         | 12       |                    | 29        | 35       |
|          | 250.0              | 13        | 19       | GIV                | 2000.0    |
|          |                    | 14        | 20       |                    | 37        | 43       |
|          |                    | 15        | 21       |                    | 38        | 44       |
|          |                    | 16        | 22       |                    | 39        | 45       |
|          |                    | 17        | 23       |                    | 40        | 46       |
|          |                    | 18        | 24       |                    | 41        | 47       |
|          |                    |           |          |                    | 42        | 48       |

Calibration curve of acetamide-2-2-2-D3 reference standard concentration ranging from 0.101 to 50.633 ppm was prepared for plasma sample analysis. The coefficient of determination ($r^2$) was between 0.99559295 to 0.99838482 during all the analytical runs (acceptance criteria: $r^2 \geq 0.98$).
The dose formulation analysis was performed following the validated method (JRF Study N° 228-2-14-17729; “Validation of Analytical method for Determination of Acetamide Concentration, Homogeneity and Stability in Vehicle”).

The plasma samples analysis was performed following the validated method (JRF Study N°228-2-14-18476; “Validation of Bioanalytical Method for Determination of Acetamide Concentration using Acetamide-D3 as Reference Standard in Mice and Rat Plasma”). Concentrations were obtained as per below table:

| Animal N° | Sex | Concentration (ppm) | Animal N° | Sex | Concentration (ppm) |
|-----------|-----|---------------------|-----------|-----|---------------------|
| T1        | M   | 0.269               | T13       | M   | 48.667              |
| T2        | M   | 0.334               | T14       | M   | 52.371              |
| T3        | M   | 0.374               | T15       | M   | 41.246              |
| T4        | M   | 0.585               | T16       | M   | 12.423              |
| T5        | M   | 0.370               | T17       | M   | 29.650              |
| T6        | M   | 0.432               | T18       | M   | 29.964              |
| T7        | F   | 0.399               | T19       | M   | 3.677               |
| T8        | F   | 0.401               | T20       | M   | 28.466              |
| T9        | F   | 0.589               | T21       | M   | 6.538               |
| T10       | F   | 0.486               | T22       | M   | 10.573              |
| T11       | F   | 0.374               | T23       | M   | 1.903               |
| T12       | F   | 0.403               | T24       | M   | 14.543              |

| Animal N° | Sex | Concentration (ppm) | Animal N° | Sex | Concentration (ppm) |
|-----------|-----|---------------------|-----------|-----|---------------------|
| T25       | M   | 323.102             | T37       | M   | 102.238             |
| T26       | M   | 89.722              | T38       | M   | 96.394              |
| T27       | M   | 62.629              | T39       | M   | 220.194             |
| T28       | M   | 255.482             | T40       | M   | 187.772             |
| T29       | M   | 91.289              | T41       | M   | 177.226             |
| T30       | M   | 205.819             | T42       | M   | 316.967             |
| T31       | F   | 44.515              | T43       | F   | 151.431             |
| T32       | F   | 120.778             | T44       | F   | 127.048             |
| T33       | F   | 142.52              | T45       | F   | 68.048              |
| T34       | F   | 47.157              | T46       | F   | 198.855             |
| T35       | F   | 30.879              | T47       | F   | 105.888             |
| T36       | F   | 23.310              | T48       | F   | 37.282              |
1. INTRODUCTION

1.1 Dose formulation analysis

The objective of the analysis was to estimate the concentration of acetamide in dose formulation by GC-MS for JRF Study Number: 485-1-06-17727.

The samples analysis details are provided below:

| Sample analysis                  | Date of samples analyses |
|----------------------------------|--------------------------|
| Dose formulation analysis        | September 18, 2017       |

1.2 Plasma sample analysis

The objective of the analysis was to estimate the concentration of acetamide in the plasma of different groups of healthy, young adult mice Hsd:ICR(CD1) (*Mus musculus*) strain treated with acetamide by GC-MS for JRF Study Number: 485-1-06-17727.

The samples analysis details are provided below:

| Sample analysis                  | Date of samples analyses |
|----------------------------------|--------------------------|
| Plasma sample analysis           | September 25, 2017       |
| Repeat samples analysis GI-T1-M and GII-T14-M | September 27, 2017       |
2. ANALYTICAL METHOD

All samples were analysed by following the validated methods (“Validation of Analytical Method for Determination of Acetamide Concentration, Homogeneity and Stability in Vehicle”, JRF Study N° 228-2-14-17729, Khanvilkar T., 2017 and “Validation of Bioanalytical Method for Determination of Acetamide Concentration using Acetamide-D3 as Reference Standard in Mice and Rat Plasma”, and JRF Study N° 228-2-14-18476, Khanvilkar T., 2017).

2.1 Instrumental Parameters

2.1.1 Instrument parameters for dose concentration analysis of acetamidozide GC-MS

| Column                          | Agilent VF-5MS, 0.25mm i.d., 30m length, 0.25μm film thickness |
|--------------------------------|---------------------------------------------------------------|
| Carrier Gas                    | Helium                                                        |
| Injection Volume (μL)          | 2.0                                                           |
| Injector Temperature (°C)      | 250                                                           |
| Flow Rate (mL/minute)          | 1.2                                                           |
| Oven Temperature               | 40 °C (Hold 2.0 min.) to 20.0 to 300, (hold for 10 min) -Total of 25 min. |
| Mass Spectrometry              | Electron Ionization mode with 70 eV                           |
|                                | SIM Mode                                                      |
|                                | Solvent Delay Time : 4.0 min                                 |
|                                | Quadruple Temperature : 150°C                                 |
| Data Acquisition               | Selected Ion Monitoring (SIM) for masses                      |
|                                | Xanthyl-acetamide : 239                                      |
|                                | Xanthyl-propinamide : 253                                    |
2.1.2 Instrument parameters for plasma concentration analysis of acetamide on GC-MS

| Parameter                  | Specification                                                                 |
|----------------------------|-------------------------------------------------------------------------------|
| Column                     | Agilent VF-5MS, 0.25mm i.d., 30m length, 0.25μm film thickness                |
| Carrier Gas                | Helium                                                                        |
| Injection Volume (μL)      | 2.0                                                                            |
| Injector Temperature (°C) | 250                                                                            |
| Flow Rate (mL/minute)      | 1.2                                                                            |
| Oven Temperature           | 40 °C (Hold 2.0 min.) to 20.0 °C to 300 °C, (hold for 10 min) - Total of 25 min. |
| Mass Spectrometry          | Electron Ionization mode with 70 eV                                            |

Data Acquisition

- Selected Ion Monitoring (SIM) for masses
  - Xanthyl-acetamide : 239
  - Xanthyl-3d-acetamide : 242
  - Xanthyl-propinamide : 253

Acetamide was quantified based on the response factor of xanthyl-acetamide (area of xanthyl-acetamide over the area of xanthyl-propionamide) against a calibration plot of response factor of xanthyl-3D-acetamide (area of xanthyl-3D-acetamide over the area of xanthyl-propionamide).
### 3. EXPERIMENTAL PROCEDURE

#### 3.1 Instruments and Equipment

| S. N° | Instrument                  | Model                  | Make/Supplier                  |
|-------|-----------------------------|------------------------|--------------------------------|
| 1     | GC-MS                       | 7890B/5977AMSD         | Agilent                        |
| 2     | Analytical balance          | GR-202                 | Adair & dutt                   |
| 3     | Laboratory oven             | MSI-5                  | Metalab                        |
| 4     | Refrigerated centrifuge     | Eltek MP 400R          | Electrocraft (I) Pvt. Ltd.     |
| 5     | Nitrogen gas evaporator     | Speedovap              | Takahe analytical Instrument   |
| 6     | Freezer (-80 ± 10 °C)       | Forma 900 series       | ThermoScientific               |
| 7     | Vortex mixer                | EVS-50                 | KYTOSE                         |
| 8     | Refrigerator                | Enerji                 | Siemens                        |
| 9     | Micropipette                | -                      | Eppendorf                      |
| 10    | Sonicator                   | UCB70                  | Spectralab                     |

#### 3.2 Solvents and Chemicals

| S. N° | Name                  | Grade | Source            |
|-------|-----------------------|-------|-------------------|
| 1     | Methanol              | HPLC  | J. T. baker       |
| 2     | Hydrochloric acid     | HPLC  | SDFCL             |
| 3     | Xanthydrol            | Aldrich | Sigma aldrich   |
| 4     | Sodium chloride       | AR, ACS | SDFCL             |
| 5     | Potassium hydroxide   | ExcelAR | Fisher scientific |
| 6     | Milli-Q Water         | Milli Q | Millipore        |
| 7     | RO water              | Elix10 | Millipore         |
| 8     | Ethyl acetate         | HPLC  | Qualigens         |
### 3.3 Preparation of solutions for dose formulation analysis

#### 3.3.1 Preparation of stock solutions

| Identification of Standard Stock Solution (ppm) | Solution Taken (mL) | Final Volume (mL) | Volume made up with Methanol | Obtained Concentration (ppm) | Identification of Standard Stock Solution |
|-----------------------------------------------|---------------------|-------------------|-----------------------------|-----------------------------|------------------------------------------|
| A – (1011.84)                                 | 0.250               | 1                 |                             | 252.96                      | WS6                                      |
|                                               | 0.125               | 1                 |                             | 126.48                      | WS5                                      |
|                                               | 0.050               | 1                 |                             | 50.59                       | WS4                                      |
|                                               | 0.025               | 1                 |                             | 25.30                       | WS3                                      |
|                                               | 0.0125              | 1                 |                             | 12.65                       | WS2                                      |
|                                               | 0.005               | 1                 |                             | 5.06                        | WS1                                      |
| WS6 - (252.96)                                | 0.500               | 2.5               |                             | 50.59                       | S6                                       |
| WS5 - (126.48)                                | 0.500               | 2.5               |                             | 25.30                       | S5                                       |
| WS4 - (50.59)                                 | 0.500               | 2.5               |                             | 10.12                       | S4                                       |
| WS3 - (25.30)                                 | 0.500               | 2.5               |                             | 5.06                        | S3                                       |
| WS2 - (12.65)                                 | 0.500               | 2.5               |                             | 2.53                        | S2                                       |
| WS1 - (5.06)                                  | 0.500               | 2.5               |                             | 1.01                        | S1                                       |

#### 3.3.2 Preparation of internal standard working solution

| Identification of Standard Stock Solution (ppm) | Solution Taken (mL) | Final Volume (mL) | Volume made up with Methanol | Obtained Concentration (ppm) | Identification of Standard Stock Solution |
|-----------------------------------------------|---------------------|-------------------|-----------------------------|-----------------------------|------------------------------------------|
| 10.20                                         | 99.2                | 10                |                             | 1011.84                     | A                                        |

#### 3.3.3 Preparation of working solutions for linearity

The stock dilutions were prepared with diluent as per the table given below from the stock solution ‘A’. These solutions were stored at 2 - 8 °C in refrigerator.
The reference standard working solutions (S1, S2, S3, S4, S5 and S6) were injected onto the GC-MS. The area ratio was plotted against concentrations (ppm). The correlation coefficient (r), slope (b) and intercept (a) were calculated.

### 3.3.4 Preparation of solutions of quality controls

| Weight (mg) of Test Item | Final Volume (mL) | Volume Made up With | Obtained Concentration (ppm) |
|--------------------------|-------------------|---------------------|-----------------------------|
| 25.03                    | 1                 | Vehicle             | 24829.76 (Low Dose)         |
| 200.18                   | 1                 |                     | 198578.56 (High Dose)       |

| Obtained Concentration (ppm) | Solution Taken (mL) | Final Volume (mL) | Solution Taken (mL) | Final Volume (mL) | Volume made up to the mark with | Dilution Factor (D) | Solution ID |
|-----------------------------|---------------------|-------------------|---------------------|-------------------|--------------------------------|-------------------|-------------|
| 24829.76 (Low Dose)         | 0.100               | 10                | 1                   | 10                | RO water                       | 1000              | LQC         |
| 198578.56 (High Dose)       | 0.100               | 10                | 0.1                 | 10                |                                 | 10000             | HQC         |

### 3.3.5 Preparation of sample dilution

| Dose level and conc. (mg/mL) | Replication | Solution taken (mL) | Final volume (mL) | Solution taken (mL) | Final volume (mL) | Volume made up to the mark with | Dilution factor (D) |
|------------------------------|-------------|---------------------|-------------------|---------------------|-------------------|--------------------------------|-------------------|
| Vehicle Control G1 (0.0)     | Middle      | 1                   | -                 | -                   | -                 |                                 | 1                  |
| Low dose G2 (25)             | MR1         | 0.1                 | 10                | 1                   | 10                | RO water                       | 1000              |
|                              | MR2         |                      |                   |                     |                   |                                 |                    |
|                              | MR3         |                      |                   |                     |                   |                                 |                    |
| Middle dose G3 (100)         | MR1         | 0.1                 | 10                | 0.1                 | 10                |                                 | 10000             |
|                              | MR2         |                      |                   |                     |                   |                                 |                    |
|                              | MR3         |                      |                   |                     |                   |                                 |                    |
| High dose G4 (200)           | MR1         | 0.1                 | 10                | 0.1                 | 10                |                                 | 10000             |
|                              | MR2         |                      |                   |                     |                   |                                 |                    |
|                              | MR3         |                      |                   |                     |                   |                                 |                    |
3.4 Preparation of solutions for plasma concentration analysis

3.4.1 Preparation of stock solutions

| Name of reference standards | Purity % | Weight of reference standard (mg) | Capacity of volumetric flask (mL) | Volume made up with | Obtained concentration (ppm) | Reference standard stock solution identification |
|-----------------------------|----------|---------------------------------|---------------------------------|-------------------|-------------------------------|-------------------------------------------------|
| Acetamide-d$_3$             | 99.77    | 10.15                           | 5                               | Methanol          | 2025.331                      | AD-01                                           |
|                             |          | 10.20                           |                                  |                   | 2035.308                      | AD-02                                           |
| Acetamide                   | 99.20    | 10.20                           | 10                               |                   | 1011.840                      | A-01                                            |
|                             |          | 10.25                           |                                  |                   | 1016.800                      | A-02                                            |
| Propionamide                | 100.00   | 10.25                           | 10                               |                   | 1025.000                      | IS-01                                           |

3.4.2 Preparation of internal standard working solution

| Identification of reference standard stock solution used | Solution taken (mL) | Final volume (mL) | Obtained concentration (ppm) | Identification |
|----------------------------------------------------------|---------------------|-------------------|-------------------------------|----------------|
| IS-01                                                     | 0.050               | 10                | 5.125                         | WI-01          |

3.4.3 Preparation of calibration curve spiking solutions

Stock dilution with diluents (50:50, Methanol:Milli-Q water, v/v) were prepared as per below table from Acetamide-d3 stock solution. These solutions were stored at 2-8 ºC in refrigerator.

| Stock/SS ID | Stock/SS concentration (ppm) | Stock/SS volume (mL) | Final volume made up to (mL) | SS concentration (ppm) | SS ID   |
|-------------|------------------------------|----------------------|-----------------------------|------------------------|---------|
| AD-01       | 2025.331                     | 0.005                | 10                          | 1.013                  | STD1 SS-01 |
|             |                              | 0.010                | 10                          | 2.025                  | STD2 SS-01 |
|             |                              | 0.020                | 10                          | 4.051                  | STD3 SS-01 |
|             |                              | 0.070                | 10                          | 14.177                 | STD4 SS-01 |
|             |                              | 0.245                | 10                          | 49.621                 | STD5 SS-01 |
|             |                              | 0.850                | 10                          | 172.153                | STD6 SS-01 |
|             |                              | 0.250                | 1                            | 506.333                | STD7 SS-01 |
APPENDIX 7 (Continued)

3.4.4 Preparation of spiked matrix CC standards

The above prepared CC spiking solutions were spiked in the interference free blank mice plasma in order to ranged the matrix standards concentrations as per below table.

| SS ID | SS concentration (ppm) | SS volume (mL) | Plasma volume (mL) | Matrix concentration (ppm) | Sample ID |
|-------|-------------------------|----------------|-------------------|---------------------------|-----------|
| STD1 SS-01 | 1.013 | 0.010 | 0.090 | 0.101 | STD1 |
| STD2 SS-01 | 2.025 | 0.010 | 0.090 | 0.203 | STD2 |
| STD3 SS-01 | 4.051 | 0.010 | 0.090 | 0.405 | STD3 |
| STD4 SS-01 | 14.177 | 0.010 | 0.090 | 1.418 | STD4 |
| STD5 SS-01 | 49.621 | 0.010 | 0.090 | 4.962 | STD5 |
| STD6 SS-01 | 172.153 | 0.010 | 0.090 | 17.215 | STD6 |
| STD7 SS-01 | 506.333 | 0.010 | 0.090 | 50.633 | STD7 |

3.4.5 Preparation of quality control spiking solutions

Stock dilution with diluents (50:50, Methanol:Milli-Q water, v/v) were prepared as per below table from acetamide-2-2-2-D3 stock solution for quality control samples. These solutions were stored at 2-8 ºC in refrigerator.

| Stock/SS ID | Stock/SS concentration (ppm) | Stock/SS Volume (mL) | Final volume made up to (mL) | SS concentration (ppm) | SS ID |
|-------------|-------------------------------|---------------------|-----------------------------|------------------------|-------|
| AD-02       | 2035.308                      | 0.015               | 10                          | 3.053                  | LQC SS-01 |
|             |                               | 0.750               | 10                          | 152.648                | MQC SS-01 |
|             |                               | 0.212               | 1                           | 431.485                | HQC SS-01 |

3.4.6 Preparation of spiked matrix quality control samples

The above prepared QC spiking solutions were spiked in the interference free blank mice plasma in order to ranged the concentrations as per below table.

| Stock/SS ID | SS concentration (ppm) | SS Volume (mL) | Plasma volume (mL) | Final matrix concentration (ppm) | QC ID |
|-------------|-------------------------|----------------|-------------------|---------------------------------|-------|
| LQC SS-01   | 3.053                   | 0.010          | 0.090             | 0.305                           | LQC   |
| MQC SS-01   | 152.648                 | 0.010          | 0.090             | 15.265                          | MQC   |
| HQC SS-01   | 431.485                 | 0.010          | 0.090             | 43.149                          | HQC   |
APPENDIX 7 (Continued)

3.5 Preparation of Reagent and Solution

3.5.1 0.5 M Hydrochloric acid solution

50 mL of methanol was transferred into a 100 mL volumetric flask. 4.125 mL of 37 % HCl solution was added in the same volumetric flask. Volume was made equal to mark with methanol. Solution was mixed well.

3.5.2 0.7 M KOH solution

3.9 g of KOH was transferred to a 100 mL glass bottle and 100 mL of Milli-Q water was added by using a measuring cylinder. Solution was mixed well. Solution was stored in a refrigerator until use.

3.5.3 5% Xanthydrol solution

5 g of Xanthydrol was transferred to a 100 mL volumetric flask. 50 mL of methanol was added to it and solution was mixed well. Volume was made equal to mark with methanol. Solution was stored in a refrigerator until use.

3.5.4 Diluent solution [Methanol: Milli-Q water (50:50), % v/v]

100 mL of Methanol and 100 mL of Milli-Q water were mixed in a 200 mL glass bottle using a measuring cylinder and mixed well.

3.5.5 Saturated sodium chloride solution

71 g of sodium chloride in 200 mL of Milli-Q water were mixed in 200 mL of glass bottle using a measuring cylinder and mixed well.

3.6 Sample processing procedure

3.6.1 Derivatization Procedure for dose concentration analysis

1. 2.45 mL of RO water sample/dose formulation was transferred to a 15 mL polypropylene centrifuge tube.
2. 50 μL of internal standard solution was added (50 ppm Propionamide in methanol) to the tube except blank.
3. 2.50 mL of 0.5 M HCl in methanol was added to each tube. Samples were vortexed for 15 min.
4. All samples were centrifuged at 14000 rpm for 10 min.
5. Then 200 μl of 5 % Xanthydrol solution was added and incubated in darkness at 40°C for 1.5 h.
6. After 1.5 h, 3.0 g of sodium chloride to each tube was added.
APPENDIX 7 (Continued)

7. 2.0 mL of 0.7 M KOH was added to each sample tube to neutralize.
8. 3.0 mL of ethyl acetate was added to each tube. Vortexed, sonicated and centrifuged all samples at 10,000 rpm for 5 min.
9. 1.3 mL of supernatant was transferred from each sample to a new RIA vial.
10. Samples were placed on speedovap nitrogen evaporator until all of the ethyl acetate has been removed.
11. 130 μL of ethyl acetate were added to each RIA vial. Sonicated, vortexed and centrifuged at 10000 rpm for 10 min.
12. 100 μL of the supernatant was carefully removed and placed in a GC-MS vial and injected on to GC-MS.

3.6.2 Derivatization Procedure for plasma concentration analysis
1. 100 μL of plasma was transferred to eppendorf tube.
2. 10 μL of internal standard solution (5 ppm propionamide in methanol) was added to the tube (final concentration of 0.5 ppm for the internal standard).
3. Volume was made up to 150 μL with water (this step is important specially for preparing standards).
4. 300 μL of 0.5 M HCl in methanol was added to each tube.
5. Samples were vortexed followed by storage in -80 ± 10 °C deep freezer for 1h.
6. Samples were centrifuged at 14000 rpm for 10 minutes at set temperature of 4 °C.
7. 250 μL of supernatant was transferred to RIA tube.
8. 200 μL of 5% Xanthylhrol solution was added in sample tube and incubated in darkness at 40 °C for 2 h.
9. Samples were removed after 2 h from incubator and dried at 40 °C under nitrogen.
10. 800 μL of saturated solution of sodium chloride was added to dried sample and vortexed.
11. 60 μL of 1 M KOH solution was added to all RIA tubes and vortexed.
12. 1.6mL of ethyl acetate was added to each tube.
13. Samples were vortexed at 2000 rpm for 5 minutes followed by sonication for 1 minute.
14. Samples were centrifuged the samples at 14,000 rpm for 10 minutes at set temperature of 4 °C.
15. 1.3 mL of supernatant was transferred to pre-labeled RIA vial.
16. Samples were dried at 40°C under nitrogen gas until dryness.
17. Samples were reconstituted with 0.5 mL of ethyl acetate sonicated and vortexed.
18. Samples were centrifuged at 14000 rpm for 10 minutes at set temperature of 4°C.
19. Supernatant was carefully transferred on GC-MS instrument for analysis.
3.7 Calculation

The acetamide concentration in mice plasma was calculated using the following formula by analyst software version 1.6.3:

\[
\text{Acetamide concentration (ppm)} = \frac{Y - a}{b} \times D
\]

Where,
- \(Y\) = Peak area ratio of sample
- \(a\) = Intercept
- \(b\) = Slope of the line
- \(D\) = Dilution factor

3.7.1 % RSD

\[
\% \text{ RSD} = \frac{\text{Standard deviation}}{\text{Mean content}} \times 100
\]

3.7.2 % Accuracy

\[
\text{Mean % Accuracy} = \frac{\text{Mean recovered concentration (ppm)}}{\text{Nominal concentration (ppm)}} \times 100
\]

3.8 Samples Run Details

| Sample analysis                        | Date of Samples Analyses | Accepted / Not Accepted |
|----------------------------------------|--------------------------|-------------------------|
| Dose formulation analysis              | September 18, 2017       | Accepted                |
| Plasma sample analysis                 | September 25, 2017       | Accepted                |
| Repeat samples analysis GI-T1-M and GII-T14-M | September 27, 2017      | Accepted                |
4. RESULTS

4.1 System Suitability

| TABLE-01 | System suitability in RO water for dose formulation analysis |
|----------|------------------------------------------------------------|
| Date     | 19/09/17 | 19/09/17 |
| Replicates | Area Ratio | Replicates | Bracketed System suitability |
| 1     | 56.6689 | 1      | |
| 2     | 57.8726 | 2      | |
| 3     | 56.0478 | 6      | 51.4708 |
| 4     | 56.5178 | 7      | 53.1934 |
| 5     | 57.3039 | 8      | 55.6456 |
| Mean   | 56.8822 | Mean   | 53.4366 |
| % SD   | 0.71    | % SD   | 2.10   |
| % RSD  | 1.25    | % RSD  | 3.93   |

| System suitability in mice plasma for plasma concentration analysis |
|---------------------------------------------------------------|
| Date              | 25/09/17 | 27/09/17 |
| Replicates        | Area Ratio | |
| 1     | 2.6289 | 2.5698 |
| 2     | 2.6204 | 2.5723 |
| 3     | 2.6501 | 2.5620 |
| 4     | 2.6019 | 2.5687 |
| 5     | 2.6338 | 2.5978 |
| Mean   | 2.6270 | 2.5741 |
| % SD   | 0.02    | 0.01   |
| % RSD  | 0.76    | 0.39   |
| S/N Ratio | 5.4 | 5.3 |
4.2 Linearity

### TABLE-02

#### Linearity in RO water for dose formulation analysis on 19/09/17

| Linear standard | STD1 | STD2 | STD3 | STD4 | STD5 | STD6 | Slope     | Intercept | Coefficient of determination |
|-----------------|------|------|------|------|------|------|-----------|-----------|-----------------------------|
| Nominal conc. (ppm) | 1.01 | 2.53 | 5.06 | 10.12| 25.30| 50.59| 0.068273  | 0.263698  | 0.99968339                  |

| Back calculated conc. (ppm) | 0.92 | 2.52 | 5.29 | 10.69| 24.96| 50.63| 0.068273  | 0.263698  | 0.99968339                  |

| % Accuracy | 91.09 | 99.60 | 104.55 | 105.63| 98.66| 100.08| 0.068273  | 0.263698  | 0.99968339                  |

#### Linearity in mice plasma for plasma sample analysis on 25/09/17

| Nominal conc. (ppm) | 0.101 | 0.203 | 0.405 | 1.418| 4.962| 17.215| 50.633 | 0.044661 | - 0.002807 | 0.99559295 |

| Back calculated conc. (ppm) | 0.111 | 0.209 | 0.393 | 1.301| 5.306| 15.245| 52.372 | 0.044661 | - 0.002807 | 0.99559295 |

| % Accuracy | 109.90 | 102.96 | 97.04 | 91.75| 106.93| 88.56| 103.43 | 0.044661 | - 0.002807 | 0.99559295 |

#### Linearity in mice plasma for repeat plasma sample analysis on 27/09/17

| Nominal conc. (ppm) | 0.101 | 0.203 | 0.405 | 1.418| 4.962| 17.215| 50.633 | 0.044725 | - 0.001242 | 0.99838482 |

| Back calculated conc. (ppm) | 0.120 | 0.201 | 0.358 | 1.412| 4.898| 16.068| 51.880 | 0.044725 | - 0.001242 | 0.99838482 |

| % Accuracy | 118.81 | 99.01 | 88.40 | 99.58| 98.71| 93.34| 102.46 | 0.044725 | - 0.001242 | 0.99838482 |
APPENDIX 7 (Continued)

FIGURE 1: Linearity of acetamide for dose concentration analysis

FIGURE 2: Linearity of acetamide-2,2,2-D3 reference standard for plasma sample analysis

FIGURE 3: Linearity of acetamide-2,2,2-D3 reference standard for plasma sample analysis (Repeat Analysis)
### 4.3 Concentration dose formulation analysis

| Dose level and conc. (mg/mL) | Replication | Theoretical conc. based on purity of test item (ppm) | Dilution factor (D) | Recovered concentration (ppm) | Analysed concentration (ppm) | Recovery (%) | Mean analysed concentration (ppm) | Mean Recovery (%) | SD | %CV |
|-----------------------------|-------------|-----------------------------------------------------|---------------------|-----------------------------|-----------------------------|-------------|----------------------------------|------------------|----|-----|
| Vehicle Control G1 (0.0)    | Middle      | 0.00                                                | -                   | ND                          | ND                          | -           | -                                | -                |    |     |
| Low dose G2 (25)            | MR1         | 24800.00                                            | 1000                | 26.25                       | 26250.00                    | 105.85      | 24723.33                         | 99.69            | 1868.27 | 7.56 |
|                             | MR2         | 22400.00                                            | 1000                | 22.64                       | 22640.00                    | 91.29       |                                  |                  |      |     |
|                             | MR3         | 25.28                                               |                      | 25.28                       | 25280.00                    | 101.94      |                                  |                  |      |     |
| Middle dose G3 (100)        | MR1         | 99200.00                                            | 10000               | 9.61                        | 96100.00                    | 96.88       | 99766.67                         | 100.57           | 3601.85 | 3.61 |
|                             | MR2         | 99200.00                                            | 10000               | 9.99                        | 99900.00                    | 100.71      |                                  |                  |      |     |
|                             | MR3         | 10.33                                               |                      | 10.33                       | 103300.00                   | 104.13      |                                  |                  |      |     |
| High dose G4 (200)          | MR1         | 198400.00                                           | 10000               | 21.77                       | 217700.00                   | 109.73      | 209133.33                        | 105.41           | 14065.68 | 6.73 |
|                             | MR2         | 198400.00                                           | 10000               | 21.68                       | 216800.00                   | 109.27      |                                  |                  |      |     |
|                             | MR3         | 19.29                                               |                      | 19.29                       | 192900.00                   | 97.23       |                                  |                  |      |     |

| Purity of Test Item (% w/w) | 99.20 |

ND- Not Detected
### 4.4 Concentration of acetamide in mice plasma

#### TABLE-04

| Group | Animal N° | Sex | Dilution Factor (D) | Acetamide area response | IS area response | Response ratio | Analysed Conc. (ppm) |
|-------|-----------|-----|---------------------|-------------------------|-----------------|-----------------|----------------------|
| GI    | T1        | Male | 1                   | 790                     | 1262            | 0.6262         | 14.084*              |
|       | T2        | Male | 1                   | 896                     | 74016           | 0.0121         | 0.334                |
|       | T3        | Male | 1                   | 1109                    | 79590           | 0.0139         | 0.374                |
|       | T4        | Male | 1                   | 2102                    | 90140           | 0.0233         | 0.585                |
|       | T5        | Male | 1                   | 1174                    | 85780           | 0.0137         | 0.370                |
|       | T6        | Male | 1                   | 1391                    | 84123           | 0.0165         | 0.432                |
|       | T7        | Male | 1                   | 1273                    | 84636           | 0.0150         | 0.399                |
|       | T8        | Male | 1                   | 1206                    | 80010           | 0.0151         | 0.401                |
|       | T9        | Male | 1                   | 1868                    | 79320           | 0.0235         | 0.589                |
|       | T10       | Male | 1                   | 1424                    | 75378           | 0.0189         | 0.486                |
|       | T11       | Female | 1              | 1224                   | 88289        | 0.0139        | 0.374                |
|       | T12       | Female | 1          | 1364                   | 89480        | 0.0152        | 0.403                |
| GII   | T13       | Male | 1                   | 209456                  | 96490          | 2.1707         | 48.667               |
|       | T14       | Male | 1                   | 210689                  | 88715          | 2.3749         | 53.239*              |
|       | T15       | Male | 1                   | 155158                  | 84357          | 1.8393         | 41.246               |
|       | T16       | Male | 1                   | 45887                   | 83130          | 0.5520         | 12.423               |
|       | T17       | Male | 1                   | 112217                  | 84921          | 1.3214         | 29.650               |
|       | T18       | Male | 1                   | 122554                  | 91772          | 1.3354         | 29.964               |
|       | T19       | Female | 1           | 13406                  | 83064          | 0.1614         | 3.677                |
|       | T20       | Female | 1          | 100752                 | 79428          | 1.2685         | 28.466               |
|       | T21       | Female | 1          | 22591                 | 78121          | 0.2892         | 6.538                |
|       | T22       | Female | 1          | 41489                 | 88389          | 0.4694         | 10.573               |
|       | T23       | Female | 1          | 7048                 | 85689          | 0.0822         | 1.903                |
|       | T24       | Female | 1          | 63859                 | 98743          | 0.6467         | 14.543               |

Remarks: * GI-T1_M was identified for repeat analysis due to variation in internal standard response and GII-T14_M was also identified for repeat analysis due to concentration was found to be beyond linearity range.
### Obtained Concentrations of Acetamide in mice plasma (ppm)

| Group | Animal N° | Sex  | Dilution Factor (D) | Acetamide area response | IS area response | Response ratio | Analysed Conc. (ppm) |
|-------|------------|------|---------------------|-------------------------|-----------------|------------------|-----------------------|
| GIII  | T25        | Male | 10                  | 134786                  | 93590           | 1.4402          | 323.102               |
|       | T26        |      | 10                  | 37801                   | 94993           | 0.3979          | 89.722                |
|       | T27        |      | 10                  | 26853                   | 96982           | 0.2769          | 62.629                |
|       | T28        |      | 10                  | 97260                   | 85447           | 1.1382          | 255.482               |
|       | T29        |      | 10                  | 37715                   | 93151           | 0.4049          | 91.289                |
|       | T30        |      | 10                  | 79767                   | 87039           | 0.9164          | 205.819               |
|       | T31        |      | 10                  | 18462                   | 94189           | 0.1960          | 44.515                |
|       | T32        | Female | 10                | 42603                   | 79391           | 0.5366          | 120.778               |
|       | T33        |      | 10                  | 36866                   | 58173           | 0.6337          | 142.520               |
|       | T34        |      | 10                  | 20008                   | 96280           | 0.2078          | 47.157                |
|       | T35        |      | 10                  | 12083                   | 89453           | 0.1351          | 30.879                |
|       | T36        |      | 10                  | 9120                    | 90011           | 0.1013          | 23.310                |
|       | T37        |      | 10                  | 25778                   | 56811           | 0.4538          | 102.238               |
|       | T38        |      | 10                  | 38610                   | 90284           | 0.4277          | 96.394                |
|       | T39        |      | 10                  | 85668                   | 87361           | 0.9806          | 220.194               |
|       | T40        |      | 10                  | 75531                   | 90371           | 0.8358          | 187.772               |
|       | T41        |      | 10                  | 66398                   | 84185           | 0.7887          | 177.226               |
|       | T42        |      | 10                  | 127426                  | 90197           | 1.4128          | 316.967               |
|       | T43        |      | 10                  | 55442                   | 82319           | 0.6735          | 151.431               |
|       | T44        |      | 10                  | 51853                   | 91847           | 0.5646          | 127.048               |
|       | T45        |      | 10                  | 27439                   | 91137           | 0.3011          | 68.048                |
|       | T46        |      | 10                  | 72275                   | 81638           | 0.8853          | 198.855               |
|       | T47        |      | 10                  | 24243                   | 51575           | 0.4701          | 105.888               |
|       | T48        |      | 10                  | 14746                   | 90056           | 0.1637          | 37.282                |

**Key**
- BLQ: Below quantitation limit
- LLOQ: 0.101 ppm
- ULOQ: 50.633 ppm

| Intercept of Y-axis (a) | -0.002807 |
|------------------------|-----------|
| Slope of the line (b)  | 0.044661  |

**Repeat plasma sample analysis**

| Group | Animal N° | Sex   | Dilution Factor (D) | Acetamide area response | IS area response | Response ratio | Analysed Conc. (ppm) |
|-------|------------|-------|---------------------|-------------------------|-----------------|------------------|-----------------------|
| GI    | T1         | Male  | 1                   | 923                     | 85735           | 0.0108          | 0.269                 |
| GII   | T14        | Male  | 2                   | 102898                  | 87952           | 1.1699          | 52.371                |

**Key**
- Intercept of Y-axis (a) | -0.001242 |
- Slope of the line (b)  | 0.044725  |
### 4.5 Repeat Analysis

| Sample ID  | Initial Concentration (ppm) | Repeated Concentration (ppm) | Accepted Concentration (ppm) |
|------------|-----------------------------|-------------------------------|-----------------------------|
| GI-T1-M    | 14.084                      | 0.269                         | 0.269                       |
| GII-T14-M  | 53.239                      | 52.371                        | 52.371                      |
4.6 Chromatograms

A. Blank+IS for dose concentration analysis

| Compnd                | Signal | RT  | Limits         | Response | QRatio | Limits | Final Conc |
|-----------------------|--------|-----|----------------|----------|---------|--------|------------|
| Xanthyl acetamide     | 239.0  | 13.297 | 12.617-13.945 | 0.0      |         |        | 0.000      |
| Xanthyl propionamide  | 253.0  | 13.597 | 12.921-14.281 |          |        |        |            |

![Chromatogram of Xanthyl acetamide](image1)

![Chromatogram of Xanthyl propionamide](image2)
Chromatograms (Continued)

B. Standard-6 for dose concentration analysis

| Compound          | Signal | RT    | QRatio | Final Conc |
|-------------------|--------|-------|--------|------------|
| Xanthyl acetamide | 239.0  | 13.282| 12.617-13.945| 1255009.0  |
|                   | 253.0  | 13.587| 12.921-14.281| 36033.5    |
### Chromatograms (Continued)

**C. Vehicle control for dose concentration analysis**

| Compnd               | Signal | RT   | Limits       | Response | QRatio | Limits   | FinalCon |
|----------------------|--------|------|--------------|----------|--------|----------|----------|
| Xanthyl acetamide    | 239.0  | 13.297 | 12.617-13.945 | 0.0       |        |          | 0.001    |
| Xanthyl propionamide | 253.0  | 12.921-14.281 |            |          |        |          |          |

![Chromatogram](image)

**Chromatogram**

- **Xanthyl acetamide**
  - Peak at 13.297 min.
- **Xanthyl propionamide**
  - Peak at 12.921-14.281 min.

*Note: Images of chromatograms have been omitted for this text-based representation.*
Chromatograms (Continued)

D. High dose for dose concentration analysis

| Cmpnd               | Signal | RT   | Limits       | Response     | QRatio | Limits | FinalConc |
|---------------------|--------|------|--------------|--------------|--------|--------|-----------|
| Xanthyl acetamide   | 239.0  | 13.28 | 12.617-13.945| 520586.4    |        |        | 21.773    |
| Xanthyl propionamide| 253.0  | 13.587| 12.921-14.281| 34409.4     |        |        |           |
Chromatograms (Continued)

E. Blank sample of acetamide-2,2,2-D3 for plasma concentration analysis
F. Standard zero sample of acetamide-2,2,2-D3 for plasma concentration analysis

| Compnd            | Signal | RT Limits   | Response | QRatio | Limits   | FinalConc |
|-------------------|--------|-------------|----------|--------|----------|-----------|
| Deuterated xanthyl acetamide | 242.0  | 12.927-13.905 | 0.0      |        |          |          |
| Xanthyl propionamide   | 253.0  | 13.560-14.238 | 66504.0  |        |          | 0.000     |

**Chromatograms (Continued)**

- TIC SIM 013.D (Blank+IS_01)

- Selected Ion (242.0) 013.D

- Selected Ion (253.0) 013.D
Chromatograms (Continued)

G. Standard-7 sample of acetamide-2,2,2-D3 for plasma concentration analysis

| Compd                        | Signal | RT   | Limits          | Response | QRatio | Limits | FinalConc |
|------------------------------|--------|------|-----------------|----------|--------|--------|-----------|
| Deuterated xanthyl acetamide | 242.0  | 13.238 | 12.581-13.905 | 183374.3 |        |        | 50.858    |
| Xanthyl propionamide         | 253.0  | 13.555 | 12.862-14.238  | 78493.7  |        |        |           |
H. Control sample (GI-T6_M) of acetamide for plasma concentration analysis
Chromatograms (Continued)

I. GIII-T35_F sample of acetamide for plasma concentration analysis

| Compound                | Signal | RT     | Limits        | Response | QRatio | Limits | Final Conc |
|-------------------------|--------|--------|---------------|----------|--------|--------|------------|
| Xanthyl acetamide       | 239.0  | 13.249 | 12.581-13.905 | 12083.3  |        |        |            |
| Xanthyl propionamide    | 253.0  | 13.554 | 12.882-14.238 | 89453.5  |        |        |            |
### APPENDIX I: Animal Plasma Concentration of Acetamide

#### Acetamide Concentration in Mice plasma-
- **Group I (Dose - 0.0 mg/kg)**
- **Group II (Dose - 250.0 mg/kg)**

| Animal N° | Sex | Concentration (ppm) | Animal N° | Sex | Concentration (ppm) |
|-----------|-----|----------------------|-----------|-----|---------------------|
| T1        | M   | 0.269                | T13       | M   | 48.667              |
| T2        |     | 0.334                | T14       |     | 52.371              |
| T3        |     | 0.374                | T15       |     | 41.246              |
| T4        |     | 0.585                | T16       |     | 12.423              |
| T5        |     | 0.370                | T17       |     | 29.650              |
| T6        |     | 0.432                | T18       |     | 29.964              |
| T7        | F   | 0.399                | T19       |     | 3.677               |
| T8        |     | 0.401                | T20       |     | 28.466              |
| T9        |     | 0.589                | T21       |     | 6.538               |
| T10       |     | 0.486                | T22       |     | 10.573              |
| T11       |     | 0.374                | T23       |     | 1.903               |
| T12       |     | 0.403                | T24       |     | 14.543              |

#### Acetamide Concentration in Mice plasma-
- **Group III (Dose - 1000.0 mg/kg)**
- **Group IV (Dose - 2000.0 mg/kg)**

| Animal N° | Sex | Concentration (ppm) | Animal N° | Sex | Concentration (ppm) |
|-----------|-----|----------------------|-----------|-----|---------------------|
| T25       | M   | 323.102              | T37       | M   | 102.238             |
| T26       |     | 89.722               | T38       |     | 96.394              |
| T27       |     | 62.629               | T39       |     | 220.194             |
| T28       |     | 255.482              | T40       |     | 187.772             |
| T29       |     | 91.289               | T41       |     | 177.226             |
| T30       |     | 205.819              | T42       |     | 316.967             |
| T31       | F   | 44.515               | T43       |     | 151.431             |
| T32       |     | 120.778              | T44       |     | 127.048             |
| T33       |     | 142.52               | T45       |     | 68.048              |
| T34       |     | 47.157               | T46       |     | 198.855             |
| T35       |     | 30.879               | T47       |     | 105.888             |
| T36       |     | 23.310               | T48       |     | 37.282              |
Micronucleus Test of Acetamide in Mice

APPENDIX 8: Historical Control Data

(Data of studies conducted during August 2016 to August 2017)

| Sex          | Male                  | Female                  |
|--------------|-----------------------|-------------------------|
|              | P/E ratio | % MNPCE | P/E ratio | % MNPCE |
| **Vehicle: 0.5% Carboxymethyl cellulose** |           |           |           |         |
| Mean         | 0.48      | 0.03     | -         | -        |
| (N = 1 study)|           | (N = 1 study) |           |         |
| Standard Deviation | 0.01  | 0.03     | -         | -        |
| **Vehicle: Distilled water** |           |           |           |         |
| Mean         | 0.50      | 0.02     | 0.51      | 0.02    |
| (N = 50 studies) | (N = 50 studies) | (N = 21 studies) | (N = 21 studies) |
| Standard Deviation | 0.02  | 0.02     | 0.03      | 0.02    |
| **Vehicle: Vegetable oil** |           |           |           |         |
| Mean         | 0.50      | 0.02     | 0.50      | 0.02    |
| (N = 27 studies) | (N = 27 studies) | (N = 10 studies) | (N = 10 studies) |
| Standard Deviation | 0.02  | 0.01     | 0.02      | 0.01    |
| **Positive control: Mitomycin-C @ 1.0 mg/kg body weight** |           |           |           |         |
| Mean         | 0.50      | 1.27     | 0.54      | 1.10    |
| (N = 77 studies) | (N = 77 studies) | (N = 6 studies) | (N = 6 studies) |
| Standard Deviation | 0.03  | 0.45     | 0.03      | 0.19    |

**Vehicle Control**: Distilled Water

**Details of Positive Control used**

Mitomycin-C = 1.0 mg/kg body weight

**Keys**:

- % MNPCE = Percent Micronucleated Polychromatic Erythrocytes
- P/E = Total Polychromatic Erythrocytes/Total Erythrocytes
APPENDIX 8 (Continued)

P/E Ratio (Female) - Distilled Water

% MNPCE (Female) - Distilled water
APPENDIX 8 (Continued)

### P/E Ratio (Male) Positive Control

- **Mean**: 0.529
- **+ 1 SD**: 0.606
- **+ 2 SD**: 0.682
- **+ 3 SD**: 0.758
- **- 1 SD**: 0.452
- **- 2 SD**: 0.375
- **- 3 SD**: 0.298

### %MNPCE (Male) Positive Control

- **Mean**: 0.496
- **+ 1 SD**: 0.606
- **+ 2 SD**: 0.714
- **+ 3 SD**: 0.824
- **- 1 SD**: 0.385
- **- 2 SD**: 0.293
- **- 3 SD**: 0.202
Note: QC chart of female (positive control) prepared from historical control data of year 2015 to 2017.
**MICROBIOLOGICAL ANALYSIS CERTIFICATE OF WATER SAMPLE**

| Parameters                              | Results Observed | Permissible Limits |
|-----------------------------------------|------------------|-------------------|
| Total viable organisms                  |                  |                   |
| Bacteria                                | Nil*             | <20CFU/mL         |
| Fungus                                  | Nil*             | None/100 mL       |
| * = No colony in first dilution.       |                  |                   |
| **Salmonella sp.**                      | Absent           | None/100 mL       |
| **Coliform organisms**                  | Absent           | <10/100 mL        |
| **E.coli type I**                       | Absent           | None/100 mL       |

**Conclusion:** The results of analysis indicate that the microbial load is within the permissible limit (JRF/MIC/SOP-619).

**Analysed by:** Rahul G Badgha

**Verified by:** Dr. Rajesh Posia

**Sign & Date:** [Handwritten Signature]

**Sign & Date:** [Handwritten Signature]
APPENDIX 9 (Continued)

MICROBIOLOGICAL ANALYSIS CERTIFICATE OF WATER SAMPLE

| Parameters               | Results Observed | Permissible Limits |
|--------------------------|------------------|-------------------|
| Total viable organisms   |                  |                   |
| Bacteria                 | Nil*             | <20CFU/mL         |
| Fungus                   | Nil*             | None/100 mL       |
| Salmonella sp.           | Absent           | None/100 mL       |
| Coliform organisms       | Absent           | < 10/100 mL       |
| E.coli type I            | Absent           | None/100 mL       |

* = No colony in first dilution.

Conclusion: The results of analysis indicate that the microbial load is within the permissible limit (JRF/MIC/SOP-619).

Analysed by : Rahul G Badgha

Verified by : Dr. Rajesh Posia

Sign & Date : March 22, 2017
## APPENDIX 9 (Continued)

### Test Report

**Sample Not Drawn by SGS India Pvt. Ltd.**

| Report No | CE17-001724.001 |
|-----------|------------------|
| JOE No    | CE17-001724      |
| Report Control No | CER0000142381 |

**Sample Described by Customer as:** WATER

| Client Name         | JAI RESEARCH FOUNDATION |
|---------------------|-------------------------|
| Client Address      | Off National Highway No. 8, Near Daman ganga river bridge |
| City                | Valsada - Vapi |
| Postal Code         | 396195 |
| State               | Gujarat |
| Country             | India |
| Sample Type         | WATER |
| Received Date       | 30/03/2017 |
| Sample Qty.         | 3L & 1L |
| Recd. Marks on Sample | WATER-NEW BUILDING |
| Date                | 22.03.2017 |
| Test Start/End Date | 30/03/2017 - 07/04/2017 |

| Analysis             | Method          | Result          | Unit |
|----------------------|-----------------|-----------------|------|
| Arsenic as As        | APHA 3125 B     | BDL(DL 0.005)   | mg/L |
| Cadmium as Cd        | APHA 3125 B     | BDL(DL 0.001)   | mg/L |
| Lead as Pb           | APHA 3125 B     | BDL(DL 0.005)   | mg/L |
| Mercury as Hg        | APHA 3125 B     | BDL(DL 0.001)   | mg/L |
| Aldrin               | USEPA 5101C & 8081A | BDL(DL 0.01) | µg/L |
| Dieldrin             | USEPA 5101C & 8081A | BDL(DL 0.01) | µg/L |
| Alpha Endosulfan     | USEPA 5101C & 8081A | BDL(DL 0.01) | µg/L |
| Beta Endosulfan      | USEPA 5101C & 8081A | BDL(DL 0.01) | µg/L |
| Endrin               | USEPA 5101C & 8081A | BDL(DL 0.01) | µg/L |
| Gamma HCH (Lindane)  | USEPA 5101C & 8081A | BDL(DL 0.01) | µg/L |
| Methyl parathion     | USEPA 5101C & 8141A | BDL(DL 0.01) | µg/L |
| Malathion            | USEPA 3510C & 8141A | BDL(DL 0.01) | µg/L |
| Phorate              | USEPA 3510C & 8141A | BDL(DL 0.01) | µg/L |
| Methoxychlor         | USEPA 3510C by GC/MS | BDL(DL 0.1) | µg/L |

**Remark:** BDL: Below detection limit, DL: Detection limit

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Unless otherwise stated the results shown in this test report refer only to the sample(s) tested and such sample(s) are retained for 7 days (in case of perishable items) and 30 days for all other samples. The samples from regulatory bodies are to be retained as specified. This document cannot be reproduced except in full, without prior written approval of the Company.

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SGS India Pvt. Ltd.
Multi Laboratory, 3rd Floor, 1, Mulberry Industrial Estate, Hennur Main Road, Opposite State Bank of India, Ambattur Industrial Estate, Chennai - 600 065, Tel: 91-44-65051600

Read & Co. Off.: 308 House, 4th A.S. Mehta, Vikhroli (West), Mumbai-400083, Tel: (022) 25796421 to 28 Fax: (022) 25796426 to 30, www.sgs.com
APPENDIX 9 (Continued)

Test Report

SAMPLE NOT DRAWN BY SGS INDIA PVT. LTD.

Report No : CE17-001724.001

Report Control No : CER0000142381

Print Date : 07/04/2017

JOE No : CE17-001724

Per pro SGS India Private Ltd

K. MANOKARAN
Authorized Signatory

Remark: All parameters are within acceptable limits as per the JRF/TOX SUP 2077

19/04/2017

****End of Report****
### MICROBIOLOGICAL ANALYSIS CERTIFICATE OF ANIMAL FEED

| Parameters                  | Results Observed | Permissible Limits |
|-----------------------------|------------------|--------------------|
| Total viable organisms     |                  |                    |
| Bacteria                    | Nil*             | 20000 CFU/g        |
| Fungus                      | Nil*             | 200 CFU/g          |
| Salmonella sp.              | Absent           | None/g             |
| Coliform organisms          | Absent           | < 10/g             |
| E.coli type I               | Absent           | None/g             |

* = Not detected in first dilution.

**Conclusion:** The results of analysis indicate that the microbial load is within the permissible limit (JRF/MIC/SOP/616).

Analysed by : Rahul G. Badgha  
Sign & Date : [Signature]  
Verified by : Dr. Rajesh Posia  
Sign & Date : [Signature]

Compliance with OECD Principles of GLP. Accredited by AAALAC International.
Regd. Office: Near Daman Ganga Bridge, N. H. No. 8, Valad - 396 105, Dist. Valsad, Gujarat, India.
E-mail: jrf@jrfonline.com  Web: www.jrfglobal.com
## APPENDIX 10 (Continued)

### Teklad Certified Global 16% Protein Rodent Diet (Sterilizable)

| Analysis        | Result (%) |
|-----------------|------------|
| **Proximate Analysis** |            |
| Protein         | 15.50      |
| Fat             | 3.84       |
| Fiber           | 3.62       |
| Moisture        | 12.19      |
| Ash             | 4.99       |
| Calcium         | 0.88       |
| Phosphorus      | 0.72       |

### Laboratory Diet Certification Report

The following data is a consolidation of results obtained from one or more independent testing laboratories. The actual laboratory results are available upon request.

I have reviewed this document 2017.03.29 07:33:16 -05'00'.

### Heavy Metals

| Analysis       | Result | Units | Established Maximum Concentration |
|----------------|--------|-------|-----------------------------------|
| Arsenic        | 0.12   | ppm   | 1.00                              |
| Cadmium        | < 0.10 | ppm   | 0.50                              |
| Lead           | < 0.20 | ppm   | 1.50                              |
| Mercury        | < 0.05 | ppm   | 0.20                              |
| Selenium       | 0.28   | ppm   | 0.50                              |

### Mycotoxin

| Analysis       | Result | Units |
|----------------|--------|-------|
| Aflatoxin B1, B2, G1, G2 | < 5.00 | ppb   |

### Chlorinated Hydrocarbons

| Analysis       | Result | Units |
|----------------|--------|-------|
| Aldrin         | < 0.01 | ppm   |
| Lindane        | < 0.01 | ppm   |
| Chlordane      | < 0.01 | ppm   |
| DDT & related substances | < 0.03 | ppm |
| Dieldrin       | < 0.02 | ppm   |
| Endrin         | < 0.02 | ppm   |
| Heptachlor     | < 0.01 | ppm   |
| Heptachlor Epoxide | < 0.01 | ppm |
| Toxaphene      | < 0.10 | ppm   |
| PCB’s          | < 0.10 | ppm   |
| a-BHC          | < 0.01 | ppm   |
| b-BHC          | < 0.01 | ppm   |
| d-BHC          | < 0.01 | ppm   |
| Hexachlorobenzene | < 0.01 | ppm |
| Mirex          | < 0.01 | ppm   |
| Methoxychlor   | < 0.05 | ppm   |

### Organophosphates

| Analysis       | Result | Units |
|----------------|--------|-------|
| Thimet         | < 0.15 | ppm   |
| Diazinon       | < 0.14 | ppm   |
| Disulfaton     | < 0.15 | ppm   |
| Methyl Parathion | < 0.14 | ppm |
| Malathion      | < 0.14 | ppm   |
| Parathion      | < 0.12 | ppm   |
| Thiodan        | < 0.02 | ppm   |
| Ethion         | < 0.14 | ppm   |
| Trithion       | < 0.15 | ppm   |
APPENDIX 11: Bedding Material Analysis Reports

MICROBIOLOGICAL ANALYSIS CERTIFICATE OF BEDDING MATERIAL

Name of Sample : Sterilized Rice Husk (Paddy husk)
Sample Received From : UV Room (BMR Facility)
Identification No : BM1
Date of Sample Collection : 15/03/2017

Sample Analysis
Date of Initiation : 15/03/2017
Date of Completion : 17/03/2017
Sample Analysed at : Microbiology Lab - JRF

Result:

| Parameter       | Results Observed | Permissible Limit |
|-----------------|------------------|------------------|
| 1. Total Viable Count |                 |                  |
| Bacteria        | None/Plate       | None/Plate       |
| Fungus          | None/Plate       | None/Plate       |

Conclusion: The results of analysis indicate that the microbial load is within the permissible limit as recommended in JRF/MIC/SOP-621.

Analysed by : Rahul G Badgha
Sign & Date : March 17, 2017

Verified by : Dr. Rajesh Posia
Sign & Date : March 17, 2017
### APPENDIX 11 (Continued)

**Sample Not Drawn by Laboratory**

| Report No       | CG17-006372-001          | Report Control No | CGR00000703548 |
|-----------------|--------------------------|-------------------|----------------|
| Sample described by customer as | PADDY HUSK (RICE HUSK) |                   |                |
| Customer Name   | JAI RESEARCH FOUNDATION  |                   |                |
| Customer Address| OFF N.H-8,NEAR DAMAN GANGA BRIDGE, VAIVADA | VAPI, UMBERGAON |                |
| City            | VALSAD DIST               |                   |                |
| Postal Code     | 396195                    |                   |                |
| State           | GUJARAT                   |                   |                |
| Country         | INDIA                     |                   |                |
| Sample Type     | PADDY HUSK (RICE HUSK)   |                   |                |
| Received        | 30/03/2017                |                   |                |
| Sample Qty. Recd.| 500G                     |                   |                |
| Date of Collection | 22.03.2017              |                   |                |
| Test Start      | 30/03/2017                |                   |                |
| Test End Date   | 05/04/2017                |                   |                |

| Test/Parameter          | Method          | Result         | Unit |
|-------------------------|-----------------|----------------|------|
| Lead (as Pb)            | SO-IN-MUL-TE-063| 0.03           | mg/kg|
| Arsenic (as As)         | SO-IN-MUL-TE-063| 0.07           | mg/kg|
| Cadmium (as Cd)         | SO-IN-MUL-TE-063| BLO (LOQ : 0.01)| mg/kg|
| Mercury (as Hg)         | SO-IN-MUL-TE-063| BLO (LOQ : 0.01)| mg/kg|
| Alpha-endosulfan        | AOAC 970.52     | BLO(LOQ : 0.01)| mg/kg|
| Beta-Endosulfan         | AOAC 970.52     | BLO(LOQ : 0.01)| mg/kg|
| Aldrin                  | AOAC 970.52     | BLO(LOQ : 0.01)| mg/kg|
| Dieldrin                | AOAC 970.52     | BLO(LOQ : 0.01)| mg/kg|
| Methoxychlor            | AOAC 970.52     | BLO(LOQ : 0.01)| mg/kg|
| Endrin                  | AOAC 970.52     | BLO(LOQ : 0.01)| mg/kg|
| Methyl-parathion        | AOAC 970.52     | BLO(LOQ : 0.01)| mg/kg|
| Gamma-HCH(Lindane)      | AOAC 970.52     | BLO(LOQ : 0.01)| mg/kg|
| Phorate                 | AOAC 970.52     | BLO(LOQ : 0.01)| mg/kg|
| Malathion               | AOAC 970.52     | BLO(LOQ : 0.01)| mg/kg|

Per pro SGS India Private Ltd

M Thaneemmalai
Authorized Signatory

**Remarks:** All parameters were within acceptable limits as per the JRF Tool Set 2017

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Micronucleus Test of Acetamide in Mice

APPENDIX 12: Certificate of Analysis of Acetamide (Provided by Supplier)

Certificate of Analysis

Jul 21, 2017 (JST)

TOKYO CHEMICAL INDUSTRY CO., LTD.
4-10-1 Nihonbashi-Honcho, Chuo-ku, Tokyo 103-0023 Japan

| Chemical Name: Acetamide |
|--------------------------|
| Product Number: A0007    |
| CAS: 62-53-9             |
| Lot: QYD4G               |

| Tests                      | Results          | Specifications          |
|----------------------------|------------------|-------------------------|
| Purity (GC)                | 99.2 %           | min. 98.5 %             |
| Melting point              | 81.4 deg-C       | 80.0 to 84.0 deg-C      |
| Solubility in Water        | transparency     | almost transparency     |

TCI Lot numbers are 4-5 characters in length. Characters listed after the first 4-5 characters are control numbers for internal purpose only.
The contents of the specifications are subject to change without advance notice. The specification values displayed here are the most up to date values. There may be cases where the product labels display a different specification, however, the product quality still meets the latest specification.

Customer service:
TCI Chemicals (India) Pvt. Ltd.
Tel: 044-2262 8878 / 044-2262 8878
Fax: 044-2262 8902
E-mail: Sales-in@TCIchemicals.com
Micronucleus Test of Acetamide in Mice

APPENDIX 13: Certificate of Analysis of Acetamide (Generated at JRF)

CERTIFICATE OF ANALYSIS

This Certificate of Analysis is compiled from the exact data taken from JRF Study Number: 228-2-14-17729
The analysis was conducted in compliance with OECD Principles of Good Laboratory Practice (1998).

TEST ITEM DETAILS

Test Item Name: Acetamide
Active Ingredient(s): Acetamide
CAS Number: 60-35-5
Molecular Weight: 59.07
Molecular Formula: C₃H₇NO
Batch/Lot Number: QYD4G
Retest Date: December 3, 2017
Manufactured by: Tokyo Chemical Industry Co., Ltd.
Sponsored by: Michigan State University, United States
Appearance: White Solid
Storage Condition (at JRF): Room Temperature

RESULT OF ANALYSIS

Analysis Start: September 04, 2017
Analysis End: September 04, 2017
Method of Analysis: Gas Chromatography [GC] equipped with Mass Spectrometer (GC-MS)
Mass and Confirmation Method: Not Applicable
Analysed Purity/Concentration: 99.198 % w/w

TEST FACILITY & ARCHIVES

Jai Research Foundation, Valvada, Gujarat, India

Signature & Date
Name: Tushar Khanvilkar
Study Director, JRF

Compliance with OECD Principles of GLP, Accredited by AAALAC International
Regd. Office: Near Daman Ganga Bridge, N. H. No. 8, Valvada - 396 105, Dist. Valsad, Gujarat, India.
E-mail: jrf@jrfonline.com ♦ Web: www.jrfglobal.com
GOVERNMENT OF INDIA
Department of Science and Technology
National Good Laboratory Practice (GLP) Compliance Monitoring Authority (NGCMA)

Certificate of GLP Compliance

Based on the Inspection and the subsequent follow-up actions

Jai Research Foundation
Near Daman Ganga Bridge, N. H. No. 8
Valvada-396 105, Dist. Valsad (Gujarat)

is certified capable of conducting the below-mentioned tests/studies in compliance with Organization for Economic Co-operation & Development (OECD) Principles of GLP:

- Physical-chemical Studies
- Toxicity Studies
- Mutagenicity studies
- Environmental Toxicity Studies on Aquatic and Terrestrial Organisms
- Studies on Behavior in Water, Soil and Air; Bioaccumulation Residue Studies
- Residue Studies
- Analytical and Clinical Chemistry Testing
- Others

The specific areas of expertise, types of chemicals and test systems are listed in annexure overleaf.

Validity: August 5, 2016 – August 4, 2019

This certificate is subject to the condition that the test facility complies with the Terms & Conditions of the NGCMA’s Document No. GLP-101 and OECD Principles of GLP.

Certificate No.: GLP/C-089/2016
Issue Date : 22-07-2016

Anil Relia
Head, NGCMA
APPENDIX 14 (Continued)

National GLP Compliance Monitoring Authority (NGCMA)

Annexure to Certificate of GLP Compliance No. GLP/C-089/2016

Areas of Expertise:

| Physical-chemical Testing |
|---------------------------|
| Toxicity Studies          |
| o Acute Toxicity          |
| o Sub-acute Toxicity      |
| o Chronic Toxicity        |
| o Sub-chronic Toxicity    |
| o Inhalation Toxicity studies |
| o Reproductive Studies    |
| o Skin Sensitization Studies |
| o Neurotoxicity Studies   |
| o Teratogenicity Studies  |
| o Immunotoxicity Studies  |
| o Endocrine Disruptor Assays |
| o Carcinogenicity Studies |
| o In vitro Skin Corrosion Test: Reconstructed Human Epidermis Test |
| o In vitro Skin Irritation Test |
| o Bovine Corneal Opacity and Permeability Test for Validation of Test |

| Mutagenicity Studies |
|----------------------|
| o Bacterial Reverse Mutation Assay (AMES Test) |
| o Micronucleus Test (In-vivo & In-vitro) |
| o Chromosomal Aberration Test (In-vivo & In-vitro) |
| o Cell Gene Mutation |
| o Endocrine Disruptor Assay |

| Environmental Toxicity Studies on Aquatic & Terrestrial Organisms |
|---------------------------------------------------------------|
| o Alga Growth Inhibition Test |
| o Daphnia Acute Immobilization Test |
| o Acute Fish Toxicity |
| o Acute Oral and Contract Toxicity Test to Honeybee |
| o Acute Earthworm Toxicity Test |
| o Avian Acute Oral and Dietary Toxicity Study |
| o Earthworm and Daphnia Reproduction Toxicity Test |
| o Fish: Embryo Toxicity Test |
| o Fish: Short-term Toxicity Test on Embryo and Sac-fry Stages |

| Studies on Behaviour in Water, Soil and Air: Bioaccumulation |
|-------------------------------------------------------------|
| Residue Studies                                            |
| Analytical and Clinical Chemistry Testing                  |
| Others                                                     |
| o Impurity Profile and Five Batch Analyses                  |
| o Bio-analytical and Toxicokinetics                         |
| o Drug Metabolism & Pharmacokinetics and Tissue distribution |
| o Safety Pharmacology                                       |

Types of Chemicals:
Industrial Chemicals, Pesticides, Pharmaceuticals, Veterinary Drugs, Cosmetics, Food Additives, and Feed Additives.

Test Systems:
Rat, Mouse, Rabbit, Guinea Pig, Dog, Fish, Algae, Daphnia, Honeybee, Earthworm, Japanese Quail, Mallard Duck, Cornea, Human Lymphocytes, CHO-K1 Cell Line, H295R Cell Line, HeLa 9903 Cell Line, J774A.1 Cell Line, Mouse Lymphoma Cell Line, BALB/3T3 Clone A31, KeratinoSens Cell Line, Human Monocyte Cell Line THP-1, Human Myeloid U937 Cells, Caco-2, Colo 205, Salmonella typhimurium and Escherichia coli

(Anil Relia)
Head, NGCMA