Original Final Report 2 of 2

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 TROWBRIGE 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.

AKSolanki November 18, 2017
Avani K. Solanki, M.Sc. Date
Study Director

November 18, 2017
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.

Inspection/Audit			Reporting Dates to	
N°	**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)

Inspection			**Reporting Dates to**	
N°	**Date**	**Details**	**Study Director**	**Facility Management**
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.

Patel

HEMANGINI PATEL, M. Pharm.
QUALITY ASSURANCE OFFICER, JRF
DATE: November 15, 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.

Indrajitsinh. M. Barad, M.Sc.
Geeta J. Singh, M.Sc.
Akash D. Dhangar, M.Sc.
Statistical Analyst : Ranjit Singh, M.Sc. (Statistics)

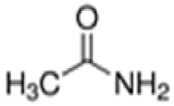
1.6 Archives

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	C_2H_5NO
Molecular Weight	59.07 g/mol
Molecular Structure	
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 N°	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)
NaH_2PO_4	Merck (Lot # QH3Q631840)
Na_2HPO_4	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 (Capable of measuring 10 mg to 210 g)
Electronic Balance	Electronic Weighing Scale - SMART (Capable of measuring 5 g to 3 kg)
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 (CO_2 Chamber)
Centrifuges	Thermo scientific

Binocular Microscopes : 1. Eclipse E600, Nikon
2. Eclipse 80i, Nikon
3. Eclipse Ni-U (Fluorescence), Nikon
4. Eclipse Ci, Nikon
Multiportable Meter : Hach, USA
Tattoo Machine : AIMS^{TM} Tattoo Machine
Microprobe Thermometer : Physitemp Instruments Inc.
Refrigerators : LG Electronics Inc.
Bench Top Autoclave : Kumar
Deep-Freezer (-20 °C) : Vestfrost Solutions
Deep-Freezer (-80 °C) : SANYO
Micropipette : Eppendorf AG (100 – 1000 µL, 20-200 µL)
Millipore Water Purification System : Merck Millipore
Vortex Mixer : Remi Elecrotechnik
RO Systems : Hitech Water Technology Pvt. Ltd., Kent RO System
Ultrasonic Cleaning Bath : Cole Parmer, USA

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

Animal Room	:	BMR Facility Room No. 31, Department of Toxicology
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

Caging	:	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.
Water Bottle	:	Each cage was supplied with a polypropylene water bottle (capacity 300 mL) with a stainless steel nozzle.
Housing	:	3 animals per cage.
Room Sanitation	:	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

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 CO_2 asphyxiation (MacGregor *et al.* 1987) and the positive control group (group V) was sacrificed 24 hour after the last treatment by CO_2 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.

5. REFERENCES

Asanami, S., and Shimono, K., 1997a: Hypothermia induces micronuclei in mouse bone marrow cells. Mutation Research. 393, pp. 91–98.

Asanami, S., and Shimono, K., 1997b: High body temperature induces micronuclei in mouse bone marrow. Mutation Research. 390, pp. 79–83.

Asanami, S., Shimono, K., and Kaneda, S., 1998: Transient hypothermia induces micronuclei in mice. Mutation Research. 413, 7–14.

Chieli, E., F. Aliboni, M. Saviozzi and G. Malvaldi 1987: Induction of micronucleated erythrocytes by primary thioamides and their metabolites in the mouse, Mutation Research 192, pp. 141-143.

Dybing et al., 1987: Studies on the Mechanism of Acetamide Hepatocarcinogenicity, Pharmacology & Toxicology, 60, pp. 9-16.

G. Krishna and M. Hayashi, 2000: In vivo Rodent Micronucleus Assay: Study plan Conduct and Data Interpretation, Mut.res. 455, pp. 155-166.

Gad, S.C. and Cassidy, C.D., 2006: Non-clinical Vehicles Use in Studies by Multiple Routes in Multiple Species, International Journal of Toxicology, 25, pp. 499 – 521

Gad, S.C. and Weil, C.S., 1994: “Statistics for Toxicologists”. In: Principles and Methods of Toxicology, 3rd edition, Hayes A.W. (Ed), Raven press Ltd., New York, pp. 221-274.

Heddle, J.A., Stuart, E. and Salamone, M.F., 1984: The Bone Marrow Micronucleus Test, In: Hand Book of Mutagenicity Test Procedures. Second Edn., Kilbey B.J., M. Legator, W. Nichols, C. Ramel (Eds.) Elsevier, New York, pp. 441-457.

Khanvilkar T., 2017: "Validation of Analytical Method for Determination of Acetamide Concentration, Homogeneity and Stability in Vehicle", JRF Study Number: 228-2-14-17729, November 08, 2017, Study Report of Michigan State University, USA.

Khanvilkar T., 2017: "Validation of Analytical Method for Determination of Acetamide Concentration and Stability in Plasma Samples", JRF Study Number: 228-2-14-18476, November 13, 2017, Study Report of Michigan State University, USA.

MacGregor, J.T., Heddle, J.A., Margolin, B.H., Ramel, C., Salamone, M.F., Tice, R.R. and Wild D., 1987: Guidelines for the Conduct of Micronucleus Assays in Mammalian Bone Marrow Erythrocytes, Mut. Res., 189, pp. 103-112.

Michael, R., Johannes, E., Ute, M., Markus, F. T., Simon, P., Heidrun Ellinger-Ziegelbauer, Andreas Z., 2014: Cross-Platform Toxicogenomics for the Prediction of Non-Genotoxic Hepatocarcinogenesis in Rat, Plos One, volume 9, Issue 5, May 2014.

Mirkova, E.T., 1996: Activities of the rodent carcinogens thioacetamide and acetamide in the mouse bone marrow micronucleus assay, Mutation Research 352, pp. 23-30.

OECD, 1998 : OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1, “OECD Principles on Good Laboratory Practice”, ENV/MC/CHEM(98)17 (as revised in 1997).

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.

Putcha, L et al., 1984: Disposition of ^{14}C-Acetohydroxamic acid and ^{14}C- Acetamide in the Rat, Drug Metabolism and Disposition, The American Society for Pharmacology and Experimental Therapeutics, Volume 12, No. 4, pp. 438-443.

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.

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**	
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	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**	
G I 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	37.1	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	37.1	37.2
	SD	0.2	0.2	0.1	0.2	0.2	0.2	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 **Refer: APPENDIX 3**

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 (Distilled water)	**Mean**	38.67	39.00	39.33	28.83	29.00	28.83
	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	Male					Female				
	Total PCE	MNPCE			P/E Ratio (Mean ± SD)	Total PCE	MNPCE			P/E Ratio (Mean ± SD)
		Total	Mean ± SD	%MNPCE (Mean ± SD)			Total	Mean ± SD	%MNPCE (Mean ± SD)	
G I Vehicle control (Distilled water)	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
G II (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
G III (1000 mg/kg body weight)	27043	4	0.667 ± 0.816	0.013 ± 0.016	0.498 ± 0.018	27095	6	1.000 ± 0.894	0.020 ± 0.018	0.522 ± 0.031
G IV (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
G V (Mitomycin-C,1.0 mg/kg body weight)	27127	355	59.167↑↑ ± 16.364	1.308↑↑ ± 0.363	0.511 ± 0.039	27066	363	60.500↑↑ ± 18.960	1.340↑↑ ± 0.422	0.499 ± 0.018

Note:

$$\% \text{ MNPCE} = \frac{\text{MNPCE x 100}}{\text{Total PCE}}$$

Keys:

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 ($p \leq 0.01$)

Micronucleus Test of Acetamide in Mice

APPENDIX 1: Individual Clinical Observations – Main Study

Group and Dose of Acetamide	Sex	Animal N°	Individual Animal Observations on Experimental Days										Before Sacrifice
			Before Dosing (Day 1)	After Dosing Day 1 (hours)				Before Dosing (Day 2)	After Dosing Day 2 (hours)				
				1	2	3	4		1	2	3	4	
G I Vehicle control (Distilled water)	M	T1	1	1	1	1	1	1	1	1	1	1	1
		T2	1	1	1	1	1	1	1	1	1	1	1
		T3	1	1	1	1	1	1	1	1	1	1	1
		T4	1	1	1	1	1	1	1	1	1	1	1
		T5	1	1	1	1	1	1	1	1	1	1	1
		T6	1	1	1	1	1	1	1	1	1	1	1
	F	T7	1	1	1	1	1	1	1	1	1	1	1
		T8	1	1	1	1	1	1	1	1	1	1	1
		T9	1	1	1	1	1	1	1	1	1	1	1
		T10	1	1	1	1	1	1	1	1	1	1	1
		T11	1	1	1	1	1	1	1	1	1	1	1
		T12	1	1	1	1	1	1	1	1	1	1	1
G II (250 mg/kg body weight)	M	T13	1	1	1	1	1	1	1	1	1	1	1
		T14	1	1	1	1	1	1	1	1	1	1	1
		T15	1	1	1	1	1	1	1	1	1	1	1
		T16	1	1	1	1	1	1	1	1	1	1	1
		T17	1	1	1	1	1	1	1	1	1	1	1
		T18	1	1	1	1	1	1	1	1	1	1	1
	F	T19	1	1	1	1	1	1	1	1	1	1	1
		T20	1	1	1	1	1	1	1	1	1	1	1
		T21	1	1	1	1	1	1	1	1	1	1	1
		T22	1	1	1	1	1	1	1	1	1	1	1
		T23	1	1	1	1	1	1	1	1	1	1	1
		T24	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 Sacrifice**
			Before Dosing (Day 1)	**After Dosing Day 1 (hours)**				**Before Dosing (Day 2)**	**After Dosing Day 2 (hours)**					
				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
		T26	1	1	1	1	1	1	1	1	1	1	1
		T27	1	1	1	1	1	1	1	1	1	1	1
		T28	1	1	1	1	1	1	1	1	1	1	1
		T29	1	1	1	1	1	1	1	1	1	1	1
		T30	1	1	1	1	1	1	1	1	1	1	1
	F	T31	1	1	1	1	1	1	1	1	1	1	1
		T32	1	1	1	1	1	1	1	1	1	1	1
		T33	1	1	1	1	1	1	1	1	1	1	1
		T34	1	1	1	1	1	1	1	1	1	1	1
		T35	1	1	1	1	1	1	1	1	1	1	1
		T36	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
		T38	1	1	1	1	1	1	1	1	1	1	1
		T39	1	1	1	1	1	1	1	1	1	1	1
		T40	1	1	1	1	1	1	1	1	1	1	1
		T41	1	1	1	1	1	1	1	1	1	1	1
		T42	1	1	1	1	1	1	1	1	1	1	1
	F	T43	1	1	1	1	1	1	1	1	1	1	1
		T44	1	1	1	1	1	1	1	1	1	1	1
		T45	1	1	1	1	1	1	1	1	1	1	1
		T46	1	1	1	1	1	1	1	1	1	1	1
		T47	1	1	1	1	1	1	1	1	1	1	1
		T48	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
		T50	-	-	-	-	-	1	1	1	1	1	1
		T51	-	-	-	-	-	1	1	1	1	1	1
		T52	-	-	-	-	-	1	1	1	1	1	1
		T53	-	-	-	-	-	1	1	1	1	1	1
		T54	-	-	-	-	-	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	Sex	Animal N°	Clinical Signs Observed after Dosing on										Before Sacrifice
			Before Dosing (Day 1)	After Dosing Day 1 (hours)				Before Dosing (Day 2)	After Dosing Day 2 (hours)				
				1	2	3	4		1	2	3	4	
G V (Mitomycin-C, 1.0 mg/kg body weight)	F	T55	1	-	-	-	-	1	1	1	1	1	1
		T56	1	-	-	-	-	1	1	1	1	1	1
		T57	1	-	-	-	-	1	1	1	1	1	1
		T58	1	-	-	-	-	1	1	1	1	1	1
		T59	1	-	-	-	-	1	1	1	1	1	1
		T60	1	-	-	-	-	1	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 N°**	**Day of Dosing**						
		Before Dosing (Day 1)	**After Dosing Day – 1 (hours)**		**Before Dosing (Day 2)**	**After Dosing Day - 2 (hours)**		**Before Sacrifice**
			2 h	**5 h**		**2 h**	**5 h**	
		°C	**°C**	**°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	37.3	37.4	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	37.3	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	36.7	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°	Day of Dosing						
		Before Dosing (Day1)	After Dosing Day – 1 (hours)		Before Dosing (Day2)	After Dosing Day – 2 (hours)		Before Sacrifice
			2 h	5 h		2 h	5 h	
		°C	°C	°C	°C	°C	°C	°C
G I Vehicle control (Distilled water)	T7	37.3	37.1	37.3	37.4	37.4	37.0	37.3
	T8	37.5	37.2	37.5	37.0	37.2	37.3	37.2
	T9	37.1	37.0	37.3	37.4	37.3	37.2	37.1
	T10	37.3	37.1	37.1	37.4	36.9	37.3	37.1
	T11	37.0	37.4	37.3	37.2	37.3	37.2	37.7
	T12	37.2	37.3	37.3	37.1	37.1	37.4	37.3
G II (250 mg/kg body weight)	T19	37.4	37.3	37.4	37.3	37.2	37.4	37.1
	T20	37.2	37.2	37.1	37.4	37.0	37.2	37.6
	T21	37.1	37.5	37.4	37.2	36.8	37.1	37.3
	T22	37.0	36.8	37.3	37.2	37.2	36.8	37.2
	T23	37.3	36.9	37.1	37.1	37.1	36.8	36.6
	T24	37.1	37.1	37.3	37.2	37.3	37.3	37.4
G III (1000 mg/kg body weight)	T31	37.3	37.1	37.4	37.3	37.0	37.3	37.1
	T32	37.3	37.0	37.1	37.1	37.3	37.0	37.2
	T33	37.0	36.9	37.3	37.0	37.0	37.2	37.1
	T34	37.4	37.1	37.4	37.3	37.1	37.2	37.2
	T35	37.1	37.4	37.3	37.0	36.9	37.3	37.6
	T36	36.8	37.1	37.0	37.1	37.0	37.2	37.3
G IV (2000 mg/kg body weight)	T43	37.3	37.3	37.4	37.1	37.0	37.2	37.6
	T44	36.8	36.9	37.2	37.2	36.9	37.2	37.4
	T45	37.1	37.5	37.3	37.3	37.4	37.3	37.2
	T46	37.2	37.1	37.2	37.0	37.0	37.0	36.9
	T47	37.0	37.4	37.4	36.9	37.2	36.8	37.5
	T48	37.4	37.2	37.5	37.3	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	37.3
	T56	-	-	-	37.2	36.9	37.2	37.0
	T57	-	-	-	37.0	37.1	37.2	37.4
	T58	-	-	-	36.8	37.0	37.0	37.6
	T59	-	-	-	37.2	37.0	37.3	37.7
	T60	-	-	-	37.1	36.9	37.3	37.4

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)		
			Day 1	Day 2	Before Sacrifice
G I Vehicle control (Distilled water)	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
	Female	T7	31	31	29
		T8	29	29	30
		T9	30	30	29
		T10	27	27	28
		T11	28	28	29
		T12	28	29	28
G II (250 mg/kg body weight)	Male	T13	43	42	42
		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 (1000 mg/kg body Weight)	Male	T25	41	41	40
		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)		
			Day 1	Day 2	Before Sacrifice
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	33	33	34
		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
		T50	-	41	40
		T51	-	39	40
		T52	-	39	40
		T53	-	39	39
		T54	-	34	34
	Female	T55	-	29	29
		T56	-	31	31
		T57	-	28	28
		T58	-	29	29
		T59	-	29	29
		T60	-	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 N°	Total PCE Scored	PCE Corr. to NCE	NCE Scored	Total Erythrocytes	P/E Ratio
G I Vehicle control (Distilled water)	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 (250 mg/kg body weight)	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
		T24	4501	296	236	532	0.556
G III (1000 mg/kg body weight)	Male	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 N°	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
G IV (2000 mg/kg body weight)	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
G V (Mitomycin-C,1.0 mg/kg body weight)	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
	Female	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 N°	Total Number of PCE Scored	Number of MNPCE	Percent MNPCE
G I Vehicle control (Distilled water)	Male	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)	Male	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)	Male	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 N°**	**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
		T41	4519	1	0.02
		T42	4513	0	0.00
	Female	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

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.

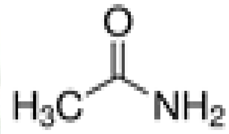
APPENDIX 6 (Continued)

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_2H_5NO
Molecular Weight	59.07 g/mol
Molecular Structure	
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 N^{o} 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.

APPENDIX 6 (Continued)

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.

APPENDIX 6 (Continued)

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.

APPENDIX 6 (Continued)

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.

APPENDIX 6 (Continued)

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).

APPENDIX 6 (Continued)

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.

APPENDIX 6 (Continued)

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 CO_2 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 CO_2 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 Bio-statistics 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 JRFs 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.

APPENDIX 6 (Continued)

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.

APPENDIX 6 (Continued)

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

APPENDIX 6 (Continued)

- 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

APPENDIX 6 (Continued)

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

Asanami, S., and Shimono, K., 1997a: Hypothermia induces micronuclei in mouse bone marrow cells. Mutation Research. 393, pp. 91–98.

Asanami, S., and Shimono, K., 1997b: High body temperature induces micronuclei in mouse bone marrow. Mutation Research. 390, pp. 79–83.

Asanami, S., Shimono, K., and Kaneda, S., 1998: Transient hypothermia induces micronuclei in mice. Mutation Research. 413, 7–14. G. Krishna and M. Hayashi, 2000: *In vivo* Rodent Micronucleus Assay: Study plan Conduct and Data Interpretation, Mut.res. 455, pp. 155-166.

Chieli, E., F. Aliboni, M. Saviozzi and G. Malvaldi 1987: Induction of micronucleated erythrocytes by primary thioamides and their metabolites in the mouse, Mutation Research 192, pp. 141-143.

Dybing et al., 1987: Studies on the Mechanism of Acetamide Hepatocarcinogenicity, Pharmacology & Toxicology, 60, pp. 9-16.

Gad, S.C. and Cassidy, C.D., 2006: Non-clinical Vehicles Use in Studies by Multiple Routes in Multiple Species, International Journal of Toxicology, 25, pp. 499 – 521

Gad, S.C. and Weil, C.S., 1994: “Statistics for Toxicologists”. In: Principles and Methods of Toxicology, 3rd edition, Hayes A.W. (Ed), Raven press Ltd., New York, pp. 221-274.

Heddle, J.A., Stuart, E. and Salamone, M.F., 1984: The Bone Marrow Micronucleus Test, In: Hand Book of Mutagenicity Test Procedures. Second Edn., Kilbey B.J., M. Legator, W. Nichols, C. Ramel (Eds.) Elsevier, New York, pp. 441-457.

MacGregor, J.T., Heddle, J.A., Margolin, B.H., Ramel, C., Salamone, M.F., Tice, R.R. and Wild D., 1987: Guidelines for the Conduct of Micronucleus Assays in Mammalian Bone Marrow Erythrocytes, Mut. Res., 189, pp. 103-112.

Michael, R., Johannes, E., Ute, M., Markus, F. T., Simon, P., Heidrun Ellinger-Ziegelbauer, Andreas Z., 2014: Cross-Platform Toxicogenomics for the Prediction of Non-Genotoxic Hepatocarcinogenesis in Rat, Plos One, volume 9, Issue 5, May 2014.

APPENDIX 6 (Continued)

Mirkova, E.T., 1996: Activities of the rodent carcinogens thioacetamide and acetamide in the mouse bone marrow micronucleus assay, Mutation Research 352, pp. 23-30.

OECD, 1998 : OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1, “OECD Principles on Good Laboratory Practice”, ENV/MC/CHEM(98)17 (as revised in 1997).

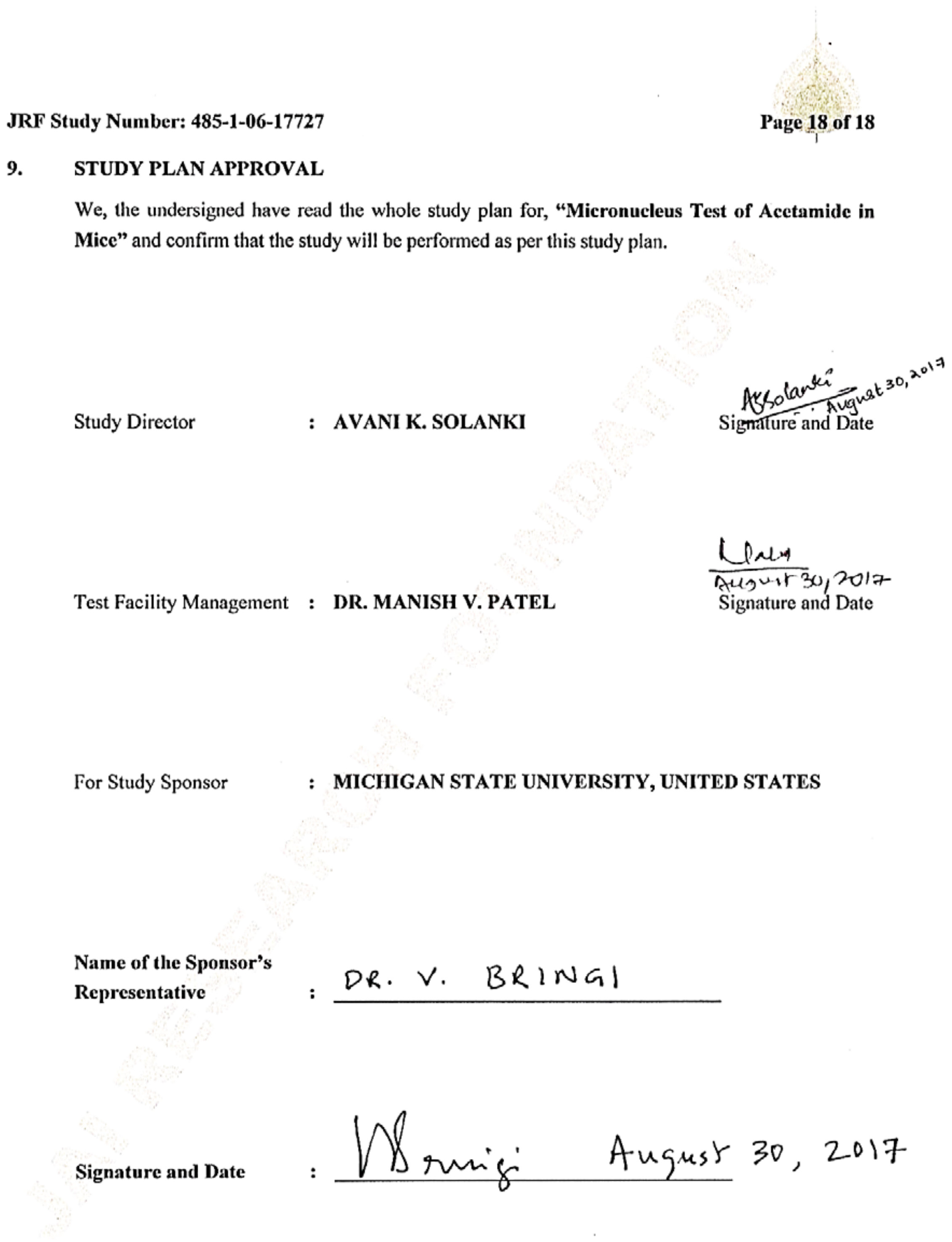
OECD, 2016: The Organisation for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals, Volume II, OECD 474, Mammalian Erythrocyte Micronucleus Test, adopted by the Council on July 29, 2016.

Putcha, L et al., 1984: Disposition of ^{14}C-Acetohydroxamic acid and ^{14}C- Acetamide in the Rat, Drug Metabolism and Disposition, The American Society for Pharmacology and Experimental Therapeutics, Volume 12, No. 4, pp. 438-443.

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.

APPENDIX 6 (Continued)



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** ASolanki August 30, 2017 Signature and Date

Test Facility Management : **DR. MANISH V. PATEL** August 30, 2017 Signature and Date

For Study Sponsor : **MICHIGAN STATE UNIVERSITY, UNITED STATES**

Name of the Sponsor's Representative : DR. V. BRINGI

Signature and Date : VBringi August 30, 2017

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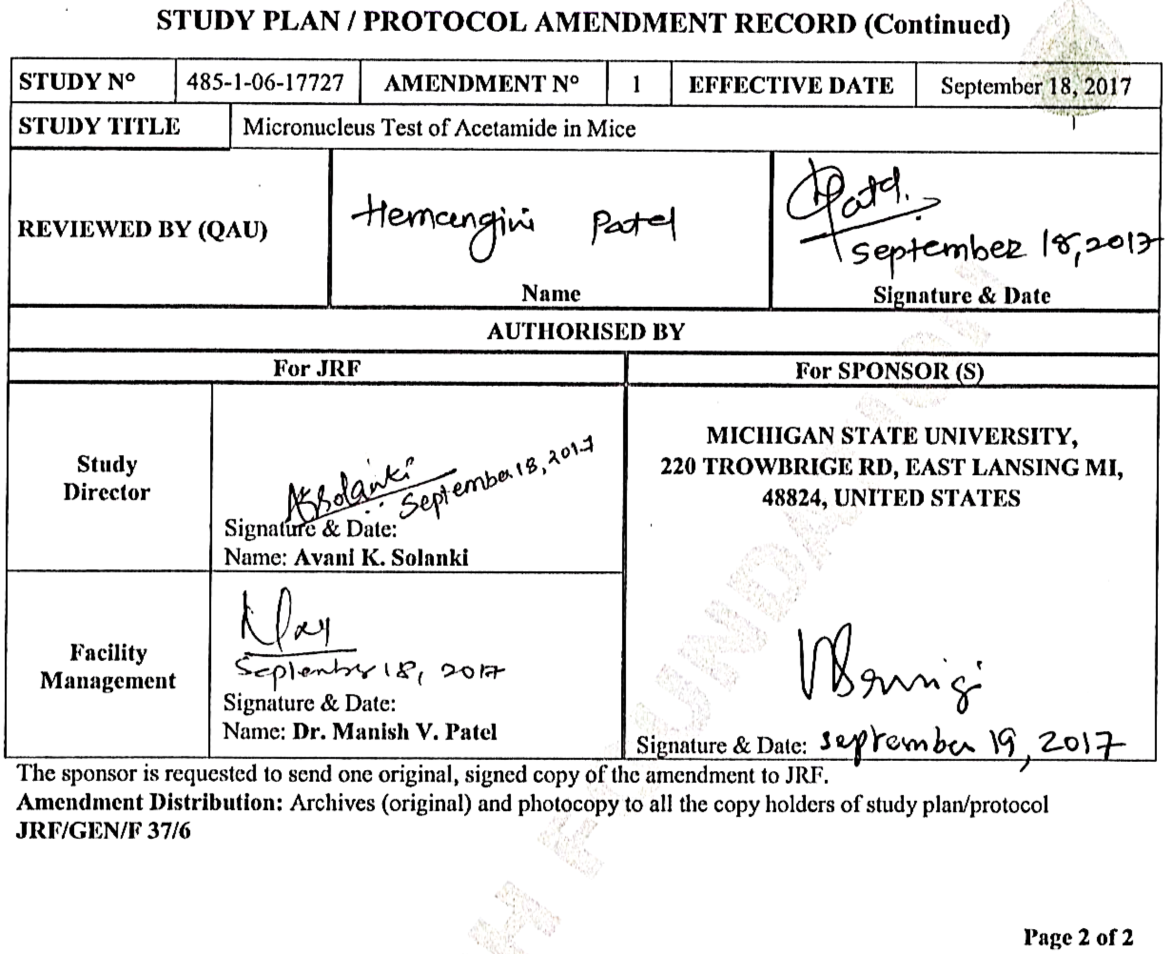
STUDY PLAN / PROTOCOL AMENDMENT RECORD

STUDY N°	485-1-06-17727	**AMENDMENT N°**	1	**EFFECTIVE DATE**	September 18, 2017
STUDY TITLE	Micronucleus Test of Acetamide in Mice				

ORIGINAL DETAILS*	DETAILS AMENDED	REASON FOR AMENDMENT
Study Plan Page 9 of 18 **5.10 Dose Formulation Preparation, Sampling and Analysis**	**Addition** 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 Quadruple Temperature : 150 °C Data Acquisition : Selected Ion Monitoring (SIM) for masses 239 (Xanthyl-acetamide) and 253 (Xanthyl-Propionamide)	Addition of analytical method parameters for dose formulation analysis.
Study Plan Page 11 of 18 **5.12 Evidence of Tissue Exposure**	**Addition** 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)	Addition of analytical method parameters for plasma sample analysis.

* Reference of page N°, paragraph number etc.

APPENDIX 6 (Continued)



STUDY PLAN / PROTOCOL AMENDMENT RECORD (Continued)

STUDY N°	485-1-06-17727	**AMENDMENT N°**	1	**EFFECTIVE DATE**	September 18, 2017
STUDY TITLE	Micronucleus Test of Acetamide in Mice				

REVIEWED BY (QAU)	Hemangini Patel **Name**	Patel September 18, 2017 **Signature & Date**

AUTHORISED BY

For JRF		**For SPONSOR (S)**
Study Director	AKSolanki September 18, 2017 Signature & Date: Name: **Avani K. Solanki**	**MICHIGAN STATE UNIVERSITY, 220 TROWBRIGE RD, EAST LANSING MI, 48824, UNITED STATES**
Facility Management	September 18, 2017 Signature & Date: 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.
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JRF/GEN/F 37/6

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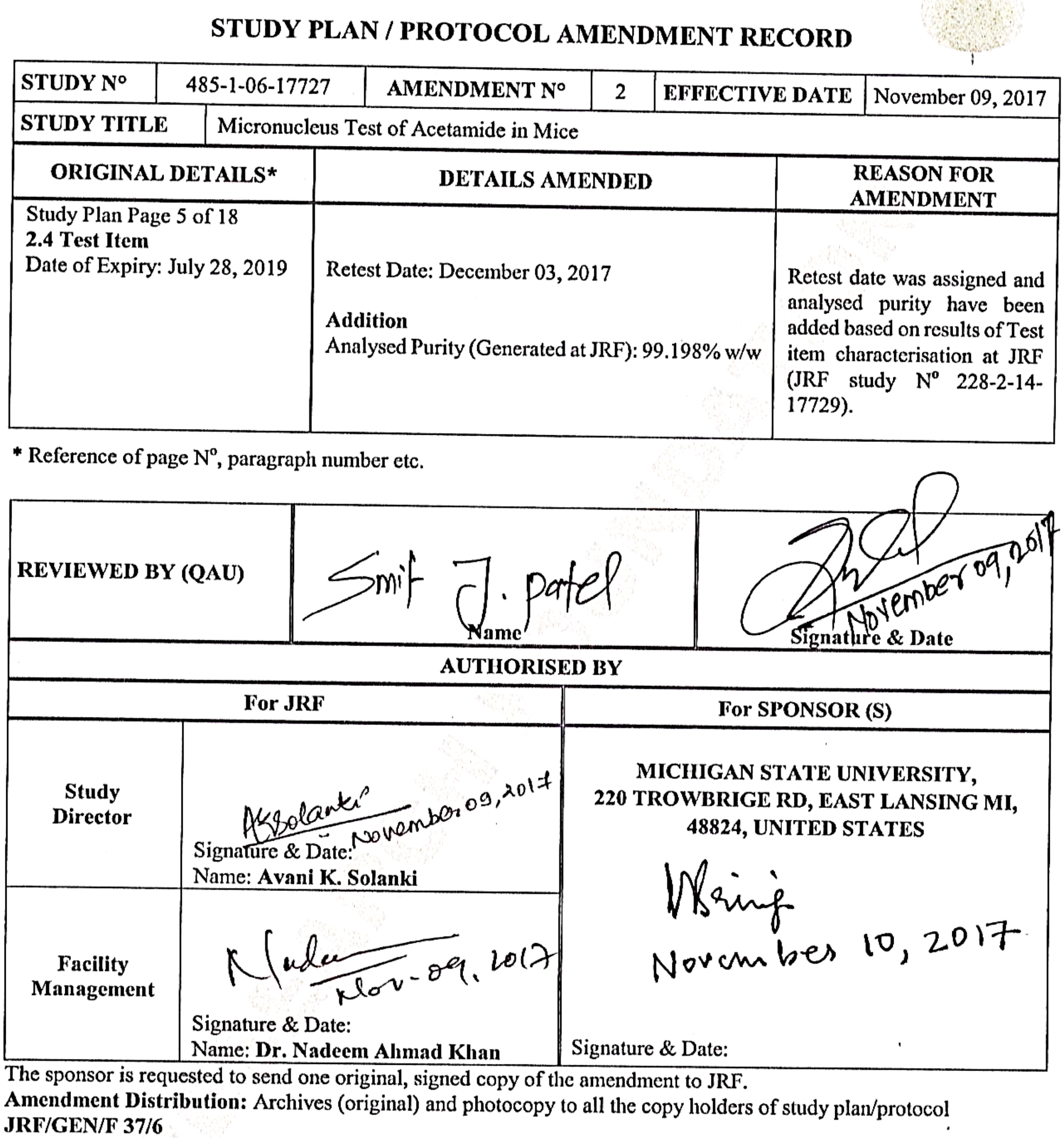

STUDY PLAN / PROTOCOL AMENDMENT RECORD

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

ORIGINAL DETAILS*	**DETAILS AMENDED**	**REASON FOR AMENDMENT**
Study Plan Page 5 of 18 **2.4 Test Item** Date of Expiry: July 28, 2019	Retest Date: December 03, 2017 **Addition** Analysed Purity (Generated at JRF): 99.198% w/w	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).

* Reference of page N°, paragraph number etc.

REVIEWED BY (QAU)	Smit J. patel Name	November 09, 2017 Signature & Date
AUTHORISED BY		

For JRF		**For SPONSOR (S)**
Study Director	AKSolanki November 09, 2017 Signature & Date: Name: **Avani K. Solanki**	**MICHIGAN STATE UNIVERSITY,** **220 TROWBRIGE RD, EAST LANSING MI,** **48824, UNITED STATES** November 10, 2017 Signature & Date:
Facility Management	Nov-09, 2017 Signature & Date: 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 37/6

APPENDIX 7: Dose Formulation Analysis and Plasma Sample Analysis Report

November 18, 2017

ABHISHEK TATER
ANALYST

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ABBREVIATIONS

% - Percent
°C - Degree Centigrade
CC - Calibration Curve
µL - Microlitre(s)
AR - Analytical Reagent
C_{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 - Milliliter(s)
mm - Millimeter
MQC - Mid Quality Control
N° - Number
RE - Relative Error
RSD - Relative Standard Deviation
SD - Standard Deviation
SS - Spiking Solution
T_{max} - Time of maximum observed peak plasma concentration
ULOQ - Upper Limit of Quantification

APPENDIX 7 (Continued)

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)	Middle	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: $r^2 \geq 0.98$).

APPENDIX 7 (Continued)

C. Plasma sample analysis

Five groups (comprising 6 animals/sex/group) were used for this study. Group I was serve 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/kgb.wt.)**	**Animal N°**		**Group N°**	**Dose (mg/kgb.wt.)**	**Animal N°**	
		Male	**Female**			**Male**	**Female**
GI	**Vehicle control**	1	7	**GIII**	**1000.0**	25	31
		2	8			26	32
		3	9			27	33
		4	10			28	34
		5	11			29	35
		6	12			30	36
GII	**250.0**	13	19	**GIV**	**2000.0**	37	43
		14	20			38	44
		15	21			39	45
		16	22			40	46
		17	23			41	47
		18	24			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: $r^2 \geq 0.98$).

APPENDIX 7 (Continued)

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:

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

APPENDIX 7 (Continued)

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

APPENDIX 7 (Continued)

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 acetamideon 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

APPENDIX 7 (Continued)

2.1.2 Instrument parameters for plasma concentration analysis of acetamide on 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 °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).

APPENDIX 7 (Continued)

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
		Lab oven	Laboratory Instruments
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

APPENDIX 7 (Continued)

3.3 Preparation of solutions for dose formulation analysis

3.3.1 Preparation of stock solutions

Preparation of Stock Solutions					
Weight (mg) of Standard	Purity (%)	Final Volume (mL)	Volume made up with	Obtained Concentration (ppm)	Identification of Standard Stock Solution
10.20	99.2	10	Methanol	1011.84	A

3.3.2 Preparation of internal standard working solution

Preparation of Stock Solutions					
Weight (mg) of Standard	Purity (%)	Final Volume (mL)	Volume made up with	Obtained Concentration (ppm)	Identification of Standard Stock Solution
10.10	100	10	Methanol	1010.00	IS

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 ºC in refrigerator.

Identification of Standard Solution (ppm)	**Solution Taken (mL)**	**Final Volume (mL)**	**Volume made up to the mark with**	**Obtained Concentration (ppm)**	**Identification of Standard Working Solutions**
A – (1011.84)	0.250	1	RO water	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

APPENDIX 7 (Continued)

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	-	-	-	RO water	1
Low dose G2 (25)	MR1	0.1	10	1	10		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						

APPENDIX 7 (Continued)

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.

Preparation of spiking solutions					
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 in to 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 100 mL glass bottle and 100 mL of Milli-Q water was added by using measuring cylinder. Solution was mixed well. Solution was stored in refrigerator until use.

3.5.3 5% Xanthydrol solution

5 g of Xanthydrol was transferred to 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 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 200 mL of glass bottle using measuring cylinder and mixed well.

3.5.5 Saturated sodium chloride solution

71g of sodium chloride in 200 mL of Milli-Q water were mixed in 200 mL of glass bottle using 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^{0}C for 1.5h.
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% Xanthydrol solution was added in sample tube and incubated in darkness at 40 ^{0}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.

APPENDIX 7 (Continued)

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

APPENDIX 7 (Continued)

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**		
1	56.6689	**Replicates**	**Bracketed System suitability**
2	57.8726		
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**

APPENDIX 7 (Continued)

4.2 Linearity

<table>
<tr><th colspan="11">TABLE-02</th></tr>
<tr><th colspan="11">Linearity in RO water for dose formulation analysis on 19/09/17</th></tr>
<tr><th>Linearity standards</th><th>STD1</th><th>STD2</th><th>STD3</th><th>STD4</th><th>STD5</th><th>STD6</th><th colspan="2">Slope</th><th>Intercept</th><th>Coefficient of determination</th></tr>
<tr><td>Nominal conc. (ppm)</td><td>1.01</td><td>2.53</td><td>5.06</td><td>10.12</td><td>25.30</td><td>50.59</td><td colspan="2" rowspan="3">0.068273</td><td rowspan="3">0.263698</td><td rowspan="3">0.99968339</td></tr>
<tr><td>Back calculated conc. (ppm)</td><td>0.92</td><td>2.52</td><td>5.29</td><td>10.69</td><td>24.96</td><td>50.63</td></tr>
<tr><td>% Accuracy</td><td>91.09</td><td>99.60</td><td>104.55</td><td>105.63</td><td>98.66</td><td>100.08</td></tr>
<tr><th colspan="11">Linearity in mice plasma for plasma sample analysis on 25/09/17</th></tr>
<tr><td>Nominal conc. (ppm)</td><td>0.101</td><td>0.203</td><td>0.405</td><td>1.418</td><td>4.962</td><td>17.215</td><td>50.633</td><td rowspan="3">0.044661</td><td rowspan="3">- 0.002807</td><td rowspan="3">0.99559295</td></tr>
<tr><td>Back calculated conc. (ppm)</td><td>0.111</td><td>0.209</td><td>0.393</td><td>1.301</td><td>5.306</td><td>15.245</td><td>52.372</td></tr>
<tr><td>% Accuracy</td><td>109.90</td><td>102.96</td><td>97.04</td><td>91.75</td><td>106.93</td><td>88.56</td><td>103.43</td></tr>
<tr><th colspan="11">Linearity in mice plasma for repeat plasma sample analysis on 27/09/17</th></tr>
<tr><td>Nominal conc. (ppm)</td><td>0.101</td><td>0.203</td><td>0.405</td><td>1.418</td><td>4.962</td><td>17.215</td><td>50.633</td><td rowspan="3">0.044725</td><td rowspan="3">- 0.001242</td><td rowspan="3">0.99838482</td></tr>
<tr><td>Back calculated conc. (ppm)</td><td>0.120</td><td>0.201</td><td>0.358</td><td>1.412</td><td>4.898</td><td>16.068</td><td>51.880</td></tr>
<tr><td>% Accuracy</td><td>118.81</td><td>99.01</td><td>88.40</td><td>99.58</td><td>98.71</td><td>93.34</td><td>102.46</td></tr>
</table>

JAI RESE

APPENDIX 7 (Continued)

FIGURE 1: Linearity of acetamide for dose concentration analysis

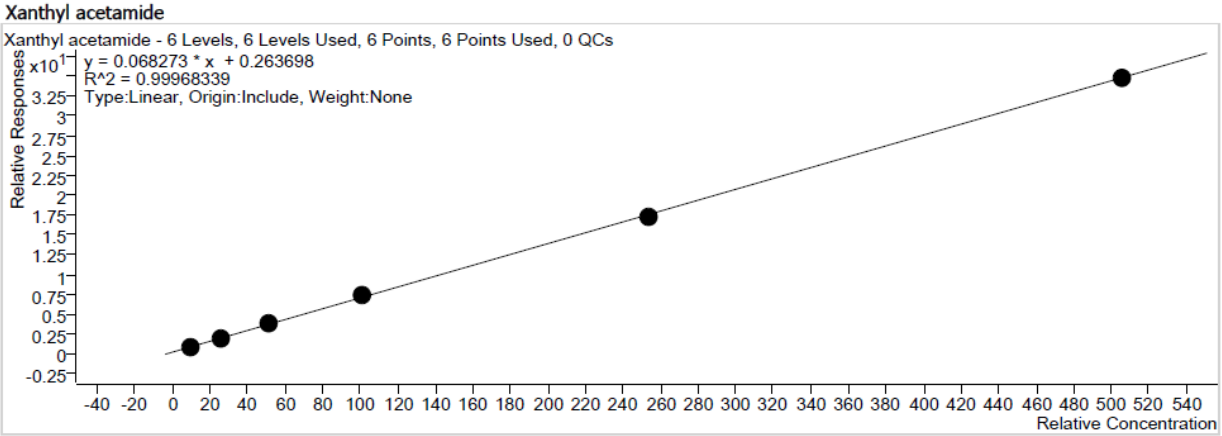


FIGURE 2: Linearity of acetamide-2,2,2-D3 reference standard for plasma sample analysis

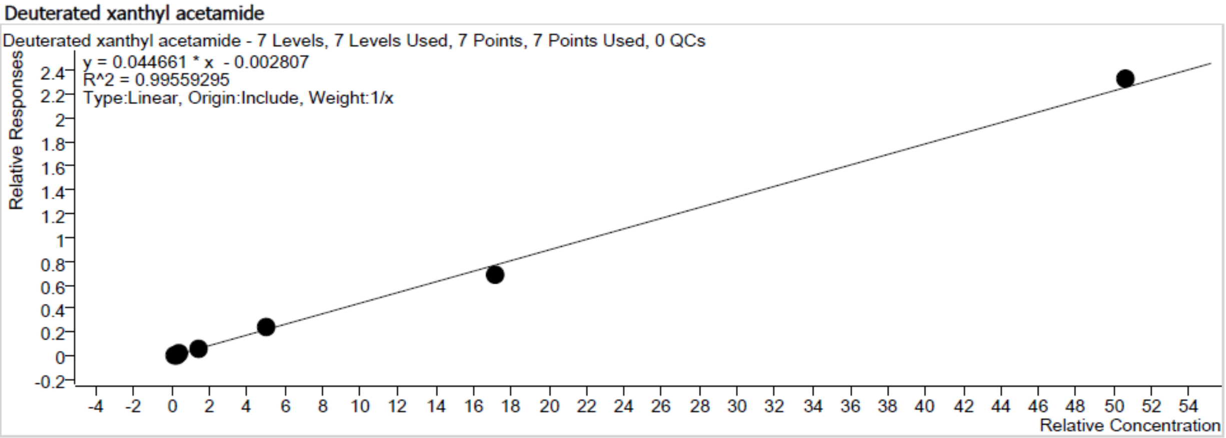
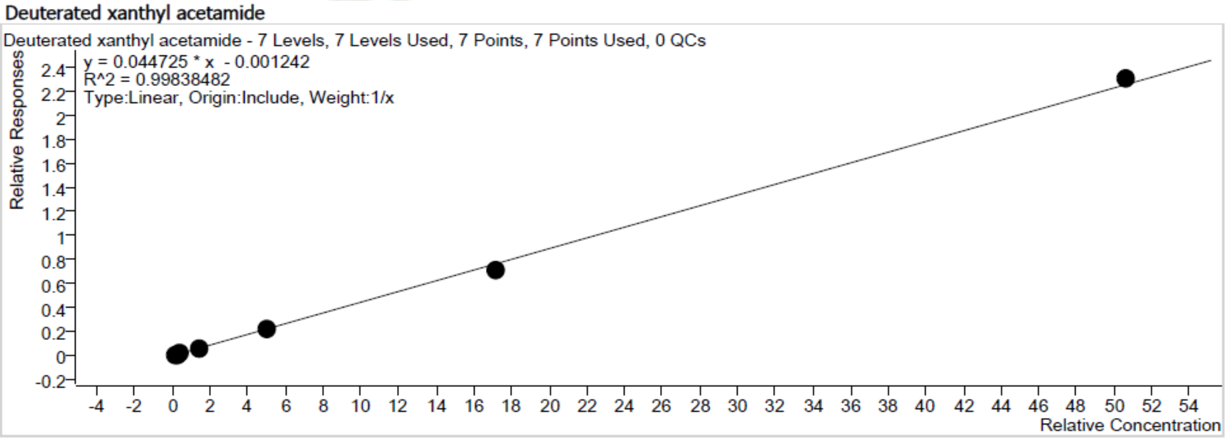


FIGURE 3: Linearity of acetamide-2,2,2-D3 reference standard for plasma sample analysis (Repeat Analysis)



APPENDIX 7 (Continued)

4.3 Concentration dose formulation analysis

TABLE-03

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 concen-tration (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			22.64	22640.00	91.29				
	MR3			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			9.99	99900.00	100.71				
	MR3			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			21.68	216800.00	109.27				
	MR3			19.29	192900.00	97.23				
Purity of Test Item (% w/w)					**99.20**					

ND- Not Detected

APPENDIX 7 (Continued)

4.4 Concentration of acetamide in mice plasma

TABLE-04

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

APPENDIX 7 (Continued)

TABLE-04 (Continued)

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	Female	10	18462	94189	0.1960	44.515
	T32		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
GIV	T37	Male	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	Female	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
Intercept of Y-axis (a)	**-0.002807**						
Slope of the line (b)	**0.044661**						
Repeat plasma sample analysis							
GI	T1	Male	1	923	85735	0.0108	0.269
GII	T14	Male	2	102898	87952	1.1699	52.371
Intercept of Y-axis (a)	**-0.001242**						
Slope of the line (b)	**0.044725**						

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

APPENDIX 7 (Continued)

4.5 Repeat Analysis

Sample ID	Initial Concentration (ppm)	Repeated Concentration (ppm)	Accepted Concentration (ppm)
		1st	
GI-T1-M	14.084	0.269	0.269
GII-T14-M	53.239	52.371	52.371

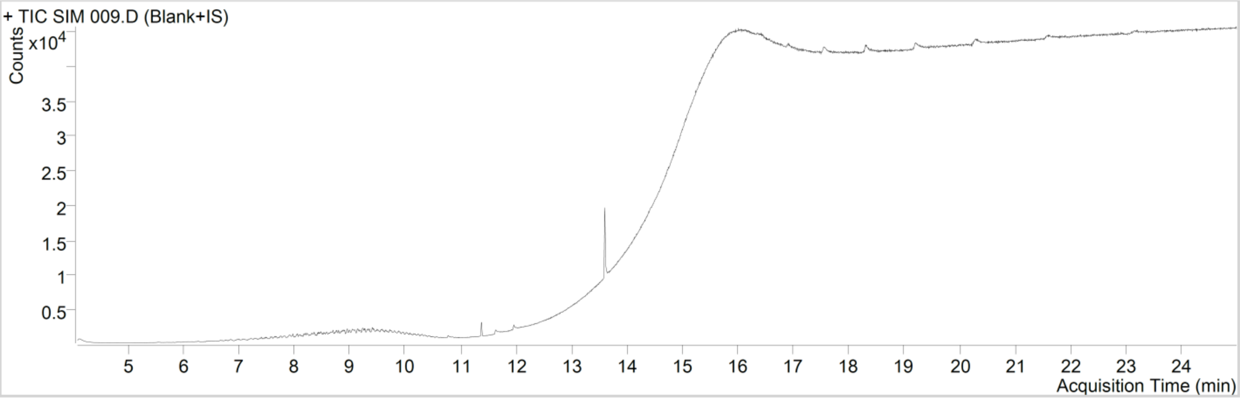
APPENDIX 7 (Continued)

4.6 Chromatograms

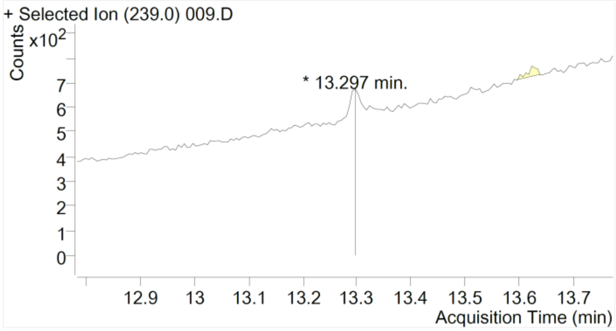
A. Blank+IS for dose concentration analysis

Data File : 009.D
Operator : PC204\abhishek.0552
Acq Method Name : Acetamide
Acquisition Date : 9/19/2017 4:19:42 PM
Vial : 3
Dilution : 1
Sample Info :
Tune File : ATUNE.U
Tune Date :
Quant Batch Version : Batch was analyzed in B.07.01, Report was generated in B.07.01
Last Calib Update : 10/2/2017 10:22:06 AM

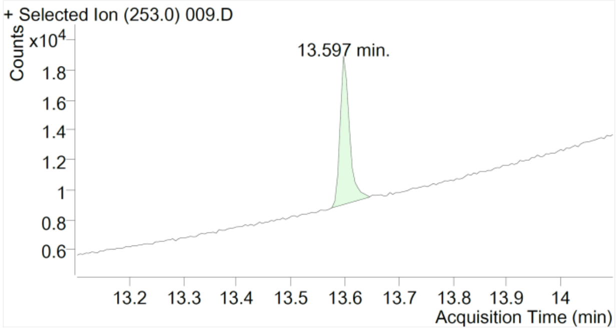
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
Xanthyl acetamide	239.0	13.297	12.617-13.945	0.0			0.000
Xanthyl propionamide	253.0	13.597	12.921-14.281	12052.6			



Xanthyl acetamide



Xanthyl propionamide



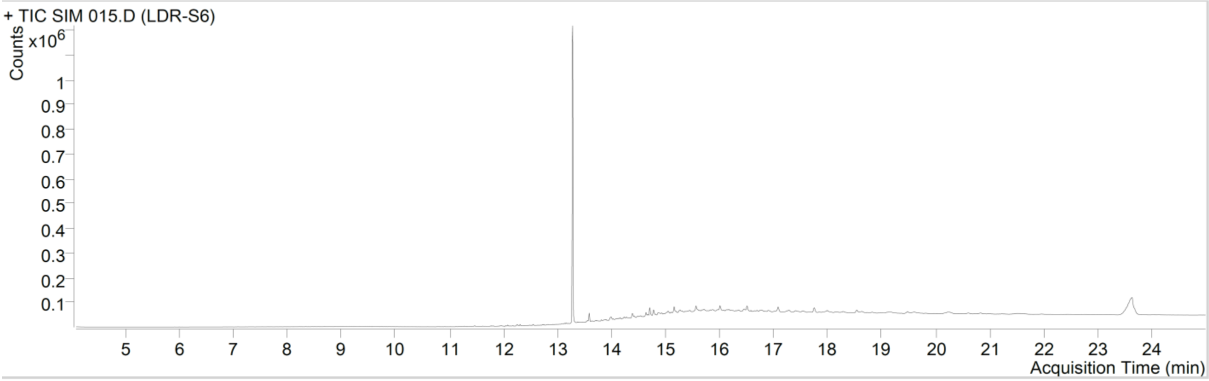
APPENDIX 7 (Continued)

Chromatograms (Continued)

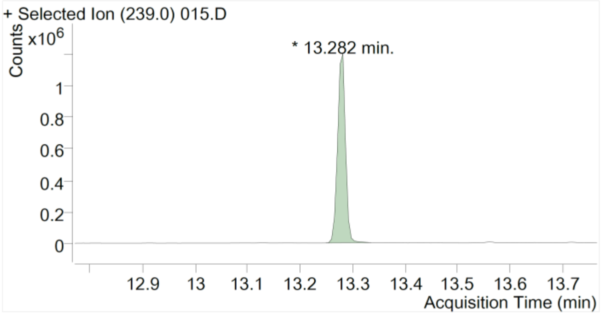
B. Standard-6 for dose concentration analysis

Data File : 015.D
Operator : PC204\abhishek.0552
Acq Method Name : Acetamide
Acquisition Date : 9/19/2017 7:41:59 PM
Vial : 9
Dilution : 1
Sample Info :
Tune File : ATUNE.U
Tune Date :
Quant Batch Version : Batch was analyzed in B.07.01, Report was generated in B.07.01
Last Calib Update : 10/2/2017 10:22:06 AM

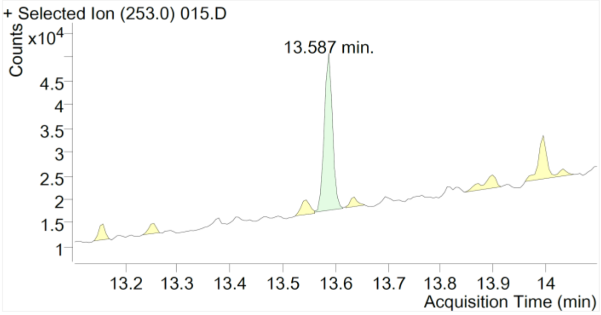
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
Xanthyl acetamide	239.0	13.282	12.617-13.945	1255009.0			50.628
Xanthyl propionamide	253.0	13.587	12.921-14.281	36033.5			



Xanthyl acetamide



Xanthyl propionamide



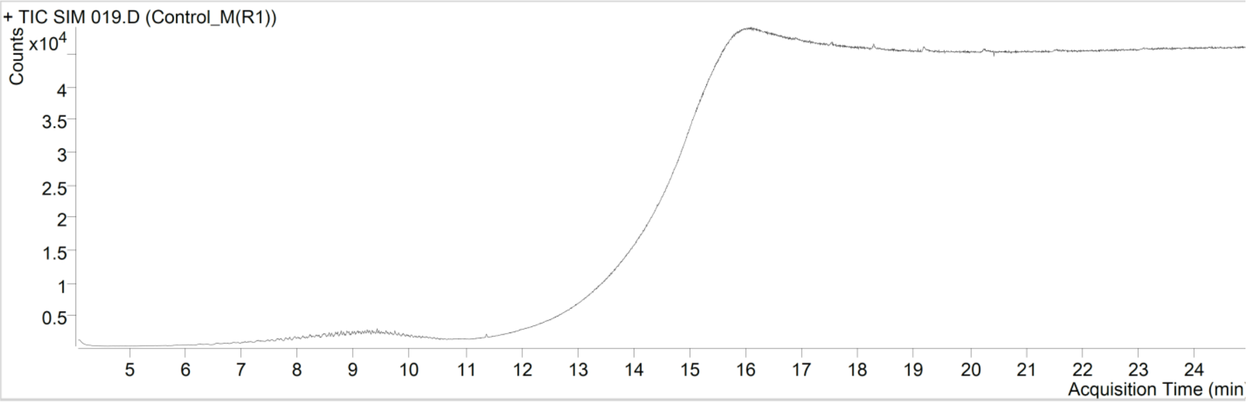
APPENDIX 7 (Continued)

Chromatograms (Continued)

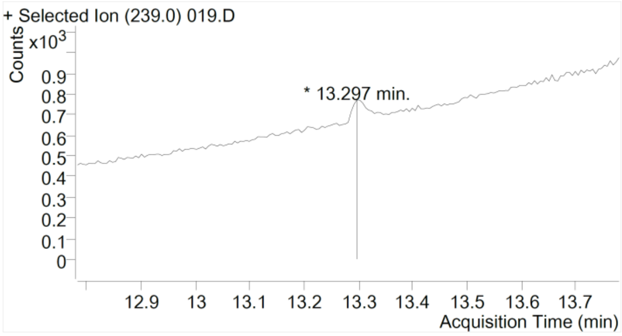
C. Vehicle control for dose concentration analysis

Data File : 019.D
Operator : PC204\abhishek.0552
Acq Method Name : Acetamide
Acquisition Date : 9/19/2017 9:56:42 PM
Vial : 12
Dilution : 1
Sample Info :
Tune File : ATUNE.U
Tune Date :
Quant Batch Version : Batch was analyzed in B.07.01, Report was generated in B.07.01
Last Calib Update : 10/2/2017 10:22:06 AM

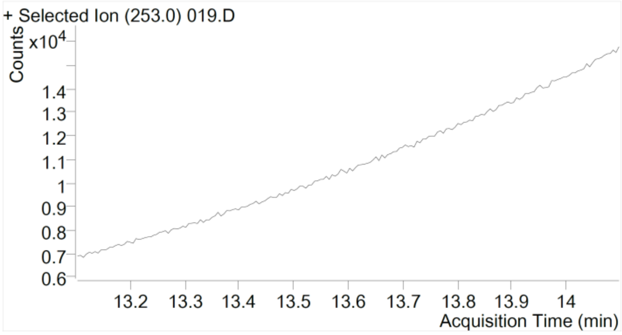
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalCon
Xanthyl acetamide	239.0	13.297	12.617-13.945	0.0			0.000
Xanthyl propionamide	253.0		12.921-14.281				



Xanthyl acetamide



Xanthyl propionamide



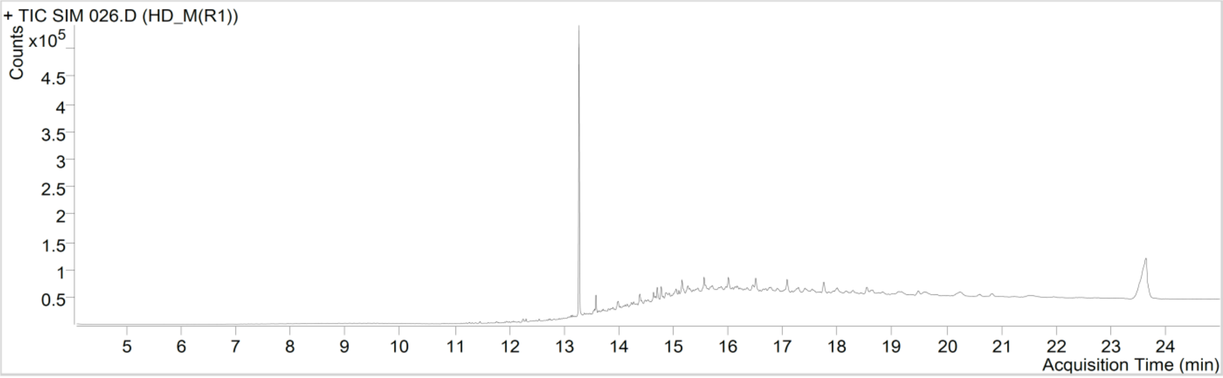
APPENDIX 7 (Continued)

Chromatograms (Continued)

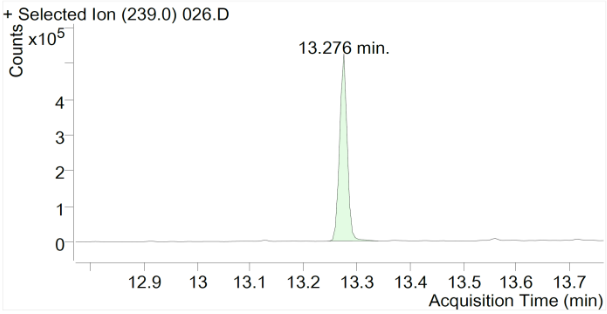
D. High dose for dose concentration analysis

Data File : 026.D
Operator : PC204\abhishek.0552
Acq Method Name : Acetamide
Acquisition Date : 9/20/2017 1:52:37 AM
Vial : 19
Dilution : 1
Sample Info :
Tune File : ATUNE.U
Tune Date :
Quant Batch Version : Batch was analyzed in B.07.01, Report was generated in B.07.01
Last Calib Update : 10/2/2017 10:22:06 AM

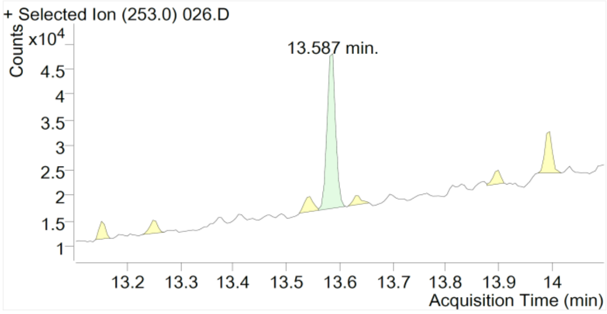
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
Xanthyl acetamide	239.0	13.276	12.617-13.945	520586.4			21.773
Xanthyl propionamide	253.0	13.587	12.921-14.281	34409.4			



Xanthyl acetamide



Xanthyl propionamide



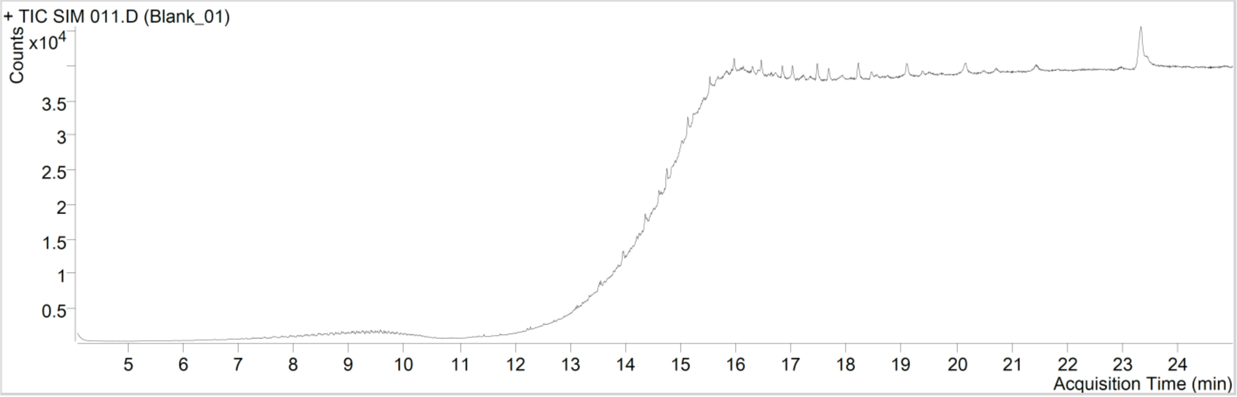
APPENDIX 7 (Continued)

Chromatograms (Continued)

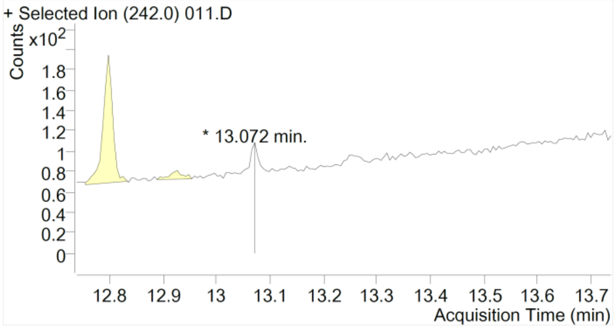
E. Blank sample of acetamide-2,2,2-D3 for plasma concentration analysis

Data File : 011.D
Operator : PC204\abhishek.0552
Acq Method Name : Acetamide
Acquisition Date : 9/25/2017 11:07:09 PM
Vial : 4
Dilution : 1
Sample Info :
Tune File : ATUNE.U
Tune Date :
Quant Batch Version : Batch was analyzed in B.07.01, Report was generated in B.07.01
Last Calib Update : 10/2/2017 10:03:47 AM

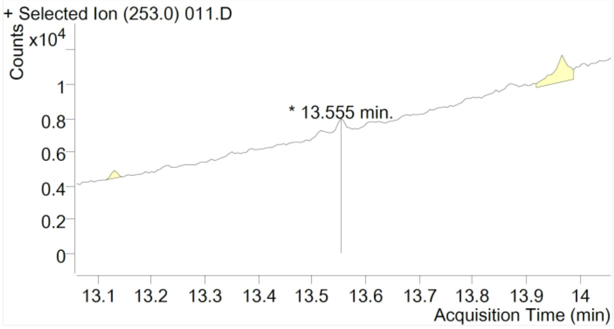
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
Deuterated xanthyl acetamide	242.0	13.072	12.581-13.905	0.0			0.000
Xanthyl propionamide	253.0	13.555	12.882-14.238	0.0			0.000



Deuterated xanthyl acetamide



Xanthyl propionamide



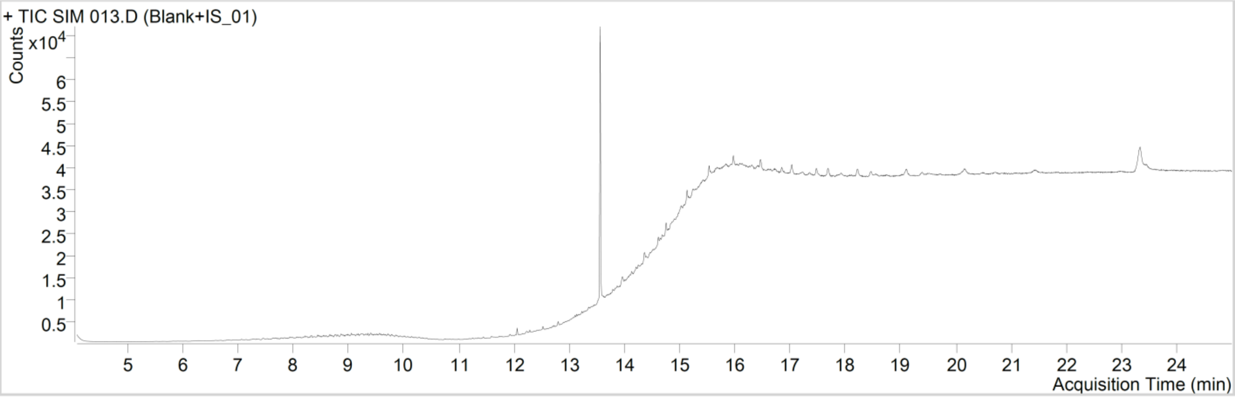
APPENDIX 7 (Continued)

Chromatograms (Continued)

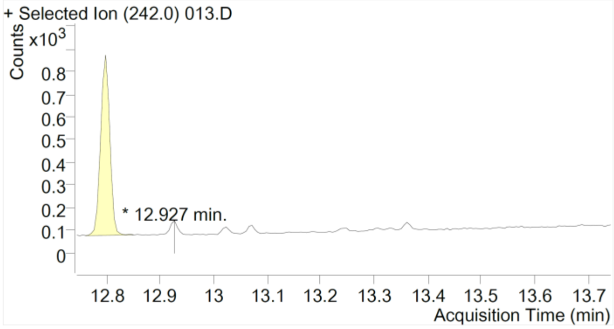
F. Standard zero sample of acetamide-2,2,2-D3 for plasma concentration analysis

Data File : 013.D
Operator : PC204\abhishek.0552
Acq Method Name : Acetamide
Acquisition Date : 9/26/2017 12:14:54 AM
Vial : 6
Dilution : 1
Sample Info :
Tune File : ATUNE.U
Tune Date :
Quant Batch Version : Batch was analyzed in B.07.01, Report was generated in B.07.01
Last Calib Update : 10/2/2017 10:03:47 AM

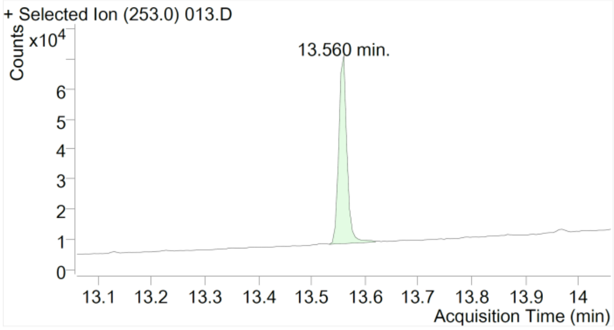
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
Deuterated xanthyl acetamide	242.0	12.927	12.581-13.905	0.0			0.000
Xanthyl propionamide	253.0	13.560	12.882-14.238	66504.0			



Deuterated xanthyl acetamide



Xanthyl propionamide



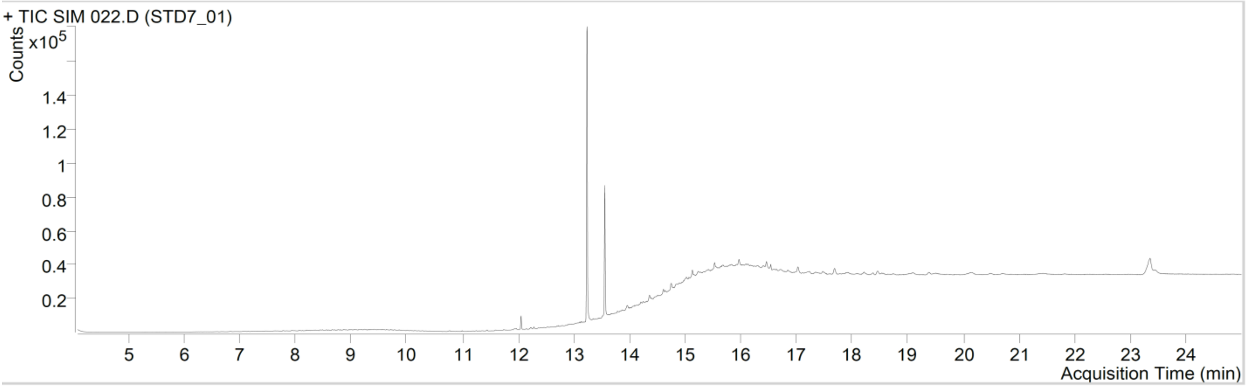
APPENDIX 7 (Continued)

Chromatograms (Continued)

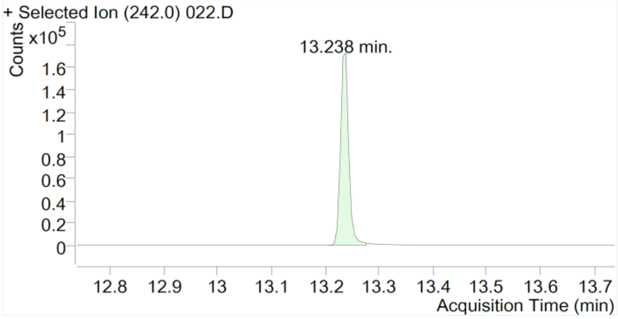
G. Standard-7 sample of acetamide-2,2,2-D3 for plasma concentration analysis

Data File : 022.D
Operator : PC204\abhishek.0552
Acq Method Name : Acetamide
Acquisition Date : 9/26/2017 5:18:27 AM
Vial : 15
Dilution : 1
Sample Info :
Tune File : ATUNE.U
Tune Date :
Quant Batch Version : Batch was analyzed in B.07.01, Report was generated in B.07.01
Last Calib Update : 10/2/2017 10:03:47 AM

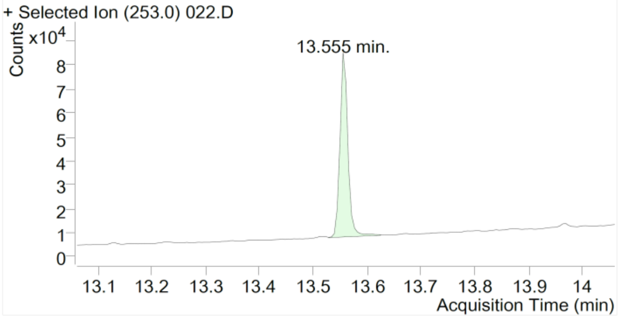
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
Deuterated xanthyl acetamide	242.0	13.238	12.581-13.905	183374.3			50.858
Xanthyl propionamide	253.0	13.555	12.882-14.238	78493.7			



Deuterated xanthyl acetamide



Xanthyl propionamide



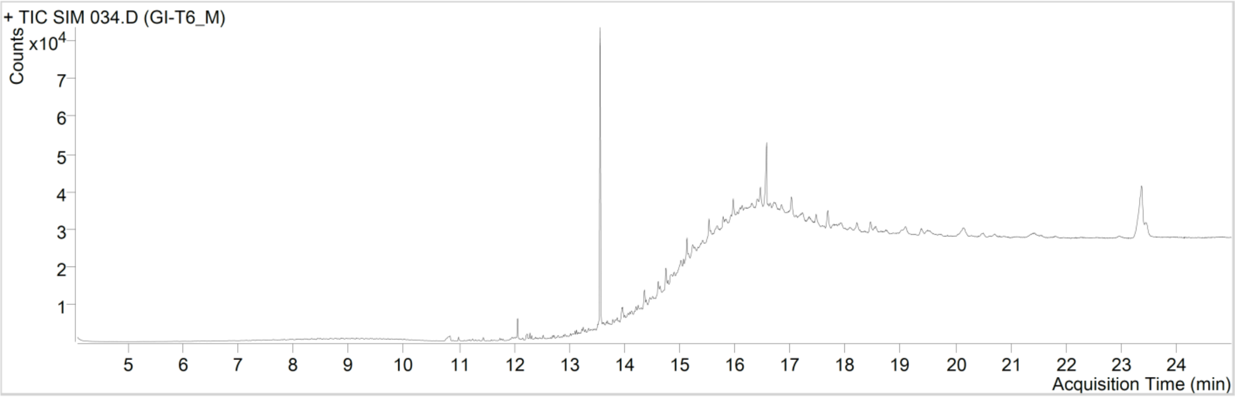
APPENDIX 7 (Continued)

Chromatograms (Continued)

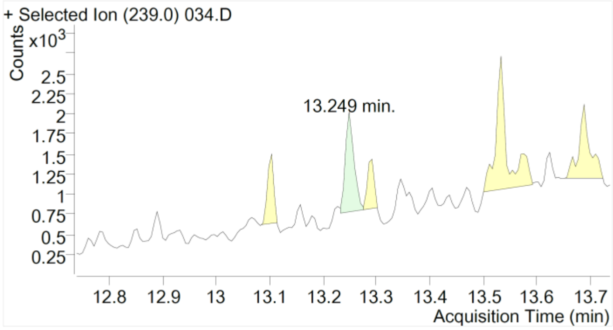
H. Control sample(GI-T6_M) of acetamide for plasma concentration analysis

Data File : 034.D
Operator : PC204\abhishek.0552
Acq Method Name : Acetamide
Acquisition Date : 9/26/2017 12:19:27 PM
Vial : 25
Dilution : 1
Sample Info :
Tune File : ATUNE.U
Tune Date :
Quant Batch Version : Batch was analyzed in B.07.01, Report was generated in B.07.01
Last Calib Update : 10/2/2017 10:03:47 AM

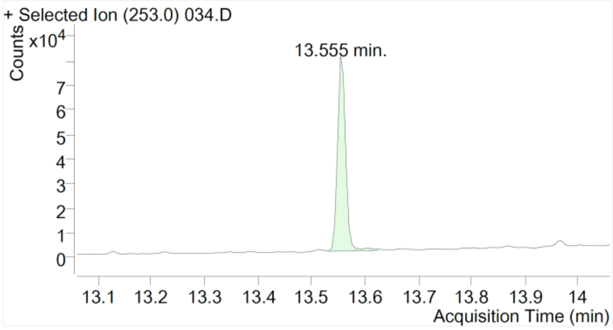
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
Xanthyl acetamide	239.0	13.249	12.581-13.905	1390.7			
Xanthyl propionamide	253.0	13.555	12.882-14.238	84122.6			



Xanthyl acetamide



Xanthyl propionamide



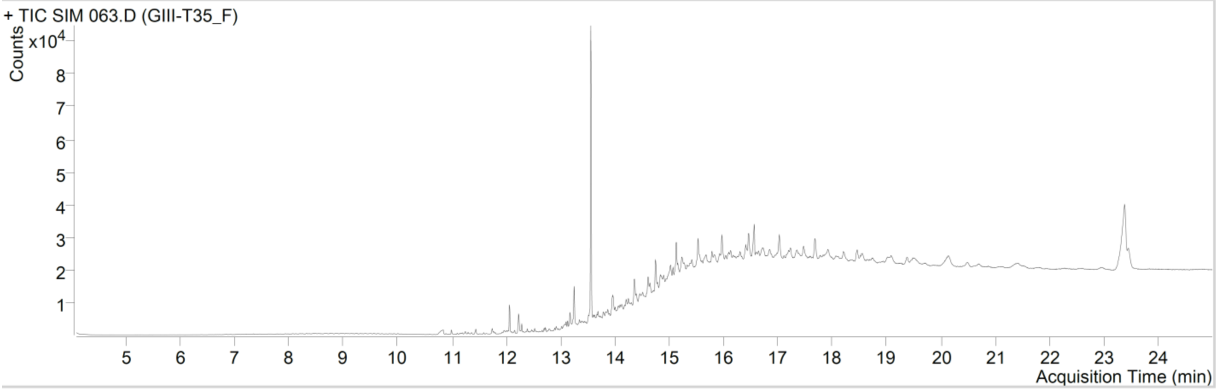
APPENDIX 7 (Continued)

Chromatograms (Continued)

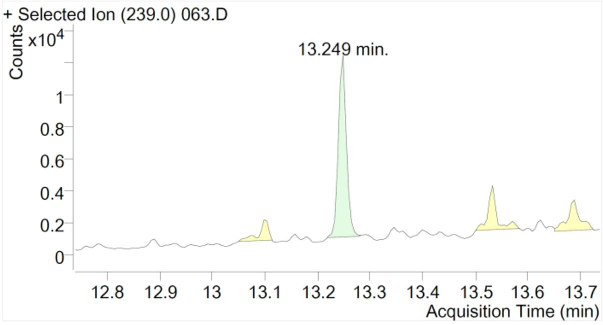
I. GIII-T35_F sample of acetamide for plasma concentration analysis

Data File : 063.D
Operator : PC204\abhishek.0552
Acq Method Name : Acetamide
Acquisition Date : 9/27/2017 4:38:35 AM
Vial : 5
Dilution : 1
Sample Info :
Tune File : ATUNE.U
Tune Date :
Quant Batch Version : Batch was analyzed in B.07.01, Report was generated in B.07.01
Last Calib Update : 10/2/2017 10:03:47 AM

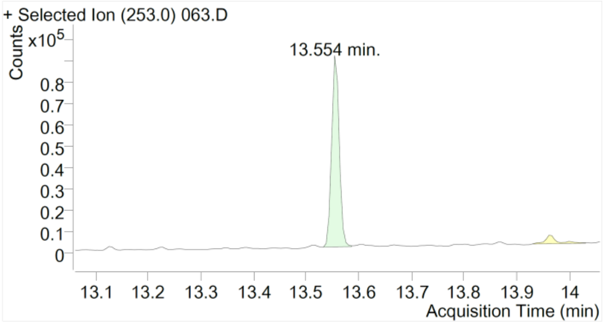
Cmpnd	Signal	RT	Limits	Response	QRatio	Limits	FinalConc
Xanthyl acetamide	239.0	13.249	12.581-13.905	12083.3			
Xanthyl propionamide	253.0	13.554	12.882-14.238	89453.5			



Xanthyl acetamide



Xanthyl propionamide



APPENDIX 7 (Continued)

APPENDIX I: Animal Plasma Concentration of Acetamide

Acetamide Concentration in Mice plasma-Group I (Dose - 0.0 mg/kg)			Acetamide Concentration in Mice plasma-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	F	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)			**Acetamide Concentration in Mice plasma-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	F	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 (N = 1 study)	0.03 (N = 1 study)	-	-
Standard Deviation	0.01	0.03	-	-
Vehicle: Distilled water				
Mean	0.50 (N = 50 studies)	0.02 (N = 50 studies)	0.51 (N = 21 studies)	0.02 (N = 21 studies)
Standard Deviation	0.02	0.02	0.03	0.02
Vehicle: Vegetable oil				
Mean	0.50 (N = 27 studies)	0.02 (N = 27 studies)	0.50 (N = 10 studies)	0.02 (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 (N = 77 studies)	1.27 (N = 77 studies)	0.54 (N = 6 studies)	1.10 (N = 6 studies)
Standard Deviation	0.03	0.45	0.03	0.19

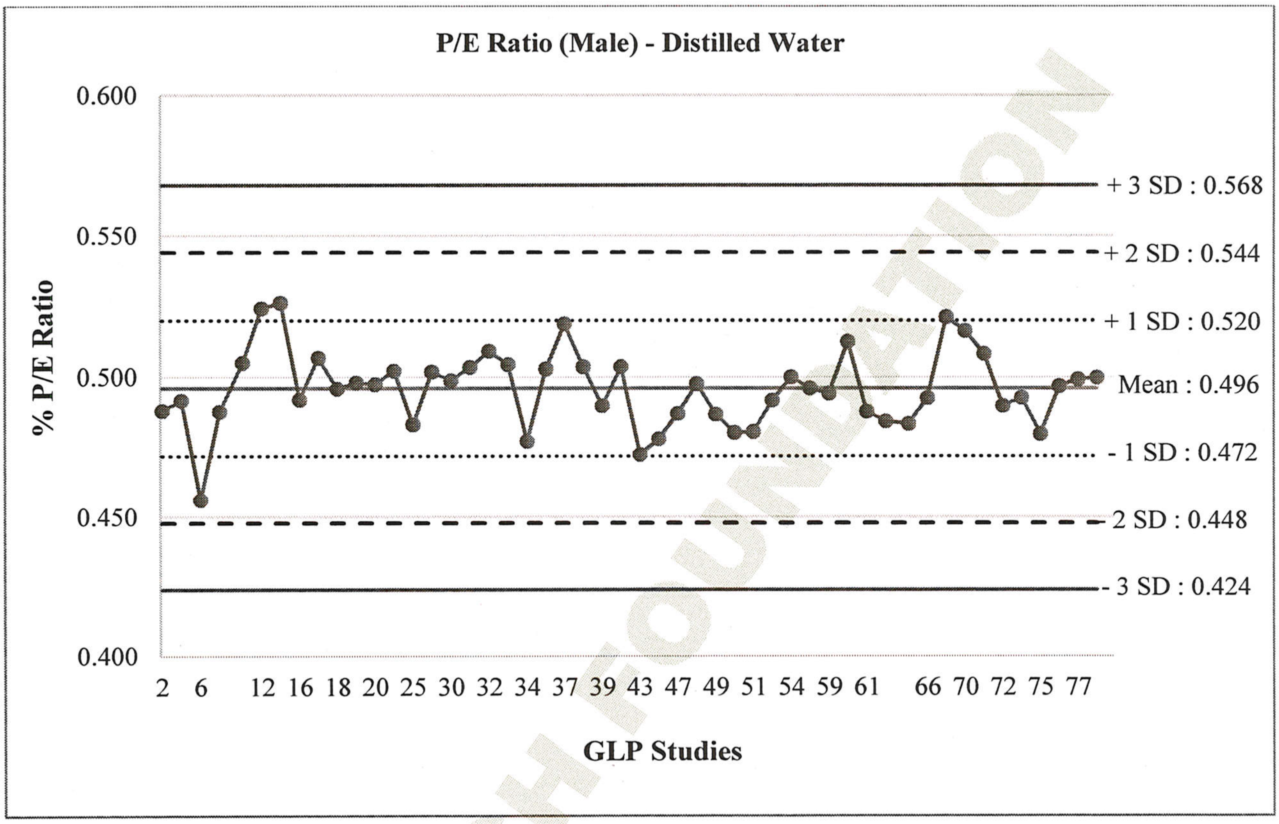
Vehicle Control: Distilled Water

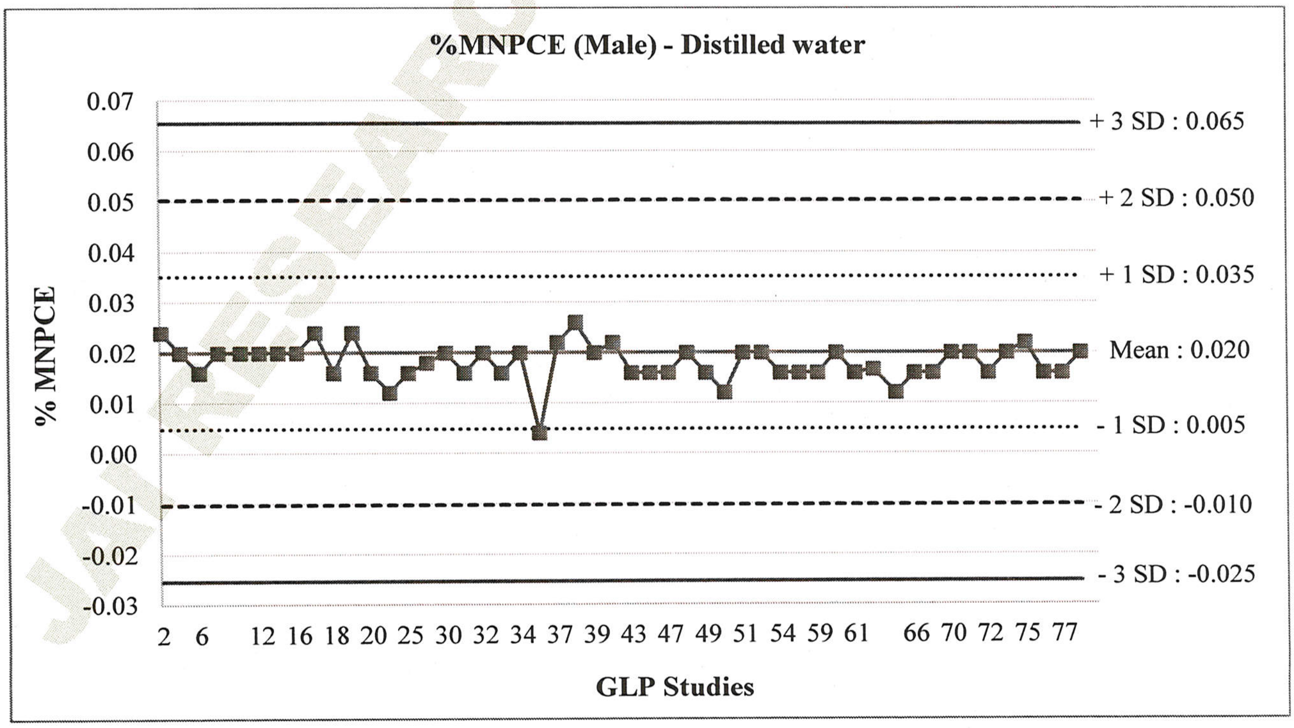
Details of Positive Control used

Mitomycin-C = 1.0 mg/kg body weight

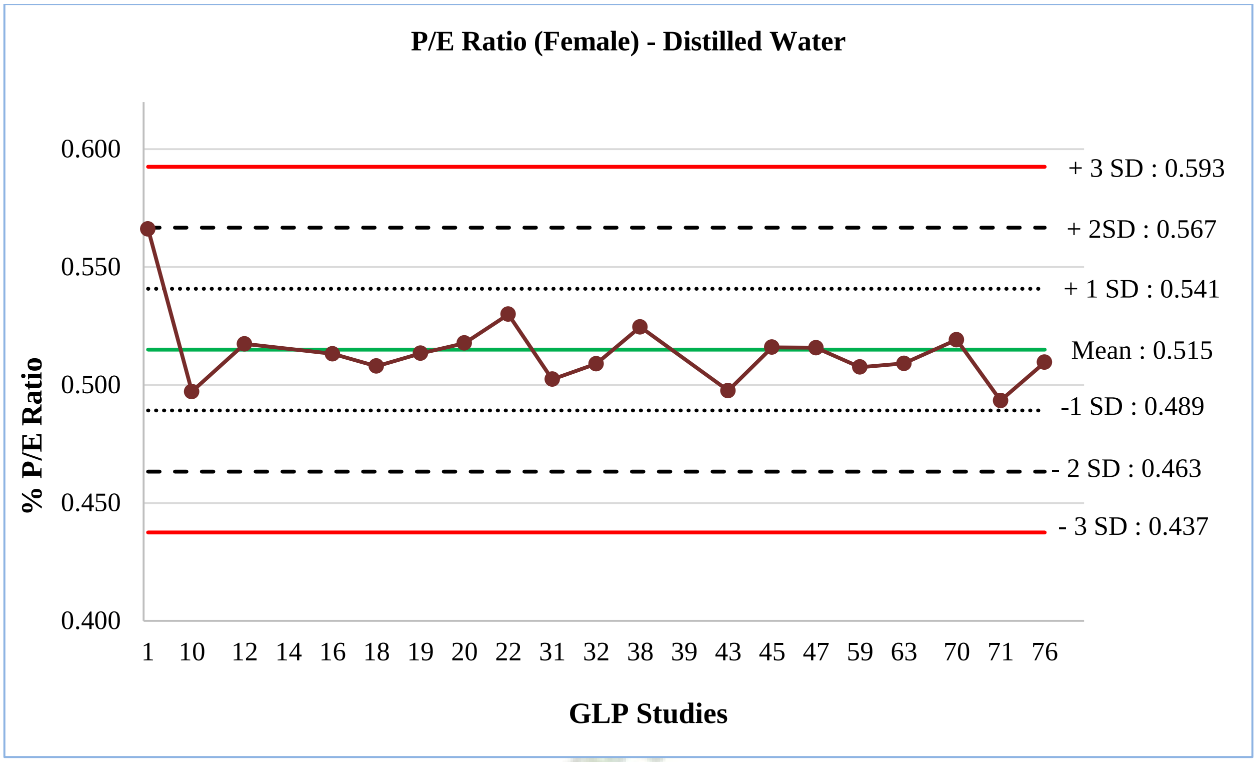
Keys: % MNPCE = Percent Micronucleated Polychromatic Erythrocytes
P/E = Total Polychromatic Erythrocytes/Total Erythrocytes

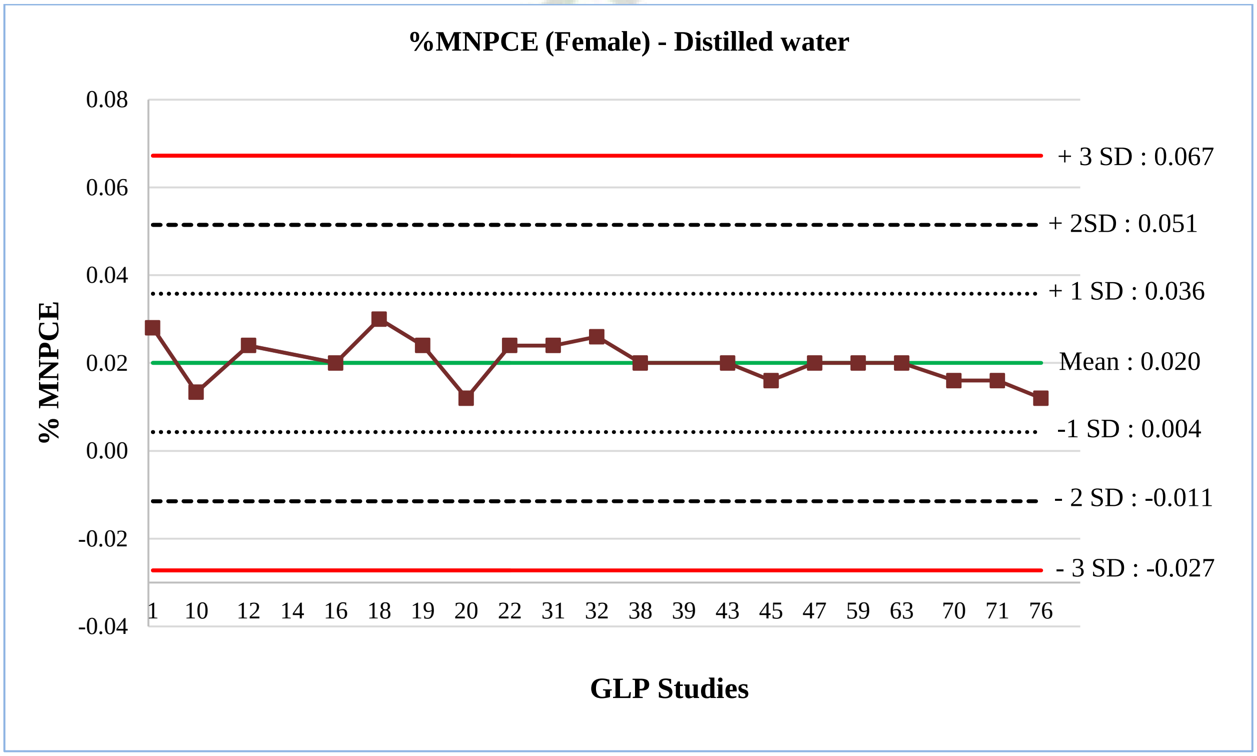
APPENDIX 8 (Continued)



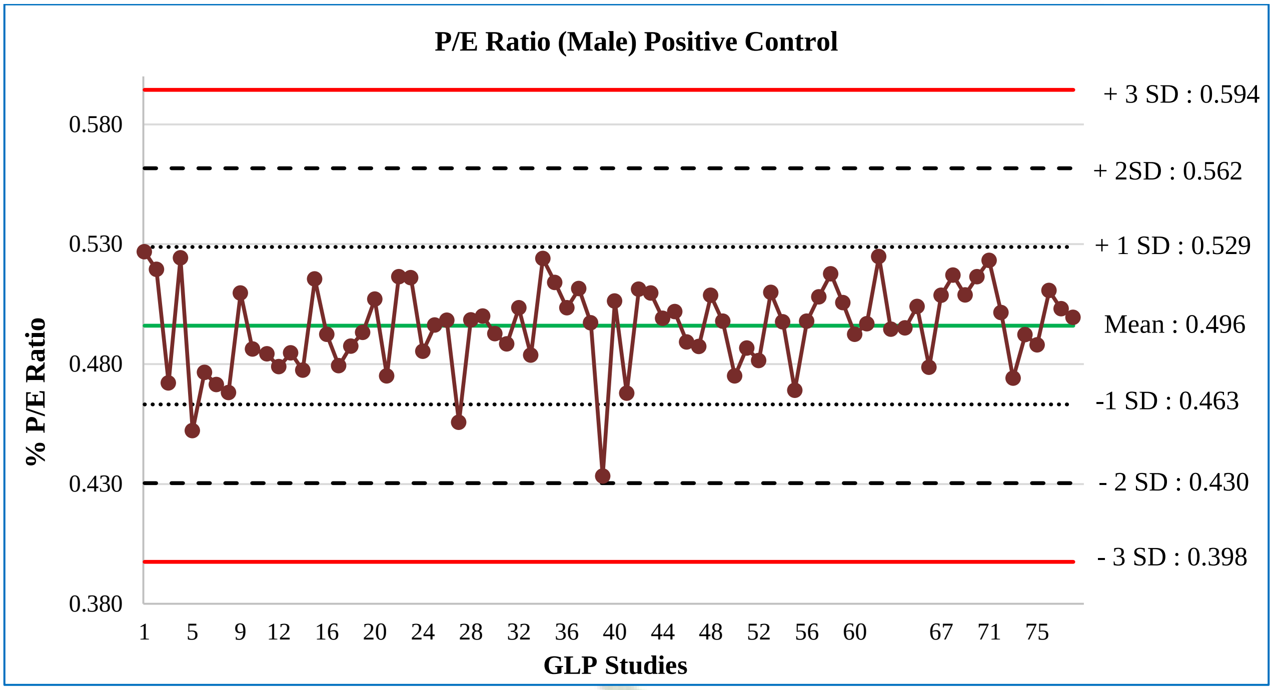


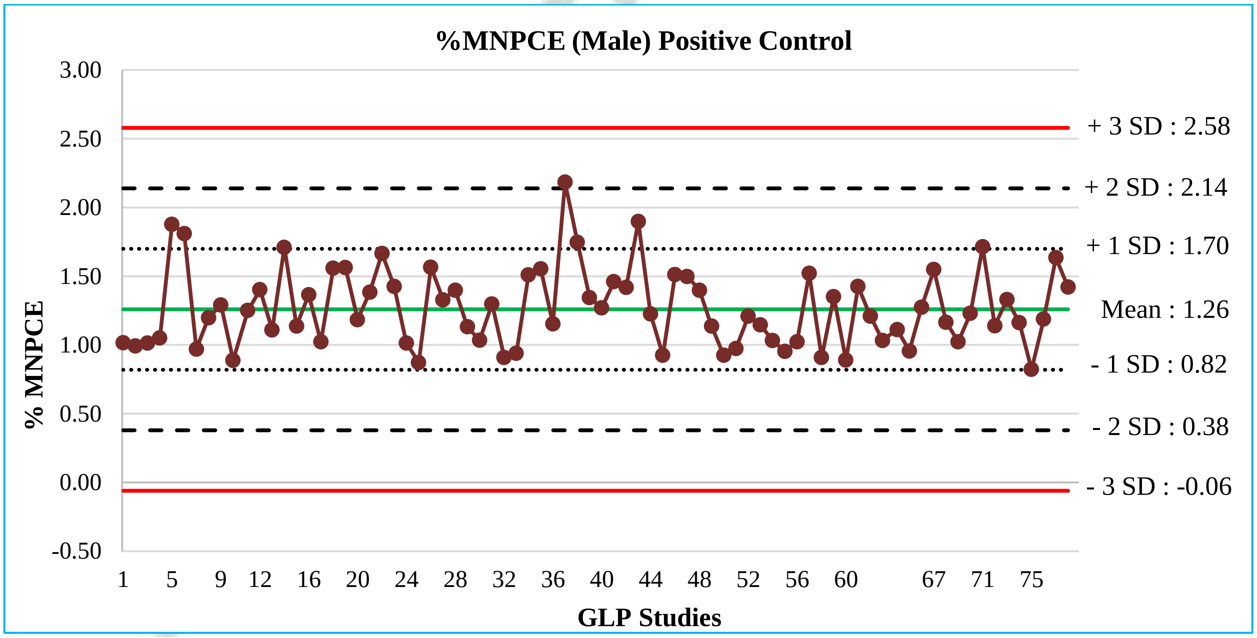
APPENDIX 8 (Continued)



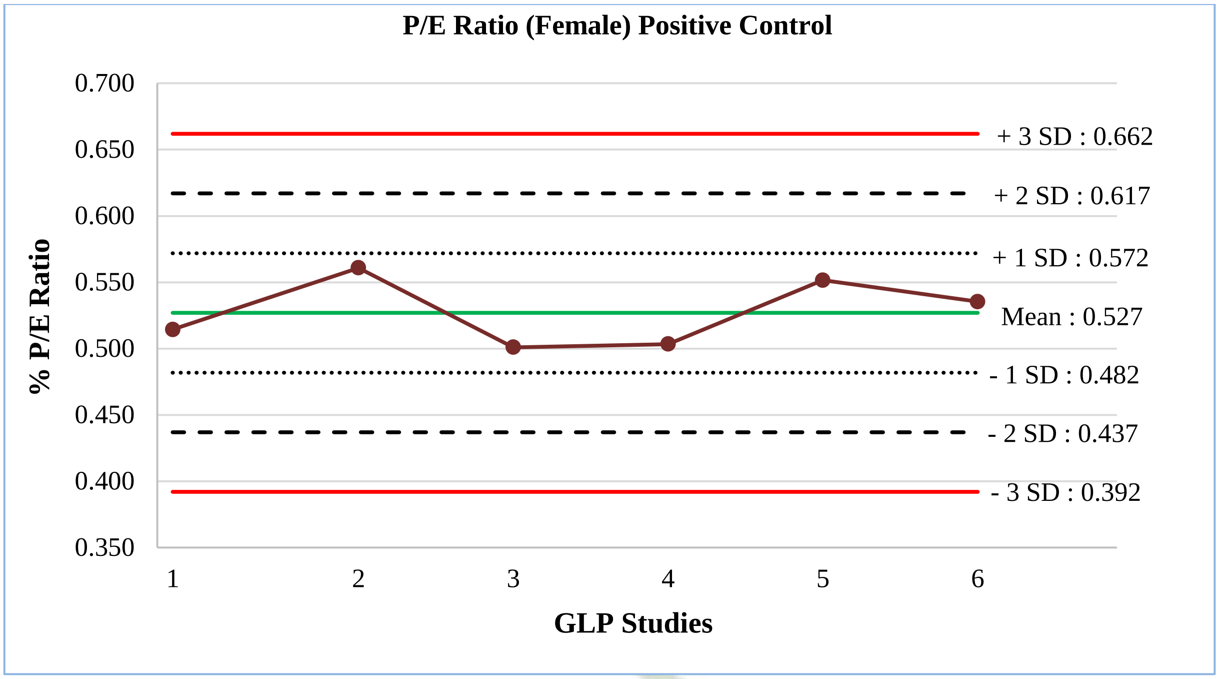


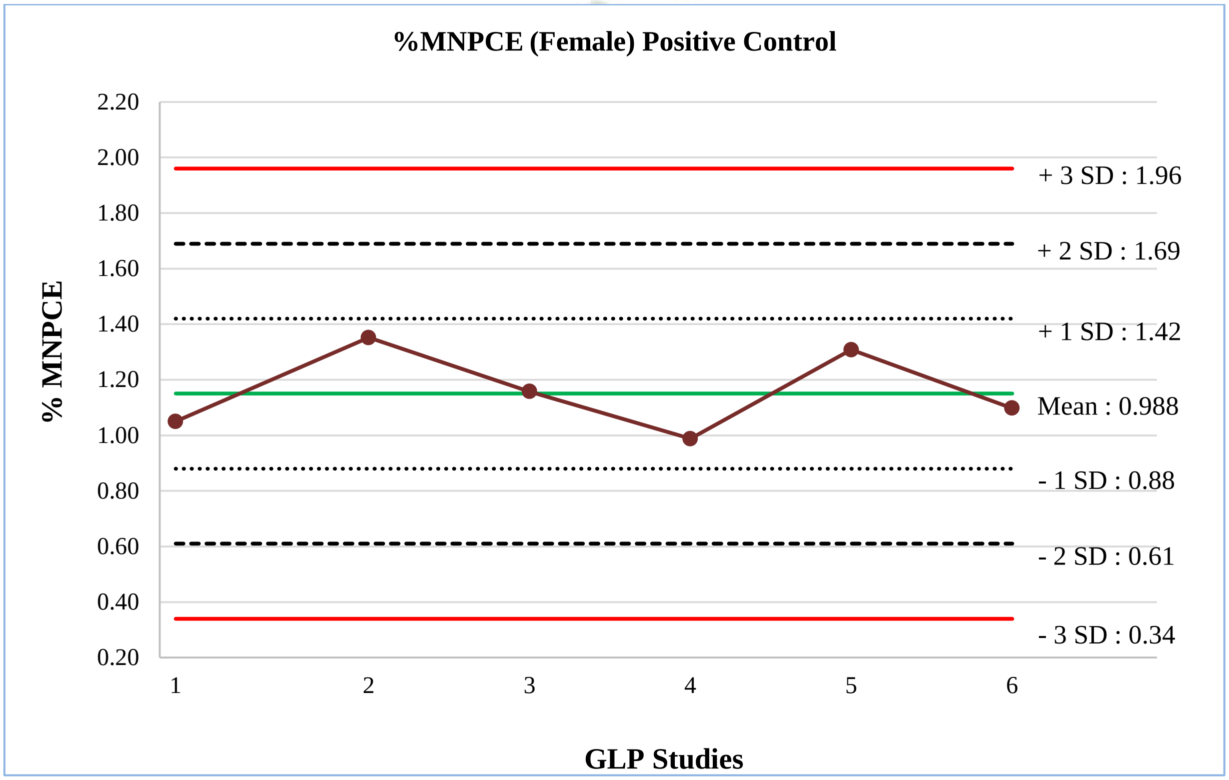
APPENDIX 8 (Continued)





APPENDIX 8 (Continued)





Note: QC chart of female (positive control) prepared from historical control data of year 2015 to 2017.

APPENDIX 9: Water Analysis Reports

JAI RESEARCH
FOUNDATION

MICROBIOLOGICAL ANALYSIS CERTIFICATE OF WATER SAMPLE

Sample Type : **RO Water**
Sample Received From : **BMR Facility**
Identification N° : **JRF/BMF/58**
Date of Sample Collection : **20/03/2017**
Sample Analysis
Date of Initiation : **20/03/2017**
Date of Completion : **22/03/2017**
Sample Analysed At : **Microbiology Lab - JRF**

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
Sign & Date : March 22, 2017

Verified by : Dr. Rajesh Posia
Sign & Date : March 22, 2017

APPENDIX 9 (Continued)

JAI RESEARCH
FOUNDATION

MICROBIOLOGICAL ANALYSIS CERTIFICATE OF WATER SAMPLE

Sample Type : **RO Water**
Sample Received From : **BMR Facility**
Identification N° : **JRF/BMF/43**
Date of Sample Collection : **20/03/2017**

Sample Analysis

Date of Initiation : **20/03/2017**
Date of Completion : **22/03/2017**
Sample Analysed At : **Microbiology Lab - JRF**

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).

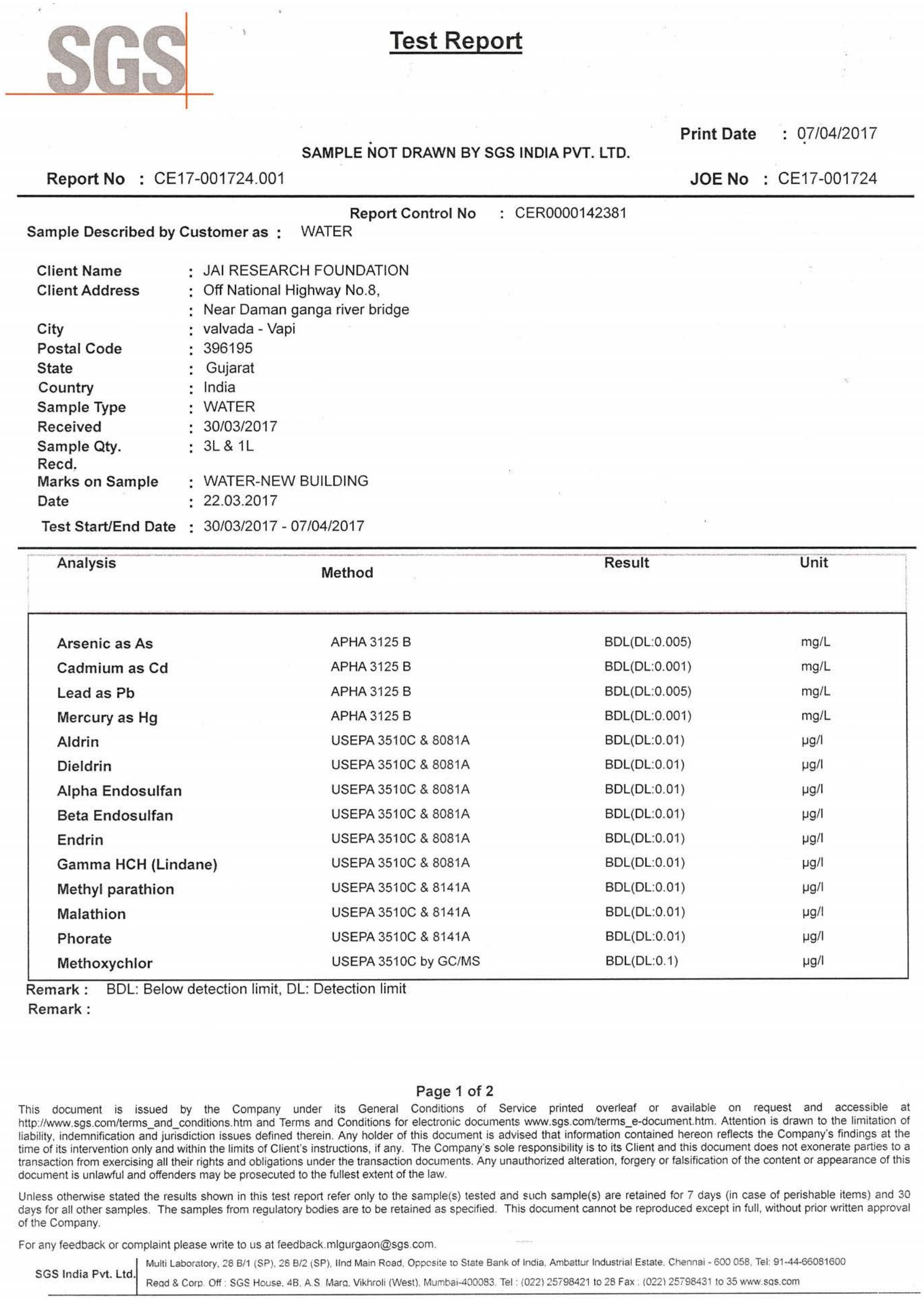
Analysed by : Rahul G Badgha

Sign & Date : RBadgha
March 22, 2017

Verified by : Dr. Rajesh Posia

Sign & Date :
March 22, 2017

APPENDIX 9 (Continued)



SGS

Test Report

Print Date : 07/04/2017

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 : valvada - Vapi
Postal Code : 396195
State : Gujarat
Country : India
Sample Type : WATER
Received : 30/03/2017
Sample Qty. Recd. : 3L & 1L
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 3510C & 8081A	BDL(DL:0.01)	µg/l
Dieldrin	USEPA 3510C & 8081A	BDL(DL:0.01)	µg/l
Alpha Endosulfan	USEPA 3510C & 8081A	BDL(DL:0.01)	µg/l
Beta Endosulfan	USEPA 3510C & 8081A	BDL(DL:0.01)	µg/l
Endrin	USEPA 3510C & 8081A	BDL(DL:0.01)	µg/l
Gamma HCH (Lindane)	USEPA 3510C & 8081A	BDL(DL:0.01)	µg/l
Methyl parathion	USEPA 3510C & 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

Remark :

Page 1 of 2

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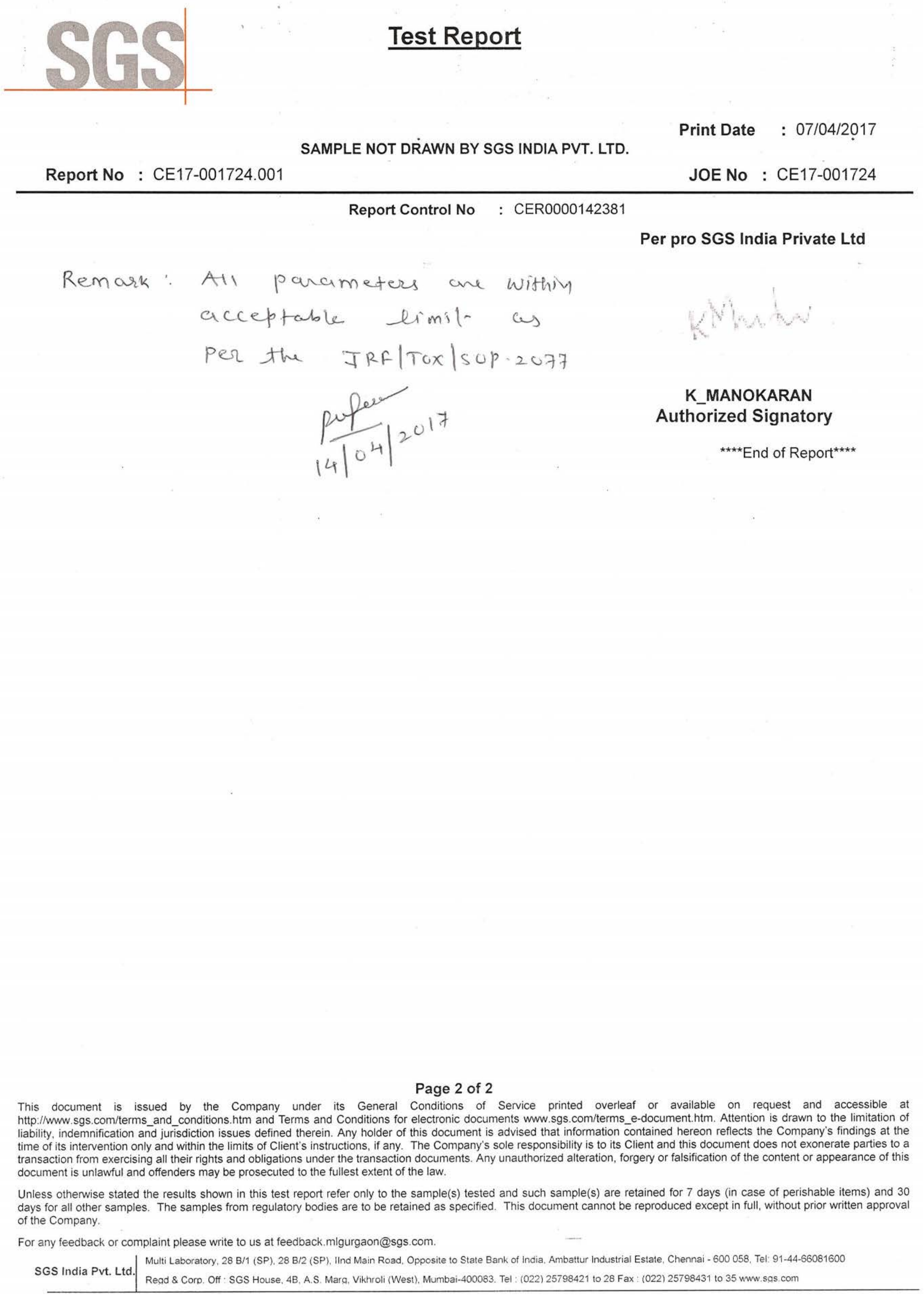
SGS India Pvt. Ltd.
Multi Laboratory, 28 B/1 (SP), 28 B/2 (SP), IInd Main Road, Opposite to State Bank of India, Ambattur Industrial Estate, Chennai - 600 058, Tel: 91-44-66081600
Regd & Corp. Off : SGS House, 4B, A.S. Marg, Vikhroli (West), Mumbai-400083. Tel : (022) 25798421 to 28 Fax : (022) 25798431 to 35 www.sgs.com

APPENDIX 9 (Continued)



SGS

Test Report

Print Date : 07/04/2017

SAMPLE NOT DRAWN BY SGS INDIA PVT. LTD.

Report No : CE17-001724.001

JOE No : CE17-001724

Report Control No : CER0000142381

Per pro SGS India Private Ltd

Remark: All parameters are within
acceptable limit as
per the JRF|Tox|SOP-2077

14|04|2017

K_MANOKARAN
Authorized Signatory

****End of Report****

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APPENDIX 10: Feed Analysis Reports

JAI RESEARCH
FOUNDATION

MICROBIOLOGICAL ANALYSIS CERTIFICATE OF ANIMAL FEED

Name of Sample : **Teklad Certified Global 16% Protein Rodent Diet (Sterilizable)**
Sample Received From : **UV Room-BMR Facility**
Date of Sample Collection : **24/05/2017**
Batch N° : **2016SC-031517MA**

Sample Analysis

Date of Initiation : **24/05/2017**
Date of Completion : **26/05/2017**
Sample Analysed At : **Microbiology Lab - JRF**

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

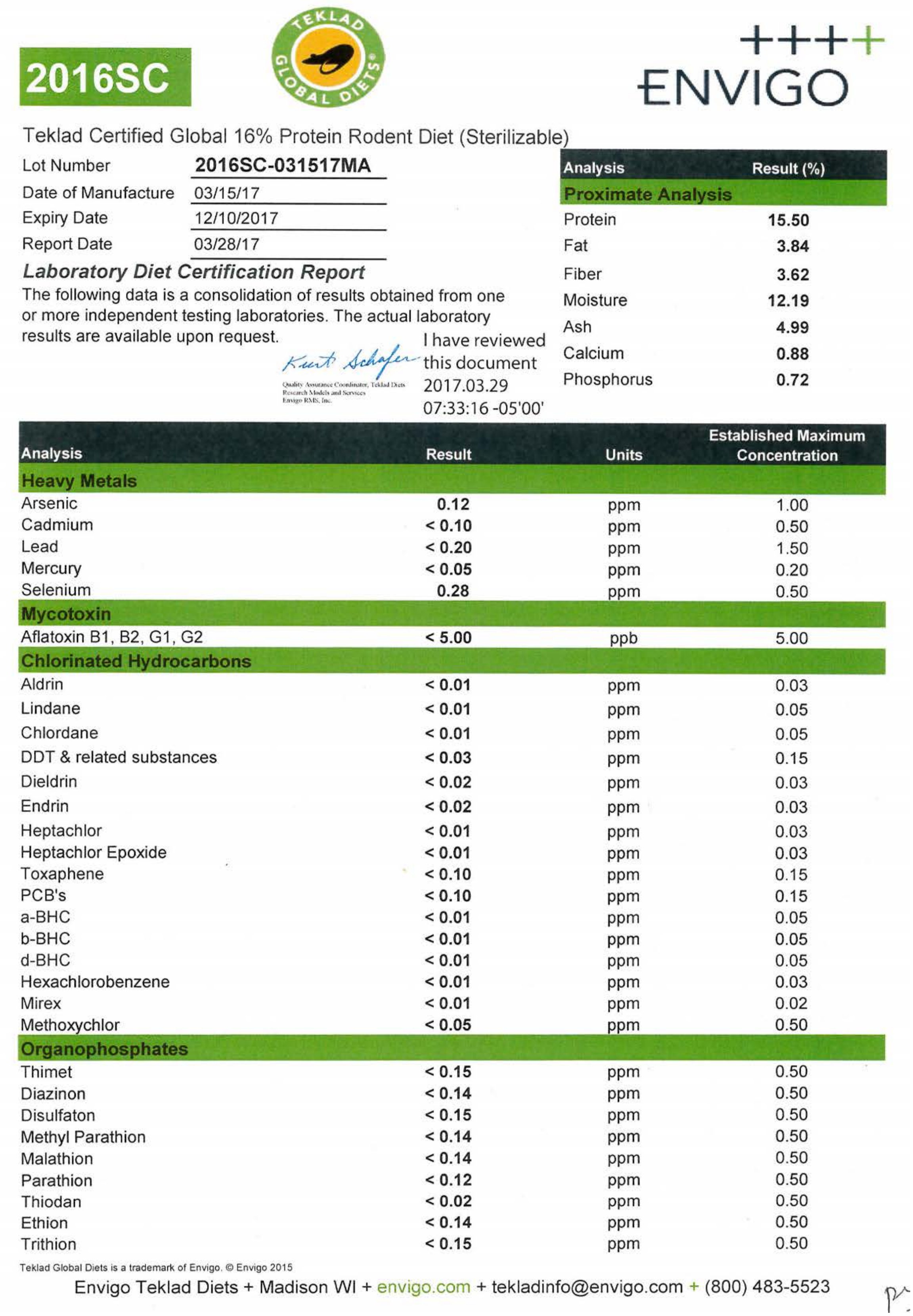
*= Not detected in first dilution.

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 : RBadgha May 26, 2017

Verified by : Dr. Rajesh Posia
Sign & Date : May 27, 2017

APPENDIX 10 (Continued)



2016SC

ENVIGO

Teklad Certified Global 16% Protein Rodent Diet (Sterilizable)

Lot Number	**2016SC-031517MA**
Date of Manufacture	03/15/17
Expiry Date	12/10/2017
Report Date	03/28/17

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.

Kurt Schafer

Quality Assurance Coordinator, Teklad Diets
Research Models and Services
Envigo RMS, Inc.

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

Analysis	Result	Units	Established Maximum Concentration
Heavy Metals			
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			
Aflatoxin B1, B2, G1, G2	**< 5.00**	ppb	5.00
Chlorinated Hydrocarbons			
Aldrin	**< 0.01**	ppm	0.03
Lindane	**< 0.01**	ppm	0.05
Chlordane	**< 0.01**	ppm	0.05
DDT & related substances	**< 0.03**	ppm	0.15
Dieldrin	**< 0.02**	ppm	0.03
Endrin	**< 0.02**	ppm	0.03
Heptachlor	**< 0.01**	ppm	0.03
Heptachlor Epoxide	**< 0.01**	ppm	0.03
Toxaphene	**< 0.10**	ppm	0.15
PCB's	**< 0.10**	ppm	0.15
a-BHC	**< 0.01**	ppm	0.05
b-BHC	**< 0.01**	ppm	0.05
d-BHC	**< 0.01**	ppm	0.05
Hexachlorobenzene	**< 0.01**	ppm	0.03
Mirex	**< 0.01**	ppm	0.02
Methoxychlor	**< 0.05**	ppm	0.50
Organophosphates			
Thimet	**< 0.15**	ppm	0.50
Diazinon	**< 0.14**	ppm	0.50
Disulfaton	**< 0.15**	ppm	0.50
Methyl Parathion	**< 0.14**	ppm	0.50
Malathion	**< 0.14**	ppm	0.50
Parathion	**< 0.12**	ppm	0.50
Thiodan	**< 0.02**	ppm	0.50
Ethion	**< 0.14**	ppm	0.50
Trithion	**< 0.15**	ppm	0.50

Teklad Global Diets is a trademark of Envigo. © Envigo 2015

Envigo Teklad Diets + Madison WI + envigo.com + tekladinfo@envigo.com + (800) 483-5523

APPENDIX 11: Bedding Material Analysis Reports

JAI RESEARCH
FOUNDATION

MICROBIOLOGICAL ANALYSIS CERTIFICATE OF BEDDING MATERIAL

Name of Sample : **Sterilized Rice Husk (Paddy husk)**
Sample Received From : **UV Room (BMR Facility)**
Identification N° : **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.

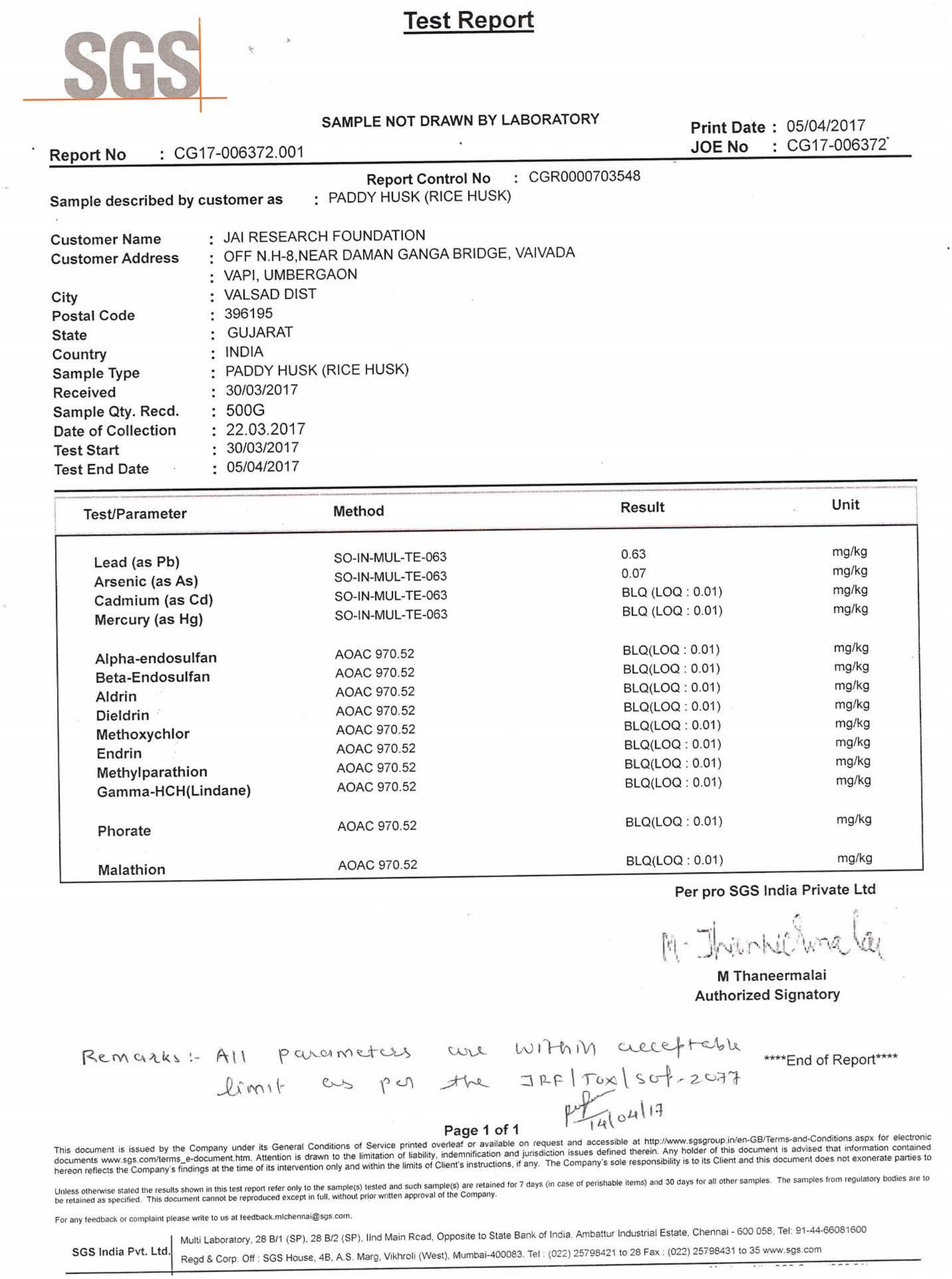
Analysed by : Rahul G Badgha

Sign & Date : March 17, 2017

Verified by : Dr. Rajesh Posia

Sign & Date : March 17, 2017

APPENDIX 11 (Continued)



Test Report

SAMPLE NOT DRAWN BY LABORATORY

Print Date : 05/04/2017
JOE No : CG17-006372

Report No : CG17-006372.001

Report Control No : CGR0000703548

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	0.63	mg/kg
Arsenic (as As)	SO-IN-MUL-TE-063	0.07	mg/kg
Cadmium (as Cd)	SO-IN-MUL-TE-063	BLQ (LOQ : 0.01)	mg/kg
Mercury (as Hg)	SO-IN-MUL-TE-063	BLQ (LOQ : 0.01)	mg/kg
Alpha-endosulfan	AOAC 970.52	BLQ(LOQ : 0.01)	mg/kg
Beta-Endosulfan	AOAC 970.52	BLQ(LOQ : 0.01)	mg/kg
Aldrin	AOAC 970.52	BLQ(LOQ : 0.01)	mg/kg
Dieldrin	AOAC 970.52	BLQ(LOQ : 0.01)	mg/kg
Methoxychlor	AOAC 970.52	BLQ(LOQ : 0.01)	mg/kg
Endrin	AOAC 970.52	BLQ(LOQ : 0.01)	mg/kg
Methylparathion	AOAC 970.52	BLQ(LOQ : 0.01)	mg/kg
Gamma-HCH(Lindane)	AOAC 970.52	BLQ(LOQ : 0.01)	mg/kg
Phorate	AOAC 970.52	BLQ(LOQ : 0.01)	mg/kg
Malathion	AOAC 970.52	BLQ(LOQ : 0.01)	mg/kg

Per pro SGS India Private Ltd

M. Thaneermalai

M Thaneermalai
Authorized Signatory

Remarks :- All parameters are within acceptable limit as per the JRF/Tox/SOP-2077
14/04/17

****End of Report****

Page 1 of 1

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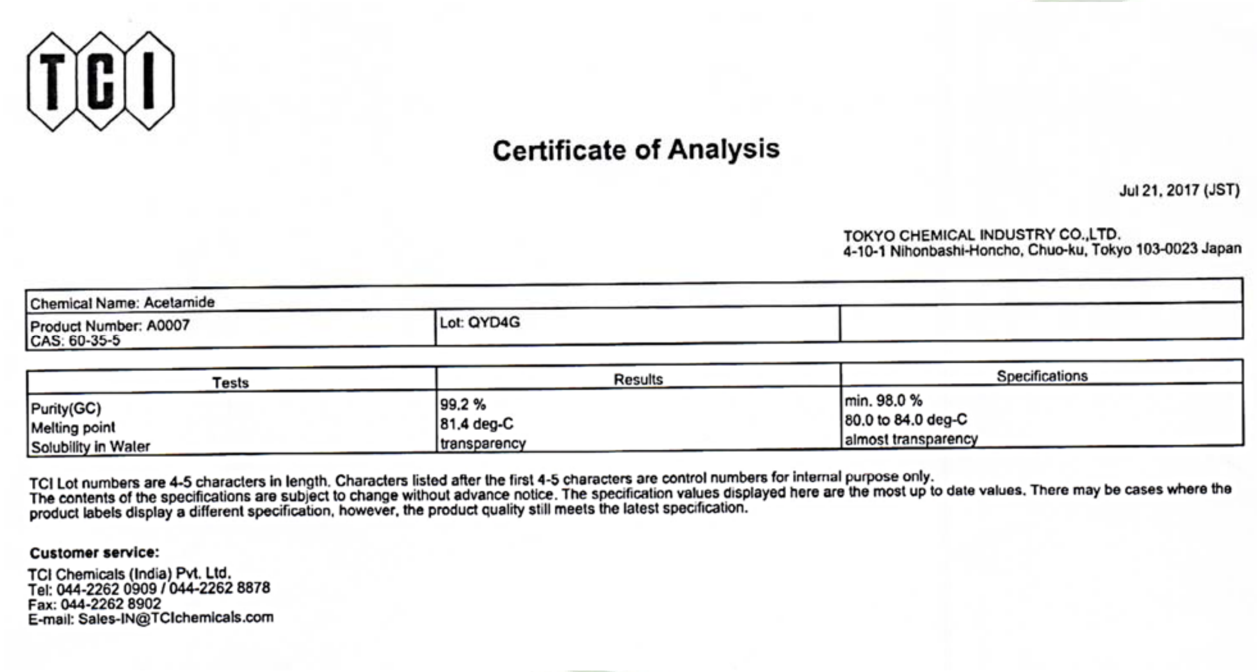
APPENDIX 12: Certificate of Analysis of Acetamide (Provided by Supplier)



TCI

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: 60-35-5	Lot: QYD4G	

Tests	Results	Specifications
Purity(GC)	99.2 %	min. 98.0 %
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 0909 / 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)

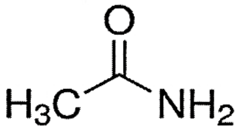
JAI RESEARCH
FOUNDATION

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 : C_2H_5NO
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



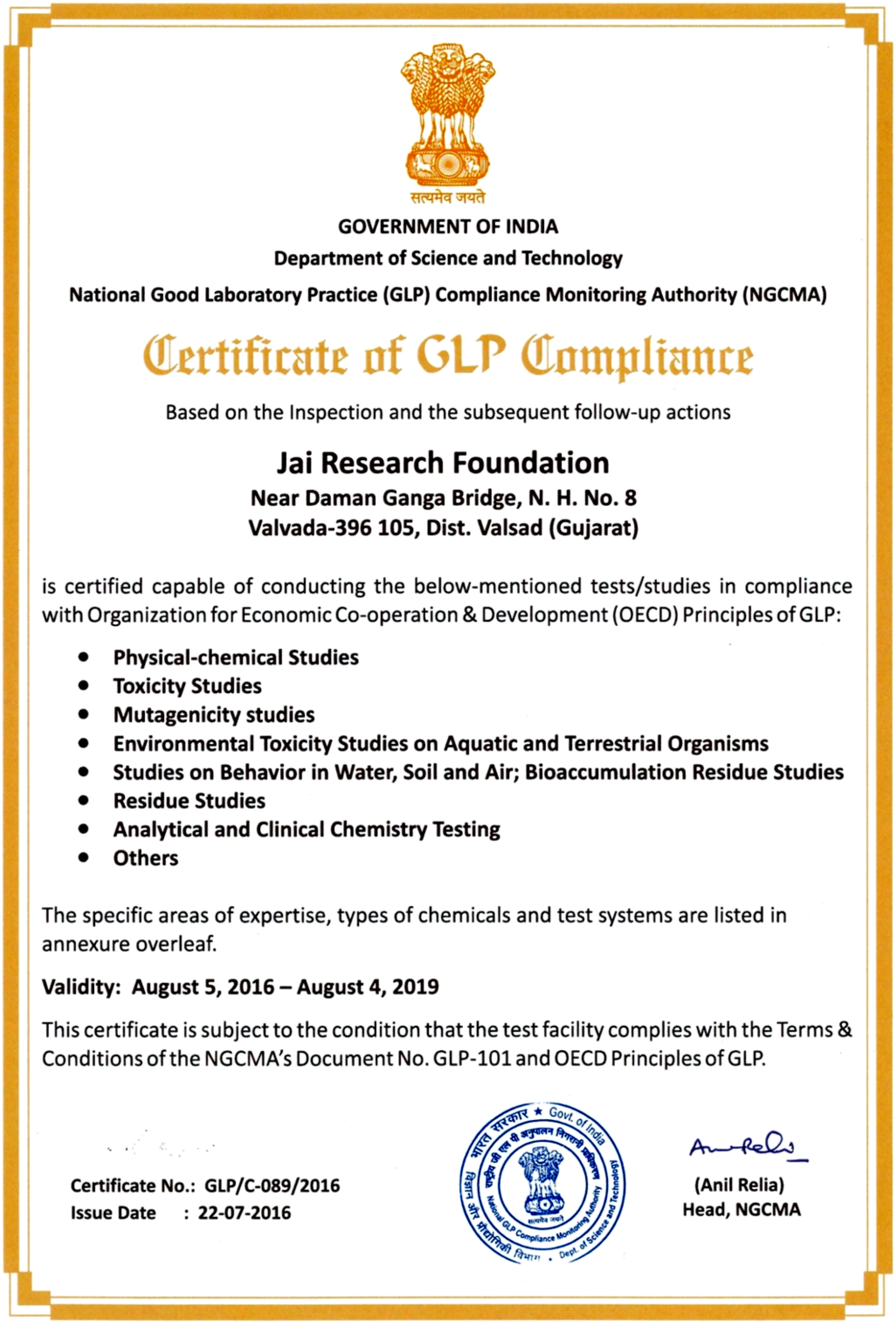
RESULT OF ANALYSIS

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**

November 08, 2017
Signature & Date
Name: Tushar Khanvilkar
Study Director, JRF

APPENDIX 14: GLP Endorsement of Compliance



सत्यमेव जयते

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.

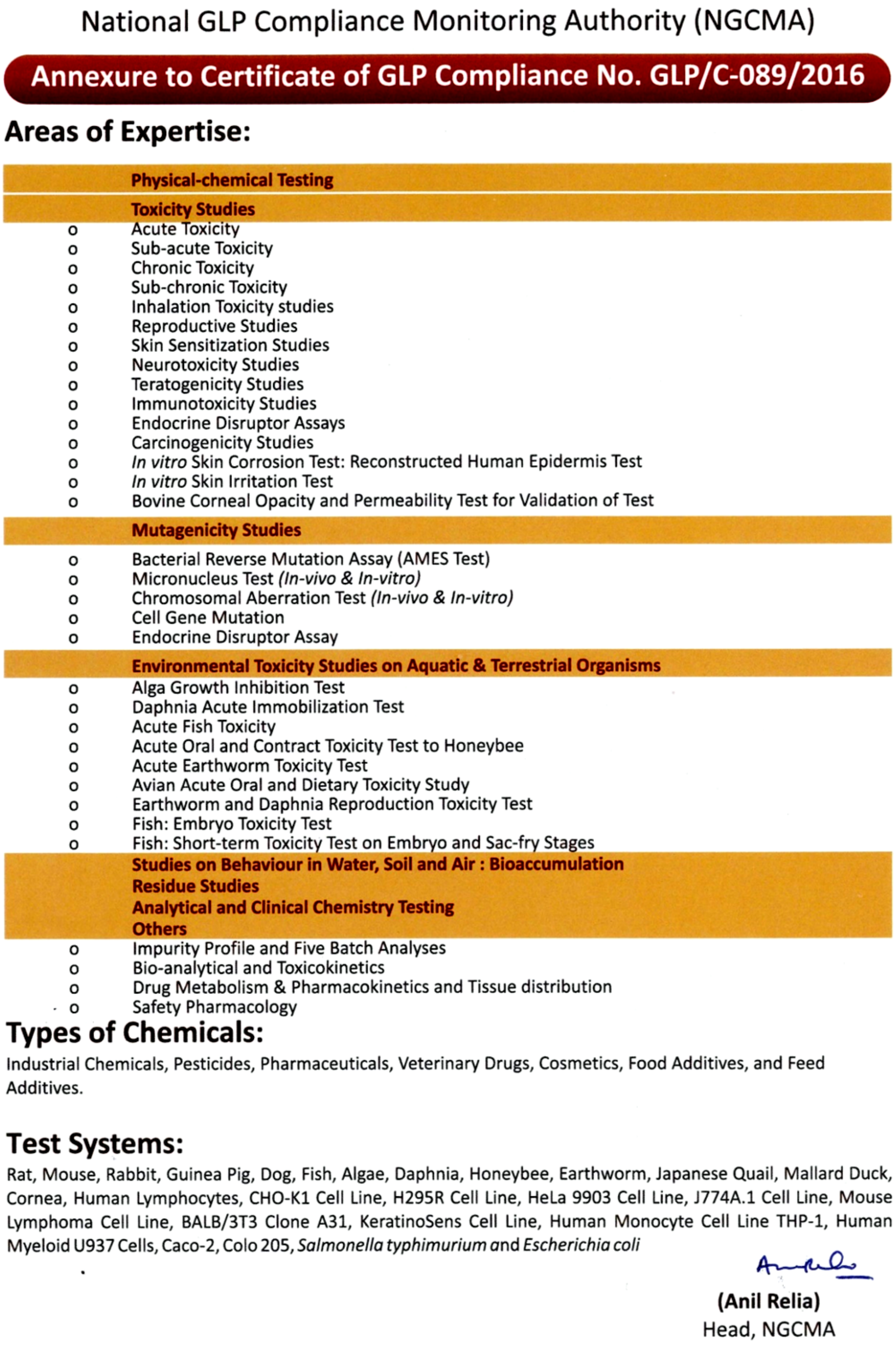
Validity: August 5, 2016 – August 4, 2019

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

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

Mutagenicity Studies

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

Environmental Toxicity Studies on Aquatic & Terrestrial Organisms

- Alga Growth Inhibition Test
- Daphnia Acute Immobilization Test
- Acute Fish Toxicity
- Acute Oral and Contract Toxicity Test to Honeybee
- Acute Earthworm Toxicity Test
- Avian Acute Oral and Dietary Toxicity Study
- Earthworm and Daphnia Reproduction Toxicity Test
- Fish: Embryo Toxicity Test
- 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

- Impurity Profile and Five Batch Analyses
- Bio-analytical and Toxicokinetics
- Drug Metabolism & Pharmacokinetics and Tissue distribution
- 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