FINAL REPORT

Study Title

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST

Test Article

Triclocarban

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Study Completion Date

22 October 2002

Performing Laboratory

BioReliance
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Laboratory Study Number

AA55XE.331.BTL

Sponsor Project Number

2002-01-TCC

Sponsor

Soap and Detergent Association
1500 K Street, NW
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STATEMENT OF COMPLIANCE

Study AA55XE.331.BTL was conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the US EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The stability of the test article has not been determined by the testing facility or the Sponsor.

Analyses of the stability of the test article mixtures were not performed by the testing facility or the Sponsor.

Ramadevi Gudi — 22 Oct 2002

Ramadevi Gudi, Ph.D.
Study Director

Date

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BioReliance Study Management

Date

Quality Assurance Statement

Study Title: *IN VITRO* MAMMALIAN CHROMOSOME ABERRATION TEST

Study Number: AA55XE.331.BTL

Study Director: Ramadevi Gudi, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Regulations, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

Inspect On	25-Feb-02 - 26-Feb-02 To Study Dir 26-Feb-02 To Mgmt 26-Feb-02
Phase	Protocol Review
Inspect On	29-Jul-02 - 31-Jul-02 To Study Dir 31-Jul-02 To Mgmt 12-Aug-02
Phase	Draft Report
Inspect On	28-Oct-02 - 28-Oct-02 To Study Dir 28-Oct-02 To Mgmt 28-Oct-02
Phase	Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Hussain S. Shaffi, B.S.
QUALITY ASSURANCE

28 OCT 2002
DATE

BIORELIANCE

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST

FINAL REPORT

Sponsor:	**Soap and Detergent Association** **1500 K Street, NW** **Suite 300** **Washington, DC 20005**
Authorized Representative:	**Alvaro DeCarvalho**
Performing Laboratory:	**BioReliance** **9630 Medical Center Drive** **Rockville, Maryland 20850**
Test Article I.D.:	**Triclocarban**
Test Article Lot Number:	**K0217**
Test Article Purity:	**100%, (provided by Sponsor)**
Sponsor Project Number:	**2002-01-TCC**
BioReliance Study No.:	**AA55XE.331.BTL**
Test Article Description:	**White powder**
Storage Conditions:	**Room temperature, protected from exposure to light and moisture**
Test Article Receipt/Login Date:	**19 February 2002 / 20 February 2002**
Study Initiation:	**25 February 2002**
Experimental Start Date:	**27 February 2002**
Experimental Completion Date:	**01 July 2002**

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SUMMARY

The test article, Triclocarban, was tested in the chromosome aberration assay using Chinese hamster ovary (CHO) cells in both the absence and presence of an Aroclor-induced S9 activation system. A preliminary toxicity test was performed to establish the dose range for the chromosome aberration assay. The chromosome aberration assay was used to evaluate the clastogenic potential of the test article.

Dimethyl sulfoxide (DMSO) was determined to be the solvent of choice based on information provided by the Sponsor and compatibility with the target cells. The test article was soluble in DMSO at a concentration of 316 mg/mL, the maximum concentration prepared in the assay.

In the preliminary toxicity assay, the maximum dose tested was 3160 μg/mL (10 mM). Visible precipitate was observed in treatment medium at dose levels ≥ 94.8 μg/mL. Dose levels ≤ 31.6 μg/mL were soluble in treatment medium. Selection of dose levels for the chromosome aberration assay was based on cell growth inhibition relative to the solvent control. Substantial toxicity (i.e., at least 50% cell growth inhibition, relative to the solvent control) was observed at dose level 3160 μg/mL in the non-activated 20 hour exposure group. Substantial toxicity was not observed at any dose level in the non-activated and S9 activated 4 hour exposure groups. Based on these findings, the doses chosen for the chromosome aberration assay ranged from 31.3 to 2000 μg/mL for all three treatment groups.

In the chromosome aberration assay, the cells were treated for 4 and 20 hours in the non-activated test system and for 4 hours in the S9 activated test system, and all cells were harvested at 20 hours after treatment initiation. Visible precipitate was observed in treatment medium at the beginning of the treatment period at dose levels ≥ 125 μg/mL. Dose levels 31.3 and 62.5 μg/mL were soluble in treatment medium at the beginning of the treatment period. At the end of the treatment period, visible precipitate was observed in treatment medium at dose levels ≥ 62.5 μg/mL in the S9 activated and non-activated 4 hour exposure groups and at dose levels ≥ 125 μg/mL in the non-activated 20 hour exposure group. Dose level 31.3 μg/mL was soluble in treatment medium at the end of the treatment period in the S9 activated and non-activated 4 hour exposure groups. Dose levels 31.3 and 62.5 μg/mL were soluble in treatment medium at the end of the treatment period in the non-activated 20 hour exposure group.

In the absence of substantial toxicity (≥ 50% cell growth inhibition, relative to the solvent control) in both the preliminary and chromosome aberration assays, the dose levels selected for analysis in the non-activated and the S9-activated 4 hour exposure groups were based on test article precipitate in the treatment medium. In the S9-activated 4 hour exposure group, the 54% cell growth inhibition observed at dose level 1500 μg/mL was not consistent with the lack of toxicity in the preliminary toxicity assay at dose levels ≤ 3160 μg/mL. Therefore, it was considered to be an error in cell counts.

The dose levels selected for analysis in the non-activated 20 hour exposure group were based on mitotic index, the lack of scorable cells and excessive reduction in the number of metaphases at 250, 500 and 1500 μg/mL.

The percentage of cells with structural aberrations in the test article-treated groups was not significantly increased above that of the solvent control at any dose level in any of the treatment groups ($p>0.05$, Fisher's exact test). The percentage of cells with numerical aberrations in the test article-treated groups was not significantly increased above that of the solvent control at any dose level in the S9 activated 4 hour exposure group or in the non-activated 20 hour exposure group ($p>0.05$, Fisher's exact test). The percentage of cells with numerical aberrations in the test article-treated groups was significantly increased above that of the solvent control at dose level 125 µg/mL in the non-activated 4 hour exposure group ($p\leq0.05$, Fisher's exact test). The Cochran-Armitage test was negative for a dose response ($p>0.05$). However, the percentage of cells with numerical aberrations in the test article-treated group (5.0) was within the historic solvent control range of 0.0% to 7.5%. Therefore, it is not considered to be biologically significant.

Treatment Time	Recovery Time	Harvest Time	S9	Toxicity* at highest dose scored (µg/mL)	Mitotic Index Reduction**	LED (lowest effective dose) for Structural Aberrations[1]	LED for Numerical Aberrations[1]
4 hr	16 hr	20 hr	-	6% at 125	7%	None	None
20 hr	0 hr	20 hr	-	41% at 125	67%	None	None
4 hr	16 hr	20 hr	+	40% at 125	91%	None	None

* cell growth inhibition

** relative to solvent control at high dose evaluated for chromosome aberrations

[1] µg/mL

Based on the findings of this study, Triclocarban was concluded to be negative for the induction of structural and numerical chromosome aberrations in CHO cells.

PURPOSE

The purpose of this study was to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in CHO cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, Triclocarban, was received by BioReliance on February 19, 2002 and was assigned the code number AA55XE. The test article was characterized by the Sponsor as a white solid that should be stored at ambient temperature, protected from exposure to moisture. Upon receipt, the test article was described as a white powder and was stored at room temperature, protected from exposure to light and moisture. The identity, strength, purity composition or other characteristics to define the test article have been determined by the Sponsor. A copy of the Certificate of Analysis is included in Appendix III.

The solvent used to deliver Triclocarban to the test system was dimethyl sulfoxide (DMSO; CAS No.: 67-68-5), obtained from Fisher Scientific. Aliquots of the dosing solution preparations from the chromosome aberration assay were sent to BioPharmaceutical Research, Inc. for chemical analysis. The concentration of the test article mixtures was determined by BioPharmaceutical Research, Inc. A copy of the dosing solution analysis draft report is included in Appendix IV. Since the concentration of all the dosing solution preparations was within 10% of their target concentrations, the test article was proven to be stable in the test article mixtures for the study.

Mitomycin C (MMC; CAS No.: 50-07-7), was obtained from the Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 100 and 200 µg/mL for use as the positive control in the non-activated test system. Cyclophosphamide (CP; CAS No.: 6055-19-2), was obtained from Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 1 and 2 mg/mL for use as the positive control in the S9 activated test system. For each positive control, one dose with sufficient scorable metaphase cells was selected for analysis. The solvent for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups.

The negative and positive control articles have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control articles and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

MATERIALS AND METHODS

Test System

Chinese hamster ovary (CHO-K_1) cells (repository number CCL 61) were obtained from American Type Culture Collection, Manassas, VA. In order to assure the karyotypic stability

of the cell line, working cell stocks were not used beyond passage 20. The freeze lot of cells was tested using the Hoechst staining procedure and found to be free of mycoplasma contamination. This cell line has an average cell cycle time of 10-14 hours with a modal chromosome number of 20. The use of CHO cells has been demonstrated to be an effective method of detection of chemical clastogens (Preston et al., 1981).

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at $\leq$-70°C until used. Each bulk preparation of S9 was assayed for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µL S9 per milliliter medium (McCoy's 5A serum-free medium supplemented with 100 units penicillin/mL, 100 µg streptomycin/mL, and 2 mM L-glutamine).

Preliminary Toxicity Assay

The preliminary toxicity assay was performed for the purpose of selecting dose levels for the chromosome aberration assay and consisted of an evaluation of test article effect on cell growth. CHO cells were seeded for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 16-24 hours. Treatment was carried out by refeeding the flasks with 5 mL complete medium (McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 units penicillin/mL, 100 µg streptomycin/mL, and 2 mM L-glutamine) for the non-activated study or S9 reaction mixture (4 mL serum-free medium plus 1 mL of S9/cofactor pool) for the activated study, to which was added 50 µL dosing solution of test article in solvent or solvent alone. The osmolality in treatment medium of the solvent and of the highest test article concentration, the lowest precipitating test article concentration and the highest soluble test article concentration were measured. The pH of the highest concentration of dosing solution in the treatment medium was measured using test tape.

The cells were exposed to solvent alone and to nine concentrations of the test article for 4 hours in both the presence and absence of S9 activation, and for 20 hours continuously in the absence of S9 activation. The cells were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. At completion of the 4 hour exposure period, the treatment medium was removed, the cells washed with calcium and magnesium-free phosphate buffered saline (CMF-PBS), refed with 5 mL complete medium and returned to the incubator for a total of 20 hours from the initiation of treatment. At 20 hours after the initiation of treatment the cells were harvested by trypsinization and counted using a Coulter counter. The presence of test article precipitate was assessed using the unaided eye. Cell viability was determined by trypan blue dye exclusion. The cell counts and percent viability were used to determine cell growth

inhibition relative to the solvent control.

Chromosome Aberration Assay

The chromosome aberration assay was performed using standard procedures (Evans, 1976), by exposing duplicate cultures of CHO cells to the test article as well as positive and solvent controls. For the chromosome aberration assay, CHO cells were seeded at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 16-24 hours. Treatment was carried out by refeeding duplicate flasks with 5 mL complete medium (McCoy's 5A medium supplemented with 10% FBS, 100 units penicillin/mL and 100 µg streptomycin/mL, and 2 mM L-glutamine) for the non-activated study or 5 mL S9 reaction mixture for the S9 activated study, to which was added 50 µL of dosing solution of test or control article in solvent or solvent alone. The osmolality in treatment medium of the solvent and of the highest test article concentration, the lowest precipitating test article concentration and the highest soluble test article concentration were measured. The pH of the highest concentration of dosing solution in the treatment medium was measured using test tape.

In the non-activated study, the cells were exposed to the test article for 4 hours or continuously for 20 hours up to the cell harvest at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air (Swierenga et al., 1991). In the 4 hour exposure group, after the exposure period, the treatment medium was removed, the cells washed with CMF-PBS, refed with complete medium and returned to the incubator. Two hours prior to the scheduled cell harvest, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 µg/mL and the flasks returned to the incubator until cell collection.

In the S9 activated study, the cells were exposed for 4 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air (Swierenga et al., 1991). After the exposure period, the treatment medium was removed, the cells washed with CMF-PBS, refed with complete medium and returned to the incubator. Two hours prior to the scheduled cell harvest, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 µg/mL and the flasks were returned to the incubator until cell collection.

A concurrent toxicity test was conducted in both the non-activated and the S9 activated test systems. After cell harvest an aliquot of the cell suspension was removed from each culture and counted using a Coulter counter. The presence of test article precipitate was assessed using the unaided eye. Cell viability was determined by trypan blue dye exclusion. The cell counts and percent viability were used to determine cell growth inhibition relative to the solvent control.

Collection of Metaphase Cells

Two hours after the addition of Colcemid®, metaphase cells were harvested for both the non-activated and S9 activated studies by trypsinization. Cells were collected approximately 20 hours after initiation of treatment (Galloway et al., 1994). The cells were collected by centrifugation at approximately 800 rpm for 5 minutes. The cell pellet was resuspended in 2-4 mL 0.075 M potassium chloride (KCl) and allowed to stand at room temperature for 4-8 minutes. The cells were collected by centrifugation, the supernatant aspirated and the cells fixed with two washes of approximately 2 mL Carnoy's fixative (methanol:glacial acetic acid,

3:1, v/v). The cells were stored overnight or longer in fixative at approximately 2-8°C.

Slide Preparation

To prepare slides, the fixed cells were centrifuged at approximately 800 rpm for 5 minutes, the supernatant was aspirated, and 1 mL fresh fixative was added. After additional centrifugation (at approximately 800 rpm for 5 minutes) the supernatant fluid was decanted and the cells resuspended to opalescence in fresh fixative. A sufficient amount of cell suspension was dropped onto the center of a glass slide and allowed to air dry. Slides were identified by the study number, date prepared and the treatment condition. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

Selection of Dose Levels for Analysis

In the absence of substantial toxicity ($\geq$ 50% cell growth inhibition, relative to the solvent control) in both the preliminary and chromosome aberration assays, the dose levels selected for analysis in the non-activated and the S9-activated 4 hour exposure groups were based on test article precipitate in the treatment medium. In the S9-activated 4 hour exposure group, the 54% cell growth inhibition observed at dose level 1500 µg/mL was not consistent with the lack of toxicity in the preliminary toxicity assay at dose levels $\leq$ 3160 µg/mL. Therefore, it was considered to be an error in cell counts.

The dose levels selected for analysis in the non-activated 20 hour exposure group were based on mitotic index due to the lack of scorable cells and excessive reduction in the number of metaphases at 250, 500 and 1500 µg/mL.

Evaluation of Metaphase Cells

Slides were coded using random numbers by an individual not involved with the scoring process. To ensure that a sufficient number of metaphase cells were present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) was determined for each treatment group. Metaphase cells with 20±2 centromeres were examined under oil immersion without prior knowledge of treatment groups. Initially, the non-activated and S9 activated 4 hour exposure groups were evaluated for chromosome aberrations and since a negative result was obtained in the non-activated 4 hour exposure group, the non-activated 20 hour continuous exposure group was then also evaluated for chromosome aberrations. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate flask) were examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). Due to a lack of scorable metaphases, only 177 metaphase spreads were scored for aberrations at dose level 62.5 µg/mL in the S9 activated 4 hour exposure group. The number of metaphase spreads that were examined and scored per duplicate flask was reduced if the percentage of aberrant cells reached a statistically significant level before 100 cells were scored. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with

an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥10 aberrations) were also recorded. Chromatid gaps (an aligned achromatic region in one chromatid, the size of which is equal to or smaller than the width of the chromatid) and isochromatid gaps (an aligned, achromatic region in both chromatids, the size of which is equal to or smaller than the width of the chromatids) were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage. Polyploid and endoreduplicated cells were evaluated from each treatment flask per 100 metaphase cells scored.

Controls

MMC was used as the positive control in the non-activated study at final concentrations of 0.1 and 0.2 µg/mL. CP was used as the positive control in the S9 activated study at final concentrations of 10 and 20 µg/mL. For both positive controls, one dose level exhibiting a sufficient number of scorable metaphase cells was selected for analysis. The solvent for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups.

Evaluation of Test Results

The toxic effects of treatment were based upon cell growth inhibition relative to the solvent-treated control and are presented for the toxicity and aberration studies. The number and types of aberrations found, the percentage of structurally and numerically damaged cells (percent aberrant cells) in the total population of cells examined, and the mean aberrations per cell were calculated and reported for each group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells was performed using the Fisher's exact test. Fisher's test was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

All conclusions were based on sound scientific basis; however, as a guide to interpretation of the data, the test article was considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically significant ($p \leq 0.05$). However, values that are statistically significant but do not exceed the range of historic solvent controls may be judged as not biologically significant. Test articles not demonstrating a statistically significant increase in aberrations will be concluded to be negative. Negative results with metabolic activation may need to be confirmed on a case-by-case basis. In those cases where confirmation of negative results is not necessary, justification will be provided.

Criteria for a Valid Test

The frequency of cells with structural chromosome aberrations in the solvent control must

be within the range of the historical solvent control. The percentage of cells with chromosome aberrations in the positive control must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control.

Deviations

No known deviations from the protocol or assay method SOPs occurred during the conduct of this study.

Archives

All raw data, the protocol, all reports, and stained and coded slides will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850. Paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied and the copy will be retained in the BioReliance archives for a minimum of 10 years.

RESULTS AND DISCUSSION

Solubility

Dimethyl sulfoxide (DMSO) was determined to be the solvent of choice based on information provided by the Sponsor and compatibility with the target cells. The test article was soluble in DMSO at a concentration of 316 mg/mL, the maximum concentration prepared in the assay.

Preliminary Toxicity Assay

Dose levels for the chromosome aberration assay were selected following a preliminary toxicity test and were based upon a reduction of cell growth (cell growth inhibition) relative to the solvent control. The results of the evaluation of cell growth inhibition are presented in Tables 1-3. CHO cells were exposed to solvent alone and to nine concentrations of test article ranging from 0.316 to 3160 µg/mL in the absence and presence of an S9 reaction mixture. Visible precipitate was observed in treatment medium at dose levels $\geq$ 94.8 µg/mL. Dose levels $\leq$ 31.6 µg/mL were soluble in treatment medium. The osmolality in treatment medium of the highest concentration tested, 3160 µg/mL, was 395 mmol/kg. The osmolality in treatment medium of the lowest precipitating concentration, 94.8 µg/mL, was 434 mmol/kg. The osmolality in treatment medium of the highest soluble concentration, 31.6 µg/mL, was 407 mmol/kg. The osmolality of the solvent (DMSO) in treatment medium was 416 mmol/kg. The pH of the highest concentration of test article in treatment medium was approximately 7.0.

Substantial toxicity (i.e., at least 50% cell growth inhibition, relative to the solvent control) was not observed at any dose level in the non-activated and S9 activated 4 hour exposure groups. Substantial toxicity was observed at dose level 3160 µg/mL in the non-activated 20 hour continuous exposure group. Based upon the results of the toxicity study, the dose levels

selected for testing in the chromosome aberration assay were as follows:

Treatment Condition	Treatment Time	Recovery Time	Dose levels (μg/mL)
-S9	4 hr	16 hr	31.3, 62.5, 125, 250, 500, 1000, 1500, 2000
	20 hr	0 hr	31.3, 62.5, 125, 250, 500, 1000, 1500, 2000
+S9	4 hr	16 hr	31.3, 62.5, 125, 250, 500, 1000, 1500, 2000

Chromosome Aberration Assay

In the chromosome aberration assay, visible precipitate was observed in treatment medium at the beginning of the treatment period at dose levels ≥ 125 μg/mL. Dose levels 31.3 and 62.5 μg/mL were soluble in treatment medium at the beginning of the treatment period. At the end of the treatment period, visible precipitate was observed in treatment medium at dose levels ≥ 62.5 μg/mL in the S9 activated and non-activated 4 hour exposure groups and at dose levels ≥ 125 μg/mL in the non-activated 20 hour exposure group. Dose level 31.3 μg/mL was soluble in treatment medium at the end of the treatment period in the S9 activated and non-activated 4 hour exposure groups. Dose levels 31.3 and 62.5 μg/mL were soluble in treatment medium at the end of the treatment period in the non-activated 20 hour exposure group.

The osmolality in treatment medium of the highest concentration tested, 2000 μg/mL, was 390 mmol/kg. The osmolality in treatment medium of the lowest precipitating concentration at the beginning of the treatment period, 125 μg/mL, was 425 mmol/kg. The osmolality in treatment medium of the highest soluble concentration at the beginning of the treatment period, 62.5 μg/mL, was 422 mmol/kg. The osmolality of the solvent (DMSO) in treatment medium was 420 mmol/kg. The pH of the highest concentration of test article in treatment medium was approximately 7.0.

Toxicity of Triclocarban (cell growth inhibition relative to the solvent control) in CHO cells when treated for 4 hours in the absence of S9 activation was 6% at 125 μg/mL, the highest test concentration evaluated for chromosome aberrations (Table 4). The activity of Triclocarban in the induction of chromosome aberrations is presented by treatment flask in Table 5 and summarized by group in Table 10. The mitotic index at the highest dose level evaluated for chromosome aberrations, 125 μg/mL, was 7% reduced relative to the solvent control. The dose levels selected for microscopic analysis were 31.3, 62.5 and 125 μg/mL. The percentage of cells with structural aberrations in the test article-treated groups was not significantly increased above that of the solvent control at any dose level ($p>0.05$, Fisher's exact test). The percentage of cells with numerical aberrations in the test article-treated groups was significantly increased above that of the solvent control at dose level 125 μg/mL ($p\leq0.05$, Fisher's exact test). The Cochran-Armitage test was negative for a dose response ($p>0.05$). However, the percentage of cells with numerical aberrations in the test article-treated group (5.0) was within the historic

solvent control range of 0.0% to 7.5%. Therefore, it is not considered to be biologically significant. The percentage of structurally damaged cells in the MMC (positive control) treatment group (14.5%) was statistically significant.

Toxicity of Triclocarban (cell growth inhibition relative to the solvent control) in CHO cells when treated for 4 hours in the presence of S9 activation was 40% at 125 µg/mL, the highest test concentration evaluated for chromosome aberrations (Table 6). The activity of Triclocarban in the induction of chromosome aberrations is presented by treatment flask in Table 7 and summarized by group in Table 10. The mitotic index at the highest dose level evaluated for chromosome aberrations, 125 µg/mL, was 91% reduced relative to the solvent control. The dose levels selected for microscopic analysis were 31.3, 62.5 and 125 µg/mL. Due to a lack of scorable metaphases, only 177 metaphase spreads were scored for aberrations at dose level 62.5. The percentage of cells with structural and numerical aberrations in the test article-treated groups was not statistically increased above that of the solvent control at any dose level ($p>0.05$, Fisher's exact test). The percentage of structurally damaged cells in the CP (positive control) treatment group (15.0%) was statistically significant.

In the absence of a positive response in the non-activated 4 hour exposure group, slides from the non-activated 20 hour exposure group were evaluated for chromosome aberrations. Toxicity of Triclocarban (cell growth inhibition relative to the solvent control) was 41% at 125 µg/mL, the highest test concentration evaluated for chromosome aberrations in the non-activated 20 hour continuous exposure group (Table 8). The activity of Triclocarban in the induction of chromosome aberrations is presented by treatment flask in Table 9 and summarized by group in Table 10. The mitotic index at the highest dose level evaluated for chromosome aberrations, 125 µg/mL, was 67% reduced relative to the solvent control. The dose levels selected for microscopic analysis were 31.3, 62.5 and 125 µg/mL. The percentage of cells with structural and numerical aberrations in the test article-treated groups was not significantly increased above that of the solvent control at any dose level ($p>0.05$, Fisher's exact test). The percentage of structurally damaged cells in the MMC (positive control) treatment group (13.0%) was statistically significant.

CONCLUSION

The positive and solvent controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, Triclocarban was concluded to be negative for the induction of structural and numerical chromosome aberrations in CHO cells.

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TABLE 1

PRELIMINARY TOXICITY TEST USING Triclocarban IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment (µg/mL)	Cell Count ($x10^6$)	Cell Viability (%)	Viable Cells/ Flask ($x10^6$)	Cell Growth Index (%)	Cell Growth Inhibition (%)
DMSO	2.28	99%	2.26	100%	
Triclocarban 0.316	2.14	99%	2.12	94%	6%
0.948	2.08	100%	2.08	92%	8%
3.16	1.93	99%	1.91	85%	15%
9.48	2.08	98%	2.04	90%	10%
31.6	1.68	98%	1.65	73%	27%
94.8	1.95	99%	1.93	86%	14%
316	1.66	98%	1.63	72%	28%
948	1.27	98%	1.25	55%	45%
3160	1.74	97%	1.69	75%	25%

Treatment: CHO cells were treated in the absence of an exogenous source of metabolic activation for 4 hours at 37±1°C.
Cell Viability: determined by trypan blue dye exclusion.
Viable Cells/Flask = cell count x % viable cells
Cell Growth Index = (cells per flask treated group/cells per flask control group), expressed as a percentage.
Cell Growth Inhibition = 100% - % cell growth index; not calculated for negative controls.

TABLE 2

PRELIMINARY TOXICITY TEST USING Triclocarban IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment (µg/mL)	Cell Count ($x10^6$)	Cell Viability (%)	Viable Cells/ Flask ($x10^6$)	Cell Growth Index (%)	Cell Growth Inhibition (%)
DMSO	1.82	99%	1.80	100%	
Triclocarban 0.316	2.12	100%	2.12	118%	-18%
0.948	1.77	100%	1.77	98%	2%
3.16	1.95	99%	1.93	107%	-7%
9.48	1.71	98%	1.67	93%	7%
31.6	1.41	99%	1.39	77%	23%
94.8	1.37	99%	1.35	75%	25%
316	1.57	98%	1.54	85%	15%
948	0.95	97%	0.93	51%	49%
3160	1.44	97%	1.40	78%	22%

Treatment: CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C.
Cell Viability: determined by trypan blue dye exclusion.
Viable Cells/Flask = cell count x % viable cells
Cell Growth Index = (cells per flask treated group/cells per flask control group), expressed as a percentage.
Cell Growth Inhibition = 100% - % cell growth index; not calculated for negative controls.

TABLE 3

PRELIMINARY TOXICITY TEST USING Triclocarban IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

20 HOUR CONTINUOUS TREATMENT

Treatment (µg/mL)	Cell Count ($x10^6$)	Cell Viability (%)	Viable Cells/ Flask ($x10^6$)	Cell Growth Index (%)	Cell Growth Inhibition (%)
DMSO	1.51	99%	1.49	100%	
Triclocarban 0.316	1.73	98%	1.70	114%	-14%
0.948	1.56	99%	1.54	103%	-3%
3.16	1.21	99%	1.20	80%	20%
9.48	1.07	100%	1.07	72%	28%
31.6	1.20	98%	1.17	78%	22%
94.8	1.13	97%	1.10	73%	27%
316	0.84	98%	0.83	55%	45%
948	1.01	97%	0.98	66%	34%
3160	0.16	0%*	0.00	0%	100%

Treatment: CHO cells were treated in the absence of an exogenous source of metabolic activation for 20 hours at 37±1°C.
Cell Viability: determined by trypan blue dye exclusion.
Viable Cells/Flask = cell count x % viable cells
Cell Growth Index = (cells per flask treated group/cells per flask control group), expressed as a percentage.
Cell Growth Inhibition = 100% - % cell growth index; not calculated for negative controls.
* No cells observed.

TABLE 4

CONCURRENT TOXICITY TEST USING Triclocarban IN
THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment (µg/mL)	Flask	Cell Count Averages ($x10^6$)	Cell Viability (%)	Mean Cells per Flask ($x10^6$)	Cell Growth Index (%)	Cell Growth Inhibition (%)
DMSO	A	1.56	100%			
	B	1.36	99%	1.45	100%	
Triclocarban						
31.3	A	1.26	99%			
	B	1.29	98%	1.25	86%	14%
62.5	A	1.22	97%			
	B	1.27	98%	1.21	83%	17%
125	A	1.43	99%			
	B	1.35	99%	1.37	94%	6%
250	A	0.89	97%			
	B	0.81	98%	0.83	57%	43%
500	A	1.03	98%			
	B	1.03	96%	1.00	69%	31%
1000	A	1.03	97%			
	B	0.98	98%	0.98	67%	33%
1500	A	0.82	95%			
	B	0.84	96%	0.80	55%	45%
2000	A	1.21	96%			
	B	1.27	97%	1.20	82%	18%
MMC, 0.1	A	1.75	98%			
	B	1.61	99%	1.66	114%	-14%
MMC, 0.2	A	1.90	99%			
	B	1.79	97%	1.81	124%	-24%

Treatment: CHO cells were treated in the absence of an exogenous source of metabolic activation for 4 hours at 37±1°C.
Cell Viability: determined by trypan blue dye exclusion.
Viable Cells/Flask = cell count x % viable cells, reported as mean of Flasks A and B.
Cell Growth Index = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage.
Cell Growth Inhibition = 100% - % cell growth index; not calculated for negative controls.

TABLE 5

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH Triclocarban IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment (µg/mL)	Flask	Mitotic Index (%)	Cells Scored	% Aberrant Cells		Total Number of Structural Aberrations						Severely Damaged Cells	Average Aberrations Per Cell
						Gaps	Chromatid		Chromosome				
				Numerical	Structural		Br	Ex	Br	Dic	Ring		
DMSO	A	1.2	100	1	0	0	0	0	0	0	0	0	0.000
	B	1.6	100	2	0	0	0	0	0	0	0	0	0.000
Triclocarbon													
31.3	A	1.6	100	4	0	0	0	0	0	0	0	0	0.000
	B	1.4	100	3	0	1	0	0	0	0	0	0	0.000
62.5	A	2.6	100	3	1	0	1	0	0	0	0	0	0.010
	B	2.2	100	4	0	2	0	0	0	0	0	0	0.000
125	A	1.4	100	5	1	0	0	0	0	1	0	0	0.010
	B	1.2	100	5	0	1	0	0	0	0	0	0	0.000
MMC,	A	3.0	100	4	15	1	10	1	3	2	0	0	0.160
0.2	B	3.2	100	4	14	1	10	2	2	3	0	0	0.170

Treatment: CHO cells were treated for 4 hours at 37±1°C in the absence of an exogenous source of metabolic activation. Dose levels 250, 500, 1000, 1500 and 2000 µg/mL were not analyzed due to test article precipitation in the treatment medium.

Mitotic index = number mitotic figures x 100/500 cells counted.

%Aberrant Cells: numerical cells include polyploid and endoreduplicated cells; structural cells exclude cells with only gaps.

Chromatid breaks (Br) include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Ex) include quadriradials, triradials and complex rearrangements.

Chromosome breaks (Br) include breaks and acentric fragments; Dic, dicentric chromosome.

Severely damaged cells includes cells with one or more pulverized chromosome and cells with 10 or more aberrations.

Average aberrations per cell: severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 6

CONCURRENT TOXICITY TEST USING Triclocarban IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment (µg/mL)	Flask	Cell Count Averages ($x10^6$)	Cell Viability (%)	Mean Cells per Flask ($x10^6$)	Cell Growth Index (%)	Cell Growth Inhibition (%)
DMSO	A	1.38	98%			
	B	1.35	97%	1.33	100%	
Triclocarban						
31.3	A	1.27	97%			
	B	1.18	99%	1.20	90%	10%
62.5	A	0.87	98%			
	B	0.82	98%	0.83	62%	38%
125	A	0.84	96%			
	B	0.80	99%	0.80	60%	40%
250	A	0.83	98%			
	B	0.88	97%	0.84	63%	37%
500	A	0.89	97%			
	B	0.94	97%	0.89	67%	33%
1000	A	0.74	95%			
	B	0.75	97%	0.71	54%	46%
1500	A	0.65	94%			
	B	0.61	98%	0.61	46%	54%
2000	A	0.98	97%			
	B	0.99	98%	0.96	72%	28%
CP, 10	A	1.59	96%			
	B	1.57	97%	1.52	114%	-14%
CP, 20	A	1.41	98%			
	B	1.49	98%	1.42	107%	-7%

Treatment: CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C.
Cell Viability: determined by trypan blue dye exclusion.
Viable Cells/Flask = cell count x % viable cells, reported as mean of Flasks A and B.
Cell Growth Index = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage.
Cell Growth Inhibition = 100% - % cell growth index; not calculated for negative controls.

TABLE 7

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH Triclocarban IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment (µg/mL)	Flask	Mitotic Index (%)	Cells Scored	% Aberrant Cells		Total Number of Structural Aberrations						Severely Damaged Cells	Average Aberrations Per Cell
				Numerical	Structural	Gaps	Chromatid		Chromosome				
							Br	Ex	Br	Dic	Ring		
DMSO	A	1.8	100	2	0	1	0	0	0	0	0	0	0.000
	B	2.8	100	2	0	0	0	0	0	0	0	0	0.000
Triclocarbon													
31.3	A	2.6	100	4	1	1	0	1	0	0	0	0	0.010
	B	2.8	100	3	0	2	0	0	0	0	0	0	0.000
62.5	A	0.2	100	3	0	2	0	0	0	0	0	0	0.000
	B	0.2	77	3	1	0	0	0	0	2	0	0	0.026
125	A	0.2	100	4	2	4	1	1	0	0	0	0	0.020
	B	0.2	100	4	2	1	0	0	1	0	1	0	0.020
CP,	A	1.2	100	4	14	2	10	1	2	1	1	0	0.150
10	B	1.6	100	5	16	2	11	5	3	0	2	0	0.210

Treatment: CHO cells were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation. Dose levels 250, 500, 1000, 1500 and 2000 µg/mL were not analyzed due to due to test article precipitation in the treatment medium.

Mitotic index = number mitotic figures x 100/500 cells counted.

%Aberrant Cells: numerical cells include polyploid and endoreduplicated cells; structural cells exclude cells with only gaps.

Chromatid breaks (Br) include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Ex) include quadriradials, triradials and complex rearrangements.

Chromosome breaks (Br) include breaks and acentric fragments; Dic, dicentric chromosome.

Severely damaged cells includes cells with one or more pulverized chromosome and cells with 10 or more aberrations.

Average aberrations per cell: severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 8

CONCURRENT TOXICITY TEST USING Triclocarban IN
THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

20 HOUR CONTINUOUS TREATMENT

Treatment (µg/mL)	Flask	Cell Count Averages ($x10^6$)	Cell Viability (%)	Mean Cells per Flask ($x10^6$)	Cell Growth Index (%)	Cell Growth Inhibition (%)
DMSO	A	1.31	100%			
	B	1.42	100%	1.36	100%	
Triclocarban						
31.3	A	1.12	99%			
	B	1.19	99%	1.14	84%	16%
62.5	A	0.91	99%			
	B	1.05	98%	0.96	71%	29%
125	A	0.84	98%			
	B	0.79	99%	0.80	59%	41%
250	A	0.95	98%			
	B	0.89	98%	0.90	66%	34%
500	A	0.88	97%			
	B	0.97	96%	0.90	66%	34%
1000	A	0.92	99%			
	B	0.87	95%	0.87	64%	36%
1500	A	0.72	92%			
	B	0.67	97%	0.66	48%	52%
2000	A	0.55	95%			
	B	0.42	96%	0.47	34%	66%
MMC, 0.1	A	1.70	98%			
	B	1.60	99%	1.62	119%	-19%
MMC, 0.2	A	1.63	99%			
	B	1.67	99%	1.63	120%	-20%

Treatment: CHO cells were treated in the absence of an exogenous source of metabolic activation for 20 hours at 37±1°C.
Cell Viability: determined by trypan blue dye exclusion.
Viable Cells/Flask = cell count x % viable cells, reported as mean of Flasks A and B.
Cell Growth Index = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage.
Cell Growth Inhibition = 100% - % cell growth index; not calculated for negative controls.

TABLE 9

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH Triclocarban IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

20 HOUR CONTINUOUS TREATMENT

Treatment (µg/mL)	Flask	Mitotic Index (%)	Cells Scored	% Aberrant Cells		Total Number of Structural Aberrations						Severely Damaged Cells	Average Aberrations Per Cell
				Numerical	Structural	Gaps	Chromatid		Chromosome				
							Br	Ex	Br	Dic	Ring		
DMSO	A	3.6	100	0	0	0	0	0	0	0	0	0	0.000
	B	6.0	100	1	0	0	0	0	0	0	0	0	0.000
Triclocarbon													
31.3	A	2.0	100	0	0	0	0	0	0	0	0	0	0.000
	B	1.6	100	0	0	0	0	0	0	0	0	0	0.000
62.5	A	1.4	100	0	0	0	0	0	0	0	0	0	0.000
	B	1.8	100	0	0	0	0	0	0	0	0	0	0.000
125	A	0.8	100	1	0	0	0	0	0	0	0	0	0.000
	B	2.4	100	0	0	0	0	0	0	0	0	0	0.000
MMC,	A	2.4	100	0	14	0	11	2	0	1	0	0	0.140
0.1	B	3.0	100	3	12	0	13	1	0	0	0	0	0.140

Treatment: CHO cells were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation. Dose levels 250, 500 and 1500 µg/mL were not analyzed due to a lack of scorable metaphases. Dose level 1000 µg/mL was not necessary for analysis. Dose level 2000 µg/mL was not analyzed due to excessive cell growth inhibition.

Mitotic index = number mitotic figures x 100/500 cells counted.

%Aberrant Cells: numerical cells include polyploid and endoreduplicated cells; structural cells exclude cells with only gaps.

Chromatid breaks (Br) include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Ex) include quadriradials, triradials and complex rearrangements.

Chromosome breaks (Br) include breaks and acentric fragments; Dic, dicentric chromosome.

Severely damaged cells includes cells with one or more pulverized chromosome and cells with 10 or more aberrations.

Average aberrations per cell: severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 10

SUMMARY

Treatment (µg/mL)	S9 Activation	Treatment Time	Mean Mitotic Index	Cells Scored	Aberrations Per Cell (Mean +/- SD)		Cells With Aberrations Numerical (%)	Structural (%)
DMSO	-	4	1.4	200	0.000	±0.000	1.5	0.0
Triclocarbon								
31.3	-	4	1.5	200	0.000	±0.000	3.5	0.0
62.5	-	4	2.4	200	0.005	±0.071	3.5	0.5
125	-	4	1.3	200	0.005	±0.071	5.0*	0.5
MMC, 0.2	-	4	3.1	200	0.165	±0.423	4.0	14.5**
DMSO	+	4	2.3	200	0.000	±0.000	2.0	0.0
Triclocarbon								
31.3	+	4	2.7	200	0.005	±0.071	3.5	0.5
62.5	+	4	0.2	177	0.011	±0.150	2.8	0.6
125	+	4	0.2	200	0.020	±0.140	4.0	2.0
CP, 10	+	4	1.4	200	0.180	±0.468	4.5	15.0**
DMSO	-	20	4.8	200	0.000	±0.000	0.5	0.0
Triclocarbon								
31.3	-	20	1.8	200	0.000	±0.000	0.0	0.0
62.5	-	20	1.6	200	0.000	±0.000	0.0	0.0
125	-	20	1.6	200	0.000	±0.000	0.5	0.0
MMC, 0.1	-	20	2.7	200	0.140	±0.389	1.5	13.0**

Treatment: Cells from all treatment conditions were harvested at 20 hours after the initiation of the treatments.

Aberrations per Cell: Severely damaged cells were counted as 10 aberrations.

Percent Aberrant Cells: *, $p \leq 0.05$; **, $p \leq 0.01$; using Fisher's exact test.

APPENDIX I

Historical Control Data

IN VITRO MAMMALIAN CYTOGENETIC TEST USING
CHINESE HAMSTER OVARY (CHO) CELLS

HISTORICAL CONTROL VALUES
STRUCTURAL ABERRATIONS

1999-2001

NON-ACTIVATED TEST SYSTEM

Historical Values	Solvent (%)	Positive Control[2] (%)
Mean	1.3	21.2
±SD[1]	1.4	12.1
Range	0.0-5.5	2.5-87.0

S9-ACTIVATED TEST SYSTEM

Historical Values	Solvent (%)	Positive Control[3] (%)
Mean	1.6	33.2
±SD[1]	1.5	18.1
Range	0.0-6.5	7.0-84.0

1 SD = standard deviation.

2 Positive control for non-activated studies, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.75-2 µg/ml), and Mitomycin C (MMC, 0.08-0.15 µg/ml).

3 Positive control for S9-activated studies, cyclophosphamide (CP, 10-50 µg/ml), and benzo(α)pyrene, (B[α]P, 30 µg/ml).

IN VITRO MAMMALIAN CYTOGENETIC TEST USING
CHINESE HAMSTER OVARY (CHO) CELLS

HISTORICAL CONTROL VALUES
COMBINED NUMERICAL ABERRATIONS
(POLYPLOID AND ENDOREDUPLICATED CELLS)
1999-2001

NON-ACTIVATED TEST SYSTEM

Historical Values	Solvent (%)	Positive Control[2] (%)
Mean	2.1	2.8
±SD[1]	1.4	1.6
Range	0.0-7.5	0.0-8.0

S9-ACTIVATED TEST SYSTEM

Historical Values	Solvent (%)	Positive Control[3] (%)
Mean	2.8	2.6
±SD[1]	1.9	1.5
Range	0.0-11.0	0.0-6.0

1 SD = standard deviation.

2 Positive control for non-activated studies, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.75-2 µg/ml), and Mitomycin C (MMC, 0.08-0.15 µg/ml).

3 Positive control for S9-activated studies, cyclophosphamide (CP, 10-50 µg/ml), and benzo(α)pyrene, (B[α]P, 30 µg/ml).

APPENDIX II

Study Protocol

PROTOCOL AMENDMENT 1

Sponsor: **Soap and Detergent Association**

Test Article I.D.: **Triclocarban**

BioReliance Study No.: **AA55XE.331.BTL**

Sponsor Study No.: **2002-01-TCC**

Protocol Title: **In Vitro Mammalian Chromosome Aberration Test**

QA APPROVED EB 2/27/02

1. **LOCATION:** Page 2, § 5; 0

AMENDMENT: Add test schedule dates:

Proposed Experimental Initiation Date: February 28, 2002

Proposed Experimental Completion Date: April 4, 2002

Proposed Report Date: April 18, 2002

REASON FOR THE AMENDMENT: Dates not originally included in protocol.

APPROVALS:

Ramadevi Gudi — Study Director — 26 Feb 2002 — Date

[signature] — Study Management — 26 Feb 2002 — Date

Alvaro J. DeCarvalho — Sponsor Representative — 10/10/02 — Date

BioReliance

QA APPROVED 2/26/02

Received by RAIOA 2/25/02

BioReliance Study Number: AA55XE.331.BTL

In Vitro Mammalian Chromosome Aberration Test

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

2.0 SPONSOR

2.1 Name: Soap and Detergent Association

2.2 Address: 1500 K Street, NW
Suite 300
Washington, DC 20005

2.3 Representative: Alvaro DeCarvalho

2.4 Sponsor Project #: 2002-01-TCC

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: Triclocarban

3.2 Controls: Solvent: DMSO
Positive: Mitomycin C (MMC)
Cyclophosphamide (CP)

3.3 Determination of Strength, Purity, etc.

Unless alternate arrangements are made, the testing facility at BioReliance will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article, and the stability and strength of the test article in the solvent (or vehicle).

3.4 Test Article Retention Sample

The retention of a reserve sample of the test article will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Toxicology Testing Facility
BioReliance

4.2	Address:	9630 Medical Center Drive Rockville, MD 20850
4.3	Study Director:	Ramadevi Gudi, Ph.D. Phone: 301-610-2169 Fax: 301-738-2362 E-mail: rgudi@bioreliance.com

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date:

5.2 Proposed Experimental Completion Date:

5.3 Proposed Report Date:

6.0 TEST SYSTEM

The CHO-K_1 cell line is a proline auxotroph with a modal chromosome number of 20 and a population doubling time of 10-14 hours. CHO-K_1 cells were obtained from the American Type Culture Collection (repository number CCL 61), Manassas, VA. The stability of the modal chromosome number of the cell line is routinely checked and the cell line is routinely tested and determined to be free from mycoplasma contamination. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (Preston et al., 1981).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The chromosome aberration assay will be conducted using standard procedures (Evans, 1976), by exposing cultures of CHO cells to a minimum of four concentrations of the test article as well as to positive and solvent controls. In the non-activated test system, treatment will be for 4 hours and for 20 hours; in the S9 activated test system, exposure will be for 4 hours (Swierenga et al., 1991). To ensure evaluation of first division metaphase cells the dividing cells will be arrested in metaphase and harvested for microscopic evaluation of chromosome aberrations at approximately 20 hours (1.5 normal cell cycles) after the initiation of treatment (Galloway et al., 1994). The clastogenic potential of the test article will be measured by its ability to increase structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group. In the event of a positive response in the 4 hour non-activated study, the prolonged exposure non-activated study may not be scored. The test article will also be assessed for its ability to induce numerical chromosome aberrations.

7.1 Solubility Determination

Unless the Sponsor has indicated the test article vehicle, a solubility determination will be conducted to determine the maximum soluble concentration or workable

suspension up to a maximum of 50 mg/mL for aqueous vehicles and 500 mg/mL for organic vehicles. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice will be the solvent, selected in order of preference, which permits preparation of the highest workable/soluble stock concentration, up to 50 mg/mL for aqueous vehicles and 500 mg/mL for organic vehicles.

7.2 Preliminary Toxicity Test for Selection of Dose Levels

Selection of the dose levels for the cytogenetics assay will be based upon post-treatment toxicity (cell growth inhibition relative to the solvent control) and solubility of the test article. CHO cells will be exposed to solvent alone and to at least nine concentrations of test article. The highest concentration tested will be 5 mg/ml or 10 mM whichever is lower for freely soluble test articles, or the maximum concentration resulting in a workable suspension for poorly soluble test articles not to exceed 5 mg/ml. The pH will be measured at the highest test article treatment condition and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest dose level, lowest precipitating dose level (where applicable) and the highest soluble dose level (where applicable) in treatment medium will also be measured. Cells seeded 16-24 hours earlier will be exposed for 4 hours in the absence and presence of S9 and for 20 hours in the absence of S9. Just prior to trypsinization the cell cultures will be visually inspected for the extent of monolayer confluency relative to the solvent control. Twenty hours after treatment initiation the cells will be harvested by trypsinization and counted using an automatic cell counter and the cell viability will be assessed using trypan blue dye exclusion. The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control.

Whenever possible, the high dose to evaluate chromosome aberrations will be selected to give at least 50% toxicity (cell growth inhibition relative to the solvent control) irrespective of solubility but not to exceed 5 mg/ml or 10 mM. At least two additional dose levels, demonstrating minimal or no toxicity, will be included. In the event the test article cannot be dissolved at a high enough concentration in an appropriate solvent to be toxic, then the highest dose to be tested in the chromosome aberration assay will be the concentration resulting in minimum precipitation in test medium. Precipitation will be determined by direct visual inspection. In the event the test article demonstrates a dose-responsive increase in toxicity at concentrations that exceed solubility in treatment medium, then the highest dose to be tested will be the maximum concentration that results in at least 50% toxicity. In the event that neither cytotoxicity nor insolubility is observed in the preliminary test, the highest dose in the chromosome aberration assay will be 5 mg/ml or 10 mM whichever is lower. If excessive precipitation of the test article-solvent solution occurs upon addition to treatment medium, or if the osmolality of the treatment medium is considered excessive, the Sponsor will be consulted.

7.3 Frequency and Route of Administration

Target cells will be treated for 4 hours in the absence and presence of S9, and for 20 hours in the absence of S9, by incorporation of the test article-solvent mixture into the treatment medium. This technique has been demonstrated to be an effective method of detection of chemical clastogens in this test system (Evans, 1976).

If the Sponsor is aware of specific metabolic requirements, then this information will be utilized in the preparation of the study design. Verification of a clear positive response is not required. Negative results will not be confirmed when justification can be provided. Equivocal results may be confirmed, upon consultation with the Sponsor, and may employ a modification of the study design. This guidance is based on the OECD Guideline 473 (1997) and ICH Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (1996).

7.4 Activation System

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used. Each batch preparation of S9 will be assayed for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with cofactors to contain 2 mM magnesium chloride ($MgCl_2$,) 6 mM potassium chloride (KCl), 1mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µl S9 per ml serum free medium.

7.5 Controls

7.5.1 Solvent (or Vehicle) Control

The solvent for the test article will be used as the solvent control. For solvents other than water, physiological buffer, or medium, the final concentration in treatment medium will not exceed 1%.

7.5.2 Positive Controls

Mitomycin C will be used at a concentration within 0.05-0.3 µg/ml as the positive control in the non-activated study. Cyclophosphamide will be used at a concentration within 10-50 µg/ml as the positive control in the S9-activated study.

7.6 Preparation of Target Cells

Exponentially growing CHO-K_1 cells will be seeded in complete medium (McCoy's 5A medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml and 100 µg streptomycin/ml) for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask. The flasks will be incubated at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air for 16-24 hours.

7.7 Identification of Test System

Using a permanent marking pen, the treatment flasks will be identified by the BioReliance study number and a code system to designate the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in duplicate by refeeding the flasks with 5 ml complete medium for the non-activated exposure or 5 ml S9 reaction mixture for the S9-activated exposure, to which will be added 50 µl of dosing solution of test or control article in solvent or solvent alone. Larger volumes of dosing solution may be used if water, physiological buffer, or medium is used as the solvent.

In the non-activated study, the cells will be treated for 4 hours and for 20 hours; in the S9-activated study the cells will be treated for 4 hours. Treatment will be carried out at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air. After the 4 hour treatment period in the non-activated and the S9-activated studies, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium and returned to the incubator.

A concurrent toxicity test to determine cell growth inhibition relative to the solvent control will be conducted in both the non-activated and the S9-activated studies.

7.9 Collection of Metaphase Cells

Cells will be collected approximately 20 hours after initiation of treatment. This post-treatment harvest time represents approximately 1.5 normal cell cycles and was selected to ensure that the cells are analyzed in the first division metaphase after initiation of treatment. Two hours prior to cell harvest, Colcemid® will be added to the cultures at a final concentration of 0.1 µg/ml.

Cells will be harvested by trypsinization, collected by centrifugation and an aliquot will be removed for counting using an automatic cell counter and trypan blue dye exclusion. The remainder of the cells will be swollen with 0.075M KCl, washed with two consecutive changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 2-8°C. The cell counts and percent viability will be used to determine cell growth inhibition relative to the

solvent control (% toxicity). To prepare slides, the cells will be collected by centrifugation and resuspended in fresh fixative. The suspension of fixed cells will be applied to glass microscope slides and air-dried. The slides will be identified by the experiment number, treatment condition and date. The slides will be stained with Giemsa and permanently mounted.

7.10 Scoring for Metaphase Aberrations

To ensure that a sufficient number of metaphase cells are present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) will be determined and recorded for each coded treatment group selected for scoring chromosome aberrations. Slides will be coded using random numbers by an individual not involved with the scoring process. In the event of a positive response in the 4 hour non-activated study, the prolonged exposure non-activated study may not be scored. Metaphase cells with 20 ± 2 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads from each dose level (100 per duplicate flask) will be examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). The number of metaphase spreads that will be examined and scored per duplicate flask may be reduced if the percentage of aberrant cells reaches a statistically significant level before 100 cells are scored. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells ($\geq$ 10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with a structural aberration will be recorded using a calibrated microscope stage. The percent polyploid and endoreduplicated cells will be evaluated per 100 cells for each dose level analyzed for structural aberrations.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Solvent Control

The frequency of cells with structural chromosome aberrations in the solvent control must be within the range of the historical solvent control.

8.2 Positive Control

The percentage of cells with aberrations must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control.

9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon cell growth inhibition relative to the solvent control and will be presented for the toxicity and aberration studies. The number and types of aberrations found, the percentage of structurally and numerically damaged cells (percent aberrant cells) in the total population of cells examined, and the mean aberrations per cell will be calculated and reported for each treatment group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell. Statistical analysis of the percentage of aberrant cells will be performed using the Fisher's exact test. The Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's exact test at any test article dose level, the Cochran-Armitage test will be used to measure dose-responsiveness. All conclusions will be based on sound scientific basis; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically significant ($p \leq 0.05$). However, values that are statistically significant but do not exceed the range of historic negative or solvent controls may be judged as not biologically significant. Test articles not demonstrating a statistically significant increase in aberrations will be concluded to be negative.

10.0 REPORT

A report of the results of this study will be prepared by BioReliance and will accurately describe all methods used for generation and analysis of the data.

Results presented will include, but not be limited to:

• Test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test article, if known.

• Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test article in solvent/vehicle, if known.

• Source of cells, karyotype features (modal chromosome number) and suitability of the cell type used, absence of mycoplasma, cell cycle length, passage number.

• Test conditions: composition of medium; CO_2 concentration; incubation time; cell seeding density; solvent and solvent selection rationale; concentration of test article and concentration selection rationale; composition and acceptability criteria for the metabolic activation (S9) system; duration of treatment; duration of treatment with and concentration of Colcemid®; type of metabolic activation system used; positive and solvent controls; methods of slide preparation; number of cell cultures; criteria for scoring aberrations and criteria for considering studies positive, negative.

• Results: description of precipitation; pH and osmolality of the treatment medium; cell growth inhibition relative to the solvent control; mitotic index and number of metaphases analyzed; type and number of aberration (structural and numerical) given separately for each treated and control culture; concentration-response relationship; statistical analysis; historical control data.

11.0 RECORDS AND ARCHIVES

All raw data, the protocol and all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at: BioReliance, 14920 Broschart Road, Rockville, MD 20850. Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied and the copy will be retained in the BioReliance archives for a minimum of 10 years.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 473 (*In Vitro* Mammalian Chromosome Aberration Test), July 1997 and with the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (1996 and 1997).

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies (GLPs). The protocol, an in-process phase, the raw data, and report(s) will be audited per the Standard Operating Procedures (SOPs) of BioReliance by the Quality Assurance Unit of BioReliance for compliance with GLPs, the SOPs of BioReliance and the study protocol. At least one, study-specific, in-process inspection will be performed for this study. A signed QA statement will be included in the final report. This statement will list the study-specific phases inspected, the dates of each inspection, and the dates the results of each inspection were reported to the Study Director and the Study Director's management. In addition, a signed GLP compliance statement will be included in the final report. This statement will cite the GLP guideline(s) with which the study is compliant and any exceptions to this compliance, if applicable, including the omission of characterization or stability analyses of the test or control articles or their mixtures.

Will this study be submitted to a regulatory agency? yes

If so, to which agency or agencies? EPA

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), Chemical Mutagens, Principles and Methods for their Detection, vol. 4. Plenum Press, New York, NY.

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OECD Guideline for the Testing of Chemicals, Guideline 473 (*In Vitro* Mammalian Chromosome Aberration Test), July 1997.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian in vivo and in vitro cytogenetic assays: a report of the Gene-Tox Program, Mutation Research, 87:143-188.

Scott, D., N.D. Danford, B.J. Dean and D.J. Kirkland. 1990. Metaphase Chromosome Aberration Assays In Vitro. In: Basic Mutagenicity Tests: UKEMS Recommended Procedures. D.J Kirkland (ed). Cambridge University Press, New York, NY.

Swierenga S.H.H., J.A. Heddle, E.A. Sigal, J.P.W. Gilman, R.L. Brillinger, G.R. Douglas and E.R. Nestmann (1991) Recommended protocols based on a survey of current practice in genotoxicity testing laboratories, IV. Chromosome aberration and sister-chromatid exchange in Chinese hamster ovary, V79 Chinese lung and human lymphocyte cultures, Mutation Research 246:301-322.

14.0 APPROVAL

Alvaro J DeCarvalho — 02/07/02
Sponsor Representative — Date

Alvaro J DeCarvalho
(Print or Type Name)

Ramakrishna Gudi — 25 Feb 2002
BioReliance Study Director — Date

[signature] — 25 Feb 2002
BioReliance Study Management — Date

APPENDIX III

Certificate of Analysis

BAYER CORPORATION
INDUSTRIAL CHEMICALS DIVISION
SPECIALTY PRODUCTS
PITTSBURGH PA 15205-9741

CERTIFICATE OF ANALYSIS

PRODUCT: PREVENTOL SB EXTRA TRICLOCARBA PRODUCT CODE: N117
LOT: K0217
DOCUMENT #: 0000000 P.O.#: 0
CUSTOMER #: 000000-000 AMOUNT SHIPPED: 0
CUSTOMER CODE: DATE SHIPPED:
CONTAINER DESCRIPTION:
DATE OF MFG: 08/29/2001

PROPERTY/SUB-PROPERTY	SPECIFICATIONS	RESULTS
ASSAY (LIQUID CHROMATOGR	MIN 97	99.0
SUM OF TRIARYLBIURETS	MAX 1.0	0.2
SUM OF CHLORDANILINES	MAX 450	< 450
TOLUENE	MAX 2000	1100
COLOR NUMBER INTEGRT ABS	MAX 6.0	< 0.2
SOLUBILITY IN CARBOWAX 3		PASS

SUE DELSTANCO
QEM COORDINATOR
1-800-662-2927 X4560

APPENDIX IV

Dosing Solution Analysis

BRI Biopharmaceutical Research Inc.
Bioanalytical Biopharmaceutics & Drug Discovery

3650 Wesbrook Mall
Vancouver, B.C.
Canada V6S 2L2

Tel: (604) 432-9237
Fax: (604) 432-9239
www.bripharm.com

BRI Biopharmaceutical Research Inc.

3650 Wesbrook Mall, Vancouver, B.C., Canada, V6S 2L2
Phone: (604) 432-9237, Fax: (604) 432-9239

BRI Study No: BIO-2002-002

Study Title: Validation of a HPLC Method for Quantitation of Trichlorocarbanilide (TCC) in DMSO

Sponsor:
The Soap and Detergent Association
1500 K Street, NW Suite 300
Washington, DC, USA 20005

Toxicology Testing Facility:
BioReliance Corporation
9630 Medical Center Drive
Rockville, MD, USA 20850

Study Report No: RPT-BIO-2002-002

BRI Study Initiation: April 29, 2002

Draft Report Submitted: July 4, 2002

Final Report Submitted: August 20, 2002

This report contains 48 pages

BRI Study No: BIO-2002-002

Study Title:

Validation of a HPLC Method for Quantitation of Trichlorocarbanilide (TCC) in DMSO

AUTHENTICATION

This laboratory study was performed under my supervision in accordance with the study protocol described herein conducted in compliance with Good Laboratory Practices as described in the Code of Federal Regulations, title 21: part 58. This report provides a correct and faithful record of the results obtained.

BRI Analytical Investigator
David Kwok, Ph.D.

08/20/02
Date

BRI Laboratory Supervisor
Huey Kuan, B.Sc.

08/20/02
Date

BRI Research Associate
Lori Ptachyk, B.Sc.

08/20/02
Date

BRI Research Assistant
Cherly Ann Diaz, B.Sc.

08/20/02
Date

BRI personnel initials

BRI Quality Assurance Statement

This study and this report have been inspected and examined by the BRI Quality Assurance Unit. This laboratory study was conducted in compliance with Good Laboratory Practices as described in the Code of Federal Regulations, title 21: part 58. The final report accurately describes the procedures employed during the course of this study and accurately reflects the raw data.

Date(s) of Audit	Study Phase	Date Reported to BRI Management	Date Reported to Study Director	QA Initials
24-Apr-02	Protocol review	24-Apr-02	04-Jul-02	
06-Jun-02	In-progress: validation batches data review	07-Jun-02	04-Jul-02	
13-Jun-02	In-progress: dosing sample data review	13-Jun-02	04-Jul-02	
01-Jul-02	In-progress: report table review	03-Jul-02	04-Jul-02	
28-Jun-02 to 01-Jul-02	Draft report review	03-Jul-02	04-Jul-02	

Jessica Yeung, B.Sc., B.A.
Quality Assurance Manager

08/20/02
Date

BRI personnel initials

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List of Abbreviations

ACN	Acetonitrile
Conc	Concentration
CV	Coefficient of variation
DAD	Diode array detector
dd	Deionized distilled
DMSO	Dimethyl sulfoxide
GLP	Good Laboratory Practice
HPLC	High performance liquid chromatograph
ICH	International Conference on Harmonisation
LC	Liquid chromatograph
LOD	Limit of detection
LOQ	Limit of quantitation
µL	Microlitre
µm	Micrometer
MeOH	Methanol
mg	Milligram
mL	Millilitre
mm	Millimeter
n/ap	Not applicable
N/D	Not detected
QA	Quality Assurance
QC	Quality Control
r^2	Coefficient of determination
%RE	Percent relative error
RSD	Relative standard deviation
SD/stdev	Standard deviation
STD	Standard
TCC	Trichlorocarbanilide
USP	United States Pharmacopeia
UV	Ultraviolet
Vol	Volume

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1.0 EXECUTIVE SUMMARY

1.1 Study Objectives

The objectives of this study are to validate an HPLC assay method for TCC in DMSO dosing samples in support of toxicology studies and to quantitate the amount of TCC in dosing samples used for toxicology investigations.

1.2 Study Sponsor

Alvaro J. DeCarvalho, Ph.D.
The Soap and Detergent Association
1500 K Street, NW
Suite 300
Washington, DC
USA 20005
Phone: (202)-662-2516
Fax: (202)-347-4110

1.3 Study Director and Toxicology Testing Facility

Ramadevi Gudi, Ph.D.
BioReliance Corporation
9630 Medical Center Drive
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USA 20850
Phone: (301)-610-2169
Fax: (301)-738-2362

1.4 Analytical Investigator and Analytical Testing Facility

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BRI Biopharmaceutical Research Inc.
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Vancouver, BC
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Phone: (604)-432-9237
Fax: (604)-432-9239

1.5 Experimental Conduct

A quantitative potency HPLC assay has been previously developed by the Study Sponsor for the determination of TCC in DMSO dosing samples. The assay was slightly modified by BRI before undergoing validation. These modifications include the use of an analytical column with a slightly larger internal diameter (4 mm vs. 3 mm), incresing the flow rate (1.0 mL/min vs. 0.7 mL/min), using a different solvent for sample preparation (DMSO and methanol vs. dioxane and methanol), and shortening the sample runtime (8 minutes vs. 30 minutes). The BRI assay involves the use of an isocratic HPLC system with a Lichrosphere 60 RP, 250 x 4.0 mm, 5.0 µm column and UV detection at 262 nm.

Based on the formulation used for method validation, the assay appeared to be selective for TCC. The assay was found to be linear over the concentration range from 0.05 to 0.12 mg/mL. Assay precision and accuracy were determined to be ≤ 5% in QC samples, demonstrating acceptable precision and accuracy.

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In view of the above information, BRI has performed the following in-house method validation of an isocratic HPLC potency assay method for TCC in support of toxicological investigations.

1.6 TCC HPLC Method Performance Acceptance Criteria and Method Specifications

Method validation studies for TCC were carried out as proposed in an approved Study Protocol, with reference to FDA and ICH regulatory guidelines.

1.7 Assay Range

A total of three batches of TCC standard samples were prepared in methanol. Two batches were prepared with single calibration standards and one batch was prepared with calibration standards in triplicate. Calibration standards at 0.05, 0.06, 0.08, 0.10, and 0.12 mg/mL provide an assay range from 50 to 120% of the nominal TCC concentration of 0.10 mg/mL.

1.8 Assay Accuracy

The within-batch assay accuracy of the HPLC/UV method at TCC concentrations of 0.08, 0.10, and 0.12 mg/mL were observed to be within ±1.1% relative error (%RE, n=3 replications) with reference to the expected concentrations. The between-batch accuracy was within ±1.0% (n=3 batches).

1.9 Assay Precision

The within-batch assay precision was evaluated at TCC concentrations of 0.08, 0.10, and 0.12 mg/mL and resulted in a coefficient of variation (CV) within 1.0% (n=3 replications). The between-batch assay precision was within a CV of 0.9% (n=3 batches).

1.10 Assay Linearity

Standard curves were obtained using 1/y weighted linear regression and observed to be linear based on back-interpolated concentrations with a r^2 greater than 0.99 calculated from 5-level calibration standard samples at TCC concentrations of 0.05, 0.06, 0.08, 0.10, and 0.12 mg/mL. All back-interpolated concentrations were generally observed within ±0.8% deviation from the standard curve.

1.11 Limit of Quantitation

A theoretical limit of quantitation (LOQ) was determined based on 10x(standard deviation of the response factor) divided by the average slope of the calibration curve. A theoretical assay LOQ was calculated to be 0.0045 mg/mL.

1.12 Limit of Detection

A theoretical limit of detection (LOD) was determined based on 3.3x(standard deviation of the response) divided by the average slope of the calibration curve. A theoretical assay LOD was calculated to be 0.0015 mg/mL.

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1.13 Assay Robustness

The TCC method assay accuracy and precision robustness was evaluated by performing the HPLC assay with two analysts independently preparing separate batches of samples and mobile phase. The observed between-batch accuracy and precision data were shown to be within the assay performance criteria, demonstrating acceptable assay robustness.

1.14 Assay Specificity

The specificity of the HPLC assay for TCC in DMSO dosing samples was qualitatively assessed for potential interference from the dosing vehicle. The following specificity samples were evaluated:

a) Dosing vehicle alone (DMSO)
b) HPLC mobile phase alone (70% ACN)
c) TCC in mobile phase (70% ACN)
d) TCC in methanol
e) TCC in dosing vehicle (DMSO)

Based on the evaluation of the HPLC chromatograms, the assay was observed to be specific for TCC without interference.

1.15 TCC Stock Solution Stability

TCC stock solutions were prepared at the start of the study and kept refrigerated and protected from light during the course of the validation experiments. Stability profiling and stability studies were not required for this study.

1.16 HPLC System Suitability

The chromatographic peak area response, peak tailing factor, and column efficiency of the HPLC assay were determined using a TCC system suitability sample at the nominal concentration with n = 6 replicates. All replicates were found to meet the acceptance criteria.

1.17 HPLC Assay of AF 2364 in DMSO Dosing Samples

A total of nine DMSO dosing samples were assayed in triplicate for BioReliance Study# AA55XE.331.BTL (BRI samples BIO-2002-002-3730 to BIO-2002-001-3738). The samples consisted of 8 different concentrations of TCC in DMSO, 3.13, 6.25, 12.5, 25.0, 50.0, 100.0, 150.0, and 200.0 mg/mL, and blank dosing sample. The resultant % label claim values for these samples were determined to range from 96.8% to 105.6%.

1.18 Conclusion

An HPLC method was slightly modified from a previously developed method for the determination of TCC in DMSO dosing samples in support of toxicological investigations. The method for TCC has been validated to provide analytical support of toxicological investigations.

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2.0 INTRODUCTION

Trichlorocarbanilide (TCC) is the test article being investigated as detailed in the Study Protocol approved by BioReliance as the Toxicology Testing Facility. It is the intention of the Study Director to conduct a validation study of an HPLC method in support of the determination of TCC in dosing samples as required during the course of toxicology studies. An HPLC method for TCC, previously developed by the Study Sponsor, was provided to BRI for this method validation study. Some minor changes to the Sponsor's method were made before validation. An analytical column with a slightly larger internal diameter (4 mm vs. 3 mm) was used, as a 4 mm internal diameter column having the same specifications as the Sponsor's column was available at BRI. The flow rate was increased from 0.7 mL/min to 1.0 mL/min to compensate for the increased column internal diameter. DMSO and methanol were used for stock solution preparation instead of dioxane and methanol, in order to more closely represent the sample matrix. The method provided by the Sponsor was originally intended to quantify impurities in addition to TCC, leading to a long runtime. The Sponsor determined that it was no longer necessary to quantify the impurities, allowing the runtime to be shortened to 8 minutes from 30 minutes. This report describes the validation of an HPLC method for the determination of TCC in DMSO dosing samples.

3.0 MATERIALS AND METHODS

3.1 Reference Standard

Test Article	Supplier	Lot #	BRI Reference #
3,4,4'-Trichlorocarbanilide (TCC)	BioReliance	AA55XE S4	BIO-2002-002-3134(RFS:115)

3.2 Chemicals and Reagents

Chemical	Supplier	Lot #
Acetonitrile	EM Science	41330147, 42067202
ddH_2O	in-house	n/ap
DMSO	BDH	41080126
Methanol	EM Science	42021204, 42038207, 42056210

3.3 Test Samples

A total of 9 dosing samples of TCC in DMSO for BioReliance Study# AA55XE.331.BTL were supplied by BioReliance (BRI reference # BIO-2002-002-3730 to –3738).

3.4 Sample Processing Equipment

- Balance: Mettler Toledo model AG285 (BRI# 150)
- Glass volumetric pipettes :10 mL, 7 mL, 3 mL, 1 mL
- Hamilton syringes: 100 µL, 500 µL
- Vortex mixer: VWR Vortex-Genie II (BRI# 035), VWR Multi-Tube Vortexer (BRI# 033)

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3.5 Sample Preparation

3.5.1 TCC in DMSO Standard Stock Solution at 20.0 mg/mL

1. Weigh accurately 200 mg of TCC into a screw cap test tube and dissolve in 10.0 mL of DMSO to provide a TCC concentration of 20.0 mg/mL.
2. Vortex mix to dissolve.

3.5.2 TCC in MeOH Standard Stock Solution at 0.2 mg/mL

1. Pipette 1.0 mL of 20.0 mg/mL TCC in DMSO standard stock solution into a 100 mL volumetric flask.
2. Make up to volume with MeOH.
3. Mix by inverting several times.

3.5.3 MeOH:DMSO 2:1 Solution

1. Measure 100 mL of MeOH using a graduated cylinder.
2. Measure 50 mL of DMSO using a graduated cylinder.
3. Mix the MeOH with the DMSO.

3.5.4 TCC in MeOH:DMSO 2:1 Stock Solution at 20.0 mg/mL

1. Weigh accurately 60 mg of TCC into a screw cap test tube and dissolve in 3.0 mL of MeOH:DMSO 2:1 to provide a TCC concentration of 20.0 mg/mL.
2. Vortex mix to dissolve.

3.5.5 TCC in MeOH:DMSO 2:1 Stock Solution at 0.2 mg/mL

1. Pipette 1.0 mL of 20.0 mg/mL TCC in MeoH:DMSO 2:1 stock solution into a 100 mL volumetric flask.
2. Make up to volume with MeOH:DMSO 2:1.
3. Mix by inverting several times.

3.5.6 TCC in 70% ACN Stock Solution at 0.2 mg/mL

1. Weigh accurately 2.0 mg of TCC into a screw cap test tube and dissolve completely in 7.0 mL of ACN.
2. Add 3.0 mL of ddH_2O.
3. Mix by inverting several times.

3.5.7 Standards and Quality Control Samples

1. A total of 3 validation batches are required. The standards are prepared in singles for two of the batches and the third batch in triplicates. All QCs are prepared in triplicate.
2. To individual LC vials and using a Hamilton syringe, add the appropriate volume of MeOH, as in **Table 1**.
3. Add 0.2 mg/mL TCC in MeOH standard stock solution with a syringe to the vials to obtain the concentrations in **Table 1**.
4. Cap vials and mix by inverting several times.

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3.5.8 Dosing Samples in DMSO

1. Using MeOH, make sample dilutions to the nominal concentration, as in **Table 2.**
2. Mix all solutions by inverting several times.
3. Transfer ~1 mL of each solution to LC vials and cap.

3.5.9 Specificity Samples

1. All specificity samples are prepared in triplicate.
2. For DMSO dosing vehicle alone*, transfer ~ 1 mL of MeOH:DMSO 2:1 to LC vials and cap.
3. For HPLC mobile phase alone, add 0.7 mL of ACN and 0.3 mL of ddH_2O into LC vials; cap and mix.
4. For TCC in mobile phase, add 0.5 mL of 0.2 mg/mL TCC in 70% ACN stock solution, 0.35 mL of ACN, and 0.15 mL of ddH_2O into LC vials; cap and mix.
5. For TCC in MeOH, add 0.5 mL of 0.2 mg/mL TCC in MeOH stock solution and 0.5 mL of MeOH into LC vials; cap and mix.
6. For TCC in in DMSO dosing vehicle, add 0.5 mL of 0.2 mg/mL TCC in MeOH:DMSO 2:1 stock solution and 0.5 mL of MeOH:DMSO 2:1 into LC vials; cap and mix.

*Note: 2:1 MeOH:DMSO was selected to represent the DMSO dosing vehicle as this ratio ofMeOH:DMSO would be the same as the smallest ratio ofMeOH:DMSO in the dosing samples after dilution.

3.5.10 System Suitability Samples

1. One system suitability sample was prepared for each batch, using the same procedure used for the nominal concentration standard at 0.10 mg/mL.
2. Perform n=6 injections.

3.5.11 Blank Samples

MeOH is used for blank samples.

3.5.12 HPLC Mobile Phase

Solvent A: use HPLC grade ddH_2O that has been filtered through a 0.2 µm filter
Solvent B: use HPLC grade ACN that has been filtered through a 0.2 µm filter

3.6 Analytical Instrumentation

- Hewlett Packard 1100 Series Model G1329A autosampler
- Hewlett Packard 1100 Series Model G1311A quaternary pump
- Hewlett Packard 1100 Series Model G1322A vacuum degasser
- Agilent 1100 Series Model G1316A thermostatted column compartment
- Agilent 1100 Series Model G1315B diode array detector (DAD)

Instrument Parameters:
Analytical Column: Lichrosphere 60 RP, 250 x 4.0 mm, 5.0 µm column
Supplier: Jones Chromatography
Mobile phase: Isocratic
30% ddH2O : 70% ACN
Injection volume: 5 µL
Run time: 8 min

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Refer to **Attachment 2** for more detailed instrument parameters.

3.7 Analytical Data Storage

- Chromatographic data were printed on hardcopy and stored according to BRI Standard Operating Procedures.
- Electronic copies of all raw data and resulting spreadsheets are stored in a read-only access section of the BRI server.
- Electronic data backup is scheduled at the end of each day via BRINET NT-Server on backup tape.
- All electronic data are archived onto durable media such as CD-ROM or Iomega ZIP disk. Electronic and hardcopy data pertaining to this Study will be archived at BRI 3650 Wesbrook Mall, Vancouver, B.C., Canada, V6S 2L2

3.8 Analytical Data Treatment

- Test sample inventory record: TS-BIO-2002-002-241, -284
- Laboratory notebook logsheets: LN-BIO-7, -8, -9, -25
- Analytical instrument log sheet record: AI-BIO-7; AI-BIO-8; AI-BIO-9; AI-BIO-25
- Analytical sequence log sheet record:
  AS-BIO-7; AS-BIO-8, 8b; AS-BIO-9; AS-BIO-25

4.0 STUDY PROTOCOL DEVIATIONS

According to the Protocol, one standard stock solution at 1 mg/mL of TCC in MeOH was to be prepared. However, since the dosing samples are prepared in DMSO then diluted with MeOH, it was determined that using a 1:100 DMSO in MeOH standard stock solution would be a better representation of the dosing sample matrix. Therefore, the standard stock solution for TCC was prepared as 20 mg/mL TCC in DMSO then diluted with MeOH to make a 0.2 mg/mL TCC standard stock solution. Using a similar matrix as the dosing samples also helped exclude any posible matrix interactions or effects. A 0.2 mg/mL stock solution concentration was selected over 1 mg/mL to simplify dilutions required for the standard curve.

Documentation for this Study Protocol deviation and changes may be viewed in **Attachment 3, CC-127**.

5.0 RESULTS AND DISCUSSION

5.1 TCC HPLC Method Performance Acceptance Criteria and Method Specifications

Method validation studies for TCC were carried out as proposed with reference to FDA and ICH regulatory guidelines and to an approved Study Protocol, presented in **Attachment 1**.

5.2 Assay Range

A total of three batches of TCC standard samples were prepared in methanol. Two batches were prepared with single calibration standards and one batch was prepared with calibration standards in triplicate. Calibration standards at 0.05, 0.06, 0.08, 0.10, and 0.12 mg/mL provide an assay range from 50 to 120% of the nominal TCC concentration of 0.10 mg/mL.

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5.3 Assay Accuracy

The within-batch assay accuracy of the HPLC/UV method at TCC concentrations of 0.08, 0.10, and 0.12 mg/mL were observed to be within ±1.1% relative error (%RE, n=3 replications) with reference to the expected concentrations as presented in **Table 3.** The between batch accuracy was within ±1.0% (n=3 batches), as presented in **Table 4.**

5.4 Assay Precision

The within-batch assay precision was evaluated at TCC concentrations of 0.08, 0.10, and 0.12 mg/mL and resulted in a coefficient of variation (CV) within 1.0% (n=3 replications), as seen in **Table 3.** The between-batch assay precision was within a CV of 0.9% (n=3 batches), as seen in **Table 4.**

5.5 Assay Linearity

Standard curves were obtained using 1/y weighted linear regression and observed to be linear based on back-interpolated concentrations with a r^2 greater than 0.99 calculated from 5-level calibration standard samples at TCC concentrations of 0.05, 0.06, 0.08, 0.10, and 0.12 mg/mL. Calibration curve data are presented in **Table 5.** A representative standard curve is presented in **Figure 1.** All back-interpolated concentrations were generally observed within ±0.8% deviation from the standard curve.

5.6 Limit of Quantitation

A theoretical limit of quantitation (LOQ) was determined based on the standard deviation of the response and the average slope of the calibration standard curves from all three batches. The LOQ is expressed as

LOQ = 10 x (SD of Response factor)/Response factor

where SD is the standard deviation. A theoretical assay LOQ was calculated to be 0.0045 mg/mL. Data are presented in **Table 6.**

5.7 Limit of Detection

A theoretical limit of detection (LOD) was determined based on the standard deviation of the response and the average slope of the calibration standard curves from all three batches. The LOQ is expressed as

LOD = 3.3 x (SD of Response factor)/Response factor

where SD is the standard deviation. A theoretical assay LOD was calculated to be 0.0015 mg/mL. Data are presented in **Table 6.**

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5.8 Assay Robustness

The TCC method assay accuracy and precision robustness was evaluated by performing the HPLC assay with two analysts independently preparing separate batches of samples and mobile phase. The observed between-batch accuracy and precision data were shown to be within the assay performance criteria, demonstrating acceptable assay robustness.

5.9 Assay Specificity

The specificity of the HPLC assay for TCC in DMSO dosing samples was qualitatively assessed for potential interference from the dosing vehicle. The following specificity samples were evaluated:

a) Dosing vehicle alone (DMSO)
b) HPLC mobile phase alone (70% ACN)
c) TCC in mobile phase (70% ACN)
d) TCC in methanol
e) TCC in dosing vehicle (DMSO)

Based on the evaluation of the HPLC chromatograms, the assay was observed to be specific for TCC without interference. Representative chromatograms are presented in **Figures 2** to **6**.

5.10 TCC Stock Solution Stability

TCC stock solutions were prepared at the start of the study and kept refrigerated and protected from light during the course of the validation experiments. Stability profiling and stability studies were not required for this study.

5.11 HPLC System Suitability

The chromatographic peak area response, peak tailing factor, and column efficiency of the HPLC assay were determined using a TCC system suitability sample at the nominal concentration with n = 6 replicates. All replicates were found to meet the acceptance criteria as presented in **Table 7**.

5.12 HPLC Assay of TCC in DMSO Dosing Samples

A total of nine DMSO dosing samples were assayed in triplicate for BioReliance Study# AA55XE.331.BTL (BRI samples BIO-2002-002-3730 to BIO-2002-001-3738). The samples consisted of 8 different concentrations of TCC in DMSO, 3.13, 6.25, 12.5, 25.0, 50.0, 100.0, 150.0, and 200.0 mg/mL, and blank dosing sample. The resultant % label claim values for these samples were determined to range from 96.8% to 105.6%. A summary of the TCC concentrations in the DMSO dosing samples is presented in **Table 8**.

6.0 CONCLUSION

An HPLC method was slightly modified from a previously developed method for the determination of TCC in DMSO dosing samples in support of toxicological investigations. The method for TCC has been validated to provide analytical support of toxicological investigations.

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7.0 TABLES

(Pages 19 to 26)

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Table 1: Standard and Quality Control Sample Dilutions

Sample ID	Nominal Standard Conc (mg/mL)	Vol of 0.2 mg/mL TCC in MeOH Stock Solution (mL)	Vol MeOH (mL)	Final Vol (mL)
STD-0	0	0	1.0	1.0
STD-0.05	0.05	0.25	0.75	1.0
STD-0.06	0.06	0.3	0.7	1.0
STD-0.08	0.08	0.4	0.6	1.0
STD-0.10	0.10	0.5	0.5	1.0
STD-0.12	0.12	0.6	0.4	1.0
	Nominal QC Conc (mg/mL)			
QC-0.08-1	0.08	0.4	0.6	1.0
QC-0.08-2	0.08	0.4	0.6	1.0
QC-0.08-3	0.08	0.4	0.6	1.0
QC-0.10-1	0.10	0.5	0.5	1.0
QC-0.10-2	0.10	0.5	0.5	1.0
QC-0.10-3	0.10	0.5	0.5	1.0
QC-0.12-1	0.12	0.6	0.4	1.0
QC-0.12-2	0.12	0.6	0.4	1.0
QC-0.12-3	0.12	0.6	0.4	1.0

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Table 2: TCC in DMSO Dosing Sample Dilutions for BioReliance Study# AA55XE.331.BTL

			Dilution 1			Dilution 2		
Label Claim (mg/mL)	Theoretical Conc after dilutions (mg/mL)	Dilution Factor	Volume sample used (mL)	Volume MeOH (mL)	Total Volume (mL)	Volume diluted sample used (mL)	Volume MeOH (mL)	Total Volume (mL)
0.0	0.000	101	0.100	10.0	10.100	-	-	-
3.13	0.061	51	0.200	10.0	10.200	-	-	-
6.25	0.062	101	0.100	10.0	10.100	-	-	-
12.5	0.099	126	0.100	10.0	10.100	0.800	0.200	1.00
25.0	0.099	253	0.100	10.0	10.100	0.400	0.600	1.00
50.0	0.099	505	0.100	10.0	10.100	0.200	0.800	1.00
100.0	0.099	1010	0.100	10.0	10.100	0.100	0.900	1.00
150.0	0.104	1443	0.100	10.0	10.100	0.070	0.930	1.00
200.0	0.099	2020	0.100	10.0	10.100	0.050	0.950	1.00

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Table 3: Within-Batch Precision and Accuracy of TCC Assay Method

Sample ID	Batch 1			Batch 2			Batch 3		
	Datafile (Y:\Bio\BIO42902)	Nominal Conc of TCC (mg/mL)	Assayed Conc (mg/mL)	Datafile (Y:\Bio\BIO43002)	Nominal Conc of TCC (mg/mL)	Assayed Conc (mg/mL)	Datafile (Y:\Bio\BI050102)	Nominal Conc of TCC (mg/mL)	Assayed Conc (mg/mL)
QC-0.08 -1	BIO00016	0.08	0.08	BIO00023	0.08	0.08	BIO00016	0.08	0.08
QC-0.08 -2	BIO00020	0.08	0.08	BIO00038	0.08	0.08	BIO00020	0.08	0.08
QC-0.08 -3	BIO00024	0.08	0.08	BIO00054	0.08	0.08	BIO00024	0.08	0.08
		Mean	**0.08**		**Mean**	**0.08**		**Mean**	**0.08**
		%CV	**0.6**		**%CV**	**0.7**		**%CV**	**0.6**
		%RE	**-0.3**		**%RE**	**0.0**		**%RE**	**-0.7**
QC-0.10 -1	BIO00017	0.10	0.10	BIO00024	0.10	0.10	BIO00017	0.10	0.10
QC-0.10 -2	BIO00021	0.10	0.10	BIO00039	0.10	0.10	BIO00021	0.10	0.10
QC-0.10 -3	BIO00025	0.10	0.10	BIO00055	0.10	0.10	BIO00025	0.10	0.10
		Mean	**0.10**		**Mean**	**0.10**		**Mean**	**0.10**
		%CV	**0.2**		**%CV**	**1.0**		**%CV**	**0.6**
		%RE	**-0.3**		**%RE**	**-0.1**		**%RE**	**0.2**
QC-0.12 -1	BIO00018	0.12	0.12	BIO00025	0.12	0.12	BIO00018	0.12	0.12
QC-0.12 -2	BIO00022	0.12	0.12	BIO00040	0.12	0.12	BIO00022	0.12	0.12
QC-0.12 -3	BIO00026	0.12	0.12	BIO00056	0.12	0.12	BIO00026	0.12	0.12
		Mean	**0.12**		**Mean**	**0.12**		**Mean**	**0.12**
		%CV	**0.7**		**%CV**	**0.2**		**%CV**	**0.2**
		%RE	**0.2**		**%RE**	**0.6**		**%RE**	**1.1**

*Note: Batch 1 and Batch 2 prepared by Analyst 1 and Batch 3 prepared by Analyst 2

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Table 4: Between-Batch Precision and Accuracy of TCC Assay Method

Sample ID	Batch 1				Batch 2				Batch 3						
	Datafile (Y:\Bio\Bio\042902)	Nominal Conc of TCC (mg/mL)	Assayed Conc (mg/mL)	% Recovery	Datafile (Y:\Bio\Bio\043002)	Nominal Conc of TCC (mg/mL)	Assayed Conc (mg/mL)	% Recovery	Datafile (Y:\Bio\Bio\050102)	Nominal Conc of TCC (mg/mL)	Assayed Conc (mg/mL)	% Recovery	Mean TCC Conc (mg/mL)	%CV (Based on Recovery)	%RE
QC-0.08 -1	BIO00016	0.08	0.08	99.2	BIO00023	0.08	0.08	100.1	BIO00016	0.08	0.08	98.8	0.08	0.7	-0.7
QC-0.08 -2	BIO00020	0.08	0.08	99.7	BIO00038	0.08	0.08	100.7	BIO00020	0.08	0.08	99.2	0.08	0.8	-0.1
QC-0.08 -3	BIO00024	0.08	0.08	100.3	BIO00054	0.08	0.08	99.2	BIO00024	0.08	0.08	100.0	0.08	0.5	-0.2
QC-0.10 -1	BIO00017	0.10	0.10	99.6	BIO00024	0.10	0.10	101.6	BIO00017	0.10	0.10	99.6	0.10	0.8	0.0
QC-0.10 -2	BIO00021	0.10	0.10	99.6	BIO00039	0.10	0.10	99.8	BIO00021	0.10	0.10	100.3	0.10	0.4	-0.1
QC-0.10 -3	BIO00025	0.10	0.10	99.9	BIO00055	0.10	0.10	98.9	BIO00025	0.10	0.10	100.6	0.10	0.9	-0.2
QC-0.12 -1	BIO00018	0.12	0.12	99.9	BIO00025	0.12	0.12	100.3	BIO00018	0.12	0.12	101.3	0.12	0.7	0.5
QC-0.12 -2	BIO00022	0.12	0.12	101.0	BIO00040	0.12	0.12	100.7	BIO00022	0.12	0.12	101.2	0.12	0.2	1.0
QC-0.12 -3	BIO00026	0.12	0.12	99.7	BIO00056	0.12	0.12	100.6	BIO00026	0.12	0.12	100.8	0.12	0.6	0.4

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Table 5: Calibration Curve Accuracy for TCC Assay Method

	Batch 1 (n=1)				Batch 2 (n=3)				Batch 3 (n=1)			
Sample ID	Datafile (Y:\Bio\BIO42902)	Nominal Conc of TCC (mg/mL)	Assayed Conc (mg/mL)	% RE	Datafile (Y:\Bio\BIO43002 or Y:\Bio\BIO50102 for STD-x-3)	Nominal Conc of TCC (mg/mL)	Assayed Conc (mg/mL)	% RE	Datafile (Y:\Bio\BIO50102)	Nominal Conc of TCC (mg/mL)	Assayed Conc (mg/mL)	% RE
	r^2 = 0.99992				r^2 = 0.99980				r^2 = 0.99998			
STD-0.05-1	BIO00010	0.05	0.05	-0.3	BIO00010	0.05	0.05	-0.5	BIO00010	0.05	0.05	-0.2
STD-0.05-2					BIO00017	0.05	0.05	0.1				
STD-0.05-3					BIO00003	0.05	0.05	0.0				
STD-0.06-1	BIO00011	0.06	0.06	0.1	BIO00011	0.06	0.06	0.8	BIO00011	0.06	0.06	0.3
STD-0.06-2					BIO00018	0.06	0.06	-0.7				
STD-0.06-3					BIO00004	0.06	0.06	-0.2				
STD-0.08-1	BIO00012	0.08	0.08	0.5	BIO00012	0.08	0.08	0.1	BIO00012	0.08	0.08	-0.1
STD-0.08-2					BIO00019	0.08	0.08	0.7				
STD-0.08-3					BIO00005	0.08	0.08	0.4				
STD-0.10-1	BIO00013	0.10	0.10	-0.3	BIO00013	0.10	0.10	-0.1	BIO00013	0.10	0.10	-0.1
STD-0.10-2					BIO00020	0.10	0.10	-0.2				
STD-0.10-3					BIO00006	0.10	0.10	-0.3				
STD-0.12-1	BIO00014	0.12	0.12	0.0	BIO00014	0.12	0.12	0.6	BIO00014	0.12	0.12	0.0
STD-0.12-2					BIO00021	0.12	0.12	-0.3				
STD-0.12-3					BIO00007	0.12	0.12	-0.4				

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Table 6: Limit of Quantitation and Limit of Detection

			LOQ (mg/mL)	LOD (mg/mL)
batch-1	response=	13.04	**0.0045**	**0.0015**
batch-2	response=	18.24		
batch-3	response=	32.94		
	average response=	**21.41**		
	stdev response=	**10.3**		
batch-1	slope=	22754		
batch-2	slope=	22585		
batch-3	slope=	22832		
	average slope=	**22724**		
	stdev slope=	**126.4**		

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Table 7: System Suitability Data for TCC Assay Method

Datafile	Sample ID	TCC Peak Area Response	USP Peak Tailing ($T=W_{0.05}/2f$)	Column Efficency (Theoretical plates, $5.54(t/W_{h/2})^2$
Assay Specifications		**RSD < 5.0%**	**run and report**	**run and report**
Batch 1 (Y:\Bio\BIO42902)				
BIO00002	ssuit (0.10)-1	2298.8	0.84	12317
BIO00003	ssuit (0.10)-2	2300.0	0.84	12321
BIO00004	ssuit (0.10)-3	2297.4	0.84	12318
BIO00005	ssuit (0.10)-4	2316.6	0.84	11952
BIO00006	ssuit (0.10)-5	2291.2	0.84	12321
BIO00007	ssuit (0.10)-6	2289.6	0.84	11608
	Mean	**2299**	**0.84**	**12140**
	stdev	**9.6**	**0.0**	**299**
	%CV	**0.4**	**0.0**	**2.5**
Batch 2 (Y:\Bio\BIO43002)				
BIO00002	Sys Suit -1	2276.9	0.88	13487
BIO00003	Sys Suit -2	2284.8	0.88	13470
BIO00004	Sys Suit -3	2278.7	0.88	12653
BIO00005	Sys Suit -4	2275.5	0.88	13530
BIO00006	Sys Suit -5	2273.5	0.88	13424
BIO00007	Sys Suit -6	2315.1	0.88	12656
	Mean	**2284**	**0.9**	**13203**
	stdev	**15.7**	**0.0**	**426**
	%CV	**0.7**	**0.0**	**3.2**
Batch 3 (Y:\Bio\BI050102)				
BIO00002	ssuit (0.10)-1	2313.7	0.86	12325
BIO00003	ssuit (0.10)-2	2327.4	0.86	12378
BIO00004	ssuit (0.10)-3	2315.2	0.86	12384
BIO00005	ssuit (0.10)-4	2338.8	0.86	12412
BIO00006	ssuit (0.10)-5	2318.4	0.86	12383
BIO00007	ssuit (0.10)-6	2348.0	0.86	12391
	Mean	**2327**	**0.9**	**12379**
	stdev	**13.9**	**0.0**	**29.0**
	%CV	**0.6**	**0.0**	**0.2**

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Table 8: Determination of TCC in DMSO Dosing Samples for BioReliance Study# AA55XE.331.BTL

Datafile (Y:\Bio\... \Bio61002)	BRI Sample ID	Label Claim (mg/mL)	calculated TCC conc (mg/mL)	Mean	% CV	Average % Label Claim
BIO00020	BIO-2002-002-3730-1	0.0	0.0			
BIO00021	BIO-2002-002-3730-2	0.0	0.0			
BIO00022	BIO-2002-002-3730-3	0.0	0.0	**0.0**	**n/ap**	**n/ap**
BIO00023	BIO-2002-002-3731-1	3.13	3.28			
BIO00024	BIO-2002-002-3731-2	3.13	3.28			
BIO00025	BIO-2002-002-3731-3	3.13	3.31	**3.29**	**0.6**	**105.1**
BIO00026	BIO-2002-002-3732-1	6.25	6.62			
BIO00027	BIO-2002-002-3732-2	6.25	6.59			
BIO00028	BIO-2002-002-3732-3	6.25	6.59	**6.60**	**0.2**	**105.6**
BIO00029	BIO-2002-002-3733-1	12.5	12.89			
BIO00030	BIO-2002-002-3733-2	12.5	12.83			
BIO00031	BIO-2002-002-3733-3	12.5	12.90	**12.87**	**0.3**	**103.0**
BIO00032	BIO-2002-002-3734-1	25.0	25.14			
BIO00033	BIO-2002-002-3734-2	25.0	25.19			
BIO00034	BIO-2002-002-3734-3	25.0	25.14	**25.16**	**0.1**	**100.6**
BIO00040	BIO-2002-002-3735-1	50.0	49.30			
BIO00041	BIO-2002-002-3735-2	50.0	49.66			
BIO00042	BIO-2002-002-3735-3	50.0	49.13	**49.37**	**0.5**	**98.7**
BIO00043	BIO-2002-002-3736-1	100.0	97.19			
BIO00044	BIO-2002-002-3736-2	100.0	96.60			
BIO00045	BIO-2002-002-3736-3	100.0	96.82	**96.87**	**0.3**	**96.9**
BIO00046	BIO-2002-002-3737-1	150.0	145.6			
BIO00047	BIO-2002-002-3737-2	150.0	145.2			
BIO00048	BIO-2002-002-3737-3	150.0	144.8	**145.2**	**0.3**	**96.8**
BIO00050	BIO-2002-002-3738-1	200.0	197.9			
BIO00051	BIO-2002-002-3738-2	200.0	196.7			
BIO00052	BIO-2002-002-3738-3	200.0	197.2	**197.2**	**0.3**	**98.6**

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8.0 FIGURES

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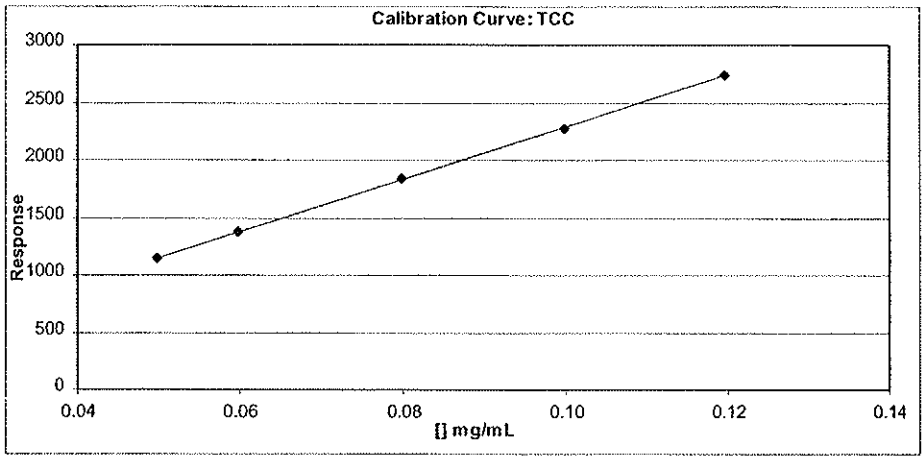

Figure 1: Representative TCC Standard Curve

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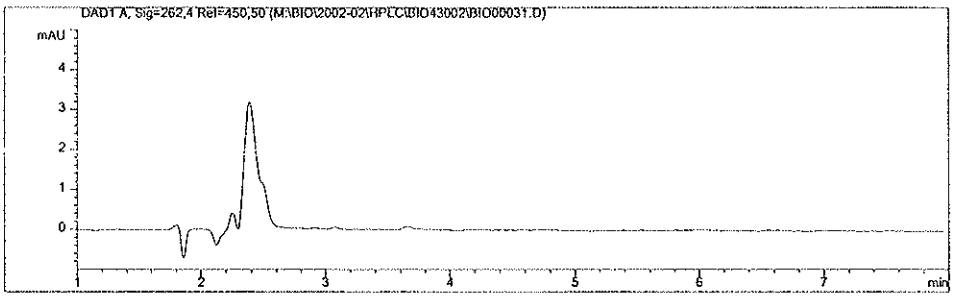

Figure 2: Representative HPLC Chromatogram of DMSO Sample Dosing Vehicle

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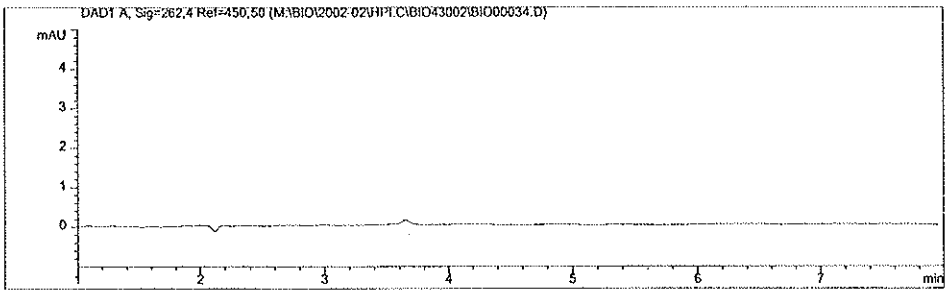

Figure 3: Representative HPLC Chromatogram of Mobile Phase (70% ACN)

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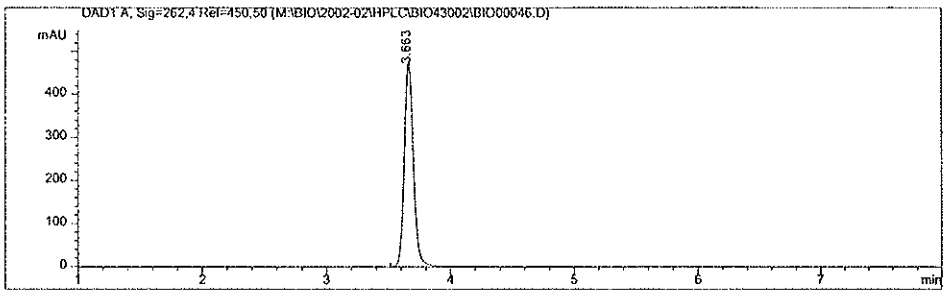

Figure 4: Representative HPLC Chromatogram of TCC in Mobile Phase (70% ACN)

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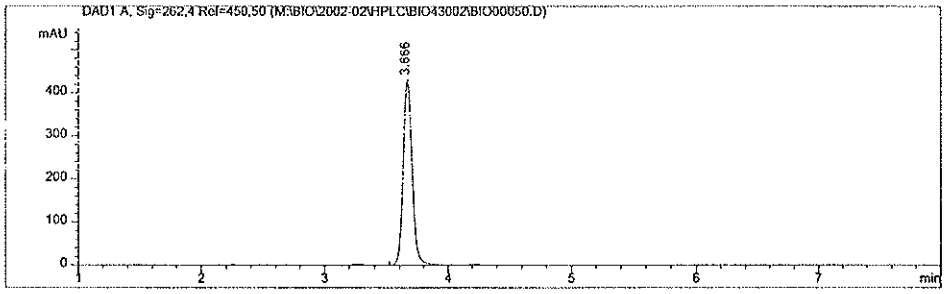

Figure 5: Representative HPLC Chromatogram of TCC in Methanol

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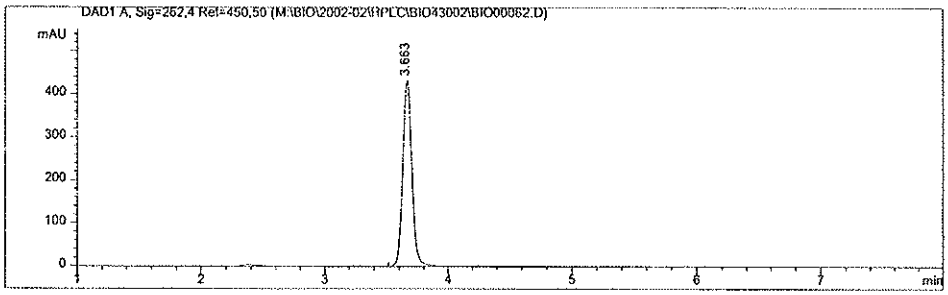

Figure 6: Representative HPLC Chromatogram of TCC in DMSO Sample Dosing Vehicle

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9.0 ATTACHMENTS

(Pages 35 to 48)

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Attachment 1: Study Protocol

(Pages 36 to 42)

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May 9, 2002

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD USA 20850

Study Title:	**Validation of a HPLC Method for Quantitation of Trichlorcarbanilide (TCC) in DMSO**
BRI Study No:	**BIO-2002-002**
Sponsor Representative:	**Ramadevi Gudi, Ph.D.** **BioReliance**
Sponsor Rep. Study No:	**AA55XE.331.BTL**
BRI Study Protocol No:	**Protocol-BIO-2002-002 (Version 2)**

Study Protocol Approval:

Alvaro DeCarvalho — 14 MAY 2002
Alvaro DeCarvalho, Ph.D.
Sponsor — Date

Ramadevi Gudi — 13 May 2002
Ramadevi Gudi, Ph.D.
Study Director
BioReliance Corporation — Date

14 May, 2002
David Kwok, Ph.D.
Analytical Investigator
BRI Biopharmaceutical Research Inc. — Date

14 May 2002
Jessica Yeung, B.A., B.Sc.
Manager, Quality Assurance
BRI Biopharmaceutical Research Inc. — Date

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Study Protocol

Sponsor Rep. Study No:	AA55XE.331.BTL
BRI Study Protocol No:	Protocol-BIO-2002-002 (Version 1)
Study Title:	Validation of a HPLC Method for Quantitation of Trichlorcarbanilide (TCC) in DMSO
Objective:	To perform validation of a HPLC assay method for TCC in DMSO dosing samples in support of toxicology studies. To perform determination of TCC in dosing samples using the validated method for toxicology investigations.
Analytical Facilities:	BRI Biopharmaceutical Research Inc. 3650 Wesbrook Mall, Vancouver, BC, V6S 2L2 Phone: (604)-432-9237 Fax: (604)-432-9239
Analytical Investigator:	David Kwok, Ph.D. BRI Biopharmaceutical Research Inc. 3650 Wesbrook Mall Vancouver, BC, V6S 2L2 Phone: (604)-432-9237 (ext 225) Fax: (604)-432-9239 Email: dkwok@bripharm.com
Study Director:	Ramadevi Gudi, Ph.D. BioReliance Corporation 9630 Medical Center Drive Rockville, MD USA 20850 Phone: (301)-610-2169 Fax: (301)-738-2362

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1. BACKGROUND

Toxicological investigations of TCC are being conducted by BioReliance Corporation. During the course of toxicology studies, dosing samples are expected to be shipped to BRI for analytical determination of TCC using a validated HPLC method. A HPLC method for TCC was previously developed by the Study Sponsor and method performance data provided by the Sponsor's representative suggested that the method is sufficiently developed for the conduct of a method validation study.

2. OBJECTIVE

2.1 To perform validation of a HPLC assay method for TCC in DMSO dosing samples in support of toxicology studies.

2.2 To perform determination of TCC in dosing samples using the validated method for toxicology investigations.

3. HPLC METHOD VALIDATION STUDIES

The following FDA and ICH guidelines have been referenced during the preparation of this protocol:

i. US FDA Analytical Procedures and Methods Validation, Chemistry, Manufacturing, and Controls Documentation (2000)
ii. ICH Q2B Validation of Analytical Procedures: Methodology (1996)
iii. FDA CDER Reviewer Guidance: Validation of Chromatographic Methods (1994)
iv. Note For Guidance on Validation of Analytical Procedures (ICH publication CPMP/ICH/281/95)

3.1 Validation of a HPLC Method for TCC in DMSO

TCC in DMSO quantitation method will be validated based on the analytical method numbered 2011-0553202-99E. Modification might be necessary due to the matrix difference between method numbered 2011-0553202-99E and the method to be validated. Equivalent HPLC column and instrument may be used based on the information given in the analytical method numbered 2011-0553202-99E.

A method for quantitation of TCC in DMSO is validated based on the following parameters. Please see **Attachment 1** for the draft analytical method provided by the Sponsor. As per the Sponsor's instruction, DMSO will be used as the dosing solution.

Method Parameters	Method Performance Acceptance Criteria
Validation Samples	All calibration standards and QC samples are prepared in DMSO dosing sample matrix.
System Suitability Injection repeatability Peak Symmetry Theoretical plates	i. Injection precision: RSD for peak area response ≤ 5.0% from 6 injections of one standard sample. ii. Column performance: theoretical plates (n) Run & Report. iii. Peak tailing factor at 5% peak height Run & Report.

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Range	Expected TCC dosing sample concentrations will be assayed over a calibration range from minimum of 80 to 120% of a nominal target concentration of 0.10 mg/ml. Calibration samples will be prepared in triplicate for batch-1, single samples for batch-2 and batch-3.
Linearity	Back-interpolated standard accuracy of relative error (RE) within +/- 15% of expected concentrations. Coefficient of determination (r^2) exceeding 0.99. Calibration samples at 0, 0.05, 0.06, 0.08, 0.10 and 0.12 mg/ml will be prepare in triplicate for batch-1, single samples for batch-2 and batch-3.
Accuracy	i. A target concentration 0.10 mg/ml will be established within the quantitation range of 0.08 to 0.12 mg/ml. ii. Within-batch (n=3 replications) and between-batch (n=3 batches) accuracy of the assay will be assessed for dosing samples at each of 0.08, 0.10 and 0.12 mg/ml and reported as percent relative error of the expected value (%RE) within an acceptance criteria of ±5% theoretical concentrations. QC samples in DMSO will be diluted to 0.08, 0.10 and 0.12 mg/ml using methanol.
Precision	Assay repeatability within-batch (n=3 replications) and between-batch (n=3 batches) will be assessed at each of 0.08, 0.10 and 0.12 mg/ml within an acceptance criteria of 5%CV.
Specificity	Specificity of the HPLC assay method for TCC will be assessed in n=3 replicate samples qualitatively. No interference should be observed in individual chromatograph. i. Dosing vehicle alone (DMSO) ii. HPLC mobile-phase alone iii. TCC in mobile-phase iv. TCC in methanol v. TCC in dosing vehicles (DMSO)
Stability	TCC stock solution will be prepared at the start of the study and used within 5 days during the course of the validation experiments. All stock solutions will be kept refrigerated and protected from light. A separated stability study on TCC in methanol stock solutions will not be required.
Limit of Quantitation	A theoretical limit of quantitation will be estimated based on the standard deviation of the response and slope of the calibration standard curve as DL=10 (SD of Response factor)/Response factor.

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Limit of Detection	A theoretical limit of detection will be estimated based on the standard deviation of the response and slope of the calibration standard curve as DL=3.3 (SD of Response factor)/Response factor.
Assay Robustness	Perform assay of QC samples at three concentrations using the following criteria: i. Using two separate analysts. ii. Using mobile-phase prepared by two separate analysts.

3.2 Sample Preparation Procedures for Dosing Samples

HPLC assay of TCC will follow the procedures below:

3.2.1 Sample Diluent

Methanol will be used for dilutions of dosing samples to achieve an expected concentration of 0.10 mg/ml as the nominal target assay concentration.

3.2.2 Blank Sample

Methanol will be used as blank samples.

3.2.3 Validation Calibration Standards and QC samples

Prepare a TCC reference standard stock solution accurately at 1 mg/ml by quantitatively transfer 50 mg to a 50ml volumetric flask. Sonicate reference standard stock solution for 5 min before use. Protect from light. Perform serial dilutions to provide calibration standards at 0.05, 0.06, 0.08, 0.10 and 0.12 mg/ml.

3.2.4 Validation Quality Control Samples

Prepare a TCC reference standard stock solution accurately at 1 mg/ml (quantitatively transfer 50 mg to 50 ml volumetric flask) and label for 'QC samples'. Sonicate reference standard stock solution for 5 min before use. Protect from light. Perform serial dilutions to provide QC samples at 0.08, 0.10 and 0.12 mg/ml using methanol.

QC samples in DMSO dosing sample matrix will be diluted to 0.08, 0.10 and 0.12 mg/ml using methanol and assayed along with QC samples similarly prepared in methanol for evaluation of potential matrix effect in DMSO.

3.2.5 Study Samples

Using an adjustable pipette, based on the label claim of each dosing sample, accurately transfer an aliquot of each dosing sample to a suitable volume Class A volumetric flask. Dilute the sample to mark with methanol and thoroughly mix to produce assay samples at a nominal target concentration of 0.10 mg/ml.

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Following the method validation study, during routine quantitation of DMSO dosing samples, assay of TCC will be performed following dilution of samples to a target concentration of 0.1 mg/ml. A minimum of 5-levels of calibration standards (n=1) and 1 level of QC (n=3) will be prepared along with the DMSO dosing samples. Homogeneity and stability study of the test compound in DMSO is not required for this study.

4. STUDY MATERIALS REQUIRED

A 500 mg supply of TCC reference standard with Certificate of Analysis is required from BioReliance prior to the commencement of the HPLC method validation study.

If a Certificate of Analysis for TCC is not available, the purity and stability of TCC will be subjected to chemical purity profiling before and after the conduct of the validation experiments. The purity profiling will be performed using LC/MS with the LC conditions listed in the draft analytical method (**Attachment 1**). Relative % of each observed minor and major components in the TCC reference standard will be determined and a relative % purity of the major TCC component calculated. A separate costing proposal will be provided to the Sponsor upon request.

5. GLP COMPLIANCE

All analytical determinations carried out at BRI will be conducted under a Good Laboratory Practice (GLP) environment in compliance with the requirements of the US FDA and the Canadian HPFB operating guidelines.

BRI QA audit reports will be prepared for the protocol review, in-process audits, and for the draft and final versions of the report. In-process audits will be conducted for each validation batch and after analysis of the study samples. Copies of all audit reports will be submitted to the Study Director for review and approval.

Any changes or deviations from the protocol will be documented according to BRI standard operating procedures and submitted to the Study Director for review and approval. The Sponsor will be also notified of any changes or deviations from the protocol.

6. MATERIALS AND EQUIPMENT

An Agilent Model 1100 HPLC system equipped with a UV diode-array detector will be used for this study. A detail description of all materials and supplies utilized during the course of this study will be recorded in the Study Report.

7. ANALYTICAL AND STATISTICAL DETERMINATION

HPLC data analysis will be performed using ChemStation for LC 3D© Rev A.08.0.[847] and Microsoft Excel 97.

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8. REPORTING

Upon completion of the study, a draft analytical study report will be provided to the Study Sponsor within 2-weeks following completion of the study. Subject to the review and comments from the Sponsor, a final study report will be prepared and released by the Quality Assurance Manager of BRI within one week from receiving a reply from the Study Sponsor.

9. DATA STORAGE

All electronic and hard copies of the data generated at BRI will be maintained and archived at 3650 Wesbrook Mall (Vancouver) according to BRI standard operating procedures unless otherwise requested by the Sponsor.

10. ATTACHMENT

END of STUDY PROTOCOL

Attachment 2: HPLC Method Parameters

(Pages 44 to 46)

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Method Information

Method Change History

Operator	Date	Change Information
Cherly	4/29/02 2:32:44 PM	
Cherly	4/29/02 2:39:53 PM	
Lori P.	4/29/02 3:51:39 PM	
Lori P.	4/29/02 4:10:15 PM	
Lori P.	5/1/02 4:06:23 PM	
Lori P.	6/10/02 9:58:22 AM	
Lori P.	6/10/02 11:35:41 AM	
Lori P.	6/10/02 11:41:28 AM	

Run Time Checklist

Pre-Run Cmd/Macro:	off
Data Acquisition:	on
Standard Data Analysis:	off
Customized Data Analysis:	off
Save GLP Data:	off
Post-Run Cmd/Macro:	off
Save Method with Data:	off

000045

1100 Quaternary Pump 1

Control

Column Flow	:	1.000 ml/min
Stoptime	:	8.00 min
Posttime	:	Off

Solvents

Solvent A	:	30.0 % (H2O)
Solvent B	:	70.0 % (ACN)
Solvent C	:	Off
Solvent D	:	Off

PressureLimits

Minimum Pressure	:	0 bar
Maximum Pressure	:	350 bar

Auxiliary

Maximal Flow Ramp	:	100.00 ml/min^2
Primary Channel	:	Auto
Compressibility	:	100*10^-6/bar
Minimal Stroke	:	Auto

Store Parameters

Store Ratio A	:	Yes
Store Ratio B	:	Yes
Store Ratio C	:	Yes
Store Ratio D	:	Yes
Store Flow	:	Yes
Store Pressure	:	Yes

Timetable

Time	Solv.B	Solv.C	Solv.D	Flow	Pressure
0.00	70.0	0.0	0.0	1.000	350
8.00	70.0	0.0	0.0	1.000	350

Agilent 1100 Diode Array Detector 1

Signals

Signal	Store	Signal,Bw	Reference,Bw	[nm]
A:	Yes	262 4	450 50	
B:	No	254 16	360 100	
C:	No	210 8	360 100	
D:	No	230 16	360 100	
E:	No	280 16	360 100	

Spectrum

Store Spectra	:	None

Time

Stoptime	:	9.00 min
Posttime	:	Off

Required Lamps

UV lamp required	:	Yes
Vis lamp required	:	Yes

Autobalance

Prerun balancing	:	Yes

```
        Postrun balancing       :       No
        Margin for negative Absorbance: 100 mAU

    Peakwidth                   :        >  0.1 min
    Slit                        :             4 nm

    Analog Outputs
        Zero offset ana. out. 1:              5 %
        Zero offset ana. out. 2:              5 %
        Attenuation ana. out. 1:           1000 mAU
        Attenuation ana. out. 2:           1000 mAU

    Timetable is empty


    ================================================================================
                          Agilent 1100 Autosampler 1
    ================================================================================

    Injection
        Injection Mode          :    Standard
        Injector volume         :          5.0 µl

    Auxiliary
        Store temperature       :      No
        Drawspeed               :          200 µl/min
        Ejectspeed              :          200 µl/min
        Draw position           :          1.0 mm

    Time
        Stoptime                :      As Pump
        Posttime                :      Off


    ================================================================================
                        Agilent 1100 Column Thermostat 1
    ================================================================================

    Temperature settings
        Left temperature        :      40.0°C
        Right temperature       :      Same as left
        Enable analysis         :      When Temp. is within setpoint +/-  0.8°C
        Store left temperature  :      Yes
        Store right temperature:       No

    Time
        Stoptime                :      As pump
        Posttime                :      Off

    Column Switching Valve      :      Column 1

    Timetable is empty
```

Attachment 3: Study Protocol Deviations and Change Control Form

(Page 48)

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(BRI) Biopharmaceutical Research Inc.

Form Number: CC-127

SOP Form-QA-001-2
Change Control / Study Deviation Form

	Change Control / Deviations
Document	☐
Test Method	☐
Reports	☐
Protocol / Proposals	X

Title: Validation of a HPLC Method for Quantitation of Triclorcarbanilide (TCC) in DMSO

Sponsor (If applicable): Soap & Detergent Association Study #: BIO-2002-002

Contact: Dr. Ramadevi Gudi Notified on: 06/10/02 by: Phone☐ Writing ☐ Other ☑ fax

Description and Justification for Change or Deviation:

According to the Protocol, one standard stock solution at 1 mg/mL TCC in MeOH was to be prepared. However, since the dosing samples are prepared in DMSO then diluted with MeOH, it was determined that using a 1:100 DMSO in MeOH standard stock solution would be a better representation of the dosing sample matrix. Therefore, the standard stock solution for TCC was prepared as 20 mg/mL TCC in DMSO then diluted with MeOH to make a 0.2 mg/mL TCC standard stock solution. Using a similar matrix as the dosing samples also helped exclude any posible matrix interactions or effects. A 0.2 mg/mL stock solution concentration was selected over 1 mg/mL to simplify dilutions required for the standard curve.

Completed by: Lori Ptachyk LP Date: 06/07/02

Study Director Signature: Ramadevi Gudi Date: 6/10/02

Analytical Investigator Sponsor Signature: Date: 06/07/02

QA Officer verification: Date: 06/07/02

Further Affected Documents	Section	Date updated

Version 1.5 December 21, 2000