Chemical Name: Tetrabromobisphenol A
Trade Name(s): BA-59P
CAS No: 79-94-7
Lab Study ID No: AA47PV.341.BTL (In Vitro Mammalian Chromosome Aberration Test using HPBL)

FINAL REPORT ACTION ITEM CHECK-OFF LIST

☒ Reviewed for possible:
- ☐ FIFRA 6 (a) (2) and/or
- ☒ TSCA Section 8 (e) reporting

☐ Copy of FIFRA 6 (a) (2) and/or TSCA Section 8 (e) letter to the following Agency(ies), if applicable:
- ☐ EPA-FIFRA
- ☐ EPA-TSCA
- ☐ California [FIFRA 6 (a) (2)s]
- ☐ Other States [FIFRA 6 (a) (2)s]: _____

☐ Confidentiality Statement page addressed, signed, and dated in FIFRA reports

☐ GLP Compliance page signed and dated in FIFRA reports

☐ Flagging Statement page addressed, signed and dated in FIFRA reports

☒ Copy of report submitted to the Agency(ies) in conjunction and/or support of one or more of the following:
- ☐ TSCA Consent Order/Agreement
- ☐ FIFRA Registration or Re-registration
- ☐ California Registration
- ☐ EU Notification
- ☐ Japanese MITI Notification
- ☐ Japanese MAFF Notification
- ☐ Canadian (DSL) Notification
- ☐ FIFRA 6 (a) (2) Submission
- ☐ TSCA 8 (e) Submission
- ☐ TSCA 8 (d) Data-Call-In
- ☐ PMN Submission
- ☒ Other: EU Risk Assessment (Sara Davenport @ UK)

☒ All information regarding the chemical and the study report entered into the IUCLID Toxicity Data Base

☒ Study Report reviewed for MSDS information — Copied to product file for addition to MSDS @ next update. JJD 10/23/01

☒ Copy of Cover & Summary report pages to Business Unit MSDS information center (domestic and international) — Dieter Drakmann

☐ Active Study file merged with final report in Regulatory Affairs file room

FINAL REPORT

Study Title

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST

Test Article

Tetrabromobisphenol A (TBBPA)

Authors

Ramadevi Gudi, Ph.D.
Caren M. Brown, M.S.

Study Completion Date

October 15, 2001

Performing Laboratory

BioReliance
9630 Medical Center Drive
Rockville, Maryland 20850

Laboratory Study Number

AA47PV.341.BTL

Sponsor

American Chemistry Council
Brominated Flame Retardant Industry Panel (BFRIP)
1300 Wilson Boulevard
Arlington, VA 22209

STATEMENT OF COMPLIANCE

Study AA47PV.341.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 identity, strength, purity and composition or other characteristics to define the control articles have not been determined by the testing facility. The control articles have been characterized as per the Certificates of Analysis on file with the testing facility.

The stability of the test and control articles has not been determined by the testing facility.

Analyses to determine the uniformity (as applicable), or concentration of the test or control mixtures were not performed by the testing facility. The Sponsor has indicated that they have not performed these analyses on the test article mixtures.

The stability of the test and control articles in the test and control mixtures, respectively, has not been determined by the testing facility. The Sponsor has indicated that they have not performed these analyses on the test article mixtures.

Ramadevi Gudi 15 Oct 2001

Ramadevi Gudi, Ph.D. — Date
Study Director

15 Oct 2001

BioReliance Study Management — Date

Quality Assurance Statement

Study Title: *IN VITRO* MAMMALIAN CHROMOSOME ABERRATION TEST

Study Number: AA47PV.341.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-Jul-01 - 25-Jul-01 To Study Dir 25-Jul-01 To Mgmt 25-Jul-01
Phase	Protocol Review
Inspect On	28-Aug-01 - 28-Aug-01 To Study Dir 28-Aug-01 To Mgmt 30-Aug-01
Phase	Preparation of slides
Inspect On	30-Sep-01 - 01-Oct-01 To Study Dir 01-Oct-01 To Mgmt 03-Oct-01
Phase	Draft Report
Inspect On	15-Oct-01 - 15-Oct-01 To Study Dir 15-Oct-01 To Mgmt 15-Oct-01
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.

Jennifer Klopsis — 15 Oct 2001

Jennifer Klopsis
QUALITY ASSURANCE

DATE

BIORELIANCE™

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST

FINAL REPORT

Sponsor:	**American Chemistry Council Brominated Flame Retardant Industry Panel (BFRIP) 1300 Wilson Boulevard Arlington, VA 22209**
Authorized Representative:	**Wendy Sherman, M.S.**
Performing Laboratory:	**BioReliance 9630 Medical Center Drive Rockville, Maryland 20850**
Test Article I.D.:	**Tetrabromobisphenol A (TBBPA)**
Test Article Lot Number:	**Wildlife International No. 5381**
Test Article Purity:	**98.91%, (provided by Sponsor)**
BioReliance Study No.:	**AA47PV.341.BTL**
Test Article Description:	**White solid**
Storage Conditions:	**Room temperature, protected from exposure to light and moisture**
Test Article Receipt/Login Date:	**23 July 2001**
Study Initiation:	**24 July 2001**
Experimental Start Date:	**27 July 2001**
Experimental Completion Date:	**09 September 2001**

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SUMMARY

The test article, Tetrabromobisphenol A (TBBPA), was tested in the *in vitro* mammalian chromosome aberration test using human peripheral lymphocytes (HPBL) 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 testing in the cytogenetic test. 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 the solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at a concentration of 500 mg/mL, the maximum concentration tested.

In the preliminary toxicity assay, the maximum dose tested was 5000 µg/mL. Human peripheral blood lymphocytes were treated in the absence and presence of an Aroclor-induced S9 activation system for 4 hours, and continuously for 20 hours in the absence of S9 activation. Visible precipitate was observed in treatment medium at concentrations ≥ 150 µg/mL. Concentrations of ≤ 50 µg/mL were soluble in treatment medium. Selection of dose levels for the chromosome aberration assay was based on a reduction in the mitotic index relative to the solvent control. Substantial toxicity, i.e., at least a 50% reduction in mitotic index, was observed at doses ≥ 150 µg/mL in the non-activated 4 and 20 hour exposure groups, respectively. Substantial toxicity was observed at dose levels ≥ 50 µg/mL in the S9 activated 4 hour exposure group. Based on these findings, the doses chosen for the chromosome aberration assay ranged from 1.5 to 200 µg/mL for the non-activated 4 and 20 hour exposure groups, and from 1.5 to 150 µg/mL for the S9 activated 4 hour exposure group.

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. The test article was soluble in treatment medium at all concentrations tested. Toxicity (mitotic inhibition) was approximately 54% and 59% at the highest dose level evaluated for chromosome aberrations, 100 µg/mL and 75 µg/mL, in the non-activated 4 hour and 20 hour exposure groups, respectively. Toxicity (mitotic inhibition) was 58% at the highest dose level evaluated for chromosome aberrations, 50 µg/mL, in the S9 activated study. Initially, the non-activated and S9 activated 4 hour exposure groups were scored for structural and numerical chromosome aberrations. No statistically significant increases in structural and numerical chromosome aberrations were observed in the non-activated or S9 activated 4 hour exposure groups relative to the solvent control group, regardless of dose level ($p>0.05$, Fisher's exact test). In the absence of a positive response in the non-activated 4 hour exposure group, the non-activated 20 hour continuous exposure group was evaluated for structural and numerical chromosome aberrations. No statistically significant increases in structural and numerical chromosome aberrations were observed in the non-activated 20 hour continuous exposure group relative to the solvent control group, regardless of dose level ($p>0.05$, Fisher's exact test).

Based on the findings of this study, Tetrabromobisphenol A (TBBPA) was concluded to be negative for the induction of structural and numerical chromosome aberrations in the *in vitro* mammalian chromosome aberration test using human peripheral lymphocytes.

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 human peripheral lymphocytes.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, Tetrabromobisphenol A (TBBPA), was received by BioReliance on 23 July 2001 and was assigned the code number AA47PV. The test article was characterized by the Sponsor as a white crystalline powder that should be stored at ambient temperature, protected from exposure to light and moisture. An expiration date of August 1, 2002 was assigned by the Sponsor. Upon receipt, the test article was described as a white solid 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 in a GLP-compliant laboratory. A copy of the characterization report is included in Appendix III.

The solvent used to deliver Tetrabromobisphenol A (TBBPA) to the test system was dimethyl sulfoxide (DMSO; CAS No.: 67-68-5), obtained from Fisher Scientific.

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 30 and 60 µg/mL for use as the positive control in the non-activated test system. Cyclophosphamide (CP), CAS No.: 6055-19-2, was obtained from the Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 2 and 4 mg/mL for use as the positive control in the S9 activated test system. For each positive control, 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.

MATERIALS AND METHODS

Test System

Peripheral blood lymphocytes were obtained from a healthy non-smoking 24 year old adult male on July 25, 2001 for the preliminary toxicity assay, from a healthy non-smoking 26 year old adult male on August 22, 2001 for the definitive assay. Neither donor had a recent history of radiotherapy, viral infection or the administration of drugs. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (Preston et al., 1981).

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 ≤-70°C until used. Each bulk preparation of S9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethyl-benz(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 (RPMI 1640 serum-free medium supplemented with 100 units penicillin and 100 µg streptomycin/mL, and 2 mM L-glutamine).

Solubility Test

A solubility test was conducted to select the solvent. The test was conducted using purified water and dimethyl sulfoxide (DMSO). The test article was tested to determine the solvent, selected in order of preference, that permitted preparation of the highest soluble or workable stock concentration, up to 50 mg/mL for aqueous solvents and up to 500 mg/mL for organic solvents.

Preliminary Toxicity Assay

The toxicity test was performed for the purpose of selecting concentrations for the chromosome aberration assay and consisted of an evaluation of test article effect on mitotic index. Approximately 0.6 mL heparinized blood was inoculated into centrifuge tubes containing 9.4 mL RPMI-1640 complete medium supplemented with 1% PHA. The tubes were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 44-48 hours. The pH and osmolality of the highest treatment condition were measured, and the pH was adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. At the time of test article treatment the culture tubes were centrifuged, the supernatant was aspirated, and the cells were resuspended in either 10 mL of fresh RPMI-1640 complete medium containing 1% PHA for the non-activated study or 10 mL S9 reaction mixture (8 mL serum free medium containing 1% PHA + 2 mL of S9 cofactor pool), to which was added 100 µL test article dosing solution in solvent or solvent alone.

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 the 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 RPMI-1640 complete medium and returned to the incubator for an additional 16 hours. Two hours prior to the scheduled cell harvest, Colcemid® was added to the cultures at a final concentration of 0.1 µg/mL and the cultures were returned to the incubator until cell collection.

Cells were collected by centrifugation, treated with hypotonic potassium chloride (0.075M KCl), fixed, stained and the number of cells in mitosis per 500 cells scored was determined in order to evaluate test article effect on mitotic index.

Chromosome Aberration Assay

The chromosome aberration assay was performed using standard procedures (Evans, 1976; Evans and O'Riordan, 1975) by exposing duplicate cultures of human peripheral blood lymphocytes (HPBL) to at least 4 concentrations of the test article as well as positive and solvent controls. The dividing cells were harvested at approximately 20 hours from the initiation of treatment.

For the chromosome aberration assays, 0.6 mL heparinized blood was inoculated into centrifuge tubes containing 9.4 mL complete medium supplemented with 1% PHA. The tubes were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 44-48 hours. Treatment was carried out by refeeding with approximately 10 mL fresh complete medium or S9 reaction mixture to which was added 100 µL of dosing solution of test or control article in solvent or solvent alone.

In the non-activated study, the cells were exposed for 4 or 20 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. In the 4 hour exposure group, after the exposure period, the treatment medium was removed, the cells washed with calcium and magnesium-free phosphate buffered saline (CMF-PBS), refed with complete medium containing 1% PHA and returned to the incubator for an additional 16 hours. Two hours prior to the scheduled cell harvest at 20 hours after treatment initiation, Colcemid® was added to the cultures at a final concentration of 0.1 µg/mL. In the 20 hour exposure group treatment was continuous until the time of cell collection. Two hours prior to the scheduled cell harvest at 20 hours after treatment initiation, Colcemid® was added to the cultures at a final concentration of 0.1 µg/mL.

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. After the exposure period, the treatment medium was removed, the cells washed with calcium and magnesium-free phosphate buffered saline (CMF-PBS), refed with complete medium containing 1% PHA and returned to the incubator for an additional 16 hours. Two hours prior to the scheduled cell harvest at 20 hours after treatment initiation, Colcemid® was added to the cultures at a final concentration of 0.1 µg/mL.

Collection of Metaphase Cells

Two hours after the addition of Colcemid®, metaphase cells were harvested for both the activated and non-activated studies by centrifugation. The cells were collected by centrifugation at approximately 1200 rpm for about 5 minutes. The cell pellet was resuspended in 5 mL 0.075 M KCl and incubated at 37±1°C for 20 minutes. At the end of the KCl treatment and immediately prior to centrifuging, the cells were gently mixed and approximately 0.5 mL of fixative (methanol:glacial acetic acid, 3:1 v/v) was added to each tube. The cells were collected by centrifugation, the supernatant aspirated, and the cells were fixed with two washes with approximately 3-5 mL of fixative and stored in fixative overnight or longer at approximately 2-8°C.

Slide Preparation

To prepare slides, the fixed cells were centrifuged at approximately 1200 rpm for 5 minutes, the supernatant was aspirated, and the cells were resuspended in 1 mL cold fresh fixative. The cells were collected by centrifugation and the supernatant aspirated, leaving 0.1 to 0.3 mL fixative above the cell pellet. An aliquot of the cell suspension was dropped onto a glass slide and allowed to air dry overnight. Slides were identified by the study number, dose level, activation condition, harvest time, replicate tube designation and date prepared. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

Selection of Dose Levels for Analysis

The selection of dose levels for analysis of chromosome aberrations in HPBL was based upon toxicity. The highest dose level selected for evaluation was the dose which induced at least 50% toxicity, as measured by mitotic inhibition, relative to the solvent control, with a sufficient number of scorable metaphase cells. Two additional lower dose levels were included in the evaluation.

Evaluation of Metaphase Cells

Slides were coded using random numbers by an individual not involved with the scoring process. Initially, the non-activated and S9 activated 4 hour exposure groups were evaluated for chromosome aberrations and if a positive result was obtained in the non-activated 4 hour exposure group, the non-activated 20 hour continuous exposure group was not necessarily evaluated for chromosome aberrations. Metaphase cells with 46 centromeres were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate treatment condition) were examined and scored for chromatid-type and chromosome-type aberrations (Scott et al., 1990). 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 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 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 ($\geq$10 aberrations) also were recorded. Chromatid and isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage. The mitotic index was recorded as the percentage of cells in mitosis per 500 cells counted. The percent polyploid and endoreduplicated cells was evaluated per 100 cells.

Controls

MMC was used as the positive control in the non-activated study at final concentrations of 0.3 and 0.6 µg/mL. CP was used as the positive control in the S9 activated study at final concentrations of 20 and 40 µg/mL. For both positive controls one dose level exhibiting a

sufficient number of scorable metaphase cells was selected for analysis. The solvent vehicle 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 are based upon mitotic inhibition relative to the solvent-treated control and are presented for the preliminary toxicity test and the chromosome aberration assay. The number and types of aberrations per cell, the percentage of structurally and numerically damaged cells (percent aberrant cells), and the frequency of structural aberrations per cell (mean aberrations per cell) in the total population of cells examined was 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 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 judgement; however, as a guide to interpretation of the data, the test article was considered to induce a positive response when the percentages of cells with aberrations were increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). A reproducible significant increase at the high dose only with no dose response or a reproducible significant increase at one dose level other than the high dose with no dose response will be considered positive. The test article was concluded to be negative if no statistically significant increase was observed relative to the solvent control.

Criteria for Determination of a Valid Test

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

Dimethyl sulfoxide (DMSO) was determined to be the solvent of choice based on the solubility of the test article, and compatibility with the target cells. The test article was soluble in DMSO at a concentration of 500 mg/mL, the maximum concentration tested.

Preliminary Toxicity Assay

Dose levels for the chromosome aberration assay were selected following a preliminary toxicity test and were based upon a reduction in mitotic index relative to the solvent control. The results of the evaluation of mitotic inhibition are presented in Tables 1, 2, and 3. HPBL cells were first exposed to nine concentrations of Tetrabromobisphenol A (TBBPA) ranging from 0.5 µg/mL to 5000 µg/mL, as well as solvent controls, in both the absence and presence of an Aroclor-induced S9 activation system for 4 hours, or continuously for 20 hours in the absence of S9 activation. The test article was soluble in treatment medium at concentrations $\leq$ 150 µg/mL. The osmolality and pH of the highest concentration tested, 5000 µg/mL, were 347 mmol/kg and approximately 7.0, respectively. The osmolality of the lowest precipitating concentration, 150 µg/mL, was 404 mmol/kg. The osmolality pH of the highest soluble concentration, 50 µg/mL, was 417 mmol/kg. The osmolality of the solvent (DMSO) in the treatment medium was 412 mmol/kg.

Substantial toxicity (mitotic inhibition in excess of 50%, relative to the solvent control) was observed at concentrations $\geq$ 150 µg/mL in the non-activated 4 and 20 hour exposure groups. Substantial toxicity was observed at concentrations $\geq$ 50 µg/mL in the S9 activated exposure group. Based on the results of the preliminary toxicity test, the dose levels selected for testing in the chromosome aberration assay were as follows:

Treatment Condition	Treatment Time	Recovery Time	Dose levels (µg/mL)
Non-activated	4 hr	16 hr	1.5, 3.125, 6.25, 12.5, 25, 50, 75, 100, 150, 200
	20 hr	0 hr	1.5, 3.125, 6.25, 12.5, 25, 50, 75, 100, 150, 200
S9 activated	4 hr	16 hr	1.5, 3.125, 6.25, 12.5, 25, 50, 75, 100, 150

Chromosome Aberration Assay

In the chromosome aberration assay, the test article was soluble in treatment medium at all concentrations tested. The osmolality in treatment medium of the highest concentration

tested, 200 µg/mL, was 401 mmol/kg. The osmolality of the solvent (DMSO) in treatment medium was 381 mmol/kg. The pH of the highest concentration of test article in treatment medium was approximately 7.0.

The findings of the cytogenetic analysis of the non-activated 4 hour exposure group are presented by treatment flask in Table 4 and summarized by group in Table 7. At the highest test concentration evaluated microscopically for chromosome aberrations, 100 µg/mL, mitotic inhibition was 54%, relative to the solvent control. The dose levels selected for analysis of chromosome aberrations were 6.25, 25, and 100 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, regardless of dose level ($p>0.05$, Fisher's exact test). The percentage of structurally damaged cells in the MMC (positive control) group was found to be statistically significant (12.0%).

The findings of the cytogenetic analysis of the S9 activated group are presented by treatment flask in Table 5 and summarized by group in Table 7. At the highest test concentration evaluated microscopically for chromosome aberrations, 50 µg/mL, mitotic inhibition was 58%, relative to the solvent control. The dose levels selected for analysis of chromosome aberrations were 3.125, 12.5, and 50 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, regardless of dose level ($p>0.05$, Fisher's exact test). The percentage of structurally damaged cells in the CP (positive control) group was found to be statistically significant (9.0%).

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. The findings of the cytogenetic analysis of the non-activated 20 hour exposure group are presented by treatment flask in Table 6 and summarized by group in Table 7. At the highest test concentration evaluated microscopically for chromosome aberrations, 75 µg/mL, mitotic inhibition was 59%, relative to the solvent control. The dose levels selected for analysis of chromosome aberrations were 6.25, 25, and 75 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, regardless of dose level ($p>0.05$, Fisher's exact test). The percentage of structurally damaged cells in the MMC (positive control) group was found to be statistically significant (12.0%).

CONCLUSION

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

Under the conditions of the assay described in this report, Tetrabromobisphenol A (TBBPA) was concluded to be negative for the induction of structural and numerical chromosome aberrations in the non-activated and S9 activated test systems in the *in vitro* mammalian chromosome aberration test using human peripheral lymphocytes.

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.

Evans, H.J. and M.L. O'Riordan. 1975. Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. Mutation Res. 31:135-148.

Galloway, S.M., M.J. Aardema, M. Ishidate Jr., J.L. Ivett, D.J. Kirkland, T. Morita, P. Mosesso and T. Sofuni (1994) Report from working group on in vitro tests for chromosomal aberrations, Mutation Research 312(3):241-261.

International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. S2A document recommended for adoption at step 4 of the ICH process on July 19, 1995. Federal Register 61:18198-18202, April 24, 1996.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B document recommended for adoption at step 4 of the ICH process on July 16, 1997. Federal Register 62:16026-16030, November 21, 1997.

OECD Guideline for the Testing of Chemicals, Guideline 473 (*In Vitro* Mammalian Chromosome Aberration Test), Revised Draft Document, Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

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.

TABLE 1

PRELIMINARY TOXICITY TEST USING Tetrabromobisphenol A (TBBPA) IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT

TREATMENT (-S9)	MITOTIC INDEX (%)	PERCENT CHANGE (%)
DMSO	9.0	
Tetrabromobisphenol A (TBBPA)		
0.5 µg/mL	7.8	-13
1.5 µg/mL	8.8	-2
5 µg/mL	7.8	-13
15 µg/mL	7.6	-16
50 µg/mL	9.0	0
150 µg/mL	2.0	-78
500 µg/mL	0.0	-100
1500 µg/mL	0.0	-100
5000 µg/mL	0.0	-100

Treatment: Human peripheral blood lymphocyte cells were treated in the absence of an exogenous source of metabolic activation for 4 hours at 37±1°C. Metaphase cells were collected following a 16 hour recovery period.

Mitotic Index = (Cells in mitosis/500 cells scored) x 100.

Percent change = (Treatment mitotic index - control mitotic index)/control mitotic index, expressed as a percentage.

TABLE 2

PRELIMINARY TOXICITY TEST USING Tetrabromobisphenol A (TBBPA) IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT

TREATMENT (+S9)	MITOTIC INDEX (%)	PERCENT CHANGE (%)
DMSO	8.2	
Tetrabromobisphenol A (TBBPA)		
0.5 µg/mL	8.2	0
1.5 µg/mL	8.0	-2
5 µg/mL	9.0	10
15 µg/mL	8.4	2
50 µg/mL	3.0	-63
150 µg/mL	0.0	-100
500 µg/mL	0.0	-100
1500 µg/mL	0.0	-100
5000 µg/mL	0.0	-100

Treatment: Human peripheral blood lymphocyte cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C. Metaphase cells were collected following a 16 hour recovery period.

Mitotic Index = (Cells in mitosis/500 cells scored) x 100.

Percent change = (Treatment mitotic index - control mitotic index)/control mitotic index, expressed as a percentage.

TABLE 3

PRELIMINARY TOXICITY TEST USING Tetrabromobisphenol A (TBBPA) IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

20 HOUR TREATMENT

TREATMENT (-S9)	MITOTIC INDEX (%)	PERCENT CHANGE (%)
DMSO	9.2	
Tetrabromobisphenol A (TBBPA)		
0.5 µg/mL	9.0	-2
1.5 µg/mL	7.6	-17
5 µg/mL	7.0	-24
15 µg/mL	8.2	-11
50 µg/mL	7.4	-20
150 µg/mL	0.0	-100
500 µg/mL	0.0	-100
1500 µg/mL	0.0	-100
5000 µg/mL	0.0	-100

Treatment: Human peripheral blood lymphocyte cells were treated in the absence of an exogenous source of metabolic activation for 20 hours at 37±1°C. Metaphase cells were collected following a 16 hour recovery period.

Mitotic Index = (Cells in mitosis/500 cells scored) x 100.

Percent change = (Treatment mitotic index - control mitotic index)/control mitotic index, expressed as a percentage.

TABLE 4

CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED WITH Tetrabromobisphenol A (TBBPA)

IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

DEFINITIVE ASSAY: 4 HOUR TREATMENT, 20 HOUR HARVEST

Treatment	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	12.0	100	0	1	0	1	0	0	0	0	0	0.010
	B	11.0	100	0	0	0	0	0	0	0	0	0	0.000
Tetrabromobisphenol A (TBBPA)													
6.25 ug/mL	A	10.4	100	0	0	0	0	0	0	0	0	0	0.000
	B	10.0	100	0	0	0	0	0	0	0	0	0	0.000
25 ug/mL	A	7.0	100	0	0	0	0	0	0	0	0	0	0.000
	B	7.2	100	0	0	0	0	0	0	0	0	0	0.000
100 ug/mL	A	5.0	100	0	0	0	0	0	0	0	0	0	0.000
	B	5.6	100	0	0	0	0	0	0	0	0	0	0.000
MMC	A	7.0	100	2	14	0	10	3	2	0	0	0	0.150
0.6 ug/mL	B	7.6	100	0	10	0	4	6	0	0	0	0	0.100

Treatment: Human peripheral blood lymphocytes were treated for 4 hours at 37± 1∘C in the absence of an exogenous source of metabolic activation. Additional dose levels of 1.5, 3.125, 12.5, 50 and 75 µg/mL were tested as a safeguard against excessive toxicity at higher dose levels but were not required for microscopic examination. Dose levels 150 and 200 µg/mL were not analyzed due to excessive toxicity.

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

% Aberrant Cells: numerical includes polyploid and endoreduplicated cells; structural excludes cells with only gaps.

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

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

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

Average Aberrations Per Cell: severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 5

CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED WITH Tetrabromobisphenol A (TBBPA)

IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

DEFINITIVE ASSAY: 4 HOUR TREATMENT, 20 HOUR HARVEST

Treatment	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	7.6	100	1	0	0	0	0	0	0	0	0	0.000
	B	8.4	100	0	0	0	0	0	0	0	0	0	0.000
Tetrabromobisphenol A (TBBPA)													
3.125 ug/mL	A	7.0	100	0	0	0	0	0	0	0	0	0	0.000
	B	7.8	100	0	0	0	0	0	0	0	0	0	0.000
12.5 ug/mL	A	5.6	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
50 ug/mL	A	3.0	100	0	0	0	0	0	0	0	0	0	0.000
	B	3.8	100	0	0	0	0	0	0	0	0	0	0.000
CP	A	2.0	100	2	7	0	2	4	0	1	0	0	0.070
20 ug/mL	B	2.2	100	1	11	0	4	3	5	0	0	0	0.120

Treatment: Human peripheral blood lymphocytes were treated for 4 hours at 37 ± 1°C in the presence of an exogenous source of metabolic activation. Additional dose levels of 1.5, 6.25 and 25µg/mL were tested as a safeguard against excessive toxicity at higher dose levels but were not required for microscopic examination. Dose levels 75, 100 and 150 µg/mL were not analyzed due to excessive toxicity.

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

% Aberrant Cells: numerical includes polyploid and endoreduplicated cells; structural excludes cells with only gaps.

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

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

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

Average Aberrations Per Cell: severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 6

CYTOGENETIC ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED WITH Tetrabromobisphenol A (TBBPA)

IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

DEFINITIVE ASSAY: 20 HOUR TREATMENT, 20 HOUR HARVEST

Treatment	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	11.8	100	1	1	0	1	0	0	0	0	0	0 010
	B	12.6	100	0	0	0	0	0	0	0	0	0	0 000
Tetrabromobisphenol A (TBBPA)													
6.25 ug/mL	A	11.0	100	0	0	0	0	0	0	0	0	0	0 000
	B	10.0	100	0	0	0	0	0	0	0	0	0	0.000
25 ug/mL	A	9.6	100	0	0	0	0	0	0	0	0	0	0.000
	B	8.8	100	2	1	0	0	1	0	0	0	0	0.010
75 ug/mL	A	4.8	100	2	0	0	0	0	0	0	0	0	0.000
	B	5.2	100	3	0	0	0	0	0	0	0	0	0.000
MMC	A	5.8	100	0	9	0	5	7	0	0	0	0	0 120
0.3 ug/mL	B	6.6	100	1	15	0	6	11	2	0	0	0	0 190

Treatment: Human peripheral blood lymphocytes were treated for 20 hours at 37 ± 1°C in the absence of an exogenous source of metabolic activation. Additional dose levels of 1.5, 3.125, 12.5 and 50 µg/mL were tested as a safeguard against excessive toxicity at higher dose levels but were not required for microscopic examination. Dose levels 100, 150 and 200 µg/mL were not analyzed due to excessive toxicity.

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

% Aberrant Cells: numerical includes polyploid and endoreduplicated cells; structural excludes cells with only gaps.

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

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

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

Average Aberrations Per Cell: severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 7

SUMMARY

Treatment	S9 Activation	Treatment Time	Mean Mitotic Index	Cells Scored	Aberrations Per Cell (Mean +/- SD)		Cells With Aberrations Numerical (%)	Cells With Aberrations Structural (%)
DMSO	-	4	11.5	200	0.005	±0.071	0.0	0.5
Tetrabromobisphenol A (TBBPA)								
6.25 ug/mL	-	4	10.2	200	0.000	±0.000	0.0	0.0
25 ug/mL	-	4	7.1	200	0.000	±0.000	0.0	0.0
100 ug/mL	-	4	5.3	200	0.000	±0.000	0.0	0.0
MMC 0.6 ug/mL	-	4	7.3	200	0.125	±0.346	1.0	12.0**
DMSO	+	4	8.0	200	0.000	±0.000	0.5	0.0
Tetrabromobisphenol A (TBBPA)								
3.125 ug/mL	+	4	7.4	200	0.000	±0.000	0.0	0.0
12.5 ug/mL	+	4	5.8	200	0.000	±0.000	0.5	0.0
50 ug/mL	+	4	3.4	200	0.000	±0.000	0.0	0.0
CP 20 ug/mL	+	4	2.1	200	0.095	±0.311	1.5	9.0**
DMSO	-	20	12.2	200	0.005	±0.071	0.5	0.5
Tetrabromobisphenol A (TBBPA)								
6.25 ug/mL	-	20	10.5	200	0.000	±0.000	0.0	0.0
25 ug/mL	-	20	9.2	200	0.005	±0.071	1.0	0.5
75 ug/mL	-	20	5.0	200	0.000	±0.000	2.5	0.0
MMC 0.3 ug/mL	-	20	6.2	200	0.155	±0.471	0.5	12.0**

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 the Fisher's exact test.

APPENDIX I

Historical Control Data

IN VITRO MAMMALIAN CYTOGENETIC TEST USING
HUMAN PERIPHERAL BLOOD LYMPHOCYTES

HISTORICAL CONTROL VALUES
STRUCTURAL CHROMOSOME ABERRATIONS
1998-2000

NON-ACTIVATED TEST SYSTEM

Historical Values	Percent Aberrant Cells (%)		
	Untreated Control	Solvent Control[1]	Positive Control[2]
Mean	0.2	0.1	13.9
Standard Deviation	0.4	0.3	6.1
Range	0.0-1.5	0.0-1.0	6.0-37.0

S9 ACTIVATED TEST SYSTEM

Historical Values	Percent Aberrant Cells (%)		
	Untreated Control	Solvent Control[1]	Positive Control[3]
Mean	0.1	0.2	13.8
Standard Deviation	0.2	0.4	5.6
Range	0.0-0.5	0.0-2.0	8.0-33.5

[1]Solvents include water, saline, DMSO, ethanol, acetone, and other non-standard and Sponsor-supplied vehicles.
[2]Positive control for non-activated studies is mitomycin C (MMC).
[3]Positive control for S9 activated studies is cyclophosphamide (CP).

IN VITRO MAMMALIAN CYTOGENETIC TEST USING
HUMAN PERIPHERAL BLOOD LYMPHOCYTES

HISTORICAL CONTROL VALUES
NUMERICAL CHROMOSOME ABERRATIONS
1998-2000

NON-ACTIVATED TEST SYSTEM

Historical Values	Percent Aberrant Cells (%)		
	Untreated Control	Solvent Control[1]	Positive Control[2]
Mean	0.1	0.0	0.1
Standard Deviation	0.2	0.2	0.3
Range	0.0-0.5	0.0-1.5	0.0-1.0

S9 ACTIVATED TEST SYSTEM

Historical Values	Percent Aberrant Cells (%)		
	Untreated Control	Solvent Control[1]	Positive Control[3]
Mean	0.1	0.1	0.2
Standard Deviation	0.2	0.2	0.4
Range	0.0-0.5	0.0-1.0	0.0-1.5

[1]Solvents include water, saline, DMSO, ethanol, acetone, and other non-standard and Sponsor-supplied vehicles.
[2]Positive control for non-activated studies is mitomycin C (MMC).
[3]Positive control for S9 activated studies is cyclophosphamide (CP).

APPENDIX II

Study Protocol

PROTOCOL AMENDMENT 1

Sponsor: **American Chemistry Council**
Brominated Flame Retardant Industry Panel (BFRIP)

Test Article I.D.: **Tetrabromobisphenol A (TBBPA)**

BioReliance Study No.: **AA47PV.341.BTL**

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

QA 7/25/01 APPROVED

1. **LOCATION:** Page 1, § 2.1, Sponsor Name

   AMENDMENT: Add "(BFRIP)" after Brominated Flame Retardant Industry Panel

   REASON FOR THE AMENDMENT: Sponsor's request

2. **LOCATION**: Page1, § 3.1, Test Article

   AMENDMENT: Add "(TBBPA)" after Tetrabromobisphenol A

   REASON FOR THE AMENDMENT: Sponsor's request

3. **LOCATION**: Page7, § 10.0, Report

   AMENDMENT: Add "Protocol and Amendments" to the list of items included in the report.

   REASON FOR THE AMENDMENT: Sponsor's request

APPROVALS:

for RG — 25 July 2001
Study Director — Date

25 July 2001
Study Management — Date

Wendy K. Sherman — August 2, 2001
Sponsor Representative — Date

BIORELIANCE

QA APPROVED 8/23/01

PROTOCOL AMENDMENT 2

Sponsor: **American Chemistry Council**
Brominated Flame Retardant Industry Panel (BFRIP)

Test Article I.D.: **Tetrabromobisphenol A**

BioReliance Study No.: **AA47PV.341.BTL**

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

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1. **LOCATION:** Page 4 of 9, § 7.5.2; Positive Controls, first sentence

   AMENDMENT: Change the range of Mitomycin C concentrations to be used from 0.1-0.5 µg/mL to 0.3-0.6 µg/mL.

   REASON FOR THE AMENDMENT: The higher concentrations of Mitomycin C provide sufficient positive response to validate the assay.

APPROVALS:

Ramadevi Gudi — 22 Aug 2001
BIORELIANCE STUDY DIRECTOR — **DATE**

[signature] — 22 Aug 2001
BIORELIANCE STUDY MANAGEMENT — **DATE**

Wendy K. Sherman — August 30, 2001
SPONSOR REPRESENTATIVE — **DATE**

BioReliance

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 human peripheral blood lymphocytes (HPBL).

2.0 SPONSOR

2.1	Name:	American Chemistry Council Brominated Flame Retardant Industry Panel
2.2	Address:	1300 Wilson Boulevard Arlington, VA 22209
2.3	Representative :	Wendy Sherman, M.S.

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1	Test Article:		Tetrabromobisphenol A
3.2	Controls:	Solvent:	Test Article Vehicle
		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: July 27, 2001

5.2 Proposed Experimental Completion Date: September 24, 2001

5.3 Proposed Report Date: October 3, 2001

6.0 TEST SYSTEM

Peripheral blood lymphocytes will be obtained from healthy adults without a recent history of either radiotherapy, viral infections or the administration of drugs. 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 assay will be conducted using standard procedures, by exposing human lymphocytes 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). 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 in order to ensure evaluation of first-division metaphase cells (Galloway et al., 1994). The clastogenic potential of the test article will be measured by its ability to increase chromosome aberrations in a dose-responsive manner when compared to a control group. The 4 hour non-activated and S9-activated studies will be scored initially. 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 Solvent Selection

7.1.1 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 inhibition of mitosis after treatment as determined in a cytotoxicity study. Cells will be exposed to solvent alone and to at least nine concentrations of test article, the highest concentration being 5000 µg/ml or 10 mM whichever is lower. The pH of the highest test article dosing solution will be measured, and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest dosing solution, lowest precipitating dose level (where applicable) and the highest soluble dose level (where applicable) will also be measured. Peripheral blood cells will be cultured in RPMI-1640 containing 15% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin and 100 ug streptomycin/ml and 1% phytohemagglutinin. Cells seeded approximately 46 hours earlier will be exposed for 4 hours in the absence and presence of S9 and for 20 hours in the absence of S9. After exposure the cultures will be grown in complete medium for 16 hours. Eighteen hours after treatment initiation Colcemid® (0.1 µg/ml) will be added to the cultures. Cells will be collected at 20 hours after the initiation of treatment by centrifugation, treated with hypotonic KCl solution and fixed with methanol-glacial acetic acid. Metaphase preparations will be made and stained. The percentage of cells in mitosis per 500 cells scored (mitotic index) will be determined for each treatment group.

Whenever possible, the high dose for the chromosome aberration assay will be selected to give at least 50% toxicity (mitotic inhibition relative to the solvent control). At least two additional dose levels, demonstrating minimal or no toxicity, will be evaluated in the chromosome aberration assay. 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 will be the concentration resulting in minimum precipitation in test medium. Precipitation will be determined with the unaided eye. In the event the test article demonstrates a dose-responsive increase in toxicity (mitotic inhibition relative to the solvent control) at concentrations that exceed solubility in treatment medium, then the highest dose to be evaluated for chromosome aberrations will be the concentration resulting in minimum precipitation in test medium. 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 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 demonstrated to be an effective method of detection of chemical clastogens in this test system (Evans, 1975).

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 Metabolic 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 a cofactor pool. The final concentration of the cofactors and S9 in the reaction vessel will be 2 mM $MgCl_2$, 6 mM KCl, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate and 20 µl S9 per ml RPMI-1640 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 two concentrations within the range of 0.1-0.50 µg/ml as the positive control for the non-activated test system. For the S9-activated system, cyclophosphamide will be used at two concentrations within the range of 10-75 µg/ml. One dose level of each positive control will be evaluated microscopically for chromosome damage.

7.6 Preparation of Target Cells

Peripheral blood lymphocytes will be cultured in complete medium (RPMI-1640 containing 15% fetal bovine serum, 2mM L-glutamine, 100 units penicillin and 100 µg streptomycin/ml, and 1% phytohemagglutinin) by adding 0.6 ml heparinized blood to a centrifuge tube containing 9.4 ml complete medium. The tubes will be incubated upright at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air for 44-48 hours.

7.7 Identification of Test System

Using a permanent marking pen, the test system will be identified by the BioReliance study number, treatment condition and date.

7.8 Treatment of Target Cells

Forty-four to 48 hours after culture initiation, duplicate centrifuge tubes will be refed with approximately 10 ml complete medium for the non-activated exposure or 10 ml S9 reaction mixture for the activated exposure to which will be added 100 µl of dosing solution of test or control article in solvent. Larger volumes of dosing solution may be used if water or medium is used as the solvent.

For the S9-activated exposure, the cells will be treated for 4 hours in the presence of an S9 reaction mixture, washed free of chemical and cultured for an additional 16 hours with Colcemid® (0.1 µg/ml) present for the last 2 hours. For the non-activated exposure, treatment will be for 4 hours followed by a 16 hour recovery period and for 20 hours continuously with Colcemid® (0.1 µg/ml) present for the last 2 hours.

7.9 Collection of Metaphase Cells

Cells will be collected approximately 20 hours after initiation of treatment (about 64-68 hours after culture initiation). This time is selected to represent the first-division metaphase after initiation of test article treatment. Two hours prior to harvest, Colcemid® will be added to the cultures at a final concentration of 0.1 µg/ml. The cells will be collected by centrifugation, treated with 0.075M KCl, washed with two changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 2-8°C. To prepare slides, the cells will be collected by centrifugation and resuspended in fresh fixative. An aliquot of fixed cells will be applied dropwise onto a microscope slide and air-dried. The slide will be identified by the experiment number, treatment condition and date. At least two slides will be prepared from each treatment tube. The slides will be stained with Giemsa and permanently mounted.

7.10 Scoring for Metaphase Aberrations

Slides will be coded using random numbers by an individual not involved with the scoring process. At least 3 dose levels in each harvest will be evaluated in the aberration assay and will be selected according to the criteria described in section 7.2. The 4 hour non-activated and S9-activated studies will be scored initially. In the event of a positive response in 4 hour non-activated study, slides from the extended non-activated exposure may not be scored. Metaphase cells will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads containing 46 centromeres from each dose level (100 per duplicate treatment tube) 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 or gap will be recorded using a calibrated microscope stage. The mitotic index will be recorded as the percentage of cells in mitosis per 500 cells counted. The percent polyploid and endoreduplicated cells will be evaluated per 100 metaphase cells for each dose level analyzed for structural aberrations.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Solvent Controls

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

8.2 Positive Controls

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

9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon inhibition of mitosis and will be reported for the cytotoxicity and chromosome aberration study. The number and types of aberrations (structural and numerical) found, the percentage of structurally damaged cells in the total population of cells examined (percent aberrant cells), the percentage of numerically damaged cells in the total population of cells examined, and the average number of structural aberrations per cell (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 average number of 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 judgement; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response if the percent aberrant cells is increased in a dose-responsive manner with one or more concentrations being statistically significant ($p \leq 0.05$). A reproducible significant increase

at the high dose only with no dose response or a reproducible significant increase at one dose level other than the high dose with no dose response will be considered positive. 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 the Testing Laboratory 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 and time the cells were obtained, karyotype features (modal chromosome number) and suitability of the cell type used.

- test conditions: composition of medium; CO_2 concentration; incubation time; 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 positve, negative or equivocal.

- results: description of precipitation; pH and osmolarity of the treatment medium; mitotic inhibition relative to the solvent control; mitotic index and number of metaphases analyzed; type and number of aberrations (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), Revised Draft Document, 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-TSCA, EU

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. and M.L. O'Riordan. 1975. Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. Mutation Res. 31:135-148.

Galloway, S.M., M.J. Aardema, M. Ishidate Jr., J.L. Ivett, D.J. Kirkland, T. Morita, P. Mosesso and T. Sofuni (1994) Report from working group on in vitro tests for chromosomal aberrations, Mutation Research 312(3):241-261.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. S2A document recommended for adoption at step 4 of the ICH process on July 19, 1995. Federal Register 61:18198-18202, April 24, 1996.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B document recommended for adoption at step 4 of the ICH process on July 16, 1997. Federal Register 62:16026-16030, November 21,

1997.

OECD Guideline for the Testing of Chemicals, Guideline 473 (*In Vitro* Mammalian Chromosome Aberration Test), Revised Draft Document, 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* cytogenetics assays: A report of the US EPA's Gene-Tox Program. Mutation Res. 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

Wendy K. Sherman 7/23/01
Sponsor Representative Date

Wendy K. Sherman
(Print or Type Name)

Ramadevi Gudi 7/24/01
BioReliance Study Director Date

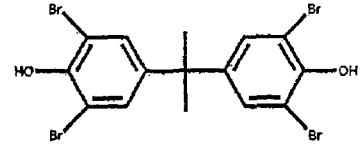
25 Jul 2001
BioReliance Study Management Date

APPENDIX III

Test Article Characterization Report

CONCLUSIONS AND TEST ARTICLE ANALYTICAL DATA.

CHEMICAL NAME: Tetrabromobisphenol-A
C.A.S. No.: 79-94-7
MOLECULAR FORMULA: $C_{15}H_{12}Br_4O_2$
PHYSICAL FORM: White Powder
CHEMICAL STRUCTURE:



ANALYSIS	RESULTS			ANALYSIS DATES	ANALYST
FT-IR	The sample FT-IR spectrum matched that of the reference spectrum. All spectra are on file with the original data.			01/04/01	W. T. Cobb
HPLC				01/05/01	J. S. Arroyave
Sample	Purity (area% TBBPA)	Average	Difference (%) from average		
top center	98.92	98.91	<5%		
middle center	98.89	98.91	<5%		
bottom center	98.91	98.91	0		

CONCLUSION: Based on these analytical data, the test article identity was confirmed as tetrabromobisphenol-A. The composite sample was shown to be homogeneous with a purity of 98.91%.

Conclusions and Test Article Data. 2.

Characterization of Test Article by HPLC (Area%)

	Top Center	Middle Center	Bottom Center	Average
Tetrabromobisphenol-A	98.92	98.89	98.91	98.91
o,p'-Tetrabromobisphenol-A	0.05	0.05	0.06	0.05
2,4,6-Tribromophenol	<0.01	<0.01	<0.01	<0.01
Tribromobisphenol-A	1.03	1.06	1.03	1.04