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SYNTHETIC ORGANIC CHEMICAL MANUFACTURERS ASSOCIATION

May 25, 2007

**By Courier:**

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Re: TSCA Section 8(e) Notification - #8EHQ-07-16707

Dear TSCA Section 8(e) Coordinator:

This final study report entitled, "Cobalt Naphthenate: *In Vitro* Mammalian Chromosome Abberation Test in Chinese Hamster Ovary Cells," is being submitted pursuant to Section 8(e) of the Toxic Substances Control Act ("TSCA") by the Synthetic Organic Chemical Manufacturers Association (SOCMA) on behalf of OM Group, Inc., The Shepherd Chemical Company, and Troy Corporation (the "Company Sponsors"). Notification of the intent to submit a copy of this report was sent and received by your office on December 27, 2006.

Company Sponsors have commissioned certain toxicity testing for cobalt naphthenate (CAS No. 61789-51-3) to fulfill their voluntary commitment under the U.S. High Production Volume Chemical (HPV) Challenge program. On December 13, 2006, Dupont Haskell Laboratory notified the Company Sponsors of positive *in vitro* genotoxicity test results based on preliminary data. On May 14, 2007, the study report was finalized by Dupont Haskell Laboratory.

**Company Sponsors**

The following companies are the sponsors for the genotoxicity test:

OM Group, Inc.  
127 Public Square  
1500 Key Tower  
Cleveland, OH 44114  
Contact: Scott L. Grove, Manager, Corporate Product Stewardship



The Shepherd Chemical Company  
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Contact: Bayard Pelsor, EHS/QS Manager



304900





Troy Corporation  
8 Vreeland Road  
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Contact: Adrian Krygsman, Director, Product Registration

Correspondence related to this 8(e) report filing should be directed to me at 202-721-4157 or jonesl@socma.com.

Sincerely,

A handwritten signature in cursive script that reads "Lynne Jones Batshon".

Lynne Jones Batshon  
Manager, SOCMA Visions

Cc: S. L. Grove, OM Group, Inc.  
C. T. Helmes, SOCMA Visions  
A. Krygsman, Troy Corporation  
B. Pelsor, The Shepherd Chemical Company



*Study Title*

Cobalt Naphthenate:  
*In Vitro* Mammalian Chromosome Aberration Test in Chinese Hamster Ovary Cells

**TEST GUIDELINES:** U.S. EPA Health Effects Test Guidelines, OPPTS 870.5375 (1998)

OECD Guidelines for the Testing of Chemicals, No. 473 (1998)

EC Commission Directive 2000/32/EC Annex 4A-B10 No. L 136

MAFF Japan 59 Nousan Number 4200 Agriculture Chemicals Laws and Regulations (1985)

**AUTHOR:** Christine M. Glatt, M.S.

**STUDY COMPLETED ON:** May 9, 2007

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**LABORATORY PROJECT ID:** DuPont-21243

**WORK REQUEST NUMBER:** 16642

**SERVICE CODE NUMBER:** 531

**SPONSOR:** The Metal Carboxylates Coalition  
1850 M Street, N.W., Suite 700  
Washington, D.C. 20036  
U.S.A.

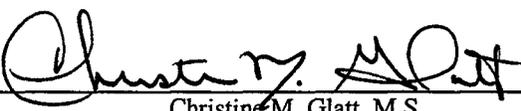
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### GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. EPA FIFRA (40 CFR part 160) and TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are compatible with current OECD and MAFF (Japan) Good Laboratory Practices, except for the items documented below. None of the items listed impact the validity of the study.

- The test substance was characterized by the sponsor prior to the initiation of this study. Although the characterization was not performed under Good Laboratory Practice Standards, the accuracy of the data is considered sufficient for the purposes of this study.
- Neither the vehicle, solvent, nor the positive controls were characterized by the testing facility or the sponsor. However, the vehicle and positive controls were purchased from a reputable vendor and showed results consistent with historical control data. The solvent was provided by the sponsor and also showed results consistent with historical control data.
- The concentrations of the positive control were not confirmed analytically; however, the solutions were prepared by trained personnel to ensure the accuracy of the concentrations.

**Applicant/Sponsor:** The Metal Carboxylates Coalition  
1850 M Street, N.W., Suite 700  
Washington, D.C. 20036  
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**Study Director:**  09-May-2007  
Christine M. Glatt, M.S.  
Senior Staff Toxicologist  
Date

**Applicant/Sponsor:**  5/14/07  
Applicant/Sponsor Representative  
Date

QUALITY ASSURANCE STATEMENT

Work Request Number: 16642  
Study Code Number: 531

<i>Phase Audited</i>	<i>Audit Dates</i>	<i>Date Reported to Study Director</i>	<i>Date Reported to Management</i>
Protocol:	November 1, 2006	November 1, 2006	November 5, 2006
Conduct:	November 3, 2006 November 9, 2006	November 3, 2006 November 9, 2006	November 3, 2006 November 9, 2006
Report/Records:	January 18-19, 23, 2007	January 23, 2007	February 07, 2007

Reported by:

  
\_\_\_\_\_  
Donna M. Johnston  
Quality Assurance Auditor

09 May 2007  
\_\_\_\_\_  
Date

**CERTIFICATION**

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Reviewed and Approved by: E. Maria Donner 08-May-2007  
E. Maria Donner, Ph.D. Date  
Senior Research Toxicologist and Manager

Issued by Study Director: Christine M. Glatt 09-May-2007  
Christine M. Glatt, M.S. Date  
Senior Staff Toxicologist

This report is approved by the sponsor.

Approved by: James F. Hobson 4/5/2007  
James F. Hobson, Ph.D., D.A.B.T. Date  
Sponsor Study Monitor

C. Tucker Helmes 4/24/07  
C. Tucker Helmes, Ph.D. Date  
Sponsor Representative

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### STUDY INFORMATION

Substance Tested: • Cobalt Naphthenate  
• 61789-51-3 (CAS Number)

Haskell Number: 27606

Composition: 53% Cobalt Naphthenate  
47% Mineral spirits (CAS# 8032-32-4)

Percent Metal: 5.93% Cobalt

Purity: See composition, above

Physical Characteristics: Purple liquid

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Study Initiated/Completed: October 23, 2006 / (see report cover page)

Experimental Start/Termination: October 26, 2006 / December 1, 2006

## SUMMARY

The test substance, cobalt naphthenate, was evaluated for its ability to induce chromosome aberrations *in vitro* in Chinese hamster ovary (CHO) cells in both the absence and presence of an exogenous S9 metabolic activation system (Aroclor-induced rat liver S9). Numerical aberrations were also recorded. To establish a concentration range for the chromosome aberration assay, a preliminary toxicity assay was initially conducted.

The test substance was supplied as a 53% solution of cobalt naphthenate (5.93% cobalt) in 47% mineral spirits. Further dilutions were prepared in ethanol as this vehicle was determined to be the solvent of choice based on solubility of the test substance and compatibility with the target cells. The test substance, as supplied by the sponsor, had a concentration of approximately 500 mg/mL and was used undiluted for the highest stock concentration on this study. It was a purple liquid in the mineral spirits. The test substance was soluble in ethanol at all subsequent dilutions.

In the preliminary toxicity assay, the cells were treated for 4 and 20 hours in the non-activated test conditions and for 4 hours in the S9-activated test condition. All cells were harvested 20 hours after treatment initiation. A vehicle control group (1% ethanol) and a solvent control group (0.47% mineral spirits) were included in each test condition.

In the preliminary toxicity assay, the highest concentration tested was 5000 µg/mL. The cells were exposed to nine concentrations of the test substance ranging from 5 to 5000 µg/mL, as well as the vehicle and solvent controls. A visible precipitate was observed in the treatment medium at concentrations  $\geq 250$  µg/mL in the beginning and end of the treatment periods. The difference in pH and osmolality of the highest test substance concentration in medium compared to the mineral spirits and ethanol controls was such that it did not affect the integrity of the data.

The test substance concentrations for the chromosome aberration assay were selected based on an assessment of the reduction in cell growth in the treated cultures relative to the ethanol vehicle control. Substantial toxicity (greater than a 50% reduction in cell growth relative to the ethanol vehicle control) was observed at concentrations  $\geq 50$  µg/mL in the 4- and 20-hour non-activated test conditions and at concentrations  $\geq 100$  µg/mL in the 4-hour activated test condition. Based on the findings from the preliminary toxicity assay, the highest concentrations initially chosen for the chromosome aberration assay were 35 µg/mL for the 4- and 20-hour non-activated test conditions and 100 µg/mL for the 4-hour activated test condition.

In the chromosome aberration assay, the cells were treated for 4 and 20 hours in the non-activated test condition and for 4 hours in the S9-activated test condition. All cells were harvested 20 hours after treatment initiation. A vehicle control group (1% ethanol), a solvent control group (0.01% mineral spirits), and two positive control groups were included in each test condition.

The concentrations initially (trial 1) chosen for the chromosome aberration assay were 1, 5, 10, 30, and 35 µg/mL for the 4- and 20-hour non-activated test conditions, and 5, 10, 25, 50, and 100 µg/mL for the 4-hour activated test condition. The difference in pH and osmolality of the highest test substance concentration in medium compared to the mineral spirits and ethanol

controls was such that it did not affect the integrity of the data. No visible precipitate was observed in the treatment medium at the beginning or end of the treatment periods at any concentration tested. Substantial toxicity was observed at concentrations  $\geq 30$   $\mu\text{g/mL}$  in the 4- and 20-hour non-activated test conditions (67% and 81% reduction, respectively), and at 100  $\mu\text{g/mL}$  only in the 4-hour activated test condition (98% reduction). A decrease in mitotic index of 100% was observed at concentrations  $\geq 30$   $\mu\text{g/mL}$  in the 20-hour non-activated test condition and at 100  $\mu\text{g/mL}$  in the 4-hour activated test condition. Because of this excessive toxicity, the assay was repeated (trial 2) for these two test conditions.

The concentrations chosen for trial 2 of the chromosome aberration assay were 1, 5, 10, 20, and 25  $\mu\text{g/mL}$  for the 20-hour non-activated test condition, and 10, 20, 50, 70, and 90  $\mu\text{g/mL}$  for the 4-hour activated test condition. In trial 2, no visible precipitate was observed in the treatment medium at the beginning or end of the treatment periods at any concentration tested. Substantial toxicity was observed at 90  $\mu\text{g/mL}$  in the 4-hour activated test condition (63% reduction in cell growth and 74.5% mitotic index inhibition). No substantial toxicity was observed at any concentration in the 20-hour non-activated test condition; however there was a reduction in mitotic index of 52.3% at the 25  $\mu\text{g/mL}$  concentration level. Although the reduction in growth was less than 50%, this dose level was the highest dose level scored for chromosomal aberrations since the reduction in mitotic index was substantial. Selection of doses for microscopic analysis was therefore based on these dose concentration levels from trials 1 and 2.

Cytogenetic evaluations were conducted at 5, 10, and 30  $\mu\text{g/mL}$  for the 4-hour non-activated test condition, at 20, 50, and 90  $\mu\text{g/mL}$  for the 4-hour activated test condition, and at 5, 10, and 25  $\mu\text{g/mL}$  for the 20-hour non-activated test condition. These concentrations were chosen based on the scorability of the slides (i.e., metaphase quality, chromosome morphology, and a sufficient amount of metaphases present). The percentage of cells with numerical aberrations in the 20-hour non-activated test substance-treated group was increased above that of the vehicle control at 5, 10, and 25  $\mu\text{g/mL}$  ( $p < 0.05$ , Fisher's exact test and Cochran-Armitage trend test). These observed changes were within the historical control range for numerical aberrations and were not considered biologically significant. The percentage of cells with structural aberrations in the 4-hour activated test substance-treated group was increased above that of the vehicle control at 50 and 90  $\mu\text{g/mL}$  ( $p < 0.05$ , Fisher's exact test and Cochran-Armitage trend test). These observed changes were dose-dependent, outside the historical control range of 0-5% (non-activated) and 0-6% (S9-activated) for structural aberrations, and are considered biologically significant.

All criteria for a valid study were met. Under the conditions of this study, cobalt naphthenate was found to induce structural chromosome aberrations in the *in vitro* mammalian chromosome aberration test in Chinese hamster ovary cells in the S9-activated test system at 4 hours only. It was concluded that the test substance was positive in this *in vitro* test.

## INTRODUCTION

The objectives of this study were to evaluate the test substance, cobalt naphthenate, for its ability to induce structural chromosome aberrations in Chinese hamster ovary (CHO) cells *in vitro*. Numerical aberrations were recorded. The assessment was done both in the presence and absence of an exogenous S9 metabolic activation system.

## MATERIALS AND METHODS

### A. Testing Guidelines

This study was conducted in compliance with the following guidelines except as noted below:

- U.S. Environmental Protection Agency (EPA), Health Effects Test Guidelines, OPPTS 870.5375, *In Vitro* Mammalian Chromosome Aberration Test. (August, 1998).
- Ninth Addendum to the OECD (Organisation for Economic Cooperation and Development) Guidelines for the Testing of Chemicals, *In Vitro* Mammalian Chromosome Aberration Test, No. 473. (February, 1998)
- European Commission Directive 2000/32/EC of May 19, 2000, Annex 4A-B10. Mutagenicity - *In Vitro* Mammalian Chromosome Aberration Test. No. L 136.
- MAFF Japan, Agriculture Chemicals Laws and Regulations, Japan (II), (59 Nousan Number 4200) (1985).

### Exception:

The highest treatment concentration in the 20-hour non-activated test condition (25 µg/mL) did not reduce cell growth by more than 50%; the cell growth reduction was 28%. Higher concentration levels (30 and 35 µg/mL) that did reduce cell growth by more than 50% resulted in unscorable slides (i.e., mitotic inhibition of 100%). The integrity and outcome of the study were not affected by this guideline deviation.

### B. Test Substance and Controls

#### 1. Identification

The test substance, cobalt naphthenate, was a purple liquid. The test batch used for this study was assigned Haskell identification number 27606. Additional information regarding the test substance is located on the study information page of this report.

#### 2. Characterization

The test substance was characterized by the sponsor prior to this study. The Certificate of Analysis (COA) of the test substance is included in this report (Appendix A).

### 3. Sample Preparation, Stability, and Analytical Verification of Test Substance Concentrations

The sponsor-reported purity for the cobalt naphthenate was 53% active ingredient in mineral spirits. A correction factor of 1.887 was used for preparation of the dosing solutions. Aliquots were taken from the vehicle control (ethanol), solvent control (mineral spirits), high, intermediate, and low test substance dose preparations to confirm test substance concentration and stability. The samples were sent to CCAS for analytical analysis (Appendix B)

### 4. Controls

Negative:	Ethanol (EtOH, CAS# 64-17-5, Aldrich) Mineral Spirits (Petroleum ether 35/60, CAS# 8032-32-4, Alfa Aesar)
Positive:	Mitomycin C (MMC, CAS# 50-07-7, Sigma) Cyclophosphamide (CP, CAS# 6055-19-2, Sigma)

The positive controls were dissolved in sterile water (CAS# 7732-18-5, molecular grade, Mediatech Inc.). The positive controls were assumed to be stable during this assay and no evidence of instability was observed.

## C. Test System

The CHO-K<sub>1</sub> cell line was originally derived as a subclone from a parental CHO cell line. The cells require proline in the medium for growth, and have a modal chromosome number of 20. The population doubling time is 10-14 hours. The cell line was obtained from the American Type Culture Collection (ATCC number CCL 61), Manassas, Virginia. The karyotype and the absence of mycoplasma infection are routinely checked by Haskell Laboratory. This test system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals.<sup>(1)</sup>

## D. Experimental Design and Methodology

The study was conducted according to published procedures.<sup>(1-5)</sup> The test substance, as well as positive and negative (vehicle and solvent) controls, was administered in the presence and absence of an exogenous S9 metabolic activation system to cell cultures by addition to the culture medium. In the non-activated test system, the treatment times were approximately 4 and 20 hours, and in the S9-activated test system, approximately 4 hours. The dividing cells were arrested in metaphase approximately 18 hours after initiation of the treatment and harvested at approximately 20 hours. This harvest timepoint represents approximately 1.5 normal cell cycles, and is determined to ensure assessment of clastogenicity in first-division metaphase cells.<sup>(4)</sup> Cytogenetic analyses were conducted on the 4- and 20-hour non-activated and 4-hour activated assays. If a positive response was observed in the 4-hour non-activated assay, the 20-hour non-activated assay may not be scored. The cytogenetic assessment also included recording of numerical aberrations. Based on OECD 473, a clear positive response does not require verification. Negative results do not require confirmation, but are justified. Equivocal results may need to be confirmed, and may require a modified study design.

### 1. Solubility Determination and Selection of Vehicle and Solvent

The test substance was provided by the sponsor in mineral spirits. The exact mineral spirits could not be obtained; therefore, a sample of a commercially-available, comparable mineral spirits (CAS #8032-32-4; H-27635) was purchased from Alfa Aesar. This sample was considered 100% mineral spirits. A solubility determination was conducted to determine the maximum soluble concentration of a 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, included, but were not limited to, culture medium or sterile water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), or ethanol (CAS 64-17-5). The vehicle of choice for this study was ethanol, which permitted preparation of the highest workable/soluble stock concentration. The ethanol was purchased from a reliable vendor by Haskell Laboratory and did not contain any contaminants that interfered with the conduct of the study. The vehicle was assumed to be stable under the conditions of this study and was stored at room temperature. Under the conditions of this test system, the final concentration of solvents other than water, physiological buffer, or medium did not exceed 1% of the treatment medium. After the addition of the dosing solution, the treatment medium was observed for precipitation (with the naked eye).

### 2. Exogenous Metabolic Activation

Liver homogenate (S9), prepared from male Sprague-Dawley rats induced with Aroclor 1254, were purchased commercially (Moltox, Inc., Boone, North Carolina) and stored frozen at approximately -70°C until used. The protein concentration was 41.1 mg/mL as reported by the vendor for the preliminary toxicity assay and trial 1 of the chromosome aberration assay. The concentration was 43.2 mg/mL as reported by the vendor for trial 2 of the chromosome aberration assay.

Immediately prior to use, the S9 liver homogenate was thawed and mixed with a cofactor pool. The final concentration of the cofactors and S9 in the metabolic activation system (S9 mixture) was 4 mM nicotinamide adenine dinucleotide phosphate (NADP), 5 mM glucose-6-phosphate, 8 mM MgCl<sub>2</sub>, 33 mM KCl, 100 mM sodium phosphate buffer, pH 7.4, and 10% S9. The S9 mixture was prepared immediately before use and kept on ice until used. The metabolic activity of the S9 was demonstrated by the response of the CP-treated cultures.

### 3. Flask Identification

Using computer generated labels or a permanent marker, each flask or tube was labeled with the work request number, the Haskell number, dose level, replicate indicator (A or B), metabolic activation system (+/-S9), exposure period, and date.

### 4. Frequency and Route of Administration of the Dosing Solutions

Cell cultures were treated once for approximately 4 hours in the absence and presence of S9 metabolic activation, and for 20 hours in the absence of metabolic activation. The test substance was added to the treatment medium in a test system-compatible vehicle. This frequency and

route of administration has been demonstrated to be effective in the detection of chemically-induced mutagenesis in this test system.<sup>(2-3)</sup>

#### 5. Preparation of Target Cells for the Preliminary Toxicity Assay

Exponentially growing CHO-K<sub>1</sub> cells were seeded in labeled, sterile flasks. Approximately  $5 \times 10^5$  cells/25 cm<sup>2</sup> flask were inoculated in complete medium (McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units penicillin/mL and 100 µg streptomycin/mL). Cultures were incubated at  $37 \pm 2^\circ\text{C}$  in a humidified atmosphere of  $5 \pm 2\%$  CO<sub>2</sub> in air for 16-24 hours.

#### 6. Preliminary Toxicity Assay to Select Dose Levels

The selection of dose levels for the cytogenetics assay was based on a preliminary toxicity assay. At the start of the assay, cell cultures seeded 16-24 hours earlier (at least one culture per concentration level) were exposed to 9 concentrations of the test substance and the vehicle and solvent control substances. The day when the cells were first exposed to the test substance was designated as test day 0. The dividing cells were harvested at a single timepoint, approximately 20 hours after the initiation of treatment (~1.5 times the normal cell cycle). The exposure times were approximately 4 and 20 hours in the absence of S9 metabolic activation, and approximately 4 hours in the presence of S9 metabolic activation. After the 4-hour exposure period only, the treatment medium was removed and replaced with complete McCoy's 5A culture medium. Approximately 20 hours after the initiation of exposure to the test substance, the cell cultures were microscopically inspected for the extent of monolayer confluency relative to the vehicle control. The cells were also harvested by trypsinization and counted by an automatic cell counter. Cell viability data were obtained, but not reported. The cell counts were used to determine cell growth inhibition relative to the solvent control.

Whenever possible, the highest concentration selected for the chromosome aberration assay induced greater than a 50% cell growth inhibition relative to the vehicle control. At least two additional dose levels, demonstrating limited toxicity or no toxicity were also evaluated. In cases where there was little or no cytotoxicity, the highest dose level tested and at least two lower dose levels were selected for analysis. In cases where there was little or no cytotoxicity, but a precipitate in the treatment medium was observed (with the naked eye), the lowest dose level demonstrating a precipitate and two other lower dose levels were selected for analysis.

If neither cytotoxicity nor precipitation was observed in the preliminary toxicity assay, the highest concentration was the limit dose of 5000 µg/mL or 10 mM, whichever was the lower.

The osmolality and pH of the vehicle and solvent controls, as well as the highest soluble test substance concentration in the culture media, were determined. Precipitation and pH were evaluated both at the beginning and the end of the treatment period by visual determination.

## 7. Chromosome Aberration Assay

### a. Preparation of Target Cells for the Chromosome Aberration Assay

Exponentially growing CHO-K<sub>1</sub> cells were seeded in labeled, sterile flasks. Approximately  $5 \times 10^5$  cells/25 cm<sup>2</sup> flask were inoculated in complete medium (McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units penicillin/mL and 100 µg streptomycin/mL). Cultures were incubated at  $37 \pm 2^\circ\text{C}$  in a humidified atmosphere of  $5 \pm 2\%$  CO<sub>2</sub> in air for 16-24 hours.

### b. Negative Control

The test substance vehicle (ethanol) was used as the concurrent negative control. Mineral spirits was used as a solvent control. The final concentration of vehicle/solvent in the treatment medium did not exceed 1%.

### c. Positive Control

The positive control substances were mitomycin-C (MMC) for the non-activated system and cyclophosphamide (CP) for the S9-activated system. The concentrations for MMC were 0.2 and 0.4 µg/mL, and for CP, 5 and 10 µg/mL. Both positive control substances were dissolved in sterile water. Two test concentrations of each positive control substance were used to ensure a valid assay; however, only one of the concentrations of each positive control was included in the cytogenetic analysis. The exposure periods for MMC were approximately 4 and 20 hours, and the exposure period for CP was approximately 4 hours. Exposure to the positive control substances was included in the chromosome aberration assay, but not in the preliminary cytotoxicity portion of the study. The positive control were not expected to contain any contaminants that would interfere with the conduct of the study, and were expected to be stable under the conditions of administration. Concentration verification of the positive control substances was not conducted. No positive control substances were used for numerical aberrations (polyploidy or endoreduplication, or both).

### d. Treatment of Target Cells

The day when the cells were first exposed to the test substance was designated as test day 0. Sixteen to twenty-four hours after seeding the CHO cultures, the culture medium was discarded and replaced with approximately 5 mL complete medium for the non-activated test condition, and 4 mL complete medium +1 mL of the S9 mixture for the activated test condition. The volumes were selected such that addition of the test substance volume (50 µL) resulted in a total volume of approximately 5 mL.

Sets of duplicate cultures were then administered an aliquot of the test substance (five concentrations were applied), the vehicle control, the solvent control, or two positive control substance concentrations for each test condition. The treatment medium was McCoy's medium for all test conditions. The cells were treated for approximately 4 and 20 hours in the non-activated test conditions, and for approximately 4 hours in the S9-activated test condition. After completion of the 4-hour exposure periods only, the cells were collected by centrifugation, washed once with phosphate buffered saline, fed with complete medium, and incubated until cell

harvest. The incubations were conducted at  $37 \pm 2^\circ\text{C}$  in a humidified atmosphere of  $5 \pm 2\%$   $\text{CO}_2$  in air.

A visual evaluation of all cultures was made at the beginning and end of the treatment period to assess both pH and precipitation. If necessary, the pH of the treatment medium was adjusted to maintain a neutral pH based on visual inspection. Documentation was made in the study records.

#### e. Collection of Metaphase Cells and Cell Harvest

The cells were arrested in metaphase approximately 18 hours after treatment initiation by adding Colcemid<sup>®</sup> to the cultures at a  $0.1 \mu\text{g}/\text{mL}$  final concentration in the culture media.

Approximately 20 hours after treatment initiation, the cultures were washed with PBS, trypsinized, collected by centrifugation, and the cells were resuspended in 5 mL fresh medium. A concurrent cytotoxicity measurement determining total cell growth inhibition (%) relative to the solvent control was conducted for all assays and test conditions using an automated cell counter. In addition, a physical examination of cell growth (monolayer cell confluency) was conducted for all test conditions. The cells were treated with 0.075M KCl hypotonic buffer, fixed once in methanol and 3 times in methanol:glacial acetic acid (3:1 v/v), and stored frozen. To prepare slides, the cells were collected by centrifugation and resuspended in fresh fixative. One slide per culture was prepared by applying an aliquot of the fixed cells onto clean microscope slides and air-drying them. The slides were stained by Giemsa and permanently mounted.

#### 8. Identification of the Slides

The slides were identified by the work request number, the Haskell number, dose level, replicate indicator (if applicable, i.e., A, B, C, etc.), metabolic activation system (+/-S9), exposure period (4 or 20 hours), and date.

#### 9. Cytogenetic Analyses

Cytogenetic analyses were conducted for at least three test substance concentrations, the vehicle control, the solvent control, and the positive control ( $0.2 \mu\text{g}/\text{mL}$  MMC or  $5 \mu\text{g}/\text{mL}$  CP). The highest test substance concentration level that was analyzed was that which caused a cell growth inhibition as close to or greater than 50% when compared to the vehicle control and that also had a sufficient amount of scorable metaphases or, if the test substance lacked toxicity, the highest scorable concentration used in the test. The percentage of cells in metaphase per at least 1000 cells scored per concentration level (at least 500 from each duplicate culture) was determined prior to coding the slides. After selection of the slides for cytogenetic analyses, the slides were coded and scored. Metaphase cells were selected for scoring based on good chromosome morphology and staining characteristics. Only metaphase cells with  $20 \pm 2$  centromeres were analyzed for structural aberrations. At least 200 metaphases per concentration level (100 from each duplicate culture), when available, were analyzed for structural aberrations.<sup>(5)</sup> Numerical aberrations were recorded as well. The number of metaphases evaluated per duplicate flask was less if 10 or more aberrant cells were observed among the first 25 cells scored. Chromatid-type aberrations included chromatid and isochromatid breaks and exchange figures. Chromosome-type aberrations included chromosome breaks and exchange

figures. Pulverized chromosome(s) and cells, and severely damaged cells (i.e., cells with  $\geq 10$  aberrations per cell) were recorded, and included in the analyses. The XY coordinates for the microscope stage were recorded for cells with structural aberrations.

#### **E. Criteria for Determination of a Valid Test**

An assay was considered acceptable for evaluation of test results only if all of the following criteria were satisfied. The metabolically activated and non-activated assays of the test are independent and, if necessary, were repeated separately.

##### **1. Negative Controls**

The frequency of cells with structural chromosome aberrations was in the frequency range of the historical control vehicle.

##### **2. Positive Controls**

The percentage of cells with structural chromosome aberrations must be statistically significantly greater ( $p < 0.05$ , Fisher's exact test) than the vehicle control response.

#### **F. Evaluation of Test Results and Statistical Analyses**

The clastogenic potential of the test substance was assessed based on its ability to induce structural chromosome aberrations. The experimental unit is the cell; therefore the percentage of cells with structural aberrations was used for the assessment.

Data was evaluated using scientific judgment. Statistical analysis was used as a guide to determine whether or not the test substance induced a positive response. Interpretation of the statistical analysis also relied on additional considerations including the magnitude of the observed test substance response relative to the vehicle control response and the presence of a dose responsive trend. Statistical analysis consisted of a Cochran-Armitage test for dose responsiveness and a Fisher's exact test to compare the percentage of cells with structural or numerical aberrations (or the percentage of cells with more than one aberration, if required) in the test substance treated groups with the vehicle control response.<sup>(6-7)</sup> At the discretion of the study director, statistical analyses may be conducted on the percentage of cells with numerical aberrations as well.

The following conditions were used as a guide to determine a positive response:

- A statistically significant increase ( $p < 0.05$ , Fisher's exact test) in the percentage of cells with structural aberrations was seen in one or more treatment groups relative to the vehicle control response.
- The observed increased frequencies were accompanied by a concentration-related increase.
- A statistically significant increase was observed at the highest dose only.

- Note: Statistically significant values that did not exceed the historical control range for the negative/vehicle control may be judged as not being biologically significant.

The following condition was used as a guide to determine an equivocal response:

- Results observed in any of the assays resulted in statistically significant elevations in structural chromosome aberrations at more than one test concentration level, except the highest dose, without demonstrating a dose-responsive trend.

The test substance was judged negative if the following condition was met:

- There was no statistically significant increase in the percentage of cells with structural aberrations in any treatment group relative to the vehicle control group.

### **G. Data Presentation**

The data was summarized in tables containing cell counts, cell growth inhibition, mitotic index, percent polyploidy/endoreduplication (numerical aberrations), number of cells analyzed, types of structural aberrations, frequencies of structural aberrations per cell, and the percentage of cells with structural aberrations. Chromatid and chromosome gaps were listed, but not added to the totals for structural aberration evaluation (gaps are not considered true structural damage).

## **RESULTS AND DISCUSSION**

### **A. Dosing Solution Analysis**

(Appendix B)

The test substance dosing solution analysis was conducted by CCAS. Data from the analysis of the samples during the study indicate that the test substance was at the targeted concentrations and within acceptable stability concentration range ( $\pm 20\%$  of nominal) when stored at the room temperature for >5 hours in the vehicle. Test substance was not found in the 0 mg/mL samples.

### **B. Solubility**

Ethanol was determined to be the solvent of choice based on the solubility of the test substance and compatibility with the target cells. The test substance was supplied as a 53% solution of cobalt naphthenate in 47% mineral spirits and further dilutions were prepared in ethanol. The test substance, as supplied, had a concentration of approximately 500 mg cobalt naphthenate/mL and was used undiluted for the highest stock concentration on this study and was a dark purple solution in the mineral spirits. The test substance was soluble in ethanol at all subsequent dilutions.

### C. Preliminary Toxicity Assay

(Table 1)

Concentrations for the chromosome aberration assay were selected based on the results from a preliminary toxicity test assessing the cell growth inhibition relative to the ethanol vehicle control. The cultures were microscopically inspected for the extent of monolayer confluency relative to the vehicle control. The data are not included in the report; the assessment is mainly an aid for study conduct. In the preliminary toxicity assay CHO cells were exposed to a total of nine concentrations of the test substance ranging from 5 to 5000  $\mu\text{g}$  cobalt naphthenate/mL as well as a vehicle (1% ethanol) and solvent control (0.47% mineral spirits). The cells were exposed for 4 hours in both the absence and presence of an exogenous metabolic activation system (Aroclor-induced S9), or for 20 hours in the absence of S9 activation. A visible precipitate was observed in the treatment medium at concentrations  $\geq 250$   $\mu\text{g}/\text{mL}$  in the beginning and end of the treatment periods. Osmolality and pH measurements were taken from the highest test substance concentration (5000  $\mu\text{g}/\text{mL}$ ) and the vehicle and solvent controls' media. Based on visual inspection of the pH-sensitive treatment medium at the beginning and end of the treatment periods, the pH of the highest test substance concentration in media was similar to the pH of the vehicle and solvent controls and the pH did not change during the treatment period. For example, in the non-activated test system, the measured pH for the highest test substance concentration in media was 7.19 compared to 7.49 and 7.57 for the ethanol and mineral spirits controls, respectively. In the S9-activated test system, the measured pH for the highest test substance concentration in media was 7.10 compared to 7.20 and 7.21 for the ethanol and mineral spirits controls, respectively. The osmolality of the highest test substance concentration tested in treatment media was 302 and 312 mmol/kg in the non-activated and activated test condition, respectively. The osmolality of the ethanol in the treatment medium was 496 and 503 mmol/kg in the non-activated and activated test condition, respectively. The osmolality of the mineral spirits in the treatment medium was 317 and 328 mmol/kg in the non-activated and activated test condition, respectively. The observed changes in osmolality between the test substance and the mineral spirits control were  $\leq 20\%$  and were not considered significant. The observed changes in osmolality between the test substance and the ethanol control were  $\geq 20\%$  but were not considered significant as a decrease in osmolality in the highest test substance concentration tested in treatment media compared to the ethanol control has no impact on the integrity or outcome of the study. Furthermore, the highest test substance concentration was supplied in mineral spirits and no ethanol was added, so this difference was expected. Substantial toxicity (greater than a 50% reduction in cell growth relative to the ethanol vehicle control) was observed at concentrations  $\geq 50$   $\mu\text{g}/\text{mL}$  in the 4- and 20-hour non-activated test conditions and at concentrations  $\geq 100$   $\mu\text{g}/\text{mL}$  in the 4-hour activated test condition. Based on the findings from the preliminary toxicity assay, the highest concentrations initially chosen for the chromosome aberration assay were 35  $\mu\text{g}/\text{mL}$  for the 4- and 20-hour non-activated test conditions and 100  $\mu\text{g}/\text{mL}$  for the 4-hour activated test condition.

#### D. Chromosome Aberration Assay

(Tables 2-10)

Based on the findings from the preliminary toxicity assay, the concentrations initially (trial 1) chosen for the chromosome aberration assay were 1, 5, 10, 30, and 35  $\mu\text{g}$  cobalt naphthenate/mL for the 4- and 20-hour non-activated test conditions, and 5, 10, 25, 50, and 100  $\mu\text{g}$  cobalt naphthenate/mL for the 4-hour activated test condition. Cells were also exposed to two concentrations of a positive control substance as well as a vehicle (1% ethanol) and solvent (0.01% mineral spirits) control. The test substance was soluble in the treatment medium at all concentrations tested. No precipitation was observed at the beginning or end of the treatment periods. Osmolality and pH measurements were taken from the highest test substance concentration (100  $\mu\text{g}/\text{mL}$ ) and the vehicle and solvent controls' media. Based on visual inspection of the pH-sensitive treatment medium at the beginning and end of the treatment periods, the pH of the highest test substance concentration in media was similar to the pH of the vehicle and solvent controls and the pH did not change during the treatment period. For example, in the non-activated test system, the measured pH for the highest test substance concentration in media was 7.41 compared to 7.36 and 7.38 for the ethanol and mineral spirits controls, respectively. In the S9-activated test system, the measured pH for the highest test substance concentration in media was 7.18 compared to 7.23 and 7.22 for the ethanol and mineral spirits controls, respectively. The osmolality of the highest test substance concentration tested in treatment media was 484 and 519 mmol/kg in the non-activated and activated test condition, respectively. The osmolality of the ethanol in the treatment medium was 492 and 503 mmol/kg in the non-activated and activated test condition, respectively. The osmolality of the mineral spirits in the treatment medium was 496 and 510 mmol/kg in the non-activated and activated test condition, respectively. The observed changes in osmolality between the test substance and the vehicle and solvent controls were  $\leq 20\%$  and were not considered significant. The cultures were also microscopically inspected for the extent of monolayer confluence relative to the vehicle control. These data are not included in the report; the assessment was mainly an aid for study conduct. In addition, the uncoded slides were microscopically observed for the presence of mitotic cells and the mitotic cells were counted to assess a mitotic index to ensure the selection of scorable test substance concentrations (Tables 2-4).

The concurrent toxicity data for trial 1 are presented in Tables 2-4. Substantial toxicity (greater than a 50% reduction in cell growth relative to the ethanol vehicle control) was observed at concentrations  $\geq 30$   $\mu\text{g}/\text{mL}$  in the 4- and 20-hour non-activated test conditions (67% and 81% reduction, respectively), and at 100  $\mu\text{g}/\text{mL}$  only in the 4-hour activated test condition (98% reduction). No mitotic figures were observed at concentrations  $\geq 30$   $\mu\text{g}/\text{mL}$  in the 20-hour non-activated test condition and at 100  $\mu\text{g}/\text{mL}$  in the 4-hour activated test condition. Because of this excessive toxicity, the assay was repeated (trial 2) for these two test conditions.

The concentrations chosen for trial 2 were 1, 5, 10, 20, and 25  $\mu\text{g}/\text{mL}$  for the 20-hour non-activated test condition, and 10, 20, 50, 70, and 90  $\mu\text{g}/\text{mL}$  for the 4-hour activated test condition. Cells were also exposed to two concentrations of a positive control substance as well as a vehicle (1% ethanol) and solvent (0.01% mineral spirits) control. The test substance was soluble in the treatment medium at all concentrations tested. No precipitation was observed at the beginning or end of the treatment periods. The cultures were also microscopically inspected for the extent of

monolayer confluence relative to the vehicle control. These data are not included in the report; the assessment was mainly an aid for study conduct. In addition, the uncoded slides were microscopically observed for the presence of mitotic cells and the mitotic cells were counted to assess a mitotic index to ensure the selection of scorable test substance concentrations (Tables 5 and 6).

The concurrent toxicity data for trial 2 are presented in Tables 5 and 6. Substantial toxicity was observed at 90  $\mu\text{g/mL}$  in the 4-hour activated test condition (63% reduction in cell growth and 74.5% mitotic index inhibition). No substantial toxicity was observed at any concentration in the 20-hour non-activated test condition; however there was a reduction in mitotic index of 52.3% at the 25  $\mu\text{g/mL}$  concentration level. Although the reduction in growth was less than 50%, this dose level was the highest dose level scored for chromosomal aberrations since the reduction in mitotic index was substantial.

Based on the concurrent toxicity assay and counting the mitotic index, concentrations selected for chromosome aberration analyses were 5, 10, and 30  $\mu\text{g/mL}$  (4-hour non-activated test condition) 20, 50, and 90  $\mu\text{g/mL}$  (4-hour activated test condition), and 5, 10, and 25  $\mu\text{g/mL}$  (20-hour non-activated test condition).

The cytogenetic analysis findings from the individual treatment cultures in the non-activated 4-hour exposure group are presented in Table 7 and summarized by group in Table 10. At the highest test concentration evaluated microscopically for chromosome aberrations, 30  $\mu\text{g/mL}$ , a 67% growth inhibition in relation to the ethanol control was observed (Table 2). The mitotic inhibition was 68.4% relative to the ethanol control. The percentage of cells with structural or numerical aberrations in the test substance-treated groups was not increased above that of the ethanol control group at any concentration ( $p \geq 0.05$ , Fisher's exact test). The percentage of cells with structurally damaged chromosomes in the MMC (positive control) treatment group (48%) was statistically significant ( $p < 0.05$ , Fisher's exact test).

The cytogenetic analysis findings from the individual treatment cultures in the S9-activated 4-hour exposure group are presented in Table 8 and summarized by group in Table 10. At the highest test concentration evaluated microscopically for chromosome aberrations, 90  $\mu\text{g/mL}$ , a 63% growth inhibition in relation to the ethanol control was observed (Table 5). The mitotic inhibition was 74.5% relative to the ethanol control. The percentage of cells with numerical aberrations in the test substance-treated groups was not significantly increased above that of the ethanol control group at any concentration ( $p \geq 0.05$ , Fisher's exact test). The percentage of cells with structural aberrations in the test substance-treated groups was increased above that of the ethanol control group at 50 (28%) and 90  $\mu\text{g/mL}$  (36%) ( $p < 0.05$ , Fisher's exact test and Cochran-Armitage trend test). These observed changes were dose-dependent, outside the historical control range of 0-6% for structural aberrations, and are considered biologically significant. The percentage of cells with structurally damaged chromosomes in the CP (positive control) treatment group (42%) was statistically significant ( $p < 0.05$ , Fisher's exact test).

The cytogenetic analysis findings from the individual treatment cultures in the non-activated 20-hour exposure group are presented in Table 9 and summarized by group in Table 10. At the highest test concentration evaluated microscopically for chromosome aberrations, 25  $\mu\text{g/mL}$ , a 28% growth inhibition in relation to the ethanol control was observed (Table 6). The mitotic

inhibition was 52.3% relative to the ethanol control. The percentage of cells with numerical aberrations in the test substance-treated groups was significantly increased above that of the ethanol control group at 5 (5%), 10 (5.5%), and 25 $\mu$ g/mL (4%) ( $p < 0.05$ , Fisher's exact test and Cochran-Armitage trend test). These observed changes were within the historical control range of 0-5% for numerical aberrations and were not considered biologically significant. The percentage of cells with numerical or structural aberrations in the test substance-treated groups was not significantly increased above that of the ethanol control group at any concentration ( $p \geq 0.05$ , Fisher's exact test). The percentage of cells with structurally damaged chromosomes in the MMC (positive control) treatment group (44%) was statistically significant ( $p < 0.05$ , Fisher's exact test).

### CONCLUSION

All criteria for a valid study were met. Under the conditions of this study, cobalt naphthenate was found to induce structural chromosome aberrations in the *in vitro* mammalian chromosome aberration test in Chinese hamster ovary cells in the S9-activated test system at 4 hours only. It was concluded that the test substance was positive in this *in vitro* test.

### RECORDS AND SAMPLE STORAGE

Specimens (if applicable), raw data, the protocol, amendments (if any), and the final report will be retained at Haskell Laboratory, Newark, Delaware, and will be returned to the sponsor six months after the last study in the metal carboxylates project, unless arrangements are made for further archiving.

Data recorded and archived electronically, and laboratory-specific raw data such as personnel files, instrument, equipment, refrigerator and/or freezer raw data will be retained at the facility where the work was done.

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**TABLES**

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TABLES

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EXPLANATORY NOTES

ABBREVIATIONS:

% Aberrant Cells	Cells with numerical aberrations include polyploid and endoreduplicated cells; cells with structural aberrations exclude cells with only gaps
Aberrations Per Cell	Cells with severely damaged chromosomes or with 10 or more structural aberrations were counted as 10 aberrations
Br	break
Cell Growth Index	(cells per flask treated group/cells per flask control group), expressed as a percentage
Cell Growth Inhibition	(cell growth index control group – cell growth index treated group)
CHO	Chinese hamster ovary
Chromatid Breaks	include chromatid and isochromatid breaks and fragments (Br); chromatid exchange figures (Ex) include quadriradials, triradials and complex rearrangements
Chromosome Breaks	include breaks and acentric fragments (Br); Dic, dicentric chromosome
Ex	exchange
Mitotic Index	(cells in mitosis / # cells scored), expressed as a percentage
SD	standard deviation
Severely Damaged Cells	includes cells with one or more pulverized chromosome and cells with 10 or more structural aberrations

Note: All calculated values are rounded.

**Table 1: Preliminary toxicity test using cobalt naphthenate in the absence or presence of exogenous metabolic activation**

Treatment* ( $\mu\text{g/mL}$ )	4 Hours						20 Hours					
	S9-			S9+			S9-			S9+		
	Cell Count (cells/mL x $10^6$ )	Cell Growth Index (%)	Cell Growth Inhibition (%)	Cell Count (cells/mL x $10^6$ )	Cell Growth Index (%)	Cell Growth Inhibition (%)	Cell Count (cells/mL x $10^6$ )	Cell Growth Index (%)	Cell Growth Inhibition (%)	Cell Count (cells/mL x $10^6$ )	Cell Growth Index (%)	Cell Growth Inhibition <sup>b</sup> (%)
Vehicle <sup>c</sup>	0.88	100	NA	0.82	100	NA	0.78	100	NA	0.78	100	NA
Solvent <sup>d</sup>	0.87	99	1	0.74	90	10	0.79	101	10	0.79	101	-1
5	0.85	97	3	0.63	77	23	0.78	100	23	0.78	100	0
10	0.78	89	11	0.80	98	2	0.76	97	2	0.76	97	3
25	0.66	75	25	0.69	84	16	0.53	68	16	0.53	68	32
50	0.01	1	99	0.54	66	34	0.03	4	34	0.03	4	96
100	0.00	0	100	0.36	44	56	0.02	2	56	0.02	2	98
250 <sup>e</sup>	0.01	1	99	0.03	3	97	0.02	3	97	0.02	3	97
500	0.01	1	99	0.02	2	98	0.04	4	98	0.04	4	96
1000	0.00	0	100	0.03	3	97	0.02	2	97	0.02	2	98
5000	0.00	0	100	0.01	1	99	0.01	1	99	0.01	1	99

<sup>a</sup>CHO cells were treated at 37°C.

<sup>b</sup>Negative value indicates growth increase and therefore no growth inhibition.

<sup>c</sup>Ethanol

<sup>d</sup>0.47% mineral spirits

<sup>e</sup>Lowest precipitating dose.

**Table 2: Concurrent toxicity test using cobalt naphthenate in the absence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)**

Treatment <sup>a</sup> (µg/mL)	Flask	Cell Count (cells/mL x 10 <sup>6</sup> )	Cell Growth Index (%)	Cell Growth Inhibition <sup>b</sup> (%)	Mitotic Index (%)	Mitotic Inhibition (%)
Vehicle <sup>c</sup>	A	0.60	100	NA	11.4	NA
	B	0.61				
Solvent <sup>d</sup>	A	0.41	79	21	8.1	28.9
	B	0.54				
1	A	0.68	104	-4	8.6	24.6
	B	0.58				
5	A	0.63	107	-7	8.3	27.2
	B	0.66				
10	A	0.64	104	-4	7.4	35.1
	B	0.62				
30	A	0.19	33	67	3.6	68.4
	B	0.21				
35	A	0.01	3	97	0.0	100.0
	B	0.02				
MMC 0.2	A	0.45	75	25	7.6	33.3
	B	0.46				
MMC 0.4	A	0.39	71	29	6.7	41.2
	B	0.47				

<sup>a</sup>CHO cells were treated at 37°C.

<sup>b</sup>Negative value indicates growth increase and therefore no growth inhibition.

<sup>c</sup>Ethanol

<sup>d</sup>0.01% mineral spirits

**Table 3: Concurrent toxicity test using cobalt naphthenate in the presence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period) (Trial 1)**

Treatment <sup>a</sup> (µg/mL)	Flask	Cell Count (cells/mL x 10 <sup>6</sup> )	Cell Growth Index (%)	Cell Growth Inhibition <sup>b</sup> (%)	Mitotic Index (%)	Mitotic Inhibition (%)
Vehicle <sup>c</sup>	A	0.52	100	NA	9.7	NA
	B	0.61				
Solvent <sup>d</sup>	A	0.59	99	1	8.5	12.4
	B	0.53				
5	A	0.56	103	-3	9.5	2.1
	B	0.60				
10	A	0.57	98	2	9.3	4.1
	B	0.54				
25	A	0.44	83	17	9.0	7.2
	B	0.50				
50	A	0.38	63	37	6.4	34.0
	B	0.33				
100	A	0.01	2	98	0.0	100.0
	B	0.02				
CP 5	A	0.39	67	33	5.3	45.4
	B	0.37				
CP 10	A	0.36	63	37	2.5	74.2
	B	0.35				

<sup>a</sup>CHO cells were treated at 37°C.

<sup>b</sup>Negative value indicates growth increase and therefore no growth inhibition.

<sup>c</sup>Ethanol

<sup>d</sup>0.01% mineral spirits

**Table 4: Concurrent toxicity test using cobalt naphthenate in the absence of exogenous metabolic activation (20-hour continuous treatment) (Trial 1)**

Treatment <sup>a</sup> ( $\mu\text{g/mL}$ )	Flask	Cell Count (cells/mL x $10^6$ )	Cell Growth Index (%)	Cell Growth Inhibition (%)	Mitotic Index (%)	Mitotic Inhibition (%)
Vehicle <sup>b</sup>	A	0.74	100	NA	10.1	NA
	B	0.69				
Solvent <sup>c</sup>	A	0.50	69	31	4.70	53.5
	B	0.49				
1	A	0.71	97	3	7.0	30.7
	B	0.67				
5	A	0.65	87	13	7.5	25.7
	B	0.59				
10	A	0.59	76	24	5.7	43.6
	B	0.50				
30	A	0.17	19	81	0.0	100.0
	B	0.10				
35	A	0.02	3	97	0.0	100.0
	B	0.03				
MMC 0.2	A	0.54	71	29	6.2	38.6
	B	0.47				
MMC 0.4	A	0.43	62	38	4.7	53.5
	B	0.45				

<sup>a</sup>CHO cells were treated at 37°C.

<sup>b</sup>Ethanol

<sup>c</sup>0.01% mineral spirits

**Table 5: Concurrent toxicity test using cobalt naphthenate in the presence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period (Trial 2))**

Treatment <sup>a</sup> (µg/mL)	Flask	Cell Count (cells/mL x 10 <sup>6</sup> )	Cell Growth Index (%)	Cell Growth Inhibition <sup>b</sup> (%)	Mitotic Index (%)	Mitotic Inhibition (%)
Vehicle <sup>c</sup>	A	0.43	100	NA	9.8	NA
	B	0.39				
Solvent <sup>d</sup>	A	0.41	110	-10	6.9	29.6
	B	0.49				
10	A	0.40	110	-10	7.8	20.4
	B	0.50				
20	A	0.33	83	17	8.3	15.3
	B	0.35				
50	A	0.22	62	38	5.8	40.8
	B	0.29				
70	A	0.30	84	16	5.5	43.9
	B	0.39				
90	A	0.17	37	63	2.5	74.5
	B	0.13				
CP 5	A	0.34	84	16	4.2	57.1
	B	0.35				
CP 10	A	0.30	74	26	3.0	69.4
	B	0.31				

<sup>a</sup>CHO cells were treated at 37°C.

<sup>b</sup>Negative value indicates growth increase and therefore no growth inhibition.

<sup>c</sup>Ethanol

<sup>d</sup>0.01% mineral spirits

**Table 6: Concurrent toxicity test using cobalt naphthenate in the absence of exogenous metabolic activation (20-hour continuous treatment) (Trial 2)**

Treatment <sup>a</sup> ( $\mu\text{g/mL}$ )	Flask	Cell Count (cells/mL $\times 10^6$ )	Cell Growth Index (%)	Cell Growth Inhibition <sup>b</sup> (%)	Mitotic Index (%)	Mitotic Inhibition (%)
Vehicle <sup>c</sup>	A	0.49	100	NA	8.8	NA
	B	0.58				
Solvent <sup>d</sup>	A	0.54	107	-7	7.6	13.6
	B	0.61				
1	A	0.52	102	-2	7.2	18.2
	B	0.57				
5	A	0.46	93	7	6.3	28.4
	B	0.54				
10	A	0.50	90	10	6.3	28.4
	B	0.46				
20	A	0.40	72	28	4.5	48.9
	B	0.37				
25	A	0.36	72	28	4.2	52.3
	B	0.41				
MMC 0.2	A	0.38	75	25	2.7	69.3
	B	0.42				
MMC 0.4	A	0.42	77	23	2.3	73.9
	B	0.40				

<sup>a</sup>CHO cells were treated at 37°C.

<sup>b</sup>Negative value indicates growth increase and therefore no growth inhibition.

<sup>c</sup>Ethanol

<sup>d</sup>0.01% mineral spirits

**Table 7: Cytogenetic analysis of CHO cells treated with cobalt naphthenate in the absence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)**

Treatment <sup>a</sup> ( $\mu\text{g}/\text{mL}$ )	Flask	Mitotic Index (%)	Cells Scored		% Aberrant Cells <sup>b</sup>	Structural		Gaps	Total Number of Structural Aberrations				Severely Damaged Cells	Average Aberrations Per Cell
			Numerical	Structural		Numerical	Structural		Chromatid	Chromosome	Br	Ex		
Vehicle <sup>c</sup>	A	9.8	100	100	5	6	1	5	0	1	0	0	0.060	
	B	13.0	100	100	1	8	4	5	0	2	2	0	0.090	
Solvent <sup>d</sup>	A	7.8	100	100	2	5	3	3	0	2	0	0	0.050	
	B	8.4	100	100	3	3	3	1	1	1	0	0	0.030	
5	A	7.6	100	100	3	5	0	4	0	0	1	0	0.050	
	B	9.0	100	100	3	5	1	3	1	1	0	0	0.050	
10	A	7.2	100	100	8	6	1	4	0	1	1	0	0.060	
	B	7.6	100	100	5	3	3	2	0	1	0	0	0.030	
30	A	3.2	100	100	5	14	1	13	1	2	0	0	0.160	
	B	4.0	100	100	3	10	3	7	0	4	0	0	0.110	
MMC 0.2	A	7.8	25	25	0	40	0	7	4	0	0	0	0.440	
	B	7.4	25	25	0	56	1	17	2	2	0	0	0.840	

<sup>a</sup>CHO cells were treated at 37°C.

<sup>b</sup>Excluding cells with only gaps.

<sup>c</sup>Ethanol

<sup>d</sup>0.01% mineral spirits

**Table 8: Cytogenetic analysis of CHO cells treated with cobalt naphthenate in the presence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)**

Treatment <sup>a</sup> (µg/mL)	Flask	Mitotic Index (%)	Cells Scored		% Aberrant Cells <sup>b</sup>	Total Number of Structural Aberrations		Severely Damaged Cells	Average Aberrations Per Cell			
			Numerical	Structural		Gaps	Chromatid			Chromosome		
						Br	Ex	Br	Ex			
Vehicle <sup>c</sup>	A	9.0	100	100	3	2	1	1	0	0	0	0.020
	B	10.6	100	100	2	4	4	0	0	0	0	0.040
Solvent <sup>d</sup>	A	7.4	100	100	1	7	3	5	1	0	1	0.070
	B	6.4	100	100	3	7	3	6	0	0	1	0.070
20	A	9.4	100	100	1	9	4	9	1	2	0	0.120
	B	7.2	100	100	2	4	1	3	1	0	0	0.040
50	A	7.0	100	100	3	16	6	12	1	4	0	0.170
	B	4.6	25	25	0	40	3	13	2	1	0	0.640
90	A	3.0	100	100	2	28	9	36	3	3	0	0.420
	B	2.0	25	25	0	44	3	10	0	2	10 <sup>e</sup>	0.880
CP 5	A	4.4	25	25	1	40	1	13	4	1	0	0.720
	B	4.0	25	25	0	44	0	8	4	3	0	0.600

<sup>a</sup>CHO cells were treated at 37°C.

<sup>b</sup>Excluding cells with only gaps.

<sup>c</sup>Ethanol

<sup>d</sup>0.01% mineral spirits

<sup>e</sup>One cell containing 10 or more aberrations (i.e., severely damaged cells).

**Table 9: Cytogenetic analysis of CHO cells treated with cobalt naphthenate in the absence of exogenous metabolic activation (20-hour continuous treatment)**

Treatment <sup>a</sup> (µg/mL)	Flask	Mitotic Index (%)	Cells Scored		% Aberrant Cells <sup>b</sup>		Total Number of Structural Aberrations				Severely Damaged Cells	Average Aberrations Per Cell	
			Numerical	Structural	Numerical	Structural	Gaps	Chromatid	Chromosome	Ex			
Vehicle <sup>c</sup>	A	9.0	100	100	0	5	1	4	0	0	1	0	0.050
	B	8.6	100	100	1	4	1	4	0	0	0	0	0.040
Solvent <sup>d</sup>	A	8.4	100	100	3	2	1	2	0	0	0	0	0.020
	B	6.8	100	100	4	3	2	3	0	0	0	0	0.030
5	A	6.6	100	100	5	2	2	1	0	1	0	0	0.020
	B	6.0	100	100	5	1	5	1	0	0	0	0	0.010
10	A	7.4	100	100	2	7	6	6	0	1	0	0	0.070
	B	5.2	100	100	9	3	3	1	0	2	0	0	0.030
25	A	4.2	100	100	6	6	3	5	0	1	0	0	0.060
	B	4.2	100	100	2	4	6	3	0	1	0	0	0.040
MMC 0.2	A	2.8	25	25	8	40	0	6	1	3	0	0	0.400
	B	2.6	25	25	0	48	0	7	2	3	0	0	0.480

<sup>a</sup>CHO cells were treated at 37°C.

<sup>b</sup>Excluding cells with only gaps.

<sup>c</sup>Ethanol

<sup>d</sup>0.01 % mineral spirits

Table 10: Summary

Treatment <sup>a</sup> µg/mL	S9 Activation	Treatment Time	Mitotic Index (%)	Cell Growth Inhibition <sup>b</sup> (%)	Cells Scored		Aberrations Per Cell		Cells with Aberrations <sup>c</sup>	
					Numerical	Structural	Mean	SD	Numerical (%)	Structural (%)
Vehicle <sup>d</sup>	-S9	4	11.4	NA	200	200	0.075	0.021	3.0	7.0
Solvent <sup>e</sup>	-S9	4	8.1	21	200	200	0.040	0.014	2.5	4.0
5	-S9	4	8.3	-7	200	200	0.050	0.000	3.0	5.0
10	-S9	4	7.4	-4	200	200	0.045	0.021	6.5	4.5
30	-S9	4	3.6	67	200	200	0.135	0.035	4.0	12.0
MMC 0.2	-S9	4	7.6	25	50	50	0.640	0.283	0.0	48.0 <sup>f</sup>
Vehicle	+S9	4	9.8	NA	200	200	0.030	0.014	2.5	3.0
Solvent	+S9	4	6.9	-10	200	200	0.070	0.000	2.0	7.0
20	+S9	4	8.3	17	200	200	0.080	0.057	1.5	6.5
50	+S9	4	5.8	38	125	125	0.405	0.332	1.5	28.0 <sup>fg</sup>
90	+S9	4	2.5	63	125	125	0.650	0.325	1.0	36.0 <sup>fg</sup>
CP 5	+S9	4	4.2	16	50	50	0.660	0.085	0.5	42.0 <sup>f</sup>
Vehicle	-S9	20	8.8	NA	200	200	0.045	0.007	0.5	4.5
Solvent	-S9	20	7.6	-7	200	200	0.025	0.007	3.5	2.5
5	-S9	20	6.3	7	200	200	0.015	0.007	5.0 <sup>fg</sup>	1.5
10	-S9	20	6.3	10	200	200	0.050	0.028	5.5 <sup>fg</sup>	5.0
25	-S9	20	4.2	28	200	200	0.050	0.014	4.0 <sup>fg</sup>	5.0
MMC 0.2	-S9	20	2.7	25	50	50	0.440	0.057	4.0 <sup>f</sup>	44.0 <sup>f</sup>

<sup>a</sup>CHO cells were treated at 37°C.

<sup>b</sup>Negative value indicates growth increase and, therefore, no growth reduction.

<sup>c</sup>Excluding cells with only gaps.

<sup>d</sup>Ethanol

<sup>e</sup>0.01% mineral spirits

<sup>f</sup>Statistically significant difference from control at  $p < 0.05$  by Fisher's exact test.

<sup>g</sup>Statistically significant difference from control at  $p < 0.05$  by Cochran-Armitage trend test.

**APPENDICES**

**Appendix A:**  
**Certificate of Analysis**

## Certificate of Analysis

THE RIGHT CHEMICALS  
THE RIGHT CHEMISTRY<sup>®</sup>

Cobalt naphthenate, approximately 53% in mineral spirits (6% Co)

Stock Number: 40387  
Lot Number: G08Q10

### Analysis

Appearance	Purple liquid
Co	5.93 %
Specific gravity	0.95

Certified by:

*Paul V Conolly*

Quality Control

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**Appendix B:**  
**Dosing Solution Analysis**

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*Study Title*

Analysis for H27606 in Dosing Samples

for

Cobalt Naphthenate: *In Vitro* Mammalian Chromosome Aberration Test in Chinese Hamster  
Ovary Cells

**AUTHOR:** Charles R. Powley, Ph.D.

**ANALYTICAL COMPLETED ON:** February 1, 2007

**PERFORMING LABORATORY:** E.I. du Pont de Nemours and Company  
DuPont Experimental Station (CCAS)  
Wilmington DE 19803  
U S A.

**STUDY NUMBER** DuPont-21243

**HASKELL WORK REQUEST NUMBER:** 16642

**HASKELL SERVICE CODE NUMBER:** 531

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DuPont-21243 AN

**CERTIFICATION**

We, the undersigned, declare that this report provides an accurate evaluation of data  
obtained from this study

CCAS  
Principle  
Investigator:

Jane B. Ramsey  
Jane B. Ramsey  
DuPont Analytical Solutions

Date: 01-Feb-2007

CCAS  
Management:

Roger A. Leach  
Roger A. Leach, Ph.D.  
DuPont Analytical Solutions

Date: 01-Feb-2007

Analytical Experimental Start Date: November 3, 2006

Analytical Experimental Termination Date: November 21, 2006

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#### SUMMARY

Samples from dosing formulations prepared November 2, 2006 containing cobalt naphthenate at concentrations of 0.1, 2.5 and 10.0 mg/mL were submitted for concentration verification analysis. A second set of samples containing 0.1, 2.0 and 9.0 mg/mL cobalt naphthenate was prepared on November 16, 2006 and submitted for analysis. The vehicle for the dosing formulation of the study was ethanol.

Concentrations of the cobalt naphthenate in separate dosing formulation samples were measured by inductively coupled plasma (ICP) spectroscopy.

The data for samples collected on both dates indicate that the test substance was at the targeted concentrations in the samples ( $\pm 20\%$  of nominal).

Cobalt naphthenate was not detected in the ethanol blank or in the 1% mineral spirits blank.

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## MATERIALS AND METHODS

### A. Samples

Samples from dosing formulations prepared November 2, 2006 containing cobalt naphthenate at concentrations of 0.1, 2.5, and 10.0 mg/mL were received at ambient temperature on November 3, 2006 for concentration verification analysis. A second set of samples prepared November 16, 2006 containing cobalt naphthenate at concentrations of 0.1, 2.0 and 9.0 mg/mL were received at ambient temperature on November 17, 2006 for the same analysis.

The vehicle for the dosing formulation for the study was ethanol. The test substance (H27606) was a 53% solution of cobalt naphthenate in mineral spirits (petroleum ether). Therefore blank solutions of both ethanol and 1% mineral spirits in ethanol were submitted with each set of study samples.

### B. Sample Analyses

#### 1. Dosing Formulation Treatment

An accurately-weighed aliquot of each dosing formulation sample (0.3 g nominal) was diluted with 3 mL of concentrated nitric acid and approximately 50 mL of deionized water. The 10.0 mg/mL samples were then diluted 1:9 with 2% nitric acid in water.

The samples were prepared and analyzed per the method "Analysis of Cobalt and Zirconium in Formulations of Metal Carboxylate Compounds by ICP-AES." Quality control samples run with each set were a sample of the blank dosing vehicle (ethanol), the 1% mineral spirits in ethanol, one of the formulated samples prepared in duplicate, and one of the samples prepared by doubling the initial amount of formulated sample weighed. A laboratory control spike at 1 ppm was also prepared by diluting nominally 0.3 g of the ethanol blank and 0.5 g of the 100 ppm working standard to 50 mL with 2% nitric acid and analyzed.

The density of each sample was determined by filling a 1-mL tared volumetric flask to the line with formulated sample and recording the weight in grams. The density of each sample was determined in triplicate.

Aliquots of submitted samples were analyzed one to five days after the formulations were prepared.

#### Instrumental Conditions

Concentrations of the cobalt naphthenate in dosing formulation samples were measured by ICP spectroscopy:

ICP Instrument:	Perkin-Elmer ICP Radial View Spectrometer
Power:	1300 W

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Nebulizer Flow: 0.8 L/min  
Auxiliary Flow: 0.2 L/min  
Plasma Flow: 15 L/min  
Pump Rate: 1.50 mL/min  
Equilibration Time: 15 seconds  
Torch View: Radial  
Wavelengths:  
Sc 361.383 nm Internal Standard  
Co 228.616 nm Analyte (quantification wavelength)  
Co 238.892 nm Analyte (confirmatory wavelength)  
Co 230.786 nm Analyte (confirmatory wavelength)

## 2. Calibration and Quantitation

A 1000-ppm stock solution of the cobalt analytical standard was purchased commercially. This solution was diluted with concentrated nitric acid and deionized water to produce a working standard of 100 ppm cobalt and calibration standards of 0.1, 0.5, 1, 5 and 10 ppm cobalt in approximately 2% nitric acid. Calibration standards and diluted dose samples were analyzed and spectral intensities were determined electronically.

The calibration standard curve was generated by regression analysis using the intensities of the calibration standard solutions (see Figure 1 for a representative calibration curve). Measured cobalt concentrations for dosing formulation samples were determined by applying the intensities from triplicate samplings of each sample to the calibration curve.

The cobalt concentrations were then used to calculate the corresponding mg/mL of cobalt naphthenate in ethanol using the dilution volumes, known percentage of Co in the test substance, known percentage of cobalt naphthenate in the test substance and the measured density of the formulated dose.

## RESULTS AND DISCUSSION

Cobalt naphthenate was not detected in the ethanol blank or in the 1% mineral spirits in ethanol solution.

Detailed analytical results from dosing formulation samples prepared November 2 and 16, 2006 for concentration verification are in Table 1. The test substance was at the targeted concentration in the samples ( $\pm 20\%$  of nominal) one to five days after formulation.

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**RECORDS AND SAMPLE STORAGE**

All primary data and the original report will be archived at DuPont Haskell Laboratory. All personnel and site-specific records will be archived at DuPont Analytical Solutions, DuPont Experimental Station.

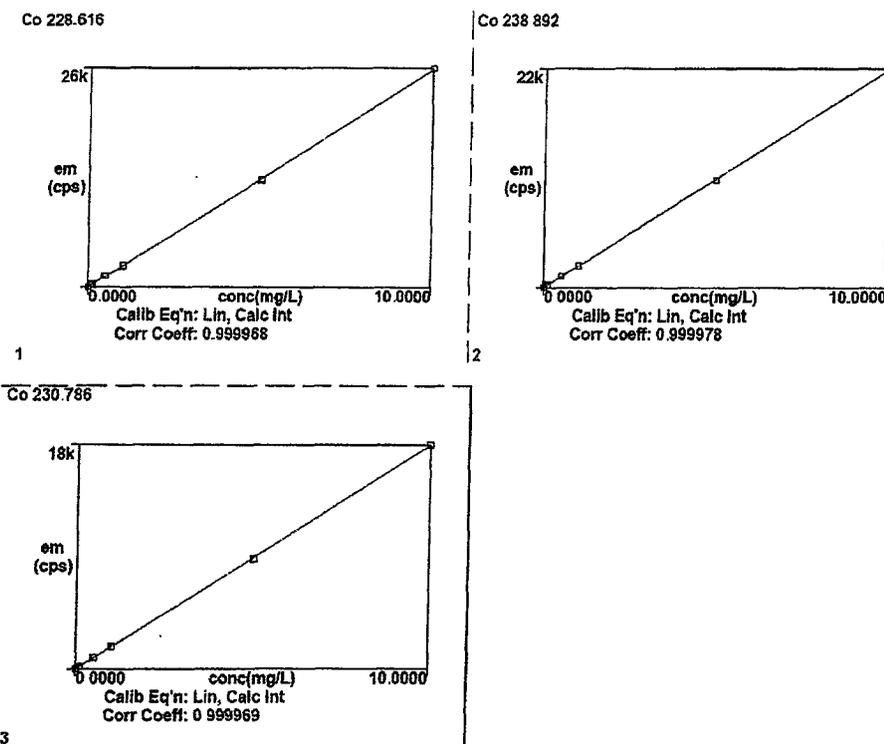
**Table 1: Concentration Verification Results of Cobalt Napthenate in Dosing Formulations**

<b>Test Substance</b>	H27606, Cobalt Napthenate (53% solution in mineral spirits)				
<b>% metal in test substance*</b>	5.9				
<b>Date of preparation</b>	02-Nov-2006				
<b>Samples</b>	Co (µg/g)	Average density (g/mL)	mg Co napthenate per mL formulation**	% of nominal***	Date of Analysis
1% Mineral Spirits Blank	ND	0.783	ND		03-Nov-2006
Ethanol Blank	ND	0.787	ND		03-Nov-2006
0.1 mg/mL H27606	15.3	0.785	0.11	110	03-Nov-2006
2.5 mg/mL H27606	369	0.789	2.62	105	03-Nov-2006
10 mg/mL H27606	1500	0.780	10.5	105	03-Nov-2006
10 mg/mL H27606 duplicate	1450	0.780	10.2	102	03-Nov-2006
10 mg/mL H27606 spike	1480	0.780	10.4	104	03-Nov-2006
10 mg/mL H27606 spike duplicate	1560	0.78	10.9	109	03-Nov-2006
<b>Lab Control Spike</b>	Co (µg/g)	Fortification level (ppm)	% Recovery		Date of Analysis
Lab control Sample - 1 ppm spike	0.96	1.00	95.6		03-Nov-2006
<b>Date of preparation</b>	16-Nov-2006				
<b>Samples</b>	Co (µg/g)	Average density (g/mL)	mg Co napthenate per mL formulation**	% of nominal***	Date of Analysis
1% Mineral Spirits Blank	ND	0.783	ND		21-Nov-2006
Ethanol Blank	ND	0.780	ND		21-Nov-2006
0.1 mg/mL H27606	15.1	0.786	0.11	110	21-Nov-2006
2.0 mg/mL H27606	295	0.782	2.07	104	21-Nov-2006
9.0 mg/mL H27606	1420	0.798	10.2	113	21-Nov-2006
9.0 mg/mL H27606 duplicate	1410	0.798	10.1	112	21-Nov-2006
9.0 mg/mL H27606 spike	1350	0.798	9.68	108	21-Nov-2006
<b>Lab Control Spike</b>	Co (µg/g)	Fortification level (ppm)	% Recovery		Date of Analysis
Lab control Sample - 1 ppm spike	1.06	1	106.00		21-Nov-2006

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Figure 1: Representative Analytical Calibration Curve



Representative calibration curves showing linear fit (line) to replicate intensity measurements (squares) for analytical standard solutions of cobalt over a concentration range of 0.1 to 10 ppm. 1: 228.616 nm, quantification signal; 2: 238.892 nm, confirmatory signal; 3: 230.786 nm, confirmatory signal

**Appendix C:**  
**Historical Control Data**

**HISTORICAL CONTROL DATA<sup>a</sup>**

Historical Values	Non-Activated Test System		S9-Activated Test System	
	Solvent Control (%)	Positive Control <sup>b</sup> (%)	Solvent Control (%)	Positive Control <sup>c</sup> (%)
<b>Structural Chromosome Aberrations</b>				
Mean	1.10	37.6	1.45	42.7
Standard Deviation	1.32	15.3	1.86	17.0
Range	0 - 5	10 - 62	0 - 6	9.5 - 68
<b>Numerical Chromosome Aberrations</b>				
Mean	1.41	0.77	1.32	0.40
Standard Deviation	1.84	1.68	1.60	0.44
Range	0 - 5	0 - 4	0 - 5	0 - 2

<sup>a</sup> Data are based on studies conducted 2003-2006. Data include all control solvents or diluents and metabolic activation systems based on Aroclor-induced rat liver S9.

<sup>b</sup> Mitomycin C (MMC)

<sup>c</sup> Cyclophosphamide (CP)