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FINAL REPORT

Primedica Redfield

Test Article: Potassium Perfluorobutane Sulfonate

SITEK Study No. 0623-3110

July 26, 2001

SITEK RESEARCH LABORATORIES
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FINAL REPORT

Study Title

Test for Chemical Induction of Chromosome Aberration in Cultured Chinese Hamster Ovary (CHO) Cells With and Without Metabolic Activation

Test Article

Potassium Perfluorobutane Sulfonate

Author

Jing Xu, M.D.

Study Initiation Date

July 31, 2000

Study Completion Date

July 26, 2001

Testing Facility

SITEK Research Laboratories
15235 Shady Grove Road, Suite 303
Rockville, Maryland 20850

Laboratory Project ID

Sponsor's Study No. 132-009
SITEK Study No. 0623-3110

Sponsor

Primedica Redfield
100 E. Boone Street
Redfield, Arkansas 72132

GLP COMPLIANCE STATEMENT

Study No. 0623-3110

Sponsor's Test Article I.D. Potassium Perfluorobutane Sulfonate

This study was conducted in compliance with the following Good Laboratory Practice standards:

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 1998.

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45 [ENV/MC/CHEM(98)17], Paris 1998,

Except for the stability of the test article and control substances under the experimental conditions, which was not determined by SITEK Research Laboratories. The dosing solutions were analyzed by Southern Research Institute, Birmingham, Alabama, however, the analysis was not performed under GLP conditions.

Signature _____ 7/26/2001

Jing Xu, M.D.
Study Director

Date

QUALITY ASSURANCE UNIT'S STATEMENT

Study No. 0623-3110

Sponsor's Test Article I.D. Potassium Perfluorobutane Sulfonate

The performance of this study was audited for adherence to the Good Laboratory Practice regulations for nonclinical laboratory studies by the Quality Assurance Unit of SITEK Research Laboratories. In this context, the facilities, equipment, personnel, methods, practices, controls, original data and reports have been inspected as per SITEK's Quality Assurance Unit's Standard Operating Procedures. The information contained within this report accurately reflects the raw data generated from this study.

Protocol Review Date: 08/01/00

The following phases were inspected for this study:

Inspection Date	Phases Inspected	Date Findings Reported to Study Director	Date Findings Reported to Management
08/22/00	Removal of the Test Article	08/22/00	08/22/00
10/17/00	Workbook Audit	10/17/00	10/20/00
10/20/00	Draft Report Audit	10/20/00	10/25/00
7/25/01	Final Report Audit	7/25/01	7/25/01

Signature Marcie A. Bauernschub 7/26/01

Marcie A. Bauernschub, B.S. Date
Manager, Quality Assurance Unit

STUDY DIRECTOR'S SIGNATURE PAGE

This study was performed under the supervision of Jing Xu, M.D., Study Director for In Vitro Cytogenetic Assays, at SITEK Research Laboratories, 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

The Final Report on this study was written by the Study Director and released on July 26, 2001.

Signature

Jing Xu, M.D.
Study Director

Jing Xu Date 7/26/2001

ABSTRACT

The test article, Potassium Perfluorobutane Sulfonate, was tested for its potential to induce chromosome aberrations in cultured Chinese hamster ovary (CHO) cells with and without exogenous metabolic activation.

A Range Finding Test (RFT) was performed to assess the toxicity of Potassium Perfluorobutane Sulfonate to CHO cells in this system. Potassium Perfluorobutane Sulfonate was dissolved in dimethyl sulfoxide (DMSO), therefore, DMSO was used as the solvent. Eight concentrations of the test article were tested ranging from 5.0-5000 μg/mL both with and without metabolic activation. DMSO and water were included in both systems as the solvent and untreated controls, respectively. The treatment time was 3 hours for both the non-activated and activated systems. Toxicity was assessed by the reduction in the Relative Cell Growth (RCG).

Based on the RCG results, concentrations of 500, 1000, 2500 and 5000 μg/mL were selected in both systems for the definitive Chromosome Aberration Assay (B1). In the definitive Chromosome Aberration Assay, Potassium Perfluorobutane Sulfonate was tested with concurrent untreated (water only), solvent (DMSO) and positive controls in both systems. Mitomycin-C (MMC), at concentrations of 0.08 and 0.2 μg/mL, was used as the positive control in the non-activated system. Cyclophosphamide (CP), at concentrations of 7.5 and 12.5 μg/mL, was used as the positive control in the activated system.

The parallel toxicity was determined by the reduction in the RCG and/or Relative Mitotic Index (RMI). Based on the RCG and RMI results, chromosome aberrations were scored from the three highest concentrations of 1000, 2500 and 5000 μg/mL in both systems. The corresponding untreated, solvent and positive controls (MMC at 0.2 μg/mL and CP at 7.5 μg/mL) were also scored. One hundred (100) metaphases were scored from each of the duplicate cultures at each concentration and the controls.

A confirmatory assay (B2) was performed without activation only, since the results from the definitive assay (B1) were negative in both systems. The procedures followed were the same as in the B1. The concentrations tested were 50, 100, 500, 1000, 2500 and 5000 μg/mL. A continuous treatment up to the harvest time of 1.5 x normal cell cycle time (18 hours) was performed, and the harvest time was 18 hours after the initiation of treatment. The parallel toxicity was determined by reduction in the RCG and/or RMI. Based on the RCG and RMI results, chromosome aberrations from the confirmatory assay were scored from the cells treated with the three concentrations of 500, 1000 and 5000 μg/mL. The corresponding untreated, solvent and positive controls (MMC at 0.2 μg/mL) were also scored. One hundred (100) metaphases were scored from each of the duplicate cultures at each concentration level and the controls.

The data from the definitive and confirmatory assays were statistically analyzed and evaluated. The results indicate that Potassium Perfluorobutane Sulfonate did not induce a statistically significant increase in the percentage of cells with aberrations at any of the concentrations tested, both with and without metabolic activation, in either assay when compared to the solvent controls.

Under the conditions of this study and according to the criteria set for evaluating the test results, Potassium Perfluorobutane Sulfonate was negative in the *in vitro* Chromosome Aberration Assay in CHO cells when tested with and without an exogenous metabolic activation system, and is not considered to be a clastogenic agent.

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INTRODUCTION

This study was conducted by Jing Xu, M.D., Keyan Wang, M.D., Dione Washington, B.A. and Weiyu Xie, M.D., at SITEK Research Laboratories from August 9, 2000 to September 25, 2000. The experimental procedures used to perform this study are described in the protocol.

The purpose of this study was to evaluate the test article, potassium perfluorobutane salfonate for its potential to cause genetic damage as manifested by induced chromosome aberrations in cultured Chinese hamster ovary (CHO) cells (1-5).

MATERIALS

TEST ARTICLE

1. Source (Supplier):	Primedica Redfield Laboratories (Sponsor)
2. Name:	Potassium Perfluorobutane Sulfonate
3. CAS No.:	29420-49-3
4. Lot No.:	2
5. Physical Appearance:	White powder
6. Date Received:	June 8, 2000 and September 7, 2000
7. Storage Conditions:	Room Temperature
8. Purity Information:	100%
9. Expiration Date:	04/06/01

CONTROL SUBSTANCES

Positive Controls

Mitomycin-C (MMC), which induces chromosome aberrations in the absence of metabolic activation, was used at 0.08 and 0.2 µg/mL in the non-activated system. Information on the MMC used in this study is provided below:

1. Source:	Sigma Chemical Company
2. CAS Registry No.:	50-07-7
3. Lot No.:	48H2511
4. Storage Conditions:	1-5°C
5. Expiration Date:	December 10, 2000

Cyclophosphamide (CP), which induces chromosome aberrations in the presence of metabolic activation, was used at 7.5 and 12.5 µg/mL in the activated system. Information on the CP used in this study is provided below:

1. Source:	Fluka Biochemika
2. CAS Registry No.:	6055-19-2
3. Lot No.:	299456/1
4. Storage Conditions:	1-5°C
5. Expiration Date:	July 18, 2002 and Jan. 24, 2004

MMC and CP were dissolved in sterile, deionized, distilled water (DDH_2O), diluted to 40 µg/mL (MMC) and 1.25 and 1.50 mg/mL (CP), dispensed in small aliquots, and stored at -10 to -20°C. One vial of each was thawed just prior to treatment and used in treating the cells. Information on the DDH_2O used in this study is provided below:

1. Source:	SITEK Research Laboratories
2. Batch No.:	24
3. Storage Conditions:	1-5°C
4. Expiration Date:	July 14, 2001

The stability of MMC and CP, under the experimental conditions, was not determined by SITEK Research Laboratories. However, both substances were used before their expiration dates.

Solvent Control

DMSO was used to dissolve the test article and to prepare the dosing solutions. Therefore, it was also used as the solvent control. Information on the DMSO used this study is provided below:

1. Source:	Fisher
2. CAS Registry No.:	67-68-5
3. Lot No.:	963769
4. Storage Conditions:	Room Temperature
5. Expiration Date:	December 12, 2001

INDICATOR CELLS

Source

The clone CHO-W-B1 of the CHO cell line, used in this study, originated at Litton Bionetics and was obtained by SITEK through the Environmental Health Research and Testing Laboratories, Lexington, Kentucky, in 1988. The doubling time of this cell line is approximately 12 hours, and its modal chromosome number is 21. The karyotype analysis of the cell line is periodically performed and documented at SITEK Research Laboratories.

Stock Cultures

The CHO cells were propagated in antibiotic-free medium to obtain a sufficient number of cells for freezing a large number of stock ampules. The cells were cryopreserved in McCoy's 5A medium, supplemented with 10% heat-inactivated fetal bovine serum and 8% DMSO, and stored in liquid nitrogen. Prior to using the stock cultures for the test,

representative ampules were tested for contaminating microorganisms, including mycoplasma, and found free of the above. Cell cultures, initiated from the stock ampules and maintained by subculturing for a maximum of 15 passages, were used in the assays.

CULTURE MEDIUM

The modified McCoy's 5A medium used in this study was obtained in powder form. The liquid culture medium (SITEK Batch No. 39) was prepared at SITEK Research Laboratories.

Information on the medium used in this study is provided below:

1. Source:	Gibco BRL
2. Lot No.:	1019960
3. Storage Conditions:	1-5°C
4. Expiration Date:	April 12, 2001

The fetal bovine serum was obtained from Summit Biotechnology, heat inactivated and used in this study. The antibiotics (penicillin and streptomycin) and the supplement (L-glutamine), used in this study were obtained from Gibco BRL. The lot numbers were recorded in the 0623-3110 study notebook.

METABOLIC ACTIVATION SYSTEM

The metabolic activation mixture consisted of phenobarbital/ß-naphthoflavone-induced rat liver homogenate (S-9) and the cofactor pool (6). The S-9 was made by SITEK Research Laboratories. Information on S-9 used in this study is provided below:

Source	S-9 Batch No.	Protein Content	Expiration Date
SITEK	101299	36.8 mg/mL	October 12, 2002

Prior to use in the assay, the S-9 was evaluated for its potential to induce an acceptable level of aberrations in CHO cells with CP. Immediately prior to use, the S-9 was thawed at room temperature and mixed with the cofactor pool to form the metabolic activation mixture, which consisted of 4mM NADP, 5mM glucose-6-phosphate, 30mM KCl, 10mM $MgCl_2$, 50mM sodium phosphate (pH 7.4) and 100 µL/mL of S-9 fraction. This mixture was diluted 1:4 by volume with serum-free medium before refeeding the cultures.

EXPERIMENTAL PROCEDURES

SOLUBILITY TEST

The test article had previously been tested for its solubility in water (in another study) and was insoluble. Therefore, DMSO was selected as the solvent for the solubility test.

One hundred (100) mg of the test article was weighed and DMSO was added in 0.1 mL increments until the test article was dissolved or until 1.5 mL of the solvent had been added. Based on the solubility of the test article in DMSO, the solubility of the test article in culture medium was determined. Twenty five (25) μL of test article at a concentration of 500 μg/mL in DMSO was added to 2.5 mL of culture medium in order to determine the appropriate concentrations to be used for the Range Finding Test.

DETERMINATION OF pH

To determine the pH of the test article 25 μL of test article at a concentration of 500 mg/mL in DMSO was added to 2.5 mL of complete medium, resulting in a final test article concentration of 5000 μg/mL in medium. If the test article caused a change in color of the medium, thereby altering the medium's pH and thus affecting the growth of cells, a buffered medium would have been used in the assay (7,8).

DETERMINATION OF OSMOLALITY

The osmolalities of the untreated, solvent controls and the highest test article concentration (5000 μg/mL) used in treating the cells in the Range Finding Test, were measured using a vapor pressure osmometer. Osmolalities in 500 mOsmol/kg of water and above are considered likely to cause DNA damage and would not be used in the assay (9).

PREPARATION OF DOSING SOLUTIONS

Dilution schemes were prepared prior to each treatment. The specified amount of test article was measured and dissolved in the required volume of DMSO to reach the highest concentration in the Range Finding Test and Chromosome Aberration Assay. The remaining concentrations were made by subsequent dilution. The dilution of the test article and the preparation of the dosing solutions were done immediately prior to treatment of the cells.

The stability of Potassium Perfluorobutane Sulfonate under the experimental conditions and the concentration of the test article dosing solutions were not analytically determined by

SITEK Research Laboratories. However, dosing solution samples (2.0 mL of each concentration) from the definitive and confirmatory Chromosome Aberration Assays (B1 and B2, respectively) were saved, stored frozen (-10 to -20°C) and shipped to the Sponsor on dry ice for analysis.

PREPARATION OF TEST CULTURES

Stock cultures, growing in T-75 cm^2 tissue culture flasks in antibiotic-free medium and showing 50-70% confluency, were harvested and used to prepare the test cultures. The culture medium from the T-75 cm^2 flasks was discarded, and the cells were washed with Ca^{++}- and Mg^{++}-free phosphate buffered saline (PBS). The cells were then dissociated by incubation at 37 ± 1°C with 0.05% trypsin. The cells were resuspended in complete culture medium containing 10% HIFBS, 2mM L-glutamine, 50 units/mL of penicillin and 50 µg/mL of streptomycin, the cell suspensions were pooled, and an aliquot of the cell suspension was diluted to the appropriate concentration and counted using a cell counter.

Based on the cell counts, a separate cell suspension with $1x10^5$ cells/mL was prepared in complete medium. Five (5.0) mL of this suspension was seeded in each T-25 cm^2 tissue culture flask to give $5x10^5$ cells per flask. These cultures were used in the Range Finding Test and the Chromosome Aberration Assays. The flasks were gassed with a mixture of approximately 5% CO_2 and 95% air, tightly capped, and incubated for approximately 20-24 hours prior to treatment.

TEST SYSTEM IDENTIFICATION

All of the test cultures were labeled in indelible ink with the SITEK study number, the activation system, the test article concentrations/controls, code number for the concentrations, A or B for distinguishing between tubes receiving the same treatment, and date of harvest. The slides were labeled with SITEK's study number, the code numbers for the concentrations tested, followed by A or B for the same treatment conditions, and the dates the slides were prepared.

RANGE FINDING TEST (A1)

In order to determine the toxicity of the test article, a Range Finding Test was performed.

Test cultures seeded approximately 24 hours earlier were used in the Range Finding Test. Two replicate cultures were used at each concentration level in both systems. The cells

were treated with concentrations of 5.0, 10, 50, 100, 500, 1000, 2500 and 5000 μg/mL in both the non-activated and activated systems, along with the untreated (water only) and solvent (DMSO) controls.

In the non-activated and activated systems, the culture medium was removed from the flasks and 5.0 mL of fresh, complete medium or 5.0 mL of serum-free medium with the S-9 activation mixture were added to each flask, respectively. The cells were exposed to the test article for 3 hours. The medium was then removed, and the cells were rinsed with PBS containing Ca^{++} and Mg^{++}, refed with 5.0 mL of complete medium, and incubated for an additional 15 hours, with 0.1 μg/mL Colcemid present during the final 2 hours.

All of the cultures were harvested 18 hours after the initiation of treatment (1.5 x normal cell cycle time). The medium with dividing cells was transferred into labeled centrifuge tubes, and the monolayer of cells was washed with PBS, dissociated with 0.05% trypsin, and resuspended in the collected medium. An aliquot of this cell suspension was counted using an electronic cell counter. The number of cells per flask was calculated for each concentration, and the Relative Cell Growth (RCG) was calculated according to the following formula:

$$RCG = \frac{\text{No. Cells in Test Flask}}{\text{No. Cells in Solvent Flask}} \times 100$$

The cytotoxicity was evaluated on the basis of the reduction in the RCG (10, 11). If possible, a concentration causing greater than 50% reduction in RCG was selected as the highest test concentration for the Chromosome Aberration Assay. In addition, three or more lower concentrations were included in the Assay. If no cytotoxicity was observed at the maximum concentration tested, the Chromosome Aberration Assay was performed at four decreasing concentrations starting with the maximum soluble concentration or one or two concentrations with precipitate.

DEFINITIVE CHROMOSOME ABERRATION ASSAY (B1)

The definitive Chromosome Aberration Assay was conducted with a single harvest at 1.5 x normal cell cycle time. Parallel toxicity was assessed by a reduction in the RCG and/or the Relative Mitotic Index (RMI). The slides for the RMI were also used for the determination of chromosome aberrations.

The test cultures were prepared as described earlier. Two replicate cultures, seeded with 500,000 cells each approximately 20-24 hours earlier, were treated at each concentration level in the non-activated and activated systems. The cells were treated at concentrations of 500, 1000, 2500 and 5000 μg/mL of the test article in both the non-activated and activated

systems. MMC was used as the positive control at 0.08 and 0.2 μg/mL in the non-activated system, and CP was used at 7.5 and 12.5 μg/mL in the activated system. Untreated and solvent controls were included in each system.

In the non-activated and activated systems, the culture medium was removed from the flasks and 5.0 mL of fresh, complete medium or 5.0 mL of serum-free medium with the S-9 activation mixture was added to each flask, respectively. The cells were exposed to the test article for 3 hours. The medium was then removed, and the cells were rinsed with PBS containing Ca^{++} and Mg^{++}, refed with 5.0 mL of complete medium, incubated for an additional 15 hours, with 0.1 μg/mL Colcemid present during the final two hours, and harvested 18 hours after the initiation of the treatment (1.5 x normal cell cycle time).

The cells were processed to determine the RCG as described in the Range Finding Test. The remaining cell suspension was processed to determine the RMI as described below.

The cells were collected by centrifugation (800 rpm), swelled in hypotonic KCl (0.075M), and fixed in methanol:glacial acetic acid (3:1) fixative. The fixed cells were stored at 1-5°C. The cells were then collected again by centrifugation, resuspended in a small volume of fresh fixative, and dropped on microslides. The slides were air dried, stained in 5% Giemsa stain, and mounted in Permount using #1 cover glasses. The coded slides were scored for Mitotic Index (MI). A total of 1000 cells were scored from each concentration (500 from each duplicate flask), and the number of dividing cells were recorded. The MI for each concentration was calculated using the following formula:

$$MI = \frac{\text{No. of Dividing Cells from 1000 Cells}}{10}$$

The RMI was calculated as shown below:

$$RMI = \frac{\text{Test Concentration MI}}{\text{Solvent Control MI}} \times 100$$

Based on the RCG and RMI results, the chromosome aberrations were scored from the 3 highest concentrations of 1000, 2500 and 5000 μg/mL in both the non-activated system and activated system. The corresponding controls were also scored. The concentrations of 0.2 μg/mL for MMC and 7.5 μg/mL for CP were scored from the positive controls in the non-activated and activated systems, respectively. One hundred (100) metaphases were scored for chromosome aberrations from each of the two duplicate flasks, thus providing 200 metaphases per concentration level. Only cells with 19-23 chromosomes were scored, and the microscope coordinates of each cell with aberrations were recorded. In addition, the number of polyploid and endoreduplicated cells in a total of 100 dividing cells was scored and recorded for each culture.

The types of chromosome aberrations scored and the corresponding abbreviations used are given below (12, 13, 14):

1. Chromatid-type Aberrations

Simple:

tg - Chromatid gap - an achromatic region occurring along the length of a chromatid in which there is no misalignment.

tb - Chromatid break - a discontinuity occurring along the length of either of the two chromatids in which there is a misalignment.

isb - Isochromatid break - a discontinuity occurring in both the chromatids at the same locus showing complete rejoining or sister chromatid union at both the broken ends or incomplete rejoining, i.e., only at one of the two broken ends.

Complex:

qr - Quadriradial - chromatid interchanges between chromosomes leading to four-armed configurations. This could be asymmetrical with formation of a dicentric and an acentric chromatid, if union is complete, or symmetrical where there is no formation of a dicentric and an acentric chromatid.

tr - Triradial - isochromatid-chromatid exchanges resulting in three-armed configurations and sometimes fragments. The latter should not be scored as an independent aberration. The triradial could be monocentric or dicentric.

id - Interstitial deletion - intra-arm intra-changes resulting in deletion of small fragments which, however, stay in association with the parent chromatid.

ci - Chromatid intrachange - exchanges occurring between arms of the same chromosome resulting in asymmetrical (rings) or symmetrical configurations.

cr - Complex interchanges - multiarmed configurations resulting from breakage and reunion of two or more chromosomes.

2. Chromosome-type Aberrations

Simple:

sg - Chromosome gap - an achromatic region occurring in both chromatids of the chromosome at the same locus with no misalignment.

sb - Chromosome break - a discontinuity at the same locus in both chromatids, giving one acentric fragment which may be misaligned and a shortened monocentric chromosome, and where there is no sister chromatid union.

Complex:

d - Dicentric - an asymmetrical exchange between two chromosomes resulting in a chromosome with two centromeres with or without an accompanying acentric fragment which should not be score as a second aberration.

r - Ring - inter-arm intrachange happening within the chromosome, leading to formation of a centric ring with or without a chromosome fragment. The fragment should not be scored as a second aberration.

dm - Double minutes - intra-arm intrachanges leading to tight acentric paired rings.

3. Other Aberrations

pu - Pulverized chromosome or chromosomes - shattering of chromatid material resulting in several minute pieces. The identity of the chromosome is not decipherable. Considered as a single aberration.

sd - Severely damaged cell - cell with 10 or more aberrations.

pp - Polyploid cells - metaphases with multiples or approximate multiples of the haploid set of chromosomes. Not scored for structural aberrations.

e - Endoreduplication - metaphases with paired duplicated chromosomes or diplochromosomes; they are not scored for structural aberrations.

The chromosome aberration data from the score sheets were consolidated on a Summary Table. The number of aberrations per cell and the percentage of cells with one or more aberrations for each concentration level were calculated. The data were consolidated separately for the two cultures at each concentration, then pooled and presented together. Chromatid gaps and chromosome gaps were scored, but they were not included in calculating the percentage of cells with aberrations and the number of aberrations per cell. Of the remaining aberrations, each aberration scored was counted as one, except severely damaged cell (sd), which was considered equal to 10 aberrations in calculating the number of aberrations per cell. Endoreduplicated and polyploid cells were recorded separately in percentages.

CONFIRMATORY CHROMOSOME ABERRATION ASSAY (B2)

A confirmatory assay (B2) was performed in the non-activated system only, since the results from the B1 showed a negative response in both systems. The procedures were the same as in the B1, except that the test article treatment period was extended to 18 hours (1.5 x normal cell cycle time). The concentrations tested were 50, 100, 500, 1000, 2500 and 5000 μg/mL. The cells were harvested 18 hours (1.5 x normal cell cycle time) after the initiation of treatment. Parallel toxicity was determined by the RCG and RMI. Based on the RCG and RMI results, chromosome aberrations were scored from cells treated with three concentrations of 500, 1000 and 5000 μg/mL. One hundred (100) metaphases were scored from each of the two replicate cultures at each concentration level and the controls.

STATISTICAL ANALYSIS

The data for the percentage of cells with aberrations for each concentration were compared to the solvent control values using the Chi-square test. A validated statistical package (Epistat) was used to calculate the p values for the Chi-square test. Results were considered significant if $p \leq 0.05$. Statistical analysis was not performed if the test concentration value was equal to or less than the concurrent or historical solvent control.

If a positive response was indicated by the Chi-square test, the Cochran-Armitage test (trend test) was performed for evidence of a concentration-related response (15). The trend test was considered positive if $p \leq 0.05$. The trend test was not performed for this study.

CRITERIA FOR A VALID ASSAY

1. In the solvent control, the percentage of cells with aberrations should not have exceeded 4%.

2. At least 25% of the cells scored in the positive control should have showed one or more chromosome aberrations.

3. At least one of the test concentrations scored should have showed greater than 50% reduction in the RCG and/or RMI. This requirement should not be applied to test articles where no apparent toxicity could be achieved at the maximum soluble concentration or the highest allowable concentration.

EVALUATION OF TEST RESULTS

Positive Response

The test article was considered to have caused a positive response in this assay if the test article showed a positive dose-response trend and a statistically significant increase over that of the solvent controls in the percentage of cells with aberrations at one or more concentrations.

Negative Response

The test article was considered to have caused a negative response if none of the test concentrations showed a statistically significant increase in the percentage of aberrant cells.

Equivocal Response

The test article was considered to have caused an equivocal response if there was a statistically significant increase in the percentage of cells with aberrations without an accompanying positive dose-response trend.

RESULTS

SOLUBILITY TEST

One hundred (100) mg of the test article was dissolved in 0.2 mL of DMSO yielding a solution with a concentration of 500 mg/mL in DMSO. The test article was soluble in medium at 5000 μg/mL. Based on the solubility in the medium, 5000 μg/mL was selected as the highest concentration for the Range Finding Test.

DETERMINATION OF pH

It was not necessary to determine the pH of the test article, since the color of the pH indicator in the complete medium did not change at the highest test article concentration of 5000 μg/mL.

OSMOLALITY TEST

The osmolalities of the untreated, solvent and test article-containing medium used in treating the cells at 5000 μg/mL in the Range Finding Test are given below:

	OSMOLALITY (mOsmol/kg water)	
Concentration	Without Activation	With Activation
Untreated (water)	277	290
Solvent (DMSO)	382	334
5000 μg/mL	394	314

Even at the highest concentration tested, changes in osmolality did not exceed 500 mOsmol/kg of water, above which DNA damage is considered probable. Therefore, no effect on osmolality was caused by the addition of the test article at the maximum concentration.

RANGE FINDING TEST (A1)

The results of the Range Finding Test (A1) are summarized and presented in Table 1 (RCG) (Appendix I).

The results showed that there was no obvious reduction (more than 50% versus that of the solvent control) in RCG even at the highest concentration of 5000 μg/mL in both the non-

activated and activated systems. The RCGs ranged from 84%-109% and 128-304%, for the non-activated and activated systems, respectively, for the test article concentrations of 5.0-5000 μg/mL.

DEFINITIVE CHROMOSOME ABERRATION ASSAY (B1)

Based on the toxicity results from the Range Finding Test, the concentrations of 500, 1000, 2500 and 5000 μg/mL were selected for the Chromosome Aberration Assay (B1) in both the non-activated and activated systems.

The parallel toxicity results, as determined by the reduction in the RCG and/or RMI of the treated cells in the non-activated and activated systems, are presented in Tables 2 (RCG) and 3 (RMI) (Appendix I).

The RCG results showed that no obvious reduction (more than 50%) was observed at any concentration in either system. The RCGs ranged from 104-133% in the non-activated system, and from 84-110% in the activated system. The RMI data showed that the RMIs ranged from 100-144% in the non-activated system, and from 61-94% in the activated system. Therefore, chromosome aberrations were scored from the 3 highest concentrations of 1000, 2500 and 5000 μg/mL in both the non-activated and activated systems. In addition, the corresponding untreated (water), solvent (DMSO) and positive controls (MMC at 0.2 μg/mL and CP at 7.5 μg/mL) were also scored. One hundred (100) metaphases were scored from each of the two replicate cultures at each concentration and the controls.

The results of the Chromosome Aberration Assay (B1) in the non-activated and activated systems are summarized and presented in Tables 4 and 5, respectively (Appendix I).

The averages of the percentage of cells with aberrations scored from the assay are summarized below:

	Average Percentage of Cells with Aberrations	
Treatment	**Without Activation**	**With Activation**
Untreated (water)	0.0%	0.0%
Solvent Control (DMSO)	0.0%	0.0%
Test Article Concentrations (1000, 2500 and 5000 μg/mL)	0.0%	0.0%
Positive Control	25.5%*	26.0%*

* Statistically significant response using the Chi-square test.

CONFIRMATORY CHROMOSOME ABERRATION ASSAY (B2)

The parallel toxicity results of the confirmatory assay (B2) without activation are presented in Tables 6 (RCG) and 7 (RMI) (Appendix I). The results showed that the RCGs ranged from 46-165%. The RMIs ranged from 17-88%. No metaphases were found at the concentration of 2500 μg/mL. Therefore, chromosome aberrations were scored from the three concentrations of 500, 1000 and 5000 μg/mL. In addition, the corresponding untreated, solvent and positive (MMC at 0.2 μg/mL) controls were also scored. One hundred (100) metaphases were scored from each of the two replicate cultures at each concentration and the controls.

The results of the confirmatory Chromosome Aberration Assay (B2) are summarized and presented in Table 8 (Appendix I).

The averages of the percentage of cells with aberrations scored in this assay are summarized below:

Treatment	Average Percentage of Cells with Aberrations Without Activation
Untreated (water)	0.0%
Solvent Control (DMSO)	0.0%
Test Article Concentrations (500, 1000 and 5000 μg/mL)	0.0-0.5%
Positive Control (MMC at 0.2 μg/mL)	29.0%*

* Statistically significant response using the Chi-square test

STATISTICAL ANALYSIS

Statistical analysis showed that, none of the test article concentrations tested in the non-activated or activated systems, in either assay, were found to have induced a statistically significant increase in the percentage of cells with aberrations over the solvent controls.

The percentage of polyploidy (pp) and endoreduplicated cells (e) was in the normal range (0-5.0% and 0-1.0% for pp and e, respectively).

All of the criteria for a valid assay were met.

ANALYSIS OF DOSING SOLUTIONS

The test article dosing solutions were analyzed by Southern Research Institute and the results are presented in Appendix IV. Dosing solution samples ranging in concentration from 5.0 to 500 mg/mL were analyzed from the definitive and confirmatory Chromosome Aberration Assays. All dosing solution samples were found to be in the acceptable range of ±10% of the labeled (targeted) concentration, except two; 100 mg/mL from the definitive assay was 111% of the targeted concentration and 250 mg/mL from the confirmatory was 117%. The latter sample was analyzed a second time and found to have a concentration of 116% of the targeted value. However, this had no effect on the study outcome.

CONCLUSIONS

Test article, Potassium Perfluorobutane Sulfonate, was tested for its potential to induce chromosome aberrations in cultured CHO cells with and without exogenous metabolic activation in the Chromosome Aberration Assay.

Under the conditions of this study, and according to the criteria set for evaluating the test results, Potassium Perfluorobutane Sulfonate was negative in the Chromosome Aberration Assay with and without exogenous metabolic activation and is not considered to be a clastogenic agent.

ARCHIVES

The raw data, documentation, protocol, protocol amendments/deviations and the Final Report along with an electronic files containing data tables and the Final Report of the study will be maintained, for at least 10 years, at SITEK Research Laboratories' archives at 15235 Shady Grove Road, Suite 303, Rockville, Maryland, 20850. After that time, the Sponsor will be notified for further arrangements regarding this study's records.

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APPENDIX I

DATA TABLES

TABLE 1
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
RCG - RANGE FINDING TEST

TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SPONSOR: Primedica Redfield
SOLVENT: DMSO
SITEK STUDY NO.: 0623-3110
TRIAL NO.: A1

WITHOUT ACTIVATION					WITH ACTIVATION				
Test Article Conc. (µg/mL)		No. of Cells per Flask	Mean No. of Cells X 10^6	RCG*	Test Article Conc. (µg/mL)		No. of Cells per Flask	Mean No. of Cells X 10^6	RCG*
Untreated	A	2.34	2.28	165%	Untreated	A	2.61	2.49	311%
Untreated	B	2.22			Untreated	B	2.37		
Solvent	A	1.11	1.38	100%	Solvent	A	1.33	0.80	100%
Solvent	B	1.64			Solvent	B	0.26		
5.0	A	1.57	1.51	109%	5.0	A	1.25	1.58	198%
5.0	B	1.45			5.0	B	1.90		
10	A	0.80	1.24	90%	10	A	1.37	1.65	206%
10	B	1.68			10	B	1.92		
50	A	1.07	1.16	84%	50	A	1.73	1.69	211%
50	B	1.25			50	B	1.64		
100	A	1.45	1.27	92%	100	A	1.07	1.24	155%
100	B	1.08			100	B	1.41		
500	A	1.27	1.19	86%	500	A	0.82	1.15	144%
500	B	1.11			500	B	1.47		
1000	A	1.48	1.32	96%	1000	A	0.78	1.42	178%
1000	B	1.15			1000	B	2.06		
2500	A	1.82	1.28	93%	2500	A	1.37	1.02	128%
2500	B	0.73			2500	B	0.67		
5000	A	1.51	1.50	109%	5000	A	2.15	2.43	304%
5000	B	1.49			5000	B	2.70		

*RCG = Relative Cell Growth = $\frac{\text{No. of Cells in the Test Flask}}{\text{No. of Cells in the Solvent Flask}}$ X 100

TABLE 2
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
RCG - DEFINITIVE ASSAY

TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SPONSOR: Primedica Redfield
SOLVENT: DMSO
SITEK STUDY NO.: 0623-3110
TRIAL NO.: B1

WITHOUT ACTIVATION					WITH ACTIVATION				
Test Article Conc. (µg/mL)		No. of Cells per Flask	Mean No. of Cells X 10^6	RCG*	Test Article Conc. (µg/mL)		No. of Cells per Flask	Mean No. of Cells X 10^6	RCG*
Untreated	A	2.54	2.54	155%	Untreated	A	2.20	2.22	102%
Untreated	B	2.54			Untreated	B	2.23		
Solvent	A	1.40	1.64	100%	Solvent	A	2.44	2.18	100%
Solvent	B	1.88			Solvent	B	1.92		
500	A	2.06	2.18	133%	500	A	2.24	1.83	84%
500	B	2.29			500	B	1.41		
1000	A	1.87	1.71	104%	1000	A	2.38	2.37	109%
1000	B	1.54			1000	B	2.35		
2500	A	2.03	1.99	121%	2500	A	2.55	2.40	110%
2500	B	1.95			2500	B	2.24		
5000	A	2.02	2.07	126%	5000	A	2.25	2.25	103%
5000	B	2.11			5000	B	2.25		

*RCG = Relative Cell Growth = $\frac{\text{No. of Cells in the Test Flask}}{\text{No. of Cells in the Solvent Flask}}$ X 100

TABLE 3

CHROMOSOME ABERRATION ASSAY IN CHO CELLS

MITOTIC INDEX - DEFINITIVE ASSAY

SPONSOR : Primedica Redfield SOLVENT : DMSO STUDY NO. : 0623-3110

TEST ARTICLE : Potassium Perfluorobutane Sulfonate TRIAL NO.: B1

Without Activation - Treatment: 3 Hours Harvest: 18 Hours						With Activation - Treatment: 3 Hours Harvest: 18 Hours					
Test Article Concentration (µg/mL)	Tube No.		No. of Dividing Cells/50	Mean Mitotic Index	Relative Mitotic Index	Test Article Concentration (µg/mL)	Tube No.		No. of Dividing Cells/500	Mean Mitotic Index	Relative Mitotic Index
Untreated	58	A	42	8.0	145%	Untreated	27	A	47	7.2	200%
		B	38					B	25		
Solvent	77	A	20	5.5	100%	Solvent	67	A	15	3.6	100%
		B	35					B	21		
500	25	A	45	7.9	144%	500	22	A	10	2.2	61%
		B	34					B	12		
1000	53	A	32	7.4	135%	1000	99	A	13	2.5	69%
		B	42					B	12		
2500	55	A	16	5.5	100%	2500	59	A	17	3.4	94%
		B	39					B	17		
5000	19	A	26	5.9	107%	5000	52	A	16	3.1	86%
		B	33					B	15		
MMC 0.08	60	A	17	3.6	45%	CP 7.5	5	A	10	1.5	21%
		B	19					B	5		
MMC 0.20	74	A	12	1.8	23%	CP 12.5	44	A	5	1.3	18%
		B	6					B	8		

The positive controls were compared to Untreated since the solvent for MMC and CP was water.

$$MI = \frac{\text{No. of dividing cells scored from 1000 cells}}{10}$$

$$RMI = \frac{\text{Test Dose MI}}{\text{Solvent Control MI}} \times 100$$

TABLE 4

CHROMOSOME ABERRATION ASSAY IN CHO CELLS

CHROMOSOME ABERRATIONS - DEFINITIVE ASSAY

TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SPONSOR: Primedica Redfield
SOLVENT: DMSO

TREATMENT TIME: 3 Hours
HARVEST TIME: 18 Hours

SITEK STUDY NO.: 0623-3110
TRIAL NO.: B1
METABOLIC ACTIVATION: Yes ( ) No (X)

TREATMENT AND CONC. (µg/mL)	CELLS Scored	NUMBER AND TYPE OF ABERRATIONS																	NO. OF ABS. PER CELL	% CELLS WITH ABS.	P-VALUE IN CHI-SQUARE**
		NOT COMPUTED				Chromatid Type							Chromosme Type								
						Simple		Complex					Simple	Complex			Others				
		tg	sg	%e	%pp	tb	isb	tr	qr	cr	id	ci	sb	d	r	dm	pu	sd*			
Untreated A	100			0	0														0.00	0.0	
Untreated B	100			0	0														0.00	0.0	
Untreated A+B	200			0.0	0.0														0.000	0.0	
Solvent A	100			0	0														0.00	0.0	
Solvent B	100			0	0														0.00	0.0	
Solvent A+B	200			0.0	0.0														0.000	0.0	
1000 A	100			0	0														0.00	0.0	
1000 B	100			0	0														0.00	0.0	
1000 A+B	200			0.0	0.0														0.000	0.0	= SOLVENT
2500 A	100			0	0														0.00	0.0	
2500 B	100			0	0														0.00	0.0	
2500 A+B	200			0.0	0.0														0.000	0.0	= SOLVENT
5000 A	100			0	0														0.00	0.0	
5000 B	100			0	0														0.00	0.0	
5000 A+B	200			0.0	0.0														0.000	0.0	= SOLVENT
MMC 0.2 A	100			0	0	7		9	3	1			9						0.29	25.0	
MMC 0.2 B	100		1	0	0	4	1	5	8	4			5						0.27	26.0	
MMC 0.2 A+B	200		1	0.0	0.0	11	1	14	11	5			14						0.280	25.5	< 0.0001

* sd = 10 aberrations in calculations.

**Statistical analyses done on the % cells with aberrations.
In the Chi-square test, MMC was compared to SITEK's historical Untreated control data (0.62%) since the solvent for MMC was water and the concurrent value was 0%.

TABLE 5

CHROMOSOME ABERRATION ASSAY IN CHO CELLS

CHROMOSOME ABERRATIONS - DEFINITIVE ASSAY

TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SPONSOR: Primedica Redfield
SOLVENT: DMSO

TREATMENT TIME: 3 Hours
HARVEST TIME: 18 Hours

SITEK STUDY NO.: 0623-3110
TRIAL NO.: B1
METABOLIC ACTIVATION: Yes (X) No ( )

TREATMENT AND CONC. (µg/mL)	CELLS Scored	NUMBER AND TYPE OF ABERRATIONS																	NO. OF ABS. PER CELL	% CELLS WITH ABS.	P-VALUE IN CHI-SQUARE**
		NOT COMPUTED				Chromatid Type							Chromosme Type								
						Simple		Complex					Simple	Complex			Others				
		tg	sg	%e	%pp	tb	isb	tr	qr	cr	id	ci	sb	d	r	dm	pu	sd*			
Untreated A	100			0	0														0.00	0.0	
Untreated B	100			0	0														0.00	0.0	
Untreated A+B	200			0.0	0.0														0.000	0.0	
Solvent A	100			0	0														0.00	0.0	
Solvent B	100			0	0														0.00	0.0	
Solvent A+B	200			0.0	0.0														0.000	0.0	
1000 A	100			0	0														0.00	0.0	
1000 B	100			0	0														0.00	0.0	
1000 A+B	200			0.0	0.0														0.000	0.0	= SOLVENT
2500 A	100			0	0														0.00	0.0	
2500 B	100			0	0														0.00	0.0	
2500 A+B	200			0.0	0.0														0.000	0.0	= SOLVENT
5000 A	100			0	0														0.00	0.0	
5000 B	100			0	0														0.00	0.0	
5000 A+B	200			0.0	0.0														0.000	0.0	= SOLVENT
CP 7.5 A	100			0	0	2		15	2	4			8						0.31	26.0	
CP 7.5 B	100			0	0	1	1	12	3	7	1		8						0.33	26.0	
CP 7.5 A+B	200			0.0	0.0	3	1	27	5	11	1		16						0.320	26.0	0.0001

* sd = 10 aberrations in calculations.

**Statistical analyses done on the % cells with aberrations .

In the Chi-square test, CP was compared to SITEK's *historical Untreated control data* (0.60%) since the solvent for CP was water and the concurrent value was 0%.

TABLE 6
CHROMOSOME ABERRATION ASSAY IN CHO CELLS
RCG - CONFIRMATORY ASSAY

TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SPONSOR: Primedica Redfield
SOLVENT: DMSO
SITEK STUDY NO.: 0623-3110
TRIAL NO.: B2

WITHOUT ACTIVATION					WITH ACTIVATION
Test Article Conc. (µg/mL)		No. of Cells per Flask	Mean No. of Cells X 10^6	RCG*	
Solvent	A	3.22	2.84	100%	The Activated System Was not perfomed in the B2.
Solvent	B	2.46			
50	A	3.57	4.68	165%	
50	B	5.79			
100	A	4.71	3.89	137%	
100	B	3.06			
500	A	2.28	1.30	46%	
500	B	0.31			
1000	A	2.41	1.37	48%	
1000	B	0.33			
2500	A	2.46	2.05	72%	
2500	B	1.64			
5000	A	1.97	1.72	61%	
5000	B	1.46			

*RCG = Relative Cell Growth = $\frac{\text{No. of Cells in the Test Flask}}{\text{No. of Cells in the Solvent Flask}}$ X 100

TABLE 7

CHROMOSOME ABERRATION ASSAY IN CHO CELLS

MITOTIC INDEX - CONFIRMATORY ASSAY

SPONSOR : Primedica Redfield SOLVENT : DMSO STUDY NO. : 0623-3110

TEST ARTICLE : Potassium Perfluorobutane Sulfonate TRIAL NO.: B2

Without Activation - Treatment: 18 Hours Harvest: 18 Hours						With Activation - Treatment: N/A Hours Harvest: N/A Hours
Test Article Concentration (µg/mL)	Tube No.		No. of Dividing Cells/500	Mean Mitotic Index	Relative Mitotic Index	
Untreated	44	A	28	4.8	117%	Activated System was not performed in B2
		B	20			
Solvent	39	A	16	4.1	100%	
		B	25			
50	99	A	13	3.5	85%	
		B	22			
100	58	A	12	3.6	88%	
		B	24			
500	72	A	22	3.3	80%	
		B	11			
1000	48	A	16	3.0	73%	
		B	14			
2500	88	A	0	NA	NA	
		B	0			
5000	12	A	3	0.7	17%	
		B	4			
MMC 0.08	75	A	17	3.2	78%	
		B	15			
MMC 0.2	21	A	10	1.6	39%	
		B	6			

The positive controls were compared to the Solvent since the solvent for MMC was water.

$$MI = \frac{\text{No. of dividing cells scored from 1000 cells}}{10}$$

$$RMI = \frac{\text{Test Dose MI}}{\text{Solvent Control MI}} \times 100$$

TABLE 8

CHROMOSOME ABERRATION ASSAY IN CHO CELLS

CHROMOSOME ABERRATIONS - CONFIRMATORY ASSAY

TEST ARTICLE: Potassium Perfluorobutane Sulfonate | TREATMENT TIME: 18 Hours | SITEK STUDY NO.: 0623-3110

SPONSOR: Primedica Redfield | HARVEST TIME: 18 Hours | TRIAL NO.: B2

SOLVENT: DMSO | METABOLIC ACTIVATION: Yes ( ) No (X)

TREATMENT AND CONC. (µg/mL)	CELLS Scored	NUMBER AND TYPE OF ABERRATIONS																	NO. OF ABS. PER CELL	% CELLS WITH ABS.	P-VALUE IN CHI-SQUARE**
		NOT COMPUTED				Chromatid Type							Chromosme Type								
						Simple		Complex					Simple	Complex			Others				
		tg	sg	% e	%pp	tb	isb	tr	qr	cr	id	ci	sb	d	r	dm	pu	sd*			
Untreated A	100			0	0														0.00	0.0	
Untreated B	100			0	0														0.00	0.0	
Untreated A+B	200			0.0	0.0														0.000	0.0	
Solvent A	100			0	0														0.00	0.0	
Solvent B	100			0	0														0.00	0.0	
Solvent A+B	200			0.0	0.0														0.000	0.0	
500A	100	1		0	0														0.00	0.0	
500 B	100			0	0														0.00	0.0	
500 A +B	200	1		0.0	0.0														0.000	0.0	= SOLVENT
1000 A	100			0	0														0.00	0.0	
1000 B	100			0	0														0.00	0.0	
1000 A+B	200			0.0	0.0														0.000	0.0	= SOLVENT
5000 A	100	3		0	0														0.00	0.0	
5000 B	100	1		0	0													1	0.10	1.0	
5000 A+B	200	4		0.0	0.0													1	0.050	0.5	0.5987
MMC 0.2 A	100		1	0	0	5	2	15	2	10			11						0.45	30.0	
MMC 0.2 B	100			0	0	7		10	3	10			3	1				1	0.44	28.0	
MMC 0.2 A+B	200		1	0.0	0.0	12	2	25	5	20			14	1				1	0.445	29.0	< 0.0001

* sd = 10 aberrations in calculations.

**Statistical analyses was done on the % cells with aberrations and with SITEK'S historical data (0.67%) since the solvent control was 0%.
In the Chi-square test, MMC was compared to SITEK's historical Untreated control data (0.62%) since the solvent for MMC was water and the concurrent value was 0%.

APPENDIX II

SITEK'S HISTORICAL DATA

HISTORICAL DATA FOR NEGATIVE CONTROL (UNTREATED) CHO IN VITRO CHROMOSOME ABERRATION ASSAY

NON-ACTIVATED SYSTEM

STUDY NUMBER	# OF METAPHASES SCORED	%CELLS WITH ABS.
0212-3112	200	2.00
0212-3112	200	0.00
0212-3112	200	0.00
0212-3112	200	0.00
0212-3112	200	1.00
0212-3112	200	1.00
0221-3112	200	0.50
0221-3112	200	2.00
0221-3112	200	0.00
0221-3112	200	0.00
0226-3112	200	1.00
0226-3112	200	0.50
0226-3112	200	1.00
0226-3112	200	1.50
0227-3112	200	1.00
0227-3112	200	0.50
0227-3112	200	0.50
0227-3112	200	1.00
0231-3112	200	1.00
0231-3112	200	0.50
0231-3112	200	0.50
0231-3112	200	0.50
0232-3113	200	1.00
0232-3113	200	0.00
0232-3113	200	0.50
0236-3113	200	1.00
0236-3113	200	0.50
0242-3112	200	0.50
0242-3112	200	0.50
0242-3112	200	1.50
0242-3112	200	0.50
0244-3112	200	1.00
0244-3112	200	0.50
0244-3112	200	0.00
0244-3112	200	0.00
0256-3114	200	0.50
0256-3114	200	0.50
0256-3114	200	1.50
0256-3114	200	0.00
0258-3114	200	0.00
0258-3114	200	0.00
0258-3114	200	0.50
0265-3113	200	0.50
0265-3113	200	0.50
0265-3113	200	0.50
0268-3110	200	0.00
0271-3114	200	0.50
0271-3114	200	1.00
0271-3114	200	1.00
0273-3113	200	0.50
0273-3113	200	0.50
0273-3113	200	1.00
0276-3110	200	0.50
0283-3110	200	0.00
0289-3114	200	0.50

HISTORICAL DATA FOR NEGATIVE CONTROL (UNTREATED)
CHO IN VITRO CHROMOSOME ABERRATION ASSAY

NON-ACTIVATED SYSTEM

STUDY NUMBER	# OF METAPHASES SCORED	%CELLS WITH ABS.
0289-3114	200	0.50
0289-3114	200	0.50
0327-3114	200	0.00
0327-3114	200	1.00
0327-3114	200	0.50
0334-3114	200	0.50
0334-3114	200	1.00
0334-3114	200	1.50
0336-3110	200	1.50
0346-3114	200	1.00
0346-3114	200	0.50
0346-3114	200	1.00
0347-3114	200	1.00
0347-3114	200	0.50
0347-3114	200	0.00
0351-3114	200	0.00
0351-3114	200	0.00
0351-3114	200	0.50
0352-3114	200	0.50
0352-3114	200	0.50
0352-3114	200	1.00
0362-3114	200	0.00
0362-3114	200	0.50
0362-3114	200	1.00
0372-3114	200	0.00
0372-3114	200	1.00
0372-3114	200	1.00
0384-3114	200	1.00
0384-3114	200	0.50
0384-3114	200	0.00
0387-3114	200	1.00
0387-3114	200	1.50
0387-3114	200	0.50
0389-3114	200	0.50
0389-3114	200	1.00
0389-3114	200	0.50
0389-3114	200	0.50
0389-3114	200	2.00
0389-3114	200	0.50
0396-3114	200	0.50
0397-3114	200	2.00
0398-3114	200	1.00
0399-3114	200	0.00
0402-3114	200	0.50
0403-3114	200	2.00
0404-3114	200	1.50
0406-3110	200	0.00
0415-3110	200	0.50
0415-3110	200	1.00
0415-3110	200	1.50
0425-3110	200	0.00
0426-3110	200	0.00
0426-3110	200	1.00
0428-3110	200	0.00
0428-3110	200	0.50
0432-3110	200	0.50

HISTORICAL DATA FOR NEGATIVE CONTROL (UNTREATED)
CHO IN VITRO CHROMOSOME ABERRATION ASSAY

NON-ACTIVATED SYSTEM

STUDY NUMBER	# OF METAPHASES SCORED	%CELLS WITH ABS.
0432-3110	200	1.00
0437-3110	200	0.00
0452-3114	200	1.00
0452-3114	200	0.50
0460-3114	200	0.50
0460-3114	200	0.50
0470-3110	200	0.00
0470-3110	200	0.50
0470-3110	200	1.00
0475-3110	200	0.00
0475-3110	200	0.50
0490-3110	200	0.00
0490-3110	200	0.50
0493-3110	200	0.50
0493-3110	200	0.50
0504-3110	200	0.00
0504-3110	200	0.00
RANGE : 0.0 – 1.5%	MEAN ± S.D.:	0.62 ± 0.51

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HISTORICAL DATA FOR NEGATIVE CONTROL (UNTREATED) CHO IN VITRO CHROMOSOME ABERRATION ASSAY

ACTIVATED SYSTEM

STUDY NUMBER	# OF METAPHASES SCORED	%CELLS WITH ABS.
0212-3112	200	0.50
0212-3112	200	0.50
0212-3112	200	1.00
0212-3112	200	0.00
0212-3112	200	0.50
0221-3112	200	0.50
0221-3112	200	1.50
0221-3112	200	0.50
0221-3112	200	0.00
0226-3112	200	1.00
0226-3112	200	0.50
0226-3112	200	0.50
0226-3112	200	0.50
0227-3112	200	1.50
0227-3112	200	1.50
0227-3112	200	0.50
0227-3112	200	1.00
0231-3112	200	0.50
0231-3112	200	1.00
0231-3112	200	0.00
0231-3112	200	0.50
0232-3113	200	0.00
0232-3113	200	0.50
0232-3113	200	0.00
0236-3112	200	1.50
0236-3112	200	1.00
0242-3112	200	1.00
0242-3112	200	0.50
0242-3112	200	0.50
0242-3112	200	0.50
0244-3112	200	0.00
0244-3112	200	0.50
0244-3112	200	0.50
0244-3112	200	0.50
0256-3114	200	1.00
0256-3114	200	1.00
0256-3114	200	1.00
0256-3114	200	0.50
0258-3114	200	1.00
0258-3114	200	0.00
0258-3114	200	0.00
0265-3113	200	0.00
0265-3113	200	0.50
0265-3113	200	0.00

HISTORICAL DATA FOR NEGATIVE CONTROL (UNTREATED) CHO IN VITRO CHROMOSOME ABERRATION ASSAY

ACTIVATED SYSTEM

STUDY NUMBER	# OF METAPHASES SCORED	%CELLS WITH ABS.
0268-3110	200	0.00
0271-3114	200	0.50
0271-3114	200	0.50
0271-3114	200	0.50
0273-3113	200	1.00
0273-3113	200	0.50
0273-3113	200	1.00
0276-3110	200	1.50
0283-3110	200	0.50
0289-3114	200	0.50
0289-3114	200	0.50
0327-3114	200	0.00
0327-3114	200	1.00
0327-3114	200	1.50
0334-3114	200	0.50
0334-3114	200	0.00
0334-3114	200	0.00
0336-3110	200	1.00
0346-3114	200	0.50
0346-3114	200	1.00
0346-3114	200	1.00
0347-3114	200	1.50
0347-3114	200	0.00
0347-3114	200	1.00
0351-3114	200	1.00
0351-3114	200	0.50
0351-3114	200	0.00
0352-3114	200	1.00
0352-3114	200	1.00
0352-3114	200	1.50
0362-3114	200	0.50
0362-3114	200	1.00
0362-3114	200	0.00
0372-3114	200	0.50
0372-3114	200	0.00
0372-3114	200	1.00
0384-3114	200	1.50
0384-3114	200	0.50
0384-3114	200	0.50
0387-3114	200	1.00
0387-3114	200	1.50
0387-3114	200	1.00
0389-3114	200	1.00
0389-3114	200	0.00
0389-3114	200	0.50

HISTORICAL DATA FOR NEGATIVE CONTROL (UNTREATED) CHO IN VITRO CHROMOSOME ABERRATION ASSAY

ACTIVATED SYSTEM

STUDY NUMBER	# OF METAPHASES SCORED	%CELLS WITH ABS.
0389–3114	200	0.00
0389–3114	200	1.00
0396–3114	200	0.00
0397–3114	200	0.50
0398–3114	200	0.00
0399–3114	200	0.00
0402–3114	200	0.50
0403–3114	200	0.50
0406–3110	200	0.00
0415–3110	200	0.50
0415–3110	200	0.50
0415–3110	200	0.50
0425–3110	200	1.00
0426–3110	200	1.00
0426–3110	200	0.00
0428–3110	200	0.50
0432–3110	200	0.50
0437–3110	200	0.50
0452–3114	200	1.00
0452–3114	200	1.00
0453–3114	200	1.00
0453–3114	200	1.00
0460–3114	200	0.50
0460–3114	200	0.00
0470–3110	200	0.50
0470–3110	200	0.50
0470–3110	200	1.00
0475–3110	200	0.50
0490–3110	200	0.00
0493–3110	200	0.00
0504–3110	200	0.00

RANGE : 0.0 – 1.5% MEAN ± S.D.: 0.60 ± 0.45

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HISTORICAL DATA FOR DMSO
CHO IN VITRO CHROMOSOME ABERRATION ASSAY

NON-ACTIVATED SYSTEM

STUDY NUMBER	# OF METAPHASES SCORED	%CELLS WITH ABS.
0212-3112	200	1.00
0212-3112	200	0.50
0212-3112	200	1.50
0212-3112	200	0.50
0212-3112	200	0.00
0212-3112	200	0.50
0221-3112	200	1.50
0221-3112	200	0.50
0221-3112	200	0.50
0221-3112	200	1.00
0231-3112	200	0.50
0231-3112	200	1.00
0231-3112	200	0.50
0231-3112	200	0.50
0242-3112	200	0.00
0242-3112	200	0.50
0242-3112	200	1.50
0242-3112	200	0.50
0256-3114	200	0.00
0256-3114	200	0.00
0256-3114	200	0.50
0256-3114	200	0.50
0268-3110	200	0.50
0289-3114	200	0.50
0289-3114	200	1.00
0289-3114	200	0.50
0289-3114	200	1.00
0327-3114	200	0.50
0327-3114	200	0.50
0327-3114	200	1.00
0336-3110	200	1.00
0346-3114	200	0.50
0346-3114	200	1.50
0346-3114	200	1.00
0346-3114	200	1.50
0346-3114	200	2.50
0346-3114	200	0.00
0347-3114	200	1.00
0347-3114	200	1.00
0347-3114	200	1.50
0347-3114	200	0.50
0347-3114	200	0.00
0347-3114	200	1.00
0351-3114	200	0.50
0351-3114	200	0.50

HISTORICAL DATA FOR DMSO
CHO IN VITRO CHROMOSOME ABERRATION ASSAY

NON-ACTIVATED SYSTEM

STUDY NUMBER	# OF METAPHASES SCORED	%CELLS WITH ABS.
0351-3114	200	0.50
0352-3114	200	0.50
0352-3114	200	1.00
0352-3114	200	0.50
0404-3114	200	1.00
0415-3110	200	0.50
0415-3110	200	0.50
0415-3110	200	0.50
0425-3110	200	0.00
0426-3110	200	0.00
0426-3110	200	1.00
0428-3110	200	0.50
0428-3110	200	1.00
0432-3110	200	0.50
0432-3110	200	0.50
0437-3110	200	0.50
0460-3114	200	0.50
0470-3110	200	0.50
0470-3110	200	1.50
0475-3110	200	0.00
0475-3110	200	0.50
0493-3110	200	0.50
0493-3110	200	1.00
0504-3110	200	0.50
0504-3110	200	0.00
RANGE : 0.0 – 1.5%	MEAN ± S.D.:	0.67 ± 0.47

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APPENDIX III

STUDY PROTOCOL AND PROTOCOL AMENDMENTS

SITEK RESEARCH LABORATORIES

TEST FOR CHEMICAL INDUCTION OF CHROMOSOME ABERRATION IN CULTURED CHINESE HAMSTER OVARY (CHO) CELLS WITH AND WITHOUT METABOLIC ACTIVATION

This protocol is presented in two parts. Part One is designed to collect specific information pertaining to the test article and study. Part Two describes the study design in detail. Please complete all sections in Part One and sign section 8.0 to approve the protocol.

PART ONE

1.0 SPONSOR

1.1 Name: Primedica Redfield

1.2 Address: 100 E. Boone Street
Redfield, AR 72132

1.3 Sponsor's Study Coordinator: John Seng, Ph.D.

2.0 TESTING FACILITY

2.1 Name: SITEK Research Laboratories

2.2 Address: 15235 Shady Grove Road, Suite 303
Rockville, Maryland 20850

* 2.3 Study Director: Jing Xu, M.D.

3.0 STUDY NUMBERS

* 3.1 Testing Facility's Study No.: 0623-3110

3.2 Sponsor's Study No.: 132-009

4.0 TEST ARTICLE

4.1 Identification

Name: Potassium Perfluorobuntane Sulfonate, CAS #29420-49-3

Batch/Lot No.: Lot #2

4.2 Description

Color: White

Physical Form: Solid, free flowing powder

* To be completed by the Testing Facility.

SITEK RESEARCH LABORATORIES

4.3 Analysis

Purity Information: 100% pure

Does the Sponsor require the use of a correction factor to account for impurity?

___ Yes _X_ No

If yes, what is the correction factor? _____

Determination of the test article characteristics as defined by Good Laboratory Practices will be the responsibility of the Sponsor. The specific GLP references for U.S. agencies are: FDA = 21 CFR, 58.105; EPA TSCA = 40 CFR, 792.105 and EPA FIFRA = 40 CFR 160.105.

4.4 Stability

Storage Conditions (check one):

_X_ Room Temperature ___ Refrigerated (1-5°C)

___ Frozen (-10 to -20°C)

___ Other (please specify): _____

Expiration Date: 04-06-01

4.5 Preferred Solvent (check one):

___ H_2O ___ Culture Medium ___ DMSO ___ Acetone ___ Ethanol

___ Other (please specify): _____

_X_ To be decided by the Testing Facility

4.6 Special Handling Instructions:

Standard precaution (MSDS attached)

5.0 REGULATORY AGENCY SUBMISSION

This study will be conducted in compliance with the following Good Laboratory Practice standards:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 1997.

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 1998.

Japanese Ministry of Agriculture, Forestry and Fisheries, 59 Nohsan, Notification No. 3850, Agriculture Production Bureau, August 10, 1984.

SITEK RESEARCH LABORATORIES

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984.

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45, Paris 1992.

Will this study be submitted to a regulatory agency?

_X_ Yes ___ No

If so, which agency(ies)? OECD

6.0 DOSING SOLUTIONS

The Sponsor will be responsible for determining the strength and stability of the dosing solutions. The U.S. requirements for analysis of dosing solutions are specified in: FDA = 21 CFR, 58.113; EPA TSCA = 40 CFR, 792.113 and EPA FIFRA = 40 CFR 160.113.

Does the Sponsor want dosing solution analysis?

_X_ Yes** ___ No

If yes, please complete the rest of this section.

If requested by the Sponsor, SITEK Research Laboratories will determine the strength and stability of the dosing solutions. The method of analysis may be provided by the Sponsor, or if requested by the Sponsor, SITEK Research Laboratories will develop the method of analysis.

Alternatively, the Sponsor will be responsible for determining the strength and stability of the dosing solutions.

Dosing solution analysis will be performed by:

___ SITEK Research Laboratories _X_ Sponsor

What dosing solutions will be analyzed?

From the Range Finding Test?

___ Yes _X_ No

From the Assay?

_X_ Yes ___ No

Which concentration(s)? All

** Additional charges will apply. See Special Services price schedule.

SITEK RESEARCH LABORATORIES

What amount of each concentration? 2.0 mL

At what temperature should the dosing solutions be stored?

___ Room Temperature _X_ Frozen (-10 to -20°C)

___ Refrigerated (1-5°C)

7.0 STUDY DATES

* 7.1 Proposed Experimental Start Date: 8/8/00

Defined as the first date the test article is applied to the test system.

* 7.2 Proposed Experimental Completion Date: 9/22/00

Defined as the last date on which data are collected directly from the study.

* Proposed Draft Report Date: 10/20/00

8.0 PROTOCOL APPROVAL

* Study Director	Date 7/31/00
Study Coordinator	Date 7/18/00
Sponsor's Authorized Representative	Date July 12, 2000
* Quality Assurance Manager	Date 8/1/00
* Safety Officer	Date 8/1/00

* To be completed by the Testing Facility.

SITEK RESEARCH LABORATORIES

STUDY DESIGN

PART TWO

9.0 PURPOSE

The purpose of this study is to evaluate the test article for its potential to cause genetic damage as manifested by induced chromosome aberrations in cultured Chinese hamster ovary (CHO) cells.

10.0 JUSTIFICATION FOR SELECTION OF TEST SYSTEM

The CHO cells have been used extensively in the Chromosome Aberration Assay and have been demonstrated to be effective in detecting the clastogenic activity of chemicals from a wide range of chemical classes (1-5).

11.0 ABBREVIATIONS

CHO	-	Chinese Hamster Ovary
CP	-	Cyclophosphamide
DMSO	-	Dimethyl Sulfoxide
G-6-P	-	Glucose-6-phosphate
HEPES	-	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIFBS	-	Heat-Inactivated Fetal Bovine Serum
KCl	-	Potassium Chloride
MMC	-	Mitomycin-C
MI	-	Mitotic Index
NADP	-	Nicotinamide-adenine Dinucleotide Phosphate (Sodium Salt)
DPBS	-	Dulbecco's Phosphate Buffered Saline (with Ca^{++} and Mg^{++})
PBS	-	Phosphate Buffered Saline (without Ca^{++} and Mg^{++})
RMI	-	Relative Mitotic Index

Complete Culture Medium - McCoy's 5A medium supplemented with 10% HIFBS, 2mM L-glutamine, 50 units/mL of penicillin and 50 μg/mL of streptomycin

Antibiotic-Free Medium - McCoy's 5A medium supplemented with 10% HIFBS and 2mM L-glutamine

12.0 INDICATOR CELLS

12.1 Source

The clone CHO-W-BI of the CHO cell line, used in this study, originated at Litton Bionetics and was obtained by SITEK through the Environmental Health Research and Testing Laboratories, Lexington, Kentucky, in 1988. The doubling time of this cell line is approximately 12 hours, and its modal chromosome number is 21. The karyotype analysis of the cell line is periodically performed and documented at SITEK Research Laboratories.

12.2 Culture Conditions

The stock cultures of CHO cells are routinely grown in sterile, plastic tissue culture flasks in antibiotic-free medium. The test cultures are grown in T-25 cm^2 plastic tissue culture flasks in complete medium. The cultures are kept in a humidified incubator maintained at approximately 37°C in an atmosphere of approximately 5% CO_2 and 95% air.

The stock cultures are routinely subcultured before confluency using 0.05% trypsin for dissociating the cells.

12.3 Stock Cultures

The CHO cells were propagated in antibiotic-free medium to obtain a sufficient number of cells for freezing a large number of stock ampules. The cells were cryopreserved in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) and 8% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. Prior to using the stock cultures for the test, representative ampules will be tested for contaminating microorganisms, including mycoplasma and also for karyotype stability. Stock ampules free of contaminating organisms will be used to initiate the stock cultures for the test. The cell cultures obtained from the stock ampules will be maintained by subculturing for a maximum of 15 passages and used to initiate cultures for the assays.

13.0 ROUTE OF ADMINISTRATION OF TEST ARTICLE

The test article will be administered in vitro directly or through a solvent compatible with the test system. This is the only route of administration available in this test system.

14.0 TEST SYSTEM IDENTIFICATION

All test cultures will be labeled in indelible ink with the SITEK study number, the test article concentrations/controls, the activation system, code number for the concentrations, A or B/C or D designating tubes receiving the same treatment, date of harvest and any other information that is pertinent to the Assay. Slides will be labeled with the SITEK study number, the code numbers for the concentrations tested, followed by A or B/C or D for the same treatment conditions and the date the slides are prepared.

15.0 CONTROL SUBSTANCES

15.1 Positive Controls

Mitomycin-C (MMC), which causes chromosome aberrations without metabolic activation, will be dissolved in water and used at 0.08 and/or 0.2 μg/mL in the non-activated system.

Cyclophosphamide (CP), which requires metabolic activation, will be dissolved in water and used at 7.5 and/or 12.5 μg/mL in the activated system.

If necessary, other appropriate positive controls can be used with the approval of the Sponsor.

15.2 Solvent Controls

The solvents used for dissolving the test article and positive controls will be used as the solvent controls. Culture medium, deionized, distilled water, DMSO (CAS #67-68-5), ethanol (CAS #64-17-5), and acetone (CAS #67-64-1) are some of the solvents which are compatible with this test system. If there is a need to use other solvents, the approval of the Sponsor will be obtained prior to their use.

16.0 DOCUMENTATION

Detailed documentation of the procedures, results, and methods used for the analysis of the results of this study will be entered in a study notebook. The study notebook also includes copies of the protocol, protocol amendments and deviations, study reports, and all relevant communications with the Sponsor.

17.0 EXPERIMENTAL PROCEDURE

17.1 Determination of Solubility/Miscibility

In order to determine the appropriate vehicle for delivering the test article to the test system, or to determine the maximum achievable concentration in the solvent requested by the Sponsor, a solubility/miscibility test will be performed.

The test article will be tested for its solubility/miscibility in deionized, distilled water, DMSO, acetone, ethanol and/or other appropriate solvents. Solid and viscous liquid test articles will be tested for solubility in weight per volume, and nonviscous liquids will be tested for miscibility in volume or weight per volume. The solubility/miscibility test will be performed as described below.

For solid and viscous liquid test articles, the solubility test will consist of weighing out 25-100 mg aliquots of test article and adding solvent in 0.1 mL increments, with thorough mixing between additions, until the test article is dissolved or until 1.5 mL of solvent has been added to the vessel. If the test article does not dissolve in 1.5 mL of solvent, more solvent will be added in aliquots of 0.5 mL until 5.0 mL has been added. The volume of solvent required for complete dissolution, and any additional observations, will be recorded in the study workbook. Test articles that do not dissolve in 5.0 mL of solvent will be recorded as either "not soluble," "partially soluble forming a homogeneous suspension," or "partially soluble not forming a homogeneous suspension."

For nonviscous liquid test articles, a miscibility test will be conducted. 0.5 mL of each of the preferred solvents in 0.1 mL increments will be added to 0.5 mL aliquots of the test article. If the test article does not dissolve in 1.5 mL of solvent, more solvent will be added in 0.5 mL increments until 5.0 mL has been added. The resulting solution will be thoroughly mixed and observed for miscibility. The test article will be rated as either "not miscible," "partially miscible," or "completely miscible" in each of the preferred solvents. The miscibility rating and any additional observations will be recorded in the study workbook.

The solubility/miscibility test need not be performed if adequate information regarding the solvent and maximum soluble concentration is available.

The solubility or miscibility of the test article in culture medium will also be checked to determine the appropriate concentrations for the tests.

17.2 Preparation of Test Cultures

The CHO stock cultures grown in antibiotic-free medium and showing approximately 50-70% confluency will be harvested and used to prepare the test cultures for the Assay. The culture medium from the flasks will be discarded, and the cells will be washed with phosphate buffered saline (PBS). The cells will then be dissociated by trypsin at 37 ± 1.-0°C, and resuspended in fresh complete culture medium. An aliquot of the cell suspension will be diluted to the appropriate concentration and counted using a cell counter. Based on the cell counts, a separate cell suspension in complete culture medium with $1x10^5$ cells/mL will be prepared to seed the test flasks. An appropriate number of T-25 cm^2 tissue culture flasks will be seeded with 5.0 mL of cell suspension to obtain test cultures with $5x10^5$ cells/flask. The cultures to be maintained beyond 48 hours after their initiation, will be seeded with an appropriately reduced number of cells (250,000-400,000 cells per flask) in order to avoid overgrowth of the monolayer. In the case of test articles which react with plastic, 60 mL sterile glass culture flasks will be used instead of T-25 cm^2 plastic culture flasks. The flasks will be incubated for approximately 20-24 hours before treatment.

17.3 Preparation of Metabolic Activation System

The metabolic activation mixture will consist of phenobarbital/ß-naphthoflavone induced rat liver homogenate (S-9 fraction) (6) and the cofactor pool. The S-9 fraction will be stored at or below -70°C in small aliquots. The S-9 will be validated for acceptable levels of protein content and metabolic activity. Immediately prior to use, the S-9 will be thawed at room temperature and mixed with the cofactor pool to form the metabolic activation mixture which will consist of 4mM NADP, 5mM glucose-6-phosphate, 30mM KCl, 10mM $MgCl_2$, 50mM sodium phosphate (pH 7.4) and 100 µL/mL of S-9 fraction. This mixture will be diluted 1:4 by volume with serum-free medium and used in refeeding the cultures.

17.4 Preparation of Test Article

The desired amount of the test article will be weighed as specified in the dilution scheme which will be prepared prior to treatment for either the Range Finding Test or Assay. The stock solution of the highest concentration will be prepared by adding the appropriate volume of solvent to the test article just prior to use and thoroughly mixing the resulting solution until the desired dissolution is achieved. The remaining stock solutions specified in the dilution scheme will be prepared by a subsequent dilution or by dissolving the required amount of test article in the solvent at each concentration. In all treatments, the amount of solvent delivered to the target cultures will be limited to a level which has no significant cytotoxic effect on the cells. If necessary, the test article may be added directly to the culture medium. If the test article is found to alter the pH of the culture medium to an extent that is toxic to the cells (7-8), either HEPES buffered medium will be used during treatment time or necessary adjustments will be made to the stock solution(s) or treatment medium prior to chemical exposure. A record of the pH measurements will be maintained in such cases.

17.5 Range Finding Test

If sufficient information is not available regarding the toxicity of the test article, a Range Finding Test will be performed in order to determine the test article concentrations that will produce 0-100% cytotoxicity. The test article will be weighed and a serial dilution will be prepared. If there is no solubility limitation, prior knowledge of cytotoxicity indicates differently, or the Sponsor specifies differently, the test article will be tested at eight to ten concentrations at a maximum concentration of 5000 µg/mL and lower concentrations

covering four log dilutions. A solvent control will also be included in both the non-activated and activated systems. An untreated control (exposed only to water) will be included if a solvent other than water or culture medium is used. If a narrower concentration range or lower concentrations are required to determine the desired cytotoxic range, the Range Finding Test will be repeated.

The test cultures seeded approximately 20-24 hours earlier and are in the log phase will be used in the Range Finding Test. Duplicate cultures will be used at each concentration level.

In the non-activated system, the culture medium will be removed, and 5 mL of fresh complete medium will be added to each of the culture flasks. The cells will then be exposed to the test article for 3 hours. After the exposure period, the cells will be washed with DPBS, refed with complete medium, allowed to grow for 15 hours with 0.1 µg/mL Colcemid present during the final 2 hours, and harvested 18 hours after the initiation of the treatment (1.5 x normal cell cycle time).

In the activated system, the medium will be removed and 5 mL of serum-free medium containing S-9 will be added to each of the culture flasks prior to treatment. The cells will be exposed to the test article for 3 hours by adding appropriate volumes of test article or dosing solutions to the culture medium. After the exposure period, the cells will be washed with DPBS, refed with complete culture medium, allowed to grow for 15 hours with 0.1 µg/mL Colcemid present during the final 2 hours, and harvested 18 hours after the initiation of the treatment (1.5 x normal cell cycle time).

17.5.1 Determination of Relative Cell Growth (RCG) (10-11)

After the Colcemid exposure, the medium with dividing cells in each flask will be transferred into labeled centrifuge tubes, the monolayer of cells will be washed with PBS, dissociated with 0.05% trypsin and resuspended in the collected medium. An aliquot of this cell suspension will be counted using an electronic cell counter. The number of cells per flask will be calculated for each concentration, and the Relative Cell Growth (RCG) will be calculated according to the following formula:

$$RCG = \frac{\text{No. Cells in Test Flask}}{\text{No. Cells in Solvent Flask}} \times 100$$

The cytotoxicity will be evaluated on the basis of the cell number (RCG). If possible, a concentration causing greater than 50% reduction in RCG will be selected as the highest test concentration for the Chromosome Aberration Assay. In addition, four or more lower concentrations will be included in the Assay. If no cytotoxicity is observed at the maximum concentration tested, the Chromosome Aberration Assay will be performed at four decreasing concentrations starting with the maximum soluble concentration or one or two concentrations with precipitate. The actual concentrations for the assay, once determined, will be added to the protocol in the form of an amendment.

Concentrations exceeding an osmolality of 500 mOsmol/kg of water will be excluded from the Chromosome Aberration Assay (9).

17.6 Chromosome Aberration Assay

The Chromosome Aberration Assay will be performed with a single harvest at 1.5 x normal cell cycle time.

Parallel Toxicity will be determined by the RCG of treated cells in comparison with solvent control. The procedure is the same as in the Range Finding Test.

The test cultures will be prepared as described in Section 17.2. Duplicate cultures will be treated and used at each concentration in each system in the evaluation of induced chromosome aberrations, RCG and the Relative Mitotic Index (RMI).

The treatment procedures for the Chromosome Aberration Assay will be the same as in the Range Finding Test. The cells will be treated with four or more concentrations of the test article, two concentrations each of the two positive controls and the solvent control in both the activated and non-activated systems. Untreated controls (only exposed to water) will be included in the Assay if a solvent other than water or culture medium is used.

In the non-activated system, the cells will be treated in complete medium for three hours. After the exposure period, the medium will be removed, the cells will be washed with DPBS, refed with complete medium and incubated for 15 hours with 0.1 μg/mL Colcemid present during the last 2 hours. The cells will be harvested 18 hours after the initiation of treatment (1.5 x normal cell cycle time).

In the activated system, the cells will be treated in serum-free, S-9 containing medium for 3 hours. The removal procedure and incubation and harvest times are the same as in the non-activated system described above.

After the Colcemid exposure, the cell suspension will be processed to determine the RCG first as described in the Range Finding Test, section 17.5.1. The remaining cell suspension will be processed to determine the Relative Mitotic Index (RMI) and chromosome aberrations as described below:

The cells will be collected by centrifugation, swelled in hypotonic KCl (0.075M), and fixed in methanol:glacial acetic acid (3:1) fixative. The fixed cells will be kept at 1-5°C. The cells will then be collected by centrifugation, resuspended in a small volume of fresh fixative, and dropped onto microslides to prepare chromosome spreads. The slides will be air dried, stained in Giemsa stain, and mounted in Permount using #1 cover glasses.

The slides will be scored for Mitotic Index (MI). A total of 1000 cells will be scored from each concentration (500 from each duplicate flask) and the number of dividing cells recorded. The MI for each concentration level will be calculated using the following formula:

$$MI = \frac{\text{No. of Dividing Cells from 1000 Cells}}{10}$$

The RMI will be calculated as shown below:

$$RMI = \frac{\text{Test Concentration MI}}{\text{Solvent Control MI}} \times 100$$

The same slides will be used to score chromosome aberrations, and scored "blind" in order to avoid bias on the part of the scorer(s). A total of three test concentrations, if possible, the highest of which causes more than 50% reduction in RCG, one positive control concentration, the solvent and untreated controls will be scored from the activated and non-activated systems. Whenever possible, 100 metaphases will be scored from each of the two duplicate flasks. Consequently, 200 metaphases will be scored for each concentration for chromosome aberrations. Only cells with 19-23 chromosomes will be scored, and the microscope coordinates of each cell with findings will be recorded. In addition, the number of endoreduplicated and polyploid cells in a total of 100 metaphases per culture will be scored and recorded.

The types of Chromosome Aberrations scored and the corresponding abbreviations used are given below (12-14):

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1. Chromatid-type Aberrations

Simple:

tg - Chromatid gap - an achromatic region occurring along the length of a chromatid in which there is no misalignment.

tb - Chromatid break - a discontinuity occurring along the length of either of the two chromatids, in which there is a misalignment.

isb - Isochromatid break - a discontinuity occurring in both the chromatids at the same locus showing complete rejoining or sister chromatid union at both the broken ends or incomplete rejoining, i.e., only at one of the two broken ends.

Complex:

qr - Quadriradial - chromatid interchanges between chromosomes leading to four-armed configurations. This could be asymmetrical with formation of a dicentric and an acentric chromatid, if union is complete, or symmetrical where there is no formation of a dicentric and an acentric chromatid.

tr - Triradial - isochromatid-chromatid exchanges resulting in three-armed configurations and sometimes fragments. The latter should not be scored as an independent aberration. The triradial could be monocentric or dicentric.

id - Interstitial deletion - intra-arm intrachanges resulting in deletion of small fragments which, however, stay in association with the parent chromatid.

ci - Chromatid intrachange - exchanges occurring between arms of the same chromosome resulting in asymmetrical (rings) or symmetrical configurations.

cr - Complex interchanges - multiarmed configurations resulting from breakage and reunion of two or more chromosomes.

2. Chromosome-type Aberrations

Simple:

sg - Chromosome gap - an achromatic region occurring in both chromatids of the chromosome at the same locus with no misalignment.

sb - Chromosome break - a discontinuity at the same locus in both chromatids, giving one acentric fragment which may be misaligned and a shortened monocentric chromosome, and where there is no sister chromatid union.

Complex:

d - Dicentric - an asymmetrical exchange between two chromosomes resulting in a chromosome with two centromeres with or without an accompanying acentric fragment which should not be scored as a second aberration.

r - Ring - inter-arm intrachange happening within the chromosome, leading to formation of a centric ring with or without a chromosome fragment. The fragment should not be scored as a second aberration.

dm - Double minutes - intra-arm intrachanges leading to tight acentric paired rings.

3. Other Aberrations

pu - Pulverized chromosome or chromosomes - shattering of chromatid material resulting in several minute pieces. The identity of the chromosome is not decipherable. Considered as a single aberration.

sd - Severely damaged cell - cell with ten or more aberrations.

pp - Polyploid cells - metaphases with multiples or approximate multiples of the haploid set of chromosomes. Not scored for structural aberrations.

e - Endoreduplicated cells - metaphases with paired duplicated chromosomes or diplochromosomes. They are not scored for structural aberrations.

The chromosome aberration data from the score sheets will be consolidated on a Summary Table. The number of aberrations per cell and the percentage of cells with one or more aberrations will be calculated separately for each duplicate culture and then pooled for each concentration. Chromatid gaps and chromosome gaps will not be included in calculating the percentage of cells with aberrations and the number of aberrations per cell. Of the remaining aberrations, each aberration scored will be counted as one, except a severely damaged cell (sd) which will be considered equal to ten aberrations in calculating the number of aberrations per cell. Endoreduplicated and polyploid cells will be recorded separately in percentages.

17.7 Statistical Analysis

The data for the percentage of cells with aberrations for each concentration will be compared to the solvent control values using a Chi-square test. The results will be considered significant if $p \leq 0.05$.

If the solvent control value is 0%, the data will be analyzed using the historical solvent control values. Statistical analysis will not be performed if the test concentration value is equal to or less than the concurrent or historical solvent control.

If a positive response is indicated by the Chi-square test, the Cochran-Armitage test (trend test) will be performed for evidence of a dose-related response (15). The trend test will be considered positive if $p \leq 0.05$.

17.8 Confirmatory Chromosome Aberration Assay

A confirmatory assay will not be performed if the first assay is positive either with and/or without activation.

A confirmatory Chromosome Aberration Assay without activation will be performed if the results of the first assay without activation produced a negative response. A continuous treatment up to the harvest time of 1.5 x normal cell cycle time (18 hours) will be performed. Negative results for the first assay with activation may require a confirmation on a case by case basis.

The RCG and RMI determination, harvest and chromosome aberration scoring procedures will be the same as in the definitive assay. Parameters, such as test concentrations, may be adjusted in the confirmatory assay.

A confirmatory chromosome aberration assay with and without activation will be performed, if the first assay produces an equivocal response.

17.9 Criteria for a Valid Assay

1. In the solvent control, the percentage of cells with aberrations should not exceed 4%.

2. At least 25% of the cells scored in the positive control should show one or more chromosome aberrations.

3. At least one of the test concentrations scored should show greater than 50% reduction in the RCG. This requirement should not be applied to test articles where no apparent toxicity could be achieved at the maximum soluble concentration or highest allowable concentration.

17.10 Evaluation of Test Results

17.10.1 Positive Response

The test article will be considered to have caused a positive response in this assay if the test article shows a positive dose-response trend and a statistically significant increase over that of the solvent controls in the percentage of cells with aberrations at one or more concentrations.

17.10.2 Negative Response

The test article will be considered to have caused a negative response if none of the test concentrations shows a statistically significant increase in the percentage of cells with aberrations.

17.10.3 Equivocal Response

The test article will be considered to have caused an equivocal response if one of the test concentrations shows a statistically significant increase in the percentage of cells with aberrations without an accompanying positive dose-response trend.

17.10.4 Other Considerations

The above criteria will be used as guidelines in evaluating the test results. However, the Study Director may take other factors into consideration in evaluating the test results.

18.0 PROTOCOL AMENDMENTS AND DEVIATIONS

If changes in the approved protocol are necessary, such changes will be documented in the form of protocol amendments and protocol deviations. Protocol amendments will be generated when changes in the protocol are made prior to performing a study or part of a study affected by the changes. In such cases, a verbal agreement to make such changes will be made between the Study Director and the Sponsor. These changes and the reasons for them will be documented and attached to the protocol as an addendum. Protocol deviations will be generated when the procedures used to perform the study do not conform to the approved protocol. The Sponsor will be informed of these deviations, and as soon as practical, such changes, along with their reasons or explanations, will be documented and kept in the study notebook.

19.0 REPORT OF RESULTS

19.1 Content

The results of the study will be submitted to the Sponsor in the form of a final report. A draft report will be submitted before the final report is issued. The report will include, but not be limited to, the following:

1. Name and address of the testing facility and the dates on which the study was initiated and completed, terminated or discontinued.

2. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

3. Methods used to analyze the data.

4. The test and control substances.

5. Description of the methods used to perform the study.

6. The name of the Study Director and the names of other technical personnel or other professionals who participated in performing the study.

7. A description of the transformations, calculations or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis.

8. The signed and dated reports of the Study Director or other professionals involved in the study.

9. The location where the raw data and reports are to be stored.

10. A statement from the Quality Assurance Unit.

19.2 Changes and Corrections to the Final Report

All changes to the final report will be in the form of report amendments which will include the reason(s) for the change, and these amendments will be added to the final report as an addendum.

20.0 ARCHIVES

The raw data, slides, protocol, documentation, electronic file containing the data tables, and final report of the study will be maintained in the SITEK Research Laboratories Archives, 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850 according to the terms and conditions of the study.

21.0 REFERENCES

1. Evans, H.J. Cytological Methods for Detecting Chemical Mutagens, In: Chemical Mutagens, Principles and Methods for their Detection, Vol. 4, Hollaender, A. (ed) Plenum Press, New York and London, pp. 1-29 (1976).

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PROTOCOL AMENDMENTS

Amendment Nos.:	1-3
Sponsor:	Primedica Redfield 100 E. Boone Street Redfield, Arkansas 72132
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
Sponsor's Study No.:	132-009
SITEK's Study No.:	0623-3110
Test Article I.D.:	Potassium Perfluorobutane Sulfonate
Protocol Title:	Test for Chemical Induction of Chromosome Aberration in Cultured Chinese Hamster Ovary (CHO) Cells With and Without Metabolic Activation

Amendment No. 1: Protocol Page 1, Section 4.1, Identification – The test article name should be Potassium Perfluorobutane Sulfonate instead of Potassium Perfluorobuntane Sulfonate.

Reason for Amendment No. 1: To correct a misspelled name.

Amendment No. 2: Protocol Page 3, Section 5.0, Regulatory Agency Submission: The GLP standard for the OECD should be Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45 [ENV/MC/CHEM(98)17], Paris 1998.

Reason for Amendment No. 2: The OECD GLP standard was updated.

Amendment No. 3: Protocol Page 9, Section 17.5.1, Determination of Relative Cell Growth (RCG) (10-11) - The Chromosome Aberration Assays were performed with the following concentration:

Definitive Assay (B1): 500, 1000, 2500 and 5000 μg/mL in both the non-activated and activated systems.

Confirmatory Assay (B2): 50, 100, 500, 1000, 2500 and 5000 μg/mL in the non-activated system only.

Reason for Amendment No. 3: As specified in the protocol, to be added in the form of an amendment once determined.

APPROVAL:

Jing Xu, M.D.
Study Director

10/25/2000
Date

John Seng, Ph.D.
Sponsor's Study Coordinator

10/31/00
Date

APPENDIX IV

DOSING SOLUTION ANALYSIS REPORT

DOSE FORMULAION ANALYSIS OF PERFLUOROBUTANESULFONATE IN DMSO BY HPLC/MS

STUDY ID: A239.1

SPONSOR STUDY NO. 132-009

Southern Research Institute
2000 Ninth Avenue South
P.O. Box 55537
Birmingham, AL 35255-5537

1. OBJECTIVE

The objective of this study was to determine the dose concentration of PFBS in the supplied dosing solutions received from the study director.

2. SAFETY

All necessary procedures to ensure safety of the analysts were based on information contained in the Material Safety and Data Sheets (MSDS), provided by the study director for the test article used in this study.

3. Compliance

This work was performed using a validated analytical method (BACG 3533), which along with the validation report is included in the appendix of this report. While this work was not audited in compliance with GLP regulations, it was performed in the spirit of the regulations using calibrated and validated instrumentation.

4. EXPERIMENTAL

4.1 Analytical Procedures

The sample preparation and analysis procedures as described in the analytical method BACG 3533 were employed for all analyses. Each sample was allowed to warm to room temperature and was then vortexed well before an aliquot was taken. Duplicated aliquots were taken from each sample and diluted as described in the method. Two standard curves was prepared over a concentration range of 500 to 10,000 ng/mL and analyzed along with the samples. A single composite curve with a correlation coefficient of 0.9991 was used to quantitative the samples. No outliers in the calibration standards were noted and subsequently no standards were dropped from either of the calibration curves.

4.2 Results

The results of the analysis are presented in the Table I at the end of the report.

5.0 Conclusion

A total of 12 dose formulation samples ranging in concentration from 5 to 500 mg/mL were analyzed by BACG 3533. All samples except two, 100 mg/mL B1, 8/22/00 (111%) and 250 mg/mL B2, 9/12/00 (117% of target) were found to be within ± 10 % of the reported

concentration. The latter sample was analyzed a second time and found to have a concentration of 116 % of target.

Table I
Dose Formulation Analysis of PFBS in DMSO

Sample	Date Prepared	Measured Conc. (mg/mL)*	% of Target
Vehicle B1	8/22/00	ND	NA
Vehicle B2	9/12/00	ND	NA
5 mg/mL B2	9/12/00	5.07	101
10 mg/mL B2	9/12/00	10.1	101
50 mg/mL B1	8/22/00	49.6	99.2
50 mg/mL B2	9/12/00	54.4	109
100 mg/mL B2	9/12/00	103.8	104
100 mg/mL B1	8/22/00	110.5	111
250 mg/mL B1	8/22/00	253.2	101
250 mg/mL B2	9/12/00	290.0	116**
500 mg/mL B1	8/22/00	484.8	97.0
500 mg/mL B2	9/12/00	521.2	104

ND = not detected
NA = not applicable
* = average of duplicate analysis
** = sample analyzed twice producing same result

Quality Assurance Statement

Final Report On

Dose Formulation Analysis of Perfluorobutane Sulfonate (PFBS) In DMSO

A239.1
Sponsor Study No. 132-009

This study was performed using a method validated for accuracy, precision (repeatability), linearity, range and specificity. No audits were performed during the course of the study, either as the analyses were performed, of the resulting data, or the report.

C.L. Marsh, Manager, Quality Assurance/Quality Control

7/3/07
Date

SUMMARY

A total of 12 formulated dose samples including blanks and vehicles ranging in concentration from 5 to 500 mg/mL were analyzed by analytical method BACG 3533 to determine the concentration of perfluorobutanesulfonate (PFBS) in the formulated mixture. All dose formulations were found to be within ± 10% of the reported concentration except for two samples, 100 mg/mL B1 dated 8/22/00 and 250 mg/mL B2 dated 9/12/00. This former sample had a formulation concentration of 110.5 % and the latter sample was initially found to be 117 % of target and upon reanalysis it was found to be 116 % of target.

KEY PERSONNEL

James D. Johnson, M.S., MBA
Manager
Bioanalytical Chemistry Group

Gregory S. Gorman, Ph.D.
Staff Chemist
Bioanalytical Chemistry Group

6.0 Approvals

Gregory S. Gorman, Ph.D.
Staff Chemist
Bioanalytical Chemistry Group

7-3-01
Date

James D. Johnson, M.S., MBA
Manager
Bioanalytical Chemistry Group

7/3/01
Date

METHOD VALIDATION REPORT

VALIDATION OF ANALYTICAL METHOD FOR DOSE FORMULATION ANALYSIS OF PERFLUOROBUTANE SULFONATE (PFBS) IN 1% CARBOXYMETHYL CELLULOSE (CMC)

STUDY ID: A098.1

Southern Research Institute
2000 Ninth Avenue South
P.O. Box 55537
Birmingham, AL 35255-5537

SUMMARY

Southern Research Institute has successfully validated for 3M an analytical method (BACG 3533) entitled "Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC Mass Spectrometry". Calibration standards were prepared by spiking solvent solutions with known amounts of test article, PFBS, and internal standard, perfluoropentanoic acid. The calibration standards were prepared over a concentration range of 500 to 10,000 ng/mL and the carboxymethyl cellulose (CMC) concentration was adjusted to equal that of each diluted sample in each set. Additionally, three standard curves were prepared and analyzed which contained no CMC as controls. A total of 6 calibration curves were generated during the study which produced correlation coefficients ranging from 0.9992 to 0.9999.

KEY PERSONNEL

James D. Johnson, M.S.
Manager
Bioanalytical Chemistry Group

Gregory S. Gorman, Ph.D.
Research Chemist III
Bioanalytical Chemistry Group

Lester Williams, B.S.
Associate Chemist II
Bioanalytical Chemistry Group

1. OBJECTIVE

The objective of this study was to provide a validated analytical method for the determination of PFBS concentration in dose formulation samples containing 1% CMC.

2. SAFETY

All necessary procedures to ensure safety of the analysts were based on information contained in the Material Safety and Data Sheets (MSDS), provided by 3M for the test article used in this study.

3. EXPERIMENTAL

3.1 Analytical Procedures

The sample preparation and analysis procedures as described in the analytical method BACG 3533 were employed for all analyses. For the preparation of the calibration standards, a known volume of a solvent (e.g., 1 mL) containing CMC was spiked with a known amount of test article and internal standard and vortexed briefly to ensure mixing. Each formulation sample was diluted to a final concentration of 3200 ng/mL and an aliquot of this was placed into autosampler vials for analysis.

3.2 Method Validation

Validation for BACG 3533 "Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC Mass Spectrometry" consisted of analyzing three standard curves containing CMC at the concentration present in the diluted 4 mg/mL dose formulation samples (0.001%) and three curves prepared without CMC. The concentration range for both sets ranged from 500 to 10,000 ng/mL. A total of 7 calibration levels in each curve containing CMC and 6 in each of the three without CMC were evaluated. The summaries are given below:

<u>Calibration Results 0% CMC</u>

A total of three standard curves each containing 6 individual standards encompassing a range of 500 to 10,000 ng/mL were analyzed in duplicate. The correlation coefficient for the composite curve comprising a total of 36 data points was found to be 0.9992. No data points were dropped from the analysis. A statistical summary for the composite curve is shown below:

STANDARD COMPOSITE CURVE PFBS (0% CMC)

Concen. (ng/mL)	500	1000	3000	5000	8000	10,000
# of Standards	6	6	6	6	6	6
Mean % Accur.	97.71	102.9	99.73	99.52	100.1	100.2
Std. Deviation	19.47	55.85	110.6	187.8	288.2	356.4

Calibration Results (CMC = 0.001%)

Three standards curves containing a total of 21 single standards encompassing a range of 500 to 10,000 ng/mL were analyzed. The correlation coefficient for the calibration curve was found to be 0.9997. No data points were dropped from the analysis. A statistical summary for the composite curve is shown below:

STANDARD COMPOSITE CURVE PFBS (0.001% CMC)

Concen. (ng/mL)	500	1000	3000	3200	5000	8000	10,000
# of Standards	3	3	3	3	3	3	3
Mean % Accur.	98.11	101.5	101	101	98.44	99.31	100.7
Std. Deviation	18.19	17.72	48.33	49.67	81.61	76.38	203.4

3.3 Calculations

Calculations were performed using TurboQuan (Version 1.0). The amount of analyte in the diluted dose formulation samples (ng/mL) was back calculated using a calibration curve generated from a set of calibration standards containing the equivalent amount of CMC as in the diluted samples. The calibration curve was generated by a regression analysis to determine the best fit curve (e.g., linear, quadratic etc.) and amount of weighting. A quadratic fit with 1/X weighting was determined to be the best fit :

$$y = ax^2 + bx + c$$

where:

y = Peak area response of test article

x = Concentration of the test article in standards.

a, b, c = Constants derived from the regression analysis.

4.0 Conclusion

A quantitative method (BAGC 3533) has been developed and validated for the determination of PFBS in dose formulation solutions containing 1% CMC. Quantitation for this method is based on internal standard which produces standard curves with a correlation coefficients of 0.9992 or greater over a concentration range of 500 to 10,000 ng/mL.

5.0 Approvals

Gregory S. Gorman, Ph.D.
Staff Chemist
Bioanalytical Chemistry Group

7-5-01
Date

James D. Johnson, MS, MBA
Manager
Bioanalytical Chemistry Group

7/5/01
Date

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

1.0 PRINCIPLE

Dose formulation samples of perfluorobutane sulfonate (PFBS) in 1 % carboxymethyl cellulose (CMC) are diluted down to 3200 ng/mL and analyzed by HPLC/MS. Quantitation is based on an internal standard using back calculated values from a calibration curve encompassing a concentration range from 500 to 10,000 ng/mL and containing the same concentration of CMC as the dose formulations in the diluted form.

2.0 REAGENTS AND SOLUTIONS

The listed reagents or their equivalents may be used.

2.1 Neat Reagents

2.1.1 Water, deionized and organic free (from in-house purification system; e.g., Ingalls 210N) –

2.1.2 Methanol, HPLC grade

2.1.3 Perfluorobutanesulfonate (analyte), as provided by the client

2.1.4 Perfluoropentanoic acid (internal standard), 97%

2.1.5 Ammonium acetate, HPLC grade

2.2 Prepared Solutions

Appropriate changes in the solutions may be made at the discretion of the analyst

2.2.1 5 mM Ammonium acetate in organic free water

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

2.2.1.1 For example, to prepare 4 liters, measure out ammonium acetate (e.g., 1.542 g) and add in organic-free water (e.g., 4 L). Mix well and filter through HPLC mobile phase filtration apparatus.

3.0 INSTRUMENTS, MATERIALS, AND APPARATUS

The following or their equivalents may be used.

3.1 HPLC pump(s), autosampler, and single quadrupole mass spectrometer

3.2 Autosampler vials with inserts

3.3 Vortex mixers (e.g., touch mixer and IKA-Vibrax ® platform mixer)

3.4 HPLC mobile phase filtration apparatus

3.5 Filters for HPLC mobile phase filtration apparatus (e.g., Nylon-66, 0.20 μm)

3.7 Analytical balance

3.8 Volumetric flasks (e.g., 10 and 25 mL)

3.9 Disposable Pasteur pipets

3.10 Micropipettor(s) with tips

3.11 Culture tubes with teflon-lined caps

3.12 Assorted glassware and syringes

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

4.0 PREPARATION OF STOCKS AND WORKING STOCKS

Appropriate changes in the concentrations of the solutions may be made at the discretion of the analyst. Actual dilutions will be documented on the preparation sheets.

4.1 Main Stock Solution of PFBS ~1000 µg/mL

4.1.1 Prepare an ~1000 µg/mL solution of PFBS in mobile phase (e.g., accurately weigh about 10 mg PFBS into a 10-mL volumetric flask). Add mobile phase to dissolve. Dilute to the mark. Alternatively, weigh the compound into an appropriate vessel (e.g., culture tube) and add 10 mL of mobile phase. Mix well. Transfer the solution to a clean vessel if desired.

4.2 Spiking Stock Solution of PFBS ~50 µg/mL

4.2.1 Prepare an ~50 µg/mL solution of PFBS in mobile phase (e.g., measure about 500 µL of ~1000 µg/mL PFBS into a 10-mL volumetric flask). Add mobile phase to dissolve. Dilute to the mark. Alternatively, weigh the compound into an appropriate vessel (e.g., culture tube) and add 10 mL of mobile phase. Mix well. Transfer the solution to a clean vessel if desired.

4.3 Stock Solution of Internal Standard (PFPA), ~200 µg/mL

4.3.1 Prepare an ~200 µg/mL solution of PFPA in deionized organic-free water (e.g., accurately weigh about 10 mg into a 50-mL volumetric flask). Add deionized organic-free water to dissolve and dilute to the mark with deionized organic-free water. Alternatively, weigh the compound into a an appropriate vessel (e.g., culture tube) and add 50 mL of deionized organic-free water. Mix well. Transfer the solution to a clean vessel if desired.

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

4.3 **Working Stock Solutions of PFBS**

4.3.1 To prepare working stock solutions, make the proper dilutions as shown in the following table. Prepare in 10-mL volumetric flasks or other appropriate glassware. If desired a modified dilution scheme can be used and documented in the study records.

Working Stock Level (WSL) Approximate Concentration (ng/mL)	Volume of Internal Standard (µL)	Volume of PFBS spiking solution (µL)	Volume of mobile phase (µL)
500	200	10	790
1000	200	20	780
3000	200	60	740
3200	200	64	736
5000	200	100	700
8000	200	160	640
10000	200	200	600

Note: The mobile phase used for each standard curve should contain the same concentration of CMC as the samples. For example, the dilution factor of a 4 mg/mL sample is 1000 (prior to addition of IS). The mobile phase for a 4 mg/mL sample prepared in 1% CMC should contain 1/1000% CMC to account for the dilution.

5.0 PREPARATION OF SPIKED STANDARDS AND BLANKS

Appropriate changes in the concentrations of the solutions may be made at the discretion of the analyst.

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

5.1 Multiple (e.g., about three) sets of standards blanks (blank + IS) are analyzed with each set of unknown samples. A double blank (blank-IS) may also be analyzed if desired. Standards may be prepared as shown in the table in 4.3.1.

6.0 PREPARATION OF SAMPLES

6.1 All samples are to be diluted to approximately the middle of the curve (e.g. 3200 ng/mL). Dilutions are to be made with mobile phase. For example, for a 4 mg/mL sample, take 1 mL and dilute with mobile phase in a 10 mL volumetric flask. Then take 100 µL of the diluted sample and dilute that with mobile phase in a 10 mL volumetric flask. Take 800 µL of the second dilution, place in an autosampler vial, add 200 µL of IS, mix, and analyze.

7.0 ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY/MASS SPECTROMETRY (HPLC/MS/MS)

7.1 Conditions are to be optimized if necessary.

7.1.1 HPLC Conditions

Analytical Column:	none
Guard Column:	Fluofix 120E 10 mm x 2 mm
Elution Flow rate:	1000 µL/min.
Injection volume:	5 µL
Mobile phase:	A: 5mM ammonium acetate buffer B: methanol
Elution Profile:	Isocratic 30% A : 70% B
Temperature:	Ambient

7.1.2 PE Sciex API 150EX Single Quadrupole Mass Spectrometer Conditions

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

Software: PE Sciex TurboQuan

Turboion Spray Source
Note: Values listed under "MS/MS Acquisition Conditions" override parameters in this table.

Auxiliary Gas: Air (e.g., Grade 0.1) at 85 pounds per square inch

Parameter	Value
IS	-5000
NC	0
TEM	450
OR	-25
RNG	-170
Q0	10
IQ1	11
ST	16
RO1	11
DF	300
CEM	2400
NEB	15
CUR	8
CAD	0
QPE	0
POL	0
VCM	0
IPE	0

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

Masses requested:

PFBS:

Q1 Mass (amu)	Dwell Time (ms)
299.2	200

PFPA (IS)

Q1 Mass (amu)	Dwell Time (ms)
218.9	200

8.0 CALCULATIONS

8.1 At the end of the analytical run, review each chromatogram to ensure the retention time, peak shape, and peak height and peak area determination of the test article and the IS are acceptable. The data may be smoothed as appropriate. For quantitation, use the ion profiles at the following mass-to-charge ratios:

Analyte	Ion Profile
PFBS	299.2
PFPA	218.9

8.2 Plot the peak area response of PFBS divided by the peak area response of the IS (PFPA) from all standards versus the concentration of the test article in the standards. Alternatively, the peak heights may be used instead of peak areas. Obtain the best curve fit of the data (e.g., quadratic fit weighted with 1/concentration of the test article or a quadratic fit). Note: The best curve fit may be dependent on the range of the standard curve and it may be necessary to have more than one standard curve for various concentration ranges using the following:

$$y = ax^2 + bx + c$$

where y = Peak height response of PFBS divided by peak height response of the IS (PFPA) in standards.

x = Concentration of the PFBS in standards.

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

a, b, c = Constants derived from the regression analysis.

8.3 Using the standard curve, calculate the level of PFBS in each unknown sample. Correct the results of samples for any dilutions.

9.0 ACCEPTANCE AND REJECTION CRITERIA

9.1 Refer to SOP SRI 91-3 for acceptance/rejection criteria except acceptable accuracy for standards is 80-120% of theoretical.

10.0 REPORTING

10.1 Results of all analyses are tabulated, and the raw data, original chromatograms, and reports are to be filed in the appropriate study file.

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