

FINAL REPORT

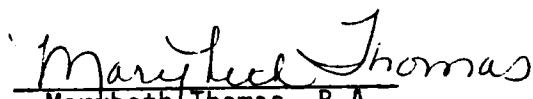
CHO/HGPRT In vitro Mammalian
Cell Mutation Assay on
SC-19129

Bioassay Systems Corporation
Project Number 12158

Prepared for:

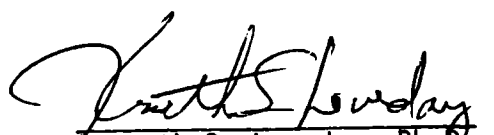
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March 20, 1985
Date

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TITLE: CHO/HGPRT Mammalian Cell Mutation Assay on SC-19129

Author: Marybeth Thomas, B.A.
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Study Number: S.A. 2463

Date: March 20, 1985

Type of Report: Final

Summary:

SC-19129 was investigated for the potential to induce mutations at the HGPRT gene locus in Chinese Hamster Ovary (CHO) cells in the presence and absence of a rat liver homogenate metabolic activation system. The eight intended test concentrations that were analyzed for induction of mutations ranged from 0.10 up to 2.00 mg/ml. The actual test concentrations based on analysis of the stock solution were 100.4% of the target concentrations. No evidence of a significant mutagenic response was seen. The responses obtained from the negative and positive controls demonstrated that the test system was capable of detecting chemical mutagens.

These results lead to the conclusion that SC-19129 is non-mutagenic under the conditions of the CHO/HGPRT mammalian cell mutagenesis assay.

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1.0 INTRODUCTION

1.1 Objective of the study

The purpose of this study was to assess the ability of SC-19129 to induce mutations at the HGPRT gene locus in Chinese hamster ovary (CHO) cells. Aliquots of the test sample were received 10/19/84 and 10/31/84. Range-finding experiments were initiated 11/14/84 and completed 11/26/84. Mutagenesis testing was initiated 12/6/84, completed 12/27/84. All assays were conducted according to FDA Good Laboratory Practice Regulations (21 CFR 58.1-58.219, 1979).

1.2 Principles of the assay

Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) is a purine salvage enzyme which provides a scavenger pathway for the synthesis of purine nucleotides. HGPRT mutants, deficient in HGPRT activity, are selected by growing mammalian cells in medium containing 6-thioguanine (6TG). This purine analogue is an HGPRT substrate and is converted to toxic nucleotides, thus killing the wild type cells. Mutant cells cannot metabolize the purine analogue and thus survive the selection step.

The HGPRT system utilizes optimal selection conditions for crucial parameters such as the mutagen exposure time, phenotypic expression time, 6TG concentration and the cell density which permits maximum mutant recovery. The CHO HGPRT assay is a well characterized mammalian cell culture system which measures the frequency of mutations induced at a specific gene locus.

2.0 MATERIALS

2.1 Test Substance

Name:	SC-19129
BSC No.:	84-1226A and 84-1226B
Lot/Batch No.:	84K-047-101
Physical State:	solid; powder
Color:	white
Density:	not applicable
Purity:	greater than 99%
Composition:	determined by Sponsor
Stability:	determined by Sponsor
Stability of Formulations:	determined by Sponsor
Solubility:	Dimethylsulfoxide (greater than 100 mg/ml)
Storage Conditions:	ambient, protect from light
Safety Precautions:	routine

The test sample was assayed in solution using warm (37°C) dimethylsulfoxide as a vehicle. A stock solution with a target concentration of 200 mg/ml was used for activated and nonactivated assays. Concentration analysis (Appendix B) of the test article mixture confirmed the target stock solution concentration (100.4%). All further dilutions were made in dimethylsulfoxide. The stock solution and dilutions (one for each concentration) prepared for the activated assay were stored at 37°C and used within a 4 hour period for the nonactivated assay. At the time of use the 200 mg/ml stock solution appeared cloudy, all the dilutions were clear. Aliquot 84-1226A was used for the range finding experiments; aliquot 84-1226B was used for the mutagenesis assays.

The Sponsor assumed responsibility for determining the identity, strength, purity, composition and stability of the test article and the stability of the formulations.

2.2 Negative Control Substance

Name:	Dimethylsulfoxide (DMSO)
Supplier:	J.T. Baker Chemical Co.
Lot/Batch No.:	327701
Physical State:	liquid
Color:	clear
Purity:	reagent grade
Composition:	on file with manufacturer
Stability:	indefinite
Storage conditions:	room temperature
Safety precautions:	avoid topical and respiratory contact

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2.3 Positive Control Substances

Activated Assay

Name:	9,10-Dimethyl-1,2-Benzanthracene (DMBA)
Lot No.:	13F-0543
Supplier:	Sigma
Physical State:	solid
Color:	yellow tint
Purity:	at least 98%
Solubility:	soluble in DMSO
Composition:	on file with manufacturer
Stability:	at least 1 yr. (solid)
Stability of formulation:	1 month at 4°C (solution)
Storage conditions:	room temperature
Safety Precautions:	avoid topical and respiratory contact

Non-Activated Assay

Name:	Ethylmethanesulfonate(EMS)
Lot No.:	83F-0279
Supplier:	Eastman Kodak
Physical State:	liquid
Color:	yellow tint
Purity:	reagent grade
Composition:	on file with manufacturer
Stability:	at least one year
Stability of formulations:	5 hours at room temperature
Specific Gravity:	1.167
Solubility:	soluble in H ₂ O (culture medium)
Storage Conditions:	4°C
Safety Precautions:	avoid topical and respiratory contact

Stock solutions of the positive control agents were prepared and aliquots added to the exposure medium. A 1.5 mg/ml stock solution of DMBA in DMSO was used to provide a final concentration of 15 ug/ml. EMS was dissolved directly in the exposure medium at a concentration of 11.7 mg/ml (final concentration 234 ug/ml). Stock solutions of DMBA are stored at 4°C. Stock solutions of EMS are prepared fresh prior to use.

2.4 CHO Cell Culture

Cells used in this assay were obtained from Dr. Samuel Latt at the Children's Hospital Medical School, Boston, MA. Dr. Latt originally obtained the line from Dr. Arthur Pardee at the Sydney Farber Cancer Center, Boston, MA.

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Master vials are stored in liquid nitrogen or in a freezer at -70°C; stock cultures are replaced from the frozen vials. All the frozen cultures have been prescreened for mycoplasma contamination and the spontaneous background mutant frequency is acceptably low. Working and experimental cultures are maintained in cell culture incubators in F12 medium (without hypoxanthine) containing 5% dialyzed fetal calf serum. The exposure medium was supplemented with HEPES buffer (20 mM).

2.5 Microsomal Activation System

An S9 microsome fraction prepared from the liver of Aroclor 1254 induced (500 mg/kg) Sprague-Dawley rats was used in the activated assays. The S9 fraction was combined with a solution of cofactors and culture medium to form the metabolic activation system. A mixture of 10% 10X Isocitrate Cofactors and 90% serum-free F12 medium was prepared and used as the exposure medium. Following the addition of the test sample, an aliquot of the S9 fraction was added to each flask. The final concentrations in each flask were 4.5 mg/ml Isocitric acid (trisodium salt), 2.4 mg/ml NADP and 20 ul/ml S9 fraction. The cofactor/medium mixture was prepared just prior to use and the S9 fraction was maintained on ice until use.

The following S9 fraction was used in the study:

Source:	Litton Bionetics
	Kensington, MD
Lot/Batch No.:	02069
Storage Conditions:	-80°C

2.6 Identification of Test System

All the experimental vessels were labeled with the last three digits of the project number and a code number. The key to code numbers are given in the raw data sheets.

3.0 EXPERIMENTAL DESIGN

3.1 Toxicity Determination

Initial range-finding experiments delineated the concentrations used in subsequent mutagenesis assays. Parallel toxicity and mutagenicity studies were conducted to confirm the toxicity of each concentration used in assays with and without activation. The following method of toxicity assessment was used for the range-finding studies and also to confirm toxicity in mutagenesis studies:

Half a million (5×10^5) CHO cells were seeded in plastic T25 flasks. Approximately 24 hours later, the cells were exposed to varying SC-19129 concentrations. Exposure times for the activated and nonactivated assays were 4 and 16 hours, respectively. Immediately

after the exposure in the nonactivated assays and the day following the exposure for activated assays 200 cells (total count) were seeded in duplicate petri dishes. After at least one week of incubation the cells were fixed, stained and scored.

3.2 Mutation Induction

CHO cells were seeded at a density of approximately 1.5×10^6 in T75 flasks and grown for 1 day before being exposed to the test sample and controls. The exposure times were 4 and 16 hours for activated and nonactivated assays, respectively. All test sample concentrations and the positive control were tested in duplicate flasks. Two sets of duplicate flasks were performed for the negative control. All flasks were maintained independently throughout the expression period. Immediately after the removal of the test sample in the nonactivated assay and the day following the removal of the test sample in the activated assay, the cells were replated for toxicity and expression time. The expression flasks were replated as necessary to maintain their maximum growth rate (usually every 2-3 days); the cells were maintained for at least 7 days. The cells were then plated at 2×10^5 per dish (usually six dishes per flask) in medium containing 2 ug/ml 6-thioguanine (6TG). Concomitantly with the selection step 200 cells from each flask were plated in duplicate petri dishes in medium without 6TG to determine the cloning efficiency of the cells. After at least 1 week the colonies were fixed with methanol and stained with Giemsa. The plates were scored by a trained technician. A group of cells containing a minimum of 50 cells was counted as a mutant colony. The activated part of the assay was performed by exposing cells in serum-free medium to the test sample and controls in the presence of a metabolic activation system (optimal concentrations of the S9 microsomal fraction mixture).

$$\text{Mutant Frequency} = \frac{\text{No. of Mutants}}{\text{No. of cells plated in selective medium} \times \text{PE}}$$

$$\text{PE (plating efficiency)} = \frac{\text{Avg. No. Colonies}}{\text{No. of plated cells}}$$

$$\text{Relative Percent survivors} = 100 \times \frac{\text{Avg. No. Colonies of Sample}}{\text{Avg. No. Colonies of Negative Control}}$$

4.0 RESULTS

Range-finding Experiments

The effects of SC-19129 on the survival of CHO cells in the absence of the activation system are presented in Table 1 and in the presence of the activation system in Table 3. No toxicity was

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observed under activated or nonactivated conditions. Since the test sample did not exhibit any signs of toxicity, the concentrations used in the mutagenesis assays were based on the solubility limit of the test sample in DMSO.

Nonactivated Mutagenesis Experiment

The effects of SC-19129 on the induction of HGPRT mutants in CHO cells in the absence of the activation system are presented in Table 2. Eight concentrations ranging from 0.10-2.00 mg/ml were tested; all survived the expression period. The background mutant frequencies of the 1% DMSO controls were 2.9, 10.9, 19.9 and 13.0 x 10⁻⁶. Two test sample concentrations (0.76 and 0.50 mg/ml) had single points with mutant frequencies noticeably higher than the negative controls (46.2 and 31.9 x 10⁻⁶). In each case, however, the mutant frequency of the duplicate flask was much lower (5.1 and 5.5 x 10⁻⁶, respectively) and these responses are considered to be within the variability of the assay. A concentration-related response was not observed. The positive control, EMS (234 ug/ml) induced significant increases in mutant frequency (272 and 132 x 10⁻⁶).

Activated Mutagenesis Experiment

The test sample SC-19129 was also tested in the presence of a metabolic activation system (Table 4). Eight concentrations ranging from 0.10-2.00 mg/ml were tested; all survived the expression period. The background mutant frequencies of the 1% DMSO + S9 controls were 6.9, 9.4, 23.2 and 32.2 x 10⁻⁶. One test sample concentration (2.00 mg/ml) had a single point with a mutant frequency higher than the negative controls (40.3 x 10⁻⁶); however, the duplicate flask had a much lower mutant frequency (13.1 x 10⁻⁶). None of the other test sample concentrations had mutant frequencies different from the negative controls. The positive control DMBA (15 ug/ml) + S9 induced significant increases in mutant frequency (231 and 167 x 10⁻⁶).

5.0 CONCLUSION

The ability of SC-19129 to induce mutations at the HGPRT locus in CHO cells was evaluated in the presence and absence of a metabolic activation system. Under the conditions of the assay employed, the compound did not exhibit mutagenic activity.

Table 1
The Effect of SC-19129
on the Survival of CHO cells

Range-finding Experiment

Nonactivated Assay

Sample	Conc. (mg/ml)	No. of Cells Remaining in Flask ^a	No. of Plated Cells/dish	No. of Colonies Per dish	Relative Percent Survivors ^b (%)
SC-19129	1	2.3×10^6	200	120/117	108.5
	0.3	2.6×10^6	200	95/94	86.5
	0.1	2.8×10^6	200	107/114	101.1
	0.03	2.4×10^6	200	121/108	104.8
	0.01	2.4×10^6	200	156/160	144.6
	0.003	2.5×10^6	200	108/105	97.5
	0.001	3.9×10^6	200	96/87	83.8
	0.0003	2.8×10^6	200	81/104	84.7
DMSO	1%	2.4×10^6	200	107/107	100
		2.9×10^6		108/115	

^aCounts taken at time of toxicity plating

^bRelative percent survivors calculated using average number of colonies exposed to 1% DMSO as 100% survivors.

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TAL 2
The Effect of SC-19129 on the Induction of
HGPRT Mutants in CHO Cells, Nonactivated Assay

Toxicity Data					Mutagenicity Data				
Sample	Conc. (mg/ml)	No. of Cells Remaining in Flask ^a	No. of Plated cells	No. of colonies	Relative Percent Survivors %	No. of Colonies (out of 200)	No. of Cells Plated in Selective Medium (x 10 ⁶)		Mutant Freq.-6 (x 10 ⁻⁶)
							No. of Mutants		
SC-19129	2.00	4.8x10 ⁶	200	85/87	80.6	93/114	1.2	5	8.1
		6.0x10 ⁶	200	102/122	104.9	124/95	1.2	13	19.8
	1.76	7.7x10 ⁶	200	85/85	79.6	146/171	1.2	8	8.4
		5.5x10 ⁶	200	95/112	97.0	159/159	1.2	3	3.1
	1.50	5.2x10 ⁶	200	71/94	77.3	109/104	1.2	5	7.8
		4.2x10 ⁶	200	97/120	101.6	93/78	1.2	4	7.8
	1.26	7.8x10 ⁶	200	70/72	66.5	111/85	1.2	13	22.1
		6.4x10 ⁶	200	111/122	109.1	134/116	1.2	20	26.7
	1.00	5.4x10 ⁶	200	95/105	93.7	113/134	1.2	8	10.8
		5.6x10 ⁶	200	116/121	111.0	126/114	1.2	3	4.2
	0.76	6.3x10 ⁶	200	79/85	76.8	92/103	1.2	27	46.2
		5.8x10 ⁶	200	112/121	109.1	60/70	1.2	2	5.1
	0.50	4.6x10 ⁶	200	87/104	89.5	117/124	1.2	4	5.5
		6.0x10 ⁶	200	95/99	90.9	117/113	1.2	22	31.9
	0.10	4.9x10 ⁶	200	116/126	113.3	74/66	1.2	1	2.4
		3.7x10 ⁶	200	155/162	148.5	106/110	1.2	6	9.3
DMSO	1%	6.4x10 ⁶	200	97/123	100	116/115	1.2	2	2.9
		5.5x10 ⁶	200	111/123		138/107	1.2	8	10.9
		5.5x10 ⁶	200	99/103		93/108	1.2	12	19.9
		5.7x10 ⁶	200	93/105		125/132	1.2	10	13.0
EMS	0.234	5.4x10 ⁶	200	104/134	111.5	50/53	1.2	84	272
		5.6x10 ⁶	200	82/94	82.4	57/64	1.2	48	132
DMSO - Dimethyl sulfoxide, EMS - Ethylmethane Sulfonate									

^aCounts taken at time of toxicity plating

^bRelative percent survivors was calculated using the average number of colonies exposed to 1% DMSO as a 100% survival.

Table 3

The Effect of SC-19129
on the Survival of CHO cells

Range-finding Experiment

Sample	Conc. (mg/ml)	No. of Cell Remaining in Flask ^a	<u>Activated Assay</u>		Relative Percent Survivors ^b (%)
			No. of Plated Cells/dish	No. of Colonies Per dish	
SC-19129	1	1.9×10^6	200	115/111	86.3
	0.3	3.1×10^6	200	96/94	72.5
	0.1	2.7×10^6	200	101/82	69.8
	0.03	2.5×10^6	200	103/99	77.1
	0.01	1.6×10^6	200	64/81	55.3
	0.003	2.2×10^6	200	96/114	80.2
	0.001	2.8×10^6	200	116/129	93.5
	0.0003	2.5×10^6	200	124/141	101.1
DMSO+S9	1%	2.5×10^6	200	135/155	100.0
		2.0×10^6		109/125	

^aCounts taken at time of toxicity plating

^bRelative percent survivors calculated using average number of colonies exposed to DMSO as 100% survivors.

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TAR 4

The Effect of SC-1912y on the Induction of HGPRT Mutants in CHO Cells, Activated Assay

Sample	Conc. (mg/ml)	Toxicity Data			Relative Percent Survivors %	Mutagenicity Data			Mutant Freq. (x 10 ⁻⁶)
		No. of Cells Remaining in Flask	No. of Plated cells	No. of colonies		No. of Colonies (out of 200)	No. of Cells Plated in Selective Medium (x 10 ⁶)	No. of Mutants	
SC-19129	2.00	4.1x10 ⁶	200	84/107	89.3 108.9	148/131 122/93	1.2 1.2	11 26	13.1 40.3
		4.9x10 ⁶	200	108/125					
	1.76	6.2x10 ⁶	200	132/129	122.0 92.1	94/125 139/124	1.2 1.2	11 12	16.7 15.2
		5.0x10 ⁶	200	103/94					
	1.50	2.4x10 ⁶	200	116/120	110.3 105.6	143/157 128/155	1.2 1.2	4 18	4.4 21.2
		4.4x10 ⁶	200	97/129					
	1.26	5.7x10 ⁶	200	103/111	100.0 91.6	104/108 130/135	1.2 1.2	15 10	23.6 12.6
		5.5x10 ⁶	200	97/99					
	1.00	5.3x10 ⁶	200	127/144	126.6 66.8	110/106 103/91	1.2 1.2	17 16	26.2 27.5
		6.1x10 ⁶	200	75/68					
	0.76	5.2x10 ⁶	200	144/117	122.0 102.3	70/89 141/161	1.2 1.2	12 16	25.2 17.7
		5.3x10 ⁶	200	105/114					
	0.50	6.5x10 ⁶	200	99/107	96.3 121.0	128/110 125/138	1.2 1.2	9 2	12.6 2.5
		4.7x10 ⁶	200	110/149					
	0.10	5.9x10 ⁶	200	143/146	135.0 146.3	113/132 116/119	1.2 1.2	6 18	8.2 25.5
		6.3x10 ⁶	200	151/162					
DMSO+S9	1%	7.4x10 ⁶	200	91/96	100	91/102 123/125	1.2 1.2	4 7	6.9 9.4
		5.1x10 ⁶	200	122/129					
		8.2x10 ⁶	200	98/114	1.2 1.2	126/118 106/122	1.2 1.2	17 22	23.2 32.2
		7.5x10 ⁶	200	93/113					
DMBA+S9	0.015	5.2x10 ⁶	200	102/116	101.9 126.6	148/152 83/97	1.2 1.2	208 90	231 167
		3.6x10 ⁶	200	132/139					
DMSO - Dimethylsulfoxide, DMBA, 9,10-Dimethyl-1,2-benzanthracene									

^aCounts taken at time of toxicity plating

^bRelative percent survivors was calculated using the average number of colonies exposed to 1% DMSO as a 100% survival.

BIOASSAY SYSTEMS CORPORATION

Quality Assurance Report

Study Title: CHO/HGPRT In Vitro Mammalian Cell Mutation Assay on SC-19129

Sponsor: G.D. Searle & Co.

BSC Project No.: 12158

BSC Sample No.: 84-1226A, 84-1226B

<u>Date(s) of Inspection</u>	<u>Date Findings Reported to Study Director</u>	<u>Date Findings Reported To Management</u>
10/31/84	10/31/84	11/5/84
12/6/84	12/7/84	12/10/84
1/29/85	1/29/85	1/29/85
3/14/85	3/14/85	3/20/85

Date: 3/20/85

Quality Assurance Officer: Susan M. O'Connor

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APPENDIX A

APPENDIX A

1. Supervisory Personnel

Kenneth S. Loveday, Ph.D., Director of Genetic Toxicology
Marybeth Thomas, B.A., Study Director
Susan M. O'Connor, B.S., Manager, Quality Assurance

2. Storage Location Information

Raw Data: BSC Archives
Final Report: BSC Archives

APPENDIX B

REPORT OF ANALYSIS

BSC PROJECT NO(s): 12158

DATE OF ANALYSIS: 12/5-6/84

SPONSOR: G. D. Searle

TEST SAMPLE IDENTIFICATION

Sponsor Identification: SC19129 B-APM

BSC Sample No.: 84-1226B

CHEMICAL VEHICLE: Dimethyl sulfoxide (DMSO)
Source: Baker Chemical Co.
Lot No.: 327701


METHOD OF ANALYSIS: BSC Standard Operating Procedure # 103-146

SUMMARY OF RESULTS:

1. System Suitability: Mean Standard Peak Area: 33.7894
Relative Standard Deviation (RSD): 2.9%
Number of Injections: 6
2. Standard Check - Percent of Theory: 102.0%
3. Control Sample - Percent Recovery: 93.3%
4. Test Samples - Concentrations Measured/% Recovery
 - a. Formulation Lot No. 12-6-84: 200.9 mg/ml DMSO
100.4% Recovery

REVIEW OF ANALYSIS

The results reported above have been reviewed and found to accurately represent the data collected during this analysis.


Theodore A. Olsson III
Manager, Chemistry

Date 3-18-85

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REPORT OF ANALYSIS

BSC PROJECT NO(s): 12158

DATE OF ANALYSIS: 12/5-6/84

SUMMARY OF DATA

Sample/Standard Identification	Preparation of Sample/Standard ^c	Injection No.	Peak Area	Mean Peak Area
Standard	0.100g to 100 ml with mobile phase	1(a)	34.5254	33.7894 (2.9% RSD)
		2(a)	35.0809	
		3(a)	33.3606	
		4(a)	32.8844	30.6060
		5(a)	32.6080	
		6(a)	34.2771	
		1(b)	31.2076	
		2(b)	37.3094*	
		9(b)	30.0044	
Standard Check	0.1000g to 100 ml with mobile phase	3(b)	30.5881	30.0013
		4(b)	29.4144	
Control Sample	0.1000g +1ml DMSO to 100 ml with mobile phase	5(b)	27.5852	28.5561
		6(b)	29.5270	
Test Sample (Form. Lot # 12-6-84)	Diluted 1:1 with DMSO, then 1 ml to 100 ml with mobile phase ^d	10(b)	30.3411	30.7366
		11(b)	31.1321	

(a) System suitability check-prior to analysis of test samples

(b) Standard and sample chromatographic runs for analysis of test sample

(c) All standards and samples were diluted 1.0 ml to 10.0 ml with mobile phase after the preparations noted below.

(d) Due to technician error, 1.0 ml DMSO was also added to the first 1.0 ml to 100ml dilution of the sample. Although this resulted in twice the concentration of DMSO in the test sample dilution, no effect was observed upon the quantitation of the test sample.

* Not used - this standard vial previously used for "system suitability" - evaporation suspected.

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REPORT OF ANALYSIS

BSC PROJECT NO(s).: 12158

DATE OF ANALYSIS: 12/5-6/84

SUMMARY OF CALCULATIONS:

1. Standard Check - Percent of Theory

$$\begin{aligned} \% \text{ STD} &= \frac{R \text{ STD}}{R \text{ STDCK}} \times \frac{W \text{ STD CK}}{W \text{ STD}} \times 100\% \\ &= \frac{30.6060}{30.0013} \times \frac{100.0 \text{ mg}}{100.0 \text{ mg}} \times 100 \\ &= 102.0\% \end{aligned}$$

2. Control Sample - Percent Recovery

$$\begin{aligned} \% \text{ Recovery} &= \frac{R \text{ CS}}{R \text{ STD}} \times \frac{\text{Conc. STD}}{\text{Conc. CS}} \times 100\% \\ &= \frac{28.5561}{30.6060} \times \frac{1.000 \text{ mg/ml}}{1.000 \text{ mg/ml}} \times 100\% \\ &= 93.3\% \end{aligned}$$

3. Concentrations of Test Sample

$$\text{Conc. Test Sample} = \frac{R \text{X}}{R \text{STD}} \times \text{Conc. STD} \times \text{dilution factor}$$

(SC19129 mg/ml DMSO)

a) Test Sample Form. Lot No. 12-6-84

$$\begin{aligned} \text{Conc. Test Sample} &= \frac{30.7366}{30.6060} \times 1.000 \text{ mg/ml} \times 200 \\ &= 200.9 \text{ mg/ml DMSO} \end{aligned}$$

$$\begin{aligned} \text{Expected Conc.} &= 200 \text{ mg/ml DMSO} \\ \% \text{ Recovery} &= 100.4\% \end{aligned}$$

Note: R STD = mean peak height or area for Standard
 R STD CK = mean peak height or area for Standard Check
 W STD = weight of test sample in Standard (mg)
 W STD CK = weight of test sample in Standard Check (mg)
 R CS = mean peak height or area for Control Sample
 Conc STD = concentration of test sample in Standard (mg/ml equivalent DMSO)
 Conc CS = concentration of test sample in Control Sample (mg/ml DMSO)

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1.0 Study Title

CHO/HGPRT In Vitro Mammalian Cell Mutation Assay on SC-19129

2.0 Purpose of Study

To determine the mutagenicity of the test sample in Chinese hamster ovary cells (CHO) at the HGPRT locus.

3.0 Management of Study

3.1 Sponsor's Name and Address:

G.D. Searle & Co.
4901 Searle Parkway
Skokie, IL 60077

3.2 Sponsor's Study Coordinator: Charles E. Piper, Ph.D.

3.3 Testing Laboratory's Name and Address:

Bioassay Systems Corporation
225 Wildwood Avenue
Woburn, MA 01801

Bioassay Systems Project Number: 12158

3.4 Supervisory Personnel:

Director of Genetic Toxicology: Kenneth S. Loveday, Ph.D.

Study Director: Marybeth Thomas, B.A.

3.5 Manager, Quality Assurance: Susan O'Connor, B.S.

3.6 Proposed Study Schedule

3.6.1 Test Substance Received:	10/19/84
3.6.2 Study Initiated:	11/5/84
3.6.3 Study Completed:	1/4/85

4.0 Test Material Data

4.1 Test Sample Description:

	Test Sample
4.1.1 Identification:	SC-19129
4.1.2 BSC No.:	To be specified in final report
4.1.3 Lot No.:	84K-047-101
4.1.4 Physical State:	Solid; powder
4.1.5 Color:	White
4.1.6 Density:	Not applicable
4.1.7 Purity:	>99%
4.1.8 Composition:	To be determined by Sponsor
4.1.9 Stability of Bulk Chemical:	To be determined by Sponsor
4.1.10 Stability of Formulations:	To be determined by Sponsor
4.1.11 Solubility:	Dimethylsulfoxide (100 mg/ml)
4.1.12 Storage Conditions:	Ambient, protect from light
4.1.13 Safety Precautions:	Routine

The Sponsor will determine the identity, strength, purity, composition and stability of the test article. Bioassay Systems will determine the concentration of the test article in the carrier. The Sponsor will analyze the test article for identity (at a minimum) upon return from the testing laboratory.

4.2 Positive Control Material Characterization (activated assay)

4.2.1 Name: 9,10-Dimethyl-1,2-benzanthracene (DMBA)
4.2.2 Supplier: Sigma
4.2.3 Lot No.: to be specified in final report
4.2.4 Physical State: solid
4.2.5 Color: yellow tint
4.2.6 Purity: at least 98%
4.2.7 Composition: on file with manufacturer
4.2.8 Stability of Bulk Compound: at least 1 yr. (solid)
4.2.9 Stability of Formulation: 1 month at 4°C (solution)
4.2.10 Solubility: soluble in DMSO
4.2.11 Storage Conditions: room temperature
4.2.12 Safety Precautions: avoid topical and respiratory contact

4.3 Positive Control Material Characterization (nonactivated assay)

4.3.1 Name: ethylmethanesulfonate, EMS
4.3.2 Supplier: Eastman Kodak
4.3.3 Lot No.: to be specified in final report
4.3.4 Physical State: liquid
4.3.5 Color: yellow tint
4.3.6 Purity: reagent grade
4.3.7 Composition: on file with manufacturer
4.3.8 Stability of Bulk Compound: at least 1 yr.
4.3.9 Stability of Formulations: 5 hours at room temp.
4.3.10 Solubility: soluble in water (culture medium)
4.3.11 Specific Gravity: 1.167
4.3.12 Storage Conditions: 4°C
4.3.13 Safety Precautions: avoid topical and respiratory contact

4.4 Negative Control Material Characterization

4.4.1 Name: Dimethylsulfoxide (DMSO)
4.4.2 Supplier: J.T. Baker Chemical Co.
4.4.3 Lot No.: To be specified in final report
4.4.4 Physical State: Liquid
4.4.5 Color: Clear
4.4.6 Purity: Reagent grade
4.4.7 Composition: on file with manufacturer
4.4.8 Stability: Indefinite
4.4.9 Storage Conditions: Room temperature
4.4.10 Safety Precautions: Avoid topical and respiratory contact

5.0 Test System Specifications

5.1 Cell Line:

Chinese hamster ovary cells (CHO) were obtained from Dr. Samuel Latt at the Children's Hospital Medical School Boston, MA. Dr. Latt originally obtained the line from Dr. Arthur Pardee at the Sydney Farber Cancer Center, Boston, MA. Routine cell culture methods, preparation of media, S9 mix, and quality control measures are detailed in Bioassay Systems Corporation's standard operating procedures.

5.2 Storage:

Master vials are stored in liquid nitrogen or in a -70°C freezer. All the frozen cultures have been prescreened for mycoplasma contamination. To prevent accumulation of HGPRT mutants in stock cultures, the latter are replaced by stocks from the frozen vials. Working and experimental cultures are maintained in cell culture incubators in F12 medium without hypoxanthine containing 5% dialyzed fetal calf serum. The exposure medium is supplemented with HEPES buffer (20 mM).

5.3 Justification for Selection

There are several reasons for using the HGPRT system for assaying compounds for mutagenic activity in mammalian cells in vitro. (1)It is one of the most intensively studied genes in cultured mammalian cells. (2)It is relatively easy to select HGPRT⁻ mutants. Since the gene is located on the X chromosome, HGPRT⁻ cells are created by a single mutagenic event. (3)The mutagenic effect is dose-dependent for several classes of mutagens, such as ethylmethanesulfonate (EMS) (induction of point mutations), ICR-191 (induction of frame shift mutations), metallic compounds and irradiation by X-rays (induction of a variety of types of mutations including deletions). The system can also be used to detect mutagenicity of promutagens such as dimethylnitrosamine or benzo(a)pyrene which require activation before the mutagenic and/or carcinogenic potential is expressed. The HGPRT/CHO system utilizes optimal conditions developed by Dr. Hsie and colleagues for such crucial parameters as the mutagen exposure time, phenotypic expression time, 6-thioguanine concentration, and the cell density which permits maximum mutant recovery.

5.4 Identification of test system:

All the experimental vessels will be labelled with last three digits of the project no., and a code no. The key to the code numbers will be given in the raw data sheets.

6.0 Experimental Design

The following outline briefly summarizes the experimental design.

- A. Sample Preparation and Dosage Analysis
- B. Range-finding (toxicity curve) with and without S9.
- C. Mutagenesis in presence or absence of S9.
 - 1. Exposure to test compound (16 hours in absence of S9, 4-5 hours in presence of S9).
 - 2. Expression time and toxicity evaluation
 - 3. Selection for mutant cells

6.1 Concentration Formulation

The concentration will be formulated according to sponsor's recommendations. Concentrations used in the mutation assays will be based on the results of a range-finding assay. Test sample and control concentrations and the method of formulation will be documented in a protocol amendment.

6.2 Range-finding (toxicity curve) with and without S9

The toxicity of the test substance is initially characterized by a killing curve. CHO cells are plated at a density of 5×10^5 per T25 flask. One day later, the sample is added with and without activation mixture for 4-5 and 16 hours for activated and non-activated assays respectively. A minimum of 5 widely-spaced concentrations will be tested, ranging downwards from solubility limits. The day following the exposure in the activated assay and immediately following exposure in the non-activated assay from 200-1000 cells per dish (total count) are seeded. The cells are grown for at least 7 days, fixed, stained, and counted.

6.3 Mutagenesis in presence or absence of S9 mix

6.3.1 Exposure to test compound

A minimum of five concentrations of the test substance and positive controls (in duplicate) and 4 solvent controls are tested. The highest test concentration is that which resulted in approximately 10% survival. Survival in the lowest should be approximately that of the untreated controls. A constant amount of DMSO will be added to each exposure flask.

CHO cells are plated at a density of 1.5×10^6 per T75 flask. One day later, the test substance and controls are added with and without activation mixture for 4-5 and 16 hours for activated and non-activated assays respectively.

6.3.2 Expression time

The day following the exposure period in the activated assay and immediately after the exposure in the non-activated assay the medium is removed and the cells are detached using trypsin. A total cell count is performed and 200 cells from each flask are plated out in duplicate to evaluate the toxicity of each sample concentration used in the mutagenicity experiment. The remainder of the cells are replated in flasks and maintained in a growing phase by being subcultured every 2-3 days. This expression period lasts from 7 to 13 days.

6.3.3 Selection of mutants

After a minimum of 7 days, the cells are plated in medium containing 2 ug/ml 6-thioguanine to select for HGPRT⁻ mutants. Usually six P-100 dishes containing 2×10^5 cells each are plated for each flask. In cases where toxicity inhibits the exponential growth of the cells, the maximum number of dishes seeded will be based on the number of available cells. During the subculturing phase and the selection phase, each flask is treated separately. Cells are not mixed from the replicate flasks. Concomitantly with the selection step, 200 cells from each test dose are plated in duplicate in medium without 6-thioguanine to determine the cloning efficiency of the cells from each dose. After at least 7 days of incubation, plates are fixed, stained and scored. The above procedures are identical for activated and non-activated assays.

6.4 Interpretation

Colony counts for the test substance and controls are presented for both mutation induction and survival. Data is presented in tabular form, giving percent survival and cloning efficiencies compared to control levels. The mutation frequency is expressed as the number of mutants per 10^6 clonable cells.

A compound is considered positive when test sample mutation frequencies are greater than 25×10^{-6} and three times the average negative control values. A concentration-dependent increase and reproducibility of results between replicate flasks are also essential to declaring the results positive.

7.0 Report

At the termination of the study, a draft and final report will be prepared describing the purpose of the study, experimental design, sample properties and preparation, tabular and textual presentation of data, statistical analysis of data summary of results, conclusions, and quality assurance information (final report only).

8.0 Records to be Retained

All original data and a copy of the final report will be retained for not less than five years after completion of the study and stored in the Bioassay Systems Corporation archives. This material will be made available for inspection upon request or by permission of authorized representatives of the sponsor. The sponsor will be notified before final disposition of these items. The test sample will be returned to sponsor upon completion of testing.

9.0 Quality Assurance

This study will be monitored under provisions of the BSC Quality Assurance Program and the final report will be reviewed by BSC Quality Assurance Unit personnel. This study will be conducted in accordance with FDA Good Laboratory Practice (21 CFR 58.1-58.219, 1979).

10.0 Alterations of Study Design

All changes in or revisions of an approved protocol and the reasons therefore will be documented, signed by the Study Director, the Study Coordinator and a BSC Quality Assurance Officer, dated, and maintained with the protocol.

11.0 Protocol Approval

11.1 Bioassay Systems Corporation

By: Margaret Thomas
 Title: Study Director
 Date: 10/31/84

11.2 Bioassay Systems Corporation Quality Assurance Unit

Content Approval

By: Susan M. O'Connor
 Title: Mgr., Quality Assurance
 Date: 10/31/84

11.3 G.D. Searle & Co.

Charles E. Piper
 Charles E. Piper, Ph.D.
 Diplomate, A.B.T.
 Study Coordinator
 Product Safety Assessment

11/2/84
 Date

Frank N. Kotsonis
 Frank N. Kotsonis, Ph.D.
 Diplomate, A.B.T.
 Director, Toxicology
 Product Safety Assessment

11/2/84
 Date

Fred E. Kohn
 Fred E. Kohn
 Senior Director
 Product Safety Assessment

11/2/84
 Date

Bioassay Systems CorporationProtocol Amendment Form

Sponsor Name: G.D. Searle & Co
BSC Project Number: 12158 BSC Sample Number: 84-1226
Study Title: CHO/HGPRT Mutation Assay
Protocol Amendment Number: #1

Section 3.6 Proposed Study Schedule

The initiation date of the range-finding experiment for the CHO/HGPRT Mutation Assay is 11/14/84.

The initiation date of the mutagenesis Assay will be documented in a future amendment.

	Date
Study Director Signature: <u>Marybeth Thomas</u>	<u>11/20/84</u>
BSC Quality Assurance Officer Signature: <u>Susan M. O'Connor</u>	<u>11/20/84</u>
Sponsor Representative Signature (if applicable): <u>Charles E. Piper</u>	<u>12/5/84</u>
Telephone Authorization of Sponsor (if applicable): <u>N/A</u>	

Bioassay Systems CorporationProtocol Amendment Form

Sponsor Name: G.D. Searle and Co.
 BSC Project Number: 12158 BSC Sample Number: 84-1226
 Study Title: CHO1 HGPRT Mutation Assay
 Protocol Amendment Number: #2

Section 3.6 Proposed Study Schedule

The initiation date of the mutagenesis assay is 12/4/84.

Section 6.1 Concentration Formulation

The following test sample & control concentrations will be performed:

⊕ S9

Test sample: 0.01, 0.51, 0.75, 0.99, 1.50, 2.01, 2.49, 3.00 mg/ml
 Positive Control: 15 µg/ml DMBA
 Negative Control: 1% DMSO + S9

⊖ S9

Test sample: 0.01, 0.51, 0.75, 0.99, 1.50, 2.01, 2.49, 3.00 mg/ml
 Positive Control: 234 µg/ml EMS
 Negative Control: 1% DMSO

A 300 mg/ml stock solution of the test sample will be prepared in 100% DMSO. All dilutions of the test sample will be made in 100% DMSO. The initial stock solution will be used up to 72 hours. Dilutions will only be used on day prepared.

Study Director Signature: <u>Margaret Thomas</u>	Date: <u>12/4/84</u>
BSC Quality Assurance Officer Signature: <u>Lancey Herring</u>	<u>12/4/84</u>
Sponsor Representative Signature (if applicable): <u>Charles E. Piper</u>	<u>12/10/84</u>
Telephone Authorization of Sponsor (if applicable): <u>N/A</u>	

Bioassay Systems Corporation

Protocol Amendment Form

Sponsor Name: G.D. Searle & Co
 BSC Project Number: 12158 BSC Sample Number: 84-1226
 Study Title: CHO/HGPRT Mutation Assay
 Protocol Amendment Number: #3

Section 3.6 Proposed Study Schedule

The initiation date of the CHO/HGPRT Mutation Assay is 12/6/84 not 12/4/84 as specified in Protocol Amendment #2. The stock solution prepared 12/4/84 solidified & could not be used. The sponsor decided that the stock solution should be prepared fresh on day of use. A 300 mg/ml stock solution will be prepared on 12/6/84.

Section 4.0 Test Material Data

4.1.10 Solubility: Dimethylsulfoxide (>100 mg/ml) - to reflect actual solubility.

Section 6.1 Concentration Formulation

The following test sample & control concentrations will be used instead of those specified in Amendment #2 - due to solidification of the 300 mg/ml stock solution:

- ⊕ S9 Test sample: 0.10, 0.50, 0.76, 1.00, 1.26, 1.50, 1.76, 2.00 mg/ml
 Positive Control: 15 µg/ml DMBA + S9
 Negative Control: 1% DMSO + S9
- ⊖ S9 Test sample: 0.10, 0.50, 0.76, 1.00, 1.26, 1.50, 1.76, 2.00 mg/ml
 Positive Control: 234 µg/ml EHS
 Negative Control: 1% DMSO

A 200 mg/ml stock solution will be prepared in 100% DMSO on the day of use. All dilutions of the test sample will be made in 100% DMSO & used only on the day prepared.

Study Director Signature:	<u>Marybeth Thomas</u>	Date	<u>12/6/84</u>
BSC Quality Assurance Officer Signature:	<u>Marybeth Thomas</u>		<u>12/6/84</u>
Sponsor Representative Signature (if applicable):	<u>Charles E. Piper</u>		<u>12/10/84</u>
Telephone Authorization of Sponsor (if applicable):	<u>Charles E. Piper</u>		<u>12/5/84</u>

Bioassay Systems CorporationProtocol Amendment FormSponsor Name: G.D. Searle & CoBSC Project Number: 12158BSC Sample Number: (b) 84-384 84-1226Study Title: CNO/N6PRT Mutation AssayProtocol Amendment Number: #4 (SOP 1/18/85)

Section 3.6 Proposed Study Schedule

3.6.3 Study Completed 12/27/84

Section 4.1 Test Sample Description

Bioassay Systems determined the concentration of the test article in the carrier according to SOP Series 103, No. 146.

	Date
Study Director Signature: <u>Marybeth Thomas</u>	<u>1/10/85</u>
BSC Quality Assurance Officer Signature: <u>Harvey K. Kline</u>	<u>1/11/85</u>
Sponsor Representative Signature (if applicable): <u>Charles P. Piper</u>	<u>1/18/85</u>
Telephone Authorization of Sponsor (if applicable): <u>Dr. James J. Tim</u>	<u>1/24/84</u>

② MBT 1/10/85 Authorization applies to the authorization of the methodology used in the chemical analysis.

③ MBT 1/10/85 - msc:trv

SOP 500-032 (30 April 84)

Bioassay Systems CorporationProtocol Amendment Form

Sponsor Name: G.D. Searle & Co.
 BSC Project Number: 12158 BSC Sample Number: 84-1226
 Study Title: CHO/HGPRT Mutation Assay
 Protocol Amendment Number: #5

Section 6.2

- Clarification of the metabolic activation system used in the activated range-finding & mutagenesis experiments

An S9 microsome fraction (prepared from the livers of Aroclor 1254 induced (500 mg/kg) [♂] Sprague-Dawley rats) was combined with a solution of cofactors & serum free medium to form the metabolic activation system. A mixture of 10% Ascorbate Cofactors & 90% serum-free F12 medium was prepared & used as the exposure medium. Following the addition of the test sample, an aliquot of the S9 fraction was added to each flask. The final concentrations in each flask were 4.5 mg/ml Ascorbic acid (trisodium salt), 2.4 mg/ml NADP & 20 µl/ml S9 fraction.

The above procedures are standard operating procedures.

Study Director Signature: Marybeth Thomas Date: 1/29/85
 BSC Quality Assurance Officer Signature: Steven M. O'Connor 1/29/85
 Sponsor Representative Signature (if applicable): Charles E. Tupper 1/30/85
 Telephone Authorization of Sponsor (if applicable): _____

@mbr 1/29/85 - Misentry

Bioassay Systems CorporationProtocol Amendment FormSponsor Name: G.D. Searle & CoBSC Project Number: 12158 BSC Sample Number: 84-1226 A, BStudy Title: CHO/HGPRT Mutation AssayProtocol Amendment-Number: #6

Section 3.6.1 Test Substances Received: 10/19/84 & 10/31/84
 - Two aliquots, A & B, respectively, were received.

Section 3.6 Changes made to section 3.6 in
 Amendment No's 1, 2, 3, 4, were made to
 reflect actual initiation & completion
 dates)

Section 4.1 The addition to section 4.1 made
 in amendment #4 was to clarify the
 methodology used in the analysis of
 the test article in the carrier.

Section 6.1 The additions made to section
 6.1 in Amendment #'s 2 & 3 were
 made to complete information
 required by protocol.

Study Director Signature: Marybeth Thomas Date: 3/14/85
 BSC Quality Assurance Officer Signature: Super M. O. Green 3/16/85
 Sponsor Representative Signature (if applicable): Charles G. Pines 3-21-85
 Telephone Authorization of Sponsor (if applicable): N/A

© mbr 3/13/85 Hiscroty

R&D PRODUCT DEVELOPMENT FUNCTION
REPORT REVIEW AND RELEASE

Page 1 of 6

DEPARTMENT: Product Development Analytical

DOCUMENT NUMBER: F-318-034-06

TITLE OF REPORT: SC-19129

TYPE OF REPORT: Analytical Summary in Support of Product Safety
Assessment Study Number 2463

AUTHOR(S): Charles Demarest

Charles Demarest 3-13-85

TECHNICAL WRITER: Michele Newcomb

Michele Newcomb 3/14/85

APPROVAL: James Jiu

James Jiu 3-14-85

REVIEWED BY:	<u>Reviewer</u>	<u>Signature</u>	<u>Date</u>
	Daniel Sweeney	<u>Daniel Sweeney</u>	<u>3-12-85</u>
	Kathy Klimovitz	<u>Kathryn P. Klimovitz</u>	<u>3-12-85</u>

APPROVAL FOR RELEASE:

R. Baum
R. Baum, Director
Analytical Development3/19/85
DateLarry Hansen
L. Hansen,
Senior Director
Product Development3/19/85
DateNORTH AMERICAN PRECLINICAL RESEARCH AND DEVELOPMENT
SEARLE PHARMACEUTICALS AND CONSUMER PRODUCTS
SKOKIE, ILLINOIS

ANALYTICAL SUMMARY
Product Development Analytical Department

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Subject: SC-19129

Summary Number: F-318-034-06

Applicable to SA Study Number: 2463

Test Article Characterization and Stability

Lot 84K-047-101 was analyzed using the release methods of testing, released against the current specifications (NS-S84-015-A), and given a re-evaluation period of one year prior to use in this study.

A summary of the significant results used to characterize the SC-19129 is presented in Table 1.

Table 1

	Pre-Study	Post-Study	
Analysis Report #	84N1058	85N0093	85N0094
Completion Date	10/16/84	02/15/85	02/15/85
Identity (HPLC)	Conforms to Standard	Conforms to Standard	Conforms to Standard
Assay (HPLC)	100.0% n = 3 s = 0.2	98.9% n = 3 s = 0.8	99.1% n = 3 s = 0.5
Water	9.8%	8.7%	8.4%

These results and all other results, coupled with the use of lot 84K-047-101 within its re-evaluation period, indicate that lot 84K-047-101 of SC-19129 was suitable for use in this study.

Subject: SC-19129

Summary Number: F-318-034-06

Applicable to SA Study Number: 2463

Stability of Test Article in Carrier

The stability of SC-19129 in 100 mg/mL solutions of dimethyl sulfoxide was determined using a stability indicating HPLC method (M84-046-A). The samples were stored at ambient conditions and sampled at times 0, 1, 2, 4, 8, 24, 48, and 72 hours. The results of the analysis are presented in Table 2. The statistics, using data at $t = 0$ as reference, are based on the percent relative recovery values.

The results of the linear regression analysis (MINITAB, Reference 1) gave a t value less than the table value (Reference 2, Table A-4), indicating no significant downward trend. The correlation between the observed recovery values and the predicted values exhibited a normal probability plot (References 1 and 3). Since the slope of the regression line showed no significant downward trend, and the differences between the observed recovery values and the predicted values were normally distributed, the SC-19129 in solutions of dimethyl sulfoxide at 100 mg/mL is considered stable for at least 72 hours at ambient conditions.

Notebook Reference: K. Klimovitz PDAD61 pp. 160-176

ANALYTICAL SUMMARY
Product Development Analytical Department

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Subject: SC-19129

Summary Number: F-318-034-06

Applicable to SA Study Number: 2463

Table 2

Stability of Test Article in Carrier

100 mg of SC-19129/mL of Dimethyl Sulfoxide

Report of Analysis # 84-2365

Time (Hours)	% SC-19129 Recovered	% Relative Recovery
0	100.8 101.2 100.8 99.7	Reference $\bar{X} = 100.6$
1	100.4 102.3	99.8 101.7
2	103.9 100.8	103.3 100.2
4	101.6 100.4	101.0 99.8
8	101.5 100.5	100.9 99.9
24	100.5 100.2	99.9 99.6
48	99.1 99.4	98.5 98.8
72	101.0 100.6	100.4 100.0
Intercept		100.7
Slope		- 0.020
t-Ratio		- 1.69
t(0.95, 12 df)		1.789
Correlation Coefficient Predicted vs Observed		0.945

Subject: SC-19129

Summary Number: F-318-034-06

Applicable to SA Study Number: 2463

References:

1. Ryan, Jr., T. A., Joiner, B. L., and Ryan, B. F., "MINITAB Student Handbook", 1976, Wadsworth Publishing Co., Inc.
2. Natrella, M. G., "Experimental Statistics, National Bureau of Standards Handbook 91", 1963, US Government Printing Office
3. Filliben, J., Technometrics, 17 (1), 111 (1975)

ANALYTICAL SUMMARY
Product Development Analytical Department

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Subject: SC-19129

Summary Number: F-318-034-06

Applicable to SA Study Number: 2463

GLP Compliance Statement

To the best of our knowledge, the support activities provided by the Product Development Analytical Department for this study were conducted in compliance with the Good Laboratory Practices Regulations, as set forth in part 58, 21 CFR.