

8EHQ-0295-13322

Cardolite Corporation



500 Doremus Avenue
Newark, New Jersey 07105
201/344-5015
Telex: 325446
Fax: 201/344-1197

A

ORIGINAL

February 3, 1995

Document Control Officer
Office of Toxic Substances, TS-793
U.S. E.P.A.
401 M Street, SW
Washington, DC 20460

Re: NC-513, CAS# 68413-24-1

Contains No CBI

RECEIVED
95 FEB 10 PM 11:45

Dear Sir or Madam:

In the course of reviewing some recently completed health studies Cardolite Corporation had done on a chemical substance (NC-513) we manufacture, it was determined that these studies should be submitted as per section 8 E of T.S.C.A. We are therefore forwarding to you a copy of those results. The studies are enclosed.

Best Regards,

Chris Ford

Chris Ford
V.P. Performance Improvement

enc.



8EHQ-95-13322
INIT 02/10/95



88950000111

2/24/95

CONFIDENTIAL

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95 FEB 19 11:11:46

CARDOLITE NC-513LC:
MUTATION OF L5178Y MOUSE LYMPHOMA CELLS
AT THE THYMIDINE KINASE TK+/- LOCUS
SINGLE EXPERIMENT FLUCTUATION ASSAY
PROJECT NUMBER 661/5

Contains No CBI

Date Started: 29 April 1994

Experimental Procedures:

Date Completed: 18 May 1994

STUDY SPONSOR:

Cardolite Corporation
500 Doremus Avenue
NEWARK
NJ 07105
UNITED STATES OF AMERICA

ISSUED BY:

Safeparm Laboratories Limited
P.O. Box No. 45
DERBY
DE1 2BT
U.K.

Telephone: DERBY (0332) 792896

Facsimile: (0332) 799018

Telex: 377079 SAFPHM G

QUALITY ASSURANCE REPORT

The routine inspection of short term studies at Safeparm Laboratories is carried out as a continuous process designed to encompass all major phases of each study type once per month. Dates of the most recently completed series of monthly inspections relevant to the study type(s) in this report are given below.

Date(s) of Inspection and Reporting:

05, 16, 19, 24 May 1994

This report has been audited by Safeparm Laboratories Quality Assurance Unit. It is considered to be an accurate account of the data generated and of the procedures followed.

Date of Report Audit:

03 June 1994

J.R. Pateman C. Biol., M.I. Biol.
FOR SAFEPHARM QUALITY ASSURANCE UNIT



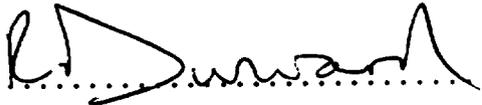
11. OCT. 1994

DATE:

GLP COMPLIANCE STATEMENT

I, the undersigned, hereby declare that the objectives laid down in the protocol were achieved and as nothing occurred to adversely affect the quality or integrity of the study, I consider the data generated to be valid. This report fully and accurately reflects the procedures used and data generated.

The work described was performed in compliance with the UK Principles of Good Laboratory Practice (The United Kingdom Compliance Programme, Department of Health 1989).

.......... DATE: 11 OCT 1994.....
R. Durward H.N.C.
Study Director
for Safepharm Laboratories

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CARDOLITE NC-513LC:

MUTATION OF L5178Y MOUSE LYMPHOMA CELLS

AT THE THYMIDINE KINASE TK+/- LOCUS

SINGLE EXPERIMENT FLUCTUATION ASSAY

SUMMARY

1. L5178Y TK+/- 3.7.2c mouse lymphoma cells (heterozygous at the thymidine kinase locus) were treated with Cardolite NC-513LC at up to six dose levels, together with vehicle (solvent) and positive controls, and in the presence and absence of an S9 metabolic activation system.

The dose range of Cardolite NC-513LC plated out for expression of mutant colonies was 62.5, 125, 250 and 500 $\mu\text{g}/\text{ml}$ without metabolic activation, and 7.81, 15.625 and 31.25 $\mu\text{g}/\text{ml}$ with metabolic activation.

2. The vehicle (solvent) controls gave mutant frequencies within the range expected for the L5178Y cell line at the TK+/- locus.
3. The positive control treatments, both in the absence and in the presence of metabolic activation, gave marked increases in the mutant frequency indicating the satisfactory performance of the test and of the activity of the metabolising system.
4. Cardolite NC-513LC demonstrated a statistically significant and dose-related increase in the mutant frequency in the absence of metabolic activation. The increase in mutant frequency was in part due to small colonies suggesting clastogenic activity resulting in structural chromosome damage. Cardolite NC-513LC was shown to be mutagenic to L5178Y cells under the conditions of the test.
5. Cardolite NC-513LC was tested in parallel with Cardolite NC-513 (Project Number 661/4) and Alkyl C₁₂-C₁₄ Glycidyl Ether (Project Number 661/6). All three test materials had comparable levels of toxicity in the presence of S9 whereas in the absence of S9 Alkyl C₁₂-C₁₄ Glycidyl Ether was more toxic to L5178Y cells by approximately an order of magnitude.

CARDOLITE NC-513LC:

MUTATION OF L5178Y MOUSE LYMPHOMA CELLS

AT THE THYMIDINE KINASE TK+/- LOCUS

SINGLE EXPERIMENT FLUCTUATION ASSAY

1. INTRODUCTION

This study was conducted according to Safepharm Standard Method Number SPL 175A and was designed to assess the potential mutagenicity of Cardolite NC-513LC on the thymidine kinase, TK+/-, locus of the L5178Y mouse lymphoma cell line.

The use of cultured mammalian cells for mutation studies may give a measure of the intrinsic response of the mammalian genome and its maintenance processes to mutagens. Such techniques have been used for many years with widely different cell types and loci. The thymidine kinase heterozygote system, TK+/- to TK-/-, was described by Clive et al., (1972) and is based upon the L5178Y mouse lymphoma cell line established by Fischer (1958). This system has been extensively validated Clive et al., (1979); Amacher et al., (1980); Jotz and Mitchell, (1981).

The test can be used to complement results obtained with bacterial mutation tests. This method has been designed as a screening test for hazard identification and not for regulatory notification purposes. The technique used is a fluctuation assay using microtitre plates and trifluorothymidine as the selective agent and is based on that described by Cole and Arlett, (1984). Two distinct types of mutant colonies can be recognised, i.e. large and small. Large colonies grow at a normal rate and represent events within the gene (base-pair substitutions or deletions) whilst small colonies represent large genetic changes involving chromosome 11b (suggesting clastogenic activity).

2. TEST MATERIAL

- | | | |
|-----------------------------|---|---|
| 1. Sponsor's identification | : | Cardolite NC-513LC |
| 2. Lot number | : | GN-1 |
| 3. Description | : | dark straw coloured slightly viscous liquid |
| 4. Date received | : | 31 March 1994 |
| 5. Container | : | metal can |
| 6. Storage conditions | : | room temperature |

2. TEST MATERIAL (contd)

Data relating to the identity, purity and stability of the test material are the responsibility of the sponsor.

3. METHODS

3.1 Cell Line

The L5178Y TK +/- 3.7.2c mouse lymphoma cell line was obtained from Dr. J. Cole of the MRC Cell Mutation Unit at the University of Sussex, Brighton, U.K. The cells were originally obtained from Dr. D. Clive of Burroughs Wellcome (U.S.A.) in October 1978 and were frozen in liquid nitrogen at that time.

3.2 Cell Culture

The stocks of cells are stored in liquid nitrogen at -196°C. Cells are routinely cultured in RPMI 1640 medium supplemented with 10% donor horse serum and 20 mM Hepes buffer (R10) at 37°C with 5% CO₂ in air. The cells have a generation time of approximately 12 hours and are sub-cultured accordingly. RPMI 1640 medium supplemented with 20 mM Hepes buffer and 20% serum (R20) and without serum (R0) were used in the assay.

3.3 Cell Cleansing

The TK+/- heterozygote cells grown in suspension spontaneously mutate at a low but significant rate. Before the stocks of cells were frozen they were cleansed of homozygous (TK-/-) mutants by culturing in THMG medium for 24 hours. This medium contained Thymidine (9 µg/ml), Hypoxanthine (15 µg/ml), Methotrexate (0.3 µg/ml) and Glycine (22.5 µg/ml). For the following 24 hours the cells were cultured in THG medium before being returned to R10 medium.

3.4 Preparation of Test and Control Materials

Cardolite NC-513LC was accurately weighed and dissolved in dimethyl sulphoxide and appropriate dilutions were made. Analysis for concentration, homogeneity and stability of the test material preparations were not a requirement of the test method and were therefore not determined.

3. METHODS (contd)

3.4 Preparation of Test and Control Materials (contd)

Vehicle and positive controls were used in parallel with the test material. Solvent (dimethyl sulphoxide) treatment groups were used as the vehicle controls, and Ethylmethanesulphonate (EMS) Sigma batch 32H0829 at 1000 $\mu\text{g/ml}$ was used as the positive control in the non-S9 cultures and Cyclophosphamide (CP) Sigma batch 72H0088 at 7.5 $\mu\text{g/ml}$ was used as the positive control for the S9 cultures.

3.5 Microsomal Enzyme Fraction

Lot No. Aro. S9/24/03/94 prepared on 24/03/94 was obtained from the British Industrial Biological Research Association on 12/04/94. It was prepared from the livers of male Sprague-Dawley rats weighing ~ 200g. These had received a single i.p. injection of Aroclor 1254 at 500 mg/kg, 5 days before S9 preparation. The S9 was stored at -196°C in a Statebourne liquid nitrogen freezer, model SXR 34.

10% S9 mix was prepared by mixing S9, NADP (5mM) and G6P (5mM) in R0 medium.

3.6 Mutagenicity Test

Several days before starting the experiment, an exponentially growing stock culture of cells was set up so as to provide an excess of cells on the morning of the experiment. The cells were counted and processed to give 0.8×10^6 cells/ml in 10 ml aliquots in R10 medium. Six dose levels of Cardolite NC-513LC were set up with vehicle and positive controls, both with and without metabolic activation (S9 mix). To each universal was added 2 ml of S9 mix if required, 0.2 ml of the treatment dilution and sufficient R0 medium to bring the total volume to 20 ml. The dose range of Cardolite NC-513LC was initially 31.25 to 1000 $\mu\text{g/ml}$ in the absence of metabolic activation and 7.81 to 250 $\mu\text{g/ml}$ in the presence of metabolic activation.

3. **METHODS (contd)**

3.6 **Mutagenicity Test (contd)**

The treatment vessels were incubated at 37°C for 3 hours with manual shaking at approximately ½-hour intervals.

At the end of the treatment period, the test material was removed by centrifugation and the cells were washed in R10 medium and resuspended in R20 medium at a cell density of 2×10^5 cells/ml.

The cultures were incubated and subcultured every 24 hours for the expression period of two days, by counting and dilution to 2×10^5 cells/ml.

On day 2 of the experiment the cells were counted, diluted to 10^4 cells/ml and plated for mutant frequency in selective medium containing 4 µg/ml trifluorothymidine in 96-well microtitre plates. Cells were also diluted to 10 cells/ml and plated for viability in non-selective medium.

3.7 **Plate Scoring**

Microtitre plates were scored after 10-14 days incubation. The number of positive wells (wells with colonies) was recorded together with the total number of scorable wells (normally 96 per plate). The numbers of small and large colonies seen in the TFT mutation plates were also recorded.

3.8 **Calculation of Plating Efficiency (Viability)**

Since the distribution of colony-forming units over the wells is described by the Poisson distribution, the plating efficiency (P.E.) was calculated using the zero term of the Poisson distribution [P(0)] method.

$$P(0) = \frac{\text{number of negative wells}}{\text{total wells plated}}$$

$$PE \% = \frac{-\ln P(0) \times 100}{\text{number of cells/well}}$$

3. METHODS (contd)

3.9 Calculation of Mutation Frequency (M.F.)

M.F. per survivor = $[(-\ln P(0) \text{ selective medium}) / (\text{cells per well in selective medium})] / \text{surviving fraction in non-selective medium.}$

3.10 Interpretation of Results

The normal range for mutant frequency per survivor is $10-125 \times 10^{-6}$ for the TK+/- locus in L5178Y cells. Vehicle control results should ideally be within this range, although minor errors in cell counting and dilution or exposure to a metabolic activation system may cause this to be slightly elevated. Experiments where the vehicle control values are greater than 150×10^{-6} mutant frequency per survivor are not acceptable and will be repeated.

Positive control chemicals should give a marked increase in mutant frequency per survivor over the vehicle controls of at least a five fold increase but preferably ten fold or greater.

For a test material to give a 'significant' result then two or more of the following criteria should be met:

- i) A greater than threefold increase in the mutant frequency per survivor over the vehicle control value.
- ii) A dose-related increase in mutant frequency per survivor.
- iii) An increase in absolute number of mutants.

A test material may be reported as equivocal if only one of the above criteria is met.

Statistical analysis of data will be performed using a dedicated statistics program which follows guidelines recommended by 1983 UKEMS.

RESULTS

The results of the microtitre plate viability and mutant frequency plates are presented in Tables 1 and 2. The maximum surviving dose level of Cardolite NC-513LC in the absence of metabolic activation was 500 $\mu\text{g/ml}$, and in the presence of metabolic activation the maximum surviving dose level was 31.25 $\mu\text{g/ml}$. It is evident from the day 2 viability (PE) results that there was some residual toxicity with Cardolite NC-513LC both in the absence of metabolic activation at 250 and 500 $\mu\text{g/ml}$ (Table 1).

Neither of the vehicle control group mutant frequency results was outside the range of 10 to 125 $\times 10^{-6}$ per survivor that is normal for the TK+/-locus in L5178Y cells. Both the positive control substances produced marked increases in the mutant frequency per survivor indicating the test system was operating satisfactorily, and that the S9 metabolic activation system was active.

Cardolite NC-513LC induced a dose-related and statistically significant increase in the mutant frequency $\times 10^{-6}$ per survivor in the absence of metabolic activation.

The numbers of small and large colonies for each dose group are presented in Table 3. In both the presence and absence of metabolic activation the increase in mutant frequency $\times 10^{-6}$ per survivor (Tables 1 and 2) was partly due to small colonies. This would suggest that part of the response is possibly due to clastogenic action resulting in structural chromosome changes.

Cardolite NC-513LC was tested in parallel with Cardolite NC-513 (Project Number 661/4) and Alkyl C₁₂-C₁₄ Glycidyl Ether (Project Number 661/6). All three test materials had comparable levels of toxicity in the presence of S9 whereas in the absence of S9 Alkyl C₁₂-C₁₄ Glycidyl Ether was more toxic to L5178Y cells by approximately an order of magnitude.

CONCLUSION

Cardolite NC-513LC produced an increase in the mutant frequency at the TK+/-locus in L5178Y cells in the absence of metabolic activation and is therefore considered to be mutagenic under the conditions of the test.

ARCHIVES

Unless instructed otherwise by the sponsor, all original data and a copy of the final report will be retained in the archives of Safeparm Laboratories for a period of ten years. After this period, the sponsor's instructions will be sought.

REFERENCES

Amacher, D.E., Paillet, S.C., Turner, G.N., Ray, V.A. and Salsburg, D.S. (1980). Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. 2. Test validation and interpretation, *Mutation Res.*, 72, 447-474.

Clive, D., Flamm, W.G., Machesko, M.R. and Bernheim, N.J. (1972). A mutational assay system using the thymidine kinase locus in mouse lymphoma cells. *Mutation Res.*, 16, 77-87.

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Cole, J. and Arlett, C.F. (1984). The detection of gene mutations in cultured mammalian cells. In: *Mutagenicity testing. A practical approach.* Eds. S. Venitt and J.M. Parry. IRL Press, Oxford. pp 233-273.

Fisher, G.A. (1958). *Ann. NY Acad. Sci.*, 76, 673-680.

Jotz, M.M. and Mitchell, A.D. (1981) Effects of 20 Coded Chemicals on the forward mutation frequency at the thymidine kinase locus in L5178Y mouse lymphoma cells. In de Serres, F.J. and Ashby J. (eds), *Evaluation of Short-Term Tests for Carcinogens.* Elsevier/North Holland, New York. pp 580-593.

Robinson, W.D. et al (1989). Statistical evaluation of bacterial/mammalian fluctuation tests. In: *Statistical Evaluation of Mutagenicity Test Data, UKEMS Sub-committee on guidelines for Mutagenicity Testing Report, Part III* (Ed. Kirkland, D.J.) Cambridge University Press, pp102 - 140.

T A B L E 1

L5178Y GENE MUTATION - MICROTITRE PLATE COUNTS

WITHOUT METABOLIC ACTIVATION

TEST MATERIAL: CARDOLITE NC-513LC

DOSE ug/ml	S9 MIX	NON-SELECTIVE MEDIUM (2 CELLS/ WELL)		SELECTIVE MEDIUM (TFT) 2 x 10 ³ CELLS/ WELL COLONIES PER INDIVIDUAL PLATES	M.F./SV x 10 ⁻⁶	LARGE COLONIES		SMALL COLONIES	
		COLONIES PER INDIVIDUAL PLATES	P.E.			% CONTROL	M.F./SV x 10 ⁻⁶	M.F./SV x 10 ⁻⁶	
0	-	$\frac{67}{96}$ $\frac{73}{96}$	65.31	$\frac{12}{96}$ $\frac{12}{96}$	102.22	75.4	24.3		
62.5	-	$\frac{62}{96}$ $\frac{65}{96}$	54.16	$\frac{7}{96}$ $\frac{9}{96}$	80.33	59.6	19.4		
125	-	$\frac{54}{94}$ $\frac{62}{94}$	53.10	$\frac{10}{96}$ $\frac{5}{96}$	76.59	50.4	24.8		
250	-	$\frac{44}{95}$ $\frac{44}{96}$	30.88	$\frac{10}{96}$ $\frac{13}{96}$	206.62	150.1	51.4		
500	-	$\frac{20}{96}$ $\frac{24}{94}$	13.17	$\frac{8}{96}$ $\frac{13}{96}$	439.73*	224.0	203.1		
EMS 1000	-	$\frac{31}{96}$ $\frac{69}{96}$	36.79	$\frac{52}{96}$ $\frac{65}{96}$	1277.69	1029.9	110.6		

Test for linear trend slope = 3.828 07 variance = 5.168 14 b²/sb = 2.833*

P.E. = Plating efficiency M.F./SV = Mutant frequency per survivor * = p < 0.05

T A B L E 2

L5178Y GENE MUTATION - MICROTITRE PLATE COUNTS
WITH METABOLIC ACTIVATION

TEST MATERIAL: CARDOLITE NC-5131C

DOSE ug/ml	S9 MIX	NON-SELECTIVE MEDIUM (2 CELLS/MELL)		SELECTIVE MEDIUM (TFT)		M.F./SV x 10 ⁻⁶	LARGE COLONIES M.F./SV x 10 ⁻⁶	SMALL COLONIES M.F./SV x 10 ⁻⁶
		COLONIES PER INDIVIDUAL PLATES	P.E. %	2 x 10 ³ CELLS/MELL COLONIES PER INDIVIDUAL PLATES				
0	+	$\frac{79}{96}$ $\frac{76}{96}$	100	$\frac{11}{96}$ $\frac{13}{96}$		81.10	59.8	19.3
7.81	+	$\frac{76}{96}$ $\frac{81}{96}$	103	$\frac{12}{96}$ $\frac{15}{96}$		89.03	75.0	12.4
15.625	+	$\frac{71}{96}$ $\frac{84}{96}$	100	$\frac{12}{96}$ $\frac{9}{96}$		70.35	56.3	12.8
31.25	+	$\frac{77}{96}$ $\frac{79}{96}$	102	$\frac{8}{96}$ $\frac{12}{96}$		65.71	45.2	19.0
CP 75	+	$\frac{51}{96}$ $\frac{60}{96}$	52	$\frac{25}{96}$ $\frac{27}{96}$		365.97	68.4	278.3

P.E. = Plating efficiency

M.F./SV = Mutant frequency per survivor

T A B L E 3

L5178Y GENE MUTATION - MUTANT COLONY NUMBERS

TEST MATERIAL: CARDOLITE NC-513LC

WITHOUT METABOLIC ACTIVATION				WITH METABOLIC ACTIVATION			
DOSE ug/ml	COLONY TYPE	NUMBER OF COLONIES PER PLATE	% SMALL COLONIES	DOSE ug/ml	COLONY TYPE	NUMBER OF COLONIES PER PLATE	% SMALL COLONIES
0	Lg	9	25	0	Lg	8	25
	Sm	3			Sm	3	
62.5	Lg	4	25	7.81	Lg	9	15
	Sm	3			Sm	3	
125	Lg	6	33	15.625	Lg	9	19
	Sm	4			Sm	3	
250	Lg	7	26	31.25	Lg	5	30
	Sm	3			Sm	3	
500	Lg	4	48	-	Lg	-	-
	Sm	4			Sm	-	
EMS 1000	Lg	45	13	CP 7.5	Lg	2	79
	Sm	7			Sm	23	

Sm = Small colonies

Lg = Large colonies

- = no data

CONFIDENTIAL

CARDOLITE NC-513:
REVERSE MUTATION ASSAY "AMES TEST"
USING SALMONELLA TYPHIMURIUM
TA98 and TA100
PROJECT NUMBER: 661/1

Experimental Procedures:

Date Started: 11 April 1994

Date Completed: 3 May 1994

STUDY SPONSOR:

Cardolite Corporation
500 Doremus Avenue
NEWARK
NJ 07105
UNITED STATES OF AMERICA

ISSUED BY:

Safeparm Laboratories Limited
P.O. Box No. 45
DERBY
DE1 2BT
U.K.

Telephone: DERBY (0332) 792896

Facsimile: (0332) 799018

Telex: 377079 SAFPHM G

QUALITY ASSURANCE REPORT

The routine inspection of short term studies at Safepharm Laboratories is carried out as a continuous process designed to encompass all major phases of each study type once per month. Dates of the most recently completed series of monthly inspections relevant to the study type(s) in this report are given below.

Date(s) of Inspection and Reporting:

07, 11, 13, 20, 26 April 1994

This report has been audited by Safepharm Laboratories Quality Assurance Unit. It is considered to be an accurate account of the data generated and of the procedures followed.

Date of Report Audit:

16 May 1994

J.R. Pateman C. Biol., M.I. Biol.
FOR SAFEPHARM QUALITY ASSURANCE UNIT



29. SEP. 1994

DATE:

GLP COMPLIANCE STATEMENT

I, the undersigned, hereby declare that the objectives laid down in the protocol were achieved and as nothing occurred to adversely affect the quality or integrity of the study, I consider the data generated to be valid. This report fully and accurately reflects the procedures used and data generated.

The work described was performed in compliance with the UK Principles of Good Laboratory Practice (The United Kingdom Compliance Programme, Department of Health 1989). These Principles are in accordance with GLP standards published as OECD Environment Monograph No. 45 (OCDE/GD(92)32); and are in conformity with, and implement, the requirements of Directives 87/18/EEC and 88/320/EEC.

These international standards are acceptable to the United States Environmental Protection Agency and Food and Drug Administration, and fulfil the requirements of 40 CFR Part 160, 40 CFR Part 792 and 21 CFR Part 58 (as amended).



29 SEP 1994

..... DATE:
P.W. Thompson H.N.C.
Study Director
for Safeparm Laboratories

C O N T E N T S

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CARDOLITE NC-513:
REVERSE MUTATION ASSAY "AMES TEST"
USING SALMONELLA TYPHIMURIUM
TA98 and TA100

SUMMARY

1. Salmonella typhimurium strains TA98 and TA100 were treated with Cardolite NC-513 by the Ames plate incorporation method at five dose levels, in triplicate both with and without the addition of a rat liver homogenate metabolising system (10% liver S9 in standard co-factors). The dose range was determined in a preliminary toxicity assay and was 8 to 5000 $\mu\text{g}/\text{plate}$ in the first experiment. The experiment was repeated on a separate day using different cultures of the bacterial strain and fresh chemical formulations. In this case the dose range of Cardolite NC-513 was 312.5 to 5000 $\mu\text{g}/\text{plate}$.
2. All solvent (dimethyl sulphoxide) control plates gave counts of revertant colonies within the normal range.
3. All positive control chemicals produced marked increases in the numbers of revertant colonies, both with and without the metabolising system.
4. Cardolite NC-513 caused no visible reduction in the growth of the bacterial lawn at any dose level in either strain of Salmonella. Cardolite NC-513 was, therefore, tested up to the maximum recommended dose of 5000 $\mu\text{g}/\text{plate}$. A precipitate was observed at the maximum dose, this did not interfere with the scoring of revertant colonies.
5. Cardolite NC-513 induced a statistically significant and reproducible increase in the frequency of revertant colonies over the appropriate concurrent solvent control in Salmonella strain TA100 (particularly with S9 mix) beginning at 1000 $\mu\text{g}/\text{plate}$ in the first experiment and 2500 $\mu\text{g}/\text{plate}$ in the second experiment. A small but statistically significant response was also observed in TA100 without S9 mix in the second experiment, this response, however, was weak and inconsistent. Cardolite NC-513 was found to be weakly mutagenic in the presence of metabolic activation under the conditions of this test.

CARDOLITE NC-513:
REVERSE MUTATION ASSAY "AMES TEST"
USING SALMONELLA TYPHIMURIUM
TA98 and TA100

INTRODUCTION

This study was conducted according to Safepharm Standard Method SPL 03B and was designed to assess the mutagenic potential of the test material using a bacterial/microsome test system. The study was based on the in vitro technique described by Ames and his co-workers (1,2,3) and Garner et al (4) in which mutagenic activity is assessed by exposing histidine auxotrophs of Salmonella typhimurium to various concentrations of the test material. A copy of the Certificate of Compliance with GLP, issued by the U.K. Department of Health is included in Appendix I.

These mutant strains of Salmonella are incapable of synthesising histidine and are, therefore, dependent for growth on an external source of this particular amino acid. When exposed to a mutagenic agent these bacteria may undergo a reverse mutation to histidine independent forms which are detected by their ability to grow on a histidine deficient medium. Using various strains of this organism, revertants produced after exposure to a chemical mutagen may arise as a result of base-pair substitution in the genetic material (miscoding) or frame-shift mutation in which genetic material is either added or deleted. In order to make the bacteria more sensitive to mutation by chemical and physical agents several additional traits have been introduced. These include a deletion through the excision repair gene (uvrB) which renders the organism incapable of DNA excision repair and a deep rough mutation (rfa) which increases the permeability of the cell wall. Since many compounds do not exert a mutagenic effect until they have been metabolised by enzyme systems, not available in the bacterial cell, the test material and the bacteria are also incubated in the presence of a liver microsomal reaction (S9) prepared from rats pre-treated with a compound known to induce an elevated level of these enzymes.

TEST MATERIAL

Sponsor's identification : Cardolite NC-513
Label : NC-513 Epoxy diluent/Flexibiliser Lot MN-3
Net .91 kg
Batch number : MN-3
Date Received : 31 March 1994
Description : dark straw coloured slightly viscous liquid
Container : metal can
Storage conditions : room temperature

Data relating to the identity, purity and stability of the test material are the responsibility of the sponsor.

METHODS

1. **Tester Strains**

The strains used in this assay were both mutants derived from Salmonella typhimurium LT2 and were among those recommended for general screening.

TA100 sensitive to agents inducing base-pair substitution
TA98 sensitive to agents inducing frame-shift mutations

These strains were obtained from the British Industrial Biological Research Association on 14th August 1987 and were stored at -196°C in a liquid nitrogen freezer. Prior to being used, characterisation checks were carried out to determine the amino-acid requirement, presence of rfa, R factors, uvr mutation and the spontaneous reversion rate.

In this assay, overnight sub-cultures of the appropriate coded stock cultures were prepared in nutrient broth (Oxoid Limited Lot no. 350 51940 11/97) and incubated at 37°C for approximately 10 hours.

2. **Preparation of Test and Control Materials**

Cardolite NC-513 was accurately weighed and dissolved in dimethyl sulphoxide and appropriate dilutions made on the day of each experiment. An analysis of the concentration, homogeneity and stability of the test material preparations is not a requirement of the test guideline and was, therefore, not determined.

2. Preparation of Test and Control Materials (contd)

Negative and positive controls were used in parallel with the test material. A solvent treatment group was used as the negative control and the positive control materials were as follows:

N-Ethyl-N'-nitro-N-nitrosoguanidine (ENNG) 3 μ g/plate for TA100
4 Nitroquinoline-1-oxide (4NQO) 0.2 μ g/plate for TA98

In addition the material Benzo(a)pyrene (BP) which is non-mutagenic in the absence of metabolising enzymes was used at 5 μ g/plate in the S9 series of plates.

3. Microsomal Enzyme Fraction

Lot No. Aro. S9/03/03/94, prepared on 03/03/94, and Lot No. Aro. S9/24/03/94, prepared on 24/03/94, was obtained from the British Industrial Biological Research Association on 15/03/94 and 12/04/94 respectively. They were prepared from the livers of male Sprague-Dawley rats weighing ~ 200g. These had received a single i.p. injection of Aroclor 1254 at 500 mg/kg, 5 days before S9 preparation.

The S9 was stored at -196°C in a Statebourne liquid nitrogen freezer, model SXR 34.

4. S9 Mix and Top Agar

The S9 Mix was prepared at 4°C as follows:

S9	5.0 ml
1.65 M KCl/0.4 M MgCl ₂	1.0 ml
0.1 M Glucose-6-phosphate	2.5 ml
0.1 M NADP	2.0 ml
0.2 M Sodium phosphate buffer (pH 7.4)	25.0 ml
Sterile distilled water	14.5 ml

Top agar was prepared using 0.6% Difco Bacto agar (lot no. 23050 12/97) and 0.5% sodium chloride. 5 ml of 1.0 mM histidine/1.0 mM biotin

4. S9 Mix and Top Agar (contd)

solution was added to each 100 ml of top agar. Base agar plates were prepared using 1.2% Oxoid Agar Technical No.3 (lot no. B326 76158 10/98) with Vogel-Bonner Medium E and 20 mg/ml D-glucose.

5. Test Procedure

a) Preliminary Toxicity Study

In order to select appropriate dose levels for use in the main study, a preliminary test was carried out to determine the toxicity of the test material to the tester organisms. 0.1 ml of bacterial suspension (TA100), 2 ml of molten, trace histidine supplemented media (histidine/biotin and top agar), 0.1 ml of test solution and 0.5 ml phosphate buffer were overlayed onto sterile plates of Vogel-Bonner Minimal Agar (30 ml/plate). Five doses of the test material and a solvent control (dimethyl sulphoxide) were tested in duplicate. After approximately 48 hours of incubation the plates were scored for revertant colonies and examined for a thinning of the background lawn.

b) Mutation Assay

Five concentrations of the test material were assayed in triplicate against each tester strain, using the direct plate incorporation method in accordance with the standard methods for mutagenicity tests using bacteria.

Test Material and Negative Controls

0.1 ml aliquots of one of the bacterial suspensions were dispensed into sets of sterile test tubes followed by 2.0 ml of molten, trace histidine supplemented, top agar at 45°C. These sets comprised two test tubes for each bacterial tester strain. 0.1 ml of the appropriately diluted test material or negative control was also added to each of the two tubes followed by either 0.5 ml of the S9 liver microsome mix or 0.5 ml of pH 7.4 buffer. The contents of each test tube were mixed and equally distributed onto the surface of Vogel-Bonner agar plates (one tube per plate). This procedure was repeated, in triplicate, for each bacterial strain and for each concentration of test material.

5. Test Procedure (contd)

b) Mutation Assay (contd)

Positive Controls

i) Without Activation

0.1 ml of one of the appropriate positive control solutions (ENNG or 4NQO) was added to a test tube containing 2.0 ml of molten, trace histidine supplemented top agar and 0.1 ml of the appropriate bacterial suspension. Finally 0.5 ml of pH 7.4 buffer was added to the tube, the contents mixed and poured onto an agar plate. This procedure was then repeated, in triplicate, for each of the positive controls.

ii) With Activation

0.1 ml of BP solution was added to a test tube containing 2.0 ml of molten, trace histidine supplemented top agar and 0.1 ml of one of the test bacterial suspensions. Finally 0.5 ml of S9 mix was added to the test tube. The procedure was then repeated, in triplicate, for each tester strain.

The plates were incubated at 37°C for approximately 48 hours and the number of revertant colonies counted.

EXPERIMENT 2

The second experiment was performed as described for experiment 1 using fresh bacterial cultures, five concentrations of test material and control solutions in triplicate.

6. Interpretation of Results

For a substance to be considered positive in this test system, it should have induced a dose-related and statistically significant increase in mutation rate in one or more strains of bacteria in the presence and/or absence of the S9 microsomal enzymes in both experiments at sub-toxic dose levels. If the two experiments give conflicting results or

6. Interpretation of Results (contd)

equivocal results are obtained then a third experiment may be used to confirm the correct response. All data are statistically analysed using the methods recommended by the UKEMS (5) and normally Dunnett's method of linear regression is used to evaluate the result. To be considered negative the number of induced revertants compared to spontaneous revertants should be less than twofold at each dose level employed, the intervals of which should be between 2 and 5 fold and extend to the limits imposed by toxicity, solubility or up to the maximum recommended dose of 5000 $\mu\text{g}/\text{plate}$. In this case the limiting factor was the maximum recommended dose.

RESULTS

a) Preliminary Toxicity Study

The dose range of Cardolite NC-513 used in the preliminary toxicity study was 0, 312.5, 625, 1250, 2500 and 5000 µg/plate. Cardolite NC-513 was non-toxic in the strain of Salmonella used (TA100).

The mean numbers of revertant colonies for the toxicity assay were:

Strain	Dose (µg/plate)					
	0	312.5	625	1250	2500	5000
TA100	132.5	144.0	131.5	146.0	128.5	169.5*

* represents p<0.05

b) Mutation Assay

The results of the checks for characteristics, viability and spontaneous reversion rate for each tester strain were all found to be satisfactory.

Individual plate counts together with the mean number of revertant colonies obtained for each tester strain following incubation with the test material, with and without metabolic activation, are shown in Tables 1 and 2 for experiment 1 and Tables 3 and 4 for experiment 2. The results for the positive controls are shown in Tables 1 to 4.

No toxicity was exhibited to either of the strains of Salmonella used. A precipitate was observed at the maximum dose, this did not interfere with the scoring of revertant colonies.

Cardolite NC-513 induced a statistically significant and reproducible increase in the frequency of revertant colonies over the appropriate concurrent solvent control in Salmonella strain TA100 (particularly with S9 mix) beginning at 1000 µg/plate in the first experiment and 2500 µg/plate in the second experiment. A small but statistically significant response was also observed in TA100 without S9 mix in the second experiment, this response, however, was weak and inconsistent.

The positive control substances all produced marked increases in the number of revertant colonies and the activity of the S9 fraction was found to be satisfactory.

CONCLUSION

The test material, Cardolite NC-513, was found to be weakly mutagenic in the presence of metabolic activation under the conditions of this test.

ARCHIVES

Unless instructed otherwise by the sponsor, all original data and a copy of the final report will be retained in the archives of Safeparm Laboratories for a period of ten years. After this period, the sponsor's instructions will be sought.

REFERENCES

1. Ames, B.N., Durston, W.E., Yamasaki, E., and Lee, F.D. Proc. Nat. Acad. Sci. USA (1970), 70, 2285
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4. Garner, R.C., Miller, E.C., and Miller, J.A. Cancer Res. (1972), 33, 2058.
5. Kirkland, D.J., (Ed). Statistical Evaluation of Mutagenicity Test Data. UKEMS Sub-committee on Guidelines for Mutagenicity Testing. Report - Part III (1989) - Cambridge University Press.

KEY TO TABLE OF TEST RESULTS

NOTES:

1. "Number of revertants" - The observed values and average value are shown in order, beginning with the lowest concentration of the test substance.

2. The following postfixes are used when required:-

C = contaminated

P = precipitate

S = sparse background lawn

V = very thin background lawn

T = toxic

X = plate unscorable

ENNG = N-Ethyl-N'-nitro-N-nitrosoguanidine

4NQO = 4-Nitroquinoline-1-oxide

BP = Benzo(a)pyrene

TABLE 1

TABLE OF TEST RESULTS: EXPERIMENT 1

NAME OF TEST SUBSTANCE: CARDOLITE NC-513

WITH (+) OR TEST SUBSTANCE		NUMBER OF REVERTANTS (number of colonies/plate)	
WITHOUT (-)	CONCENTRATION	BASE-PAIR SUBSTITUTION TYPE	FRAMESHIFT TYPE
S9 MIX	(ug/plate)	TA100	TA98
-	0	128 117 (113.0) 94	15 20 (18.0) 19
-	8.0	128 115 (123.0) 126	20 12 (17.3) 20
-	40	99 103 (102.7) 106	15 11 (15.0) 19
-	200	95 112 (111.0) 126	15 18 (17.0) 18
-	1000	111 106 (107.0) 104	14 19 (16.7) 17
-	5000	123P 135P (132.7) 140P	19P 19P (18.3) 17P
=====			
	NAME	ENMG	4NQG
POSITIVE CONTROL NOT REQUIRING S9 MIX	CONCENTRATION (ug/plate)	3.0	0.2
-	NUMBER OF COLONIES/PLATE	471 438 (480.0) 531	89 97 (96.7) 104
=====			

TABLE 2

TABLE OF TEST RESULTS: EXPERIMENT 1

NAME OF TEST SUBSTANCE: CARDOLITE NC-513

WITH (+) OR WITHOUT (-) S9 MIX	TEST SUBSTANCE CONCENTRATION (ug/plate)	NUMBER OF REVERTANTS (number of colonies/plate)	
		BASE-PAIR SUBSTITUTION TYPE TA100	FRAMESHIFT TYPE TA98
+	0	99	26
		102 (105.7)	20 (23.0)
		116	23
+	8.0	125	21
		104 (115.3)	21 (23.0)
		117	27
+	40	98	21
		112 (104.0)	27 (23.0)
		102	21
+	200	108	17
		119 (117.3)	14 (15.0)
		125	14
+	1000	154 **	10
		142 (143.3)	14 (13.7)
		134	17
+	5000	224P ***	15P
		241P (251.0)	14P (14.7)
		288P	15P

NAME	BP	BP
POSITIVE CONTROL REQUIRING S9 MIX	5.0	5.0
NUMBER OF COLONIES/PLATE	363	120
+	320 (340.7)	108 (108.3)
	339	97

** represents p<0.01
 *** represents p<0.001

TABLE 3

TABLE OF TEST RESULTS: EXPERIMENT 2

NAME OF TEST SUBSTANCE: CARDOLITE NC-513

WITH (+) OR WITHOUT (-) TEST SUBSTANCE S9 MIX	CONCENTRATION (ug/plate)	NUMBER OF REVERTANTS (number of colonies/plate)	
		BASE-PAIR SUBSTITUTION TYPE TA100	FRAMESHIFT TYPE TA98
-	0	175 150 (153.7) 136	24 28 (24.7) 22
-	312.5	169 * 180 (179.7) 190	33 21 (26.0) 24
-	625	190 176 (174.7) 158	25 25 (24.0) 22
-	1250	143 155 (153.3) 162	25 26 (23.7) 20
-	2500	180 173 (177.0) 178	26 26 (24.7) 22
-	5000	188P * 175P (179.7) 176P	29P 21P (21.7) 15P

NAME	ENNG	4NQO
POSITIVE CONTROL NOT REQUIRING S9 MIX	3.0	0.2
-	489 501 (491.7) 485	118 129 (135.7) 160

* represents p<0.05

TABLE 4

TABLE OF TEST RESULTS: EXPERIMENT 2

NAME OF TEST SUBSTANCE: CARDOLITE NC-513

WITH (+) OR TEST SUBSTANCE WITHOUT (-) CONCENTRATION S9 MIX (ug/plate)		NUMBER OF REVERTANTS (number of colonies/plate)	
		BASE-PAIR SUBSTITUTION TYPE TA100	FRAMESHIFT TYPE TA98
+	0	172	42
		167 (158.7)	26 (35.3)
		137	38
+	312.5	163	28
		171 (159.0)	34 (30.7)
		143	30
+	625	160	30
		163 (155.3)	37 (31.0)
		143	26
+	1250	158	47
		188 (167.0)	37 (43.0)
		155	45
+	2500	190 *	35
		195 (210.0)	30 (34.3)
		245	38
+	5000	214P ***	32P
		227P (230.7)	31P (29.3)
		251P	25P
NAME		BP	BP
POSITIVE CONTROL REQUIRING S9 MIX	CONCENTRATION (ug/plate)	5.0	5.0
+	NUMBER OF COLONIES/PLATE	372	135
		376 (382.3)	128 (133.7)
		399	138

* represents p<0.05

*** represents p<0.001

APPENDIX I



**THE DEPARTMENT OF HEALTH OF THE GOVERNMENT
OF THE UNITED KINGDOM**

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE

IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY

*SafePharm Laboratories Limited
P O Box No 45
Derby
DE1 2BT*

DATE OF INSPECTION

31 January 1994

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of studies performed at these facilities.

A handwritten signature in black ink, appearing to read "D. F. Moore".

16/3/94.

D. F. Moore
Director
UK GLP Monitoring Unit

CONFIDENTIAL

CARDOLITE NC-513:
MUTATION OF L5178Y MOUSE LYMPHOMA CELLS
AT THE THYMIDINE KINASE TK+/- LOCUS
SINGLE EXPERIMENT FLUCTUATION ASSAY
PROJECT NUMBER 661/4

Date Started: 29 April 1994

Experimental Procedures:

Date Completed: 24 May 1994

STUDY SPONSOR:

Cardolite Corporation
500 Doremus Avenue
NEWARK
NJ 07105
UNITED STATES OF AMERICA

ISSUED BY:

Safeparm Laboratories Limited
P.O. Box No. 45
DERBY
DE1 2BT
U.K.

Telephone: DERBY (0332) 792896

Facsimile: (0332) 799018

Telex: 377079 SAFPHM G

QUALITY ASSURANCE REPORT

The routine inspection of short term studies at Safepharm Laboratories is carried out as a continuous process designed to encompass all major phases of each study type once per month. Dates of the most recently completed series of monthly inspections relevant to the study type(s) in this report are given below.

Date(s) of Inspection and Reporting:

05, 16, 19, 24 May 1994

This report has been audited by Safepharm Laboratories Quality Assurance Unit. It is considered to be an accurate account of the data generated and of the procedures followed.

Date of Report Audit:

02 June 1994

J.R. Pateman C. Biol., M.I. Biol.
FOR SAFEPHARM QUALITY ASSURANCE UNIT



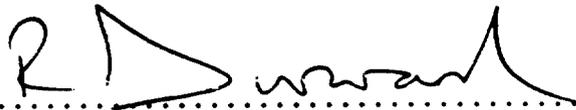
DATE:

11. OCT. 1994

GLP COMPLIANCE STATEMENT

I, the undersigned, hereby declare that the objectives laid down in the protocol were achieved and as nothing occurred to adversely affect the quality or integrity of the study, I consider the data generated to be valid. This report fully and accurately reflects the procedures used and data generated.

The work described was performed in compliance with the UK Principles of Good Laboratory Practice (The United Kingdom Compliance Programme, Department of Health 1989).



DATE: 11 OCT 1994

R. Durward H.N.C.
Study Director
for Safeparm Laboratories

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CARDOLITE NC-513:
MUTATION OF L5178Y MOUSE LYMPHOMA CELLS
AT THE THYMIDINE KINASE TK+/- LOCUS
SINGLE EXPERIMENT FLUCTUATION ASSAY

SUMMARY

1. L5178Y TK+/- 3.7.2c mouse lymphoma cells (heterozygous at the thymidine kinase locus) were treated with Cardolite NC-513 at up to seven dose levels, together with vehicle (solvent) and positive controls, and in the presence and absence of an S9 metabolic activation system.

The dose range of Cardolite NC-513 plated out for expression of mutant colonies was 31.25, 62.5, 125, 250 $\mu\text{g/ml}$ without metabolic activation, and 8, 16, 32 and 48 $\mu\text{g/ml}$ with metabolic activation.

2. The vehicle (solvent) controls gave mutant frequencies within the range expected for the L5178Y cell line at the TK+/- locus.
3. The positive control treatments, both in the absence and in the presence of metabolic activation, gave marked increases in the mutant frequency indicating the satisfactory performance of the test and of the activity of the metabolising system.
4. Cardolite NC-513 demonstrated a statistically significant and dose-related increase in the mutant frequency in the absence of metabolic activation. The increase in mutant frequency was in part due to small colonies suggesting clastogenic activity resulting in structural chromosome damage. Cardolite NC-513 was shown to be mutagenic to L5178Y cells under the conditions of the test.
5. Cardolite NC-513 was tested in parallel with Cardolite NC-513LC (Project Number 661/5) and Alkyl C₁₂-C₁₄ Glycidyl Ether (Project Number 661/6). All three test materials had comparable levels of toxicity in the presence of S9 whereas in the absence of S9 Alkyl C₁₂-C₁₄ Glycidyl Ether was more toxic to L5178Y cells by approximately an order of magnitude.

CARDOLITE NC-513:

MUTATION OF L5178Y MOUSE LYMPHOMA CELLS

AT THE THYMIDINE KINASE TK+/- LOCUS

SINGLE EXPERIMENT FLUCTUATION ASSAY

1. INTRODUCTION

This study was conducted according to Safepharm Standard Method Number SPL 175A and was designed to assess the potential mutagenicity of Cardolite NC-513 on the thymidine kinase, TK+/-, locus of the L5178Y mouse lymphoma cell line.

The use of cultured mammalian cells for mutation studies may give a measure of the intrinsic response of the mammalian genome and its maintenance processes to mutagens. Such techniques have been used for many years with widely different cell types and loci. The thymidine kinase heterozygote system, TK+/- to TK-/-, was described by Clive et al., (1972) and is based upon the L5178Y mouse lymphoma cell line established by Fischer (1958). This system has been extensively validated Clive et al., (1979); Amacher et al., (1980); Jotz and Mitchell, (1981).

The test can be used to complement results obtained with bacterial mutation tests. This method has been designed as a screening test for hazard identification and not for regulatory notification purposes. The technique used is a fluctuation assay using microtitre plates and trifluorothymidine as the selective agent and is based on that described by Cole and Arlett, (1984). Two distinct types of mutant colonies can be recognised, i.e. large and small. Large colonies grow at a normal rate and represent events within the gene (base-pair substitutions or deletions) whilst small colonies represent large genetic changes involving chromosome 11b (suggesting clastogenic activity).

2. TEST MATERIAL

- | | | |
|-----------------------------|---|---|
| 1. Sponsor's identification | : | Cardolite NC-513 |
| 2. Lot number | : | MN-3 |
| 3. Description | : | dark straw-coloured slightly viscous liquid |
| 4. Date received | : | 31 March 1994 |
| 5. Container | : | metal can |
| 6. Storage conditions | : | room temperature |

2. TEST MATERIAL (contd)

Data relating to the identity, purity and stability of the test material are the responsibility of the sponsor.

3. METHODS

3.1 Cell Line

The L5178Y TK +/- 3.7.2c mouse lymphoma cell line was obtained from Dr. J. Cole of the MRC Cell Mutation Unit at the University of Sussex, Brighton, U.K. The cells were originally obtained from Dr. D. Clive of Burroughs Wellcome (U.S.A.) in October 1978 and were frozen in liquid nitrogen at that time.

3.2 Cell Culture

The stocks of cells are stored in liquid nitrogen at -196°C. Cells are routinely cultured in RPMI 1640 medium supplemented with 10% donor horse serum and 20 mM Hepes buffer (R10) at 37°C with 5% CO₂ in air. The cells have a generation time of approximately 12 hours and are sub-cultured accordingly. RPMI 1640 medium supplemented with 20 mM Hepes buffer, 20% serum (R20) and without serum (R0) were used in the assay.

3.3 Cell Cleansing

The TK +/- heterozygote cells grown in suspension spontaneously mutate at a low but significant rate. Before the stocks of cells were frozen they were cleansed of homozygous (TK -/-) mutants by culturing in THMG medium for 24 hours. This medium contained Thymidine (9 µg/ml), Hypoxanthine (15 µg/ml), Methotrexate (0.3 µg/ml) and Glycine (22.5 µg/ml). For the following 24 hours the cells were cultured in THG medium before being returned to R10 medium.

3.4 Preparation of Test and Control Materials

Cardolite NC-513 was accurately weighed and dissolved in dimethyl sulphoxide and appropriate dilutions were made. Analysis for concentration, homogeneity and stability of the test material preparations were not a requirement of the test method and were therefore not determined.

3. **METHODS (contd)**

3.4 **Preparation of Test and Control Materials (contd)**

Vehicle and positive controls were used in parallel with the test material. Solvent (dimethyl sulphoxide) treatment groups were used as the vehicle controls, and Ethylmethanesulphonate (EMS) Sigma batch 32H0829 at 1000 $\mu\text{g/ml}$ was used as the positive control in the non-S9 cultures and Cyclophosphamide (CP) Sigma batch 72H0088 at 7.5 $\mu\text{g/ml}$ was used as the positive control for the S9 cultures.

3.5 **Microsomal Enzyme Fraction**

Lot No. Aro. S9/24/03/94 prepared on 24/03/94 was obtained from the British Industrial Biological Research Association on 12/04/94. It was prepared from the livers of male Sprague-Dawley rats weighing - 200g. These had received a single i.p. injection of Aroclor 1254 at 500 mg/kg, 5 days before S9 preparation. The S9 was stored at -196°C in a Statebourne liquid nitrogen freezer, model SXR 34.

10% S9 mix was prepared by mixing S9, NADP (5mM) and G6P (5mM) in RO medium.

3.6 **Mutagenicity Test**

Several days before starting the experiment, an exponentially growing stock culture of cells was set up so as to provide an excess of cells on the morning of the experiment. The cells were counted and processed to give approximately 1×10^6 cells/ml in 10 ml aliquots in R10 medium. Seven and six dose levels of Cardolite NC-513 were set up with vehicle and positive controls, for the with and without metabolic activation (S9 mix) cultures respectively. To each universal was added 2 ml of S9 mix if required, 0.2 ml of the treatment dilution and sufficient RO medium to bring the total volume to 20 ml. The dose range of Cardolite NC-513 was 15.625 to 500 $\mu\text{g/ml}$ for the cultures without metabolic activation, and 1 to 48 $\mu\text{g/ml}$ for the cultures with metabolic activation after proving too toxic at 15.625 to 500 $\mu\text{g/ml}$ in an initial experiment.

3. METHODS (contd)

3.6 Mutagenicity Test (contd)

The treatment vessels were incubated at 37°C for 3 hours with manual shaking at approximately ½-hour intervals.

At the end of the treatment period, the test material was removed by centrifugation and the cells were washed in R10 medium and resuspended in R20 medium at a cell density of 2×10^5 cells/ml.

The cultures were incubated and subcultured every 24 hours for the expression period of two days, by counting and dilution to 2×10^5 cells/ml.

On day 2 of the experiment the cells were counted, diluted to 10^4 cells/ml and plated for mutant frequency in selective medium containing 4 µg/ml trifluorothymidine in 96-well microtitre plates. Cells were also diluted to 10 cells/ml and plated for viability in non-selective medium.

3.7 Plate Scoring

Microtitre plates were scored after 10-14 days incubation. The number of positive wells (wells with colonies) was recorded together with the total number of scorable wells (normally 96 per plate). The numbers of small and large colonies seen in the TFT mutation plates were also recorded.

3.8 Calculation of Plating Efficiency (Viability)

Since the distribution of colony-forming units over the wells is described by the Poisson distribution, the plating efficiency (P.E.) was calculated using the zero term of the Poisson distribution [P(0)] method.

$$P(0) = \frac{\text{number of negative wells}}{\text{total wells plated}}$$

$$PE \% = \frac{-\ln P(0) \times 100}{\text{number of cells/well}}$$

3. METHODS (contd)

3.9 Calculation of Mutation Frequency (M.F.)

M.F. per survivor = $[(-\ln P(0) \text{ selective medium}) / (\text{cells per well in selective medium})] / \text{surviving fraction in non-selective medium}$.

3.10 Interpretation of Results

The normal range for mutant frequency per survivor is 10^{-12} to 125×10^{-6} for the TK \pm locus in L5178Y cells. Vehicle control results should ideally be within this range, although minor errors in cell counting and dilution or exposure to a metabolic activation system may cause this to be slightly elevated. Experiments where the vehicle control values are greater than 150×10^{-6} mutant frequency per survivor are not acceptable and will be repeated.

Positive control chemicals should give a marked increase in mutant frequency per survivor over the vehicle controls of at least a five fold increase but preferably ten fold or greater.

For a test material to give a 'significant' result then two or more of the following criteria should be met:

- i) A greater than threefold increase in the mutant frequency per survivor over the vehicle control value.
- ii) A dose-related increase in mutant frequency per survivor.
- iii) An increase in absolute number of mutants.

A test material may be reported as equivocal if only one of the above criteria is met.

Statistical analysis of data will be performed using a dedicated statistics program which follows guidelines recommended by 1983 UKEMS.

RESULTS

The results of the microtitre plate viability and mutant frequency plates are presented in Tables 1 and 2. The maximum surviving dose level of Cardolite NC-513 in the absence of metabolic activation was 250 $\mu\text{g/ml}$, and in the presence of metabolic activation the maximum surviving dose level was 48 $\mu\text{g/ml}$. It is evident from the day 2 viability (P.E.) results that there was some residual toxicity with Cardolite NC-513 in the absence of metabolic activation at 250 $\mu\text{g/ml}$ (Table 1).

Neither of the vehicle control group mutant frequency results was outside the range of 10 to 125 $\times 10^{-6}$ per survivor that is normal for the TK+/-locus in L5178Y cells. Both the positive control substances produced marked increases in the mutant frequency per survivor indicating the test system was operating satisfactorily, and that the S9 metabolic activation system was active.

Cardolite NC-513 induced a dose-related and statistically significant increase in the mutant frequency $\times 10^{-6}$ per survivor in the absence of metabolic activation.

The numbers of small and large colonies for each dose group are presented in Table 3. In the absence of metabolic activation the increase in mutant frequency $\times 10^{-6}$ per survivor (Table 1) was partly due to small colonies. This would suggest that part of the response is possibly due to clastogenic action resulting in structural chromosome changes.

Cardolite NC-513 was tested in parallel with Cardolite NC-513LC (Project Number 661/5) and Alkyl C₁₂-C₁₄ Glycidyl Ether (Project Number 661/6). All three test materials had comparable levels of toxicity in the presence of S9 whereas in the absence of S9 Alkyl C₁₂-C₁₄ Glycidyl Ether was more toxic to L5178Y cells by approximately an order of magnitude.

CONCLUSION

Cardolite NC-513 produced an increase in the mutant frequency at the TK+/-locus in L5178Y cells in the absence of metabolic activation and is therefore considered to be mutagenic under the conditions of the test.

ARCHIVES

Unless instructed otherwise by the sponsor, all original data and a copy of the final report will be retained in the archives of Safepharm Laboratories for a period of ten years. After this period, the sponsor's instructions will be sought.

REFERENCES

Amacher, D.E., Paillet, S.C., Turner, G.N., Ray, V.A. and Salsburg, D.S. (1980). Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. 2. Test validation and interpretation, *Mutation Res.*, 72, 447-474.

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Robinson, W.D. et al (1989). Statistical evaluation of bacterial/mammalian fluctuation tests. In: *Statistical Evaluation of Mutagenicity Test Data, UKEMS Sub-committee on guidelines for Mutagenicity Testing Report, Part III* (Ed. Kirkland, D.J.) Cambridge University Press, pp102 - 140.

T A B L E 1

L5178Y GENE MUTATION - MICROTITRE PLATE COUNTS

WITHOUT METABOLIC ACTIVATION

TEST MATERIAL: CARDOLITE NC-513

DOSE µg/ml	S9 MIX	NON-SELECTIVE MEDIUM (2 CELLS/WELL)			SELECTIVE MEDIUM (TFT)		M.F./SV x 10 ⁻⁶	LARGE COLONIES		SMALL COLONIES	
		COLONIES PER INDIVIDUAL PLATES	P.E.	% CONTROL	2 x 10 ³ CELLS/WELL	COLONIES PER INDIVIDUAL PLATES		M.F./SV x 10 ⁻⁶	M.F./SV x 10 ⁻⁶	M.F./SV x 10 ⁻⁶	
0	-	67 96	73 96	100	12 96	12 96	102.22	75.4	24.3		
31.25	-	65 96	74 96	99	13 96	9 96	94.54	85.5	8.1		
62.5	-	72 96	77 96	115	10 95	10 96	73.92	50.9	21.3		
125	-	61 96	72 96	90	17 96	17 96	165.18	154.5	8.9		
250	-	23 96	42 95	32	20 96	20 96	561.59*	378.9	155.1		
EMS 1000	-	31 96	69 96	56	52 96	65 96	1277.69	1029.9	110.6		

Test for Linear Trend: slope = 7.54E-07 Variance = 1.51E-13 b²/sb = 3.766*

P.E. = Plating efficiency M.F./SV = Mutant frequency per survivor * = p < 0.05

T A B L E 2

L5178Y GENE MUTATION - MICROFITRE PLATE COUNTS
WITH METABOLIC ACTIVATION

TEST MATERIAL: CARDOLITE NC-513

DOSE µg/ml	S9 MIX	NON-SELECTIVE MEDIUM (2 CELLS/WELL)			SELECTIVE MEDIUM (TFT) 2 x 10 ³ CELLS/WELL COLONIES PER INDIVIDUAL PLATES	M.F./SV x 10 ⁻⁶	LARGE COLONIES		SMALL COLONIES	
		COLONIES PER INDIVIDUAL PLATES	P.E.	% CONTROL			M.F./SV x 10 ⁻⁶	M.F./SV x 10 ⁻⁶	M.F./SV x 10 ⁻⁶	M.F./SV x 10 ⁻⁶
0	+	$\frac{76}{96}$	81.00	100	$\frac{2}{96}$	19.60	13.0	6.5		
8	+	$\frac{68}{96}$	71.44	88	$\frac{4}{96}$	45.17	22.2	22.2		
16	+	$\frac{82}{96}$	98.08	121	$\frac{5}{96}$	32.90	21.7	10.7		
32	+	$\frac{79}{96}$	96.26	119	$\frac{2}{96}$	27.78	19.3	8.2		
48	+	$\frac{67}{96}$	69.31	86	$\frac{3}{96}$	22.90	7.6	15.2		
CP 75	+	$\frac{60}{96}$	47.00	58	$\frac{26}{96}$	276.89	51.1	214.1		

P.E. = Plating efficiency

M.F./SV = Mutant frequency per survivor

T A B L E 3

L5178Y GENE MUTATION - MUTANT COLONY NUMBERS

TEST MATERIAL: CARDOLITE NC-513

WITHOUT METABOLIC ACTIVATION				WITH METABOLIC ACTIVATION			
DOSE ug/ml	COLONY TYPE	NUMBER OF COLONIES PER PLATE	% SMALL COLONIES	DOSE ug/ml	COLONY TYPE	NUMBER OF COLONIES PER PLATE	% SMALL COLONIES
0	Lg	9	25	0	Lg	1	33
	Sm	3			Sm	1	
31.25	Lg	13	9	8	Lg	2	50
	Sm	0			Sm	2	
62.5	Lg	7	30	16	Lg	3	33
	Sm	3			Sm	2	
125	Lg	16	6	32	Lg	1	30
	Sm	1			Sm	2	
250	Lg	13	30	48	Lg	2	67
	Sm	7			Sm	1	
EMS 1000	Lg	45	13	CP 7.5	Lg	5	80
	Sm	7			Sm	21	

Lg = Large colonies

Sm = Small colonies

APPENDIX I



**THE DEPARTMENT OF HEALTH OF THE GOVERNMENT
OF THE UNITED KINGDOM**

GOOD LABORATORY PRACTICE

**STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC**

LABORATORY

*SafePharm Laboratories Limited
P O Box No 45
Derby
DE1 2BT*

DATE OF INSPECTION

31 January 1994

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of studies performed at these facilities.

16/3/94.

D. F. Moore
Director
UK GLP Monitoring Unit



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

Chris Ford
V.P. Performance Improvement
Cardolite Corporation
500 Doremus Avenue
Newark, New Jersey 07105

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

APR 24 1995

EPA acknowledges the receipt of information submitted by your organization under Section 8(e) of the Toxic Substances Control Act (TSCA). For your reference, copies of the first page(s) of your submission(s) are enclosed and display the TSCA §8(e) Document Control Number (e.g., 8EHQ-00-0000) assigned by EPA to your submission(s). Please cite the assigned 8(e) number when submitting follow-up or supplemental information and refer to the reverse side of this page for "EPA Information Requests".

All TSCA 8(e) submissions are placed in the public files, unless confidentiality is claimed according to the procedures outlined in Part X of EPA's TSCA §8(e) policy statement (43 FR 11110, March 16, 1978). Confidential submissions received pursuant to the TSCA §8(e) Compliance Audit Program (CAP) should already contain information supporting confidentiality claims. This information is required and should be submitted if not done so previously. To substantiate claims, submit responses to the questions in the enclosure "Support Information for Confidentiality Claims". This same enclosure is used to support confidentiality claims for non-CAP submissions.

Please address any further correspondence with the Agency related to this TSCA 8(e) submission to:

Document Processing Center (7407)
Attn: TSCA Section 8(e) Coordinator
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
Washington, D.C. 20460-0001

EPA looks forward to continued cooperation with your organization in its ongoing efforts to evaluate and manage potential risks posed by chemicals to health and the environment.

Sincerely,

Terry R. O'Bryan

Terry R. O'Bryan
Risk Analysis Branch

Enclosure

13322A



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contains at least 50% recycled fiber

Triage of 8(e) Submissions

Date sent to triage: 12/14/95

NON-CAP

CAP

Submission number: 13322A

TSCA Inventory:

Y N D

Study type (circle appropriate):

Group 1 - Dick Clements (1 copy total)

ECO AQUATO

Group 2 - Ernie Falke (1 copy total)

ATOX SBTOX SEN w/NEUR

Group 3 - Elizabeth Margosches (1 copy each)

STOX CTOX EPI RTOX GTOX
STOX/ONCO CTOX/ONCO IMMUNO CYTO NEUR

Other (FATE, EXPO, MET, etc.): _____

Notes:

THIS IS THE ORIGINAL 8(e) SUBMISSION; PLEASE REFILE AFTER TRIAGE DATABASE ENTRY

For Contractor Use Only	
entire document: <u>0</u> 1 2 pages <u>1</u>	pages <u>1, TAPS</u>
Notes:	
Contractor reviewer: <u></u>	Date: <u>4/18/95</u>

CECATS/TRIAGE TRACKING DBASE ENTRY FORM

CECATS DATA: Submission # BEHQ 0295-13322 SEQ A

TYPE: INT SUPP FLWP

SUBMITTER NAME: Cardolite Corporation

INFORMATION REQUESTED: FLWP DATE: _____
 0501 NO INFO REQUESTED
 0502 INFO REQUESTED (TECH)
 0503 INFO REQUESTED (VOL ACTIONS)
 0504 INFO REQUESTED (REPORTING RATIONALE)
 DISPOSITION:
 0639 REFER TO CHEMICAL SCREENING
 0678 CAP NOTICE

VOLUNTARY ACTIONS:
 0400 NO ACTION RECORDED
 0402 STUDIES PLANNED (INDIVIDUAL)
 0403 NOTIFICATION OF WORK IN PROGRESS
 0404 LABEL/MSDS (TRANSFERS)
 0405 PROCESS/ANDI INC. (TRANSFERS)
 0406 APP/USE DISCONTINUED
 0407 PRODUCTION DISCONTINUED
 0408 CONFIDENTIAL

SUB DATE: 02/03/95 OTS DATE: 02/10/95 CSRAD DATE: 02/24/95

CHEMICAL NAME:

Cardolite NC-513LC

CAS# 68413-24-1

INFORMATION TYPE:	P F C	INFORMATION TYPE:	P F C	INFORMATION TYPE:	P F C
0201 ONCO (HUMAN)	01 02 04	0216 EPICLIN	01 02 04	0241 IMMUNO (ANIMAL)	01 02 04
0202 ONCO (ANIMAL)	01 02 04	0217 HUMAN EXPOS (PROD CONTAM)	01 02 04	0242 IMMUNO (HUMAN)	01 02 04
0203 CELL TRANS (IN VITRO)	01 02 04	0218 HUMAN EXPOS (ACCIDENTAL)	01 02 04	0243 CHEM/PHYS PROP	01 02 04
0204 MUTA (IN VITRO)	01 02 04	0219 HUMAN EXPOS (MONITORING)	01 02 04	0244 CLASTO (IN VITRO)	01 02 04
0205 MUTA (IN VIVO)	01 02 04	0220 ECO/AQUA TOX	01 02 04	0245 CLASTO (ANIMAL)	01 02 04
0206 REPRO/TERATO (HUMAN)	01 02 04	0221 ENV. OCCUREL/FATE	01 02 04	0246 CLASTO (HUMAN)	01 02 04
0207 REPRO/TERATO (ANIMAL)	01 02 04	0222 EMER INCI OF ENV CONTAM	01 02 04	0247 DNA DAM/REPAIR	01 02 04
0208 NEURO (HUMAN)	01 02 04	0223 RESPONSE REQUEST DELAY	01 02 04	0248 PROD/USE/PROC	01 02 04
0209 NEURO (ANIMAL)	01 02 04	0224 PRODCOMP/CHEM ID	01 02 04	0251 MSDS	01 02 04
0210 ACUTE TOX (HUMAN)	01 02 04	0225 REPORTING RATIONALE	01 02 04	0299 OTHER	01 02 04
0211 CHR. TOX (HUMAN)	01 02 04	0226 CONFIDENTIAL	01 02 04		
0212 ACUTE TOX (ANIMAL)	01 02 04	0227 ALLERG (HUMAN)	01 02 04		
0213 SUB ACUTE TOX (ANIMAL)	01 02 04	0228 ALLERG (ANIMAL)	01 02 04		
0214 SUB CHRONIC TOX (ANIMAL)	01 02 04	0229 METAB/PHARMACO (ANIMAL)	01 02 04		
0215 CHRONIC TOX (ANIMAL)	01 02 04	0240 METAB/PHARMACO (HUMAN)	01 02 04		

TRIAGE DATA: NON-CBI INVENTORY

YES

ONGOING REVIEW

YES (DROP/REFER)

CAS SR NO

IN TRIAL/INI

REFTR

NO (CONTINUE)

SPECIES

In Vitro

TOXICOLOGICAL CONCERN:

LOW

MED

HIGH

USE:

PRODUCTION:

UNCLASSIFIED Non-CBI

28)

8EHQ-0295-13322: Rank - medium.

Chemical: Cardolite NC-513LC (CAS# 68413-24-1).

Cardolite NC-513LC: Reverse Mutation Assay ":Ames Test" using *Salmonella typhimurium* TA98 and TA100, Safeparm Laboratories Ltd., Derby, UK, dated 3 May 1994: Positive for gene mutations, with a dose response, in the *Salmonella typhimurium*/mammalian microsomal (Ames) assay in strain TA100 both without and with metabolic activation.

Cardolite NC-513: Reverse Mutation Assay ":Ames Test" using *Salmonella typhimurium* TA98 and TA100, Safeparm Laboratories Ltd., Derby, UK, dated 3 May 1994: Positive for gene mutations, with a dose response, in the *Salmonella typhimurium*/mammalian microsomal (Ames) assay in strain TA100 both without and with metabolic activation.

Cardolite NC-513LC: Mutation of L5178Y mouse lymphoma cells at the thymidine kinase TK^{+/-} locus: Single experiment fluctuation assay, Safeparm Laboratories Ltd., Derby, UK, dated 18 May 1994: Positive for gene mutations, with a dose response, in the L5178Y TK^{+/-} mouse lymphoma gene mutation assay in vitro without metabolic activation.

Cardolite NC-513: Mutation of L5178Y mouse lymphoma cells at the thymidine kinase TK^{+/-} locus: Single experiment fluctuation assay, Safeparm Laboratories Ltd., Derby, UK, dated 24 May 1994: Positive for small colonies, suggesting chromosome mutations (aberrations), with a dose response, in the L5178Y TK^{+/-} mouse lymphoma gene mutation assay in vitro without metabolic activation.