SafePharm Laboratories

C6-2AL:

REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

SPL PROJECT NUMBER: 408/330

AUTHOR: P W Thompson

STUDY SPONSOR:

Asahi Glass Co., Ltd.
1-12-1, Yurakucho
Chiyoda-ku
TOKYO 100-8405
JAPAN

TEST FACILITY:

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

Telephone: (01332) 792896

Facsimile: (01332) 799018

CONFIDENTIAL

QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress.

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

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

	15 August 2001	Standard Test Method Compliance Audit
	27 May 2002	Test Material Preparation
	29 May 2002	Test System Preparation
	30 May 2002	Exposure
	30 May 2002	Assessment of Response
§	05 July 2002	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

J M. Crowther DATE: 23 OCT 2002

For Safepharm Quality Assurance Unit*

*Authorised QA Signatures:

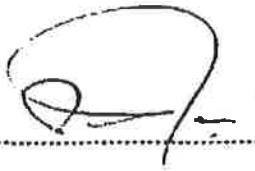
Head of Department:	JR Pateman CBiol MIBiol DipRQA FRQA
Deputy Head of Department:	JM Crowther MIScT MRQA
Senior Audit Staff:	JV Johnson BSc MRQA; G Wren ONC MRQA; R Hurst MRQA

GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 87/18/EEC (as amended by Directive 1999/11/EC) and 88/320/EEC (as amended by Directive 1999/12/EC).

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

This report fully and accurately reflects the procedures used and data generated.



..... DATE: 23 OCT 2002

P W Thompson HNC
Study Director

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SUMMARY

Introduction. The method was designed to meet the requirements of the OECD Guidelines for Testing of Chemicals No. 471 "Bacterial Reverse Mutation Test", Method B13/14 of Commission Directive 2000/32/EC and the USA, EPA (TSCA) OPPTS harmonised guidelines.

Methods. *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and *Escherichia coli* strain WP2uvrA⁻ were treated with the test material using 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 50 to 5000 µg/plate in the first experiment. The experiment was repeated on a separate day using the same dose range as Experiment 1, fresh cultures of the bacterial strains and fresh test material formulations.

Results. The vehicle (dimethyl sulphoxide) control plates gave counts of revertant colonies within the normal range. All of the positive control chemicals used in the test induced marked increases in the frequency of revertant colonies, both with and without metabolic activation. Thus, the sensitivity of the assay and the efficacy of the S9-mix were validated.

The test material caused no visible reduction in the growth of the bacterial background lawn at any dose level. The test material was, therefore, tested up to the maximum recommended dose level of 5000 µg/plate. No test material precipitate was observed on the plates at any of the doses tested in either the presence or absence of S9-mix.

No significant increases in the frequency of revertant colonies were recorded for any of the bacterial strains, with any dose of the test material, either with or without metabolic activation.

Conclusion. The test material was considered to be non-mutagenic under the conditions of this test.

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REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

1. INTRODUCTION

This study 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* and tryptophan auxotrophs of *Escherichia coli* to various concentrations of the test material. The method conforms to the guidelines for bacterial mutagenicity testing published by the major Japanese Regulatory Authorities including METI, MHLW and MAFF. It also meets the requirements of the OECD Guidelines for Testing of Chemicals No. 471 "Bacterial Reverse Mutation Test", Method B13/14 of Commission Directive 2000/32/EC and the USA, EPA (TSCA) OPPTS harmonised guidelines. A copy of the Certificate of Compliance with GLP, issued by the UK Department of Health, is included as Appendix 2.

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$^-$ *Salmonella* strains) which renders the organism incapable of DNA excision repair and deep rough mutation (rfa) which increases the permeability of the cell wall. A mutant strain of *Escherichia coli* (WP2uvrA$^-$), which requires tryptophan and which can be reverse mutated by base substitution to tryptophan independence was used to complement the *Salmonella* strains. This strain also has a deletion in an excision repair gene (uvrA$^-$). 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 preparation (S9-mix) prepared from rats pre-treated with a compound known to induce an elevated level of these enzymes.

The experimental phase of this study was performed between 24 April 2002 and 31 May 2002.

2. TEST MATERIAL

Sponsor's identification	:	C6-2AL
Description	:	clear colourless liquid
Purity	:	99.8%
Batch number	:	001002
Date received	:	25 March 2002
Storage conditions	:	room temperature, in the dark

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

3. METHODS

3.1 Tester Strains

Salmonella typhimurium TA1535, TA1537, TA98 and TA100

Escherichia coli WP2uvrA$^-$

The *Salmonella* strains were obtained from the University of California at Berkeley on culture discs on 4 August 1995 whilst *Escherichia coli* strain WP2uvrA$^-$ was obtained from the British Industrial Biological Research Association on 17 August 1987. All of the strains were stored at -196°C in a Statebourne liquid nitrogen freezer, model SXR 34. Prior to the master strains being used, characterisation checks were carried out to confirm the amino-acid requirement, presence of rfa, R factors, uvrB or uvrA 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 numbers 250177 05/06 and 257111 06/10) and incubated at 37°C for approximately 10 hours. Each culture was monitored spectrophotometrically for turbidity with titres determined by viable count analysis on nutrient agar plates.

3.2 Preparation of Test and Control Materials

The test material was accurately weighed and approximate half-log dilutions prepared in dimethyl sulphoxide by mixing on a vortex mixer and sonication for 5 minutes at room temperature on the day of each experiment. Analysis for concentration, homogeneity and stability of the test material formulations is not a requirement of the test guidelines and was, therefore, not determined. Prior to use, the solvent was dried using molecular sieves (sodium alumino-silicate) ie 2 mm pellets with a nominal pore diameter of 4×10^{-4} microns.

Vehicle and positive controls were used in parallel with the test material. A solvent treatment group was used as the vehicle control and the positive control materials used in the series of plates without S9-mix were as follows:

N-ethyl-N'-nitro-N-nitrosoguanidine(ENNG):	3 µg/plate for TA100, 5 µg/plate for TA1535 and 2 µg/plate for WP2uvrA⁻
9-Aminoacridine (9AA):	80 µg/plate for TA1537
4-Nitroquinoline-1-oxide (4NQO):	0.2 µg/plate for TA98

In addition, 2-Aminoanthracene (2AA) and Benzo(a)pyrene (BP), which are non-mutagenic in the absence of metabolising enzymes, were used in the series of plates with S9-mix at the following concentrations:

2AA at 1 µg/plate for TA100
2AA at 2 µg/plate for TA1535 and TA1537
BP at 5 µg/plate for TA98
2AA at 10 µg/plate for WP2uvrA⁻

3.3 Microsomal Enzyme Fraction

S9 was prepared in-house on 03 March 2002 (Preliminary Toxicity Test only) and 12 April 2002 from the livers of male Sprague-Dawley rats weighing ~ 250g. These had each orally received three consecutive daily doses of phenobarbitone/β-naphthoflavone (80/100 mg per kg per day) prior to S9 preparation. Before use, each batch of S9 was assayed for its ability to metabolise the indirect mutagens 2-Aminoanthracene and Benzo(a)pyrene. The S9 was stored at -196°C.

3.4 S9-Mix and Agar

The S9-mix was prepared immediately before use using sterilised co-factors and maintained on ice for the duration of the test.

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 NADPH	2.0 ml
0.1 M NADH	2.0 ml
0.2 M Sodium phosphate buffer (pH 7.4)	25.0 ml
Sterile distilled water	12.5 ml

A 0.5 ml aliquot of S9-mix and 2 ml of molten, trace histidine or tryptophan supplemented. top agar was overlaid onto a sterile Vogel-Bonner Minimal agar plate in order to assess the sterility of the S9-mix. This procedure was repeated, in triplicate, on the day of each experiment.

Top agar was prepared using 0.6% Difco Bacto agar (lot number 0305000 11/05) and 0.5% sodium chloride with 5 ml of 1.0 mM histidine and 1.0 mM biotin or 1.0 mM tryptophan solution added to each 100 ml of top agar. Vogel-Bonner Minimal agar plates were purchased from International Laboratory Services Ltd (lot numbers 813715-2 10/05 and 812139-2 01/07).

3.5 Test Procedure

3.5.1 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. The concentrations tested were 0, 0.15, 0.5, 1.5, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate. The test was performed by mixing 0.1 ml of bacterial culture (TA100 or WP2uvrA$^-$), 2 ml of molten, trace histidine or tryptophan supplemented, top agar, 0.1 ml of test material formulation and 0.5 ml of S9-mix or phosphate buffer and overlaying onto sterile plates of Vogel-Bonner Minimal agar (30 ml/plate). Ten concentrations of the test material formulation and a vehicle control (dimethyl sulphoxide) were tested. In addition, 0.1 ml of the maximum concentration of the test material and 2 ml of molten, trace histidine or tryptophan supplemented, top agar was overlaid onto a sterile Nutrient agar plate in order to assess the sterility of the test material. After approximately 48 hours incubation at 37°C the plates were assessed for numbers of revertant colonies using a Domino colony counter and examined for effects on the growth of the bacterial background lawn.

3.5.2 Mutation Study - Experiment 1

Five concentrations of the test material (50, 150, 500, 1500 and 5000 µg/plate) were assayed in triplicate against each tester strain, using the direct plate incorporation method.

Measured aliquots (0.1 ml) of one of the bacterial cultures were dispensed into sets of test tubes followed by 2.0 ml of molten, trace histidine or tryptophan supplemented, top agar, 0.1 ml of the test material formulation, vehicle or positive control and either 0.5 ml of S9-mix or phosphate buffer. The contents of each test tube were mixed and equally distributed onto the surface of Vogel-Bonner Minimal agar plates (one tube per plate). This procedure was repeated, in triplicate, for each bacterial strain and for each concentration of test material both with and without S9-mix.

All of the plates were incubated at 37°C for approximately 48 hours and the frequency of revertant colonies assessed using a Domino colony counter.

3.5.3 Mutation Study - Experiment 2

The second experiment was performed using methodology as described for Experiment 1, using fresh bacterial cultures, test material and control solutions. The test material dose range was the same as Experiment 1 (50 to 5000 µg/plate).

3.6 Acceptance Criteria

The reverse mutation assay may be considered valid if the following criteria are met:

All tester strain cultures exhibit a characteristic number of spontaneous revertants per plate in the vehicle and untreated controls. Acceptable ranges are presented in the standard test method section 3 with historical control ranges for 2000 and 2001 in Appendix 1.

The appropriate characteristics for each tester strain have been confirmed, eg rfa cell-wall mutation and pkM101 plasmid R-factor etc.

All tester strain cultures should be in the approximate range of 1 to 9.9 x 10^9 bacteria per ml.

Each mean positive control value should be at least two times the respective vehicle control value for each strain, thus demonstrating both the intrinsic sensitivity of the tester strains to mutagenic exposure and the integrity of the S9-mix. The historical control ranges for 2000 and 2001 are presented in Appendix 1.

There should be a minimum of four non-toxic test material dose levels.

There should be no evidence of excessive contamination.

3.7 Evaluation Criteria

The test material may be considered positive in this test system if the following criteria are met:

The test material should have induced a reproducible, dose-related and statistically (Dunnett's method of linear regression(5)) significant increase in the revertant count in at least one strain of bacteria.

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years, after which instructions will be sought as to further retention or disposal.

5. RESULTS

5.1 Preliminary Toxicity Study

The test material was non-toxic to the strains of bacteria used (TA100 and WP2uvrA$^-$). The test material formulation and S9-mix used in this experiment were both shown to be effectively sterile.

The number of revertant colonies for the toxicity assay were:

With (+) or without (-) S9-mix	Strain	Dose (µg/plate)										
		0	0.15	0.5	1.5	5	15	50	150	500	1500	5000
-	TA100	67	79	69	75	112	81	98	114	80	89	83
+	TA100	74	73	100	105	85	96	92	95	94	90	75
-	WP2uvrA$^-$	24	16	16	22	23	22	17	14	21	17	20
+	WP2uvrA$^-$	23	45	17	19	32	25	28	33	16	25	26

5.2 Mutation Study

Prior to use, the master strains were checked for characteristics, viability and spontaneous reversion rate (all were found to be satisfactory). These data are not given in the report. The S9-mix used in both experiments was shown to be sterile.

Results for the negative controls (spontaneous mutation rates) are presented in Table 1 and were considered to be acceptable. These data are for concurrent untreated control plates performed on the same day as the Mutation Study.

The individual plate counts, the mean number of revertant colonies and the standard deviations, for the test material, positive and vehicle controls, both with and without metabolic activation, are presented in Table 2 and Table 3 for Experiment 1 and Table 4 and Table 5 for Experiment 2.

A history profile of vehicle and positive control values for 2000 and 2001 is presented in Appendix 1.

The test material caused no visible reduction in the growth of the bacterial background lawn at any dose level. The test material was, therefore, tested up to the maximum recommended dose level of 5000 µg/plate. No test material precipitate was observed on the plates at any of the doses tested in either the presence or absence of S9-mix.

No significant increases in the frequency of revertant colonies were recorded for any of the bacterial strains, with any dose of the test material, either with or without metabolic activation.

All of the positive control chemicals used in the test induced marked increases in the frequency of revertant colonies thus confirming the activity of the S9-mix and the sensitivity of the bacterial strains.

6. CONCLUSION

The test material was considered to be non-mutagenic under the conditions of this test.

7. REFERENCES

1. Ames B N, Durston W E, Yamasaki E and Lee F D (1973b) *Proc. Natl. Acad. Sci. (USA)*, **70**, 2281-2285.

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5. Kirkland D J (Ed) (1989) Statistical Evaluation of Mutagenicity Test Data. *UKEMS Sub-committee on Guidelines for Mutagenicity Testing*, **Report - Part III**, Cambridge University Press.

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Table 1 Spontaneous Mutation Rates (Concurrent Negative Controls)

EXPERIMENT 1

Number of revertants (mean number of colonies per plate)									
Base-pair substitution type						Frameshift type			
TA100		TA1535		WP2uvrA⁻		TA98		TA1537	
79		15		21		18		5	
70	(79)	13	(14)	22	(22)	20	(18)	2	(5)
89		14		24		16		7	

EXPERIMENT 2

Number of revertants (mean number of colonies per plate)									
Base-pair substitution type						Frameshift type			
TA100		TA1535		WP2uvrA⁻		TA98		TA1537	
70		17		21		12		15	
76	(72)	24	(21)	23	(21)	11	(16)	12	(12)
69		23		20		24		8	

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Table 2 Test Results: Experiment 1 – Without Metabolic Activation

TEST PERIOD		FROM: 24 MAY 2002						TO: 27 MAY 2002					
With or without S9-Mix	Test substance concentration (µg/plate)	Number of revertants (mean number of colonies per plate)											
		Base-pair substitution type										Frameshift type	
		TA100		TA1535		WP2uvrA-		TA98		TA1537			
-	0	79 65 90	(78) 12.5#	14 17 19	(17) 2.5	34 31 37	(34) 3.0	18 20 17	(18) 1.5	3 9 12	(8) 4.6		
-	50	80 79 87	(82) 4.4	23 22 24	(23) 1.0	37 39 33	(36) 3.1	11 18 19	(16) 4.4	11 9 9	(10) 1.2		
-	150	89 96 85	(90) 5.6	18 25 23	(22) 3.6	28 25 33	(29) 4.0	17 17 23	(19) 3.5	4 4 2	(3) 1.2		
-	500	87 78 90	(85) 6.2	23 11 21	(18) 6.4	17 38 32	(29) 10.8	21 12 14	(16) 4.7	8 7 7	(7) 0.6		
-	1500	80 61 66	(69) 9.8	11 14 24	(16) 6.8	35 22 28	(28) 6.5	14 21 17	(17) 3.5	8 11 6	(8) 2.5		
-	5000	80 97 85	(87) 8.7	14 17 15	(15) 1.5	17 33 29	(26) 8.3	16 14 15	(15) 1.0	5 4 4	(4) 0.6		
Positive controls	Name	ENNG		ENNG		ENNG		4NQO		9AA			
	Concentration (µg/plate)	3		5		2		0.2		80			
S9-Mix	No. colonies per plate	506 530 503	(513) 14.8	94 126 143	(121) 24.9	214 300 332	(282) 61.0	104 89 138	(110) 25.1	1106 2406 1815	(1776) 650.9		

ENNG N-ethyl-N'-nitro-N-nitrosoguanidine
4NQO 4-Nitroquinoline-1-oxide
9AA 9-Aminoacridine
Standard deviation

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Table 3 Test Results: Experiment 1 – With Metabolic Activation

TEST PERIOD		FROM: 24 MAY 2002					TO: 27 MAY 2002				
With or without S9-Mix	Test substance concentration (µg/plate)	Number of revertants (mean number of colonies per plate)									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2uvrA-		TA98		TA1537	
+	0	99 103 93	(98) 5.0#	16 11 15	(14) 2.6	26 24 35	(28) 5.9	25 29 23	(26) 3.1	11 5 4	(7) 3.8
+	50	113 100 89	(101) 12.0	18 16 11	(15) 3.6	38 21 27	(29) 8.6	31 36 32	(33) 2.6	11 6 5	(7) 3.2
+	150	88 78 88	(85) 5.8	11 13 13	(12) 1.2	20 29 28	(26) 4.9	26 26 26	(26) 0.0	5 9 13	(9) 4.0
+	500	94 82 85	(87) 6.2	9 14 17	(13) 4.0	34 37 25	(32) 6.2	33 37 29	(33) 4.0	7 6 13	(9) 3.8
+	1500	77 94 86	(86) 8.5	13 17 12	(14) 2.6	32 31 24	(29) 4.4	26 33 31	(30) 3.6	5 9 6	(7) 2.1
+	5000	90 93 93	(92) 1.7	13 9 12	(11) 2.1	33 15 32	(27) 10.1	29 34 20	(28) 7.1	4 2 3	(3) 1.0
Positive controls	Name	2AA		2AA		2AA		BP		2AA	
S9-Mix	Concentration (µg/plate)	1		2		10		5		2	
+	No. colonies per plate	2074 1872 2053	(2000) 111.1	C 247 193	(220) 38.2	529 620 592	(580) 46.6	109 112 122	(114) 6.8	113 194 223	(177) 57.0

2AA 2-Aminoanthracene
BP Benzo(a)pyrene
C Contaminated
Standard deviation

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Table 4 Test Results: Experiment 2 – Without Metabolic Activation

TEST PERIOD		FROM: 28 MAY 2002						TO: 31 MAY 2002					
With or without S9-Mix	Test substance concentration (µg/plate)	Number of revertants (mean number of colonies per plate)											
		Base-pair substitution type								Frameshift type			
		TA100		TA1535		WP2uvrA-				TA98		TA1537	
-	0	66 60 78	(68) 9.2#	16 9 18	(14) 4.7	19 20 18	(19) 1.0	16 15 21	(17) 3.2	12 14 21	(16) 4.7		
-	50	60 61 62	(61) 1.0	11 C 20	(16) 6.4	20 18 19	(19) 1.0	14 20 13	(16) 3.8	8 18 17	(14) 5.5		
-	150	88 64 73	(75) 12.1	13 19 19	(17) 3.5	24 21 17	(21) 3.5	17 18 13	(16) 2.6	16 9 11	(12) 3.6		
-	500	66 73 70	(70) 3.5	18 18 18	(18) 0.0	17 20 18	(18) 1.5	21 20 11	(17) 5.5	22 19 17	(19) 2.5		
-	1500	69 67 62	(66) 3.6	15 8 16	(13) 4.4	19 19 29	(22) 5.8	13 17 17	(16) 2.3	9 11 16	(12) 3.6		
-	5000	61 69 70	(67) 4.9	13 13 18	(15) 2.9	15 24 29	(23) 7.1	19 17 15	(17) 2.0	11 23 20	(18) 6.2		
Positive controls	Name	ENNG		ENNG		ENNG		4NQO		9AA			
	Concentration (µg/plate)	3		5		2		0.2		80			
S9-Mix	No. colonies per plate												
-		387 433 428	(416) 25.2	261 319 234	(271) 43.4	345 332 280	(319) 34.4	66 102 143	(104) 38.5	1334 1627 1177	(1379) 228.4		

ENNG N-ethyl-N'-nitro-N-nitrosoguanidine
4NQO 4-Nitroquinoline-1-oxide
9AA 9-Aminoacridine
C Contaminated
Standard deviation

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Table 5 Test Results: Experiment 2 – With Metabolic Activation

TEST PERIOD		FROM: 28 MAY 2002						TO: 31 MAY 2002					
With or without S9-Mix	Test substance concentration (µg/plate)	Number of revertants (mean number of colonies per plate)											
		Base-pair substitution type									Frameshift type		
		TA100			TA1535			WP2uvrA-			TA98			TA1537		
+	0	68 76 75	(73) 4.4#		15 9 16	(13) 3.8		28 16 25	(23) 6.2		36 34 21	(30) 8.1		15 14 17	(15) 1.5	
+	50	68 68 69	(68) 0.6		12 17 12	(14) 2.9		29 26 29	(28) 1.7		24 25 27	(25) 1.5		15 16 15	(15) 0.6	
+	150	76 69 60	(68) 8.0		14 14 16	(15) 1.2		21 19 33	(24) 7.6		41 24 27	(31) 9.1		14 12 12	(13) 1.2	
+	500	61 64 68	(64) 3.5		15 11 15	(14) 2.3		16 24 25	(22) 4.9		29 27 28	(28) 1.0		14 17 19	(17) 2.5	
+	1500	65 68 66	(66) 1.5		19 20 8	(16) 6.7		18 21 17	(19) 2.1		19 34 35	(29) 9.0		19 14 14	(16) 2.9	
+	5000	72 74 68	(71) 3.1		14 16 17	(16) 1.5		20 11 20	(17) 5.2		24 25 26	(25) 1.0		15 24 19	(19) 4.5	
Positive controls	Name	2AA			2AA			2AA			BP			2AA		
	Concentration (µg/plate)	1			2			10			5			2		
S9-Mix	No. colonies per plate															
+		1739 1974 1771	(1828) 127.4		137 158 189	(161) 26.2		1193 1081 1129	(1134) 56.2		109 183 156	(149) 37.4		265 420 361	(349) 78.2	

2AA 2-Aminoanthracene
BP Benzo(a)pyrene
Standard deviation

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Appendix 1 History Profile of Vehicle and Positive Control Values

COMBINED VEHICLE AND UNTREATED CONTROL VALUES 2000

Strain	TA100		TA1535		WP2uvrA⁻		TA102		TA98		TA1537		TA1538		WP2uvrA⁻ pkm101		TA97a	
S9-Mix	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Mean	115	118	22	16	22	24	284	312	23	32	12	16	14	31	222	198	102	125
SD	22	22	5	4	5	6	41	35	6	6	4	4	6	3	99	42	14	4
Min	63	63	8	9	8	10	193	224	11	13	4	8	8	29	113	168	82	122
Max	198	181	40	31	45	47	381	377	55	63	25	27	21	33	377	228	123	127
Values	801	621	761	588	706	540	149	81	764	594	749	575	6	2	5	2	6	2

POSITIVE CONTROL VALUES 2000

Strain	TA100		TA1535		WP2uvrA⁻		TA102		TA98		TA1537		TA1538		WP2uvrA⁻ pkm101		TA97a	
S9-Mix	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Mean	488	1620	382	278	663	664	877	794	138	259	1071	466	225	607	2089	1389	261	685
SD	137	490	260	89	243	212	137	123	34	70	327	121	28	40	175	967	1	141
Min	234	443	125	128	203	185	522	388	89	129	290	191	193	568	1965	705	260	585
Max	1190	3079	1843	630	1520	1534	1161	1090	262	506	2160	768	245	648	2213	2073	261	785
Values	172	172	172	170	166	166	69	70	173	172	171	170	3	3	2	2	2	2

COMBINED VEHICLE AND UNTREATED CONTROL VALUES 2001

Strain	TA100		TA1535		WP2uvrA⁻		TA102		TA98		TA1537		TA1538		WP2uvrA⁻ pkm101		TA97a		TA104	
S9-Mix	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Mean	106	116	16	15	22	26	278	305	23	32	11	13	14	no data available	144	147	116	131	512	no data available
SD	23	23	4	4	5	6	45	39	7	8	4	4	5		61	14	21	24	93	
Min	58	62	8	7	11	13	195	201	10	13	2	4	6		74	131	84	114	389	
Max	178	178	37	37	41	52	405	421	56	58	23	29	20		292	164	134	134	616	
Values	817	658	790	621	656	501	244	157	814	662	793	630	5		10	4	5	2	5	

POSITIVE CONTROL VALUES 2001

Strain	TA100		TA1535		WP2uvrA⁻		TA102		TA98		TA1537		TA1538		WP2uvrA⁻ pkm101		TA97a		TA104	
S9-Mix	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Mean	505	1582	431	336	685	756	849	830	140	303	1415	428	153	501	1946	2102	618	750	825	1445
SD	190	541	412	110	323	243	179	159	43	96	713	146	60	246	794	844	62	142	209	202
Min	259	454	98	113	248	242	461	485	72	136	197	139	113	322	672	956	547	649	644	1224
Max	1829	2738	2039	990	1960	1340	1541	1303	362	679	3616	792	133	413	2790	3100	655	850	1125	1621
Values	165	163	163	161	156	155	92	93	166	164	162	160	4	4	7	7	3	2	4	3

SD = Standard deviation
Min = Minimum value
Max = Maximum value

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Appendix 2 Statement of GLP Compliance in Accordance with Directive 88/320/EEC

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 Ltd
Shardlow Business Park
London Road
Shardlow
Derbyshire
DE72 2GD

TEST TYPE

Analytical Chemistry
Environmental Fate
Environmental Toxicity
Mutagenicity
Phys/Chem Tests
Toxicology

DATE OF INSPECTION
28 February 2000

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

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

Roger G. Alexander 26/4/00

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

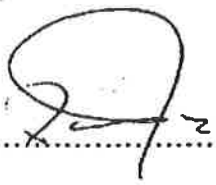
SAFEPHARM LABORATORIES LIMITED

C6-2AL:

REVERSE MUTATION ASSAY "AMES TEST" USING
SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI

SPL PROJECT NUMBER: 408/330

I verify that this is an exact copy of the original report which is located in the Archives of SafePharm Laboratories Limited, Derby, UK.

P W Thompson HNC
Study Director ..... 

DATE ..... 24 OCT 2002

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