PERFLUOROOCTANESULFONYL FLUORIDE (POSF)

BACTERIAL REVERSE MUTATION TEST

Sponsor

3M Center
3M Corporate Toxicology
Building 220-2E-02
St Paul
MN 55133-3220
USA

Research Laboratory

Huntingdon Life Sciences Ltd.
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

Report issued 23 October 2002

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice Standards, with the exceptions stated below, and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No. 3106).

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No. L 77/8).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

In line with normal practice in this type of short-term study, the protocol did not require analysis of the dose form.

The expiry date of the test sample was the Sponsor's responsibility.

K. K. May

23 October 2002

Kenneth May, B.Sc., C.Biol., M.I.Biol.,
Study Director,
Huntingdon Life Sciences Ltd.

Date

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QUALITY ASSURANCE STATEMENT

The following inspections and audits have been carried out in relation to this study:

Study Phase	Date of Inspection	Date of Reporting
Protocol Audit	16 October 2001	16 October 2001
Process Based Inspections		
S9 Preparation	15 January 2002	15 January 2002
Formulation and Treatment	31 October 2001	31 October 2001
Plate Scoring	24 October 2001	24 October 2001
Report Audit	4 February 2002	5 February 2002

Protocol Audit: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Process Based Inspections: At or about the time this study was in progress inspections of routine and repetitive procedures employed on this type of study were carried out. These were conducted and reported to appropriate Company Management as indicated above.

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results of this study to reflect the raw data.

A M Jennings — 23 October 2002

Angela M. Jennings, B.Sc., M.Sc., Ph.D., M.R.Q.A., Date
Group Manager,
Department of Quality Assurance,
Huntingdon Life Sciences Ltd.

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RESPONSIBLE PERSONNEL

Kenneth May, B.Sc., C.Biol., M.I.Biol.
Study Director

Elizabeth Farrall, B.Sc.
Scientist

SUMMARY

In this *in vitro* assessment of the mutagenic potential of Perfluorooctanesulfonyl fluoride (POSF), histidine dependent auxotrophic mutants of *Salmonella typhimurium*, strains TA1535, TA1537, TA98 and TA100, and a tryptophan dependent mutant of *Escherichia coli*, strain WP2*uvr*A/pKM101 (CM891), were exposed to the test substance.

Two independent mutation tests were performed in the presence and absence of liver preparations from Aroclor 1254-induced rats (S9 mix). Both tests involved a pre-incubation stage in airtight vessels.

Concentrations of Perfluorooctanesulfonyl fluoride (POSF) up to 5000 µg/plate were tested in the mutation tests. This is the standard limit concentration recommended in the regulatory guidelines that this assay follows. No signs of toxicity were observed towards the tester strains in either mutation test.

No evidence of mutagenic activity was seen at any concentration of Perfluorooctanesulfonyl fluoride (POSF) in either mutation test.

The concurrent positive controls demonstrated the sensitivity of the assay and the metabolising activity of the liver preparations.

It is concluded that, under the test conditions employed, Perfluorooctanesulfonyl fluoride (POSF) showed no evidence of mutagenic activity in this bacterial system.

INTRODUCTION

This report describes a study designed to assess the mutagenic potential of Perfluorooctanesulfonyl fluoride (POSF) in a bacterial system. The study was conducted in compliance with the following guidelines:

OECD Guidelines for the Testing of Chemicals. (1997) Genetic Toxicology: Bacterial Reverse Mutation Test, Guideline 471.

EC Commission Directive 2000/32/EC Annex 4D-B.13/14. Mutagenicity - Reverse mutation test in bacteria. No. L 136/57.

US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5100 Bacterial reverse mutation test. EPA 712-C-98-247.

Japan Ministry of Agriculture, Forestry and Fisheries. (1985) Notification of Director General, Agricultural Production Bureau. NohSan No. 4200.

Joint Directives of J EPA, J MHW and J MITI. (31 October 1997) Kanpoan No. 287, Eisei No. 127 and Kikyoku No. 2 (31 October 1997).

JMHW Genotoxicity Testing Guideline, PAB Notification No. 1604 (1 November 1999).

Official Notice of J MOL. (8 February 1999).

The method described was also designed to comply with ICH (1995 & 1997), and followed the recommendations of the United Kingdom Environmental Mutagen Society (Gatehouse *et al* 1990).

The *in vitro* technique described by Ames and his co-workers (Ames, McCann and Yamasaki 1975, Maron and Ames 1983) enables the mutagenic effect of a test substance to be determined by exposing specially selected strains of *Salmonella typhimurium* to the test substance. Normally *S. typhimurium* is capable of synthesising the essential amino acid, histidine, but the mutant strains used in this test are incapable of this function. When these strains are exposed to a mutagen, reverse mutation to the original histidine independent form takes place in a proportion of the population. These are referred to as revertants, and are readily detected by their ability to grow and form colonies on a histidine deficient medium (supplemented with biotin, since these strains are also incapable of biotin synthesis).

A technique based on similar principles has also been described by Green (1984). This system employs mutant strains of *Escherichia coli* that are incapable of synthesising the amino acid, tryptophan, which is required for growth.

The strains used carry additional mutations that render them more sensitive to mutagens. The *S. typhimurium* strains have a defective cell coat, which allows greater permeability of test substances into the cell. All the strains are deficient in normal DNA repair processes. In addition, three of them possess a plasmid (pKM101), which introduces an error-prone repair process, resulting in increased sensitivity to some mutagens.

Many substances do not exert a mutagenic effect until they have been metabolised by enzyme systems not available in the bacterial cell. Therefore, the bacteria and test substance are incubated in both the absence and presence of a supplemented liver fraction (S9 mix) prepared from rats previously treated with a substance (Aroclor 1254) known to induce a high level of enzyme activity.

The protocol was approved by Huntingdon Life Sciences Management on 18 July 2001, by the Sponsor on 31 July 2001 and by the Study Director on 15 October 2001.

The study was conducted at Huntingdon Life Sciences Ltd., Eye Research Centre, Eye, Suffolk, IP23 7PX, England.

Experimental start date: 21 November 2001.
Experimental completion date: 31 January 2002.

TEST SUBSTANCE

Identity:	Perfluorooctanesulfonyl fluoride (POSF)
Appearance:	Clear liquid
Storage conditions:	Room temperature
Lot number:	040227
Expiry date:	Sponsor's responsibility; assumed stable for duration of study
Purity:	>95.5%
Specific gravity:	*ca* 1.8
Date received:	14 June 2001

EXPERIMENTAL PROCEDURE

BACTERIAL STRAINS

The following strains were used:

S. typhimurium TA1535: contains a histidine missense mutation (*his*G46) but is also deficient in a DNA repair system (*uvr*B) and has a defective lipopolysaccharide coat on the cell wall (*rfa* mutation). It is reverted by many agents causing base-pair substitutions, but is not sensitive to frameshift mutagens.

S. typhimurium TA100: is the same as TA1535 but contains a resistance transfer factor conferring ampicillin resistance and increasing sensitivity to some mutagens (plasmid pKM101). In addition to base-pair substitutions, it is also able to detect certain frameshift mutagens.

S. typhimurium TA1537: bears a histidine frameshift mutation (*his*C3076). Like TA1535, it is defective in a DNA repair system and lipopolysaccharide coat. It is sensitive to agents causing frameshift mutations involving insertion or deletion of a single base-pair.

S. typhimurium TA98: contains another histidine frameshift mutation (*his*D3052). Again it has a defective DNA repair system and lipopolysaccharide coat but also contains the pKM101 plasmid. It is reverted by agents causing deletion of two adjacent base-pairs (double frameshift mutations), but not by simple alkylating agents causing base-pair substitutions.

E. coli WP2*uvr*A/pKM101: (CM891) contains an ochre mutation. It is reverted by many agents causing A-T base-pair substitutions at the *trp*E locus or by G-C base-pair substitutions in transfer RNA loci elsewhere in the chromosome. It is also deficient in a DNA repair system (*uvr*A), and is more readily reverted by certain mutagens than its parent strain WP2. It also contains the pKM101 plasmid.

The strains of *S. typhimurium* were obtained from the National Collection of Type Cultures, London, England.

The strain of *E. coli* was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland.

Batches of the strains were obtained from master stocks held in liquid nitrogen. The test batches were aliquots of nutrient broth cultures and were stored at -80°C. Dimethyl sulphoxide (DMSO) was added to the cultures at 8% v/v as a cryopreservative. Each batch of frozen strain was tested, where applicable, for cell membrane permeability (*rfa* mutation), sensitivity to UV light and the pKM101 plasmid, which confers resistance to ampicillin. The responses of the strains to a series of diagnostic mutagens were also assessed.

For use in tests, an aliquot of frozen culture was added to 25 ml of nutrient broth and incubated, with shaking, at 37°C for 10 hours. These cultures were intended to provide approximately 10^9 cells per ml, which were measured by spreading aliquots (0.1 ml) of a 10^{-6} dilution of the overnight cultures on the surface of plates of nutrient agar and counting the resultant colonies.

POSITIVE CONTROLS

In the absence of S9 mix

Identity:	Sodium azide
CAS No.:	26628-22-8
Supplier:	Sigma Chemical
Lot number:	77H0079
Purity:	min. 99.5%
Appearance:	White powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	0.5 µg/plate for strains TA1535 and TA100

Identity:	9-Aminoacridine
CAS No.:	90-45-9
Supplier:	Sigma Chemical
Lot number:	106F-06681
Purity:	> 97%
Appearance:	Yellow powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	30 µg/plate for strain TA1537

Identity:	2-Nitrofluorene
CAS No.:	607-57-8
Supplier:	Aldrich Chemical Company
Lot number:	80501-24227
Purity:	98%
Appearance:	Beige powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	1 µg/plate for strain TA98

Identity:	2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)
CAS No.:	3688-53-7
Supplier:	Wako Pure Chemical Industries Ltd.
Lot number:	PAE 1151
Purity:	98-102%
Appearance:	Red powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	0.05 µg/plate for strain WP2*uvr*A/pKM101 (CM891)

In the presence of S9 mix

Identity:	2-Aminoanthracene
CAS No.:	613-13-8
Supplier:	Aldrich Chemical Company
Lot number:	52234-024
Purity:	96%
Appearance:	Green powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	2 µg/plate for strain TA1535 10 µg/plate for strain WP2*uvr*A/pKM101 (CM891)

Identity:	Benzo[a]pyrene
CAS No.:	50-32-8
Supplier:	Aldrich Chemical Company
Lot number:	07778-105
Purity:	98%
Appearance:	Yellow powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	5 µg/plate for strains TA1537, TA98 and TA100

PREPARATION OF S9 FRACTION

Species:	Rat
Sex:	Male
Strain:	Sprague-Dawley derived
Source:	Charles River UK Ltd.
Weight:	<300 g

S9 fraction was prepared from a group of *ca* 10 animals according to the method described by Ames, McCann and Yamasaki (1975). Mixed function oxidase systems in the rat livers were stimulated by Aroclor 1254, administered as a single intra-peritoneal injection in corn oil at a dosage of 500 mg/kg body weight. On the fifth day after injection, following overnight fasting, the rats were killed by cervical dislocation and their livers aseptically removed.

The following steps were carried out at 0-4°C under aseptic conditions. The livers were placed in 0.15 M KCl (3 ml KCl : 1 g liver) before being transferred to a Potter-Elvehjem homogeniser. Following preparation, the homogenate was centrifuged at 9000 *g* for 10 minutes. The supernatant fraction (S9 fraction) was dispensed into aliquots and stored at -80°C or below. Each batch of S9 fraction was tested for sterility and efficacy.

Date of preparation: 17 July 2001 (test 1); 15 January 2002 (test 2)

PREPARATION OF S9 MIX

The S9 mix contained: S9 fraction (10% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADPH (4 mM) and NADH (4 mM). All the cofactors were filter-sterilised before use.

FORMULATION OF TEST SUBSTANCE

The solubility of the test substance was assessed in dimethyl sulphoxide (DMSO), ethanol, acetone and hexane. It was insoluble in DMSO, ethanol and acetone, and was known to be insoluble in water. Although soluble in hexane at 50 mg/ml, the volume of hexane required to administer the test substance at this concentration was found to be too toxic towards the test system. It was, therefore, decided to administer the test substance by direct addition without the use of a solvent.

All concentrations cited in this report are expressed in terms of the Perfluorooctanesulfonyl fluoride (POSF) sample as received.

MUTATION TEST PROCEDURE

First test (range-finding)

The test substance was added to cultures of the five tester strains at seven concentrations. The highest concentration of test substance tested was 5000 µg/plate (obtained by addition of 2.8 µl of the test substance). This is the standard limit concentration recommended in the regulatory guidelines this assay follows. The other concentrations were 2500 µg/plate (1.4 µl), 1750 µg/plate (1.0 µl), 1250 µg/plate (0.7 µl), 700 µg/plate (0.4 µl), 350 µg/plate (0.2 µl) and 175 µg/plate (0.1 µl). Untreated controls and the appropriate positive controls were also included.

Following the addition of the above aliquots of the test substance (or 0.1 ml of positive control solution) to airtight glass vessels, 0.5 ml S9 mix or 0.5 ml 0.1 M phosphate buffer (pH 7.4) was added, followed by 0.1 ml of a 10 hour bacterial culture. The mixtures were incubated at 37°C for 30 minutes with shaking before addition of 2 ml of agar containing histidine (0.5 mM) and tryptophan (0.5 mM). The mixtures were thoroughly shaken and overlaid onto previously prepared Petri dishes containing 25 ml minimal agar. Each Petri dish was individually labelled with a unique code corresponding to a sheet, identifying the contents of the dish. Three Petri dishes were used for each concentration. Plates were also prepared without the addition of bacteria in order to assess the sterility of the test substance, S9 mix and sodium phosphate buffer. All plates were incubated at 37°C for *ca* 72 hours. After this period the appearance of the background bacterial lawn was examined and revertant colonies counted using a Domino automated colony counter.

Any toxic effects of the test substance would be detected by a substantial reduction in revertant colony counts or by the absence of a complete background bacterial lawn. In the absence of any toxic effects the top concentration normally used in the second test would be the same as that used in the first. If toxic effects were observed a lower concentration might be chosen, ensuring that signs of bacterial inhibition are present at the top concentration. Ideally a minimum of three non-toxic concentrations should be obtained. If precipitate were observed on the plates at the end of the incubation period, at least four non-precipitating dose levels should be obtained, unless otherwise justified by the Study Director.

Second test

The second test was an exact repeat of the first test, except that only five concentrations were used. 5000 µg/plate was again chosen as the top concentration.

STABILITY AND FORMULATION ANALYSIS

The stability of the test substance and the stability and homogeneity of the test substance in the test system were not determined as part of this study. Analysis of achieved concentration was not performed as part of this study.

ASSESSMENT OF RESULTS

Acceptance

For a test to be considered valid the mean of the solvent/vehicle control revertant colony numbers for each strain should lie within the 99% confidence limits of the current historical control range of the laboratory unless otherwise justified by the Study Director. The historical range will be maintained as a rolling record over a maximum of five years. Also, the positive control compounds must cause at least a doubling of mean revertant colony numbers over the negative control.

Analysis

The mean number of revertant colonies for all treatment groups will be compared with those obtained for the solvent/vehicle control groups.

Evaluation

If exposure to a test substance produces an increase in revertant colony numbers of at least twice the concurrent solvent/vehicle controls, with some evidence of a positive dose-relationship (increased revertant colony counts at concentrations below that at which the maximal increase is obtained), in two separate experiments, with any bacterial strain either in the presence or absence of S9 mix, it will be considered to show evidence of mutagenic activity in this test system. No statistical analysis will be performed.

If exposure to a test substance does not produce an increase in revertant colony numbers in two separate experiments, with any bacterial strain either in the presence or absence of S9 mix, it will be considered to show no evidence of mutagenic activity in this test system. No statistical analysis will be performed.

If the results obtained fail to satisfy the criteria for a clear "positive" or "negative" response, even after the additional testing outlined in the mutation test procedure, the test data may be subjected to analysis to determine the statistical significance of any increases in revertant colony numbers. The statistical procedures used will be those described by Mahon *et al* (1989) and will usually be analysis of variance followed by Dunnett's test. Biological significance should always be considered along with statistical significance. It should be noted that it is acceptable to conclude an equivocal response if no clear results can be obtained.

DEVIATIONS FROM PROTOCOL

Although the protocol indicated that a solvent or vehicle would be employed, it was not possible to obtain a solution or suspension of the test substance that would be compatible with the test system. The test substance was, therefore, added directly to the test system. Since this procedure complies with the test guidelines that this study follows, this deviation does not impact on the integrity of the study.

MAINTENANCE OF RECORDS

All raw data, samples and specimens (if appropriate) arising from the performance of this study will remain the property of the Sponsor. Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

RESULTS

The results obtained with Perfluorooctanesulfonyl fluoride (POSF) and positive control compounds are presented in Tables 1 to 10. The mean values quoted have been corrected to the nearest whole number.

The absence of colonies on sterility check plates confirmed the absence of microbial contamination.

The total colony counts on nutrient agar plates (see Tables) confirmed the viability and high cell density of the cultures of the individual organisms.

The mean revertant colony counts for the solvent controls were within the 99% confidence limits of the current historical control range of the laboratory. Appropriate positive control chemicals (with S9 mix where required) induced substantial increases in revertant colony numbers with all strains, confirming sensitivity of the cultures and activity of the S9 mix.

FIRST TEST (RANGE-FINDING)

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to Perfluorooctanesulfonyl fluoride (POSF) at any concentration in either the presence or absence of S9 mix.

No visible thinning of the background lawn of non-revertant cells was obtained following exposure to Perfluorooctanesulfonyl fluoride (POSF). A maximum exposure concentration of 5000 µg/plate was, therefore, selected for use in the second test.

SECOND TEST

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to Perfluorooctanesulfonyl fluoride (POSF) at any concentration in either the presence or absence of S9 mix.

No visible thinning of the background lawn of non-revertant cells was obtained following exposure to Perfluorooctanesulfonyl fluoride (POSF).

CONCLUSION

It is concluded that, under the test conditions employed, Perfluorooctanesulfonyl fluoride (POSF) showed no evidence of mutagenic activity in this bacterial system.

REFERENCES

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TABLE 1

Results obtained with *S. typhimurium* TA98: test 1 (range-finding)

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	46	44	56	49	6
4	POSF	(2500 µg/plate)	+	45	41	42	43	2
5	POSF	(1750 µg/plate)	+	48	45	50	48	3
6	POSF	(1250 µg/plate)	+	43	51	50	48	4
7	POSF	(700 µg/plate)	+	48	50	48	49	1
8	POSF	(350 µg/plate)	+	61	35	52	49	13
9	POSF	(175 µg/plate)	+	56	55	55	55	1
10	Untreated		+	41	36	51	43	8
11	POSF	(5000 µg/plate)	-	41	37	37	38	2
12	POSF	(2500 µg/plate)	-	37	38	35	37	2
13	POSF	(1750 µg/plate)	-	43	31	41	38	6
14	POSF	(1250 µg/plate)	-	39	46	49	45	5
15	POSF	(700 µg/plate)	-	34	39	43	39	5
16	POSF	(350 µg/plate)	-	37	42	41	40	3
17	POSF	(175 µg/plate)	-	44	36	21	34	12
18	Untreated		-	30	30	38	33	5
19	Benzo[a]pyrene	(5 µg/plate)	+	566	546	542	551	13
20	2-Nitrofluorene	(1 µg/plate)	-	234	205	209	216	16
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	133	128	129	130	3

• Except plate nos. 1, 2 and 21 (total colony counts)

sd Standard deviation

TABLE 2

Results obtained with *S. typhimurium* TA98: test 2

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	43	48	39	43	5
4	POSF	(2500 µg/plate)	+	42	36	36	38	3
5	POSF	(1750 µg/plate)	+	42	42	36	40	3
6	POSF	(1250 µg/plate)	+	36	43	30	36	7
7	POSF	(700 µg/plate)	+	51	42	46	46	5
8	Untreated		+	53	44	49	49	5
9	POSF	(5000 µg/plate)	-	21	39	34	31	9
10	POSF	(2500 µg/plate)	-	34	38	23	32	8
11	POSF	(1750 µg/plate)	-	37	35	35	36	1
12	POSF	(1250 µg/plate)	-	29	31	29	30	1
13	POSF	(700 µg/plate)	-	32	38	23	31	8
14	Untreated		-	38	39	31	36	4
15	Benzo[a]pyrene	(5 µg/plate)	+	449	550	567	522	64
16	2-Nitrofluorene	(1 µg/plate)	-	504	467	443	471	31
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	106	96	117	106	11

* Except plate nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 3

Results obtained with *S. typhimurium* TA100: test 1 (range-finding)

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	125	169	137	144	23
4	POSF	(2500 µg/plate)	+	148	130	152	143	12
5	POSF	(1750 µg/plate)	+	153	164	154	157	6
6	POSF	(1250 µg/plate)	+	159	141	165	155	12
7	POSF	(700 µg/plate)	+	154	131	114	133	20
8	POSF	(350 µg/plate)	+	159	145	131	145	14
9	POSF	(175 µg/plate)	+	143	159	128	143	16
10	Untreated		+	148	140	131	140	9
11	POSF	(5000 µg/plate)	-	119	131	122	124	6
12	POSF	(2500 µg/plate)	-	125	132	88	115	24
13	POSF	(1750 µg/plate)	-	125	121	139	128	9
14	POSF	(1250 µg/plate)	-	88	118	124	110	19
15	POSF	(700 µg/plate)	-	138	97	136	124	23
16	POSF	(350 µg/plate)	-	119	118	125	121	4
17	POSF	(175 µg/plate)	-	122	122	109	118	8
18	Untreated		-	112	110	141	121	17
19	Benzo[a]pyrene	(5 µg/plate)	+	573	507	568	549	37
20	Sodium azide	(0.5 µg/plate)	-	360	453	471	428	60
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	168	160	173	167	7

* Except plate nos. 1, 2 and 21 (total colony counts)

sd Standard deviation

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TABLE 4

Results obtained with *S. typhimurium* TA100: test 2

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	131	145	119	132	13
4	POSF	(2500 µg/plate)	+	148	132	148	143	9
5	POSF	(1750 µg/plate)	+	122	154	139	138	16
6	POSF	(1250 µg/plate)	+	147	132	166	148	17
7	POSF	(700 µg/plate)	+	165	125	143	144	20
8	Untreated		+	131	145	131	136	8
9	POSF	(5000 µg/plate)	-	111	118	123	117	6
10	POSF	(2500 µg/plate)	-	126	137	121	128	8
11	POSF	(1750 µg/plate)	-	135	128	147	137	10
12	POSF	(1250 µg/plate)	-	119	128	124	124	5
13	POSF	(700 µg/plate)	-	150	124	125	133	15
14	Untreated		-	143	123	122	129	12
15	Benzo[a]pyrene	(5 µg/plate)	+	752	706	705	721	27
16	Sodium azide	(0.5 µg/plate)	-	528	501	549	526	24
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	109	110	111	110	1

* Except plate nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 5

Results obtained with *S. typhimurium* TA1535: test 1 (range-finding)

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	14	21	16	17	4
4	POSF	(2500 µg/plate)	+	20	26	16	21	5
5	POSF	(1750 µg/plate)	+	29	22	12	21	9
6	POSF	(1250 µg/plate)	+	14	26	20	20	6
7	POSF	(700 µg/plate)	+	22	23	19	21	2
8	POSF	(350 µg/plate)	+	17	23	20	20	3
9	POSF	(175 µg/plate)	+	14	20	19	18	3
10	Untreated		+	31	28	19	26	6
11	POSF	(5000 µg/plate)	-	21	15	22	19	4
12	POSF	(2500 µg/plate)	-	15	19	14	16	3
13	POSF	(1750 µg/plate)	-	20	14	24	19	5
14	POSF	(1250 µg/plate)	-	17	15	21	18	3
15	POSF	(700 µg/plate)	-	19	16	24	20	4
16	POSF	(350 µg/plate)	-	16	15	19	17	2
17	POSF	(175 µg/plate)	-	21	20	20	20	1
18	Untreated		-	22	20	21	21	1
19	2-Aminoanthracene	(2 µg/plate)	+	282	324	247	284	39
20	Sodium azide	(0.5 µg/plate)	-	231	252	251	245	12
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	184	165	175	175	10

* Except plate nos. 1, 2 and 21 (total colony counts)

sd Standard deviation

TABLE 6

Results obtained with *S. typhimurium* TA1535: test 2

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	20	13	13	15	4
4	POSF	(2500 µg/plate)	+	19	15	13	16	3
5	POSF	(1750 µg/plate)	+	12	13	13	13	1
6	POSF	(1250 µg/plate)	+	20	22	14	19	4
7	POSF	(700 µg/plate)	+	21	14	19	18	4
8	Untreated		+	22	19	19	20	2
9	POSF	(5000 µg/plate)	-	17	14	16	16	2
10	POSF	(2500 µg/plate)	-	15	27	16	19	7
11	POSF	(1750 µg/plate)	-	14	16	22	17	4
12	POSF	(1250 µg/plate)	-	21	14	16	17	4
13	POSF	(700 µg/plate)	-	22	16	27	22	6
14	Untreated		-	27	21	19	22	4
15	2-Aminoanthracene	(2 µg/plate)	+	145	137	121	134	12
16	Sodium azide	(0.5 µg/plate)	-	253	237	288	259	26
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	129	129	161	140	18

* Except plate nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 7

Results obtained with *S. typhimurium* TA1537: test 1 (range-finding)

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	23	22	15	20	4
4	POSF	(2500 µg/plate)	+	26	22	16	21	5
5	POSF	(1750 µg/plate)	+	23	27	21	24	3
6	POSF	(1250 µg/plate)	+	21	27	19	22	4
7	POSF	(700 µg/plate)	+	21	22	26	23	3
8	POSF	(350 µg/plate)	+	26	21	24	24	3
9	POSF	(175 µg/plate)	+	17	24	24	22	4
10	Untreated		+	22	22	26	23	2
11	POSF	(5000 µg/plate)	-	19	15	10	15	5
12	POSF	(2500 µg/plate)	-	15	10	13	13	3
13	POSF	(1750 µg/plate)	-	17	17	10	15	4
14	POSF	(1250 µg/plate)	-	17	19	10	15	5
15	POSF	(700 µg/plate)	-	16	16	12	15	2
16	POSF	(350 µg/plate)	-	13	9	15	12	3
17	POSF	(175 µg/plate)	-	13	13	10	12	2
18	Untreated		-	16	15	13	15	2
19	Benzo[a]pyrene	(5 µg/plate)	+	355	394	360	370	21
20	9-Aminoacridine	(30 µg/plate)	-	489	457	453	466	20
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	103	139	107	116	20

* Except plate nos. 1, 2 and 21 (total colony counts)

sd Standard deviation

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TABLE 8

Results obtained with *S. typhimurium* TA1537: test 2

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	16	22	23	20	4
4	POSF	(2500 µg/plate)	+	22	16	20	19	3
5	POSF	(1750 µg/plate)	+	24	29	20	24	5
6	POSF	(1250 µg/plate)	+	22	28	15	22	7
7	POSF	(700 µg/plate)	+	23	26	14	21	6
8	Untreated		+	20	19	26	22	4
9	POSF	(5000 µg/plate)	-	9	8	13	10	3
10	POSF	(2500 µg/plate)	-	10	14	8	11	3
11	POSF	(1750 µg/plate)	-	8	9	14	10	3
12	POSF	(1250 µg/plate)	-	10	17	12	13	4
13	POSF	(700 µg/plate)	-	13	9	13	12	2
14	Untreated		-	16	10	15	14	3
15	Benzo[a]pyrene	(5 µg/plate)	+	315	281	238	278	39
16	9-Aminoacridine	(30 µg/plate)	-	269	276	317	287	26
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	124	129	124	126	3

• Except plate nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

TABLE 9

Results obtained with *E. coli* WP2*uvr* A/pKM101 (CM891): test 1 (range-finding)

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	107	131	87	108	22
4	POSF	(2500 µg/plate)	+	119	136	96	117	20
5	POSF	(1750 µg/plate)	+	126	122	116	121	5
6	POSF	(1250 µg/plate)	+	123	96	119	113	15
7	POSF	(700 µg/plate)	+	122	144	123	130	12
8	POSF	(350 µg/plate)	+	122	138	119	126	10
9	POSF	(175 µg/plate)	+	117	121	119	119	2
10	Untreated		+	123	121	133	126	6
11	POSF	(5000 µg/plate)	-	101	112	99	104	7
12	POSF	(2500 µg/plate)	-	111	82	89	94	15
13	POSF	(1750 µg/plate)	-	132	118	116	122	9
14	POSF	(1250 µg/plate)	-	85	138	85	103	31
15	POSF	(700 µg/plate)	-	110	86	110	102	14
16	POSF	(350 µg/plate)	-	95	74	84	84	11
17	POSF	(175 µg/plate)	-	81	111	73	88	20
18	Untreated		-	88	124	123	112	21
19	2-Aminoanthracene	(10 µg/plate)	+	320	423	405	383	55
20	AF-2†	(0.05 µg/plate)	-	559	529	531	540	17
21	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	162	188	217	189	28

• Except plate nos. 1, 2 and 21 (total colony counts)

sd Standard deviation

† 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide

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TABLE 10

Results obtained with *E. coli* WP2*uvr*A/pKM101 (CM891): test 2

Plate No.	Addition		S9 mix + present - absent	Revertant colony counts* and means				
				A	B	C	Mean	sd
1+	None; S9 mix sterility check		+	0	0	0	0	0
1 -	None; buffer sterility check		-	0	0	0	0	0
2	POSF; sterility check	(5000 µg/plate)	-	0	0	0	0	0
3	POSF	(5000 µg/plate)	+	125	136	122	128	7
4	POSF	(2500 µg/plate)	+	154	122	165	147	22
5	POSF	(1750 µg/plate)	+	126	159	145	143	17
6	POSF	(1250 µg/plate)	+	132	145	172	150	20
7	POSF	(700 µg/plate)	+	184	143	166	164	21
8	Untreated		+	137	144	165	149	15
9	POSF	(5000 µg/plate)	-	147	148	128	141	11
10	POSF	(2500 µg/plate)	-	121	139	155	138	17
11	POSF	(1750 µg/plate)	-	144	131	145	140	8
12	POSF	(1250 µg/plate)	-	131	131	139	134	5
13	POSF	(700 µg/plate)	-	115	110	135	120	13
14	Untreated		-	153	152	140	148	7
15	2-Aminoanthracene	(10 µg/plate)	+	501	465	583	516	60
16	AF-2†	(0.05 µg/plate)	-	1041	899	957	966	71
17	None; 10^{-6} dilution of overnight culture, plated on nutrient agar		-	190	175	189	185	8

* Except plate nos. 1, 2 and 17 (total colony counts)

sd Standard deviation

† 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide

APPENDIX 1

Historical control data

Presented below are the historical control data from the period 1 April 1997 to 30 September 2001.

Untreated controls

Strain	TA100		TA1535		WP2*uvr*A/pKM101 (CM891)		TA98		TA1537	
S9 mix	-	+	-	+	-	+	-	+	-	+
Minimum	78	81	12	10	79	73	23	29	7	7
Maximum	149	165	33	29	167	200	48	54	23	34
Mean	109	114	18	19	121	134	38	41	12	13
No. of values	309	313	307	311	214	218	311	315	308	312
Standard deviation	16	22	3	3	17	23	4	5	3	5
Upper 99% limit	151	168	33	29	170	204	49	55	23	35
Lower 99% limit	76	78	12	10	76	69	22	28	7	6

Positive controls

Strain	TA100			TA1535			WP2*uvr*A/pKM101 (CM891)			TA98		TA1537	
S9 mix	-	-	+	-	-	+	-	-	+	-	+	-	+
	(a)	(d)	(h)	(b)	(d)	(i)	(c)	(e)	(j)	(f)	(h)	(g)	(h)
Minimum	212	237	263	53	112	63	294	244	188	123	123	39	67
Maximum	860	1327	1350	781	1130	1145	2312	1533	1704	993	1031	1933	543
Mean	405	564	561	205	419	229	1331	679	659	320	517	350	246
No. of values	346	441	808	341	434	796	112	415	549	788	810	569	810
Standard deviation	120	175	189	139	196	114	488	183	250	115	165	337	80

(a) ENNG 3 µg
(b) ENNG 5 µg
(c) ENNG 2 µg
(d) Sodium azide 0.5µg
(e) AF-2 0.05 µg
(f) 2-Nitrofluorene 1 µg
(g) 9-Aminoacridine 30 µg
(h) Benzo[a]pyrene 5 µg
(i) 2-Aminoanthracene 2 µg
(j) 2-Aminoanthracene 10 µg

APPENDIX 2

Eye Research Centre GLP Compliance Statement 2001

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	TEST TYPE
Huntingdon Life Sciences **Eye Research Centre** **Eye** **Suffolk** **IP23 7PX**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION

29th January 2001

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 J. Alexander
3/4/01

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

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