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PERFLUOROOCTANESULFONYL FLUORIDE (POSF)

T-7661.3

IN VITRO MAMMALIAN CHROMOSOME ABERRATION TEST IN HUMAN LYMPHOCYTES

Sponsor

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Report issued 22 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 substance was the responsibility of the Sponsor.

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Ms Linda Allais, DEA Tox., DESS Pharm. Vet., France
Study Director,
Department of Genetic Toxicology,
Huntingdon Life Sciences Ltd.

22 October 2002
Date

QUALITY ASSURANCE STATEMENT

The following have been inspected or audited in relation to this study.

Study Phase	Date of Inspection	Date of Reporting
Protocol Audit	11 January 2002	11 January 2002
Study Based Inspection		
Culture treatment	17 January 2002	17 January 2002
Process Based Inspections		
Formulation	14 January 2002	14 January 2002
Culture Establishment	11 February 2002	11 February 2002
Harvesting and slide preparation	7 February 2002	8 February 2002
Slide scoring	5 March 2002	5 March 2002
S-9 preparation	15 January 2002	15 January 2002
Report Audit	15 April 2002	15 April 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.

Study Based Inspection: An inspection of a phase of 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 to reflect the raw data.

L A M Jennings ..... 22 October 2002

Angela Jennings, B.Sc., M.Sc., PhD., M.R.Q.A., Date
Group Manager,
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RESPONSIBLE PERSONNEL

Linda Allais, DEA Tox., DESS Pharm. Vet., France,
Study Director,
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SUMMARY

A study was performed to assess the ability of Perfluorooctanesulfonyl Fluoride (POSF) to induce chromosomal aberrations in human lymphocytes cultured *in vitro*.

Human lymphocytes, in whole blood culture, were stimulated to divide by addition of phytohaemagglutinin, and exposed to the test substance both in the presence and absence of S9 mix derived from rat livers. Solvent and positive control cultures were also prepared. Two hours before the end of the incubation period, cell division was arrested using Colcemid®, the cells harvested and slides prepared, so that metaphase cells could be examined for chromosomal damage.

In order to assess the toxicity of Perfluorooctanesulfonyl Fluoride (POSF) to cultured human lymphocytes, the mitotic index was calculated for all cultures treated with the test substance and the solvent control. On the basis of these data, the following concentrations were selected for metaphase analysis:

First test

With and without S9 mix - 3 hours treatment, 17 hours recovery: 1.25, 2.5 and 5% v/v atmosphere.

Second test

Without S9 mix - 20 hours continuous treatment: 0.8, 1 and 2% v/v atmosphere.

With S9 mix - 3 hours treatment, 17 hours recovery: 2, 5 and 7.5% v/v atmosphere.

In both the absence and presence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused no statistically significant increase in the proportion of metaphase figures containing chromosomal aberrations at any dose level, when compared with the solvent control, in either test.

A quantitative analysis for polyploidy was made in cultures treated with the negative control and highest dose level. No statistically significant increases in the proportion of polyploid cells were seen.

All positive control compounds caused large statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

It is concluded that the test substance Perfluorooctanesulfonyl Fluoride (POSF) has shown no evidence of clastogenic activity in this *in vitro* cytogenetic test system, under the experimental conditions described.

INTRODUCTION

This report describes a study designed to assess the ability of Perfluorooctanesulfonyl Fluoride (POSF) to cause chromosomal aberrations in human lymphocytes cultured *in vitro*.

The study was conducted in compliance with the following guidelines:

> OECD Guideline for the Testing of Chemicals. (1997) Genetic Toxicology: *In Vitro* Mammalian Chromosome Aberration Test, Guideline 473.
>
> US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5375 *In Vitro* Mammalian Chromosome Aberration Test. EPA 712-C-98-223.

Human lymphocytes have been used in this type of study for a number of years (Evans and O'Riordan 1975, Scott, Dean, Danford and Kirkland 1990). They are cultured *in vitro* but do not divide unless stimulated to do so. This is achieved by adding phytohaemagglutinin (PHA) to the culture that results in a high mitotic yield (Nowell 1960).

In this study, blood taken from healthy male non-smoking donors was pooled and diluted with tissue culture medium. The cultures were incubated in the presence of PHA before being treated with the test substance. Following treatment the cells were arrested at metaphase using the mitotic inhibitor, Colcemid®. Chromosomes in these metaphase cells were then examined for the presence of chromosome aberrations. The best estimate of the aberration frequency is at the first cell division after initiation of treatment since certain types of damage may be lost during subsequent cell divisions. In this laboratory the cell cycle time for human lymphocytes in whole blood culture is approximately 13-14 hours.

The study was performed on two separate occasions. In the first test, a three hour treatment was used in both the presence and the absence of S9 mix. In the second test, a continuous treatment was used without S9 mix, and the test with S9 mix was a repeat of the first test.

Aberrations were scored according to the classification of the ISCN (1985). Traditionally gaps have been excluded from the quantitation of chromosome aberrations. Some gaps, however, have been shown to be real discontinuities in DNA (Heddle and Bodycote 1970, Satya-Prakash, Hsu and Pathak 1981). In this study the total number of cells containing aberrations both with and without gaps has been calculated.

Many substances do not exert a mutagenic effect until they have been metabolised by enzyme systems that are not found in cultured cells. Therefore the cultures and test substance were 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 enzymic activity (Maron and Ames 1983, Natarajan *et al.* 1976).

The protocol was approved by Huntingdon Life Sciences Management on 18 July 2001, by the Sponsor on 31 August 2001 and by the Study Director on 10 January 2002.

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

The experimental start and completion dates of the study were 15 January 2002 and 15 March 2002, respectively.

TEST SUBSTANCE

Identity:	Perfluorooctanesulfonyl Fluoride (POSF)
CAS No.:	307-35-7
Appearance:	Clear liquid
Storage conditions:	Room temperature
Batch number:	040227
Expiry:	Sponsor's responsibility; assumed stable for the duration of the study
Purity:	>95.5 %
Date received:	14 June 2001

EXPERIMENTAL PROCEDURE

CULTURE OF LYMPHOCYTES

Human blood was collected aseptically from healthy, non-smoking male donors, pooled and diluted with RPMI 1640 tissue culture medium supplemented with 10% foetal calf serum, 1 unit/ml Heparin, 20 I.U./ml penicillin/20 μg/ml streptomycin and 2.0 mM glutamine. Aliquots (0.4 ml blood : 4.5 ml medium : 0.1 ml phytohaemagglutinin) of the cell suspension were placed in sterile universal containers and incubated at 37°C for approximately 48 hours. The cultures were gently shaken daily to resuspend the cells.

POSITIVE CONTROLS

In the absence of S9 mix

Identity:	Mitomycin C
Supplier:	Sigma Chemical Co Ltd
Appearance:	Blue powder
Batch number:	31K2500
Solvent:	Sterile purified water
Final concentration:	0.2 μg/ml (3 hour treatment) 0.1 μg/ml (continuous treatment)

In the presence of S9 mix

Identity:	Cyclophosphamide
Supplier:	Asta Medica Ltd
Appearance:	White powder
Batch number:	ON465 (Test 1) and 1H485 (Test 2)
Solvent:	Sterile purified water
Final concentration:	10 μg/ml

PREPARATION OF S9 FRACTION

Species:	Rat
Sex:	Male
Strain:	Sprague-Dawley derived
Source:	Charles River UK
Age:	7 - 8 weeks
Weight:	<300 g

S9 fraction was prepared from a group of *ca.* 10 animals. Mixed function oxidase systems in the rat livers were stimulated by Aroclor 1254, administered as a single intraperitoneal injection in corn oil at a dosage of 500 mg/kg bodyweight. On the fifth day after injection, following an overnight starvation, the rats were killed 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 an homogeniser. Following preparation, the homogenates were centrifuged at 9000 *g* for 10 minutes. The supernatant fraction (S9 fraction) was dispensed into aliquots and stored at -80°C or below until required.

PREPARATION OF S9 MIX

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

TREATMENT OF CELLS WITH TEST SUBSTANCE - FIRST TEST

After approximately 48 hours, the cultures were centrifuged and the cells were resuspended in fresh culture medium. Atmospheres of Perfluorooctanesulfonyl Fluoride (POSF) were established in sealed glass bottles (160 ml internal volume) with septum caps to give final concentrations of 1.25, 2.5, 5, 10, 20, 40 and 70% v/v atmosphere. Air was withdrawn from each bottle and then an appropriate volume of Perfluorooctanesulfonyl Fluoride (POSF) was introduced using a syringe and needle, inserted through the septum cap. After evaporation of Perfluorooctanesulfonyl Fluoride (POSF) and equilibration of the atmospheres at 37 °C, the lymphocyte cultures were injected into the bottles. The glass bottles were then incubated on their sides at 37 °C in a roller apparatus (see appendix 3: Apparatus for vapour/gas phase exposure of cultivated mammalian cells), which rotates the bottles once every eight minutes approximately. The lymphocytes coat the inside of the bottles and were immersed in culture medium once every revolution and exposed directly to Perfluorooctanesulfonyl Fluoride (POSF) for the rest of the revolution. The solvent control (Air) was established in duplicate cultures and contained an atmosphere of air. Mitomycin C, at a final concentration of 0.2 μg/ml, was added to duplicate cultures and also contained an atmosphere of air.

Immediately before treatment of the second set of cultures, 1 ml of medium was removed from each culture and discarded. This was replaced with 1 ml of S9 mix. The cultures were then added to the appropriate glass bottle giving the same series of final concentrations as above. The duplicate solvent control cultures were established under an atmosphere of air. Cyclophosphamide was added to duplicate cultures at a final concentration of 10 μg/ml that were contained in an atmosphere of air.

Three hours after dosing, the cultures were centrifuged at 500 *g* for 5 minutes. The cells were rinsed and resuspended in fresh medium under an atmosphere of air in universal containers. They were then incubated for a further 17 hours.

HARVESTING AND FIXATION

Two hours before the cells were harvested, mitotic activity was arrested by addition of Colcemid® (Sigma) to each culture at a final concentration of 0.1 µg/ml. After 2 hours incubation, each cell suspension was transferred to a centrifuge tube and centrifuged for 5 minutes at 500 *g*. The cell pellets were treated with a hypotonic solution (0.075M KCl prewarmed at 37°C). After a 10 minute period of hypotonic incubation at 37°C, the suspensions were centrifuged at 500 *g* for 5 minutes and the cell pellets fixed by addition of freshly prepared cold fixative (3 parts methanol : 1 part glacial acetic acid). The fixative was replaced further times until it became colourless.

SLIDE PREPARATION

The pellets were resuspended, then centrifuged at 500 *g*. for 5 minutes and finally resuspended in a small volume of fresh fixative. A few drops of the cell suspensions were dropped onto pre-cleaned microscope slides which were then allowed to air-dry. The slides were then stained in 10% Giemsa, prepared in buffered water (pH 6.8). After rinsing in buffered water the slides were left to air-dry and then mounted in DPX. The remaining cultures in fixative were stored at 4°C until slide analysis was completed.

MICROSCOPIC EXAMINATION

The prepared slides were examined by light microscopy using a low power objective. The proportion of mitotic cells per 1000 cells in each culture was recorded except for positive control treated cultures. From these results the dose level causing a decrease in mitotic index of approximately 50% of the solvent control value or, if there was no decrease, the maximum achievable concentration was used as the highest dose level for the metaphase analysis. The intermediate and low dose levels were also selected.

The concentration of each positive control compound selected for analysis was the lowest concentration dosed unless a preliminary scan of metaphase figures indicated an insufficient level of aberrant cells.

The selected slides were then coded. Metaphase cells were identified using a low power objective and examined at a magnification of x1000 using an oil immersion objective. One hundred metaphase figures were examined, where possible, from each culture. This number was reduced in cultures showing a high level of aberrant cells. Chromosome aberrations were scored according to the classification of the ISCN (1985). Only cells with 44 - 48 chromosomes were analysed. Polyploid and endoreduplicated cells were noted when seen. The vernier readings of all aberrant metaphase figures were recorded.

The incidence of polyploid metaphase cells, out of 500 metaphase cells, was determined quantitatively for negative control cultures and cultures treated with the highest dose level of the test substance used in the analysis for chromosomal aberrations.

The number of aberrant metaphase cells in each treatment group was compared with the solvent control value using Fisher's test (Fisher 1973).

SECOND TEST

Cultures were initiated and maintained as previously described. In this second test a continuous treatment was used in the absence of S9 mix. In the presence of S9 mix, a three hour treatment was used, as in the first test. The harvest time was at 20 hours for both parts of the test. Concentrations of Perfluorooctanesulfonyl Fluoride (POSF) were as follows:

Without S9 mix: 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2 and 5% v/v atmosphere.

With S9 mix: 0.6, 0.8, 1, 2, 5 and 7.5% v/v atmosphere.

Duplicate cultures were used for each treatment and the solvent control. Mitomycin C, at a final concentration of 0.1 µg/ml, and Cyclophosphamide, at a final concentration of 10 µg/ml, were added to duplicate cultures.

Three hours after dosing, the cultures containing S9 mix were centrifuged. The cells were rinsed and resuspended in fresh medium under an atmosphere of air in universal containers. They were then incubated for a further 17 hours. Cultures treated in the absence of S9 mix were incubated for 20 hours.

All cultures were treated with Colcemid®, at a final concentration of 0.1 µg/ml, two hours before the end of the incubation period. They were then harvested, fixed and the slides prepared as previously described. The slides were then examined microscopically as previously described.

STABILITY, HOMOGENEITY AND FORMULATION ANALYSIS

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

ASSESSMENT OF RESULTS

An assay is considered to be acceptable if the negative and positive control values lie within the current historical control range.

The test substance is considered to cause a positive response if the following conditions are met:

Statistically significant increases ($P<0.01$) in the frequency of metaphases with aberrant chromosomes (excluding gaps) are observed at one or more test concentration.

The increases exceed the negative control range of this laboratory, taken at the 99% confidence limit.

The increases are reproducible between replicate cultures.

The increases are not associated with large changes in osmolality of the treatment medium or extreme toxicity.

Evidence of a dose-relationship is considered to support the conclusion.

A negative response is claimed if no statistically significant increases in the number of aberrant cells above concurrent control frequencies are observed, at any dose level.

A further evaluation may be carried out if the above criteria for a positive or a negative response are not met.

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

FIRST TEST

Toxicity data

Mitotic indices of cultured human lymphocytes treated with Perfluorooctanesulfonyl Fluoride (POSF) are shown in Table 2.

In the absence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused a reduction in the mitotic index to 57% of the solvent control value at dose level of 5% v/v atmosphere. The dose levels selected for the metaphase analysis were 1.25, 2.5 and 5% v/v atmosphere.

In the presence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused a reduction in the mitotic index to 52% of the solvent control value at dose level of 5% v/v atmosphere. The dose levels selected for the metaphase analysis were 1.25, 2.5 and 5% v/v atmosphere.

The quantitative analysis for polyploidy showed no increase in the number of polyploid metaphase figures when compared to the solvent control.

Metaphase analysis

The effects of Perfluorooctanesulfonyl Fluoride (POSF) on the chromosomes of cultured human lymphocytes are shown in Table 3 and summarised in Table 1.

In both the absence and the presence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused no statistically significant increase in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

Both positive control compounds, Mitomycin C and Cyclophosphamide, caused large statistically significant increases ($P<0.001$) in the proportion of aberrant cells. This demonstrated the efficacy of the S9 mix and the sensitivity of the test system.

SECOND TEST

Toxicity data

Mitotic indices of cultured human lymphocytes treated with Perfluorooctanesulfonyl Fluoride (POSF) are shown in Table 4.

In the absence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused a reduction in the mitotic index to 61% of the solvent control value at dose level of 2% v/v atmosphere. The dose levels selected for the metaphase analysis were 0.8, 1 and 2% v/v atmosphere.

In the presence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused a reduction in the mitotic index to 39% of the solvent control value at dose level of 7.5% v/v. The dose levels selected for the metaphase analysis were 2, 5 and 7.5% v/v.

The quantitative analysis for polyploidy showed no increase in the number of polyploid metaphase cells when compared to the solvent control.

Metaphase analysis

The effects of Perfluorooctanesulfonyl Fluoride (POSF) on the chromosomes of cultured human lymphocytes are shown in Table 5 and summarised in Table 1.

In both the absence and the presence of S9 mix, Perfluorooctanesulfonyl Fluoride (POSF) caused no statistically significant increase in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

Both positive control compounds, Mitomycin C and Cyclophosphamide, caused large, statistically significant increases ($P<0.001$) in the proportion of aberrant cells.

CONCLUSION

It is concluded that the test substance Perfluorooctanesulfonyl Fluoride (POSF) has shown no evidence of clastogenic activity in this *in vitro* cytogenetic test system, under the experimental conditions described.

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TABLE 1

Summary of Results

Test 1

Exposure period (hours)	S9 mix	Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	Cells with aberrations Excluding gaps			Cells with aberrations Including gaps			Relative Mitotic Index (%)
			Individual values (%)		Mean (%)	Individual values (%)		Mean (%)	
3	-	0 (Air)	1	1	1.0	2	2	2.0	100
		1.25	5	4	4.5	5	5	5.0	60
		2.5	3	0	1.5	3	2	2.5	72
		5	5	1	3.0	7	1	4.0	57
		0.2 μg/ml (Mitomycin C)	[a] 18	[a] 24	21.0***	[a] 24	[a] 28	26.0***	-
3	+	0 (Air)	2	2	2.0	4	4	4.0	100
		1.25	1	3	2.0	1	5	3.0	92
		2.5	3	5	4.0	3	6	4.5	80
		5	1	1	1.0	1	2	1.5	52
		10μg/ml (Cyclophosphamide)	[a] 20	23	22.0***	[a] 22	29	26.7***	-

Test 2

Exposure period (hours)	S9 mix	Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	Cells with aberrations Excluding gaps			Cells with aberrations Including gaps			Relative Mitotic Index (%)
			Individual values (%)		Mean (%)	Individual values (%)		Mean (%)	
20	-	0 (Air)	2	0	1.0	2	0	1.0	100
		0.8	0	2	1.0	0	3	1.5	69
		1	2	1	1.5	3	1	2.0	66
		2	0	0	0.0	0	0	0.0	61
		0.1 μg/ml (Mitomycin C)	9	7	8.0***	9	7	8.0***	-
3	+	0 (Air)	1	1	1.0	2	2	2.0	100
		2	1	1	1.0	3	2	2.5	73
		5	0	1	0.5	1	2	1.5	70
		7.5	[b] 0	1	0.7	[b] 0	2	1.3	39
		10μg/ml (Cyclophosphamide)	[a] 18	[a] 18	18.0***	[a] 20	[a] 20	20.0***	-

*** P<0.001
Otherwise P≥0.01

[a] 50 cells were analysed from these cultures due to high levels of aberrations seen
[b] 50 cells were analysed from this culture due to insufficient metaphases present on slide

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TABLE 2

Mitotic index data - first test

Without S9 mix, 3 hours treatment and 17 hours recovery

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	Mitotic index #		Relative mitotic index #	Polyploidy	
	Incidence	% Mean	(%)	Incidence	% Mean
0 (Air)	88/1000 92/1000	9.0	100	1/500 1/500	0.2
1.25	51/1000 57/1000	5.4	60		
2.5	74/1000 55/1000	6.5	72		
5	57/1000 45/1000	5.1	57	2/500 0/500	0.2
10	a a				
20	b b				
40	b b				
70	b b				

\# Calculations have been made using rounded values
a Very few metaphases present on slide
b No cells, no metaphases present on slide

TABLE 2

Mitotic index data - first test (continued)

With S9 mix, 3 hours treatment and 17 hours recovery

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	Mitotic index #		Relative mitotic index #	Polyploidy	
	Incidence	% Mean	(%)	Incidence	% Mean
0 (Air)	97/1000 100/1000	9.9	100	1/500 1/500	0.2
1.25	68/1000 113/1000	9.1	92		
2.5	64/1000 93/1000	7.9	80		
5	76/1000 26/1000	5.1	52	0/500 0/500	0.0
10	a a				
20	b b				
40	b b				
70	b -				

\# Calculations have been made using rounded values
a Very few live cells, no metaphases present on slide
b No cells, no metaphases present on slide
\- Not assessed

TABLE 3

Metaphase analysis data - first test

Without S9 mix, 3 hours treatment and 17 hours recovery

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	No. cells examined	Aberrations				No. of aberrant cells				Relative Mitotic index %
		Chromatid type	Chromosome type	Others	Gaps	Exc. gaps	Mean %	Inc. gaps	Mean %	
		ctb cte	csb cse		ctg csg					
0	100	1			1	1	1.0	2	2.0	100
(Air)	100	1			1	1		2		
1.25	100	5				5	4.5	5	5.0	60
	100	2	2		1	4		5		
2.5	100	5				3	1.5	3	2.5	72
	100				2	0		2		
5	100	4	1		2	5	3.0	7	4.0	57
	100	1				1		1		
0.2 μg/ml	50	8	2		4	9	21.0	12	26.0	-
(Mitomycin C)	50	13	3		1 1	12	***	14	***	

ctb	Chromatid break	cte	Chromatid exchange
csb	Chromosome break	cse	Chromosome exchange
ctg	Chromatid gap	csg	Chromosome gap
		others	Cells with greater than 8 aberrations, pulverised cells and pulverised chromosomes

***	P<0.001
Otherwise	P≥0.01

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TABLE 3

Metaphase analysis data - first test (continued)

With S9 mix, 3 hours treatment and 17 hours recovery

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	No. cells examined	Aberrations					No. of aberrant cells				Relative Mitotic index %
		Chromatid type		Chromosome type		Others	Gaps	Exc. gaps	Mean %	Inc. gaps	Mean %	
		ctb	cte	csb	cse		ctg csg					
0	100	2					3	2	2.0	4	4.0	100
(Air)	100	2					2	2		4		
1.25	100	1						1	2.0	1	3.0	92
	100	3		1			2	3		5		
2.5	100	5					1	3	4.0	3	4.5	80
	100	3		3			2	5		6		
5	100	1						1	1.0	1	1.5	52
	100	1					1	1		2		
10 μg/ml	50	7	1	2			2	10	22.0	11	26.7	-
(Cyclophosphamide)	100	19	2	4			11	23	***	29	***	

ctb	Chromatid break	cte	Chromatid exchange
csb	Chromosome break	cse	Chromosome exchange
ctg	Chromatid gap	csg	Chromosome gap
		others	Cells with greater than 8 aberrations, pulverised cells and pulverised chromosomes

***	P<0.001
Otherwise	P≥0.01

TABLE 4

Mitotic index data - second test

Without S9 mix, 20 hours continuous treatment

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	Mitotic index #		Relative mitotic index #	Polyploidy	
	Incidence	% Mean	(%)	Incidence	% Mean
0 (Air)	126/1000 107/1000	11.7	100	0/500 0/500	0.0
0.1	104/1000 113/1000	10.9	93		
0.2	114/1000 103/1000	10.9	93		
0.4	90/1000 97/1000	9.4	80		
0.6	105/1000 64/1000	8.5	73		
0.8	71/1000 90/1000	8.1	69		
1	72/1000 81/1000	7.7	66		
2	65/1000 77/1000	7.1	61	0/500 0/500	0.0
5	14/1000 29/1000	2.2	19		

Calculations have been made using rounded values

TABLE 4

Mitotic index data - second test (continued)

With S9 mix, 3 hours treatment and 17 hours recovery

Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	Mitotic index #		Relative mitotic index #	Polyploidy	
	Incidence	% Mean	(%)	Incidence	% Mean
0 (Air)	69/1000 94/1000	8.2	100	0/500 1/500	0.1
0.6	89/1000 74/1000	8.2	100		
0.8	72/1000 79/1000	7.6	93		
1	56/1000 83/1000	7.0	85		
2	60/1000 59/1000	6.0	73		
5	58/1000 56/1000	5.7	70		
7.5	29/1000 35/1000	3.2	39	0/71 0/241	0.0

Calculations have been made using rounded values

TABLE 5

Metaphase analysis data - second test

Without S9 mix, 20 hours continuous treatment

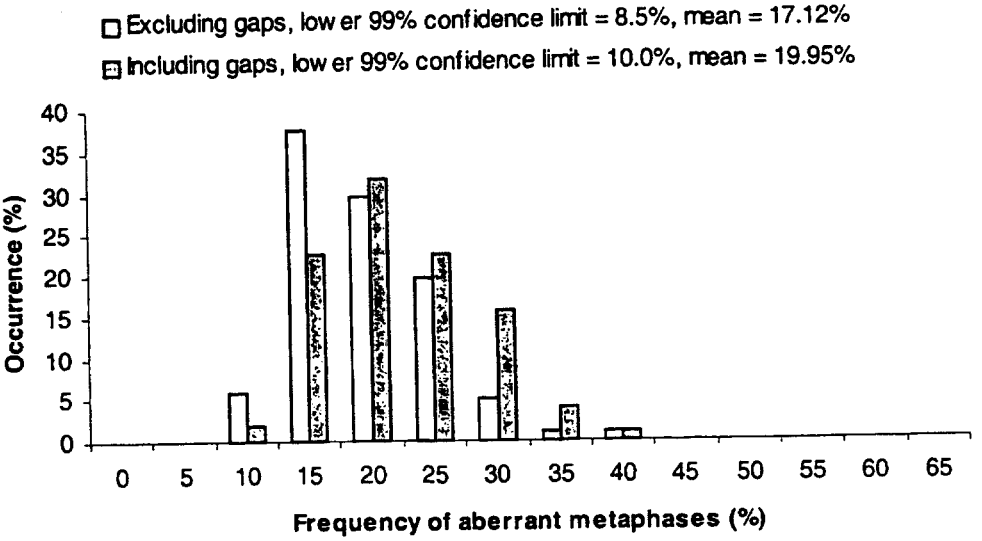
Concentration of Perfluorooctanesulfonyl Fluoride (POSF) (% v/v atmosphere)	No. cells examined	Aberrations					No. of aberrant cells				Relative Mitotic index %
		Chromatid type		Chromosome type		Others	Gaps	Exc. gaps	Mean %	Inc. gaps	Mean %	
		ctb	cte	csb	cse		ctg csg					
0	100	1		2				2	1.0	2	1.0	100
(Air)	100							0		0		
0.8	100							0	1.0	0	1.5	69
	100	2					1	2		3		
1	100	2					1	2	1.5	3	2.0	66
	100			1				1		1		
2	100							0	0.0	0	0.0	61
	100							0		0		
0.1 μg/ml	100	6	3	1				9	8.0	9	8.0	-
(Mitomycin C)	100	3	2	2			1	7	***	7	***	

ctb	Chromatid break	cte	Chromatid exchange
csb	Chromosome break	cse	Chromosome exchange
ctg	Chromatid gap	csg	Chromosome gap
		others	Cells with greater than 8 aberrations, pulverised cells and pulverised chromosomes

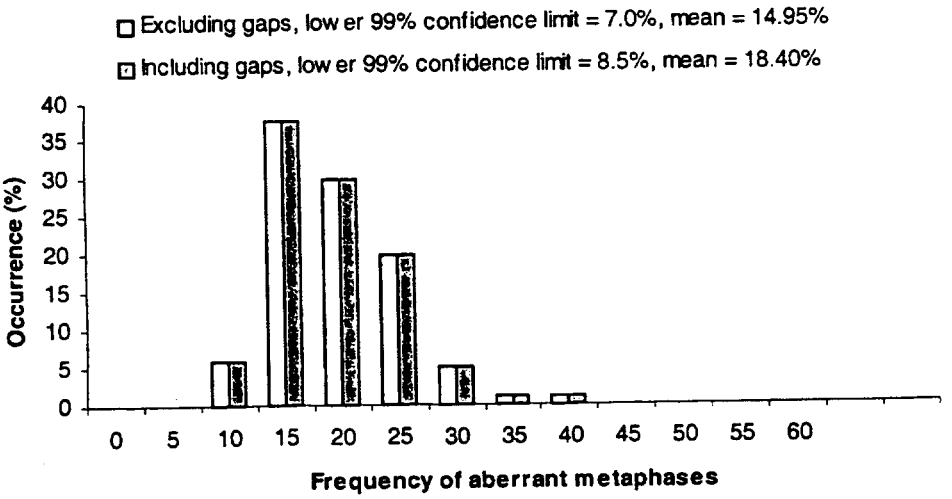
***	P<0.001
Otherwise	P≥0.01

APPENDIX 2

Historical positive control data (January 1999 - December 2001)
Without S9 mix



Historical positive control data (January 1999 - December 2001)
With S9 mix



APPENDIX 3

APPARATUS FOR VAPOUR/GAS PHASE EXPOSURE OF CULTIVATED MAMMALIAN CELLS

FIGURE 1

Culture bottle (volume 160ml)

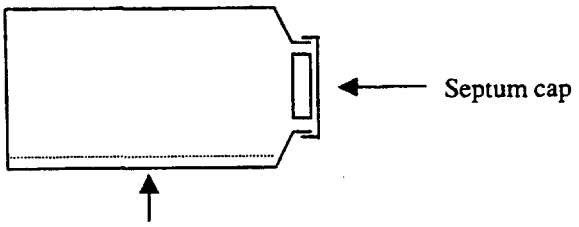
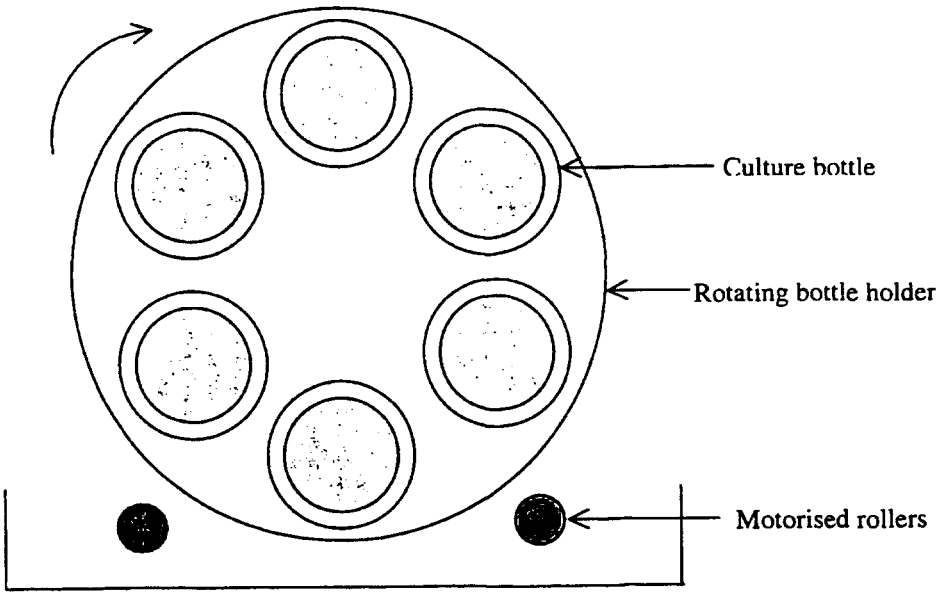


FIGURE 2

Roller apparatus



APPENDIX 4

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