Final Report

Study Title	[redacted] reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*
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STUDY DIRECTOR AUTHENTICATION AND GLP COMPLIANCE STATEMENT

[redacted] reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*

I, the undersigned, hereby declare that the work was performed by me or under my supervision and that the findings provide a true and accurate record of the results obtained.

The study was performed in accordance with the agreed protocol and with Covance Laboratories Limited Standard Operating Procedures, unless otherwise stated, and the study objectives were achieved. The study was conducted in compliance with the United Kingdom Statutory Instrument 1999 No. 3106, The Good Laboratory Practice Regulations 1999 and the OECD Principles of Good Laboratory Practice (revised 1997, issued January 1998) ENV/MC/CHEM (98) 17*.

* with the exception that the supporting data for purity and expiry (characterisation), stability and homogeneity of the test article were not available to the Study Director at the time of finalising this report. However, these data are the subject of a separate GLP compliant study conducted by the Sponsor, [redacted] study reference number AC030449.

M Johnson BSc PhD
Study Director

12 March 2004
Date

QUALITY ASSURANCE STATEMENT

[redacted] reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*

The study described in this report was subject to audit by the independent Quality Assurance Department as indicated below. The report audit confirms that the methods, procedures and observations are accurately and completely described and the reported results accurately and completely reflect the raw data of the regulatory study.

The following inspections were conducted and findings reported to the Study Director and Management as prescribed by Standard Operating Procedures. Critical procedures which are performed routinely in an operational area may be audited in accordance with a 'batch process' programme (B). This can be in addition to phases scheduled on an individual study basis (S).

Inspection programme	Inspection date	Report date
Protocol review	27 November 2003	27 November 2003
Colony counting (B)	12 December 2003	12 December 2003
Dose preparation (B)	18 December 2003	18 December 2003
Culture establishment (B)	7 January 2004	7 January 2004
Data review	January 2004	2 February 2004
Audited draft report review	January 2004	2 February 2004

D Allison BSc (Hons) MSc
QA Team Manager

12/03/04
Date

REVIEWING SCIENTIST'S STATEMENT

[redacted] **reverse mutation in five histidine-requiring strains of *Salmonella typhimurium***

I, the undersigned, hereby declare that I have reviewed this report in conjunction with the Study Director and that the interpretation and presentation of the data in the report are consistent with the results obtained.

N Dawkes BSc
Scientist

10 March 2004
Date

RESPONSIBLE PERSONNEL

[redacted] reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*

The following personnel were responsible for key elements of the study:

Study Director	M Johnson BSc PhD
Study Supervisor	K Braginton BSc

ARCHIVE STATEMENT

[redacted] reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*

All primary data, or authenticated copies thereof, and the final report will be retained in the Covance Laboratories Limited archives for **one** year after issue of the final report. At this time the Sponsor will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. Sponsors will be notified of the financial implications of each of these options at that time. Archiving of the test article is the responsibility of the Sponsor.

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SUMMARY

[redacted] was assayed for mutation in five histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102), both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9), in two separate experiments.

An initial toxicity Range-Finder Experiment was carried out in strain TA100 only, in the absence and presence of S-9, using final concentrations of [redacted] at 1.6, 8, 40, 200, 1000 and 5000 µg/plate, plus solvent and positive controls. Evidence of toxicity was observed following the top two dose treatments in the absence of S-9, and following the top dose treatment in the presence of S-9.

Experiment 1 treatments of all the test strains in the presence of S-9 retained the same test doses as employed for the Range-Finder Experiment. Treatments of all test strains in the absence of S-9 were performed up to a maximum dose of 1000 µg/plate (an estimate of the lower limit of toxicity) using doses 0.32, 1.6, 8, 40, 200, 1000 µg/plate. Evidence of toxicity was observed following the top dose treatment of all strains in the absence and presence of S-9.

Experiment 2 treatments of all strains in the absence of S-9 were performed using a maximum test dose of 1000 µg/plate (an estimate of the lower limit of toxicity) and doses of 1.638, 4.096, 10.24, 25.6, 64, 160, 400 and 1000 µg/plate. Treatments of all strains in the presence of S-9 were performed up to 5000 µg/plate using doses of 8.192, 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate. Narrowed dose ranges were employed in order to examine more closely those concentrations of [redacted] approaching the maximum test dose and therefore considered most likely to provide evidence of any mutagenic activity. In addition, pre-incubation methodology was used for all treatments in the absence and presence of S-9. Evidence of toxicity was observed following the top one or two dose treatments of all strains in the absence and presence of S-9, except strain TA98 in the presence of S-9 where no clear evidence of toxicity was seen.

The test article was completely soluble in the aqueous assay system at all concentrations treated, in each of the experiments performed.

No statistically significant, dose-related and reproducible increases in revertant numbers were observed following any strain treatments in the absence or presence of metabolic activation, and therefore this study was considered to have provided no evidence of any [redacted] mutagenic activity.

It was concluded that [redacted] did not induce mutation in five histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) when tested under the conditions of this study. These conditions included treatments at concentrations up to either 5000 µg/plate, or the lower limit of toxicity, in the absence and in the presence of a rat liver metabolic activation system (S-9).

INTRODUCTION

The Ames test is a rapid, reliable and economical method of evaluating the mutagenic potential of a test article by measuring genetic activity in one or more histidine-requiring strains of *Salmonella typhimurium* in the absence and presence of a liver metabolising system (1). A large database has been accumulated with this assay, confirming its ability to detect genetically active compounds of most chemical classes with around 80-90% sensitivity and specificity (2). The following bacterial strains were used in this study:

Organism	Strain	Type of mutation in the histidine gene
S. typhimurium	TA98	frame-shift
S. typhimurium	TA100	base-pair substitution
S. typhimurium	TA1535	base-pair substitution
S. typhimurium	TA1537	frame-shift
S. typhimurium	TA102	base-pair substitution

With the exception of strain TA102, these strains require biotin as well as histidine for growth. In strain TA102 the critical mutation in the histidine gene is located on a multicopy plasmid pAQ1. This strain is particularly sensitive to the activities of oxidative and cross-linking mutagens. The pKM101 plasmid derivatives (TA98, TA100 and TA102) have increased sensitivity to certain mutagens as the pKM101 plasmid codes for an error-prone DNA repair system (3).

When exposed to a mutagen, some of the bacteria in the treated population undergo genetic changes which revert them to a non-histidine-requiring state, and they can then grow without exogenous histidine. Different tester strains are used because each is mutated by particular chemical classes of compound. A compound that is mutagenic in one strain need not be so in another (4).

The objective of this study was therefore to evaluate the mutagenic activity of [redacted]y examining its ability to revert five histidine-requiring strains of *Salmonella typhimurium* in the absence and in the presence of a rat liver metabolising system (S-9). The procedures used in this study were in accordance with OECD Guideline 471 (adopted 1997), EEC Annex 4D Test B13/14 (2000), UKEMS Guidelines (1990) and ICH Harmonised Tripartite Guideline (1997).

This study was performed according to the protocol, with the exception of the minor deviations detailed in Appendix 7, none of which in any way prejudiced the validity of this study.

The study was initiated on 21 November 2003. Experimental work started on 11 December 2003 and was completed on 9 January 2004. The study completion date is considered to be the date on which the Study Director signs the final report.

MATERIALS

Test article

[redacted] sample number S2539801, was a [redacted] which was received on 26 November 2003. It was stored at room temperature in the dark. The expiry date and purity were to be determined in [redacted] study AC030449. Determinations of the stability and characteristics of the test article were the responsibility of the Sponsor.

Characterisation, homogeneity and stability of the test sample were to be assessed in [redacted] study AC030449. Homogeneity of the test solutions was addressed by solubility in the test system. Stability of the test solutions was not assessed as this was a short-term study and the test solutions were freshly prepared for each experiment.

Test article solutions were prepared by dissolving [redacted] in sterile purified water, with the aid of vortexing, immediately prior to assay to give the maximum required treatment solution concentration. This solution was not filter-sterilised and further dilutions were made using purified water. The test article solutions were protected from light and used within approximately 6¼ hours of the initial formulation of the test article. Solutions were used as follows overleaf:

Experiment	S-9	Concentration of treatment solution (mg/mL)	Final concentration (µg/plate)
Range-Finder Experiment and Mutation Experiment 1	– and +	0.0032[b]	0.32[b]
		0.016[ab]	1.6[ab]
		0.08[ab]	8[ab]
		0.40[ab]	40[ab]
		2.00[ab]	200[ab]
		10.00[ab]	1000[ab]
		50.00[a]	5000[a]
Mutation Experiment 2	+	0.08192	8.192
		0.2048	20.48
		0.512	51.2
		1.28	128
		3.20	320
		8.00	800
		20.00	2000
		50.00	5000
Mutation Experiment 2	-	0.01638	1.638
		0.4096	4.096
		0.1024	10.24
		0.256	25.6
		0.64	64
		1.60	160
		4.00	400
		10.00	1000

a Doses used for all Range-Finder treatments, and for the treatment of all strains in the presence of S-9 in Experiment 1.
b Doses used for the treatment of all strains in the absence of S-9 in Experiment 1.

0.1 mL volume additions of test article formulations were used for all treatments.

Controls

Control treatments were performed using the same addition volumes per plate as the test article treatments, 0.1 mL (or 0.05 mL for pre-incubation positive control treatments and additional solvent control treatments, see footnote to table on the following page). Negative controls comprised treatments with the solvent, purified water. Further control treatments employing solvents used in the formulation of positive control chemicals were performed where appropriate (refer to Appendix 7 for any exceptions). The positive control chemicals were supplied and used as tabulated overleaf:

Chemical	Source	Stock * concentration (µg/mL)#	Final concentration (µg/plate)	Use Strain(s)	S-9
2-nitrofluorene (2NF)	Aldrich Chemical Co, Gillingham, UK	50	5.0	TA98	–
Sodium azide (NaN_3)	Sigma Chemical Co, Poole, UK	20	2.0	TA100, TA1535	–
9-aminoacridine (AAC)	Aldrich Chemical Co, Gillingham, UK	500	50.0	TA1537	–
Glutaraldehyde (GLU)	Aldrich Chemical Co, Gillingham, UK	250	25.0	TA102	–
Benzo[a]pyrene (B[a]P)	Aldrich Chemical Co, Gillingham, UK	100	10.0	TA98	+
2-aminoanthracene (AAN)	Sigma Chemical Co, Poole, UK	50	5.0	TA100, TA1535, TA1537	+
		200	20.0	TA102	+

* With the exception of NaN_3 and GLU, which were prepared in water, all stock solutions were prepared in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO). All stock solutions were stored in aliquots at 1-10°C in the dark, with the exception of B[a]P which was stored in aliquots at –80°C in the dark.

\# For Experiment 2 pre-incubation treatments, stock solutions of these positive control compounds were twice the concentration stated. This enabled the volume additions to be reduced to 0.05mL (thus avoiding solvent-induced toxicity) whilst maintaining the final concentrations per plate detailed above.

Metabolic activation system

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. Batches of MolTox™ S-9 were stored frozen at –80°C, and thawed just prior to incorporation into the top agar (5). Each batch was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-O-dealkylase activities). The quality control statement, relating to the batch of S-9 preparation used, is included in Appendix 6 of this report.

Preparation of 10% S-9 mix and buffer solutions

Quantities were prepared according to the following ratios per 100 mL:

Ingredient	Concentration	Quantity (mL)	
		10% S-9 mix	Buffer solution
Sodium phosphate buffer pH 7.4	500 mM	20	20
Glucose-6-phosphate (disodium)	180 mg/mL	0.845	–
NADP (disodium)	25 mg/mL	12.6	–
Magnesium chloride	250 mM	3.2	–
Potassium chloride	150 mM	22	–
L-histidine HCl (in 250 mM $MgCl_2$)	1 mg/mL	4	4
d-biotin	1 mg/mL	4.88	4.88
S-9	as detailed above	10	–
Water	–	to volume	to volume

Bacteria

Five bacterial strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) were used in this study. All the tester strains, with the exception of strain TA102, were originally obtained from the UK NCTC. Strain TA102 was originally obtained from Glaxo Group Research Limited. For all assays, bacteria were cultured for up to 10 hours at 37±1°C (refer to minor deviations from protocol, Appendix 7) in nutrient broth (containing ampicillin for strains TA98 and TA100 and ampicillin and tetracycline for strain TA102). Incubation was carried out in a shaking incubator. Bacteria were taken from vials of frozen cultures, which had been checked for strain characteristics of histidine dependence, *uvrB* character, *rfa* character and resistance to ampicillin (TA98 and TA100) or ampicillin plus tetracycline (TA102). Checks were carried out according to Maron and Ames (3) and De Serres and Shelby (6). All experimentation commenced within 2 hours of the end of the incubation period.

METHODS

Toxicity Range-Finder Experiment

[redacted] was tested in strain TA100, at the concentrations detailed previously. Triplicate plates without and with S-9 mix were used. Negative (solvent) and positive controls were included in quintuplicate and triplicate respectively, without and with S-9 mix. These platings were achieved by the following sequence of additions to 2.5 mL molten agar at 46±1°C:

- 0.1 mL bacterial culture
- 0.1 mL test article solution or control
- 0.5 mL 10% S-9 mix or buffer solution

followed by rapid mixing and pouring on to Vogel-Bonner E agar plates. When set, the plates were inverted and incubated at 37±1°C in the dark for 3 days. Following incubation, these plates were examined for evidence of toxicity to the background bacterial lawn, and where possible revertant colonies were counted (see Colony counting).

Mutation experiments

[redacted] was tested for mutation in five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102), in two separate experiments, at the concentrations detailed previously, using triplicate plates without and with S-9. Negative (solvent) controls were included in each assay, in quintuplicate without and with S-9. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S-9. The activity of the S-9 mix used in each experiment was confirmed by AAN or B[a]P treatments (again in triplicate) of the strains in the presence of S-9. Platings were achieved as described above.

As the results of the first experiment were negative, treatments in the absence and presence of S-9 in Experiment 2 included a pre-incubation step. Quantities of test article or control solution (volumes reduced to 0.05 mL for positive control additions, and additional DMSO solvent controls), bacteria and S-9 mix or buffer solution detailed above, were mixed together and incubated for 1 hour at 37±1°C, with shaking, before the addition of 2.5 mL molten agar at 46±1°C. Plating of these

treatments then proceeded as for the normal plate-incorporation procedure. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected in the assay.

Colony counting

Colonies were counted electronically using a Seescan Colony Counter (Seescan Plc) or manually where confounding factors such as split agar, bubbles in the agar or a dirty plate affected the accuracy of the automated counter. The background bacterial lawn was inspected for signs of toxicity. Some treatment plates were counted manually for accuracy.

Analysis of results

Treatment of data

Individual plate counts from all experiments were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined.

The accepted normal ranges for mean numbers of spontaneous revertants on solvent control plates for this laboratory are presented in Appendix 4. Data for this laboratory are consistent with ranges of spontaneous revertants per plate considered acceptable elsewhere (6). The accepted normal ranges for mean numbers of induced revertants on positive control plates for this laboratory are presented in Appendix 5. The ranges quoted are based on a large volume of historical control data accumulated from experiments where the correct strain and assay functioning were considered to have been confirmed.

For evaluation of data there are many statistical methods in use, and several are acceptable (7,8). The m-statistic was calculated to check that the data were Poisson-distributed (8), and Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis (8).

Acceptance criteria

The assay was considered valid if the following criteria were met:

1. the mean negative control counts fell within the normal ranges as defined in Appendix 4 (for any exceptions refer to Appendix 7)

2. the positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S-9 preparation

3. no more than 5% of the plates were lost through contamination or some other unforeseen event.

Evaluation criteria

The test article was considered to be mutagenic if:

1. the assay was valid (see above)

2. Dunnett's test gave a significant response ($p \leq 0.01$) and the data set(s) showed a significant dose correlation

3. the positive responses described above were reproducible.

RESULTS

Toxicity, solubility and dose selection

Details of all treatment solution concentrations and final [redacted] doses are provided in the Test article section.

An initial toxicity Range-Finder Experiment was carried out in strain TA100 only, in the absence and presence of S-9, using final concentrations of [redacted] at 1.6, 8, 40, 200, 1000 and 5000 µg/plate, plus solvent and positive controls. Evidence of toxicity in the form of a marked decrease in revertant numbers (but no concurrent diminution in the background bacterial lawn) was observed following the top two dose treatments in the absence of S-9 and following the top dose treatment in the presence of S-9. This may be indicative of bacteriostasis.

Experiment 1 treatments of all the test strains in the presence of S-9 retained the same test doses as employed for the Range-Finder Experiment. Treatments of all test strains in the absence of S-9 were performed up to a maximum dose of 1000 µg/plate (an estimate of the lower limit of toxicity) using doses of 0.32, 1.6, 8, 40, 200 and 1000 µg/plate. Evidence of toxicity in the form of a marked decrease in revertant numbers (but no concurrent diminution in the background bacterial lawn, except for strain TA102 treatments in the absence of S-9) was observed following the top dose treatment in all strains in the absence and presence of S-9. This may be indicative of bacteriostasis.

Experiment 2 treatments of all strains in the absence of S-9 were performed up to 1000 µg/plate (an estimate of the lower limit of toxicity) using doses of 1.638, 4.096, 10.24, 25.6, 64, 160, 400 and 1000 µg/plate. Treatments of all strains in the presence of S-9 were performed up to 5000 µg/plate using doses of 8.192, 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate. Narrowed dose ranges were employed in order to examine more closely those concentrations of [redacted] approaching the maximum test dose and therefore considered most likely to provide evidence of any mutagenic activity. In addition, pre-incubation methodology was used for all treatments in the absence and presence of S-9. In the absence of S-9, evidence of toxicity in the form of a slight thinning of the background bacterial lawn and in most cases a marked decrease in revertant numbers was observed following the top dose treatment of strain TA98, TA1535, TA1537 and TA102, and the top two dose treatments of strain TA100. Evidence of toxicity in the form of a slight thinning of the

background bacterial lawn and/or a marked decrease in revertant numbers was observed following the top dose treatment of all strains in the presence of S-9, with the exception of strain TA98 where no clear evidence of toxicity was seen.

The test article was completely soluble in the aqueous assay system at all concentrations treated, in each of the experiments performed.

Mutation

The individual plate counts were averaged to give mean values, which are presented in Appendix 2 and Appendix 3. From the data it can be seen that mean solvent control counts fell within the normal historical ranges (Appendix 4), with any exceptions detailed in Appendix 7. The positive control chemicals all induced large increases in revertant numbers in the appropriate strains, which fell within or above the normal historical ranges (Appendix 5). Less than 5% of plates were lost, leaving adequate numbers of plates at all treatments. The study therefore demonstrated correct strain and assay functioning and was accepted as valid.

The mutation data were evaluated as follows:

No statistically significant, dose-related and reproducible increases in revertant numbers were observed following any strain treatments in the absence or presence of metabolic activation, and therefore this study was considered to have provided no evidence of any [redacted] mutagenic activity.

CONCLUSION

It was concluded that [redacted] did not induce mutation in five histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) when tested under the conditions of this study. These conditions included treatments at concentrations up to either 5000 µg/plate, or the lower limit of toxicity, in the absence and in the presence of a rat liver metabolic activation system (S-9).

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APPENDICES

Appendix 1
raw plate counts and toxicity data
Range-Finder Experiment

Summary of mean revertant colonies - Range-Finder Experiment

Substance	Dose Level µg/plate	TA100 (-S-9)	TA100 (+S-9)
		Mean ± SD	Mean ± SD
Solvent - Water	100 µl	131 ± 4	134 ± 9
	1.6	134 ± 9	145 ± 15
	8	142 ± 12	131 ± 14
	40	138 ± 6	135 ± 13
	200	150 ± 28	130 ± 4
	1000	23 ± 17	128 ± 19
	5000	0 ± 0	33 ± 12
Positive controls	Compound	NaN_3	AAN
	Dose Level	2 µg	5 µg
	Mean ± SD	662 ± 10	1319 ± 137

SD Standard deviation

NaN_3 Sodium azide
AAN 2-Aminoanthracene

[redacted] Range-Finder Experiment

Table 1

Test strain: TA100 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	131	136	126	135	129
1.6	124	137	140		
8	128	152	145		
40	131	143	139		
200	119	174	158		
1000	23	39	6		
5000	0	0	0		
Positive	653	672	662		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	131.40	5		4.16					
1.6	133.67	3	1.02	8.50	0.21	NS	1.42	0.16	NS
8	141.67	3	1.08	12.34	0.53	*	1.28	0.71	NS
40	137.67	3	1.05	6.11	0.25	NS	0.12	0.44	NS
200	150.33	3	1.14^	28.29	0.45	*	0.08	1.25	NS
1000	22.67	3	0.17	16.50	0.91	NS	-0.11	-11.39	NS
5000	0.00	3	0.00	0.00	0.82	NS	-0.03	-18.75	NS
Positive	662.33	3	5.04	9.50					
	M Statistic = 2.784								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

^ Maximum increase above control

[redacted]

Range-Finder Experiment

Table 2

Test strain: TA100 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	124	143	136	143	126
1.6	157	150	128		
8	142	116	136		
40	150	128	128		
200	130	126	134		
1000	119	150	115		
5000	20	44	34		
Positive	1171	1441	1346		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	134.40	5		9.07					
1.6	145.00	3	1.08^	15.13	0.46	NS	6.63	0.95	NS
8	131.33	3	0.98	13.61	0.19	NS	-0.66	-0.30	NS
40	135.33	3	1.01	12.70	0.07	NS	-0.05	0.08	NS
200	130.00	3	0.97	4.00	0.23	NS	-0.03	-0.40	NS
1000	128.00	3	0.95	19.16	0.25	NS	-0.01	-0.63	NS
5000	32.67	3	0.24	12.06	0.94	NS	-0.02	-12.70	NS
Positive	1319.33	3	9.82	136.96					
	M Statistic = 1.606								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

^ Maximum increase above control

Appendix 2
[redacted]raw plate counts and calculated mutagenicity data
Experiment 1

[redacted]Summary of mean revertant colonies (-S-9) - Experiment 1

Substance	Dose Level µg/plate	TA98	TA100	TA1535	TA1537	TA102
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Solvent - Water	100 µl	22 ± 4	141 ± 8	14 ± 4	10 ± 2	265 ± 11
[redacted]	0.32	26 ± 2	139 ± 4	17 ± 3	11 ± 4	252 ± 30
	1.6	22 ± 4	141 ± 3	13 ± 4	12 ± 6	290 ± 12
	8	18 ± 4	145 ± 28	19 ± 7	11 ± 3	286 ± 4
	40	21 ± 1	131 ± 9	17 ± 3	13 ± 1	302 ± 20
	200	27 ± 7	129 ± 8	18 ± 2 (M)	15 ± 5	252 ± 44
	1000	1 ± 1 (M)	1 ± 1	3 ± 3	0 ± 0 (M)	3 ± 4 (S)
Solvent - DMSO	100 µl	23 ± 3	NT	NT	11 ± 4	NT
Positive controls	Compound	2NF	NaN_3	NaN_3	AAC	GLU
	Dose Level	5 µg	2 µg	2 µg	50 µg	25 µg
	Mean ± SD	788 ± 104	742 ± 25	598 ± 25	231 ± 24	823 ± 16

SD Standard deviation

2NF 2-Nitrofluorene
NaN_3 Sodium azide
AAC 9-Aminoacridine
GLU Glutaraldehyde

S Slight thinning of background lawn
M Plates counted manually
NT Plates not treated

[redacted] Summary of mean revertant colonies (+S-9) - Experiment 1

Substance	Dose Level µg/plate	TA98	TA100	TA1535	TA1537	TA102
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Solvent - Water	100 µl	28 ± 5	119 ± 13	18 ± 5	15 ± 2	315 ± 23
[redacted]	1.6	28 ± 9	141 ± 3	24 ± 5	13 ± 5	271 ± 23
	8	26 ± 3	133 ± 8	18 ± 3	17 ± 3	289 ± 9
	40	28 ± 8	128 ± 19	23 ± 7	15 ± 3	307 ± 12
	200	30 ± 7	127 ± 12	20 ± 12	11 ± 4	324 ± 4
	1000	24 ± 6	145 ± 17	23 ± 6	8 ± 5	225 ± 42
	5000	14 ± 2	9 ± 5	3 ± 2	2 ± 2	69 ± 11
Solvent - DMSO	100 µl	29 ± 6	133 ± 16	23 ± 7	14 ± 5	317 ± 12
Positive controls	Compound	B[a]P	AAN	AAN	AAN	AAN
	Dose Level	10 µg	5 µg	5 µg	5 µg	20 µg
	Mean ± SD	190 ± 11	1445 ± 31	289 ± 35	334 ± 25	1854 ± 106

SD Standard deviation

B[a]P Benzo[a]pyrene
AAN 2-Aminoanthracene

[redacted] Experiment 1

Table 3

Test strain: TA98 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	23	27	20	16	22
0.32	28	26	24		
1.6	25	18	24		
8	16	15	23		
40	21	20	21		
200	20	28	33		
1000	0 M	1 M	2 M		
Solvent - DMSO	20	26	24	26	21
Positive	718	908	739		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	21.60	5		4.04					
0.32	26.00	3	1.20	2.00	0.58	NS	13.75	1.35	NS
1.6	22.33	3	1.03	3.79	0.01	NS	-0.06	0.24	NS
8	18.00	3	0.83	4.36	0.49	NS	-0.65	-1.18	NS
40	20.67	3	0.96	0.58	0.22	NS	-0.06	-0.25	NS
200	27.00	3	1.25^	6.56	0.38	*	0.02	1.56	NS
1000	1.00	3	0.05	1.00	0.81	NS	-0.02	-11.07	NS
Solvent - DMSO	23.40	5		2.79					
Positive	788.33	3	33.69	104.16					
	M Statistic = 0.746								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

M Plate counted manually

^ Maximum increase above control

[redacted] Experiment 1

Table 4

Test strain: TA98 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	37	25	26	25	25
1.6	39	25	21		
8	25	24	30		
40	19	33	33		
200	23	30	37		
1000	26	17	28		
5000	16	12	13		
Solvent - DMSO	26	31	37	28	22
Positive	185	182	202		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	27.60	5		5.27					
1.6	28.33	3	1.03	9.45	0.06	NS	0.46	0.09	NS
8	26.33	3	0.95	3.21	0.11	NS	-0.18	-0.26	NS
40	28.33	3	1.03	8.08	0.04	NS	0.02	0.11	NS
200	30.00	3	1.09^	7.00	0.16	NS	0.01	0.50	NS
1000	23.67	3	0.86	5.86	0.24	NS	-0.00	-0.92	NS
5000	13.67	3	0.50	2.08	0.67	NS	-0.00	-3.57	NS
Solvent - DMSO	28.80	5		5.63					
Positive	189.67	3	6.59	10.79					
	M Statistic = 1.408								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

^ Maximum increase above control

[redacted] Experiment 1

Table 5

Test strain: TA100 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	145	153	134	135	140
0.32	137	143	136		
1.6	138	141	144		
8	127	131	178		
40	141	125	128		
200	124	124	138		
1000	0	2	1		
Positive	746	716	765		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	141.40	5		7.83					
0.32	138.67	3	0.98	3.79	0.22	NS	-8.54	-0.28	NS
1.6	141.00	3	1.00	3.00	0.01	NS	0.08	-0.03	NS
8	145.33	3	1.03^	28.36	0.16	NS	0.62	0.33	NS
40	131.33	3	0.93	8.50	0.30	NS	-0.24	-1.09	NS
200	128.67	3	0.91	8.08	0.38	NS	-0.06	-1.38	NS
1000	1.00	3	0.01	1.00	0.97	NS	-0.14	-27.91	NS
Positive	742.33	3	5.25	24.70					
	M Statistic = 1.078								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

^ Maximum increase above control

[redacted] Experiment 1

Table 6

Test strain: TA100 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	137	118	102	123	117
1.6	143	138	143		
8	138	123	137 M		
40	106	140	138		
200	118	123	141		
1000	127	149	160		
5000	14	8	5		
Solvent - DMSO	106	133	141	142	144
Positive	1436	1419	1480		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	119.40	5		12.58					
1.6	141.33	3	1.18	2.89	0.76	*	13.71	2.21	NS
8	132.67	3	1.11	8.39	0.32	NS	1.18	1.36	NS
40	128.00	3	1.07	19.08	0.02	NS	0.02	0.85	NS
200	127.33	3	1.07	12.10	0.04	NS	-0.01	0.82	NS
1000	145.33	3	1.22^	16.80	0.41	*	0.02	2.56	*
5000	9.00	3	0.08	4.58	0.91	NS	-0.02	-18.10	NS
Solvent - DMSO	133.20	5		15.77					
Positive	1445.00	3	10.85	31.48					
	M Statistic = 1.439								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

M Plate counted manually

^ Maximum increase above control

[redacted] Experiment 1

Table 7

Test strain: TA1535 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	13	11	12	20	13
0.32	15	20	17		
1.6	17	13	10		
8	11	25	22		
40	14	19	17		
200	16 M	20 M	17 M		
1000	6	1	3		
Positive	574	623	598		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	13.80	5		3.56					
0.32	17.33	3	1.26	2.52	0.52	NS	11.04	1.21	NS
1.6	13.33	3	0.97	3.51	0.15	NS	-0.74	-0.16	NS
8	19.33	3	1.40^	7.37	0.40	NS	0.58	1.67	NS
40	16.67	3	1.21	2.52	0.17	NS	0.05	0.99	NS
200	17.67	3	1.28	2.08	0.20	NS	0.01	1.32	NS
1000	3.33	3	0.24	2.52	0.71	NS	-0.01	-5.12	NS
Positive	598.33	3	43.36	24.50					
	M Statistic = 1.058								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

M Plate counted manually

^ Maximum increase above control

[redacted] Experiment 1

Table 8

Test strain: TA1535 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	14	22	12	19 M	23
1.6	26	28	19		
8	21	17	15		
40	24	15	29		
200	7	26	28		
1000	28	16	24		
5000	4	1 M	3		
Solvent - DMSO	31	27	21	22	12
Positive	254	289	323		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	18.00	5		4.85					
1.6	24.33	3	1.35^	4.73	0.59	NS	3.96	1.28	NS
8	17.67	3	0.98	3.06	0.14	NS	-0.20	-0.03	NS
40	22.67	3	1.26	7.09	0.22	NS	0.07	0.93	NS
200	20.33	3	1.13	11.59	0.03	NS	0.00	0.25	NS
1000	22.67	3	1.26	6.11	0.14	NS	0.00	0.95	NS
5000	2.67	3	0.15	1.53	0.70	NS	-0.00	-4.79	NS
Solvent - DMSO	22.60	5		7.16					
Positive	288.67	3	12.77	34.50					
	M Statistic = 1.926								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

M Plate counted manually

^ Maximum increase above control

[redacted] Experiment 1

Table 9

Test strain: TA1537 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	10	12	7	9	12
0.32	8	11	15		
1.6	7	18	12		
8	14	11	9		
40	12	13	13		
200	19	9	17		
1000	0 M	0 M	0 M		
Solvent - DMSO	11	6	13	8	15
Positive	213	258	221		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	10.00	5		2.12					
0.32	11.33	3	1.13	3.51	0.27	NS	4.17	0.55	NS
1.6	12.33	3	1.23	5.51	0.28	NS	1.35	0.87	NS
8	11.33	3	1.13	2.52	0.10	NS	0.09	0.59	NS
40	12.67	3	1.27	0.58	0.23	NS	0.04	1.18	NS
200	15.00	3	1.50^	5.29	0.42	*	0.02	1.95	NS
1000	0.00	3	0.00	0.00	0.72	NS	-0.01	-9.03	NS
Solvent - DMSO	10.60	5		3.65					
Positive	230.67	3	21.76	24.01					
	M Statistic = 0.986								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

M Plate counted manually

^ Maximum increase above control

[redacted] Experiment 1

Table 10

Test strain: TA1537 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	17	13	17	13	17
1.6	9	13	18		
8	13	19	19		
40	18	13	13		
200	13	13	6		
1000	2	11	10		
5000	0 M	3	2		
Solvent - DMSO	17	5	18	14	17
Positive	333	310	360		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	15.40	5		2.19					
1.6	13.33	3	0.87	4.51	0.34	NS	-1.29	-0.64	NS
8	17.00	3	1.10^	3.46	0.28	NS	0.26	0.41	NS
40	14.67	3	0.95	2.89	0.03	NS	-0.01	-0.21	NS
200	10.67	3	0.69	4.04	0.49	NS	-0.02	-1.49	NS
1000	7.67	3	0.50	4.93	0.62	NS	-0.01	-2.75	NS
5000	1.67	3	0.11	1.53	0.77	NS	-0.00	-6.13	NS
Solvent - DMSO	14.20	5		5.36					
Positive	334.33	3	23.54	25.03					
	M Statistic = 1.191								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

M Plate counted manually

^ Maximum increase above control

[redacted] Experiment 1

Table 11

Test strain: TA102 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	274	270	246	262	272
0.32	244	227	285		
1.6	276	299	295		
8	285	290	282		
40	301	282	322		
200	271	283	201		
1000	1 S	8 S	1 S		
Positive	815	813	842		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	264.80	5		11.45					
0.32	252.00	3	0.95	29.82	0.34	NS	-40.00	-0.73	NS
1.6	290.00	3	1.10	12.29	0.57	*	17.94	1.34	NS
8	285.67	3	1.08	4.04	0.45	NS	2.91	1.12	NS
40	301.67	3	1.14^	20.01	0.56	**	0.86	1.94	NS
200	251.67	3	0.95	44.29	0.25	NS	-0.10	-0.80	NS
1000	3.33	3	0.01	4.04	0.96	NS	-0.27	-26.00	NS
Positive	823.33	3	5.84	16.20					
	M Statistic = 2.390								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

S Slight thinning of background lawn

^ Maximum increase above control

[redacted] Experiment 1

Table 12

Test strain: TA102 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	340	321	302	329	281
1.6	248	271	293		
8	285	300	283		
40	299	321	300		
200	327	326	319		
1000	217	271	188		
5000	73	78	57		
Solvent - DMSO	320	298	320	331	318
Positive	1828	1971	1764		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	314.60	5		23.35					
1.6	270.67	3	0.86	22.50	0.73	NS	-27.46	-2.50	NS
8	289.33	3	0.92	9.29	0.29	NS	-2.20	-1.40	NS
40	306.67	3	0.97	12.42	0.13	NS	0.20	-0.42	NS
200	324.00	3	1.03^	4.36	0.43	*	0.14	0.53	NS
1000	225.33	3	0.72	42.12	0.67	NS	-0.07	-5.38	NS
5000	69.33	3	0.22	10.97	0.94	NS	-0.05	-18.35	NS
Solvent - DMSO	317.40	5		11.99					
Positive	1854.33	3	5.84	105.98					
	M Statistic = 1.976								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

^ Maximum increase above control

Appendix 3
[redacted] raw plate counts and calculated mutagenicity data
Experiment 2

[redacted] Summary of mean revertant colonies (-S-9) - Experiment 2

Substance	Dose Level µg/plate	TA98	TA100	TA1535	TA1537	TA102
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Solvent - Water	100 µl	25 ± 6	149 ± 7	20 ± 2	10 ± 4	435 ± 20
[redacted]	1.638	32 ± 7	171 ± 15	16 ± 5	10 ± 4	382 ± 41
	4.096	26 ± 2	167 ± 9	25 ± 1	17 ± 1	389 ± 29
	10.24	20 ± 8	157 ± 32	18 ± 3	14 ± 10	525 ± 69
	25.6	26 ± 8	156 ± 54	19 ± 5	13 ± 6	455 ± 21
	64	29 ± 16	144 ± 25	14 ± 5	12 ± 3	342 ± 97
	160	16 ± 5	146 ± 46	22 ± 7	11 ± 4	358 ± 107
	400	27 ± 8	117 ± 38 (S)	14 ± 2	9 ± 6	370 ± 16
	1000	4 ± 3 (S)	28 ± 10 (S)	3 ± 2 (S)	1 ± 1 (S)	34 ± 27 (S)
Solvent - DMSO	50 µl	23 ± 5	NT	NT	12 ± 5	NT
Positive controls	Compound	2NF	NaN_3	NaN_3	AAC	GLU
	Dose Level	5 µg	2 µg	2 µg	50 µg	25 µg
	Mean ± SD	1691 ± 443	669 ± 48	679 ± 41	244 ± 50	645 ± 76

SD Standard deviation

2NF 2-Nitrofluorene
NaN_3 Sodium azide
AAC 9-Aminoacridine
GLU Glutaraldehyde

S Slight thinning of background lawn
NT Plates not treated

[redacted]Summary of mean revertant colonies (+S-9) - Experiment 2

Substance	Dose Level µg/plate	TA98	TA100	TA1535	TA1537	TA102
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Solvent - Water	100 µl	32 ± 6	163 ± 12	24 ± 8	18 ± 2	435 ± 33
[redacted]	8.192	29 ± 3	132 ± 19	22 ± 14	19 ± 1	311 ± 40
	20.48	30 ± 3	154 ± 13	22 ± 6	10 ± 6	343 ± 13
	51.2	23 ± 3	151 ± 9	18 ± 2	14 ± 4	341 ± 25
	128	31 ± 8	177 ± 9	17 ± 2	12 ± 4	358 ± 41
	320	22 ± 6	159 ± 20	24 ± 6	9 ± 4	328 ± 6
	800	29 ± 6	169 ± 7	19 ± 8	13 ± 4	327 ± 34
	2000	27 ± 5	155 ± 36	22 ± 5	13 ± 2	239 ± 40
	5000	22 ± 7	56 ± 12 (S)	13 ± 3	2 ± 1	99 ± 44 (S)
Solvent - DMSO	50 µl	25 ± 9	141 ± 21	21 ± 5	14 ± 4	326 ± 67
Positive controls	Compound	B[a]P	AAN	AAN	AAN	AAN
	Dose Level	10 µg	5 µg	5 µg	5 µg	20 µg
	Mean ± SD	295 ± 41	787 ± 13	83 ± 7	115 ± 4	1403 ± 79

SD Standard deviation

B[a]P Benzo[a]pyrene
AAN 2-Aminoanthracene

S Slight thinning of background lawn

[redacted] Experiment 2

Table 13

Test strain: TA98 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	19	22	19	31	32
1.638	31	40	26		
4.096	26	28	25		
10.24	25	10	24		
25.6	30	17	30		
64	13	28	45		
160	15	11	21		
400	36	22	23		
1000	6 S	5 S	1 S		
Solvent - DMSO	22	22	31	19	21
Positive	1654	2151	1268		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	24.60	5		6.43					
1.638	32.33	3	1.31^	7.09	0.54	NS	4.72	1.22	NS
4.096	26.33	3	1.07	1.53	0.14	NS	0.47	0.34	NS
10.24	19.67	3	0.80	8.39	0.37	NS	-0.66	-0.95	NS
25.6	25.67	3	1.04	7.51	0.13	NS	-0.09	0.16	NS
64	28.67	3	1.17	16.01	0.09	NS	0.03	0.45	NS
160	15.67	3	0.64	5.03	0.35	NS	-0.06	-1.66	NS
400	27.00	3	1.10	7.81	0.05	NS	-0.00	0.39	NS
1000	4.00	3	0.16	2.65	0.59	NS	-0.02	-5.01	NS
Solvent - DMSO	23.00	5		4.64					
Positive	1691.00	3	73.52	442.66					
	M Statistic = 2.534								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:
S Slight thinning of background lawn
^ Maximum increase above control

[redacted] Experiment 2

Table 14

Test strain: TA98 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	37	24	34	26	38
8.192	32	29	27		
20.48	33	28	28		
51.2	19	25	25		
128	29	39	24		
320	26	15	25		
800	22	33	31		
2000	25	33	24		
5000	29	22	15		
Solvent - DMSO	13	27	19	30	34
Positive	342	278	266		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	31.80	5		6.42					
8.192	29.33	3	0.92	2.52	0.25	NS	-0.30	-0.51	NS
20.48	29.67	3	0.93	2.89	0.21	NS	-0.11	-0.43	NS
51.2	23.00	3	0.72	3.46	0.62	NS	-0.16	-2.08	NS
128	30.67	3	0.96	7.64	0.09	NS	-0.01	-0.27	NS
320	22.00	3	0.69	6.08	0.43	NS	-0.02	-2.41	NS
800	28.67	3	0.90	5.86	0.13	NS	-0.00	-0.71	NS
2000	27.33	3	0.86	4.93	0.10	NS	-0.00	-1.01	NS
5000	22.00	3	0.69	7.00	0.32	NS	-0.00	-2.43	NS
Solvent - DMSO	24.60	5		8.50					
Positive	295.33	3	12.01	40.86					
	M Statistic = 1.151								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

[redacted] Experiment 2

Table 15

Test strain: TA100 -S-9

Treatment (µg/plate)	Revertant numbers/plate					
Solvent - Water	146	160	152	143 M	143	
1.638	165	188	159			
4.096	166	176	159			
10.24	168	183	121			
25.6	186	94	188			
64	172	124	135			
160	152	189	97			
400	74 S	144 S	134 S			
1000	18 S	38 S	29 S			
Positive	618	676	714			

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent - Water	148.80	5		7.26			
1.638	170.67	3	1.15^	15.31	0.75 *	13.35	0.90 NS
4.096	167.00	3	1.12	8.54	0.58 *	4.55	0.76 NS
10.24	157.33	3	1.06	32.35	0.10 NS	0.45	0.32 NS
25.6	156.00	3	1.05	53.70	0.02 NS	-0.06	0.16 NS
64	143.67	3	0.97	25.15	0.21 NS	-0.23	-0.25 NS
160	146.00	3	0.98	46.29	0.19 NS	-0.10	-0.23 NS
400	117.33	3	0.79	37.86	0.44 NS	-0.10	-1.54 NS
1000	28.33	3	0.19	10.02	0.84 NS	-0.13	-7.28 NS
Positive	669.33	3	4.50	48.35			
	M Statistic = 6.249						

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

S Slight thinning of background lawn
M Plate counted manually
^ Maximum increase above control

[redacted] Experiment 2

Table 16

Test strain: TA100 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	155	181	164	165	149
8.192	131	113	151		
20.48	163 M	159	139		
51.2	160	150	143		
128	179	185	167		
320	181	143	153		
800	171	162	175		
2000	179	113	172		
5000	59 S	43 S	66 S		
Solvent - DMSO	159	114	Cont	135	156
Positive	777	782	802		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	162.80	5		12.13					
8.192	131.67	3	0.81	19.01	0.76	NS	-3.80	-2.45	NS
20.48	153.67	3	0.94	12.86	0.23	NS	-0.48	-0.69	NS
51.2	151.00	3	0.93	8.54	0.11	NS	-0.09	-0.88	NS
128	177.00	3	1.09^	9.17	0.47	*	0.18	1.04	NS
320	159.00	3	0.98	19.70	0.22	NS	0.04	-0.30	NS
800	169.33	3	1.04	6.66	0.32	NS	0.02	0.49	NS
2000	154.67	3	0.95	36.25	0.05	NS	0.00	-0.71	NS
5000	56.00	3	0.34	11.79	0.78	NS	-0.02	-10.00	NS
Solvent - DMSO	141.00	4		20.93					
Positive	787.00	3	5.58	13.23					
	M Statistic = 2.027								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

S Slight thinning of background lawn
M Plate counted manually
Cont Contaminated plate
^ Maximum increase above control

[redacted] Experiment 2

Table 17

Test strain: TA1535 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	20	21	22	19	16
1.638	16	12	21		
4.096	26 M	24	26		
10.24	16	16	22		
25.6	18	24	14		
64	9	13	19		
160	14	26	27		
400	13	17	13		
1000	5 S	1 S	2 M+S		
Positive	719	681	638		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	19.60	5		2.30					
1.638	16.33	3	0.83	4.51	0.49	NS	-1.99	-1.09	NS
4.096	25.33	3	1.29^	1.15	0.55	*	1.36	1.65	NS
10.24	18.00	3	0.92	3.46	0.02	NS	-0.02	-0.51	NS
25.6	18.67	3	0.95	5.03	0.10	NS	-0.05	-0.34	NS
64	13.67	3	0.70	5.03	0.46	NS	-0.10	-2.07	NS
160	22.33	3	1.14	7.23	0.07	NS	0.01	0.70	NS
400	14.33	3	0.73	2.31	0.26	NS	-0.01	-1.74	NS
1000	2.67	3	0.14	2.08	0.73	NS	-0.02	-7.75	NS
Positive	679.33	3	34.66	40.53					
	M Statistic = 1.006								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

S Slight thinning of background lawn
M Plate counted manually
^ Maximum increase above control

[redacted] Experiment 2

Table 18

Test strain: TA1535 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	23	30	33	18	15
8.192	38	15	14		
20.48	23	28 M	16		
51.2	19	15	19		
128	18	15	17		
320	31	20	21		
800	18	12	27		
2000	17	26	22		
5000	17	11	12		
Solvent - DMSO	16	17	22	24	27
Positive	75	89	85		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	23.80	5		7.66					
8.192	22.33	3	0.94	13.58	0.08	NS	-0.18	-0.44	NS
20.48	22.33	3	0.94	6.03	0.08	NS	-0.07	-0.25	NS
51.2	17.67	3	0.74	2.31	0.30	NS	-0.12	-1.20	NS
128	16.67	3	0.70	1.53	0.37	NS	-0.06	-1.42	NS
320	24.00	3	1.01^	6.08	0.01	NS	0.00	0.09	NS
800	19.00	3	0.80	7.55	0.11	NS	-0.00	-1.00	NS
2000	21.67	3	0.91	4.51	0.01	NS	-0.00	-0.36	NS
5000	13.33	3	0.56	3.21	0.33	NS	-0.00	-2.26	NS
Solvent - DMSO	21.20	5		4.66					
Positive	83.00	3	3.92	7.21					
	M Statistic = 2.151								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:
M Plate counted manually
^ Maximum increase above control

[redacted] Experiment 2

Table 19

Test strain: TA1537 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	8	14	15	6	6
1.638	6	13	12		
4.096	16	16	18		
10.24	3	16	22		
25.6	6	18	15		
64	14	14	8		
160	10	15	8		
400	3	15	8		
1000	2 S	1 S	1 S		
Solvent - DMSO	14	6	7	14	18
Positive	188	260	284		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	9.80	5		4.38					
1.638	10.33	3	1.05	3.79	0.07	NS	0.33	0.18	NS
4.096	16.67	3	1.70^	1.15	0.65	*	1.66	1.73	NS
10.24	13.67	3	1.39	9.71	0.31	NS	0.43	0.69	NS
25.6	13.00	3	1.33	6.24	0.16	NS	0.09	0.77	NS
64	12.00	3	1.22	3.46	0.04	NS	0.01	0.63	NS
160	11.00	3	1.12	3.61	0.07	NS	-0.01	0.37	NS
400	8.67	3	0.88	6.03	0.23	NS	-0.01	-0.44	NS
1000	1.33	3	0.14	0.58	0.59	NS	-0.01	-3.29	NS
Solvent - DMSO	11.80	5		5.12					
Positive	244.00	3	20.68	49.96					
	M Statistic = 2.191								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:
S Slight thinning of background lawn
^ Maximum increase above control

[redacted] Experiment 2

Table 20

Test strain: TA1537 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	20	14	17	19	18
8.192	19	18	20		
20.48	7	17	5		
51.2	18	11	13		
128	10	17	9		
320	6	8	14		
800	12	10	18		
2000	14	15	11		
5000	2	3	1		
Solvent - DMSO	14	16	19	11	10
Positive	115	119	112		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	17.60	5		2.30					
8.192	19.00	3	1.08^	1.00	0.37	NS	0.17	0.45	NS
20.48	9.67	3	0.55	6.43	0.66	NS	-0.38	-3.11	NS
51.2	14.00	3	0.80	3.61	0.39	NS	-0.09	-1.22	NS
128	12.00	3	0.68	4.36	0.39	NS	-0.04	-1.99	NS
320	9.33	3	0.53	4.16	0.50	NS	-0.02	-3.10	NS
800	13.33	3	0.76	4.16	0.23	NS	-0.00	-1.48	NS
2000	13.33	3	0.76	2.08	0.13	NS	-0.00	-1.43	NS
5000	2.00	3	0.11	1.00	0.63	NS	-0.00	-7.37	NS
Solvent - DMSO	14.00	5		3.67					
Positive	115.33	3	8.24	3.51					
	M Statistic = 1.142								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

^ Maximum increase above control

[redacted] Experiment 2

Table 21

Test strain: TA102 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	435	459	451	415	417
1.638	428	348	370		
4.096	358	414	396		
10.24	457	594	523		
25.6	480	442	444		
64	314	450	261		
160	371	246	458		
400	351	381	377		
1000	19 S	17 S	65 S		
Positive	610	732	593		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	435.40	5		19.72					
1.638	382.00	3	0.88	41.33	0.72	NS	-32.60	-1.16	NS
4.096	389.33	3	0.89	28.59	0.56	NS	-11.49	-0.99	NS
10.24	524.67	3	1.21^	68.52	0.60	*	9.66	1.75	NS
25.6	455.33	3	1.05	21.39	0.36	NS	2.27	0.41	NS
64	341.67	3	0.78	97.49	0.34	NS	-1.10	-2.18	NS
160	358.33	3	0.82	106.57	0.38	NS	-0.55	-1.81	NS
400	369.67	3	0.85	16.29	0.32	NS	-0.19	-1.43	NS
1000	33.67	3	0.08	27.15	0.85	NS	-0.37	-13.38	NS
Positive	645.00	3	1.48	75.82					
	M Statistic = 10.044								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:

S Slight thinning of background lawn
^ Maximum increase above control

[redacted] Experiment 2

Table 22

Test strain: TA102 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent - Water	466	383	454	421	449
8.192	347	268	317		
20.48	334	357	337		
51.2	358	312	354		
128	402	321	351		
320	334	324	325		
800	307	367	308		
2000	277	198	243		
5000	108 S	52 S	138 S		
Solvent - DMSO	294	326	440	267	301
Positive	1408	1480	1322		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit	Dunnett's t value	
Solvent - Water	434.60	5		33.23					
8.192	310.67	3	0.71	39.88	0.89	NS	-15.13	-3.98	NS
20.48	342.67	3	0.79	12.50	0.64	NS	-4.61	-2.86	NS
51.2	341.33	3	0.79	25.48	0.46	NS	-1.37	-2.92	NS
128	358.00	3	0.82	40.95	0.24	NS	-0.28	-2.38	NS
320	327.67	3	0.75	5.51	0.34	NS	-0.16	-3.37	NS
800	327.33	3	0.75	34.36	0.33	NS	-0.06	-3.40	NS
2000	239.33	3	0.55	39.63	0.66	NS	-0.06	-6.65	NS
5000	99.33	3	0.23	43.65	0.88	NS	-0.05	-13.61	NS
Solvent - DMSO	325.60	5		67.31					
Positive	1403.33	3	4.31	79.10					
	M Statistic = 4.668								

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS not significant

Key to postfixes:
S Slight thinning of background lawn

Appendix 4
Historical negative (solvent) control values for *S. typhimurium* strains

Strain	S-9	N	Mean revertant numbers	Standard Deviation	Range*	
					Lower	Upper
TA98	-	30	21.8	4.4	14.8 (10.5)	34.6 (33.1)
	+	30	28.5	5.8	20.2 (13.7)	39.6 (43.3)
TA100	-	30	114.6	12.8	86.2 (81.6)	141.6 (147.6)
	+	30	117.5	18.0	88.2 (71.1)	157.8 (163.9)
TA1535	-	30	17.6	3.0	13.4 (9.8)	23.8 (25.4)
	+	30	20.3	4.5	10.0 (8.7)	32.2 (31.8)
TA1537	-	30	9.8	2.5	5.6 (3.4)	17.0 (16.3)
	+	30	12.0	3.1	7.4 (4.1)	20.6 (19.9)
TA102	-	22	276.6	66.3	212.0 (105.9)	420.6 (447.3)
	+	24	286.3	77.2	209.6 (87.5)	461.5 (485.1)

* Ranges stated are the maximum and minimum mean revertant counts from the data sets sampled, together with the 99% percentile values given in parenthesis.

The above ranges are derived from the number of data sets indicated, selected without bias over the period 23 September 2002 to 25 November 2002.

Appendix 5
Historical positive control values for *S. typhimurium* strains

Strain	S-9	Chemical	N	Mean revertant numbers	Standard Deviation	Range*	
						Lower	Upper
TA98	-	2NF	30	953.9	256.3	546.2 (293.7)	1469.2 (1614.0)
	+	B[a]P	30	270.9	57.5	149.3 (122.7)	383.1 (419.0)
TA100	-	NaN_3	30	482.0	102.4	201.7 (218.3)	637.8 (745.7)
	+	AAN	30	1024.6	425.9	327.5 -(72.6)	2147.8 (2121.9)
TA1535	-	NaN_3	30	454.7	72.0	220.9 (269.3)	605.3 (640.0)
	+	AAN	30	148.6	59.9	43.7 -(5.8)	330.6 (303.0)
TA1537	-	AAC	30	161.1	63.9	54.1 -(3.4)	313.1 (325.7)
	+	AAN	30	185.5	101.8	48.1 -(76.7)	503.2 (447.6)
TA102	-	GLU	22	279.9	90.0	144.0 (48.1)	548.9 (511.6)
	+	AAN	24	1011.0	272.5	527.1 (309.0)	1497.1 (1712.9)

* Ranges stated are the maximum and minimum mean induced revertant counts from the data sets sampled, together with the 99% percentile values given in parenthesis.

2NF 2-Nitrofluorene
NaN_3 Sodium azide
AAC 9-Aminoacridine
GLU Glutaraldehyde
B[a]P Benzo[a]pyrene
AAN 2-Aminoanthracene

The above ranges are derived from the number of data sets indicated, selected without bias over the period 23 September 2002 to 25 November 2002.

All revertant numbers used to derive the above data are induced revertant numbers, i.e. the number of revertant colonies on the positive control plates minus the number of spontaneous revertant colonies on concurrent negative control plates.

Appendix 6
Quality control statement for S-9

MOLTOX™ POST MITOCHONDRIAL SUPERNATANT (S-9) QUALITY CONTROL & PRODUCTION CERTIFICATE

LOT NO.: 1619
PART NO.: 11-101
VOLUME: 5ml
SPECIES: Rat
STRAIN: Sprague Dawley
SEX: Male
TISSUE: Liver
PREPARATION DATE: October 21, 2003
EXPIRATION DATE: October 21, 2005
BUFFER: 0.154 M KCl
INDUCING AGENT(s): Aroclor 1254 (Monsanto KL615), 500 mg/kg i.p.
REFERENCE: Maron, D & Ames, B. *Mutat Res* 113:173, 1983

BIOCHEMISTRY:

- PROTEIN

37.5 mg/ml — Assayed according to the method of Lowry et al., *JBC* 193:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	52.6
PROD	2B1	17.3
BROD	2B1	30.0
MROD	1A2	85.6

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* 34:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 68.6, 27.0, 78.6, & 11.8 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants EtBr/ TA98	CPA/ TA1535
132.4	1930

The ability of the sample to activate ethidium (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* 129:299, 1984. Data were expressed as revertants per μg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames, (*Mutat Res* 113:173, 1983).

μl S9 per plate/number *his*+ revertants per plate

Promutagen	0	1	5	10	20	50
BP (5 μg)	74	201	361	453	489	463
2-AA (2.5 μg)	76	522	743	755	657	379

MOLECULAR TOXICOLOGY, INC.
157 Industrial Park Dr.
Boone, NC 28607
(828) 264-9099

Becky L Ward

Appendix 7
Minor deviations from protocol

Protocol section	Subject	Deviation
Materials	Controls	In the Range-finder experiment, extra DMSO solvent controls were performed in the absence of S-9 instead of in the presence of S-9 (data not presented). TA100 treatments were included in Experiment 1, where the additional DMSO solvent controls were performed correctly, therefore this was not considered to adversely affect the data acceptability.
Materials	Bacteria	The bacterial cultures used for Experiment 1 treatments were grown for 6.5 hours as opposed to the 10 hours stipulated in the protocol. As all solvent and positive control counts were within acceptable limits, this was not considered to adversely affect the data acceptability.
Methods	Analysis of Results	In Experiment 2, the TA100 solvent control counts in the absence of S-9 were slightly higher than the historical data range. This was due to two out of the five plate counts being greater than the historical range, however as the mean number of revertants was comparable with the range, the data were considered acceptable.

Final Report

Study Title	[redacted] Induction of chromosome aberrations in cultured human peripheral blood lymphocytes
Author	M Lloyd BSc
Sponsor	[redacted]
[redacted] scientific representative	Miss C Bourner
[redacted] contacts	Mrs E Selbie/ Mrs C Talbot/ Mrs T Gübler Miss J Evans
[redacted] sponsor	Dr M York
Test Facility	Covance Laboratories Ltd Otley Road, Harrogate North Yorkshire HG3 1PY ENGLAND
Covance Study Number	375/291
Covance Report Number	375/291-D6172
[redacted] Study Reference Number	KC030431
Report Issued	April 2004
Page Number	1 of 44

STUDY DIRECTOR AUTHENTICATION AND GLP COMPLIANCE STATEMENT

[redacted] **Induction of chromosome aberrations in cultured human peripheral blood lymphocytes**

I, the undersigned, hereby declare that the work was performed by me or under my supervision and that the findings provide a true and accurate record of the results obtained.

The study was performed in accordance with the agreed protocol and with Covance Laboratories Limited Standard Operating Procedures, unless otherwise stated, and the study objectives were achieved. The study was conducted in compliance with the United Kingdom Statutory Instrument 1999 No. 3106, The Good Laboratory Practice Regulations 1999 and the OECD Principles on Good Laboratory Practice (revised 1997, issued January 1998) ENV/MC/CHEM (98) 17, with one exception. There was no information for expiry or purity of the test articles and thus there is an exception to GLP regarding the sample characteristics. These data are the subject of a separate GLP compliant study conducted by the Sponsor [redacted] Study Reference Number AC030449.

M Lloyd BSc
Study Director

20 April 2004
Date

QUALITY ASSURANCE STATEMENT

[redacted] **Induction of chromosome aberrations in cultured human peripheral blood lymphocytes**

This study has been reviewed by the Quality Assurance Unit of Covance Laboratories Ltd. and the report accurately reflects the raw data. The following inspections were conducted and findings reported to the study director (SD) and associated management.

Critical procedures, which are performed routinely in an operational area, may be audited as part of a "process" inspection program. This can be in addition to phases scheduled on an individual study basis. Process inspections conducted and considered applicable to this study are included below.

Inspection Dates			Date Reported to SD and SD Management
From	To	Phase	
05 Dec 2003	05 Dec 2003	Protocol Review	05 Dec 2003
02 Mar 2004	03 Mar 2004	Draft Report and Data Review	03 Mar 2004

Process

Inspection Dates			Date Reported to SD and SD Management
From	To	Phase	
09 Dec 2003	09 Dec 2003	Test Article Dilutions	16 Dec 2003
18 Dec 2003	18 Dec 2003	Dose Preparation	18 Dec 2003
07 Jan 2004	07 Jan 2004	Dose Preparation	07 Jan 2004
08 Jan 2004	08 Jan 2004	S9 Mix Preparations	08 Jan 2004
10 Feb 2004	10 Feb 2004	Dose Preparation	10 Feb 2004
11 Feb 2004	11 Feb 2004	Wash Off	11 Feb 2004
13 Feb 2004	13 Feb 2004	Media Preparation	13 Feb 2004
03 Mar 2004	03 Mar 2004	Dose Preparation	03 Mar 2004
04 Mar 2004	04 Mar 2004	Stock Solution Preparation	04 Mar 2004

D Allison BSc (Hons) MSc
QA Team Manager

20/04/04
Date

REVIEWING SCIENTIST'S STATEMENT

[redacted] **Induction of chromosome aberrations in cultured human peripheral blood lymphocytes**

I, the undersigned, hereby declare that I have reviewed this report in conjunction with the Study Director and that the interpretation and presentation of the data in the report are consistent with the results obtained.

T. S Kumar — 20 April 2004

T S Kumaravel MD PhD
Scientist

Date

RESPONSIBLE PERSONNEL

[redacted] **Induction of chromosome aberrations in cultured human peripheral blood lymphocytes**

The following personnel were responsible for key elements of the study:

Study Director	M Lloyd BSc
Study Supervisor	S Coates BSc
Slide Analysts	Y Stoddart BSc
	C Trott BSc DPhil
	S Doak BSc PhD
	C Svennevik BSc PhD
	L Hudson BSc

ARCHIVE STATEMENT

[redacted] **Induction of chromosome aberrations in cultured human peripheral blood lymphocytes**

All primary data, or authenticated copies thereof, specimens and the final report will be retained in the Covance Laboratories Limited archives for **one** year after issue of the final report. At this time the Sponsor will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. Sponsors will be notified of the financial implications of each of these options at that time.

Specimens requiring storage deep frozen are specifically excluded from the above. These will be retained for as long as the quality of the material permits evaluation, but for no longer than three months after issue of the final report. The Sponsor will be notified of the intent to destroy samples and any financial implications before specimens are destroyed on their behalf.

Archiving of the test article is the responsibility of the Sponsor.

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SUMMARY

[redacted] was tested in an *in vitro* cytogenetics assay using duplicate human lymphocyte cultures prepared from the pooled blood of three female donors in two independent experiments. Treatments covering a broad range of doses, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S-9). The test article was dissolved in water for injection (purified water) and the highest dose level used, 5000 µg/mL, was an acceptable maximum concentration for *in vitro* chromosome aberration studies according to current regulatory guidelines.

In Experiment 1, treatment in the absence and presence of S-9 was for 3 hours followed by a 17-hour recovery period prior to harvest (3+17). The S-9 used was prepared from a rat liver post-mitochondrial fraction (S-9) from Aroclor 1254 induced animals. The test article dose levels for chromosome analysis were selected by evaluating the effect of [redacted] on mitotic index. Chromosome aberrations were analysed at three dose levels (see below). The highest concentration chosen for analysis, 5000 µg/mL, induced approximately 35% and 0% mitotic inhibition (reduction in mitotic index) in the absence and presence of S-9 respectively.

S-9	Treatment + recovery (hours)	Vehicle control	Concentration (µg/mL) [redacted]	Positive control
-	3+17	0[a]	3200, 4000, 5000	MMC, 0.80 µg/mL
+	3+17	0[a]	3200, 4000, 5000	CPA, 6.25 µg/mL

[a] Vehicle control was purified water only

In Experiment 2, treatment in the absence of S-9 was continuous for 20 hours. Treatment in the presence of S-9 was for 3 hours only followed by a 17-hour recovery period prior to harvest (3+17). Chromosome aberrations were analysed at three dose levels (see overleaf) and the highest concentrations chosen for analysis, 984.4 µg/mL in the absence of S-9 and 5000 µg/mL in the presence of S-9, induced approximately 55% and 19% mitotic inhibition respectively.

S-9	Treatment + recovery (hours)	Vehicle control	Concentration (µg/mL) [redacted]	Positive control
-	20+0	0[a]	711.2, 836.7, 984.4	MMC, 0.60 µg/mL
+	3+17	0[a]	3613, 4250, 5000	CPA, 6.25 µg/mL

[a] Vehicle control was purified water only

Appropriate negative (solvent) control cultures were included in the test system in both experiments under each treatment condition. The proportion of cells with structural aberrations in these cultures fell within historical solvent control ranges Appendix 6. Mitomycin C (MMC) and cyclophosphamide (CPA) were employed as positive control chemicals in the absence and presence of liver S-9 respectively. Cells receiving these were sampled in each experiment, 20 hours after the start of treatment; both compounds induced statistically significant increases in the proportion of cells with structural aberrations.

Treatment of cultures with [redacted] for 3+17 hours in the absence and presence of S-9 resulted in frequencies of cells with structural aberrations which were similar to those in concurrent negative controls at all concentrations analysed. Numbers of aberrant cells (excluding gaps) in all treated cultures fell within historical negative control ranges.

Treatment of cultures for 20+0 hours in the absence of S-9 resulted in frequencies of cells with structural aberrations which were significantly different from those observed in concurrent negative controls at all concentrations analysed. Numbers of aberrant cells (excluding gaps) in treated cultures exceeded the historical negative control range in single cultures analysed at the lowest and highest concentrations (711.2 and 984.4 µg/mL) and in both cultures analysed at the intermediate concentration (836.7 µg/mL).

No increases in the frequency of cells with numerical aberrations, which exceeded the historical negative control range, were observed in cultures treated with [redacted] in the absence and presence of S-9 in Experiments 1 and 2.

It is concluded that [redacted] induced chromosome aberrations in cultured human peripheral blood lymphocytes, when tested to its limit of cytotoxicity, following 20+0 hour treatment in the absence of a rat liver metabolic activation system (S-9). In the same test system, [redacted] did not induce chromosome aberrations when tested to 5000 µg/mL, an acceptable top dose for *in vitro* chromosome aberration studies according to current regulatory guidelines, following 3+17 hour treatments in the absence and presence of S-9.

INTRODUCTION

Chromosome defects are recognised as the basis of a number of human genetic diseases (1).

The purpose of the *in vitro* chromosome aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells. No one assay has been extensively evaluated on the same compounds in several laboratories but there is a large database on the use of chromosomal assays for screening purposes (2). The use of human peripheral blood lymphocytes is recommended because the cells are only used in short-term culture and maintain a stable karyotype (3). Experiments with these cells can also be performed in conjunction with a rat liver metabolising system since, for short incubation periods, no toxicity is induced by the liver homogenate itself. Increases in numerical chromosome aberrations can be detected but the assay is not specifically designed to evaluate potential to induce aneuploidy or polyploidy.

In the first instance, cells are exposed to the test article both in the absence and presence of liver S-9 (from rats induced with Aroclor 1254) for 3 hours and sampled at 20 hours after the beginning of treatment. This is equivalent to approximately one and a half times the average generation time of cultured lymphocytes from the panel of donors used in this laboratory. As a number of chemicals have been reported as only exerting positive effects following prolonged treatment (4, 5, 6), however, provision was made for a second experiment involving continuous treatment for 20 hours in the absence of S-9. This, and a repeat treatment in the presence of S-9, using a different spacing of test article concentrations, is performed in the event of a negative result in Experiment 1 (6). It should be noted that some chemicals (e.g. nucleoside/tide analogs or nitrosamides) might be more readily detected by treatment/sampling times longer than 20 hours (6). Provision is therefore made for sampling at later times for test articles known to belong to either of these chemical classes.

The objective of this study was to evaluate the clastogenic potential of [redacted] by examining its effects on the chromosomes of the lymphocytes of human donors, cultured *in vitro* and treated in the absence and presence of a rat liver metabolising system (S-9). The test methodology in this study is in accordance with current literature and complies with the following regulatory guidelines: OECD Guideline 473 (1997) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (1995) (6, 7, 8).

This study was performed according to the protocol.

The study was initiated on 2 December 2003. Experimental work started on 8 December 2003 and was completed on 9 February 2004. The study completion date is considered to be the date on which the Study Director signs the final report.

MATERIALS

Test article

[redacted] sample number S2539801, was a [redacted] It was received on 26 November 2003 and stored at room temperature in the dark. Purity and expiry date were not supplied (these data are the subject of a separate GLP compliant study conducted by the Sponsor, [redacted] Study Reference Number AC030449).

Determinations of stability and characteristics of the test article were the responsibility of the Sponsor.

Characterisation, homogeneity and stability were to be addressed in [redacted] study AC030449.

Homogeneity of the test solutions was addressed by solubility. Stability of the test solutions was not assessed as this was a short-term study and the test solutions were freshly prepared for each experiment.

Preliminary solubility data indicated that [redacted] was soluble in water for injection (purified water), with the aid of vortex mixing, at a concentration of at least 50.87 mg/mL. A 10-fold dilution of this purified water solution into culture medium at a final concentration of approximately 5087 µg/mL did not result in visible precipitation. A maximum concentration of 5000 µg/mL (the maximum concentration for *in vitro* chromosome aberration studies according to current regulatory guidelines) was selected as a suitable maximum for the chromosome aberration study.

Test article stock solutions were prepared by dissolving [redacted] in purified water, with the aid of vortex mixing, to give 50 mg/mL. Dilutions were made from the stock solutions using sterile purified water. The test article solutions were protected from light and used within 3¼ hours of initial formulation as follows overleaf:

Experiment 1 Concentration of treatment solution (mg/mL)	Final concentration (µg/mL)	Hours treatment + hours recovery 3+17 -S-9	3+17 +S-9
1.126	112.6	✓	✓
1.407	140.7	✓	✓
1.759	175.9	✓	✓
2.199	219.9	✓	✓
2.749	274.9	✓	✓
3.436	343.6	✓	✓
4.295	429.5	✓	✓
5.369	536.9	✓	✓
6.711	671.1	✓	✓
8.389	838.9	✓	✓
10.49	1049	✓	✓
13.11	1311	✓	✓
16.38	1638	✓	✓
20.48	2048	✓	✓
25.60	2560	✓	✓
32.00	3200	✓	✓
40.00	4000	✓	✓
50.00	5000	✓	✓

✓ Indicates concentration tested

Experiment 2 Concentration of treatment solution (mg/mL)	Final concentration (µg/mL)	Hours treatment + hours recovery 20+0 -S-9	3+17 +S-9
4.368	436.8	✓	
5.138	513.8	✓	
6.045	604.5	✓	
7.112	711.2	✓	
8.367	836.7	✓	
9.844	984.4	✓	
11.58	1158	✓	
13.62	1362	✓	
16.03	1603	✓	
18.86	1886	✓	
22.19	2219	✓	✓
26.10	2610	✓	✓
30.71	3071	✓	✓
36.13	3613	✓	✓
42.50	4250	✓	✓
50.00	5000	✓	✓

✓ Indicates concentration tested

Measurements on post-treatment media for 3+17 hour treatments in the absence and presence of S-9 in Experiments 1 and 2 indicated that the test article generally had no marked effect on osmolality (greater than a shift of 50 mOsm/kg). The one exception to this was observed in cultures at 4250 µg/mL in the presence of S-9 in Experiment 2, where a decrease in osmolality of 63 mOsm/kg was observed, compared to concurrent vehicle controls. However, no changes of >50 mOsm/kg were

observed at 5000 µg/mL in the presence of S-9 in either experiment, and the frequency of cells with chromosome aberrations observed at 4250 µg/mL in the presence of S-9 in Experiment 2 was similar to the concurrent controls. This observation was therefore not considered biologically relevant. No changes in pH (shift of greater than 1 pH unit, compared to concurrent controls) were observed in treated cultures (9, 10).

Controls

Sterile purified water was added to cultures designated as negative (solvent) controls as described in the methods section of this report. The positive control chemicals Mitomycin C (MMC) and Cyclophosphamide (CPA) were dissolved in purified water and sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) respectively, immediately prior to use, as follows:

Chemical	Supplier	Concentration of treatment solution (mg/mL)	Final concentration (µg/mL)	S-9
Mitomycin C (MMC)	Sigma-Aldrich Chemical Co, Poole, UK*	0.040	0.40	-
		0.060	0.60	-
		0.080	0.80	-
Cyclophosphamide (CPA)	Sigma-Aldrich Chemical Co, Poole, UK	0.3125	3.125	+
		0.625	6.25	+
		1.25	12.5	+

Cells treated with 0.80 µg and 0.60 µg MMC/mL (Experiments 1 and 2 respectively) and 6.25 µg CPA/mL (both experiments) gave satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage. These were selected for analysis.

Metabolic activation system

The mammalian liver post-mitochondrial fraction (S-9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. The batches of MolTox™ S-9 were stored frozen in aliquots at -80°C and thawed just prior to use. Each batch was checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin-O-dealkylase activities). The quality control statements, relating to the batches of S-9 preparation used, are included in Appendix 7 of this report.

Preparation of S-9 mix

Glucose-6-phosphate (180 mg/mL), NADP (25 mg/mL), 150 mM KCl and rat liver S-9 were mixed in the ratio 1:1:1:2. An aliquot of the resulting S-9 mix was added to each cell culture designated for treatment in the presence of S-9 to achieve the required final concentration in a total of 10 mL. The final concentration of liver homogenate in the test system was 2%. Cultures treated in the absence of S-9 received an equal volume of 150 mM KCl.

Blood cultures

Blood from three healthy, non-smoking female volunteers was used for each experiment of this study:

Experiment	Donor Sex	Donor Identity
1	Female	SH, YH, JJ
2	Female	PH, JM, JC

No donor was suspected of any virus infection nor had been exposed to high levels of radiation or hazardous chemicals. For each experiment an appropriate volume of whole blood was drawn from the peripheral circulation within one day of culture initiation. Blood was stored refrigerated and pooled prior to use. Whole blood cultures were established in sterile disposable centrifuge tubes by placing 0.8 mL heparinised blood into 7.7 mL (Experiment 1) or 8.6 mL (Experiment 2) Hepes-buffered RPMI medium containing 20% (v/v) foetal calf serum and 50 µg/mL gentamycin. Phytohaemagglutinin (PHA, reagent grade) was included at a concentration of approximately 2% of culture volume to stimulate the lymphocytes to divide. Blood cultures were incubated for approximately 48 hours at 37°C and rocked continuously.

METHODS

Treatment

In Experiment 1, immediately prior to treatment, all positive control cultures had 0.9 mL culture medium added to give a final volume of 9.4 mL. Immediately prior to treatment in Experiment 2, all solvent and test article treated cultures were pelleted (approximately 300 x 'g', 10 minutes) and 0.9 mL of culture medium removed to give a final volume of 8.5 mL.

S-9 mix or KCl (0.5 mL) was added appropriately as detailed previously. One set of quadruplicate cultures (A, B, C and D) for each of the treatment regimes was then treated with the solvent, and one set of duplicate cultures with the test article (1 mL per culture). Additional duplicate cultures for treatments in the absence of S-9 and in its presence, were treated with 0.1 mL of the positive control chemicals. All cultures were then incubated at 37°C. This scheme is illustrated below:

Treatment	S-9	Number of cultures 3+17*	20+0*
Experiment 1			
Negative control	-	4	
	+	4	
Test article	-	2	
(all doses)	+	2	
Positive controls	-	2	
(all doses)	+	2	
Experiment 2			
Negative control	-		4
	+	4	
Test article	-		2*
(doses as appropriate)	+	2	
Positive controls	-		2
(all doses)	+	2	

Hours treatment + hours recovery

* Only one culture treated at 4250 µg/mL due to spillage of test article

Treatment media remained on cultures receiving the continuous treatment until sampling, that is, 20 hours after the beginning of treatment. Cultures received pulse treatments (both in the absence and presence of S-9) for 3 hours only. They were then pelleted (approximately 300 x 'g', 10 minutes), washed twice with sterile saline (prewarmed at 37°C) and resuspended in fresh medium containing foetal calf serum and gentamycin. Cultures were incubated for a further 17 hours before harvesting.

Summary of Treatment Conditions

Treatment		S-9	Duration of treatment (hours)	Harvest time (hours after start of treatment)
Continuous	20+0	-	20	20
Pulse	3+17	+	3	20
	3+17	-	3	20

Harvesting

Approximately 3 hours prior to harvest, colchicine was added to give a final concentration of approximately 1 µg/mL to arrest dividing cells in metaphase. At the defined sampling time cultures were centrifuged at approximately 300 x 'g' for 10 minutes; the supernatant was carefully removed and cells were resuspended in 4 mL pre-warmed hypotonic (0.075 M) KCl and incubated at 37°C for 15 minutes to allow cell swelling to occur. Cells were then fixed by dropping the KCl suspension into an equal volume of fresh, ice-cold methanol/glacial acetic acid (3:1, v/v). The fixative was changed by centrifugation (approximately 300 x 'g', 10 minutes) and resuspension. This procedure was repeated several times (centrifuging at approximately 1250 x 'g', 2-3 minutes) until the cell pellets were clean.

Preparation of metaphase spreads

Lymphocytes were kept in fixative in the refrigerator before slides were prepared but slides were not made on the day of harvest to ensure cells were adequately fixed. Cells were pelleted and resuspended in a minimal amount of fresh fixative (if required) so as to give a milky suspension. Several drops of 45% (v/v) aqueous acetic acid were added to each suspension to enhance chromosome spreading, and several drops of suspension were transferred to clean microscope slides labelled with the appropriate study details. Slides were flamed to improve metaphase spreading.

After the slides had dried the cells were stained for 5 minutes in 4% (v/v) filtered Giemsa stain in Gurr's pH 6.8 buffer. The slides were rinsed, dried and mounted with coverslips.

Selection of doses for cytogenetic analysis

Slides were examined, uncoded, for mitotic index (MI) or percentage of cells in mitosis. Slides from enough dose levels from each treatment regime were scored to determine if chemically induced mitotic inhibition had occurred. This is defined as a clear decrease in mitotic index compared with negative controls (based on at least 1000 cells counted), preferably dose-related.

Rationale for dose selection

The highest dose for chromosome analysis from cultures sampled at 20 hours should be one at which at least 50% mitotic inhibition (approximately) has occurred or should be the highest dose tested. Analysis of slides from highly cytotoxic concentrations is avoided, if possible. Slides from the highest selected dose and two lower doses, such that a range of cytotoxicity from maximum to little or none is covered, were taken for microscope analysis.

For each treatment regime, two solvent control cultures were initially to be analysed for chromosome aberrations. Slides from the remaining solvent control cultures were only to be analysed if considered necessary, for example, to help resolve an equivocal result. A single positive control dose level, which gives satisfactory responses in terms of quality and quantity of mitoses and extent of chromosomal damage, was analysed.

Mitotic index data and the results of dose selection are presented in the results section of this report.

Scoring of aberrations

Slides from MMC and CPA positive control treatments were checked to ensure that the system was operating satisfactorily. Slides from the selected treatments and from solvent and positive controls were coded using randomly generated letters by a person not connected with the scoring of the slides. Labels bearing only the study reference number, experiment number, the sex of the donor and the code were used to cover treatment details on the slides.

One hundred metaphases from each code were analysed for chromosome aberrations. Only cells with 44-46 chromosomes were considered acceptable for analysis of structural aberrations. Any cell with 47-68 chromosomes, that is hyperdiploid cells, observed during this search was noted and recorded separately. Classification of structural aberrations was based on the scheme described by ISCN (11) and is detailed

in Appendix 2. Under this scheme, a gap is defined as a discontinuity less than the width of the chromatid and no evidence of displacement of the fragment and a deletion is defined as a discontinuity greater than the width of the chromatid and/or evidence of displacement of the fragment. Observations (summarised in Appendix 3 and Appendix 4) were recorded on raw data sheets with the microscope stage co-ordinates of any aberrant cell.

Furthermore, 1000 metaphase cells per culture were analysed for polyploidy (greater than 68 chromosomes) or endoreduplication. Slides were scanned under low power and cells categorised into hyperdiploid, polyploid or endoreduplicated. Classification was based on a broad assessment of chromosome number and the microscope stage coordinates of aberrant cells was not recorded.

Slide analysis was performed off-site by analysts trained in accordance with Covance Laboratories Limited Standard Operating Procedures. Details of the analysts are included in the responsible personnel list. All slides and raw data have been returned to Covance Laboratories Limited for archiving in accordance with the archive statement in this report.

Analysis of results

Treatment of data

After completion of microscopic analysis, data were decoded. The aberrant cells in each culture were categorised as follows:

1. cells with structural aberrations including gaps

2. cells with structural aberrations excluding gaps

3. polyploid, endoreduplicated or hyperdiploid cells.

The totals for category 2 in negative control cultures were compared with the current laboratory negative control (normal) ranges to determine whether the assay was acceptable or not. The proportion of cells in category 2 in test article treated cultures were also compared with normal ranges. The statistical significance of any data set was only to be taken into consideration if the frequency of aberrant cells in both replicate cultures at one or more concentration exceeded the normal range. Under this condition, the statistical method used would be Fisher's exact test (12). Probability values of $p \leq 0.05$ were to be accepted as significant. The proportions of cells in categories 1 and 3 were also

examined in relation to historical negative control (normal) ranges and statistical analysis by Fisher's exact test may be used.

The proportions of aberrant cells in each replicate were also used to establish acceptable heterogeneity between replicates by means of a binomial dispersion test (12). Probability values of $p \leq 0.05$ were to be accepted as significant.

Acceptance criteria

The human lymphocyte assay is considered valid if the following criteria are met:

1. the binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures, and

2. the proportion of cells with structural aberrations (excluding gaps) in negative control cultures falls within the normal range, and

3. at least 160 cells out of an intended 200 are analysable at each dose level, and

4. the positive control chemicals induce statistically significant increases in the proportion of cells with structural aberrations.

Evaluation criteria

A test article is considered as positive in this assay if:

1. the proportions of cells with structural aberrations at one or more concentration exceeds the normal range in both replicate cultures, and

2. a statistically significant increase in the proportion of cells with structural aberrations (excluding gaps) occurs at these doses.

Increased incidence of cells with gaps or increased proportions of cells with structural aberrations not exceeding the normal range, or occurring only at very high or very toxic concentrations are likely to be concluded as "equivocal". Full assessment of the biological importance of such increases is likely only to be possible with reference to data from other test systems. Evidence of a dose-related effect is considered useful but not essential in the evaluation of a positive result. Cells with exchange aberrations or cells with greater than one structural aberration occur very infrequently in negative control cultures. Their appearance is therefore considered to be of particular biological significance.

RESULTS

Selection of doses for cytogenetic analysis

The results of mitotic index determinations for the treatments in Experiment 1 were as follows:

Treatment (µg/mL)	Mitotic index (%) 3+17 hours, -S-9 A/C	B/D	MIH*	3+17 hours, +S-9 A/C	B/D	MIH*
Solvent	16.4/16.5	17.5/12.2	-	5.9/7.8	8.6/9.1	-
112.6	NS	NS	-	NS	NS	-
140.7	NM	NM	-	NM	NM	-
175.9	NM	NM	-	NM	NM	-
219.9	NM	NM	-	NM	NM	-
274.9	NM	NM	-	NM	NM	-
343.6	NM	NM	-	NM	NM	-
429.5	NM	NM	-	NM	NM	-
536.9	NM	NM	-	NM	NM	-
671.1	NM	NM	-	NM	NM	-
838.9	NM	NM	-	NM	NM	-
1049	NS	NS	-	NS	NS	-
1311	NS	NS	-	NS	NS	-
1638	NS	NS	-	NS	NS	-
2048	14.3	16.3	2	NS	NS	-
2560	13.5	14.4	11	NS	NS	-
3200	13.3	13.5	14	10.8	11.3	0
4000	11.8	13.9	18	11.2	14.3	0
5000	11.3	9.2	35	10.3	10.0	0

NM = slides not made NS = not scored
*Mitotic inhibition (%) = [1 - (mean MI_T/mean MI_C)] x 100%
(where T = treatment and C = negative control)
(Slides from solvent control cultures C and D scored for mitotic index only)
A/C, B/D refers to the number of cultures treated (four [A, B, C, D] for solvent controls and two [A, B] for test article)

The following doses were selected for analysis, following consultation with the Sponsor:

	Mitotic inhibition†
3+17 hours, -S-9: 3200, 4000, 5000 µg/mL	35%
3+17 hours, +S-9: 3200, 4000, 5000 µg/mL	0%

† At highest concentration analysed

The results of mitotic index determinations for the treatments in Experiment 2 were as follows:

Treatment (µg/mL)	Mitotic index (%) 20+0 hours, -S-9 A/C	B/D	MIH*	3+17 hours, +S-9 A/C	B/D	MIH*
Solvent	13.7/12.3	10.7/9.7	-	13.2/18.3	9.3/11.0	-
436.8	NS	NS	-	NT	NT	-
513.8	NS	NS	-	NT	NT	-
604.5	11.0	12.6	0	NT	NT	-
711.2	10.5	10.4	10	NT	NT	-
836.7	4.9	6.0	53	NT	NT	-
984.4	4.6	5.9	55	NT	NT	-
1158	4.1	3.4	68	NT	NT	-
1362	3.5	2.8	73	NT	NT	-
1603	2.8	2.8	76	NT	NT	-
1886	3.3	2.7	74	NT	NT	-
2219	4.3	3.0	69	NS	NS	-
2610	1.7	2.0	84	12.2	15.4	0
3071	1.5	1.9	85	11.6	14.6	0
3613	1.7	0.5	91	10.1	10.5	20
4250	2.1	NT	82	8.1	13.0	19
5000	1.3	1.1	90	8.8	12.2	19

NT = not tested NS = not scored
*Mitotic inhibition (%) = [1 - (mean MI_T/mean MI_C)] x 100%
(where T = treatment and C = negative control)
(Slides from solvent control cultures C and D scored for mitotic index only).
A/C, B/D refers to the number of cultures treated (four [A, B, C, D] for solvent controls and two [A, B] for test article)

The following doses were selected for analysis, following consultation with the Sponsor:

	Mitotic inhibition†
20+0 hours, -S-9: 711.2, 836.7, 984.4 µg/mL	55%
3+17 hours, +S-9: 3613, 4250, 5000 µg/mL	19%

† At highest concentration analysed

Chromosome aberration analysis

Raw data

The raw data for the observations on the test article plus positive and negative controls are retained by Covance Laboratories Limited. A summary of the number of cells containing structural aberrations is given in Appendix 1 for each of the different treatment regimes in Experiment 1 and 2. The numbers and types of

structural aberrations seen per cell are given in Appendix 3. Frequencies of cells with numerical aberrations observed are given in Appendix 4.

Validity of study

The data in Appendix 1 and Appendix 6 confirm that:

1. no evidence of significant heterogeneity between replicate cultures was obtained in the binomial dispersion test (Appendix 1), and

2. the proportion of cells with structural aberrations (excluding gaps) in negative control cultures fell within the normal range (Appendix 6), and

3. at least 160 cells out of an intended 200 were analysed at each dose level (Appendix 1), and

4. the positive control chemicals MMC and CPA induced statistically significant increases in the number of cells with structural aberrations (Appendix 1).

Analysis of data

Structural aberrations

Treatment of cultures with [redacted] for 3+17 hours in the absence and presence of S-9 resulted in frequencies of cells with structural aberrations which were similar to those in concurrent negative controls (Appendix 1) at all concentrations analysed. Numbers of aberrant cells (excluding gaps) in all treated cultures fell within historical negative control ranges (Appendix 6).

Treatment of cultures for 20+0 hours in the absence of S-9 resulted in frequencies of cells with structural aberrations which were significantly different from those observed in concurrent negative controls (Appendix 1) at all concentrations analysed. Numbers of aberrant cells (excluding gaps) in treated cultures exceeded the historical negative control range (Appendix 6) in single cultures analysed at the lowest and highest concentrations (711.2 and 984.4 µg/mL) and in both cultures analysed at the intermediate concentration (836.7 µg/mL).

Numerical aberrations

No increases in the frequency of cells with numerical aberrations, which exceeded the historical negative control range, were observed in cultures treated with [redacted] in the absence and presence of S-9 (Appendix 4 and Appendix 6) in Experiments 1 and 2.

CONCLUSION

It is concluded that [redacted] induced chromosome aberrations in cultured human peripheral blood lymphocytes, when tested to its limit of cytotoxicity, following 20+0 hour treatment in the absence of a rat liver metabolic activation system (S-9). In the same test system, [redacted] did not induce chromosome aberrations when tested to 5000 µg/mL, an acceptable top dose for *in vitro* chromosome aberration studies according to current regulatory guidelines, following 3+17 hour treatments in the absence and presence of S-9.

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APPENDICES

Appendix 1
[redacted] cells with structural aberrations

Table 1
3 hour treatment -S-9, 17 hour recovery (3+17), Experiment 1
Donor sex: female

Treatment (µg/mL)	Replicate	Cells Scored	Cells with Aberrations Including Gaps	Cells with Aberrations Excluding Gaps	Mitotic Index (mean)
Solvent	A	100	2	1	16.4
	B	100	0	0	17.5
	C	ND	ND	ND	16.5
	D	ND	ND	ND	12.2
	Totals	200	2	1	(15.7)
3200	A	100	3	1	13.3
	B	100	2	1	13.5
	Totals	200	5	2	(13.4)
4000	A	100	2	2	11.8
	B	100	0	0	13.9
	Totals	200	2	2	(12.9)
5000	A	100	1	1	11.3
	B	100	1	1	9.2
	Totals	200	2	2	(10.3)
MMC, 0.80	A	100	28	26	
	B	100	35	32	
	Totals	200	63	58[a]	

Binomial Dispersion Test $\chi^2 = 3.03$, not significant
Note: solvent replicates C and D scored for mitotic index only
[a] Statistical significance $p \leq 0.001$
ND = not determined
Numbers highlighted exceed historical negative control range (Appendix 6)

Table 2
3 hour treatment +S-9, 17 hour recovery (3+17), Experiment 1
Donor sex: female

Treatment (µg/mL)	Replicate	Cells Scored	Cells with Aberrations Including Gaps	Cells with Aberrations Excluding Gaps	Mitotic Index (mean)
Solvent	A	100	2	2	5.9
	B	100	3	3	8.6
	C	ND	ND	ND	7.8
	D	ND	ND	ND	9.1
	Totals	200	5	5	(7.9)
3200	A	100	[illegible]	4	10.8
	B	100	2	2	11.3
	Totals	200	9	6	(11.1)
4000	A	100	5	3	11.2
	B	100	4	1	14.3
	Totals	200	9	4	(12.8)
5000	A	100	2	2	10.3
	B	100	0	0	10.0
	Totals	200	2	2	(10.2)
CPA, 6.25	A	100	[illegible]	[illegible]	
	B	100	[illegible]	[illegible]	
	Totals	200	87	77[a]	

Binomial Dispersion Test $\chi^2 = 3.93$, not significant
Note: solvent replicates C and D scored for mitotic index only
[a] Statistical significance $p \leq 0.001$
ND = not determined
Numbers highlighted exceed historical negative control range (Appendix 6)

Table 3
20 hour treatment -S-9, 0 hour recovery (20+0), Experiment 2
Donor sex: female

Treatment (µg/mL)	Replicate	Cells Scored	Cells with Aberrations Including Gaps	Cells with Aberrations Excluding Gaps	Significance §	Mitotic Index (mean)
Solvent	A	100	1	0		13.7
	B	100	1	1		10.7
	C	ND	ND	ND		12.3
	D	ND	ND	ND		9.7
	Totals	200	2	1		(11.6)
711.2	A	100	9	9		10.5
	B	100	6	5		10.4
	Totals	200	15	14	$p \leq 0.001$	(10.5)
836.7	A	100	8	7		4.9
	B	100	9	7		6.0
	Totals	200	17	14	$p \leq 0.001$	(5.5)
984.4	A	100	10	9		4.6
	B	100	9	6		5.9
	Totals	200	19	15	$p \leq 0.001$	(5.3)
MMC, 0.60	A	100	38	37		
	B	100	29	29		
	Totals	200	67	66	$p \leq 0.001$	

Binomial Dispersion Test $\chi^2 = 2.88$, not significant
Note: solvent replicates C and D scored for mitotic index only
§ Statistical significance (Appendix 5)
ND = not determined
NS = not significant
Numbers highlighted exceed historical negative control range (Appendix 6)

Table 4
3 hour treatment +S-9, 17 hour recovery (3+17), Experiment 2
Donor sex: female

Treatment (µg/mL)	Replicate	Cells Scored	Cells with Aberrations Including Gaps	Cells with Aberrations Excluding Gaps	Mitotic Index (mean)
Solvent	A	100	0	0	13.2
	B	100	1	1	9.3
	C	ND	ND	ND	18.3
	D	ND	ND	ND	11.0
	Totals	200	1	1	(13.0)
3613	A	100	0	0	10.1
	B	100	2	1	10.5
	Totals	200	2	1	(10.3)
4250	A	100	3	2	8.1
	B	100	2	0	13.0
	Totals	200	5	2	(10.6)
5000	A	100	4	2	8.8
	B	100	1	1	12.2
	Totals	200	5	3	(10.5)
CPA, 6.25	A	100	[illegible]	[illegible]	
	B	100	[illegible]	[illegible]	
	Totals	200	33	32[a]	

Binomial Dispersion Test $\chi^2 = 4.37$, not significant
Note: solvent replicates C and D scored for mitotic index only
[a] Statistical significance $p \leq 0.001$
ND = not determined
Numbers highlighted exceed historical negative control range (Appendix 6)

Appendix 2
Abbreviations and classification of observations

abs	=	aberrations
rep	=	replicate
tot	=	total

Gaps

csg	=	chromosome gap
ctg	=	chromatid gap

Chromosome deletions

del	=	chromosome deletion
d min	=	double minute
f	=	isolocus fragment

Chromosome exchanges

t	=	interchange between chromosomes (e.g. reciprocal translocation)
inv	=	chromosome intrachange (e.g. pericentric inversion)
dic	=	dicentric
dic+f	=	dicentric with accompanying fragment
acr	=	acentric ring
r+f	=	centric ring with accompanying fragment
r	=	centric ring

Chromatid deletions

del	=	chromatid deletion
min	=	single minute

Chromatid exchanges

qr	=	interchange between chromatids of different chromosomes (e.g. quadriradial)
cx	=	obligate complex interchange
e	=	chromatid intrachange
tr/tr+f	=	isochromatid/chromatid interchange (triradial)/with accompanying fragment
su	=	intra-arm intrachange with sister union of broken ends
nud	=	intra-arm intrachange with non-union of broken ends distally
nup	=	intra-arm intrachange with non-union of broken ends proximally

Other structural aberrations

pvz	=	pulverised cell
mabs	=	multiple aberrations (greater than 7 aberrations per cell or too many aberrations to permit accurate analysis)

Numerical aberrations

E	=	endoreduplicated
H	=	hyperdiploid (47-68 chromosomes)
P	=	polyploid (greater than 68 chromosomes)

Appendix 3

[redacted] summary of numbers and types of structural aberrations observed

Table 5

3 hour treatment -S-9, 17 hour recovery (3+17), Experiment 1

Donor sex: female

Treatment (µg/mL)	Rep	Cells *	G	Chr del	Chr exch	Ctd del	Ctd exch	Other	Abs +g	Abs -g
Solvent	A	100	1	0	0	1	0	0	2	1
	B	100	0	0	0	0	0	0	0	0
	Total	200	1	0	0	1	0	0	2	1
3200	A	100	2	1	0	0	0	0	3	1
	B	100	2	0	0	1	0	0	3	1
	Total	200	4	1	0	1	0	0	6	2
4000	A	100	0	0	0	2	0	0	2	2
	B	100	0	0	0	0	0	0	0	0
	Total	200	0	0	0	2	0	0	2	2
5000	A	100	0	1	0	0	0	0	1	1
	B	100	0	1	0	0	0	0	1	1
	Total	200	0	2	0	0	0	0	2	2
MMC, 0.80	A	100	12	12	0	22	5	0	51	39
	B	100	10	11	0	23	6	0	50	40
	Total	200	22	23	0	45	11	0	101	79

* Total cells examined for structural aberrations
Totals given for each culture may differ from values given in Appendix 1, Tables 1 - 4 if cells are observed which have more than one aberration
For abbreviations and classification see Appendix 2

Table 6
3 hour treatment +S-9, 17 hour recovery (3+17), Experiment 1
Donor sex: female

Treatment (µg/mL)	Rep	Cells *	G	Chr del	Chr exch	Ctd del	Ctd exch	Other	Abs +g	Abs -g
Solvent	A	100	0	1	0	1	0	0	2	2
	B	100	0	1	0	2	0	0	3	3
	Total	200	0	2	0	3	0	0	5	5
3200	A	100	3	1	0	3	0	0	7	4
	B	100	0	0	0	2	0	0	2	2
	Total	200	3	1	0	5	0	0	9	6
4000	A	100	2	2	0	1	0	0	5	3
	B	100	3	0	0	1	0	0	4	1
	Total	200	5	2	0	2	0	0	9	4
5000	A	100	0	0	0	2	0	0	2	2
	B	100	0	0	0	0	0	0	0	0
	Total	200	0	0	0	2	0	0	2	2
CPA, 6.25	A	100	9	11	0	40	1	0	61	52
	B	100	20	11	0	57	4	0	92	72
	Total	200	29	22	0	97	5	0	153	124

* Total cells examined for structural aberrations
Totals given for each culture may differ from values given in Appendix 1, Tables 1 - 4 if cells are observed which have more than one aberration
For abbreviations and classification see Appendix 2

Table 7
20 hour treatment -S-9, 0 hour recovery (20+0), Experiment 2
Donor sex: female

Treatment (µg/mL)	Rep	Cells *	G	Chr del	Chr exch	Ctd del	Ctd exch	Other	Abs +g	Abs -g
Solvent	A	100	2	0	0	0	0	0	2	0
	B	100	0	0	0	1	0	0	1	1
	Total	200	2	0	0	1	0	0	3	1
711.2	A	100	4	7	0	7	0	0	18	14
	B	100	4	2	0	3	0	0	9	5
	Total	200	8	9	0	10	0	0	27	19
836.7	A	100	4	3	0	5	1	0	13	9
	B	100	3	1	0	7	0	0	11	8
	Total	200	7	4	0	12	1	0	24	17
984.4	A	100	3	3	0	5	0	1	12	9
	B	100	4	5	0	6	1	0	16	12
	Total	200	7	8	0	11	1	1	28	21
MMC, 0.60	A	100	5	3	0	47	17	1	73	68
	B	100	4	4	0	32	10	2	52	48
	Total	200	9	7	0	79	27	3	125	116

* Total cells examined for structural aberrations
Totals given for each culture may differ from values given in Appendix 1, Tables 1 - 4 if cells are observed which have more than one aberration
For abbreviations and classification see Appendix 2

Table 8
3 hour treatment +S-9, 17 hour recovery (3+17), Experiment 2
Donor sex: female

Treatment (µg/mL)	Rep	Cells *	G	Chr del	Chr exch	Ctd del	Ctd exch	Other	Abs +g	Abs -g
Solvent	A	100	0	0	0	0	0	0	0	0
	B	100	0	0	0	1	0	0	1	1
	Total	200	0	0	0	1	0	0	1	1
3613	A	100	0	0	0	0	0	0	0	0
	B	100	1	0	0	1	0	0	2	1
	Total	200	1	0	0	1	0	0	2	1
4250	A	100	3	0	0	2	0	0	5	2
	B	100	2	0	0	0	0	0	2	0
	Total	200	5	0	0	2	0	0	7	2
5000	A	100	2	0	0	2	0	0	4	2
	B	100	0	0	0	1	0	0	1	1
	Total	200	2	0	0	3	0	0	5	3
CPA, 6.25	A	100	0	2	0	15	3	0	20	20
	B	100	2	1	0	14	4	0	21	19
	Total	200	2	3	0	29	7	0	41	39

* Total cells examined for structural aberrations
Totals given for each culture may differ from values given in Appendix 1, Tables 1 - 4 if cells are observed which have more than one aberration
For abbreviations and classification see Appendix 2

Appendix 4

[redacted] summary of numbers and types of numerical aberrations observed

Table 9
3 hour treatment -S-9, 17 hour recovery (3+17), Experiment 1
Donor sex: female

		Hyperdiploidy		Polyploidy and endoreduplication			
Treatment (µg/mL)	Rep	Mitotic cells observed **	H	Mitotic cells observed **	P	E	%P+E (mean)
Solvent	A	100	0	1000	0	0	0.0
	B	100	0	1000	2	0	0.2
	Total	200	0	2000	2	0	(0.10)
3200	A	100	0	1000	0	0	0.0
	B	100	0	1000	1	0	0.1
	Total	200	0	2000	1	0	(0.05)
4000	A	100	0	1000	0	1	0.1
	B	100	0	1000	0	0	0.0
	Total	200	0	2000	0	1	(0.05)
5000	A	100	0	1000	0	0	0.0
	B	100	0	1000	2	0	0.2
	Total	200	0	2000	2	0	(0.10)
MMC, 0.80	A	100	0	ND	-	-	-
	B	100	0	ND	-	-	-
	Total	200	0	-	-	-	-

** Total cells examined for numerical aberrations
ND = not determined
For abbreviations and classification see Appendix 2

Table 10
3 hour treatment +S-9, 17 hour recovery (3+17), Experiment 1
Donor sex: female

		Hyperdiploidy		Polyploidy and endoreduplication			
Treatment (µg/mL)	Rep	Mitotic cells observed **	H	Mitotic cells observed **	P	E	%P+E (mean)
Solvent	A	100	0	1000	1	0	0.1
	B	100	0	1000	0	0	0.0
	Total	200	0	2000	1	0	(0.05)
3200	A	100	0	1000	0	0	0.0
	B	100	0	1000	2	0	0.2
	Total	200	0	2000	2	0	(0.10)
4000	A	100	0	1000	0	0	0.0
	B	100	0	1000	0	0	0.0
	Total	200	0	2000	0	0	(0.00)
5000	A	101	1	1000	0	0	0.0
	B	100	0	1000	1	1	0.2
	Total	201	1	2000	1	1	(0.10)
CPA, 6.25	A	100	0	ND	-	-	-
	B	101	1	ND	-	-	-
	Total	201	1	-	-	-	-

** Total cells examined for numerical aberrations
ND = not determined
For abbreviations and classification see Appendix 2

Table 11
20 hour treatment -S-9, 0 hour recovery (20+0), Experiment 2
Donor sex: female

		Hyperdiploidy		Polyploidy and endoreduplication			
Treatment (µg/mL)	Rep	Mitotic cells observed **	H	Mitotic cells observed **	P	E	%P+E (mean)
Solvent	A	100	0	1000	0	1	0.1
	B	100	0	1000	0	0	0.0
	Total	200	0	2000	0	1	(0.05)
711.2	A	100	0	1000	1	0	0.1
	B	100	0	1000	0	0	0.0
	Total	200	0	2000	1	0	(0.05)
836.7	A	100	0	1000	0	0	0.0
	B	100	0	1000	2	0	0.2
	Total	200	0	2000	2	0	(0.10)
984.4	A	100	0	1000	0	0	0.0
	B	100	0	1000	0	0	0.0
	Total	200	0	2000	0	0	(0.00)
MMC, 0.60	A	100	0	ND	-	-	-
	B	100	0	ND	-	-	-
	Total	200	0	-	-	-	-

** Total cells examined for numerical aberrations
ND = not determined
For abbreviations and classification see Appendix 2

Table 12
3 hour treatment +S-9, 17 hour recovery (3+17), Experiment 2
Donor sex: female

		Hyperdiploidy		Polyploidy and endoreduplication			
Treatment (µg/mL)	Rep	Mitotic cells observed **	H	Mitotic cells observed **	P	E	%P+E (mean)
Solvent	A	100	0	1000	0	0	0.0
	B	100	0	1000	0	0	0.0
	Total	200	0	2000	0	0	(0.00)
3613	A	100	0	1000	0	0	0.0
	B	100	0	1000	0	0	0.0
	Total	200	0	2000	0	0	(0.00)
4250	A	100	0	1000	0	0	0.0
	B	100	0	1000	1	0	0.1
	Total	200	0	2000	1	0	(0.05)
5000	A	100	0	1000	0	0	0.0
	B	100	0	1000	1	0	0.1
	Total	200	0	2000	1	0	(0.05)
CPA, 6.25	A	100	0	ND	-	-	-
	B	100	0	ND	-	-	-
	Total	200	0	-	-	-	-

** Total cells examined for numerical aberrations
ND = not determined
For abbreviations and classification see Appendix 2

Appendix 5

[redacted] statistical analysis of test article data

Cells with structural aberrations excluding gaps

Table 13

20 hour treatment -S-9, 0 hour recovery (20+0), Experiment 2

Binomial Dispersion Test $\chi^2 = 2.88$	DF = 4
Significance NS	

Treatment (µg/mL)	Cells	Aberrant Cells	Prop-ortion	Fisher's Exact Test	Significance
Solvent	200	1	0.0050		
711.2	200	14	0.0700	0.000	$p \leq 0.001$
836.7	200	14	0.0700	0.000	$p \leq 0.001$
984.4	200	15	0.0750	0.000	$p \leq 0.001$
MMC, 0.60	200	66	0.3300	0.000	$p \leq 0.001$

NS = not significant
DF = degrees of freedom

Appendix 6
Historical ranges for solvent controls

Sex and S-9 treatment	Category	Total number of cells scored	Aberrant cells scored per 100 cells		
			Mean	Calculated normal range	Observed Range
Female -S-9	Structural aberrations including gaps	6600	2.33	3-8	0-9
	Structural aberrations excluding gaps	6600	1.50	3-6	0-7
	Polyploid and Endoreduplicated cells	28000	0.09	0-0.34	0-0.3
Female +S-9 (Aroclor 1254)	Structural aberrations including gaps	5600	1.66	2-6	0-6
	Structural aberrations excluding gaps	5600	0.95	2-4	0-4
	Polyploid and Endoreduplicated cells	28000	0.08	0-0.30	0-0.3

Calculated in October 2002

Appendix 7
Quality control statements for S-9

MOLTOX™ POST MITOCHONDRIAL SUPERNATANT (S-9) QUALITY CONTROL & PRODUCTION CERTIFICATE

LOT NO.: 1619
PART NO.: 11-101
VOLUME: 5ml

SPECIES: Rat
STRAIN: Sprague Dawley
SEX: Male
TISSUE: Liver

PREPARATION DATE: October 21, 2003
EXPIRATION DATE: October 21, 2005
BUFFER: 0.154 M KCl
INDUCING AGENT(s): Aroclor 1254 (Monsanto KL615), 500 mg/kg i.p.

REFERENCE: Maron, D & Ames, B. *Mutat Res* 113:173, 1983

BIOCHEMISTRY:

- PROTEIN

37.5 mg/ml

Assayed according to the method of Lowry et al., *JBC* 193:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold-Induction
EROD	IA1, IA2	52.6
PROD	2B1	17.3
BROD	2B1	30.0
MROD	1A2	85.6

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* 34:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 68.6, 27.0, 78.6, & 11.8 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
BtBr/	CPA/
TA98	TA1535
132.4	1930

The ability of the sample to activate ethidium (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Leaca, et al., *Mutation Res* 129:299, 1984. Data were expressed as revertants per μg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames, (*Mutat Res* 113:173, 1983).

	μl S9 per plate/number *his*+ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5 μg)	74	201	361	453	489	463
2-AA (2.5 μg)	76	522	743	755	657	379

MOLECULAR TOXICOLOGY, INC.
157 Industrial Park Dr.
Boone, NC 28607
(828) 264-9099

Becky L Ward

MOLTOX™ POST MITOCHONDRIAL SUPERNATANT (S-9) QUALITY CONTROL & PRODUCTION CERTIFICATE

LOT NO.: 1621
PART NO.: 11-101
VOLUME: 5ml
SPECIES: Rat
STRAIN: Sprague Dawley
SEX: Male
TISSUE: Liver
PREPARATION DATE: October 23, 2003
EXPIRATION DATE: October 23, 2005
BUFFER: 0.154 M KCl
INDUCING AGENT(s): Aroclor 1254 (Monsanto KL615), 500 mg/kg i.p.
REFERENCE: Maron, D & Ames, B, *Mutat Res* 113:173, 1983

BIOCHEMISTRY:

- PROTEIN

37.6 mg/ml

Assayed according to the method of Lowry et al., *JBC* 193:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	45.26
PROD	2B1	13.3
BROD	2B1	21.7
MROD	1A2	53.5

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* 34:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 68.6, 27.0, 78.6, & 11.8 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants EtBr/ TA98	CPA/ TA1535
113.2	566

The ability of the sample to activate ethidium (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* 129:299, 1984. Data were expressed as revertants per μg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames, (*Mutat Res* 113:173, 1983).

μl S9 per plate/number *his*⁺ revertants per plate

Promutagen	0	1	5	10	20	50
BP (5 μg)	189	594	1682	2245	1983	1380
2-AA (2.5 μg)	257	359	673	957	954	1050

MOLECULAR TOXICOLOGY, INC.
157 Industrial Park Dr.
Boone, NC 28607
(828) 264-9099

Becky L Ward

Final Report

Study Title	[redacted] Additional analysis of chromosome aberrations
Author	G Clare BSc PhD
Sponsor	[redacted]
[redacted] Scientific Representative	Dr A Wolfreys
[redacted] Scientific Expert	Dr L Henderson
[redacted] Contacts	Mrs E Selbie/ Mrs C Talbot/ Miss K Barron/ Miss S Buljeeon
[redacted] Sponsor	Dr M York
Test Facility	Covance Laboratories Ltd Otley Road, Harrogate North Yorkshire HG3 1PY ENGLAND
Covance Study Number	375/316
Covance Report Number	375/316-D6172
[redacted] Study Reference Number	KC040192
Report Issued	January 2005
Page Number	1 of 23

STUDY DIRECTOR AUTHENTICATION AND GLP COMPLIANCE STATEMENT

[redacted] Additional analysis of chromosome aberrations

I, the undersigned, hereby declare that the work was performed by me or under my supervision and that the findings provide a true and accurate record of the results obtained.

The study was performed in accordance with the agreed protocol and with Covance Laboratories Limited Standard Operating Procedures, unless otherwise stated, and the study objectives were achieved. The study was conducted in compliance with the United Kingdom Statutory Instrument No. 3106, the Good Laboratory Practice Regulations 1999, as amended by the Good Laboratory Practice (Codification Amendments Etc.) Regulations 2004 and the OECD Principles on Good Laboratory Practice (revised 1997, issued January 1998) ENV/MC/CHEM (98) 17 with one exception.

Purity and expiry date of the test article were not supplied as part of this study, as these data are the subject of a separate GLP compliant study conducted by the Sponsor [redacted] Study Reference Number AC030449.

Gill Clare
G Clare BSc PhD
Study Director

25 January 2005
Date

QUALITY ASSURANCE STATEMENT

[redacted] **Additional analysis of chromosome aberrations**

This study has been reviewed by the Quality Assurance Unit of Covance Laboratories Ltd. and the report accurately reflects the raw data. The following inspections were conducted and findings reported to the study director (SD) and associated management.

Critical procedures, which are performed routinely in an operational area, may be audited as part of a "process" inspection program. This can be in addition to phases scheduled on an individual study basis. Selected process inspections conducted and considered applicable to this study are included below.

Inspection Dates From	Inspection Dates To	Phase	Date Reported to SD and SD Management
26 Aug 2004	26 Aug 2004	Protocol Review	26 Aug 2004
03 Sep 2004	03 Sep 2004	Protocol Amendment Review	03 Sep 2004
07 Oct 2004	07 Oct 2004	Draft Report and Data Review	07 Oct 2004
25 Jan 2005	25 Jan 2005	Final Report Review	25 Jan 2005

Process

Inspection Dates From	Inspection Dates To	Phase	Date Reported to SD and SD Management
06 Sep 2004	06 Sep 2004	Slide Analysis	08 Sep 2004

D Allison BSc (Hons) MSc
QA Team Manager

25/01/05
Date

REVIEWING SCIENTIST'S STATEMENT

[redacted] Additional analysis of chromosome aberrations

I, the undersigned, hereby declare that I have reviewed this report in conjunction with the Study Director and that the interpretation and presentation of the data in the report are consistent with the results obtained.

M Lloyd BSc
Scientist

20 January 2005
Date

RESPONSIBLE PERSONNEL

[redacted] **Additional analysis of chromosome aberrations**

The following personnel were responsible for key elements of the study:

Study Director	G Clare BSc PhD
Analysts	L Teare
	M King BSc PhD
	D Crellin ONC

ARCHIVE STATEMENT

[redacted] **Additional analysis of chromosome aberrations**

All primary data, or authenticated copies thereof, specimens and the final report will be retained in the Covance Laboratories Limited archives for **one** year after issue of the final report. At this time the Sponsor will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. Sponsors will be notified of the financial implications of each of these options at that time.

Specimens requiring storage deep frozen are specifically excluded from the above. These will be retained for as long as the quality of the material permits evaluation, but for no longer than three months after issue of the final report. The Sponsor will be notified of the intent to destroy samples and any financial implications before specimens are destroyed on their behalf.

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QUALITY ASSURANCE STATEMENT ..... **ERROR! BOOKMARK NOT DEFINED.**

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SUMMARY

A previous study (Covance study number 375/291, [redacted] reference number KC030431) showed that a 20-hour exposure to cytotoxic concentrations of [redacted] in the absence of S-9 induced structural, but not numerical, chromosome aberrations in cultured human peripheral blood lymphocytes. Slides prepared from cultures exposed to lower concentrations than those analysed, presumed to show little or no cytotoxicity, were not analysed previously. The objective of the present study was to investigate further the clastogenic potential of [redacted]

Slides analysed were from continuous treatment for 20 hours in the absence of S-9. Chromosome aberrations were analysed at three dose levels (see below) and the highest concentration chosen for analysis, 604.5 µg/mL induced approximately 29% mitotic inhibition.

S-9	Treatment + recovery (hours)	Vehicle control	Concentration (µg/mL) [redacted]
-	20+0	0[a]	436.8, 513.8, 604.5

[a] Vehicle control was purified water only

Appropriate negative (solvent) control cultures were included for analysis for comparison, but positive controls were omitted since Mitomycin C had already proved effective functioning of the test system at the time of treatment.

Treatment of cultures with 436.8-604.5µg/mL [redacted] in the absence of S-9 resulted in frequencies of cells with structural aberrations that were similar to those in concurrent negative controls. Numbers of aberrant cells (excluding gaps) in all treated cultures fell within historical negative control ranges.

It is concluded that [redacted] did not induce structural chromosome aberrations in cultured human peripheral blood lymphocytes, at the concentrations assessed in the additional analysis, under the experimental conditions described.

INTRODUCTION

A previous study (Covance study number 375/291, [redacted] reference number KC030431) showed that a 20-hour exposure to cytotoxic concentrations of [redacted] in the absence of S-9 induced structural, but not numerical, chromosome aberrations in cultured human peripheral blood lymphocytes (1). Slides prepared from cultures exposed to lower concentrations, presumed to show little or no cytotoxicity, were not analysed. The test system has been described previously.

The objective of the present study was to investigate further the clastogenic potential of [redacted] after a 20-hour exposure in the absence of S-9, which was the only condition where a positive response was observed.

Slides from cultures exposed to 711.2, 836.7 and 984.4 µg/mL [redacted] were analysed for chromosome damage previously, along with those exposed to vehicle (purified water) and Mitomycin C, the positive control. The clastogenic potential of lower concentrations of [redacted] than those analysed previously were evaluated, using slides from the 20-hour exposure in the absence of S-9. Vehicle control slides were also analysed for comparison, but not positive controls, since Mitomycin C has already proved effective functioning of the test system at the time of treatment.

With the exception of the minor deviation detailed in Appendix 5, which in no way prejudiced the validity of the study, this study was performed according to the protocol and one amendment.

The study was initiated on 24 August 2004. Experimental work started on 9 September 2004 and was completed on 16 September 2004. The study completion date is considered to be the date on which the Study Director signs the final report.

MATERIALS

Test article

[redacted] sample number S2539801, a [redacted] was not actually used in this study, but details are included for reference purposes. It was received on 26 November 2003 and stored at room temperature in the dark. Purity and expiry date were not supplied (these data are the subject of a separate GLP compliant study conducted by the Sponsor, [redacted] Study Reference Number AC030449).

The concentrations to which the cells were exposed for 20-hours in the absence of S-9 mix in Experiment 2 in the previous study (1) were:

Concentration of treatment solution (mg/mL)	Final concentration (µg/mL)
4.368	**436.8**
5.138	**513.8**
6.045	**604.5**
7.112	711.2
8.367	836.7
9.844	984.4
11.58	1158
13.62	1362
16.03	1603
18.86	1886
22.19	2219
26.10	2610
30.71	3071
36.13	3613
42.50	4250
50.00	5000

Bold text indicates additional concentrations selected for analysis in this study.
Shading indicates concentrations selected for chromosome analysis in previous Covance study number 375/291[redacted]eference number KC030431.

Controls

Vehicle control slides were analysed for comparison, but not positive controls, since Mitomycin C has already proved effective functioning of the test system at the time of treatment.

METHODS

Treatment

Quadruplicate cultures (A, B, C and D) were treated in the absence of S-9 for 20 hours with purified water and duplicate cultures treated with the test article at appropriate concentrations. Cultures using blood from female donors retained treatment medium through to harvest, 20 hours after the start of treatment.

Selection of doses for chromosome analysis

Slides were examined for mitotic index (MI) or percentage of cells in mitosis based on at least 1000 cells counted. Slides from dose levels 0, 436.8, 513.8 and 604.5 µg/mL were scored to determine whether chemically induced mitotic inhibition had occurred. This is defined as a clear decrease in mitotic index compared with negative controls.

Scoring of aberrations

Slides from the selected test article treatments and from solvent controls that were not analysed previously (replicates C and D) were coded using randomly generated letters by a person not connected with the scoring of the slides. Labels bearing only the study reference number, experiment number, the sex of the donor and the code were used to cover treatment details on the slides.

One hundred metaphases from each code were analysed for chromosome aberrations. Only cells with 44-46 chromosomes were considered acceptable for analysis of structural aberrations. Any cell with 47-68 chromosomes, that is hyperdiploid cells, observed during this search was noted and recorded separately. Classification of structural aberrations was based on the scheme described by ISCN (2) and is detailed in Appendix 2. Under this scheme, a gap is defined as a discontinuity less than the width of the chromatid and no evidence of displacement of the fragment and a deletion is defined as a discontinuity greater than the width of the chromatid and/or evidence of displacement of the fragment. Observations (summarised in Appendix 3) were recorded on raw data sheets with the microscope stage co-ordinates of any aberrant cell.

Slide analysis was performed by competent analysts trained in the applicable Covance Laboratories Harrogate (CLEH) standard operating procedures. Although physically located remote from the CLEH facility, all analysts participating in this study were subject to CLEH management and GLP control systems, and all slides and raw data were returned to CLEH for archiving on completion of their analysis.

Analysis of results

Treatment of data

After completion of microscopic analysis, data were decoded. The aberrant cells in each culture were categorised as follows:

1. cells with structural aberrations including gaps

2. cells with structural aberrations excluding gaps

3. polyploid, endoreduplicated or hyperdiploid cells.

The totals for category 2 in negative control cultures were compared with the current laboratory negative control (normal) ranges to determine whether the assay was acceptable or not. The proportion of cells in category 2 in test article treated cultures were also compared with normal ranges. The statistical significance of any data set was only to be taken into consideration if the frequency of aberrant cells in both replicate cultures at one or more concentration exceeded the normal range. Under this condition, the statistical method used would be Fisher's exact test (3). Probability values of $p \leq 0.05$ were to be accepted as significant. The proportions of cells in categories 1 and 3 were also examined in relation to historical negative control (normal) ranges and statistical analysis by Fisher's exact test may be used.

The proportions of aberrant cells in each replicate were also used to establish acceptable heterogeneity between replicates by means of a binomial dispersion test (3). Probability values of $p \leq 0.05$ were to be accepted as significant.

Acceptance criteria

The human lymphocyte assay is considered valid if the following criteria are met:

1. the binomial dispersion test demonstrates acceptable heterogeneity between replicate cultures, and

2. the proportion of cells with structural aberrations (excluding gaps) in negative control cultures falls within the normal range, and

3. at least 160 cells out of an intended 200 are analysable at each dose level

Evaluation criteria

The data would be interpreted in conjunction with previous results. Cells with exchange aberrations or cells with greater than one structural aberration occur very infrequently in negative control cultures. Their appearance will therefore be considered to be of particular biological significance.

RESULTS

Selection of doses for cytogenetic analysis

The results of mitotic index determinations were as follows:

Treatment (µg/mL)	Mitotic index (%) 20+0 hours, -S-9 A/C	B/D	MIH*
Solvent	10.2/9.4	10.3/7.8	-
436.8	3.8	4.2	58
513.8	6.7	8.9	17
604.5	6.3	7.0	29

*Mitotic inhibition (%) = [1 - (mean MI_T/mean MI_C)] x 100%
(where T = treatment and C = negative control)
(Slides from solvent control cultures A and B scored for mitotic index only)
A/C, B/D refers to the number of cultures treated (four [A, B, C, D] for vehicle controls and two [A, B] for test article)

The following doses were selected for analysis:

	Mitotic inhibition†
20+0 hours, -S-9: 436.8, 513.8, 604.5 µg/mL	29%

† At highest concentration analysed (58% after 436.8 µg/mL)

Chromosome aberration analysis

Raw data

The raw data for the observations on the test article plus positive and negative controls are retained by Covance Laboratories Limited. A summary of the number of cells containing structural aberrations is given in Appendix 1. The numbers and types of structural aberrations seen per cell are given in Appendix 3.

Validity of study

The data in Appendix 1 and Appendix 3 confirm that:

1. no evidence of significant heterogeneity between replicate cultures was obtained in the binomial dispersion test (Appendix 1), and

2. the proportion of cells with structural aberrations (excluding gaps) in negative control cultures fell within the normal range (Appendix 4), and

3. 200 cells were analysed at each dose level (Appendix 1).

Analysis of data

Treatment of cultures with 436.8-604.5 µg/mL [redacted] in the absence of S-9 resulted in frequencies of cells with structural aberrations that were similar to those in concurrent negative controls (Appendix 1 and Appendix 3). Numbers of aberrant cells (excluding gaps) in all treated cultures fell within historical negative control ranges (expressed as the 95 percentile of the historical control values recorded in the laboratory over an extensive period, results presented in Appendix 4).

CONCLUSION

[redacted] did not induce structural chromosome aberrations in cultured human peripheral blood lymphocytes, at the concentrations assessed in the additional analysis, under the experimental conditions described.

REFERENCES

1. Lloyd M (2004) [redacted] Induction of chromosome aberrations in cultures human peripheral blood lymphocytes. Covance report number 375/291-D6172, [redacted] study reference number KC030431.

2. ISCN (1995) An International System for Human Cytogenetic Nomenclature. Editor Felix Mitelman; S Karger, Switzerland.

3. Richardson C, Williams D A, Allen J A, Amphlett G, Chanter D O and Phillips B (1989) Analysis of data from *in vitro* cytogenetic assays. In "Statistical Evaluation of Mutagenicity Test Data", (UKEMS Guidelines Sub-committee Report, Part III), Ed D J Kirkland, Cambridge University Press, pp 141-154.

APPENDICES

Appendix 1
[redacted] cells with structural aberrations

Table 1
20 hour treatment -S-9, 0 hour recovery (20+0)
Donor sex: female

Treatment (µg/mL)	Replicate	Cells Scored	Cells with Aberrations Including Gaps	Cells with Aberrations Excluding Gaps	Mitotic Index (mean)
Solvent	A	ND	ND	ND	10.2
	B	ND	ND	ND	10.3
	C	100	1	1	9.4
	D	100	1	0	7.8
	Totals	200	2	1	(9.4)
436.8	A	100	1	0	3.8
	B	100	1	1	4.2
	Totals	200	2	1	(4.0)
513.8	A	100	0	0	6.7
	B	100	1	1	8.9
	Totals	200	1	1	(7.8)
604.5	A	100	4	2	6.3
	B	100	1	1	7.0
	Totals	200	5	3	(6.7)

Binomial Dispersion Test $\chi^2 = 3.35$, not significant
Note: solvent replicates A and B scored for mitotic index only
ND = not determined

Appendix 2
Abbreviations and classification of observations

abs	=	Aberrations
rep	=	Replicate
tot	=	Total

Gaps

csg	=	chromosome gap
ctg	=	chromatid gap

Chromosome deletions

del	=	chromosome deletion
d min	=	double minute
f	=	isolocus fragment

Chromosome exchanges

t	=	interchange between chromosomes (e.g. reciprocal translocation)
inv	=	chromosome intrachange (e.g. pericentric inversion)
dic	=	Dicentric
dic+f	=	dicentric with accompanying fragment
acr	=	acentric ring
r+f	=	centric ring with accompanying fragment
r	=	centric ring

Chromatid deletions

del	=	chromatid deletion
min	=	single minute

Chromatid exchanges

qr	=	interchange between chromatids of different chromosomes (e.g. quadriradial)
cx	=	obligate complex interchange
e	=	chromatid intrachange
tr/tr+f	=	isochromatid/chromatid interchange (triradial)/with accompanying fragment
su	=	intra-arm intrachange with sister union of broken ends
nud	=	intra-arm intrachange with non-union of broken ends distally
nup	=	intra-arm intrachange with non-union of broken ends proximally

Other structural aberrations

pvz	=	pulverised cell
mabs	=	multiple aberrations (greater than 7 aberrations per cell or too many aberrations to permit accurate analysis)

Numerical aberrations

E	=	Endoreduplicated
H	=	hyperdiploid (47-68 chromosomes)
P	=	polyploid (greater than 68 chromosomes)

Appendix 3

[redacted] summary of numbers and types of structural aberrations observed

Table 2
20 hour treatment -S-9, 0 hour recovery (20+0), Experiment 1
Donor sex: female

Treatment (µg/mL)	Rep	Cells *	G	Chr del	Chr exch	Ctd del	Ctd exch	Other	Abs +g	Abs -g
Solvent	C	100	0	1	0	0	0	0	1	1
	D	100	1	0	0	0	0	0	1	0
	Total	200	1	1	0	0	0	0	2	1
436.8	A	100	1	0	0	0	0	0	1	0
	B	100	0	0	0	1	0	0	1	1
	Total	200	1	0	0	1	0	0	2	1
513.8	A	100	0	0	0	0	0	0	0	0
	B	100	0	0	0	1	0	0	1	1
	Total	200	0	0	0	1	0	0	1	1
604.5	A	100	3	2	0	0	0	0	5	2
	B	100	0	1	0	0	0	0	1	1
	Total	200	3	3	0	0	0	0	6	3

* Total cells examined for structural aberrations
Totals given for each culture may differ from values given in Appendix 1 if cells are observed which have more than one aberration
G = gaps
For abbreviations and classification see Appendix 2

Appendix 4
Historical ranges for solvent controls

		Structural aberrations observed on 100 cells scored		Numerical aberrations observed during scoring of structural aberrations	
		Structural aberrations including gaps	Structural aberrations excluding gaps	Polyploid cells	Numerical aberrations (H+E+P)
-S-9	Number of studies	38	38	34	34
	Number of cultures	175	175	157	157
	Median	1.00	1.00	0.00	0.00
	Mean	1.61	0.99	0.20	0.36
	SD	1.47	1.19	0.49	0.61
	Observed range	0 - 8	0 - 7	0 - 3	0 - 3
	95% reference range	**0 - 5**	**0 - 4**	**0 - 2**	**0 - 2**

H = Hyperdiploid, E=Endoreduplicated, P = Polyploid
Reference ranges are calculated from percentiles of the observed distributions.

Calculated in February 2004 by CLEH Statistics, from audited report data of studies started after 1 January 2003.

Appendix 5
Minor deviation from protocol

Protocol section	Subject	Deviation
Methods	Scoring of aberrations	There was a limited analysis of numerical aberrations, using the normal procedure for chromosome analysis. The analysis of numerical aberrations was not specified in the protocol, as there was no evidence that [redacted] induced numerical aberrations previously (or in the present study). The data for numerical aberrations will not be presented in the report. This deviation has no adverse impact on the scientific integrity of the study.

Final Report

Study Title	[redacted] Induction of micronuclei in the bone marrow of treated rats
Author	L Williams BSc MSc
Sponsor	[redacted]
[redacted] Scientific Representative	Dr A Wolfreys
[redacted] Science Expert	Dr L Henderson
[redacted] Contact	Mrs E Selbie / Mrs C Talbot / Miss J Evans / Miss S Buljeeon
[redacted] Sponsor	M York
Test Facility	Covance Laboratories Ltd Otley Road, Harrogate North Yorkshire HG3 1PY ENGLAND
Principal Investigator and Analytical Test Site	C Sparham [redacted]
Covance Study Number	375/299
Covance Report Number	375/299-D6172
[redacted] Reference Number	KMN040131
Report Issued	May 2005
Page Number	1 of 58

STUDY DIRECTOR AUTHENTICATION AND GLP COMPLIANCE STATEMENT

[redacted] **Induction of micronuclei in the bone marrow of treated rats**

I, the undersigned, hereby declare that I have reviewed the work and generated data and found them to be scientifically acceptable and valid. I further declare that the report provides a true and accurate record of the results obtained.

The study was performed in accordance with the agreed protocol and with Covance Laboratories Limited Standard Operating Procedures and the study objectives were achieved.

The study was conducted in compliance with the United Kingdom Good Laboratory Practice Regulations 1999, Statutory Instrument No. 3106 as amended by the Good Laboratory Practice (Codification Amendments Etc.) Regulations 2004 and the OECD Principles on Good Laboratory Practice (revised 1997, issued January 1998) ENV/MC/CHEM (98) 17, with the exception that the supporting data for purity and expiry date of the test item were not available to the Study Director at the time of finalising this report. However, these data are the subject of a separate GLP compliant study conducted by the Sponsor [redacted] Study reference Number AC030449.

L. Williams
L Williams BSc MSc
Study Director

17 May 2005
Date

QUALITY ASSURANCE STATEMENT

[redacted] **Induction of micronuclei in the bone marrow of treated rats**

This study* has been reviewed by the Quality Assurance Unit of Covance Laboratories Ltd. and the report accurately reflects the raw data. The following inspections were conducted and findings reported to the study director (SD) and associated management.

Critical procedures, which are performed routinely in an operational area, may be audited as part of a "process" inspection program. This can be in addition to phases scheduled on an individual study basis. Selected process inspections conducted and considered applicable to this study are included below.

* Excluding Appendix 8 which is the responsibility of [redacted] Quality Assurance.

Inspection Dates		Phase	Date Reported to SD and SD Management
From	To		
27 Apr 2004	27 Apr 2004	Protocol Review	27 Apr 2004
17 May 2004	17 May 2004	Protocol Amendment Review	17 May 2004
04 Aug 2004	04 Aug 2004	Draft Report and Data Review	09 Aug 2004
27 Aug 2004	27 Aug 2004	Protocol Amendment Review	27 Aug 2004
29 Sep 2004	30 Sep 2004	Draft Report and Data Review	30 Sep 2004
18 Nov 2004	18 Nov 2004	Protocol Amendment Review	18 Nov 2004
17 May 2005	17 May 2005	Final Report Review	17 May 2005

Process

Inspection Dates		Phase	Date Reported to SD and SD Management
From	To		
16 Apr 2004	16 Apr 2004	Slide Preparation	16 Apr 2004
21 Apr 2004	21 Apr 2004	Treatment	21 Apr 2004
21 Apr 2004	23 Apr 2004	Slide Analysis	23 Apr 2004
27 Apr 2004	27 Apr 2004	Animal Arrival	27 Apr 2004
27 Apr 2004	27 Apr 2004	Dose Preparation	27 Apr 2004
04 May 2004	04 May 2004	Stock Solution Preparation	04 May 2004
07 May 2004	07 May 2004	Harvest	07 May 2004
18 May 2004	18 May 2004	Slide Analysis	19 May 2004
19 May 2004	19 May 2004	Dose Preparation	19 May 2004
27 May 2004	27 May 2004	Slide Analysis	27 May 2004
01 Jun 2004	01 Jun 2004	Slide Decoding	01 Jun 2004
15 Jun 2004	15 Jun 2004	Dose Preparation	15 Jun 2004

D Allison BSc (Hons) MSc
QA Team Manager

14/05/05
Date

REVIEWING SCIENTIST'S STATEMENT

[redacted] **Induction of micronuclei in the bone marrow of treated rats**

I, the undersigned, hereby declare that I have reviewed this report in conjunction with the Study Director and that the interpretation and presentation of the data in the report are consistent with the results obtained.

J Whitwell

J Whitwell BSc
Scientist

13-5-05
Date

RESPONSIBLE PERSONNEL

[redacted] **Induction of micronuclei in the bone marrow of treated rats**

The following personnel were responsible for key elements of the study:

Study Director (from 8 November 2004 to the date of final report)	L Williams BSc MSc
Study Director (from 16 April 2004 to 8 November 2004)	C Beevers BSc PhD
Animal House	L Cooper IAT
Principal Investigator, formulations and plasma analysis	C Sparham[1]
Study Supervisor	S McGarry BSc

1 [redacted]

ARCHIVE STATEMENT

[redacted] Induction of micronuclei in the bone marrow of treated rats

All primary data, or authenticated copies thereof, generated at Covance Laboratories Limited and the final report will be retained in the Covance Laboratories Limited archives for **one** year after issue of the final report. At this time the Sponsor will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. Sponsors will be notified of the financial implications of each of these options at that time.

Specimens requiring storage deep frozen are specifically excluded from the above. These will be retained for as long as the quality of the material permits evaluation, but for no longer than three months after issue of the final report. The Sponsor will be notified of the intent to destroy samples and any financial implications before specimens are destroyed on their behalf.

Archiving of the test article is the responsibility of the Sponsor.

The raw data generated at [redacted] will be archived at Datacare Business Management Systems, 3012 Heyford Park, Upper Heyford, Buckinghamshire, OX25 5HF. Datacare is not a member of the UK GLP compliance programme. In order to assure GLP compliance, the [redacted] Quality Assurance function performs annual audits of the site according to specific SOPs. The UK Medicines and Healthcare Products Regulatory Agency have been informed of [redacted] use of the contract facility. The [redacted] Archivist manages the flow of data to and from the control facility (deposits and loans). The Archivist also manages the culling programme.

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SUMMARY

[redacted] was assayed *in vivo* in a rat bone marrow micronucleus test at three dose levels. The choice of dose levels was based on an initial toxicity range-finding study in which [redacted] formulated in purified water was administered to rats via oral gavage. The test article was administered once daily on two consecutive days to groups of three male and three female rats at doses covering the range 200 to 390 mg/kg/day. Observations were made over a 2 day period following the second administration and signs of toxicity recorded. Doses of 280 mg/kg/day and above were lethal and therefore 200 mg/kg/day was selected as the maximum dose for this study.

In the main study, [redacted] was formulated as described and administered at 50, 100 and 200 mg/kg/day to groups of six male and six female rats killed 24 hours after the second administration. One animal who received the highest dose of [redacted] died prior to the scheduled sampling times indicating that it would not have been reasonable to administer the test article at an appreciably higher dose.

In addition to the main study animals, groups of three male and three female animals were dosed twice with either vehicle control or 200 mg/kg/day [redacted] Blood samples were taken approximately 30 minutes, 1, 2 and 4 hours after the last dose administration and plasma isolated for analysis of [redacted] concentration. Plasma concentrations peaked at the first sampling time (0.5 hours) with a mean of 1.59 µg/mL in the females and 1.34 µg/mL in the males. This provides evidence of systemic exposure to [redacted] in addition to the clinical signs of toxicity observed following treatment with the test article.

The negative (vehicle) control in the study was purified water also administered via oral gavage once daily on two consecutive days. A group of six male and six female rats treated with this were killed and sampled 24 hours after the second administration. Cyclophosphamide (CPA), the positive control, was dissolved in saline and administered via oral gavage as a single dose of 20 mg/kg/day to a group of six male and six female rats which were killed after 24 hours. Samples were taken from each formulation on each day of dosing to allow for analysis of achieved dosing concentration [redacted] and the purified water control solutions only). All samples were returned to the Principal Investigator for analysis. Achieved concentrations were within 5% of the nominal concentration.

Negative (vehicle) control rats exhibited normal group mean frequencies of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) and normal frequencies of micronucleated PCE consistent with historical negative control (normal) data. Positive control animals exhibited increased numbers of micronucleated PCE such that the frequency in the positive control group was significantly greater than in concurrent controls.

Rats treated with [redacted] at all doses exhibited group mean % PCE and % micronucleated PCE values that were similar to the concurrent vehicle control group. There were no instances of statistically significant increases in micronucleus frequency for any of the groups receiving the test article.

It is concluded that [redacted] did not induce micronuclei in the polychromatic erythrocytes of the bone marrow of male and female rats treated up to 200 mg/kg/day (the maximum tolerated dose for this study) via oral gavage. [redacted] was detected in the plasma following treatment at this dose and clinical signs of toxicity were observed, providing evidence for the systemic exposure of the rats to the test article.

TEST SYSTEM

Introduction

Chromosome defects are recognised as being the basis of a number of human genetic diseases (1). Although there are a number of assays that detect genotoxic chemicals, there are relatively few that detect chemicals which either interfere with the process of mitosis or cause chromosomal aberrations.

Chemicals can be tested *in vitro* for their ability to produce chromosome aberrations in cells with which they have direct contact in a static system. Chemicals may react differently *in vivo*, however, where metabolic systems other than liver cytochrome P448/P450 operate, and where the dynamic processes of absorption, metabolism and excretion are involved. Chemicals should therefore also be tested for chromosome damaging activity *in vivo*.

In the bone marrow micronucleus test (2, 3, 4) erythroblasts undergoing their last chromosome replication are the target cell. Chromosome fragments or whole chromosomes, which are unable to attach to the spindle, are left behind as micronuclei when the main nucleus is extruded in the production of the polychromatic erythrocyte (PCE). Micronuclei can be clearly seen in these cells and are more easily counted than structural chromosome aberrations in metaphase cells. Because they are also formed from intact chromosomes, agents which affect spindle formation or function (that is, result in aneuploidy) can be detected.

With the exception of the minor deviations detailed in Appendix 9, none of which in any way prejudice the validity of the study, this study was performed according to the protocol and three amendments.

The study was initiated on 16 April 2004. Experimental work started on 26 April 2004 and was completed on 15 September 2004. The study completion date is considered to be the date on which the Study Director signs the final report.

Justification and rationale for study design

The objective of this study was to evaluate the clastogenicity/aneugenicity of [redacted] *in vivo* by examining micronuclei in the PCE of rat bone marrow.

The micronucleus test in the rat has been used in a number of collaborative trials, and correlates quite well with other systems as long as high enough doses and multiple sampling times are used.

The test article was given as two administrations, 24 hours apart and animals were sampled 24 hours after the final administration. In this way, cells exposed to the test article over the period 24 to 48 hours prior to sampling were examined. This has been shown to be of sufficient duration for the expression of any genotoxic potential (5, 6, 7, 8).

All treatments were given via oral gavage in order to maximise exposure of the target organ to the test article and to enable comparison with other toxicology studies performed orally.

The Sponsor provided the following information on the *in vivo* toxicity of [redacted] In an acute oral toxicity study in fasted female rats, doses of [redacted] at 200 and 300 mg/kg/day caused mortality within 1 hour of dose administration. No mortality or clinical signs were observed at 50 mg/kg/day. In a seven day repeat dose study no clinical signs were observed at 150 mg/kg/day.

This information was used to select appropriate doses for administration in a range-finder experiment, which were used to select an appropriate maximum dose level for the micronucleus test, in accordance with current recommendations (9, 10).

Both male and female rats were used in this study. Contradictory results were obtained in two *in vitro* chromosome aberration assays performed using two different batches of test article. One of the few differences between the two experiments was the sex of the blood donors used. In order to enable a comparison of the *in vitro* data with the *in vivo* micronucleus assay, it is considered appropriate to treat both male and female animals in this study.

Regulatory test guidelines

The test methodology used in this study was in accordance with current literature (11, 12,) and the protocol was designed to meet the known requirements of the OECD Guideline 474, 1997 (9) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests, 1995 (13).

MATERIALS

Animals and husbandry

All procedures carried out on live animals as part of this study were subject to the provisions of United Kingdom National Law, in particular the Animals (Scientific Procedures) Act, 1986.

A sufficient number of out-bred young adult male and female Han Wistar Crl:WI (Glx/BRL/Han) IGS BR rats were obtained from Charles River UK Ltd, Margate, UK. They were housed in groups of no more than four animals of the same sex in solid-floored cages, cleaned and dried before use, with Aspen wood chips (Datesand Ltd, Manchester) for bedding. Bottled water (public supply) and diet, (Special Diets Services Ltd, RM1.(E).SQC.) were provided *ad libitum*. Additionally, in order to enrich the environment and enhance the welfare of the animals, they were provided with wooden Aspen chew blocks. All of the above are routinely monitored and are not known to contain any biological or chemical entity which might interfere with the test system.

Holding rooms were set to maintained a temperature of 19-25°C and relative humidity of 40-70%. The holding rooms were illuminated continuously by fluorescent light for 12 hours out of each 24 hour cycle and designed to receive at least 15 fresh air changes per hour.

Animals were acclimatised for at least 5 days prior to dosing and were identified by numbered ear-tag. Range-finder and satellite animals were allocated to groups of up to three but were not randomised. In the micronucleus experiment, rats were individually identified and randomised to groups of six animals of the same sex using a system of random numbers. Cages were then suitably labelled (using a colour coded procedure) to clearly identify the study number, cage number, start date, species and strain, together with a description of the treatment and dose level, route of administration and proposed time of necropsy. Checks were made on the first day of treatment to ensure group weights differed from the overall mean by no more than 5% (Appendix 2). The allocation of animals to groups is detailed in Appendix 3.

	Range Finder		Main study	
Number of animals used in study	9M	9F	42M	42F
Weight range on first day of assay (g)	204-235	167-202	153-203	162-204
Approximate age on first day of assay (weeks)	7-8	8-9	6-7	8-9
Acclimatisation period (days)	6-9		7	
Temperature range during assay†	19.5-20.9°C		19.7-21.3°C	
Humidity range during assay†	51-66%		55-75%	

† From receipt to last day of sampling
M Male
F Female

Test article

[redacted] sample number S2539801 was a [redacted] It was received on 15 April 2004 and stored at room temperature in the dark. Test article purity was to be assessed in GLP compliant [redacted] study AC030449 but was assumed to be 100% for the purpose of this study. The expiry date was to be assessed in [redacted] study AC030449.

Determinations of stability and characteristics of the test article were the responsibility of the Sponsor. Archiving a sample of the test article was the responsibility of the sponsor.

The stability and homogeneity of the test article in purified water was assessed in [redacted] study AH030452. A concentration range of 0.08 to 50 mg/mL was analysed and found to be stable for one week, when stored at 2-8°C.

Test article preparation

Sufficient volume of each test article formulation required for the main study was prepared on the first day of dosing. For each concentration of dose formulation, the test article was weighed into a tared glass vessel and the vehicle (purified water) added. The dose formulations were mixed on a magnetic stirrer for a minimum of 30 minutes at ambient temperature (10-30°C) and then separated into three equal aliquots (one for each day of dosing, plus one reserve). All aliquots were clearly labelled and stored refrigerated (1-10°C in the dark) until required.

Prior to dosing, one aliquot of each dose concentration was removed from the refrigerator and stirred on a magnetic stirrer for a minimum of 30 minutes prior to use in order to equilibrate to room temperature. Dose formulations were stirred continuously throughout the dosing period in order to maintain the homogeneity of the test article in the vehicle.

Formulations were prepared in the same way for range-finder treatments, with the exception that no reserve aliquot was retained. Although no stirring times were recorded for the range-finder test article preparations, they were documented as being clear solutions and were considered homogenous prior to dosing.

Dosing preparations were prepared at the concentrations specified in the table below. The test article preparations were protected from light, and used within 28¼ hours of initial formulation as follows:

Experiment	Concentration of dosing preparation (mg/mL)	Dose administered (mg/kg/day)
Range-finder	20	200
	28	280
	39	390
Main study	5	50
	10	100
	20	200

Analysis of achieved concentration

Samples of the vehicle control and test article formulations used in the main study were taken for analysis of achieved concentration, homogeneity and stability. Duplicate 10 mL samples were taken from the top, middle and bottom of each formulation and frozen (at -20°C) immediately. Samples were taken before dosing on day one and after dosing on day two (i.e. a total of 12 samples per concentration).

At the end of the dosing phase of the study, one set of the duplicate samples was shipped frozen (on dry ice) to the Principal Investigator. The second set of duplicate samples and any remaining dose preparations were retained at Covance Laboratories and will be disposed of on issue of the final report. The results of formulations analysis are given in Appendix 8.

Controls

The negative (vehicle) control was purified water.

Cyclophosphamide (CPA, Sigma Chemical Co, Poole, UK) was freshly dissolved in saline at 2 mg/mL to serve as the positive control.

METHODS

Dosing of animals

Animals were weighed before dosing and the volume of vehicle, test article preparation or positive control solution to be administered was calculated based on a dose volume of 10 mL/kg. Animals were not fasted prior to dosing. All treatments were given via oral gavage.

Range-finder

An initial range finding study was performed using groups of three male and three female rats treated with the test article at suitable doses. Animals were dosed once daily for two consecutive days with the test article and observations made over a two day period following the second administration. Clinical signs of toxicity and body weight gain over this period were recorded and a maximum acceptable dose (10) determined based on these data. This was used as the highest dose level in the main study.

Main study

Animals were dosed once daily for two consecutive days with the test article or vehicle. The positive control was given as a single administration at 20 mg/kg/day, on the second day of dosing.

Animals were treated in the main study as follows:

Treatment group	Dose administered (mg/kg/day) [a]	Dose volume (mL/kg)	Number of animals treated [b]
Vehicle	0	10	6M 6F
[redacted]	50	10	6M 6F
	100	10	6M 6F
	200	10	6M 6F
Positive control, CPA [c]	20	10	6M 6F

a Doses administered once daily for two consecutive days, approximately 24 hours apart (except positive control)
b Animals sampled 24 hours after final dose administration
c Cyclophosphamide; administered once only
M Male
F Female

Group weights are shown in Appendix 2 and individual body weights and dosages administered are shown in Appendix 3.

Blood sampling

In addition to the micronucleus test animals, groups of three male and three female satellite animals were dosed twice with either vehicle or [redacted] by the same dose route and at the same dose volume as described above. Plasma was isolated from these animals and was used to assess systemic exposure to the test compound.

Blood samples were taken approximately 0.5, 1, 2 or 4 hours after the second dose administration according to the table below:

Treatment group (mg/kg/day)	Number of animals dosed	Bleed time (hours after second administration) 0.5	1	2	4
Vehicle (purified water)	3M 3F	√		√*	
Vehicle (purified water)	3M 3F		√		√*
[redacted] (200)	3M 3F	√		√*	
[redacted] (200)	3M 3F		√		√*

M Male
F Female
√ Blood taken via lateral tail vein
√* Blood taken via cardiac puncture under terminal anaesthesia

Approximately 1 mL of whole blood was taken from the lateral tail vein (first sample per group) or via cardiac puncture under terminal anaesthesia (second sample per group) approximately 0.5, 1, 2 or 4 hours after the final test article administration. Blood was collected at room temperature into tubes containing lithium heparin. Samples were centrifuged (800 'g', 4°C, 10 minutes) after which plasma was separated into appropriately labelled tubes and stored frozen at –80°C nominal, prior to dispatch on dry ice to the PI at the analytical test site. The results of plasma analysis are given in Appendix 8.

Clinical signs

All animals were observed daily for signs of ill health or overt toxicity. An individual record was maintained of the clinical condition of each animal.

In the range-finder experiment, post-dosing observations were performed immediately after each dose administration, at least four times in the 4 hours following each administration and prior to the second dose. Observations were also recorded at least once on each of the non-dose administration days.

In the main study post-dosing observations were performed immediately after each dose administration, at least twice in the 4 hours following each dose and prior to the

second administration. Observations were also recorded at least once on the day of bone marrow sampling. In error, clinical observations were not recorded for any satellite animals (see minor deviations from protocol, Appendix 9).

Routine health checks

All animals were examined at the beginning and the end of the working day to ensure the animals were in good health. Three range-finder animals were killed *in extremis* as documented in the Results section.

Necropsy and slide preparation

Test article and vehicle treated rats were killed in groups, 24 hours after the second administration; CPA-treated rats were killed 24 hours after the single dose. Rats were killed by asphyxiation with carbon dioxide (subsequently ensured by cervical dislocation) in the same order as they were dosed.

A single femur from each animal was exposed, removed, cleaned of adherent tissue and the ends removed from the shank. Using a syringe and needle, bone marrows were flushed from the marrow cavity with 2 mL foetal bovine serum into appropriately labelled centrifuge tubes (one per animal).

The tubes were centrifuged (1250 x 'g', 2-3 minutes) and the serum was aspirated to leave one or two drops and the cell pellet. The pellet was mixed into this small volume of serum in each tube and from each tube a small volume of suspension was placed on the end of each of two slides labelled with the appropriate study number, sampling time, sex, date of preparation and tag number. The latter served as a code so analysis could be conducted "blind". A smear was made from the drop by drawing the end of a clean slide along the labelled slide.

Slides were allowed to air-dry and then fixed for 10 minutes in absolute methanol, followed by rinsing several times in distilled water. The slides were fixed and rinsed for a second time and were stained for 5 minutes in 12.5 µg/mL acridine orange (see minor deviations from protocol, Appendix 9) made up in 0.1 M phosphate buffer pH 7.4. Slides were rinsed in phosphate buffer and allowed to dry. When dry, slides were mounted with coverslips.

Scoring

Slides from the CPA-treated rats were initially checked at Covance Laboratories Limited to ensure the system was operating satisfactorily. The slides from all control and dose groups were arranged in numerical order by sampling time and analysed by a person not connected with the dosing phase of the study.

Initially the relative proportions of polychromatic erythrocytes (PCE), seen as bright orange enucleate cells, and normochromatic erythrocytes (NCE), seen as smaller dark green enucleate cells, were determined until a total of at least 1000 cells (PCE plus NCE) had been analysed.

Counting continued (but of PCE only) until at least 2000 PCE had been observed. All PCE containing micronuclei observed during these two phases of counting were recorded. The vernier coordinates of all cells containing micronuclei were recorded to a maximum of six per slide.

Slide analysis was performed by an analyst trained in accordance with Covance Laboratories Limited Standard Operating Procedures. Details of the analyst are included in the raw data. All slides and raw data have been returned to Covance Laboratories Limited for archiving in accordance with the archive statement in this report.

In an initial analysis only 2000 PCE per animal were scored in error. The reserve slides were therefore stained and a further 2000 PCE were scored for each male animal. As the initial analysis resulted in unusual results for female animals, a further 4000 PCE (where possible) were scored from the reserve slides for female animals. The additional analysis resulted in data for a total of 4000 and 6000 PCE for male and female animals respectively and these results are presented in Appendix 6.

The data in Appendix 6 demonstrate high levels of heterogeneity, especially at the highest dose for both males and females, which made interpretation of the relevance of any increase in micronucleated PCE very complex and made comparisons between the sexes difficult. This was further exacerbated by the uneven numbers of PCE scored for each sex and it was therefore not considered possible to fully evaluate the data obtained. Consequently, the results obtained were set aside and the slides were rescored as described below.

For the vehicle control, 50 and 100 mg/kg test article-treated and positive control groups, one slide per animal was selected at random and a total of 4000 PCE scored.

For the top dose group (200 mg/kg) 4000 PCE were scored from each slide to give a total of 8000 PCE for both male and female animals. This second analysis provided less heterogeneous data and more even cell numbers between groups. These data (presented in Appendices 1, 4 and 5) were used for micronucleus assessment.

Analysis of results

Treatment of data

After completion of microscopic analysis and decoding of the data, the % PCE for each animal and the mean for each group was calculated. The individual and group mean frequency of micronucleated PCE ± standard deviation were also determined.

% PCE values were examined to see if there was any decrease in groups of treated animals that could be taken as evidence of bone marrow toxicity.

The frequencies of micronucleated PCE in vehicle control animals were compared with the historical negative control data to determine whether or not the assay was acceptable. For each group, inter-individual variation in the numbers of micronucleated PCE was estimated by means of a heterogeneity χ^2 test (14).

The numbers of micronucleated PCE in each treated group were then compared with the numbers in vehicle control groups by using a 2 x 2 contingency table to determine χ^2 (14). Probability values of $P \leq 0.05$ were to be accepted as significant. A further statistical test (for linear trend) was used to evaluate possible dose-response relationships.

If the heterogeneity χ^2 test provided evidence of significant ($P \leq 0.05$) variability between animals within at least one group, non-parametric analysis was more appropriate. Provision was made to use the Wilcoxon rank sum test (15) under these circumstances. Although significant heterogeneity was observed within some groups when the sexes were analysed separately, the combined data set demonstrated no significant heterogeneity and therefore it was considered unnecessary to perform this additional statistical test.

Acceptance criteria

The assay was considered valid if the following criteria were met:

1. the incidence of micronucleated PCE in the vehicle control group was consistent with the historical vehicle control data as given in Appendix 7, and

2. at least five animals out of each group were available for analysis, and

3. the positive control chemical (CPA) induced a statistically significant increase in the frequency of micronucleated PCE.

Evaluation criteria

A test article was considered as positive in this assay if:

1. a statistically significant increase in the frequency of micronucleated PCE occurred at least at one dose, and

2. the frequency and distribution of micronucleated PCE within the group at such a point exceeded the historical vehicle control data.

RESULTS

Selection of doses for main study

The range-finding study was performed with the following results.

Dose (mg/kg/day)	Animals dosed	Deaths	Observations
200	3M 3F	0	Day 1: Exophthalmus (3M 3F) Day 2-4: Normal (3M 3F).
280	3M 3F	1F	Day 1: Normal (3M 3F). Day 2: Normal (3M 2F). 1F was found dead 2 hours after dosing Day 3-4: Normal (3M 2F).
390	3M 3F	3M 3F	Day 1: Twitching, tremors, tachypnoea, exophthalmus (2M, 1F), animals subsequently killed *in extremis*. 1M 2F found dead within 30 minutes of dosing.

Animal observations from the range-finder experiment confirmed that there were no differences in toxicity between the sexes. Doses of 280 and 390 mg/kg/day were lethal to at least one out of six animals dosed. Therefore, although no notable clinical signs of toxicity were observed at 200 mg/kg/day, this was considered an estimate of the maximum tolerated dose for this study. The range-finder data were also consistent with observations made in previous studies with this compound (see Justification and rationale for study design section), further justifying the selection of 200 mg/kg/day as the top dose for both male and female animals in the main study.

Micronucleus assay

Raw data

The total numbers of PCE, NCE, and micronucleated PCE counted for each animal, together with calculated % PCE and % micronucleated (MN) PCE values appear in Appendix 4.

The data from Appendix 4 are summarised as group means in Appendix 1. Appendix 7 shows the current historical vehicle control data from micronucleus tests in this laboratory.

Validity of study

The acceptance criteria for the micronucleus test are listed in the acceptance criteria section. The data in Appendix 1, Appendix 4, and Appendix 7 confirm that:

1. the incidence of micronucleated PCE in the vehicle control group was consistent with the historical vehicle control data, and

2. at least five animals (males) out of each group were available for analysis, and

3. the positive control chemical (CPA) induced a statistically significant increase in the frequency of micronucleated PCE (Appendix 5). It is noted that one female animal (number 460) did not appear to respond to treatment with positive control. However, as all other animals demonstrated a clear increase in % MN PCE and as the group means were statistically significant compared to the concurrent vehicle control group, this non responding animal was not considered to have affected the validity of the study.

It is noted that the group mean % PCE values for male vehicle control and all test article treated animals fall outside of the laboratory's calculated historical control ranges, although they are within the observed range. This was considered a staining artefact as large numbers of highly fluorescent nucleated cells were present on the slides. These bright cells may mask the fluorescence of the duller NCE cells, making them difficult to score. Positive control treatments produced a characteristic decrease in % PCE compared to the vehicle control group; this is due to the cytotoxicity frequently induced by CPA. Furthermore, all micronucleated PCE frequencies for vehicle and positive controls were consistent with historical data, thus confirming that the staining artefact had not affected the scorer's ability to identify micronucleated PCE. The high % PCE values were therefore not considered to have affected the validity or integrity of the data generated.

Clinical signs

One female animal dosed at 50 mg/kg/day was observed to have chromodacryorrhoea prior to dosing on day 2. One male animal dosed at 200 mg/kg/day was found dead 2 hours after dosing on day 1. This mortality did not affect the integrity of the study, but did demonstrate that it would not have been appropriate to treat animals at higher than 200 mg/kg/day. No other clinical signs were observed in any other treated animal.

Achieved [redacted] concentrations in dose formulations and Plasma

The results of the formulations and plasma analysis are presented in Appendix 8.

Analysis of formulation samples taken before and after dosing confirmed that all preparations were both homogenous and stable during the dosing period and that the achieved concentrations were 100% ± 5% of nominal concentrations.

Analysis of plasma taken 30 minutes, 1, 2 or 4 hours after dosing confirmed animals were systemically exposed to the test article following administration of [redacted] at 200 mg/kg/day. Plasma concentrations peaked at the first sampling time (0.5 hours) with a mean of 1.59 µg/mL in females and 1.34 µg/mL in the males, and gradually decreased at later sampling times.

Analysis of micronucleus data

Groups of rats treated with [redacted] exhibited %PCE values that were similar to the vehicle controls (Appendix 1 and Appendix 4). There was no evidence of any test article-induced toxicity to the bone marrow (as would usually be indicated by a notable decrease in %PCE compared to the concurrent vehicle control groups).

Frequencies of micronucleated PCE in male and female animals were also similar to that seen in the concurrent vehicle control group (Appendix 1 and Appendix 4) and were consistent with the laboratory's historical control data (Appendix 9). The frequencies of micronucleated PCE observed in test article treated groups were not significantly different to the vehicle control group following χ^2 analysis (Appendix 5).

CONCLUSION

It is concluded that [redacted] did not induce micronuclei in the polychromatic erythrocytes of the bone marrow of male and female rats treated up to 200 mg/kg/day (the maximum tolerated dose for this study) via oral gavage. [redacted] was detected in the plasma following treatment at this dose and clinical signs of toxicity were observed, providing evidence for the systemic exposure of the rats to the test article.

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APPENDICES

Appendix 1
Summary of group mean data

Table 1

Treatment group (mg/kg/day)	Sex	%PCE	Group mean %MN PCE ± SD	
			Per sex	Per treatment group
Vehicle	M	69	0.06 ± 0.06	0.05 ± 0.05
	F	65	0.04 ± 0.02	
[redacted] (50)	M	71	0.05 ± 0.03	0.06 ± 0.05
	F	61	0.06 ± 0.06	
[redacted] (100)	M	73	0.06 ± 0.03	0.06 ± 0.03
	F	61	0.06 ± 0.04	
[redacted] (200)	M	69	0.06 ± 0.01	0.06 ± 0.03
	F	64	0.05 ± 0.04	
Positive control, CPA (20)	M	54	1.08 ± 0.20	0.79 ± 0.37
	F	44	0.50 ± 0.27	

PCE Polychromatic erythrocyte
MN Micronucleated
SD Standard deviation
CPA Cyclophosphamide; administered once only

Appendix 2
Total group weights

Table 2

Treatment (mg/kg/day)	Group number and sex	Total group weight (g)
Vehicle Control	1M	1074
	6F	1093
[redacted] (50)	2M	1103
	7F	1083
[redacted] (100)	3M	1103
	8F	1120
[redacted] (200)	4M	1064
	9F	1119
Positive control, CPA (20)	5M	1100
	10F	1079

Mean male group weight = 1089 g
Mean female group weight = 1099 g

CPA Cyclophosphamide; administered once only
M Male
F Female

Group weights checked on the first day of dosing to ensure weights of all groups differ from mean by no more than 5%.

Appendix 3
Body weights and dosages

Table 3 Male Data

Treatment (mg/kg/day)	Group	Animal number	Day 1 Body weight (g)	Day 1 Dose given (mL)	Day 2 Body weight (g)	Day 2 Dose given (mL)	Day 3 Body weight (g)
Vehicle	1	424	167	1.7	169	1.7	179
		414	174	1.7	178	1.8	185
		409	187	1.9	191	1.9	199
		423	153	1.5	157	1.6	163
		408	190	1.9	195	2.0	201
		425	203	2.0	208	2.1	214
[redacted]	2	403	191	1.9	197	2.0	201
[redacted] 50)		415	187	1.9	197	2.0	205
		411	190	1.9	199	2.0	203
		428	187	1.9	192	1.9	199
		402	180	1.8	187	1.9	194
		418	168	1.7	173	1.7	178
[redacted]	3	401	186	1.9	189	1.9	198
[redacted] 100)		422	186	1.9	192	1.9	197
		419	167	1.7	174	1.7	181
		417	196	2.0	198	2.0	210
		412	192	1.9	200	2.0	208
[redacted]	4	430	176	1.8	183	1.8	190
[redacted] 200)		405	188	1.9	192	1.9	200
		413	182	1.8	186	1.9	194
		427	186	1.9	189	1.9	200
		421	155	1.6	a	-	-
		406	182	1.8	183	1.8	193
		420	171	1.7	174	1.7	182
Positive control, CPA (20)	5	416	184	-	186	1.9	188
		429	184	-	187	1.9	194
		426	177	-	182	1.8	186
		410	177	-	177	1.8	187
		407	194	-	199	2.0	201
		404	184	-	191	1.9	193

CPA Cyclophosphamide; administered once only
a Animal died prior to the scheduled sampling time.

Table 4 Female Data

Treatment (mg/kg/day)	Group	Animal number	Day 1 Body weight (g)	Day 1 Dose given (mL)	Day 2 Body weight (g)	Day 2 Dose given (mL)	Day 3 Body weight (g)
Vehicle	6	451	191	1.9	188	1.9	193
		452	168	1.7	168	1.7	174
		456	179	1.8	181	1.8	186
		441	185	1.9	185	1.9	188
		433	174	1.7	177	1.8	176
		434	196	2.0	192	1.9	201
[redacted]	7	450	182	1.8	175	1.8	181
		436	170	1.7	167	1.7	173
(50)		446	194	1.9	199	2.0	203
		437	180	1.8	178	1.8	184
		440	162	1.6	169	1.7	173
		457	195	2.0	200	2.0	204
[redacted]	8	461	191	1.9	183	1.8	190
		448	181	1.8	182	1.8	183
(100)		458	186	1.9	188	1.9	187
		435	202	2.0	199	2.0	207
		438	181	1.8	182	1.8	184
[redacted]	9	459	179	1.8	183	1.8	191
		447	190	1.9	190	1.9	190
(200)		455	172	1.7	174	1.7	179
		444	190	1.9	187	1.9	193
		449	197	2.0	193	1.9	203
		445	194	1.9	189	1.9	184
		439	176	1.8	182	1.8	189
Positive control, CPA (20)	10	442	172	-	174	1.7	175
		453	165	-	171	1.7	171
		432	174	-	169	1.7	175
		443	180	-	179	1.8	181
		460	184	-	187	1.9	181
		454	204	-	204	2.0	201

CPA Cyclophosphamide; administered once only

Appendix 4
Individual animal data

Table 5

Treatment (mg/kg/day)	Animal Number	PCE Count	NCE Count	% PCE	Total PCE Count	MN PCE	% MN PCE
Vehicle	424 M	597	403	59.70	4000	2	0.05
	414 M	811	189	81.10	4000	0	0.00
	409 M	627	373	62.70	4000	3	0.08
	423 M	629	371	62.90	4000	1	0.03
	408 M	762	238	76.20	4000	1	0.03
	425 M	712	288	71.20	4000	7	0.18
	451 F	487	513	48.70	4000	3	0.08
	452 F	713	287	71.30	4000	1	0.03
	456 F	765	235	76.50	4000	1	0.03
	441 F	594	406	59.40	4000	1	0.03
	433 F	730	270	73.00	4000	2	0.05
	434 F	621	379	62.10	4000	1	0.03
[redacted] (50)	403 M	721	279	72.10	4000	3	0.08
	415 M	669	331	66.90	4000	4	0.10
	411 M	741	259	74.10	4000	2	0.05
	428 M	645	355	64.50	4000	1	0.03
	402 M	764	236	76.40	4000	1	0.03
	418 M	743	257	74.30	4000	1	0.03
	450 F	532	468	53.20	4000	6	0.15
	436 F	461	539	46.10	4000	2	0.05
	446 F	521	479	52.10	4000	5	0.13
	437 F	659	341	65.90	4000	1	0.03
	440 F	675	325	67.50	4000	0	0.00
	457 F	792	208	79.20	4000	1	0.03
[redacted] (100)	401 M	668	332	66.80	4000	4	0.10
	422 M	730	270	73.00	4000	1	0.03
	419 M	700	300	70.00	4000	3	0.08
	417 M	779	221	77.90	4000	3	0.08
	412 M	652	348	65.20	4000	3	0.08
	430 M	822	178	82.20	4000	1	0.03
	461 F	560	440	56.00	4000	3	0.08
	448 F	553	447	55.30	4000	4	0.10
	458 F	507	493	50.70	4000	0	0.00
	435 F	717	283	71.70	4000	3	0.08
	438 F	728	272	72.80	4000	4	0.10
	459 F	570	430	57.00	4000	1	0.03

PCE Polychromatic erythrocyte
NCE Normochromatic erythrocyte
MN Micronucleated

Table 5 (Contd)

Treatment (mg/kg/day)	Animal Number	PCE Count	NCE Count	% PCE	Total PCE Count	MN PCE	% MN PCE
[redacted] (200)	405 M	613	387	61.30	8000	3	0.04
	413 M	740	260	74.00	8000	6	0.08
	427 M	797	203	79.70	8000	5	0.06
	421 M	a	-	-	-	-	-
	406 M	768	232	76.80	8000	5	0.06
	420 M	520	480	52.00	8000	5	0.06
	447 F	583	417	58.30	8000	3	0.04
	455 F	649	351	64.90	8000	2	0.03
	444 F	530	470	53.00	8000	10	0.13
	449 F	848	152	84.80	8000	3	0.04
	445 F	633	367	63.30	8000	6	0.08
	439 F	620	380	62.00	4000	0	0.00
Positive control, CPA (20)	416 M	633	367	63.30	4000	46	1.15
	429 M	410	590	41.00	4000	49	1.23
	426 M	548	452	54.80	4000	54	1.35
	410 M	571	429	57.10	4000	36	0.90
	407 M	573	427	57.30	4000	34	0.85
	404 M	504	496	50.40	4000	39	0.98
	442 F	485	515	48.50	4000	12	0.30
	453 F	323	677	32.30	4000	34	0.85
	432 F	365	635	36.50	4000	16	0.40
	443 F	498	502	49.80	4000	19	0.48
	460 F	432	568	43.20	4000	8	0.20
	454 F	536	464	53.60	4000	32	0.80

PCE Polychromatic erythrocyte
NCE Normochromatic erythrocyte
MN Micronucleated
CPA Cyclophosphamide; administered once only
a Animal died prior to the scheduled sample time

Appendix 5
Statistical analysis of data

Table 6 Male Data

Treatment (mg/kg/day)	Cell Total	MN PCE	% MN PCE	Standard Deviation	Heterogeneity		2 x 2 Contingency	
					X^2	Significance	X^2	Significance
Vehicle	24000	14	0.06	0.06	13.44	P≤0.05		
[redacted] (50)	24000	12	0.05	0.03	4.00	NS	0.04	NS
[redacted] (100)	24000	15	0.06	0.03	3.00	NS	0.00	NS
[redacted] (200)	40000	24	0.06	0.01	1.00	NS	0.01	NS
Positive control, CPA (20)	24000	258	1.08	0.20			218.33	P≤0.001

Linear Trend: z = 0.264 NS
NS Not Significant
PCE Polychromatic erythrocyte
MN Micronucleated
CPA Cyclophosphamide; administered once only

Table 7 Female Data

Treatment (mg/kg/day)	Cell Total	MN PCE	% MN PCE	Standard Deviation	Heterogeneity		2 x 2 Contingency	
					X^2	Significance	X^2	Significance
Vehicle	24000	9	0.04	0.02	2.33	NS		
[redacted] (50)	24000	15	0.06	0.06	11.81	P≤0.05	1.04	NS
[redacted] (100)	24000	15	0.06	0.04	5.40	NS	1.04	NS
[redacted] (200)	44000	24	0.05	0.04	13.04	P≤0.05	0.52	NS
Positive control, CPA (20)	24000	121	0.50	0.27			95.03	P≤0.001

Linear Trend: z = 0.420 NS
NS Not Significant
PCE Polychromatic erythrocyte
MN Micronucleated
CPA Cyclophosphamide; administered once only

Table 8 Combined Data

Treatment (mg/kg/day)	Cell Total	MN PCE	% MN PCE	Standard Deviation	Heterogeneity		2 x 2 Contingency	
					X^2	Significance	X^2	Significance
Vehicle	48000	23	0.05	0.05	19.27	NS		
[redacted] (50)	48000	27	0.06	0.05	17.01	NS	0.18	NS
[redacted] (100)	48000	30	0.06	0.03	8.41	NS	0.68	NS
[redacted] (200)	84000	48	0.06	0.03	13.54	NS	0.28	NS
Positive control, CPA (20)	48000	379	0.79	0.37			314.81	P≤0.001

Linear Trend: z = 0.472 NS
NS Not Significant
PCE Polychromatic erythrocyte
MN Micronucleated
CPA Cyclophosphamide; administered once only

Appendix 6
Initial slide scores (Data not used for assessment of induction of micronuclei)

Table 9

Treatment (mg/kg/day)	Animal Number	PCE Count	NCE Count	% PCE	Total PCE Count	MN PCE	% MN PCE
Vehicle	424 M	627	373	62.70	4000	6	0.15
	414 M	806	194	80.60	2000	1	0.05
	409 M	628	372	62.80	4000	3	0.08
	423 M	637	363	63.70	4000	2	0.05
	408 M	727	273	72.70	4000	2	0.05
	425 M	742	258	74.20	4000	5	0.13
	451 F	482	518	48.20	6000	9	0.15
	452 F	704	296	70.40	6000	4	0.07
	456 F	751	249	75.10	6000	1	0.02
	441 F	603	397	60.30	6000	3	0.05
	433 F	717	283	71.70	6000	7	0.12
	434 F	536	464	53.60	6000	2	0.03
[redacted] (50)	403 M	738	262	73.80	4000	1	0.03
	415 M	522	478	52.20	4000	8	0.20
	411 M	746	254	74.60	4000	4	0.10
	428 M	670	330	67.00	4000	9	0.23
	402 M	776	224	77.60	4000	2	0.05
	418 M	732	268	73.20	4000	8	0.20
	450 F	554	446	55.40	6000	3	0.05
	436 F	452	548	45.20	6000	2	0.03
	446 F	541	459	54.10	6000	3	0.05
	437 F	620	380	62.00	6000	4	0.07
	440 F	701	299	70.10	6000	4	0.07
	457 F	770	230	77.00	6000	5	0.08
[redacted] (100)	401 M	651	349	65.10	4000	3	0.08
	422 M	705	295	70.50	4000	1	0.03
	419 M	735	265	73.50	4000	2	0.05
	417 M	730	270	73.00	4000	2	0.05
	412 M	667	333	66.70	4000	4	0.10
	430 M	790	210	79.00	4000	3	0.08
	461 F	585	415	58.50	6000	8	0.13
	448 F	590	410	59.00	6000	6	0.10
	458 F	484	516	48.40	6000	6	0.10
	435 F	734	266	73.40	6000	3	0.05
	438 F	726	274	72.60	6000	3	0.05
	459 F	591	409	59.10	6000	0	0.00

PCE Polychromatic erythrocyte
NCE Normochromatic erythrocyte
MN Micronucleated

Table 9 (Contd)

Treatment (mg/kg/day)	Animal Number	PCE Count	NCE Count	% PCE	Total PCE Count	MN PCE	% MN PCE
[redacted] (200)	405 M	578	422	57.80	4000	3	0.08
	413 M	811	189	81.10	4000	1	0.03
	427 M	824	176	82.40	4000	4	0.10
	421 M	a	-	-	-	-	-
	406 M	763	237	76.30	4000	13	0.33
	420 M	548	452	54.80	4000	14	0.35
	447 F	565	435	56.50	6000	8	0.13
	455 F	684	316	68.40	6000	4	0.07
	444 F	535	465	53.50	6000	8	0.13
	449 F	831	169	83.10	6000	8	0.13
	445 F	608	392	60.80	6000	8	0.13
	439 F	643	357	64.30	2000	0	0.00
Positive control, CPA (20)	416 M	651	349	65.10	4000	46	1.15
	429 M	519	481	51.90	4000	90	2.25
	426 M	562	438	56.20	4000	91	2.28
	410 M	582	418	58.20	4000	51	1.28
	407 M	577	423	57.70	4000	39	0.98
	404 M	487	513	48.70	4000	48	1.20
	442 F	526	474	52.60	6000	46	0.77
	453 F	324	676	32.40	6000	68	1.13
	432 F	404	596	40.40	2000	5	0.25
	443 F	557	443	55.70	6000	32	0.53
	460 F	433	567	43.30	6000	29	0.48
	454 F	520	480	52.00	6000	72	1.20

PCE Polychromatic erythrocyte
NCE Normochromatic erythrocyte
MN Micronucleated
CPA Cyclophosphamide; administered once only
a Animal died prior to the scheduled sample time

Appendix 7
Historical vehicle control data: Han Wistar Rat

Sex		Individual PCE %	Individual frequency of micronucleated PCE per 2000 (%)	Animals (%) with 0,1 (or more) micronuclei (for 2000 PCE scored)					
				0	1	2	3	4	5+
Males	Mean	58	0.87 (0.043)	41.0	36.2	18.1	4.8	0	0
	SD	10.3							
	Median	58							
	Observed range	33-83							
	95% confidence interval for group mean of:								
	4 values	48 – 69							
	5 values	49 – 68							
	6 values	50 – 67							
	7 values	50 – 67							
	8 values	51 – 66							
	9 values	51 – 66							
	10 values	51 – 65							

Calculated in September 2004 by CLEH Statistics, from audited report data for 105 males on 15 studies started between 1 May 2002 and 25 May 2004.

Due to the small number of data sets available for female animals, it was considered inappropriate to generated female specific historical data ranges. However, vehicle control values for female animals are consistent with male data and therefore, use of the male historical data for analysis of female data is considered acceptable.

Appendix 8

Achieved [redacted] concentrations in dose formulations and plasma

PRINCIPAL INVESTIGATORS REPORT:

[redacted]

INDUCTION OF MICRONUCLEI IN THE BONE MARROW OF TREATED RATS

(FORMULATIONS AND PLASMA ANALYSIS PHASE)

[redacted] **Study Reference Number KMN040131**
COVANCE STUDY No. 375/299

AUTHOR : CHRIS SPARHAM (PRINCIPAL INVESTIGATOR)

[redacted] **Confidential - Class 2 Circulation**

This report must not be circulated further, copied, or destroyed without reference to the Reports Administrator [redacted]

[redacted]

Date : July 2004

AUTHORISATION STATEMENT

Covance Study number	:	375/299
[redacted]tudy number	:	KMN040131
Study title	:	[redacted] Induction of micronuclei in the bone marrow of treated rats (Formulations and plasma analysis phase)

This Principal Investigator's report has been authorised for issue to the appropriate recipients.

N.R. Colson
NEIL COLSON
DEPARTMENT HEAD [redacted]

20/07/04
DATE

[redacted]

QUALITY ASSURANCE STATEMENT

[redacted] Reference Number : KMN040131

Study Title [redacted] Induction of Micronuclei in the bone marrow of treated rats

Phase of study : Formulation and Plasma Analysis.

This study was contracted to Covance Laboratories Ltd. (Covance Study Number 375/299)

The work for the above named phase of the study was conducted by [redacted]

Inspections and audits for this phase of the study were performed as detailed below and findings reported to the Principal Investigator and Facility Management. Significant findings affecting the main study are reported to the Study Director.

Audit Type	Audit Date	Report Date
Data Audit	09-Jul-2004	12-Jul-2004
Process Inspection - Measurement Procedures – Wt/Vol - Extraction - HPLC - LC/MS	05-Jan-2004	19-Mar-2004
Process Inspection - Measurement Procedures –Wt/Vol - Extraction - HPLC - LC/MS	04-May-2004	08-Jul-2004
Facility Inspection	27-Nov-2003	05-Dec-2003

As far as can reasonably be established, this document has been accepted by Quality Assurance as being an accurate presentation of the raw data and finding of the study.

20 July 2004.

H LALL
QUALITY ASSURANCE
[redacted]

DATE

AUTHENTICATION STATEMENT

Covance Study number	:	375/299
[redacted] Study number	:	KMN040131
Study title	:	[redacted] Induction of micronuclei in the bone marrow of treated rats (Formulations and plasma analysis phase)

I, the undersigned, hereby declare that the dose confirmation phase of the study has been conducted under my supervision, as Principal Investigator, in accordance with [redacted] policy on Good Laboratory Practice which is based on the UK Good Laboratory Practice Regulations 1999, Statutory Instrument No. 3106 (amended by the Good Laboratory Practice (Codification Amendments etc) Regulations 2004, Statutory Instrument No. 994) and OECD Principles on Good Laboratory Practice (as revised in 1997) ENV/MC/CHEM/ (98)17.

I also certify that this Principal Investigator's report presents a true and accurate account of the procedures used and the results obtained.

C Sparham — 20/7/04.

CHRIS SPARHAM
PRINCIPAL INVESTIGATOR

DATE

[redacted]

[redacted]
INDUCTION OF MICRONUCLEI IN THE
BONE MARROW OF TREATED RATS

(FORMULATIONS AND PLASMA ANALYSIS PHASE)

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[redacted] INDUCTION OF MICRONUCLEI IN THE BONE MARROW OF TREATED RATS

(FORMULATIONS AND PLASMA ANALYSIS PHASE)

SUMMARY

The aim of this phase of the study was to confirm satisfactory achieved concentration, homogeneity and stability at all levels of dosing using a method developed in [redacted] study number AH030452. Levels of [redacted] in plasma taken from the animals was also measured in a method developed as a course of this study.

Homogeneity (±5 % in the analysis of top, middle and bottom replicates), achieved concentration and stability (±5% of nominal) of the 5, 10 and 20 mg/mL dosing solutions were confirmed by high performance liquid chromatography/ ultra violet detection (HPLC/UV). Measured plasma concentrations in the range 0.600 – 1.97 µg/mL were determined by 100-fold dilution of plasma into mobile phase and analysis by liquid chromatography/mass spectrometry (LC/MS) in single ion monitoring mode (SIM) for *m/z* 634.2. The plasma method was demonstrated to give a mean recovery of >80% by analysis of Quality Control (QC) plasma samples spiked with [redacted] at the level of interest. Stability of the plasma samples under frozen storage conditions was demonstrated by equivalent storage and acceptable recovery obtained in the reanalysis of QC samples.

1. INTRODUCTION

The aim of this phase of the study was to confirm satisfactory achieved concentration, homogeneity and stability at all levels of dosing using a method developed in [redacted] study number AH030452. Levels of [redacted] in plasma taken from the animals was also measured in a method developed as a course of this study.

2. TEST ITEMS

The following samples were provided by Covance and analysed to provide dose confirmation.

Before dosing Day 1, sampled 18/5/04 (approximately 10 mL supplied)

[redacted]

Purified water (Top of formulation)
Purified water (Middle of formulation)
Purified water (Bottom of formulation)

After dosing Day 2, sampled 19/5/04 (approximately 10 mL supplied)

[redacted]

Purified water (Top of formulation)
Purified water (Middle of formulation)
Purified water (Bottom of formulation)

All samples were stored at –20 °C until dispatch in dry ice. Received in [redacted] on 27/5/04 and held frozen until analysis on 28/5/04.

The following plasma samples were taken by Covance after administration of the day 2 dose (19/5/04)

[redacted] Study KMN040131
Covance Study No 375/299

Animal number	Sex	Dose (mg/Kg)	Bleed time (hr)
471	female	200	0.5
472	female	200	0.5
473	female	200	0.5
483	female	200	1.0
484	female	200	1.0
485	female	200	1.0
471	female	200	2.0
472	female	200	2.0
473	female	200	2.0
483	female	200	4.0
484	female	200	4.0
485	female	200	4.0
465	female	0	0.5
466	female	0	0.5
467	female	0	0.5
477	female	0	1.0
478	female	0	1.0
479	female	0	1.0
465	female	0	2.0
466	female	0	2.0
467	female	0	2.0
477	female	0	4.0
478	female	0	4.0
479	female	0	4.0
468	male	200	0.5
469	male	200	0.5
470	male	200	0.5
480	male	200	1.0
481	male	200	1.0
482	male	200	1.0
468	male	200	2.0
469	male	200	2.0
470	male	200	2.0
480	male	200	4.0
481	male	200	4.0
482	male	200	4.0
462	male	0	0.5
463	male	0	0.5
464	male	0	0.5
474	male	0	1.0
475	male	0	1.0
476	male	0	1.0
462	male	0	2.0
463	male	0	2.0
464	male	0	2.0
474	male	0	4.0
475	male	0	4.0
476	male	0	4.0

All the above samples were stored frozen at –80°C, until dispatch on 24/5/04 in dry ice. Samples were received on 25/5/04 in [redacted] where they were held frozen (unless being thawed for analysis).

3. METHOD

3.1 Dose Confirmation

Sample preparation

The purified water samples (vehicle) were analysed directly. The 5, 10 and 20 mg/mL [redacted] dose concentrations were diluted 10, 20 and 50-fold respectively in Ultrapure water.

HPLC/UV

The main component peak area was measured by High Performance Liquid Chromatography with UV detection (HPLC/UV). The solutions were analysed by reversed phase HPLC by injecting 10µL onto a Waters Spherisorb S5 C_6 (250 x 4.6mm) column held at 35°C. An isocratic mobile phase, flowing at 1.5 mL/min., consisting of 35% acetonitrile and 65% ultrapure water containing 10 mM triethylamine and 10 mM octanesulphonic acid sodium salt was used for the analysis. The aqueous portion was made to pH 2.5 with o-phosphoric acid before adding the acetonitrile. Reagents were HPLC Grade or equivalent. Detection was by UV at 260 nm. Calibration standards, prepared in Ultrapure water, were run at concentrations of 0, 0.25, 0.50, 0.75 and 1.0 mg/mL. The calibration standards were made fresh on the day of analysis.

3.2 Plasma analysis

Sample preparation

[redacted] was spiked into plasma at 0, 1.5, 3 and 7.5 µg/mL using plasma of the Wistar Hanover type (Harlan Sera-Lab). It was also spiked at 3 µg/mL in pooled sample blanks from the 0 mg/Kg-dosed animals. These spiked samples were analysed on the day of analysis of the plasma samples and also after frozen storage for 10 days. This was to confirm frozen sample stability over a 9-day period.

100 µL of each plasma sample and spiked sample was diluted 100-fold in mobile phase, consisting of 5 mM ammonium formate pH3.0/acetonitrile (90/10 v/v). Analysis by LC/MS was then carried out as follows.

LC/MS

[redacted] concentrations were measured by LC/MS. The solutions were analysed by injecting 100µL onto an Atlantis C_{18} 5 µm (150 x 2.1mm) column held at 30°C. An isocratic mobile phase, flowing at 0.20 mL/min., consisting of 85% 5mM ammonium formate pH 3.0 (mobile phase A) and 15% acetonitrile (mobile phase B) was used for the analysis. Reagents were HPLC Grade or equivalent. The mass spectrometer was operated in positive electrospray ionisation (ESI) mode, with gas temperature 300°C, gas flow 11.0 L/min,

nebuliser pressure 30 psi and capillary voltage 3000V. Quantitation of [redacted] was carried out using single ion monitoring (SIM) for *m/z* 634.2. The calibration stock solution of [redacted] (S2539801) was prepared in Ultrapure water at 3000 mg/L (stable for 1 week in the fridge). Further dilutions in calibration standard preparation were carried out in mobile phase (90/10, A/B as for samples). Calibration standards were run at concentrations of 0, 0.0075, 0.015, 0.03, 0.045 and 0.09 µg/mL and were made fresh on each day of analysis from the stock solution.

3.3 Study dates

The analysis phase of the study including method development was conducted between 29 April and 07 June 2004.

3.4 Storage and retention of data

The protocol, any amendments, the raw data and final report will be placed in Datacare Business Management Systems, 3012 Heyford Park, Upper Heyford, Bucks, OX25 5HF. Datacare is not a member of the UK GLP compliance programme. The test item will be archived in [redacted]

4. RESULTS AND DISCUSSION

4.1 Dose Confirmation

Results are shown in Table 1. Homogeneity of the samples was shown by the good agreement obtained (± 5%) in replicates analysed from the top, middle and bottom of solutions. Achieved concentration and stability over the 2 day dosing period was also demonstrated with mean concentrations being within ±5% of the nominal, for the 5, 10 and 20 mg/mL [redacted] solutions.

A typical HPLC/UV chromatogram is shown in Figure 1.

4.2 Plasma analysis

Measured concentrations of [redacted] in plasma are shown in Table 2. Quality control data showing acceptable spike recovery and stability of [redacted] in plasma is shown in Table 3. Frozen stability for 10 days for quality control samples was also demonstrated.

A typical LC/MS (SIM) chromatogram is shown in Figure 2.

5. CONCLUSION

Homogeneity (±5 % in the analysis of top, middle and bottom replicates), achieved concentration and stability (±5% of nominal) of the 5, 10 and 20 mg/mL dosing solutions were confirmed by HPLC/UV. Measured plasma concentrations in the range 0.600 – 1.97 µg/mL were determined by 100-fold dilution of plasma into mobile phase and analysis by LC/MS (SIM for *m/z* 634.2). The plasma method was demonstrated to give a mean recovery of >80% by analysis of QC plasma samples spiked with [redacted] at the level of interest. Stability of the samples under frozen storage conditions was demonstrated by equivalent storage and acceptable recovery obtained in the reanalysis of QC samples.

TABLE 1. DOSE CONFIRMATION ANALYSIS

DOSE	REPLICATE	DOSING DAY (DATE)	MEASURED CONCENTRATION (mg/mL)	% OF NOMINAL
Vehicle	Top	1 (18/05/04)	<0.001	
Vehicle	Middle	1 (18/05/04)	<0.001	
Vehicle	Bottom	1 (18/05/04)	<0.001	
Vehicle	**Average**	**1 (18/05/04)**	**<0.001**	
5 mg/mL	Top	1 (18/05/04)	5.00	99.9
5 mg/mL	Middle	1 (18/05/04)	4.99	99.8
5 mg/mL	Bottom	1 (18/05/04)	4.97	99.3
5 mg/mL	**Average**	**1 (18/05/04)**	**4.98**	**99.7**
10 mg/mL	Top	1 (18/05/04)	9.58	95.8
10 mg/mL	Middle	1 (18/05/04)	9.63	96.3
10 mg/mL	Bottom	1 (18/05/04)	9.78	97.8
10 mg/mL	**Average**	**1 (18/05/04)**	**9.66**	**96.6**
20 mg/mL	Top	1 (18/05/04)	19.7	98.5
20 mg/mL	Middle	1 (18/05/04)	19.3	96.7
20 mg/mL	Bottom	1 (18/05/04)	20.3	101
20 mg/mL	**Average**	**1 (18/05/04)**	**19.8**	**98.8**
Vehicle	Top	2 (19/05/04)	<0.001	
Vehicle	Middle	2 (19/05/04)	<0.001	
Vehicle	Bottom	2 (19/05/04)	<0.001	
Vehicle	**Average**	**2 (19/05/04)**	**<0.001**	
5 mg/mL	Top	2 (19/05/04)	4.96	99.1
5 mg/mL	Middle	2 (19/05/04)	4.98	99.7
5 mg/mL	Bottom	2 (19/05/04)	5.00	99.9
5 mg/mL	**Average**	**2 (19/05/04)**	**4.98**	**99.6**
10 mg/mL	Top	2 (19/05/04)	9.61	96.1
10 mg/mL	Middle	2 (19/05/04)	9.59	95.9
10 mg/mL	Bottom	2 (19/05/04)	9.57	95.7
10 mg/mL	**Average**	**2 (19/05/04)**	**9.59**	**95.9**
20 mg/mL	Top	2 (19/05/04)	19.6	98.1
20 mg/mL	Middle	2 (19/05/04)	19.4	97.2
20 mg/mL	Bottom	2 (19/05/04)	19.7	98.3
20 mg/mL	**Average**	**2 (19/05/04)**	**19.6**	**97.9**

[redacted] Study KMN040131
Covance Study No 375/299

TABLE 2. PLASMA ANALYSIS

ANIMAL NO.	SEX	DOSE (mg/Kg)	BLEED TIME (HR)	MEASURED CONCENTRATION (µg/mL)
471	F	200	0.5	1.60
472	F	200	0.5	1.21
473	F	200	0.5	1.97
483	F	200	1.0	1.48
484	F	200	1.0	0.976
485	F	200	1.0	1.24
471	F	200	2.0	0.977
472	F	200	2.0	1.58
473	F	200	2.0	1.11
483	F	200	4.0	0.856
484	F	200	4.0	0.936
485	F	200	4.0	0.935
468	M	200	0.5	1.28
469	M	200	0.5	1.66
470	M	200	0.5	1.09
480	M	200	1.0	1.37
481	M	200	1.0	1.00
482	M	200	1.0	0.742
468	M	200	2.0	0.686
469	M	200	2.0	1.13
470	M	200	2.0	0.977
480	M	200	4.0	0.908
481	M	200	4.0	0.882
482	M	200	4.0	0.600
465	F	0	0.5	<0.02
466	F	0	0.5	<0.02
467	F	0	0.5	<0.02
477	F	0	1.0	<0.02
478	F	0	1.0	<0.15
479	F	0	1.0	<0.02
465	F	0	2.0	<0.02
466	F	0	2.0	<0.02
467	F	0	2.0	<0.02
477	F	0	4.0	<0.02
478	F	0	4.0	<0.02
479	F	0	4.0	<0.03
462	M	0	0.5	<0.02
463	M	0	0.5	<0.02
464	M	0	0.5	<0.02
474	M	0	1.0	<0.02
475	M	0	1.0	<0.02
476	M	0	1.0	<0.02
462	M	0	2.0	<0.02
463	M	0	2.0	<0.02
464	M	0	2.0	<0.02
474	M	0	4.0	<0.02
475	M	0	4.0	<0.02
476	M	0	4.0	<0.02

[redacted] Study KMN040131
Covance Study No 375/299

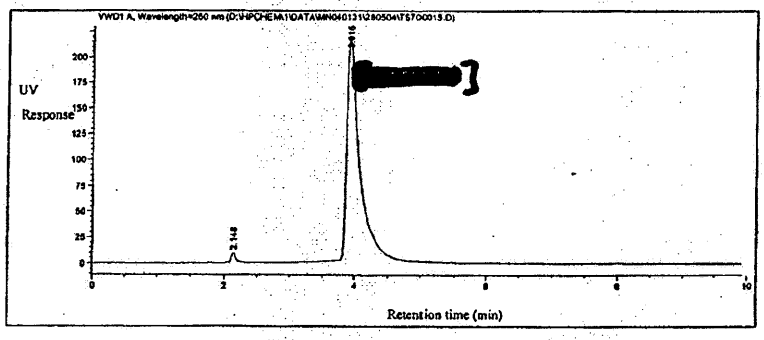
TABLE 3. PLASMA QUALITY CONTROL RESULTS

SAMPLE	REP	% OF NOMINAL CONCENTRATION (INITIAL)	% OF NOMINAL CONCENTRATION (STABILITY***)
1.5 µg/mL in plasma*	1	79.3	87.8
	2	79.9	86.8
3.0 µg/mL in plasma*	1	83.3	87.2
	2	85.6	86.7
7.5 µg/mL in plasma*	1	87.6	83.7
	2	88.8	83.6
3.0 ug/ml pooled**	1	85.8	89.0
	2	88.9	89.1

[redacted] concentrations in plasma from Harlan Sera-Lab
[redacted] concentrations in plasma pooled from sample blanks (0 mg/Kg-dosed animals)
***Quality Control samples re-analysed after 10 day frozen storage

[redacted] Study KMN040131
Covance Study No 375/299

FIGURE 1. TYPICAL HPLC/UV CHROMATOGRAM (5 mg/mL [redacted] IN PURIFIED WATER)



[redacted] Study KMN040131
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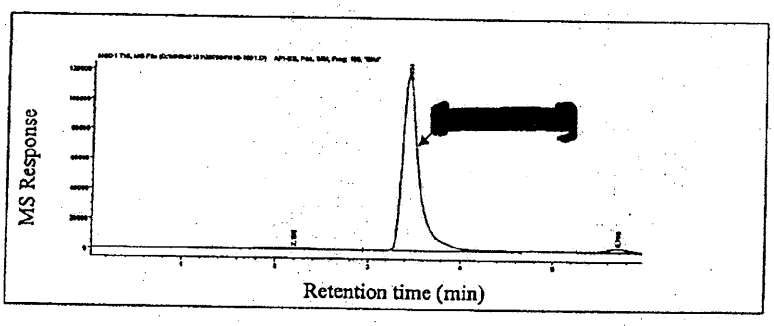
FIGURE 2. TYPICAL SAMPLE CHROMATOGRAM OF [redacted] IN PLASMA BY LC/MS (SIM *M/Z* 634.2)



Appendix 9
Minor deviations from protocol

Protocol section	Subject	Deviation
Methods	Slide staining	In error, slides were stained for 5 minutes and not 4 minutes as documented in the protocol. As all slides were easily scored this was not considered to have affected the validity of the study.
Methods	Clinical signs	In error clinical observations not recorded for satellite animals. As both the range-finder and main study confirmed that few if any clinical signs were observed with this test material, this deviation was not considered to have affected the validity of the study.

REPORT

Study Title:

ACUTE TOXICITY OF [redacted]
TO ZEBRA FISH (*DANIO RERIO*)
IN A 96-HOUR SEMI-STATIC TEST

Data Requirements / Test Guidelines:

OECD No. 203
EU Commission Directive 92/69/EEC, C.1

Study Director:

Roger Bätscher

Study Completion Date:

June 30, 2004

Test Facility:

RCC Ltd
Environmental Chemistry &
Pharmanalytics
CH–4452 Itingen / Switzerland

Sponsor:

[redacted]

Identification:

RCC Study Number: 851901
[redacted]hase Number: KAF030430

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RCC

GLP CERTIFICATE

The Swiss GLP Monitoring Authorities

Swiss Federal Office of Public Health | Swiss Agency for the Environment, Forests and Landscape | swissmedic Swissmedic Swiss Agency for Therapeutic Products

Statement of GLP Compliance

It is hereby confirmed that

during the period of November 18 – 22, 2002

the following Test Facilities of **RCC Ltd**
4452 Itingen
Switzerland

were inspected by the Federal Office of Public Health, the Swiss Agency for Therapeutic Products and the Swiss Agency for the Environment, Forests and Landscape with respect to the compliance with the Swiss legislation on Good Laboratory Practice.

Test Facilities	Areas of expertise *
- Toxicology	TOX, ACC, MUT, OTH (Safety Pharmacology)
- Environmental Chemistry and Pharmanalytics	ACC, ECT, ENF, EMN, PCT, RES, OTH (Animal metabolism)

The inspections were performed in agreement with the OECD Guidelines for National GLP Inspections and Audits. It was found that the aforementioned test facilities were operating in compliance with the Swiss Ordinance relating to Good Laboratory Practice [RS 813.016.5] at the time they were inspected.

Federal Office of Public Health
The Director

Bern, March 2003

Prof. Th. Zeltner

* TOX = Toxicology ; ACC = Analytical and Clinical Chemistry ; ECT = Environmental toxicity on aquatic and terrestrial organisms ; ENF = Behaviour in water, soil and air. Bioaccumulation ; EMN = Studies on effects on mesocosms and natural ecosystems; MUT = Mutagenicity; PCT = Physical-chemical testing ; RES = Residue studies ; OTH = Other, to be specified.

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE

RCC Study Number: 851901

Test Item: [redacted]

Study Director: Mr. Roger Bätscher

Study Title: Acute toxicity of [redacted]
to zebra fish (*Danio rerio*)
in a 96-hour semi-static test

This study (with the exception of the pre-experiments as mentioned in the report) has been performed in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted February 2nd, 2000 [RS 813.016.5]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C(97) 186/Final].

The purity and the expiry date of the test item were not available prior to administration, hence this information is excluded from the Statement of Compliance.

There were no circumstances that may have affected the quality or integrity of the data.

Study Director: Mr. Roger Bätscher

R. Bätscher

Date: June 30, 2004

SIGNATURES

Study Director: Mr. Roger Bätscher

Date: June 30, 2004

Management: Dr. Uwe Morgenroth

Date: June 30, 2004

QUALITY ASSURANCE

RCC Ltd, Environmental Chemistry & Pharmanalytics, CH-4452 Itingen / Switzerland

STATEMENT

Study Number: 851901

Test Item: [redacted]

Study Director: Mr. Roger Bätscher

Study Title: Acute toxicity of [redacted] to zebra fish (*Danio rerio*) in a 96-hour semi-static test

The general facilities and activities are inspected periodically and the results are reported to the responsible person and the management.

Study procedures (with the exception of the pre-experiments as mentioned in the report) were periodically inspected. The study plan and this report were audited by the Quality Assurance. The dates are given below.

Dates and Types of QA Inspections		Dates of Reports to the Study Director and to the Management
February 19, 2004	Study plan	February 19, 2004
May 05, 2004	Process based (application)	May 05, 2004
June 11, 2004	Report	June 11, 2004

This statement also confirms that this final report reflects the raw data.

Quality Assurance: for Mr. Henry Schneylin

D. Bürgi

Date: June 30, 2004

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ANALYTICAL PHASE REPORT

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PREFACE

GENERAL

Study Title: Acute toxicity of [redacted] to zebra fish (*Danio rerio*) in a 96-hour semi-static test

Sponsor: [redacted]

Sponsors Scientific Representative: [redacted]

[redacted] Contact: [redacted]

Test Facility: RCC Ltd
Environmental Chemistry & Pharmanalytics
Zelgliweg 1
CH-4452 Itingen / Switzerland

Lead QA: Quality Assurance RCC Ltd
Environmental Chemistry & Pharmanalytics
Zelgliweg 1
CH-4452 Itingen / Switzerland

Test Site (Analytical Phase): [redacted]

Test Site QA: [redacted]

[redacted] Phase Number: KAF030430

RESPONSIBILITIES

Study Director:	Mr. Roger Bätscher
Deputy Study Director:	Dr. Armin Peither
Technical Coordinator:	Mr. Christian Stier
Head of Lead Quality Assurance:	Mrs. Iris Wüthrich
Principal Investigator Analytical Phase:	Mr. Chris Sparham [redacted]

SCHEDULE

Experimental Starting Date:	February 23, 2004
Experimental Completion Date:	March 06, 2004
Study Completion Date:	June 30, 2004

ARCHIVING

RCC Ltd, CH-4452 Itingen, Switzerland will retain the study plan, raw data, a sample of the test item and the final report of the present study for at least ten years. Any unused test item will be returned to the sponsor. Archiving of the test item is responsibility of the sponsor.

No data will be discarded without the Sponsor's consent.

The raw data generated by the analytical phase of the study will be archived for at least 10 years at Datacare Business Management Systems, 3012 Heyford Park, Upper Heyford, Buckinghamshire, OX25 5HF, UK. Datacare is not a member of the UK GLP compliance programme. However, the [redacted] Quality Assurance performs annual audits of Datacare according to specific SOPs which have been approved by the UK GLP compliance programme. The UK Medicines and Healthcare products Regulatory Agency have been informed of [redacted] use of the contract facility. The [redacted] archivist manages the flow of data to and from the contract facility (deposits and loans). The archivist also manages the culling programme.

DATA REQUIREMENTS / TEST GUIDELINES

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

OECD Guideline for Testing of Chemicals, No. 203, Fish, Acute Toxicity Test, 1992

EU Commission Directive 92/69/EEC, Part C.1: Acute Toxicity for Fish, 1992

SUMMARY OF STUDY PLAN AMENDMENTS

There were no amendments to study plan.

SUMMARY

The acute toxicity of the test item [redacted] to zebra fish (*Danio rerio*) was determined in a 96-hour semi-static test with a daily test medium renewal according to the EU Commission Directive 92/69/EEC, Part C.1 (1992), and the OECD Guideline for Testing of Chemicals No. 203, (1992).

A limit test was performed in accordance with the guidelines to demonstrate that the test item has no toxic effect on the test organisms at test item concentration at least up to 100 mg/L. Thus, the only nominal concentration tested was 125 mg/L and a control.

The measured concentration of the test item in the stock solution was 93.9% of nominal at the start of the test and 95.9% of nominal at the last test medium renewal after 72 hours test duration. Thus, the test item in the stock solution was stable until the last application.

In the test medium samples taken at the start of the test medium renewal periods, the average measured test item concentrations were between 88.5 and 93.2% of the nominal value. This demonstrates the correct dosage of the test item. During the test medium renewal period of 24 hours, a decrease of test item concentration in the test medium was determined. At the end of the test medium renewal periods, average values of 67.6 to 69.6% of the nominal value was found. The mean measured test item concentration (calculated as the average over all measurements per test concentration) was calculated to be 100 mg/L (80.1% of the nominal value). The reported biological results are related to the mean measured test item concentration of 100 mg/L.

In the control and at the mean measured test item concentration of 100 mg/L, no mortality or other visible abnormalities were determined in the test fish during the test period of 96 hours.

Therefore, the 96-hour NOEC (highest concentration tested without toxic effects after the exposure period of 96 hours) and the 96-hour LC0 of [redacted] to zebra fish were determined to be at least 100 mg/L. The NOEC and the LC0 might even be higher than this concentration but concentrations far above 100 mg/L were not tested according to the guidelines. The 96-hour LOEC (lowest concentration with toxic effects), the 96-hour LC50, and the 96-hour LC100 were clearly higher than 100 mg/L. These values could not be quantified due to the absence of toxicity of [redacted] at the tested concentration.

1 PURPOSE

The purpose of this 96-hour toxicity test was to evaluate the acute toxicity of the test item [redacted] to fish. For this purpose, zebra fish *(Danio rerio)* were exposed in a semi-static test to an aqueous test medium at a nominal concentration of 125 mg/L under defined conditions. This limit test was based on a range-finding test and was performed in compliance with the test guidelines to demonstrate that the test item has no toxic effect on fish up to at least this concentration.

The test method and the test species are recommended by the international test guidelines.

2 MATERIALS AND METHODS

2.1 DEFINITIONS

LC0:	The highest test concentration without a significant number of dead test organisms.
LC50:	The median lethal concentration, i.e. the calculated concentration of test item which causes death in 50% of the test organisms.
LC100:	The lowest test concentration at which all test organisms are dead.
NOEC (No Observed Effect Concentration):	The highest test concentration at which no significant toxic effect on the test organisms is observed.
LOEC (Lowest Observed Effect Concentration):	The lowest test concentration at which a significant toxic effect on the test organisms is observed.

2.2 TEST ITEM

The test item and the following information concerning the test item were provided by the sponsor:

Identity:	[redacted]
Sample no.:	S2539801
Purity:	The purity of the test item was not available prior to administration, hence this information is excluded from the Statement of Compliance. However, the sponsor is addressing this in a GLP compliant study [redacted] Reference Number AC030449.
Expiration date:	The expiry date of the test item was not available prior to administration, hence this information is excluded from the Statement of Compliance. However, the sponsor is addressing this in a GLP compliant study, [redacted] Reference Number AC030449.
Aggregate state at room temperature:	Solid [redacted]
Colour:	[redacted] determined visually at RCC)
Solubility in water:	Approx. 5 percent by weight
Stability in water:	7 days in the refrigerator (based on unaudited data)
Storage conditions:	At room temperature in the dark

2.3 TEST SYSTEM

The study was performed with zebra fish *(Danio rerio)*. The test fish were obtained from Zoohaus Schaub, CH-4410 Liestal, Switzerland. In accordance with the test guidelines, the fish were held in the laboratories of RCC for more than two weeks without any medication. Prior to test start, they were acclimated for one week to the test water and temperature. During holding and acclimatisation until one day before the start of the test, the fish were fed with a commercial fish diet (Tetra Min® Hauptfutter, supplied by TETRA-Werke, D-49304 Melle, Germany). During holding and acclimatisation, the mortality rate in the test fish batch was lower than 5% and all fish were healthy.

From the acclimated test fish batch, 10 fish were measured at the start of the test: The mean body length of the fish was 2.9 ± 0.1 cm (mean ± standard deviation), the mean body wet weight was 0.25 ± 0.06 g.

2.4 STUDY DESIGN

2.4.1 Experimental Conditions

According to information given by the sponsor, the test item is not sufficiently stable in water over a period of 96 hours. Therefore, a semi-static test procedure was chosen: the test media were renewed daily to keep the concentrations of [redacted] in the test media as constant as possible during the test period of 96 hours.

Glass aquaria with 4 litres test medium were used for the single test concentration and the control. The test vessels were labelled with the RCC study number and all necessary additional information to ensure unmistakable identification. During this semi-static test, the surviving test fish were placed each day into a clean aquarium with freshly prepared test medium of the single test concentration and the control.

At the start of the test, 7 fish were introduced into each aquarium in a random order. The loading rate was 0.43 g fish wet weight per litre test medium. The test medium and the control were slightly aerated during the test period. The fish were not fed during the test.

Test duration: 96 hours

Water temperature: 21 °C during the test period (Table 6)

Light conditions: A 16-hour light to 8-hour dark photoperiod, with a 30-minute transition period (light intensity during the light period was approximately within the range of 50 to 500 Lux).

Test water: Reconstituted water: analytical grade salts were dissolved in deionized water to obtain the following nominal concentrations:

$CaCl_2 \times 2H_2O$	:	2.0 mmol/L	(= 294 mg/L)
$MgSO_4 \times 7H_2O$	:	0.5 mmol/L	(= 123 mg/L)
$NaHCO_3$	:	0.75 mmol/L	(= 65 mg/L)
KCl	:	0.075 mmol/L	(= 5.8 mg/L)
Alkalinity	:	0.8 mmol/L	

Ratio of Ca : Mg = 4 : 1 (based on molarity)
Na : K = 10 : 1 (based on molarity)

Water hardness: calculated: 2.5 mmol/L (= 250 mg/L as $CaCO_3$)
measured: 280 - 290 mg/L as $CaCO_3$ (see Table 5)

The test water was aerated prior to the preparation of the test media until oxygen saturation was reached.

2.4.2 Dosage and Concentrations

A limit test was performed in accordance with the guidelines to demonstrate that the test item has no toxic effect on the test fish up to the test item concentration of at least 100 mg/L. Thus, a single test item concentration of nominal 125 mg/L was tested. Additionally, a control was tested in parallel (test water without addition of test item).

Immediately before the start of the test, a concentrated stock solution of the test item of the nominal concentration of 12.5 g/L was prepared by dissolving 2.75 g of the test item completely in 220 mL of purified water by intense stirring for 30 minutes at room temperature. The stock solution was prepared once at the start of the test and was used for the preparation of the test medium by dilution with test water at each test medium renewal. The stock solution was stored in a refrigerator at approximately 4 °C in the dark. At each renewal period, the stock solution was removed from the refrigerator and stirred on a magnetic stirrer for 30 minutes prior to use in order to equilibrate to ambient temperature.

Before the start of the test and prior to each test medium renewal, the test medium was freshly prepared by mixing 40 mL of the stock solution into a final volume of 4000 mL test water. Also the control medium was renewed daily.

The actual concentration of the test item in the test medium was analytically determined (see Section 2.6.3).

The limit test was based on the results of a range-finding test (without GLP). However, concentrations far above 100 mg/L were not tested according to the test guidelines.

2.5 EVALUATIONS

2.5.1 Determination of the LC50, LC0, LC100, NOEC and LOEC

The test fish were observed after approximately 3, 24, 48, 72, and 96 hours test duration for mortality and visible abnormalities.

The NOEC and the LC0 were determined directly from the raw data. The LOEC, the LC50, and the LC100 at the observation times could not be quantified due to the absence of a toxic effect of the test item at the tested concentration.

2.5.2 Water Quality Criteria

The water temperature, pH-values, oxygen concentrations, and water hardness were measured at the single test concentration and the control at the start of the test and once every day during the test in the freshly prepared and old test media.

At the same dates the appearance of the test medium was recorded.

2.5.3 Analysis of the Test Item Concentrations

Triplicate samples of the concentrated stock solution of the test item was taken at the start of the test and at the last test medium preparation after 72 hours test duration.

For the determination of the test concentration in the semi-static test, triplicate samples were taken from the freshly prepared test medium and the control at the start of the test and at each test medium preparation. To confirm the maintenance of the test item concentrations during the test medium renewal periods of one day, triplicate samples were taken from the aged test medium and the control at the end of each test medium renewal period.

The samples of the test medium and the control were taken from the approximate centre of the aquaria without prior mixing of the test media. The sampling volume of the stock solution, the test media, and the control was 5 mL per sample stored in 20 mL glass vials.

The samples were deep-frozen at about -20 °C immediately after sampling. At each sampling date, the deep-frozen samples (replicate number 1 and 2) were packed in dry ice and shipped overnight to the sponsor for analysis. Sample 3 was stored deep-frozen at approximately -20 °C at RCC as insurance against possible sample losses.

The analytical procedure and the results are described in the attached analytical phase report.

3 RESULTS AND DISCUSSION

A limit test was performed in accordance with the test guidelines to demonstrate that the test item has no toxic effect on the test fish at a nominal concentration of 125 mg/L.

The measured concentration of the test item in the stock solution was 93.9% of nominal at the start of the test and 95.9% of nominal at the last test medium renewal after 72 hours test duration (see attached analytical phase report). Thus, the test item in the stock solution was stable until the last application.

In the test medium samples taken at the start of the test medium renewal periods, the average measured test item concentrations were between 88.5 and 93.2% of the nominal value (see attached analytical phase report). This demonstrates the correct dosage of the test item. During the test medium renewal period of 24 hours, a decrease of test item concentration in the test medium was determined. At the end of the test medium renewal periods, average values of 67.6 to 69.6% of the nominal value was found. The mean measured test item concentration (calculated as the average over all measurements per test concentration) was calculated to be 100 mg/L (80.1% of the nominal value). The reported biological results are related to the mean measured test item concentration of 100 mg/L.

The biological results are listed in Table 1.

In the control and in the test medium of the mean measured test item concentration of 100 mg/L, all fish survived until the end of the test and no visible abnormalities were observed in the test fish.

Therefore, the 96-hour NOEC (highest concentration tested without toxic effects after the exposure period of 96 hours) and the 96-hour LC0 of [redacted] to zebra fish were determined to be at least 100 mg/L. The 96-hour NOEC and the 96-hour LC0 might even be higher than this concentration but concentrations far above 100 mg/L were not tested according to the guidelines. The 96-hour LOEC (lowest concentration with toxic effects), the 96-hour LC50, and the 96-hour LC100 were clearly higher than 100 mg/L. These values could not be quantified due to the absence of toxicity of [redacted] at the tested concentration.

No remarkable observations were made concerning the appearance of the test medium. The test medium was a clear solution coloured [redacted] by the test item during the test medium renewal periods of 24 hours (Table 2).

The pH values in the test medium and the control ranged from 7.7 to 7.9 (Table 3). The oxygen concentration was always 8.4 mg/L or higher (Table 4), and thus higher than 60% oxygen saturation. The water hardness measured in the test medium and the control ranged from 279 to 286 mg/L as $CaCO_3$ (Table 5). The water temperature was 21 °C at all measurements (Table 6).

4 TABLES

Table 1: Mortality and visible abnormalities observed at the test fish

Abbreviations of visible abnormalities for which fish were observed during the study:

AK:	Strongly extended gills	OB:	Fish mainly at the water surface
AP:	Apathy	SA:	Mucus secretion
BA:	Distended abdomen	SR:	Fish lying on side or back on the bottom
BO:	Fish mainly at the bottom of the aquarium	SV:	Strong ventilation
		TS:	Tumbling during swimming
GA:	Exophthalmus	VF:	Changed body colour
KR:	Convulsions		

Number of fish tested at the single concentration and the control: 7

Nominal test item concentration (mg/L)	Mean measured test item concentration (mg/L)	Number of affected fish* / number of dead fish visible abnormalities				
		Observation time				
		3 hours	24 hours	48 hours	72 hours	96 hours
Control	—	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
125	100	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
LC50		> 100 mg/L (based on mean measured test item concentration)				

* number of dead fish plus number of fish with visible abnormalities

Table 2: Appearance of the freshly prepared and old test medium

Abbreviations:

0: no remarkable observations, clear test medium
1: homogeneous dispersion in the water, turbidity observable
2: noticeable turbidity caused by the test item
3: noticeable coloration caused by the test item [redacted]
4: inhomogeneous dispersion of the test item
5: precipitation of the test item
6: test item at the surface
7: test item lying at the bottom of the aquarium

Nominal test item concentration	Mean measured test item concentration	Exposure time							
		0 h	24 h		48 h		72 h		96 h
(mg/L)	(mg/L)	new	old	new	old	new	old	new	old
125	100	3	3	3	3	3	3	3	3

Table 3: pH-values in the freshly prepared and old test medium and in the control

Nominal test item concentration	Mean measured test item concentration	Exposure time							
		0 h	24 h		48 h		72 h		96 h
(mg/L)	(mg/L)	new	old	new	old	new	old	new	old
Control	—	7.8	7.9	7.9	7.8	7.8	7.8	7.8	7.8
125	100	7.8	7.8	7.7	7.8	7.8	7.8	7.8	7.8

Table 4: Oxygen concentrations (mg/L) in the freshly prepared and old test medium and in the control

Nominal test item concentration (mg/L)	Mean measured test item concentration (mg/L)	Exposure time 0 h new	24 h old	24 h new	48 h old	48 h new	72 h old	72 h new	96 h old
Control	—	8.6	8.4	8.7	8.5	8.7	8.6	8.5	8.5
125	100	8.6	8.4	8.7	8.6	8.7	8.6	8.5	8.5

Table 5: Water hardness (mg/L as $CaCO_3$) in the freshly prepared and old test medium and in the control

Nominal test item concentration (mg/L)	Mean measured test item concentration (mg/L)	Exposure time 0 h new	24 h old	24 h new	48 h old	48 h new	72 h old	72 h new	96 h old
Control	—	280	280	280	280	280	290	280	280
125	100	280	280	280	280	280	280	280	280

Table 6: Water temperatures (°C) in the freshly prepared and old test medium and in the control

Nominal test item concentration (mg/L)	Mean measured test item concentration (mg/L)	Exposure time 0 h new	24 h old	24 h new	48 h old	48 h new	72 h old	72 h new	96 h old
Control	—	21	21	21	21	21	21	21	21
125	100	21	21	21	21	21	21	21	21

APPENDIX 1

ANALYTICAL PHASE REPORT

PRINCIPAL INVESTIGATORS REPORT:

ACUTE TOXICITY OF [redacted]
TO ZEBRA FISH (*DANIO RERIO*)
IN A 96-HOUR SEMI-STATIC TEST

(ANALYTICAL PHASE)

[redacted] Study Reference Number KAF030430

RCC STUDY No. 851901

AUTHOR : CHRIS SPARHAM (PRINCIPAL INVESTIGATOR)

[redacted] Confidential - Class 2 Circulation

This report must not be circulated further, copied, or destroyed without reference to the Reports Administrator [redacted]

[redacted]

Date : April 2004

AUTHORISATION STATEMENT

RCC study number	:	RCC study No. 851901
[redacted] study reference number	:	KAF030430
Study title	:	Acute toxicity of [redacted] to zebra fish (*Danio rerio*) in a 96-hour semi-static test (Analytical phase)

This Principal Investigator's report has been authorised for issue to the appropriate recipients.

Julia Fentem

JULIA FENTEM
GROUP HEAD, APPLIED SCIENCE AND TECHNOLOGY [redacted]

30.04.04
DATE

[redacted]

AUTHENTICATION STATEMENT

RCC study number	:	RCC study No. 851901
[redacted] study reference number	:	KAF030430
Study title	:	Acute toxicity of [redacted] to zebra fish (*Danio rerio*) in a 96-hour semi-static test (Analytical phase)

I, the undersigned, as Principal Investigator hereby declare that this phase has been conducted under my supervision, in accordance with [redacted] policy on Good Laboratory Practice which is based on the UK Good Laboratory Practice Regulations 1999, No. 3106 and OECD Principles on Good Laboratory Practise (revised in 1997) ENV/MC/CHEM/ (98) 17.

I also certify that this Principal Investigator's report presents a true and accurate account of the procedures used and the results obtained.

CSparham

CHRIS SPARHAM
PRINCIPAL INVESTIGATOR

29/4/04

DATE

[redacted]

QUALITY ASSURANCE STATEMENT

[redacted] Reference Number: KAF030430

Study Title: Acute Toxicity of [redacted] to Zebra Fish (*Danio Rerio*) in a 96-Hour Semi-Static Test
(Analytical Phase)

Phase of study: Confirmation of achieved concentration, homogeneity and stability of test solutions.

This study was contracted to RCC Ltd (RCC Study No. 851901)

The work for the above named phase of the study was conducted by [redacted]

Inspections and audits for this phase of the study were performed as detailed below and findings reported to the Principal Investigator and Facility Management. Significant findings affecting the main study are reported to the Study Director.

Audit Type	Audit Date	Report Date
Data Audit	20-Apr-2004	21-Apr-2004
Process Inspection - Measurement Procedures - LC/MS	05-Jan-2004	19-Mar-2004
Facility Inspection	27-Nov-2003	05-Dec-2003

As far as can reasonably be established, this document has been accepted by Quality Assurance as being an accurate presentation of the raw data and finding of the study.

29 April 2004.

H LALL
QUALITY ASSURANCE
[redacted]

DATE

ACUTE TOXICITY OF [redacted] TO ZEBRA FISH (*DANIO RERIO*) IN A 96-HOUR SEMI-STATIC TEST

(ANALYTICAL PHASE)

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ACUTE TOXICITY OF [redacted] TO ZEBRA FISH (*DANIO RERIO*) IN A 96-HOUR SEMI-STATIC TEST

(ANALYTICAL PHASE)

SUMMARY

The aim of this phase of the study was to confirm satisfactory achieved concentration, homogeneity and stability of test solutions, using a method developed in [redacted] study AH030453. [redacted] concentrations were measured by Liquid Chromatography/mass spectrometry (LC/MS).

The homogeneity of solutions was found to be satisfactory with good agreement in the analysis of replicate samples taken by RCC. Achieved concentration, in the range 88.5 to 93.2 % of the nominal, was confirmed for solution preparation. Test solutions were not stable over 24 hours, as expected from work done in [redacted] study AH030453, with the concentrations dropping to values in the range of 67.6 to 69.6 % of the nominal.

1. INTRODUCTION

The aim of this phase of the study was to confirm satisfactory achieved concentration, homogeneity and stability of test solutions using a method developed in [redacted] study AH030453.

2. SUBSTANCES TESTED

The following samples were provided by RCC and analysed to provide achieved concentration, homogeneity and stability data. All samples were supplied as solutions of [redacted] (S2539801) in test water or test water only.

Time (hours)	Nominal test item concentration (mg/L)	Replicate number
0	0	1
0	0	2
0	125	1
0	125	2
24 old	0	1
24 old	0	2
24 new	0	1
24 new	0	2
24 old	125	1
24 old	125	2
24 new	125	1
24 new	125	2
48 old	0	1
48 old	0	2
48 new	0	1
48 new	0	2
48 old	125	1
48 old	125	2
48 new	125	1
48 new	125	2
72 old	0	1
72 old	0	2
72 new	0	1
72 new	0	2
72 old	125	1
72 old	125	2
72 new	125	1
72 new	125	2
96	0	1
96	0	2
96	125	1
96	125	2
0	12.5 g/l stock	1
0	12.5 g/l stock	2
72	12.5 g/l stock	1
72	12.5 g/l stock	2

Samples were received frozen from RCC the day after sampling. Analysis was carried out on the same day as receipt.

3. METHOD

3.1 Sample preparation

Analysis was carried out at 0 hours, 24 hours (old and new), 48 hours (old and new), 76 hours (old and new) and 96 hours to assess the satisfactory achieved concentration, homogeneity and stability of the test solutions, using methodology developed in AH030453. All samples were analysed after diluting 1 in 10 in mobile phase (5mM ammonium formate pH 3.0: acetonitrile, 81: 19, % v/v). Stock solutions were diluted 1 in 1000 in mobile phase.

3.2 LC/MS analysis

[redacted] concentrations were measured by LC/MS. The solutions were analysed by injecting 10µL onto a Luna C_8 5µm (150 x 2.0mm) column held at 30°C. An isocratic mobile phase, flowing at 0.2 mL/min., consisting of 81% 5mM ammonium formate pH 3.0 and 19% acetonitrile was used for the analysis. Reagents were HPLC Grade or equivalent. The mass spectrometer was operated in positive electrospray ionisation (ESI) mode, with gas temperature 300°C, gas flow 11.0 L/min, nebuliser pressure 30 psi and capillary voltage 3000V. Quantitation of [redacted] was carried out using single ion monitoring (SIM) for *m/z* 634.2. The calibration stock solution of [redacted] (S2539801) was prepared in Ultrapure water at 1250 mg/L (stable for 1 week in the fridge). Further dilutions in calibration standard preparation were carried out in mobile phase. Calibration standards were run at concentrations of 0, 6.25, 9.375, 12.5 and 15.625 mg/L. The calibration standards were made fresh on each day of analysis from the stock solution.

3.3 Study dates

The analysis phase of the study was conducted from 2 to 6 March 2004.

3.4 Storage and retention of data

Datacare Business Management Systems has been chosen as the test site's archive because they have been accepted as qualified contract facility. Although Datacare Management Systems is not a member of the UK GLP compliance programme, [redacted] Quality Assurance performs annual audits of the contract facility to specific SOPs which have been approved by the UK GLP authority. The UK Medicines and Healthcare products Regulatory Agency have been informed of [redacted] use of the contract facility. The [redacted] archivist manages the flow of data to and from the contract facility (deposits and loans). The archivist also manages the culling program.

4. RESULTS AND DISCUSSION

Full results are shown in Tables 1 to 3. A typical LC/MS chromatogram is shown in Figure 1.

The 125mg/L test solutions prepared at 0 hours, 24 hours, 48 hours and 72 hours had a mean measured concentration of 116, 111, 116 and 113 mg/L respectively to 3 significant figures (93.2, 88.5, 93.0 and 90.4 % of the nominal). After 24 hours under test conditions the mean measured concentration of these solutions had dropped to 86.5, 87.0, 84.5 and 86.3 mg/L respectively (69.2, 69.6, 67.6 and 69.0 % of the nominal). All control solutions (0 mg/L) were measured at < 5 mg/L. The 12.5 g/L stock solution measured at 0 and 72 hours gave concentrations of 11.7 and 12.0 g/L respectively (93.9 and 95.9 % of the nominal).

The homogeneity of solutions was found to be satisfactory with good agreement in the analysis of replicate samples taken by RCC. Achieved concentration, in the range 88.5 to 93.2 % of the nominal, was confirmed for solution preparation. Test solutions were not stable over 24 hours, as expected from previous work done i[redacted] study AH030453, with the concentrations dropping to values in the range of 67.6 to 69.6 % of the nominal.

5. CONCLUSION

The homogeneity of solutions was found to be satisfactory with good agreement in the analysis of replicate samples taken by RCC. Achieved concentration, in the range 88.5 to 93.2 % of the nominal, was confirmed for solution preparation. Test solutions were not stable over 24 hours, as expected from work done i[redacted]study AH030453, with the concentrations dropping to values in the range of 67.6 to 69.6 % of the nominal.

TABLE 1. TEST SOLUTIONS

Sample Name	Nominal Concentration (mg/L)	0 Hours		24 Hours Old		24 Hours New		48 Hours Old	
		Measured Concentration (mg/L)	% of Nominal	Measured Concentration (mg/L)	% of Nominal	Measured Concentration (mg/L)	% of Nominal	Measured Concentration (mg/L)	% of Nominal
0 mg/L Rep 1 A	0	<5	n/a	<5	n/a	<5	n/a	<5	n/a
0 mg/L Rep 1 B	0	<5	n/a	<5	n/a	<5	n/a	<5	n/a
0 mg/L Rep 1 Average	**0**	**<5**	**n/a**	**<5**	**n/a**	**<5**	**n/a**	**<5**	**n/a**
0 mg/L Rep 2 A	0	<5	n/a	<5	n/a	<5	n/a	<5	n/a
0 mg/L Rep 2 B	0	<5	n/a	<5	n/a	<5	n/a	<5	n/a
0 mg/L Rep 2 Average	**0**	**<5**	**n/a**	**<5**	**n/a**	**<5**	**n/a**	**<5**	**n/a**
0 mg/L Average	**0**	**<5**	**n/a**	**<5**	**n/a**	**<5**	**n/a**	**<5**	**n/a**
125 mg/L Rep 1 A	125	117.32	93.86	85.70	68.56	112.14	89.71	86.90	69.52
125 mg/L Rep 1 B	125	117.08	93.67	86.06	68.85	110.46	88.37	87.54	70.03
125 mg/L Rep 1 Average	**125**	**117.20**	**93.76**	**85.88**	**68.71**	**111.30**	**89.04**	**87.22**	**69.78**
125 mg/L Rep 2 A	125	115.22	92.18	87.49	69.99	108.28	86.62	85.80	68.64
125 mg/L Rep 2 B	125	116.35	93.08	86.90	69.52	111.39	89.11	87.58	70.07
125 mg/L Rep 2 Average	**125**	**115.79**	**92.63**	**87.19**	**69.75**	**109.83**	**87.87**	**86.69**	**69.35**
125 mg/L Average	**125**	**116.49**	**93.19**	**86.54**	**69.23**	**110.56**	**88.45**	**86.96**	**69.57**

n/a = not applicable

Sanitized Version

TABLE 1. TEST SOLUTIONS (CONTINUED)

Sample Name	Nominal Concentration (mg/L)	48 Hours New		72 Hours Old		72 Hours New		96 Hours	
		Measured Concentration (mg/L)	% of Nominal	Measured Concentration (mg/L)	% of Nominal	Measured Concentration (mg/L)	% of Nominal	Measured Concentration (mg/L)	% of Nominal
0 mg/L Rep 1 A	0	<5	n/a	<5	n/a	<5	n/a	<5	n/a
0 mg/L Rep 1 B	0	<5	n/a	<5	n/a	<5	n/a	<5	n/a
0 mg/L Rep 1 Average	**0**	**<5**	**n/a**	**<5**	**n/a**	**<5**	**n/a**	**<5**	**n/a**
0 mg/L Rep 2 A	0	<5	n/a	<5	n/a	<5	n/a	<5	n/a
0 mg/L Rep 2 B	0	<5	n/a	<5	n/a	<5	n/a	<5	n/a
0 mg/L Rep 2 Average	**0**	**<5**	**n/a**	**<5**	**n/a**	**<5**	**n/a**	**<5**	**n/a**
0 mg/L Average	**0**	**<5**	**n/a**	**<5**	**n/a**	**<5**	**n/a**	**<5**	**n/a**
125 mg/L Rep 1 A	125	113.92	91.14	89.37	71.49	112.99	90.39	87.83	70.26
125 mg/L Rep 1 B	125	117.87	94.30	85.06	68.04	109.78	87.82	86.13	68.90
125 mg/L Rep 1 Average	**125**	**115.90**	**92.72**	**87.21**	**69.77**	**111.38**	**89.11**	**86.98**	**69.58**
125 mg/L Rep 2 A	125	117.74	94.19	84.04	67.24	116.21	92.97	85.17	68.13
125 mg/L Rep 2 B	125	115.66	92.53	79.41	63.53	113.16	90.52	85.90	68.72
125 mg/L Rep 2 Average	**125**	**116.70**	**93.36**	**81.73**	**65.38**	**114.68**	**91.75**	**85.53**	**68.42**
125 mg/L Average	**125**	**116.30**	**93.04**	**84.47**	**67.58**	**113.03**	**90.43**	**86.25**	**69.00**

n/a = not applicable

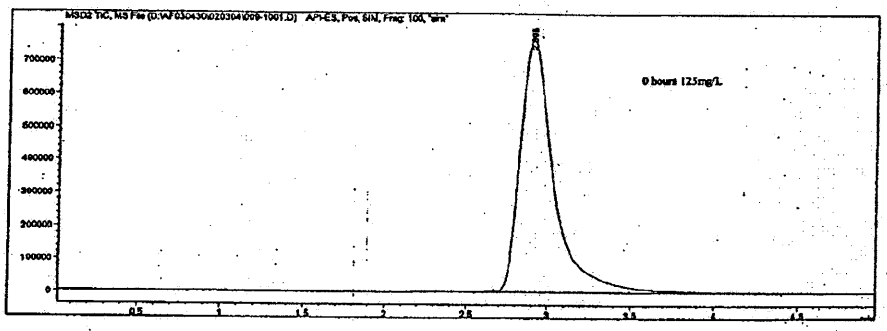
Sanitized Version

TABLE 2. STOCK SOLUTIONS

Stock	Time (hours)	Replicate	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	% of Nominal
12.5g/L stock	0	1A	12500	11478	91.82
12.5g/L stock	0	1B	12500	11837	94.69
12.5g/L stock	0	2A	12500	11577	92.62
12.5g/L stock	0	2B	12500	12031	96.25
Average 12.5g/L 0 hrs	**0**	**Average**	**12500**	**11731**	**93.85**
12.5g/L stock	72	1A	12500	12162	97.30
12.5g/L stock	72	1B	12500	12112	96.90
12.5g/L stock	72	2A	12500	12027	96.22
12.5g/L stock	72	2B	12500	11641	93.12
Average 12.5g/L 72 hrs	**72**	**Average**	**12500**	**11986**	**95.88**

Sanitized Version

FIGURE 1. TYPICAL LC/MS CHROMATOGRAM OF [redacted] IN A TEST SOLUTION, USING SIM FOR *m/z* 634.2



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Pages

STUDY REPORT

ACUTE TOXICITY OF [redacted]
TO *DAPHNIA MAGNA*

Study EAD030424

AUTHOR : J ROBERTS (STUDY DIRECTOR)

[redacted] Confidential - Class 2 Circulation

This report must not be circulated further, copied, or destroyed without reference to the Reports Administrator [redacted]

[redacted]

Date : June 2004

STUDY INFORMATION

Study title	:	Acute Toxicity of [redacted] to *Daphnia magna*
Study number	:	EAD030424
Study location	:	[redacted]

STUDY DATES

Date protocol signed	:	16 February 2004
Experimental period	:	17 February to 26 February 2004

ARCHIVING

Data and Report	:	Datacare Business Management Systems 3012 Heyford Park Upper Heyford Bucks OX25 5HF
Test Item	:	[redacted]

STUDY PERSONNEL

Study Director	:	J Roberts
Responsible Practitioner	:	C Sparham
Operators	:	Sean O'Connor (Chemical Analysis)
Scientific Reviewer	:	Roger van Egmond
QA	:	Harjit Lall

STUDY INFORMATION (CONTINUED)

CROSS REFERENCES

Project number	:	221130
Project Name	:	MP5[redacted]

AUTHORISATION STATEMENT

Study Number : EAD030424

Study Name : Acute Toxicity of [redacted] to *Daphnia magna*

This report has been authorised for issue to the appropriate recipients.

Julia Fentem — 15-06-04

JULIA FENTEM — DATE

GROUP HEAD, APPLIED SCIENCE AND TECHNOLOGY

[redacted]

[redacted]

AUTHENTICATION STATEMENT

Study number : EAD030424

Study title : Acute Toxicity of [redacted] to *Daphnia magna*

I, the undersigned, hereby declare that this study has been conducted under my supervision, as Study Director, in accordance with [redacted] policy on Good Laboratory Practice which is based on the UK Good Laboratory Practice Regulations 1999, No. 3106 and OECD Principles on Good Laboratory Practice (as revised in 1997) ENV/MC/CHEM/ (98)17.

I also certify that this report presents a true and accurate account of the procedures used and the results obtained.

J Roberts.

J ROBERTS
STUDY DIRECTOR

11/6/04

DATE

[redacted]

RESPONSIBLE PRACTITIONERS

Study number : EAD030434

Study title : Acute Toxicity of [redacted] to *Daphnia magna*

Analytical phase

C Sparham

C SPARHAM

10/6/04.

DATE

[redacted]

QUALITY ASSURANCE STATEMENT

Study Number : EAD030424

Study Title: Acute Toxicity of [redacted] to *Daphnia Magna*

This study was conducted at [redacted]

The following inspections and audits were conducted on the study. The dates on which they were performed and the dates on which any findings were reported to the Study Director and to Management are given below.

Audit Type	Audit Date	Report Date
Protocol Audit	09-Feb-2004	09-Feb-2004
Study Report Audit	25-May-2004	26-May-2004
Environment		
Process Inspection	03-Nov-2003	16-Dec-2003
- Daphnia acute Toxicity		
Chemistry		
Process Inspection	05-Jan-2004	19-Mar-2004
- Measurement Procedures (Wt/Vol)		
- LC/MS		
Environment		
Facility Inspection	24-Feb-2004	16-Mar-2004
Chemistry		
Facility Inspection	27-Nov-2003	05-Dec-2003

As far as can reasonably be established, this report has been accepted by Quality Assurance as being an accurate presentation of the raw data and findings of the study.

09 June 2004

H LALL
QUALITY ASSURANCE
[redacted]

DATE

ACUTE TOXICITY OF [redacted] TO *DAPHNIA MAGNA*

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ACUTE TOXICITY OF [redacted]
TO *DAPHNIA MAGNA*

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ACUTE TOXICITY OF [redacted] TO *DAPHNIA MAGNA*

SUMMARY

The purpose of this study was to determine the 48-hour EC50 of [redacted] to juvenile i.e. <24hrs old, *Daphnia magna.*

The test was conducted under semi-static conditions. *Daphnia* were not fed during the test.

Daphnia were exposed to a range of concentrations of [redacted] prepared by diluting a concentrated stock solution with Elendt M7 medium. Test concentrations were measured by liquid chromatography/mass spectrometry (LC/MS) by Chemical Analysis Department.

The 48-hour EC50 based on mean measured concentrations was 23.7 mg/l.

The 48-hour NOEC based on mean measured concentrations was 7.3 mg/l.

1. INTRODUCTION

The 48 hour EC_{50} of [redacted] to the freshwater cladoceran *Daphnia magna* was determined according to OECD Guideline 202 [1], EEC Directive 92/69/EEC L383 A [2] and BS EN ISO 6341:1996 [3].

2. SUBSTANCES TESTED

2.1 Test Item

The test item used in this investigation was [redacted] It is registered in compound control as sample number S2539801. The test sample is a [redacted] and was tested as 100 percent active.

2.2 Characterisation information

Characterisation, stability and homogeneity is confirmed by analysis in study number AC030449.

3. METHOD

3.1 Experimental method

A nominal 1000 mg/L stock was prepared by dissolving 1000 mg of S2539801 in 1000 mL of purified water. Test media were prepared by diluting the stock with appropriate volumes of Elendt M7 medium to obtain nominal concentrations of 0, 10, 18, 32, 56 and 100 mg/L. This concentration range was determined by the results of a range-finder study performed prior to the main experiment. Twelve litres of Elendt M7 media were isolated and adjusted to pH 7.5 using 1 M HCl for use as dilution media to ensure that the pH of the test solutions remained below pH 8. Details of the range-finder study, including raw data, can be found in the study file.

Each exposure vessel (120 mL crytallising dish) was filled with approximately 100 mL test media. Five juvenile *Daphnia* were distributed to each test vessel using a 6 mm bore glass tube. Four replicates were prepared per concentration. *Daphnia* were not fed during the test. Test media were renewed after 24 hours.

The test vessels were arranged in the test area according to a random distribution under normal laboratory light intensity with a photo-period of 16 hours light, 8 hours dark.

The pH, dissolved oxygen concentration and total hardness (as mg/L $CaCO_3$) of the test media were measured at 0, 24 (old and new) and 48 hours using the appropriate methods. The temperature in a representative sample of the test vessels was measured daily.

3.2 Statistical Analysis

The EC50 was calculated using the computer application BMPDIN. The program generates where possible, three statistical analyses of the data using the moving average method, the probit method and the binomial method.

3.3 Study dates

The study was conducted from 17 February to 26 February.

3.4 Storage and retention of data

The study protocol, any amendments, raw data, and final report will be archived on completion at Datacare Business Management Systems, 3012 Heyford Park, Upper Heyford, Bucks OX25 5HF. Datacare is not a member of the UK GLP compliance programme. Test item will be archived within [redacted] This study will be added to the Archive Index for review.

4. RESULTS

4.1 Test Conditions

The means of the water quality data for each test are given in Table 1. Full details can be found in Appendix 1.

4.2 Test Item Concentrations

Test item concentrations were measured by Liquid Chromatography/Mass Spectroscopy. Mean measured concentrations were calculated using time weighted method. Summary of test item concentrations is shown in Table 2. Full details can be found in Appendix 3.

An anomalous result was observed in the 10 mg/l (24 hr new) test solution (Appendix 3,Table 2). The original test sample supplied to Chemical Analysis had a mean measured concentration of 17.42 mg/L (174.2% of nominal conc). A second test sample of the 10mg/L (24 hr new) solution was supplied and the original sample re-analysed. The mean measured concentration of the re-analysed test sample was 15.38 mg/L (153.8 % of nominal conc) and the concentration of the second sample was 8.06 mg/L (80.6 % of nominal concentrations). Re-analysis of the original sample confirms that loss of test compound over time is not significant and suggests that this is not the reason for the lower concentration observed in second sample. It is believed that the original sample supplied may have been taken from the wrong test solution flask as the results would appear to correlate more closely with the 18mg/L test solution. Consequently a mean measured concentration of 8.06mg/L has been taken as being the correct and used in the calculation of the results.

4.3 Effect Data

The percentage immobility observed at 24 and 48 hours are summarised in Table 3, and in Figure 1. Further details can be found in Appendix 2.

5. CONCLUSIONS

The 24-hour EC50 based on measured concentrations was 36.6 mg/L.

The 24-hour NOEC based on measured concentrations was 13.5 mg/L.

The 48-hour EC50 based on measured concentrations was 23.7 mg/L.

The 48-hour NOEC based on measured concentrations was 7.3 mg/L.

The lowest concentration causing 100%-immobility at 48 hrs was 42.9 mg/L.

6. REFERENCES

[1] OECD Guidelines for Testing of Chemicals. Section 2. Guideline 202 "*Daphnia sp.*, Acute Immobilisation Test and Reproduction Test" (1993).

[2] EEC Directive 92/69/EEC, Official Journal of the EEC L383 A. : Methods for the Determination of Ecotoxicity. C.2. Acute Toxicity for *Daphnia.*

[3] BS EN ISO 6341:1996. Water Quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (*Cladocera, Crustacea*) - Acute toxicity test.

TABLE 1. MEAN WATER QUALITY MEASUREMENTS

	Mean	SD
Test Media Temperature (°C)	19.7	0.4
pH	7.5	0.3
Dissolved Oxygen (mg/L)	8.0	0.1
Total Hardness (mg/L as $CaCO_3$)	206	14

TABLE 2. SUMMARY OF MEASURED TEST CONCENTRATIONS

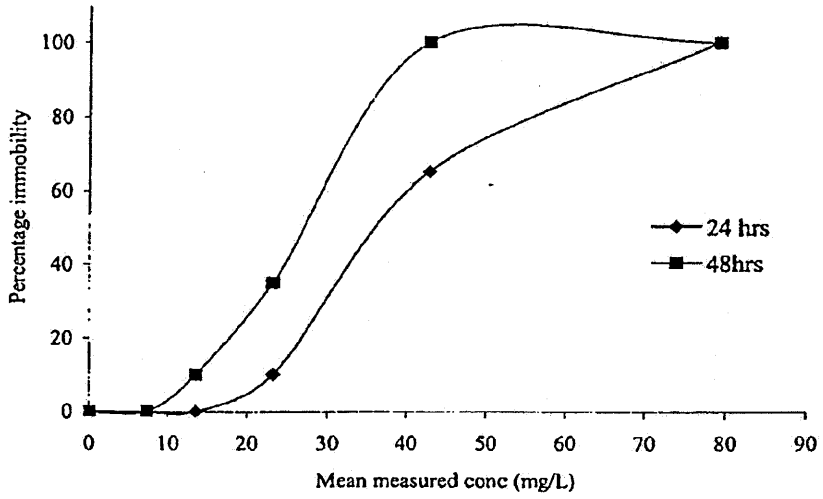
Nominal Concentration (mg/L)	*Mean Measured Concentrations (mg/L)				Mean Measured Conc (mg/L)
	0 Hours	24 Hours old	24 Hours new	48 Hours	
0	<3	<3	<3	<3	<3
10	7.89	6.49	8.06	6.85	7.30
18	12.45	12.94	16.81	12.04	13.49
32	24.59	20.83	26.45	21.20	23.19
56	45.13	40.02	48.54	38.52	42.93
100	82.44	76.48	-	-	79.42

* calculated as time weighted mean

TABLE 3. 24 & 48 HR IMMOBILITY DATA

Mean Measured Conc. (mg/L)	Perecent immobility	
	at 24 hrs	at 48 hrs
0	0	0
7.3	0	0
13.5	0	10
23.2	10	35
42.9	65	100
79.4	100	100

FIGURE 1. SUMMARY OF EFFECT DATA



APPENDIX 1. TEST CONDITIONS

Temperature °C

Time (hours)	Vessel Number 3	9	13	16	22
0	19.5	19.5	19.5	19.5	19.5
24 (old)	19.5	19.5	19.5	19.5	19.5
24 (new)	20.0	20.5	20.5	20.5	-
48	19.5	19.5	19.5	19.5	-

pH

Time (hours)	Mean Measured Concentration (mg/L) 0.0	7.3	13.5	23.2	42.9	79.4
0	7.5	7.7	7.7	7.8	7.6	7.8
24 (old)	6.9	7.2	7.4	7.4	7.4	7.4
24 (new)	8.2	7.7	7.7	7.7	7.7	-
48	7.3	7.4	7.5	7.5	7.5	-

At 24 hr renewal only the pH of the control and highest remaining concentration are required

Dissolved Oxygen (mg/l)

Time (hours)	Mean Measured Concentration (mg/L) 0.0	7.3	13.5	23.2	42.9	79.4
0	8.0	8.1	7.9	7.9	7.8	7.7
24 (old)	8.2	8.2	8.1	8.1	8.0	8.0
24 (new)	8.1	8.1	8.0	8.0	7.9	-
48	8.1	8.1	8.1	8.0	8.0	-

At 24 hr renewal only the DO of the control and highest remaining concentration are required

Total Hardness (mg/L as $CaCO_3$)

Time (hours)	Mean Measured Concentration (mg/L) 0.0	7.3	13.5	23.2	42.9	79.4
0	230	-	-	-	-	198
24 (old)	220	-	-	-	-	198
24 (new)	201	-	-	-	187	-
48	208	-	-	-	204	-

APPENDIX 2. EFFECT DATA

Mean Measured Concentration (mg/L)	Number Immobile at 24 hours					Number Immobile at 48 hours					Number Mobile at 48 hours				
	Replicate				Total	Replicate				Total	Replicate				Total
	1	2	3	4		1	2	3	4		1	2	3	4	
0.0	0	0	0	0	0	0	0	0	0	0	5	5	5	5	20
7.3	0	0	0	0	0	0	0	0	0	0	5	5	5	5	20
13.5	0	0	0	0	0	1	0	1	0	2	4	5	4	5	18
23.2	0	0	1	1	2	2	3	0	0	7	3	2	4	4	13
42.9	3	3	3	4	13	2	2	2	1	20	0	0	0	0	0
79.4	5	5	5	5	20	-	-	-	-	20	0	0	0	0	0

Immobile neonates are removed at 24 hours. 48 hour totals include effects at 24 hrs.

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APPENDIX 3. PRACTIONERS REPORT

ACUTE TOXICITY OF [redacted] TO *DAPHNIA MAGNA* RESPONSIBLE PRACTITIONER SUMMARY (CHEMICAL ANALYSIS)

INTRODUCTION

Samples were supplied to Chemical Analysis to measure achieved concentration and stability of test solutions, using a method validated in [redacted] study AH030453. [redacted] concentrations were measured by Liquid Chromatography/mass spectrometry (LC/MS).

METHOD

Sample preparation

Analysis was carried out at 0 hours, 24 hours (old and new) and 48 hours to assess the satisfactory achieved concentration and stability of the test concentrations of 0, 10 18, 32, 56 and100mg/L, using methodology developed in AH030453. All samples were analysed in duplicate after diluting 1 in 10 in the mobile phase mixture (5mM ammonium formate pH 3.0: acetonitrile, 81: 19, % v/v).

LC/MS analysis

[redacted] concentrations were measured by LC/MS. The solutions were analysed by injecting 10µL onto a Luna C_8 5µm (150 x 2.0mm) column held at 30°C. An isocratic mobile phase, flowing at 0.2 mL/min., consisting of 81% 5mM ammonium formate pH 3.0 and 19% acetonitrile was used for the analysis. Reagents were HPLC Grade or equivalent. The mass spectrometer was operated in positive electrospray ionisation (ESI) mode, with gas temperature 300°C (this was increased from 250°C after analysis of the 0 hour samples to give better source stability), gas flow 11.0 L/min, nebuliser pressure 30 psi and capillary voltage 3000V. Quantitation of [redacted] was carried out using single ion monitoring (SIM) for *m/z* 634.2. The calibration stock solution of [redacted] (S2539801) was prepared in Ultrapure water at 1000 mg/L (stable for 1 week in the fridge). Further dilutions in calibration standard preparation were carried out in mobile phase. Calibration standards were run at concentrations of 0.1, 1, 5 10 and 15 mg/L. The calibration standards were made fresh on each day of analysis from the stock solution.

Study dates

The analysis phase of the study was conducted between 24 February and 26 February 2004.

RESULTS

Full results are shown in Tables 1 and 2. Table 2 details the investigation into a high initial result for the 10 mg/L 24 hours new sample. This high result was confirmed by re-dilution of the initial sample. Another sample was supplied from Environmental Protection on the same day, which gave results in line with other data in Table 1.

TABLE 1. ANALYSIS OF TEST SOLUTIONS

Sample Name	Nominal Concentration (mg/L)	0 Hours		24 Hours Old		24 Hours New		48 Hours	
		Measured Concentration (mg/L)	% of Nominal Concentration	Measured Concentration (mg/L)	% of Nominal Concentration	Measured Concentration (mg/L)	% of Nominal Concentration	Measured Concentration (mg/L)	% of Nominal Concentration
0 mg/L A	0	<3	n/a	<3	n/a	<3	n/a	<3	n/a
0 mg/L B	0	<3	n/a	<3	n/a	<3	n/a	<3	n/a
0 mg/L Average	**0**	<3	**n/a**	<3	**n/a**	<3	**n/a**	<3	**n/a**
10 mg/L A	10	7.91	79.12	6.56	65.58	See Table 2	See Table 2	6.84	68.40
10 mg/L B	10	7.87	78.69	6.41	64.13	See Table 2	See Table 2	6.86	68.62
10 mg/L Average	**10**	**7.89**	**78.90**	**6.49**	**64.86**	See Table 2	See Table 2	**6.85**	**68.51**
18 mg/L A	18	14.30	79.42	12.89	71.61	16.83	93.50	12.00	66.68
18 mg/L B	18	10.60	58.90	12.99	72.17	16.78	93.22	12.07	67.05
18 mg/L Average	**18**	**12.45**	**69.16**	**12.94**	**71.89**	**16.81**	**93.36**	**12.04**	**66.87**
32 mg/L A	32	24.51	76.59	20.88	65.25	25.93	81.04	21.13	66.03
32 mg/L B	32	24.66	77.07	20.77	64.91	26.97	84.27	21.26	66.45
32 mg/L Average	**32**	**24.59**	**76.83**	**20.83**	**65.08**	**26.45**	**82.65**	**21.20**	**66.24**
56 mg/L A	56	47.81	85.38	39.98	71.40	48.38	86.40	38.78	69.25
56 mg/L B	56	42.45	75.81	40.06	71.53	48.70	86.97	38.26	68.32
56 mg/L Average	**56**	**45.13**	**80.59**	**40.02**	**71.47**	**48.54**	**86.68**	**38.52**	**68.79**
100 mg/L A	100	81.42	81.42	76.08	76.08	-	-	-	-
100 mg/L B	100	83.46	83.46	76.88	76.88	-	-	-	-
100 mg/L Average	**100**	**82.44**	**82.44**	**76.48**	**76.48**	-	-	-	-

n/a = not applicable

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TABLE 2. INVESTIGATION OF 10 mg/L 24 HOURS NEW RESULT

Sample	Solution description (time of analysis)	Measured Concentration (mg/L)	% of Nominal Concentration
10 mg/L (24 hours new) A	Initial analysis (12:00)	17.31	173.10
10 mg/L (24 hours new) B	Initial analysis (12:00)	17.53	175.30
10 mg/L (24 hours new) A	Repeat of original sample (16:15)	15.52	155.16
10 mg/L (24 hours new) B	Repeat of original sample (16:15)	15.24	152.38
10 mg/L (24 hours new) A	New Sample supplied by Env (16:30)	8.08	80.82
10 mg/L (24 hours new) B	New Sample supplied by Env (16:30)	8.04	80.35

Env = Environmental Protection

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STUDY REPORT

ACUTE TOXICITY OF [redacted]
TO *PSEUDOKIRCHNERIELLA SUBCAPITATA*

Study EAL030423

AUTHOR : D S NICHOLLS (STUDY DIRECTOR)

[redacted] **Confidential - Class 2 Circulation**

This report must not be circulated further, copied, or destroyed without reference to the Reports Administrator [redacted]

[redacted]

Date : May 2004

STUDY INFORMATION

Study title	:	Acute Toxicity of [redacted] to *Pseudokirchneriella subcapitata*
Study number	:	EAL030423
Study location	:	[redacted]

STUDY DATES

Date protocol signed	:	9 February 2004
Experimental period	:	13 February to 28 April 2004

ARCHIVING

Data & Report	:	Datacare Business Management Systems 3012 Heyford Park Upper Heyford Bucks OX25 5HF
Test item	:	[redacted]

STUDY PERSONNEL

Study Director	:	Darren Nicholls
Study Operator	:	Darren Nicholls
Responsible Practitioner*(s)*		Chris Sparham
Phase	:	Test substance analysis
Scientific Reviewer	:	Helen David
QA	:	Emma Butlin, Harjit Lall, Fay Williams

STUDY INFORMATION (CONTINUED)

CROSS REFERENCES

Project number	:	221130
Project name	:	MP5

AUTHORISATION STATEMENT

Study number	:	EAL030423
Study title	:	Acute Toxicity of [redacted] to *Pseudokirchneriella subcapitata*

This report has been authorised for issue to the appropriate recipients.

Julia Fentem

JULIA FENTEM
GROUP HEAD APPLIED SCIENCE & TECHNOLOGY
[redacted]

10/08/04
DATE

[redacted]

QUALITY ASSURANCE STATEMENT

Study Number : EAL030423

Study Title: Acute Toxicity of [redacted] to *Pseudokirchneriella Subcapitata*

This study was conducted at [redacted]

The following inspections and audits were conducted on the study. The dates on which they were performed and the dates on which any findings were reported to the Study Director and to Management are given below.

Audit Type	Audit Date	Report Date
Protocol Audit	09-Feb-2004	09-Feb-2004
Study Report Audit	01-Jun-2004	03-Jun-2004
Environment		
Process Inspection	01-Mar-2004	07-Jun-2004
- Algae Acute Toxicity		
Chemistry		
Process Inspection	05-Jan-2004	19-Mar-2004
- Measurement Procedures (Wt/Vol)		
- LC/MS		
Environment		
Facility Inspection	24-Feb-2004	16-Mar-2004
Chemistry		
Facility Inspection	27-Nov-2003	05-Dec-2003

As far as can reasonably be established, this report has been accepted by Quality Assurance as being an accurate presentation of the raw data and findings of the study.

10 June 2004.

H LALL
QUALITY ASSURANCE
[redacted]

DATE

AUTHENTICATION STATEMENT

Study number	:	EAL030423
Study title	:	Acute Toxicity of [redacted] to *Pseudokirchneriella subcapitata*

I, the undersigned, hereby declare that this study has been conducted under my supervision, as Study Director, in accordance with [redacted] policy on Good Laboratory Practice which is based on the UK Good Laboratory Practice Regulations 1999, No. 3106 and OECD Principles on Good Laboratory Practice (as revised in 1997) ENV/MC/CHEM/ (98)17.

I also certify that this report presents a true and accurate account of the procedures used and the results obtained.

D S NICHOLLS
STUDY DIRECTOR

10/06/04
DATE

[redacted]

RESPONSIBLE PRACTITIONERS

Study number	:	EAL030423
Study title	:	Acute Toxicity of [redacted] to *Pseudokirchneriella subcapitata*
Phase	:	Test substance analysis

CHRIS SPARHAM

10/6/04.
DATE

[redacted]

ACUTE TOXICITY OF [redacted] TO *PSEUDOKIRCHNERIELLA SUBCAPITATA*

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ACUTE TOXICITY OF [redacted]
TO *PSEUDOKIRCHNERIELLA SUBCAPITATA*

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ACUTE TOXICITY OF [redacted] TO *PSEUDOKIRCHNERIELLA SUBCAPITATA*

SUMMARY

The purpose of this study was to determine the 72hr EC50 and NOEC (No Observed Effect Concentration) values of [redacted] sample number S2539801) to *Pseudokirchneriella subcapitata.*

Two definitive experiments were run for this study. The first experiment, using a nominal concentration range based on the results of range finder tests (10 to 100 mg/l), was repeated, as not all the required EC50 and NOEC values were obtained. The repeated definitive experiment, using a revised nominal concentration range (3 to 300 mg/l) yielded all required values.

For each experiment, a range of test solutions were prepared by diluting a concentrated stock solution with ISO media. An (pre-calculated) aliquot of *Pseudokirchneriella subcapitata* inoculum was added to each test solution to give a nominal algal density of 10^4 cells/ml. Cell concentrations were determined at 0, 24, 48 and 72 hours using a Z2 Coulter Counter.

Test concentrations were measured by liquid chromatography/mass spectrometry (LC/MS).

With the 10 to 100 mg/l nominal concentration range, the geometric means of the measured results were as follows;

The 72-hour EC50 value for inhibition of growth rate was >39.43 mg/l.

The 72-hour NOEC value for inhibition of growth rate was <2.72 mg/l.

The 72-hour EC50 value for inhibition of Biomass was 5.48 mg/l.

The 72-hour NOEC value for inhibition of Biomass was <2.72 mg/l.

There was a 178 fold mean increase in the cell density of control cultures over 72 hours confirming study validity (a minimum 16-fold increase in cell density is required).

With the 3 to 300 mg/l nominal concentration range, the geometric means of the measured results were as follows;

The 72-hour EC50 value for inhibition of growth rate was 29.40 mg/l.

The 72-hour NOEC value for inhibition of growth rate was 2.77 mg/l.

The 72-hour EC50 value for inhibition of Biomass was 5.15 mg/l.

The 72-hour NOEC value for inhibition of Biomass was 0.86 mg/l.

There was a 112 fold mean increase in the control cell density over 72 hours, which confirmed study validity.

Test substance analysis showed that there was a decrease in the amount of test compound available over the 72 hour test periods. This loss cannot be attributed to absorption of compound onto the algal test species as the same phenomenon was observed amongst the blank test flasks. Hydrolysis of the principal active in [redacted] to the associated monoester was observed in analysis by LC-MS.

1. INTRODUCTION

The purpose of this study was to determine the 72hr EC50 and NOEC (No Observed Effect Effect Concentration) values of [redacted] to *Pseudokirchneriella subcapitata*. *Pseudokirchneriella subcapitata* is a unicellular green alga widely distributed in freshwaters and is easily maintained in laboratory culture in synthetic media [1]. *Pseudokirchneriella subcapitata* is recommended for use in the ISO freshwater algal growth inhibition test [2], by OECD Guideline 201 [3] and EEC Directive 92/69/EEC L383A [4].

2. SUBSTANCE TESTED

The test item used in this investigation was [redacted] It is registered in compound control as sample number S2539801 and has an activity of 97.89 percent. The sample is a [redacted]

Characterisation, stability and homogeneity were confirmed by analysis in study number AC030449.

3. METHOD

3.1 Experimental method

For each definitive experiment a nominal 1000 mg/ml stock solution was prepared by dissolving a pre-weighed aliquot of S2539801 in purified water; 500 mg into 500 ml for the 10 to 100 mg/l concentration range, 1000 mg into 1000 ml for the 3 to 300 mg/l concentration range.

Test media were prepared in volumetric flasks by adding appropriate volumes of stock, ISO concentrate and purified water to obtain the respective final nominal concentrations for each test (10, 18, 32, 56 and 100 mg/l) and (3, 10, 30, 100 and 300 mg/l). Blank flasks were prepared by removing an aliquot of test media from each volumetric flask before the *Pseudokirchneriella subcapitata* inoculum was added.

Glassware was conditioned with test compound for at least 24 hours before use in order to minimize potential losses by absorption of compound onto the glass.

Test flasks were placed in an illuminated orbital incubator according to the randomized distribution numbers generated by the Random.bas (version 3) computer software. Incubator temperature was recorded at 24-hour intervals for the duration of the test. Light intensity was not recorded.

Cell concentration was determined at 0, 24, 48 and 72 hours by a Z2 Coulter counter.

At 0 hours, the pH of the remaining test media was measured once all test flasks had been set up. At 72 hours, 20 ml from each test flask of the same concentration was pooled (excluding blanks) and a pH value recorded.

Test samples were sent for analysis at 0 and 72 hr to confirm satisfactory test solution concentration and to assess stability. Analysis was conducted using liquid chromatography/ mass spectrometry (LC/MS).

3.2 Statistical Analysis

The percentage inhibitions of biomass and growth rate were calculated using the ALGAE v.4.0 computer software. Reported figures are based on geometric means of measured concentrations.

3.3 Study dates

The study was conducted from 13 February to 28 April 2004.

3.4 Storage and retention of data

The protocol, any amendments, the raw data and final report will be placed in Datacare Business Management Systems, 3012 Heyford Park, Upper Heyford, Bucks, OX25 5HF. Datacare is not a member of the UK GLP compliance programme. The test item will be archived in [redacted]

4. RESULTS

4.1 Test Conditions

The pH values of each test solution at 0 and 72 hours are given in Tables 1 and 2 as recommended in OECD Guideline 201 [3].

During the experimental period for the 10 to 100 mg/l concentration range the incubator temperature fluctuated between 22.9 to 23.2 °C.

During the experimental period for the 3 to 300 mg/l concentration range the incubator temperature fluctuated between 22.9 to 23.0 °C.

4.2 Test Substance Concentrations

Characterisation, stability and homogeneity were confirmed by analysis in study number AC030449.

The Chemical Analysis Responsible Practitioner Summary is included as Appendix 3.

4.3 Effect Data

The test using the 10 to 100 mg/l concentration range showed a 178-fold mean increase in the cell density of control cultures over 72 hours, which confirmed study validity (a minimum 16-fold increase in control cell density is required for the study to be valid under OECD Guideline 201).

The test using the 3 to 300 mg/l concentration range produced a 112-fold mean increase in control cell density over 72 hours.

Cell densities (cells/ml) for each test flask are given in Appendices 1 and 2.

The calculated percentage inhibitions of growth rate and biomass at 72 hours for each test concentration are given in Tables 3 and 4.

Figures 1 and 2 show the percentage inhibition of growth rate for $\log_{10}$ (concentration) at 72 hours for each test concentration.

The measured concentrations of [redacted] as determined by LC-MS are detailed in Appendix 3.

4.4 Effect Concentrations

The effect concentrations, 48 and 72 hours EC50 and NOEC values are shown in Tables 5 and 6.

With the 10 to 100 mg/l concentration range, the geometric means of the measured results were as follows;

The 72-hour EC50 for inhibition of growth rate was >39.43 mg/l.

The 72-hour NOEC for inhibition of growth rate was <2.72 mg/l.

The 72-hour EC50 for inhibition of biomass was 5.48 mg/l.

The 72-hour NOEC for inhibition of biomass was <2.72 mg/l.

With the 3 to 300 mg/l concentration range, the geometric means of the measured results were as follows;

The 72-hour EC50 for inhibition of growth rate was 29.40 mg/l.

The 72-hour NOEC for inhibition of growth rate was 2.77 mg/l.

The 72-hour EC50 for inhibition of biomass was 5.15 mg/l.

The 72-hour NOEC for inhibition of biomass was 0.86 mg/l.

5. CONCLUSION

Two definitive experiments were run for this study. The first experiment, using a nominal concentration range based on the results of range finder tests (10 to 100 mg/l), was repeated, as not all the required EC50 and NOEC values were obtained. The repeated definitive experiment, using a revised nominal concentration range (3 to 300 mg/l) yielded all required values.

Using the 10 to 100 mg/l nominal concentration range, the geometric means of the measured results were as follows;

The 72-hour EC50 value for inhibition of growth rate was >39.43 mg/l.

The 72-hour NOEC value for inhibition of growth rate was <2.72 mg/l.

The 72-hour EC50 value for inhibition of Biomass was 5.48 mg/l.

The 72-hour NOEC value for inhibition of Biomass was <2.72 mg/l.

There was a 178-fold mean increase in the cell density of control cultures over 72 hours confirming study validity (a minimum 16-fold increase in cell density is required).

Using the 3 to 300 mg/l nominal concentration range, the geometric means of the measured results were as follows;

The 72-hour EC50 value for inhibition of growth rate was 29.40 mg/l.

The 72-hour NOEC value for inhibition of growth rate was 2.77 mg/l.

The 72-hour EC50 value for inhibition of Biomass was 5.15 mg/l.

The 72-hour NOEC value for inhibition of Biomass was 0.86 mg/l.

There was a 112-fold mean increase in the control cell density over 72 hours, which confirmed study validity.

Test substance analysis showed that there was a decrease in the amount of test compound available over the 72 hour test periods. This loss cannot be attributed to adsorption of compound onto the algal test species, as the same phenomenon was observed amongst the blank test flasks. Hydrolysis of the principal active in [redacted] to the associated monoester was observed in analysis by LC-MS.

6. REFERENCES

[1] Culture Centre for Algae and Protozoa. Freshwater Biological Association, Windermere Lab., The Ferry House, Ambleside, Cumbria, LA22 OLP.

[2] ISO 8692:1989 (E) Water Quality-Freshwater algal growth inhibition test with *Selenastrum capricornutum* and *Scenedesmus subspicatus*.

[3] OECD Guidelines for the Testing of Chemicals. Section 2. Guidelines 201 "Algae, Growth Inhibition Test" 1993.

[4] EEC Directive 92/69/EEC L383 A: Methods for the Determination of Ecotoxicity Algal Inhibition Test.

TABLE 1. TEST CONDITIONS: 10 TO 100 MG/L CONCENTRATION RANGE

Concentration (mg/l)	0.0	10	18	32	56	100
pH at 0 hours*	7.5	7.5	7.5	7.5	7.4	7.3
pH at 72 hours#	8.0	7.4	7.2	7.0	7.0	6.9

* pH of remaining test solution once flasks had been set up.
# pH of pooled test flasks (excluding blanks).

TABLE 2. TEST CONDITIONS: 3 TO 300 MG/L CONCENTRATION RANGE

Concentration (mg/l)	0.0	3	10	30	100	300
pH at 0 hours*	6.8	7.2	7.2	7.1	6.9	6.8
pH at 72 hours#	7.4	7.2	7.2	6.9	6.8	6.7

* pH of remaining test solution once flasks had been set up.
# pH of pooled test flasks (excluding blanks).

TABLE 3. 72 HOUR MEAN PERCENTAGE INHIBITIONS OF BIOMASS AND GROWTH RATE: 10 TO 100 MG/L CONCENTRATION RANGE

Nominal Conc. (mg/l)	Mean Measured Conc. (mg/l)	Biomass (% Inhibition)	Growth rate (% Inhibition)
10	2.72	34.42	8.83
18	5.56	48.15	13.35
32	11.14	67.64	25.61
56	19.68	81.44	40.83
100	39.43	88.39	48.11

TABLE 4. 72 HOUR MEAN PERCENTAGE INHIBITIONS OF BIOMASS AND GROWTH RATE: 3 TO 300 MG/L CONCENTRATION RANGE

Nominal Conc. (mg/l)	Mean Measured Conc. (mg/l)	Biomass (% Inhibition)	Growth rate (% Inhibition)
3	0.86	26.76	5.86
10	2.77	32.59	7.42
30	10.65	58.39	17.33
100	38.65	90.31	52.15
300	149.56	101.22	108.18

TABLE 5. 48 & 72 HOUR EC50 AND NOEC VALUES (MG/L): 10 TO 100 MG/L CONCENTRATION RANGE

	Biomass	Growth rate
48 hour EC50	12.45	>39.43
48 hour NOEC	2.72	5.56
72 hour EC50	5.48	>39.43
72 hour NOEC	<2.72	<2.72

TABLE 6. 48 & 72 HOUR EC50 HOUR AND NOEC VALUES (MG/1): 3 TO 300 MG/L CONCENTRATION RANGE

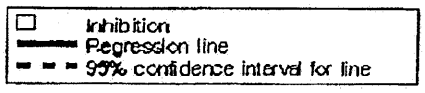
	Biomass	Growth rate
48 hour EC50	7.11	23.54
48 hour NOEC	2.77	2.77
72 hour EC50	5.15	29.40
72 hour NOEC	0.86	2.77

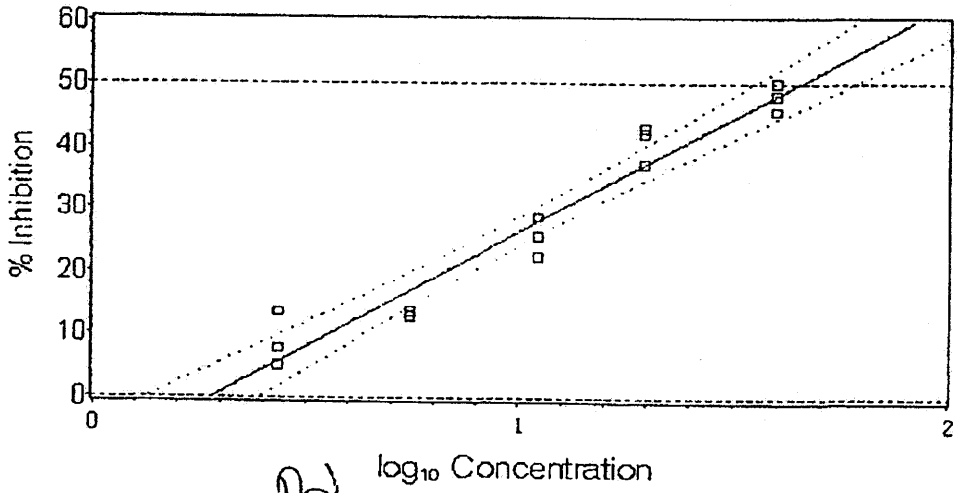
FIGURE 1. INHIBITION OF GROWTH RATE AT 72 HOURS:10 TO 100 MG/L CONCENTRATION RANGE

STUDY NO. EAL030423DEF

RESULT AT HOUR 72
Inhibition of Growth Rate

EC50 : > 39.433 mg/l, NOEC : < 2.722 mg/l
95% fiducial interval : * NOT COMPUTED *





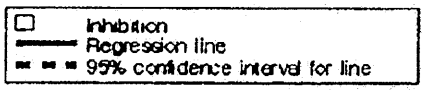
Operator: ..... ALGAE Version 4.0 (2003), 28APR2004 11:49:51

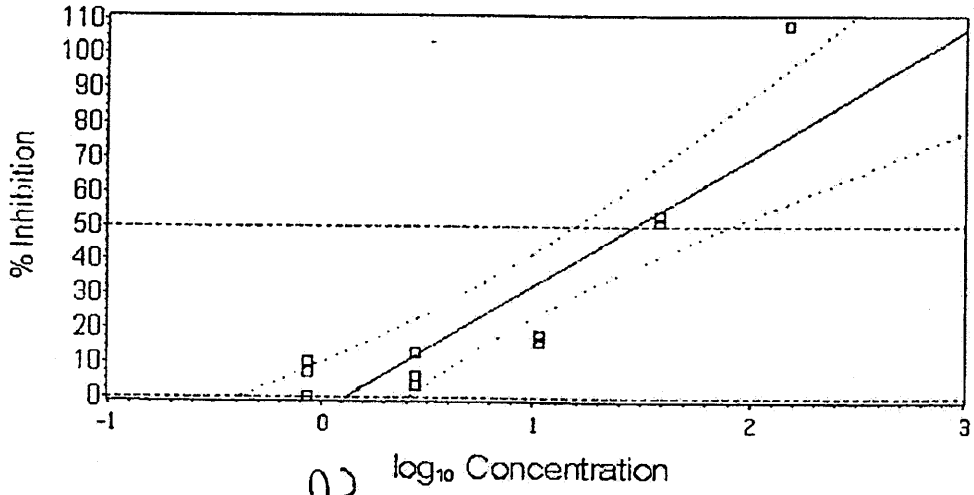
FIGURE 2. INHIBITION OF GROWTH RATE AT 72 HOURS:3 TO 300 MG/L CONCENTRATION RANGE

STUDY NO. EAL030423DEF2

RESULT AT HOUR 72
Inhibition of Growth Rate

EC50 : 29.397 mg/l, NOEC : 2.771 mg/l
95% fiducial interval : (17.155 , 60.141)





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APPENDIX 1. TABLE OF CELL DENSITIES AT 72 HOURS (CELLS/ML): NOMINAL 10 TO 100 MG/L CONCENTRATION RANGE

Flask code	Conc. mg/l	0 hours	24 hours	48 hours	72 hours
A1	0.0	9567.7	55629.9	386526.3	1972087.0
A2	0.0	9525.1	46063.2	291931.0	1501276.0
A3	0.0	10333.4	45536.6	272667.5	1544095.0
A4	0.0	9833.5	55888.6	390382.1	2119445.0
A5	0.0	10003.7	50246.1	329819.2	1681384.0
A6	0.0	9791.0	48707.8	297617.9	1713158.0
B1	10	9771.7	50447.8	318425.6	1302485.0
B2	10	9378.2	36992.1	179742.8	829443.3
B3	10	9463.2	42924.3	246794.2	1106027.0
C1	18	9261.4	40343.3	237640.1	788282.6
C2	18	9080.6	37962.1	196927.6	813087.0
C3	18	9654.9	40633.0	233141.4	796890.3
D1	32	9559.0	38773.9	225874.0	479072.4
D2	32	9633.4	35290.3	184987.5	410924.8
D3	32	9941.9	27574.7	121640.4	378624.4
E1	56	10057.3	30488.9	133776.7	235233.3
E2	56	10014.7	31291.5	116584.1	183582.6
E3	56	9982.8	31526.9	121107.4	173222.1
F1	100	7681.6	19521.7	72088.3	115436.8
F2	100	7702.9	26751.8	88455.6	103995.0
F3	100	8872.9	28516.3	90910.2	107407.0

APPENDIX 2. TABLE OF CELL DENSITIES AT 72 HOURS – (CELLS/ML): NOMINAL 3 to 300 MG/L CONCENTRATION RANGE

Flask code	Conc. mg/l	0 hours	24 hours	48 hours	72 hours
A1	0.0	10183.8	30935.1	126961.6	659705.6
A2	0.0	9715.9	38959.1	202313.3	1153796.0
A3	0.0	9407.5	43638.5	170720.2	938203.6
A4	0.0	9758.4	45185.4	278570.8	1619496.0
A5	0.0	10428.4	40911.7	216232.0	1137745.0
A6	0.0	9896.6	39355.9	215177.8	1148410.0
B1	3.0	9620.7	30807.9	141642.0	732524.9
B2	3.0	9907.9	36387.4	193567.1	1040918.0
B3	3.0	9939.8	28091.0	120168.7	663138.9
C1	10	9769.7	32061.6	124030.9	589046.4
C2	10	9578.3	25644.6	144515.7	718846.1
C3	10	9886.7	33560.6	178330.9	882731.3
D1	30	8462.9	18878.9	92969.0	356064.6
D2	30	9185.9	29603.3	126531.8	451270.4
D3	30	9100.8	29207.5	122656.0	412952.3
E1	100	8961.6	11987.3	47696.0	66822.7
E2	100	9046.7	14374.3	56944.5	73642.2
E3	100	9344.4	8356.9	53469.7	59302.8
F1	300	8443.0	5815.8	9298.6	4712.3
F2	300	8283.5	3675.7	7472.6	-2193.0
F3	300	8634.4	3026.5	4824.7	-491.8

APPENDIX 3. RESPONSIBLE PRACTITIONER SUMMARY (CHEMICAL ANALYSIS)

ACUTE TOXICITY OF [redacted] TO *PSEUDOKIRCHNERIELLA SUBCAPITATA*

Analysis was carried out at 0 and 72 hr to confirm satisfactory test solution concentration and assess stability using liquid chromatography/mass spectrometry (LC/MS) methodology developed in AH030453. The range finder was used to confirm the methodology developed so far was suitable for chemical analysis support of the main algal studies. An increase in injection volume, from that used in work carried out in AH030453, was necessary to detect the lower concentrations proposed for the study. The main Algal test was repeated to achieve the necessary toxicity data, referred to as Agal Test 1 and 2 in the following summary.

1. METHOD

1.1 Method development (Range finder)

1.1a Sample preparation

Samples were supplied at concentrations of 0, 1, 3, 10, and 100 mg/L in Isomedia with and without algae. Duplicate aliquots of each sample were filtered through a 0.45 µm PTFE filter before dilution in mobile phase as follows:

0mg/L sample were analysed after diluting 1:10 in mobile phase.
1mg/L sample were analysed after diluting 1:5 in mobile phase.
3mg/L sample were analysed after diluting 1:10 in mobile phase.
10mg/L sample were analysed after diluting 1:20 in mobile phase.
100mg/L sample were analysed after diluting 1:200 at 0hr and 1:20 at 72hrs in mobile phase.

(Where mobile phase was 81% 5mM ammonium formate pH 3.0 and 19% acetonitrile v/v)

1.1b LC/MS

[redacted] concentrations were measured by LC/MS. The solutions were analysed by injecting 100 µL onto a Luna C_8 5µm (150 x 2.0mm) column held at 30°C. An isocratic mobile phase, flowing at 0.2 mL/min., consisting of 81% 5mM ammonium formate pH 3.0 and 19% acetonitrile was used for the analysis. Reagents were HPLC Grade or equivalent. The mass spectrometer was operated in positive electrospray ionisation (ESI) mode, with gas temperature 250°C (increased to 300°C for 72 hr samples and further algal tests to aid source stability), gas flow 11.0 L/min, nebuliser pressure 30 psi and capillary voltage 3000V. Quantitation of [redacted] was carried out using single ion monitoring (SIM) for

m/z 634.2. Full scan data was also acquired to assess any degradation of the main active of [redacted] The calibration stock solution of [redacted] (S2539801) was prepared in Ultrapure water at 3000 mg/L (stable for 1 week in the fridge). Further dilutions in calibration standard preparation were carried out in mobile phase (81% 5mM ammonium formate pH 3.0 and 19% acetonitrile v/v). Calibration standards were run at concentrations of 0, 0.15, 0.3, 0.6 and 0.9 mg/L and were made fresh on each day of analysis from the stock solution.

1.2 Algal Tests

1.2a Sample preparation

Samples were supplied at concentrations of 0, 10, 18, 32, 56 and 100 mg/L (Algal Test 1) and 0, 3, 10, 30, 100 and 300 mg/L (Algal Test 2) in Isomedia with and without algae. Duplicate aliquots of each sample were filtered through a 0.45 µm PTFE filter before dilution in mobile phase as follows:

0mg/L sample were analysed after diluting 1:10 in mobile phase.
3mg/L sample were analysed after diluting 1:10 in mobile phase.
10mg/L sample were analysed after diluting 1:25 in mobile phase.
18mg/L sample were analysed after diluting 1:25 in mobile phase.
30mg/L sample were analysed after diluting 1:50 in mobile phase.
32mg/L sample were analysed after diluting 1:50 in mobile phase.
56mg/L sample were analysed after diluting 1:100 in mobile phase.
100mg/L sample were analysed after diluting 1:200 in mobile phase.
300mg/L sample were analysed after diluting 1:500 in mobile phase.

(Where mobile phase was 81% 5mM ammonium formate pH 3.0 and 19% acetonitrile)

1.2b LC/MS

As described in Section 1.1a. An additional calibration standard was included at 0.045mg/L.

2. RESULTS

2.1 Method development (Range finder)

Results for the method development stage of this study, at concentrations of 0, 1, 3, 10,and 100 mg/L [redacted] (S2539801) in Isomedia with and without algae are detailed in Table 1.

2.2 Algal Tests

Results for Algal Test 1 at concentrations of 0, 10, 18, 32, 56 and 100 mg/L [redacted] (S2539801) in Isomedia with and without algae are detailed in Table 2. Results for Agal Test 2 at concentrations of 0, 3, 10, 30, 100 and 300 mg/L [redacted] (S2539801) in Isomedia with and without algae are detailed in Table 3.

A typical chromatogram is shown in Figure 1. The data was also acquired in scan mode on the LC/MS to assess degradation of the main active of [redacted] Typical mass spectra of the main peak in the total ion chromatogram in samples containing 100 mg/L of [redacted] are presented in Figure 2. A comparison is made of spectra taken at 0hr and after 72hr under test conditions in Isomedia and Isomedia containing algae. In spectra of 72hr samples in both Isomedia (C) and Isomedia containing algae (D), an ion at *m/z* 620 is clearly visible in increased amounts compared to the respective 0hr samples (A) and (B). Hydrolysis of the principal active in [redacted] with *m/z* 634, has resulted in the formation of monoester, with characteristic ion *m/z* 620. In the absence of standards an accurate estimate of the amount of monoester present in these samples cannot be made.

TABLE 1. MEASURED [redacted] CONCENTRATIONS FROM RANGE FINDER TEST (METHOD DEVELOPMENT)

Sample details		0Hr				72Hr			
		Isomedia		Isomedia with algae		Isomedia		Isomedia with algae	
Nominal mg/L	Replicate	mg/L measured in solution	% of nominal	mg/L measured in solution	% of nominal	mg/L measured in solution	% of nominal	mg/L measured in solution	% of nominal
0	A	<0.1	-	<0.1	-	<0.1	-	<0.1	-
	B	<0.1	-	<0.1	-	<0.1	-	<0.1	-
1	A	0.8358	83.6	0.8345	83.5	0.1349	13.5	0.0456	4.96
	B	0.8316	83.2	0.8326	83.3	0.1400	14.0	0.0447	4.47
3	A	2.738	91.3	2.577	85.9	0.7078	23.6	0.0741	2.47
	B	2.547	84.9	2.647	88.2	0.5539	18.5	0.0713	2.38
10	A	9.341	93.4	8.799	88.0	1.5457	15.5	1.3203	13.2
	B	8.769	89.7	8.973	89.7	1.4054	14.1	1.3044	13.0
100	A	94.96	95.0	97.34	97.3	23.696	23.7	22.592	22.6
	B	94.66	94.7	94.09	94.1	23.529	23.5	22.967	23.0

Sanitized Version

TABLE 2. MEASURED [redacted] CONCENTRATIONS– ALGAL TEST 1

Sample details		0hr				72hr			
		Isomedia		Isomedia with algae		Isomedia		Isomedia with algae	
Nominal mg/L	Replicate	mg/L measured in solution	% of nominal	mg/L measured in solution	% of nominal	mg/L measured in solution	% of nominal	mg/L measured in solution	% of nominal
0	A	<0.1	-	<0.1	-	<0.1	-	<0.1	-
	B	<0.1	-	<0.1	-	<0.1	-	<0.1	-
10	A	7.371	73.7	7.501	75.0	1.935	19.4	1.222	12.2
	B	8.288	82.9	6.834	68.4	1.924	19.2	0.8456	8.46
18	A	13.45	74.7	13.17	73.2	2.575	14.3	2.260	12.6
	B	14.99	83.3	13.26	73.6	2.379	13.2	2.424	13.5
32	A	24.06	75.2	23.26	72.7	6.468	20.2	5.012	15.7
	B	24.72	77.2	25.90	80.9	6.222	19.4	5.080	15.9
56	A	46.66	83.3	46.14	82.4	10.91	19.5	8.429	15.1
	B	43.31	77.3	43.07	76.9	10.21	18.2	8.930	15.9
100	A	81.24	81.2	82.19	82.2	23.68	23.7	20.39	20.4
	B	88.51	88.5	74.56	74.6	22.63	22.6	19.29	19.3

Sanitized Version

TABLE 3. MEASURED [redacted] CONCENTRATIONS– ALGAL TEST 2

Sample details		0hr				72hr			
		Isomedia		Isomedia with algae		Isomedia		Isomedia with algae	
Nominal mg/L	Replicate	mg/L measured in solution	% of nominal	mg/L measured in solution	% of nominal	mg/L measured in solution	% of nominal	mg/L measured in solution	% of nominal
0	A	<0.1	-	<0.1	-	<0.1	-	<0.1	-
	B	<0.1	-	<0.1	-	<0.1	-	<0.1	-
3	A	2.519	84.0	2.384	79.5	0.5384	17.9	0.3315	11.1
	B	2.559	85.3	2.161	72.0	0.5445	18.2	0.3225	10.8
10	A	8.924	89.2	8.059	80.6	1.358	13.6	0.9840	9.84
	B	8.916	89.2	7.485	74.9	1.341	13.4	0.9469	9.47
30	A	27.38	91.3	25.31	84.4	6.718	22.4	4.717	15.7
	B	25.83	86.1	22.44	74.8	5.914	19.7	4.776	15.9
100	A	91.55	91.6	87.00	87.0	16.54	16.5	17.74	17.7
	B	91.66	91.7	81.94	81.9	16.82	16.8	17.62	17.6
300	A	297.7	99.3	253.8	84.6	87.49	29.2	89.76	29.9
	B	283.1	94.4	267.1	89.0	86.78	28.9	82.00	27.3

Sanitized Version

FIGURE 1. TYPICAL LC/MS CHROMATOGRAM OF [redacted] USING SIM FOR *m/z* 634.2

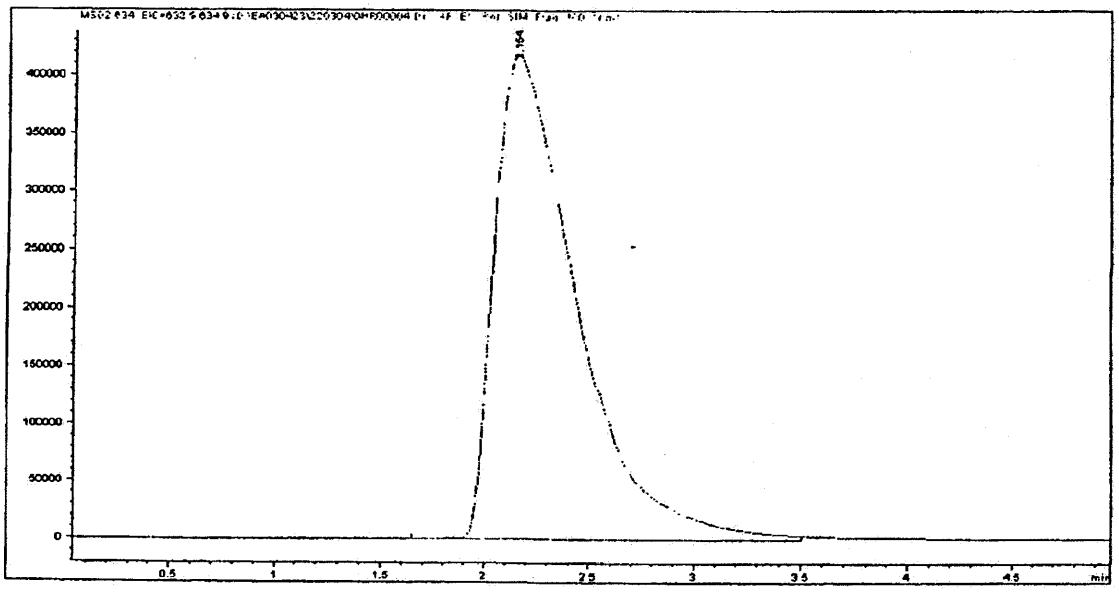
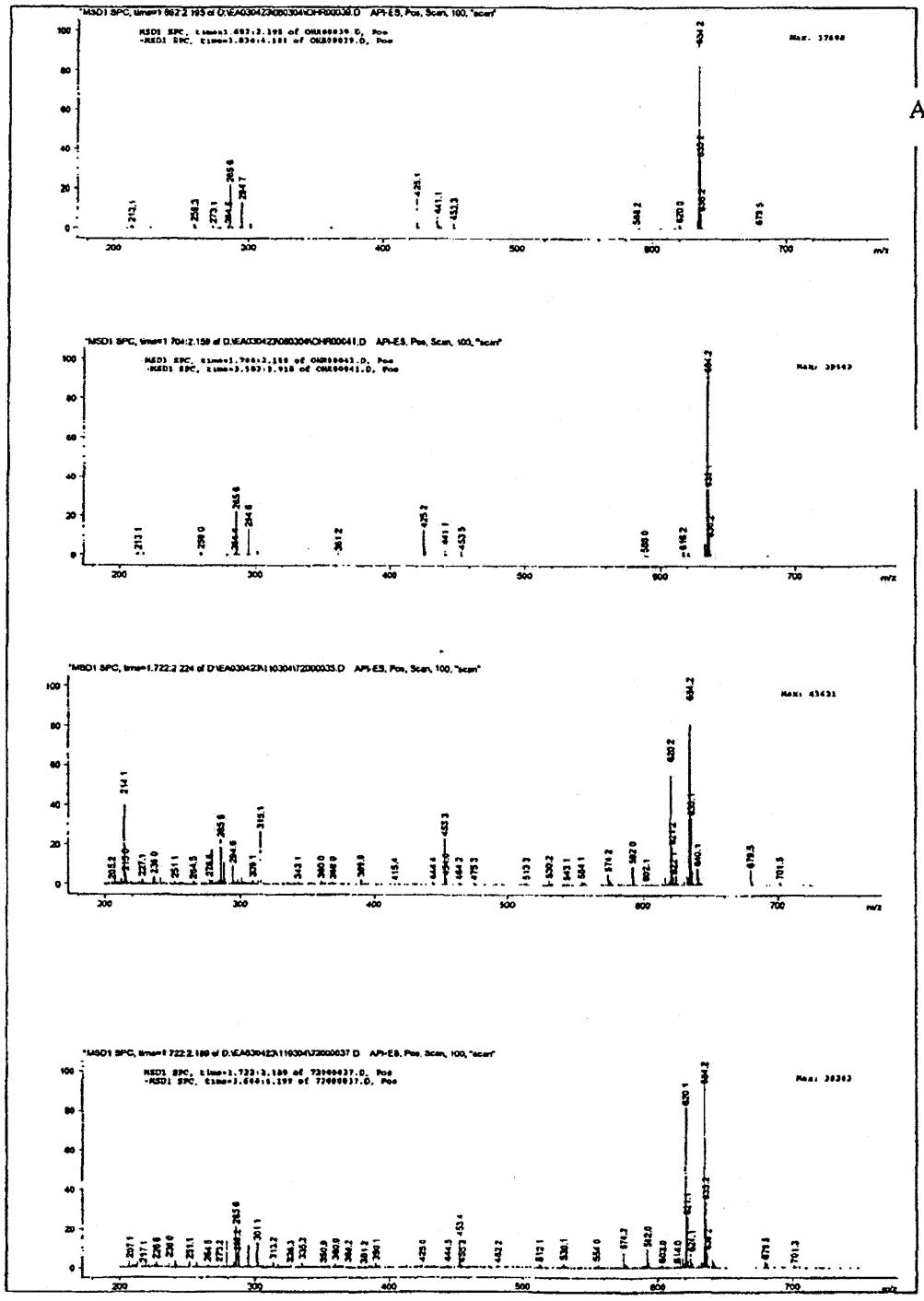


FIGURE 2. TYPICAL MASS SPECTRA OF THE MAIN PEAK ANALYSED IN 100 mg/L [redacted] TEST SOLUTIONS (ALGAL TEST 1)

A) 0HR ISOMEDIA B) 0HR ISOMEDIA PLUS ALGAE
C) 72HR ISOMEDIA D) 72HR ISOMEDIA PLUS ALGAE



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Study EAL030423

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REPORT

Study Title:

TOXICITY OF [redacted]
TO ACTIVATED SLUDGE
IN A RESPIRATION INHIBITION TEST

Data Requirements / Test Guidelines:

OECD No. 209
EU Commission Directive 88/302/EEC, C.11

Study Director:

Roger Batscher

Study Completion Date:

March 04, 2004

Test Facility:

RCC Ltd
Environmental Chemistry &
Pharmanalytics
CH–4452 Itingen/Switzerland

Sponsor:

[redacted]

Identification:

RCC Study Number: 851903
[redacted] Study Reference No.: KBI030429

Page 1 of 20

RCC

GLP CERTIFICATE

The Swiss GLP Monitoring Authorities

Swiss Federal Office of Public Health

Swiss Agency for the Environment, Forests and Landscape

swissmedic Swissmedic Swiss Agency for Therapeutic Products

Statement of GLP Compliance

It is hereby confirmed that

during the period of November 18 – 22, 2002

the following Test Facilities of

RCC Ltd
4452 Itingen
Switzerland

were inspected by the Federal Office of Public Health, the Swiss Agency for Therapeutic Products and the Swiss Agency for the Environment, Forests and Landscape with respect to the compliance with the Swiss legislation on Good Laboratory Practice.

Test Facilities	**Areas of expertise ***
- Toxicology	TOX, ACC, MUT, OTH (Safety Pharmacology)
- Environmental Chemistry and Pharmanalytics	ACC, ECT, ENF, EMN, PCT, RES, OTH (Animal metabolism)

The inspections were performed in agreement with the OECD Guidelines for National GLP Inspections and Audits. It was found that the aforementioned test facilities were operating in compliance with the Swiss Ordinance relating to Good Laboratory Practice [RS 813.016.5] at the time they were inspected.

Federal Office of Public Health
The Director

Bern, March 2003

Prof. Th. Zeltner

* TOX = Toxicology ; ACC = Analytical and Clinical Chemistry ; ECT = Environmental toxicity on aquatic and terrestrial organisms ; ENF = Behaviour in water, soil and air, Bioaccumulation ; EMN = Studies on effects on mesocosms and natural ecosystems; MUT = Mutagenicity; PCT = Physical-chemical testing ; RES = Residue studies ; OTH = Other, to be specified.

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE

RCC Study Number:	851903
Test Item:	[redacted]
Study Director:	Mr. Roger Bätscher
Study Title:	Toxicity of [redacted] to activated sludge in a respiration inhibition test

This study has been performed in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted February 2nd, 2000 [RS 813.016.5]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C(97) 186/Final].

The purity of the test item is not yet available and is therefore excluded from the statement of compliance.

There were no circumstances that may have affected the quality or integrity of the data.

Study Director: Mr. Roger Bätscher

R. Bätscher

Date: March 04, 2004

SIGNATURES

Study Director: Mr. Roger Bätscher

.....

Date: March 04, 2004

Management: Dr. Uwe Morgenroth

.....

Date: March 04, 2004

QUALITY ASSURANCE

RCC Ltd, Environmental Chemistry & Pharmanalytics, CH-4452 Itingen / Switzerland

STATEMENT

RCC Study Number: 851903

Test Item: [redacted]

Study Director: Mr. Roger Bätscher

Study Title: Toxicity of [redacted] to activated sludge in a respiration inhibition test

The general facilities and activities are inspected periodically and the results are reported to the responsible person and the management.

Study procedures were periodically inspected. The study plan and this report were audited by the Quality Assurance. The dates are given below:

Dates and Types of QA Inspections		Dates of Reports to the Study Director and to the Management
December 12, 2003	Study plan	December 12, 2003
January 08, 2004	Process based (test conditions)	January 08, 2004
January 23, 2004	Report	January 23, 2004

This statement also confirms that this final report reflects the raw data.

Quality Assurance: for Mrs. Jane Sanotra

.....

Date: March 04, 2004

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PREFACE

GENERAL

Study Title:	Toxicity of [redacted] to activated sludge in a respiration inhibition test
Sponsor:	[redacted]
Scientific Representative:	[redacted]
[redacted] Contact:	[redacted]
Test Facility:	RCC Ltd Environmental Chemistry & Pharmanalytics Zelgliweg 1 CH-4452 Itingen / Switzerland

RESPONSIBILITIES

Study Director:	Mr. Roger Bätscher
Deputy Study Director:	Dr. Birgit Seyfried
Technical Coordinator:	Mrs. Renate Wetzlinger
Head of RCC Quality Assurance:	Mrs. Iris Wüthrich

SCHEDULE

Experimental Starting Date:	February 02, 2004
Experimental Completion Date:	February 05, 2004
Study Completion Date:	March 04, 2004

ARCHIVING

RCC Ltd, CH-4452 Itingen, Switzerland will retain the study plan, raw data, a sample of the test item and the final report of the present study for at least ten years. Any unused test item will be returned to the sponsor. Archiving of the test item is the responsibility of the sponsor.

No data will be discarded without the Sponsor's consent.

DATA REQUIREMENTS / TEST GUIDELINES

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

OECD Guideline for Testing of Chemicals, No. 209, Activated Sludge, Respiration Inhibition Test, 1984.

EU Commission Directive 88/302/EEC, Part C.11, Activated Sludge Respiration Inhibition Test, 1988.

SUMMARY OF STUDY PLAN AMENDMENTS

There were no amendments to the study plan.

SUMMARY

The inhibitory effect of the test item [redacted] on the respiration rate of aerobic wastewater microorganisms of activated sludge was investigated in a 3-hour respiration inhibition test according to the EU Commission Directive 88/302/EEC, Part C.11, and the OECD Guideline for Testing of Chemicals, No. 209.

The following nominal concentrations of [redacted] were tested: 10, 32, 100, 320, and 1000 mg/L. Concentrations above 1000 mg/L were not tested.

In addition, two controls and three different concentrations of the reference item 3,5-dichlorophenol (5, 16, and 50 mg/L) were tested in parallel. The results of these treatments confirmed the suitability of the activated sludge and the method used.

Up to and including the concentration of 100 mg/L, the test item [redacted] had no significant inhibitory effect (<15%) on the respiration rate of activated sludge after the incubation period of three hours. At the higher test item concentrations of 320 and 1000 mg/L, the respiration rate was inhibited by 22 and 41%, respectively.

The 3-hour EC20 was determined to be 264 mg/L with 95% confidence limits of 171 and 364 mg/L. The 3-hour EC50 and 3-hour EC80 could not be determined but were above the highest test concentration of 1000 mg/L.

The 3-hour NOEC (determined as the calculated 3-hour EC15) was 174 mg/L with 95% confidence limits of 96 and 249 mg/L.

1 PURPOSE

The purpose of the 3-hour toxicity test was to evaluate the influence of the test item [redacted] n activated sludge microorganisms. Aerobic activated sludge was incubated for three hours in the presence of various concentrations of the test item under defined conditions. The respiration rates (oxygen consumption) were measured. The inhibitory effect of the test item was expressed as a percentage of the mean respiration rate of two controls.

The method of application and the test system are recommended by the OECD and EU test guidelines.

2 MATERIALS AND METHODS

2.1 DEFINITIONS

Respiration rate:	the oxygen consumption of wastewater microorganisms in aerobic activated sludge, expressed as mg O_2 per liter per minute
ECx:	the calculated concentration of test item at which the respiration rate is inhibited by x% when compared to the respiration rate of the control
NOEC:	the highest test concentration at which no significant toxic effect is observed (≤15% inhibition)

2.2 TEST ITEM

The test item and the following information concerning the test item were provided by the sponsor:

Identity:	
Lot no.:	S2539801
Purity:	The purity of the test item was not available prior to administration, hence this information is excluded from the Statement of Compliance. However, the sponsor is addressing this in a GLP compliant study Reference Number AC030449.
Expiration date:	January 01, 2005
Aggregate state at room temperature:	Solid
Colour:	(determined visually at RCC)
Solubility in water:	Approx. 5 percent by weight
Stability in water:	7 days in the refrigerator (based on unaudited data)
Storage conditions:	At room temperature in the dark

2.3 REFERENCE ITEM

The reference item and the following information concerning the reference item were provided by Aldrich Chemie:

Identity:	3,5-dichlorophenol
Product no.:	D7060-0
Lot no.:	02611ES
Purity:	99.1% (GLC)
Expiration date:	July 17, 2005
Storage conditions:	In tightly closed original container, at room temperature at about 20 °C

2.4 TEST SYSTEM

The study was performed with aerobic activated sludge from a wastewater treatment plant (ARA Ergolz II, Füllinsdorf, Switzerland) treating predominantly domestic wastewater. The sludge was washed twice with tap water by centrifugation and the supernatant liquid phase was decanted. A homogenized aliquot of the final sludge suspension was weighed, thereafter dried and the ratio of wet to dry weight was calculated.

Based on this ratio, an aliquot of washed sludge was suspended in tap water to obtain a concentration equivalent to 3 g dry material per liter. During the holding of three days prior to use, the sludge was fed daily with 50 mL synthetic wastewater* per liter and was kept at room temperature under continuous aeration until use. Before use, the dry weight of the activated sludge was measured again in the inoculum used in the test. The pH of the activated sludge inoculum was adjusted from 8.5 to 7.8 with a diluted sulfuric acid solution.

* Synthetic wastewater:

16	g peptone	filled up to a final volume of 1 liter with deionized water
11	g meat extract	
3	g urea	
0.7	g NaCl	
0.4	g $CaCl_2 \cdot 2H_2O$	
0.2	g $MgSO_4 \cdot 7H_2O$	
2.8	g K_2HPO_4	

2.5 STUDY DESIGN

2.5.1 Dosage and Concentrations

The final nominal test concentrations of the test item were 10, 32, 100, 320, and 1000 mg/L. Concentrations above 1000 mg of test item per liter were not tested.

A concentrated stock solution of [redacted] with a nominal concentration of 5.0 g/L was prepared: 1000 mg of [redacted] was dissolved in 200 mL of tap water and was thoroughly mixed by intense stirring for 10 minutes at room temperature. In this way, a clear solution colored [redacted] by the test item was obtained. Adequate volumes of this stock solution were diluted with tap water to prepare the test media. The exact volumes used are given in the scheme below. The test media were prepared as far as possible in the dark to avoid photolytical degradation of the test item.

Additionally, two controls (tap water, synthetic wastewater and activated sludge inoculum, without test item) and the reference item 3,5-dichlorophenol (positive control) at nominal concentrations of 5, 16, and 50 mg/L were tested in parallel under otherwise identical conditions. Thus, one replicate was tested for each test concentration of the test item and the reference item.

For test media containing the reference item, a stock solution of 3,5-dichlorophenol was prepared according to the test guidelines: 0.5 g of 3,5-dichlorophenol was dissolved in 10 mL 1 M NaOH and diluted to about 30 mL with purified water. Excess of NaOH was neutralized with 0.5 mol/L H_2SO_4 to the point of incipient precipitation. Thereafter, the mixture was made up to one liter with purified water. The pH was determined to be 7.6. The final concentration of 3,5-dichlorophenol amounted to 500 mg/L. Aliquots of this 3,5-dichlorophenol stock solution were mixed with synthetic wastewater and tap water in the respective test flasks to obtain the desired concentrations. The exact volumes used are given in the scheme below.

Composition of the test media:

Flask No.	Identification	Synthetic waste-water (mL)	Aliquot of stock solution (mL)*	Aliquot of stock solution (mL)**	Activated sludge (mL)	Tap water (mL)	Final nominal conc. (mg/L)
1	Inoculum control	16	---	---	200	284	---
2	3,5-dichlorophenol	16	---	5	200	279	5
3	3,5-dichlorophenol	16	---	16	200	268	16
4	3,5-dichlorophenol	16	---	50	200	234	50
5	Test item	16	1.0	---	200	283	10
6	Test item	16	3.2	---	200	280.8	32
7	Test item	16	10	---	200	274	100
8	Test item	16	32	---	200	252	320
9	Test item	16	100	---	200	184	1000
10	Inoculum control	16	---	---	200	284	---

* Stock solution of [redacted] (5.0 g/L)
** Stock solution of 3,5-dichlorophenol (500 mg/L)

2.5.2 Experimental Conditions

The test was performed in brown 1000-mL glass flasks. The test flasks were labeled with the necessary information to ensure unmistakable identification.

At the start of the test, synthetic wastewater and activated sludge inoculum (see Sections 2.4 and 2.5.1) were added. The sludge concentration of the inoculum was 3.5 g dry weight per liter (corresponding to about 1.4 g dry material per liter test medium). The sludge was added in time intervals of 15 minutes (an arbitrary but convenient interval) first to a control, then to the test solutions of the reference item, then to the test solutions of the test item, and finally to the second control (= start of the incubation). The final volume of the test media was 500 mL.

During the incubation period of exactly 3 hours, all test media and the controls were continuously aerated with compressed air at a flow rate of approximately one liter per minute. The concentration of dissolved oxygen did not drop below 2.5 mg/L during the incubation period, and just before the measurements of the respiration rates, the dissolved oxygen concentrations were at least 8.0 mg/L (Tables 1 and 2). The temperature in the test media, measured in one control, was 20 °C at the start and at the end of the incubation period.

2.6 EVALUATIONS

2.6.1 Measurement of the Respiration Rates

For measurement of the respiration rate, a well-mixed sample of each test medium was poured into a BOD-flask after exactly three hours incubation time, and was not further aerated. Then, the dissolved oxygen concentration was measured with an oxygen electrode (WTW TriOxmatic® 300 and an oxygen meter WTW Oxi 539, Wissenschaftlich-Technische Werkstaetten WTW, Weilheim/Germany), and was continuously recorded for about ten to fifteen minutes. During measurement, the samples were continuously stirred on a magnetic stirrer. The oxygen consumption rate (in mg O_2 L^{-1} $minute^{-1}$) was determined from the linear part of the respiration curve in the range of 6.5–2.5 mg O_2/L. In case of very rapid oxygen consumption, the range used was below the limits indicated above but always within the linear part of the respiration curve. In case of low oxygen consumption, the rate was determined from measurements over a period of at least 10 minutes.

2.6.2 Determination of the Inhibition Rates

The inhibitory effect of the test item or of the reference item on the respiration rate (oxygen consumption per minute) is expressed as a percentage of the mean respiration rate of the two controls measured at the start and at the end of the test series (flasks 1 and 10).

$$\left(1-\frac{2R_s}{R_{c1}+R_{c2}}\right)\times 100\% = \text{percent inhibition}$$

where

R_s = oxygen consumption rate (in mg O_2 L^{-1} $minute^{-1}$) at tested concentration

R_{c1} = oxygen consumption rate in control flask 1

R_{c2} = oxygen consumption rate in control flask 10

The 3-hour EC15 (NOEC) and EC20 for the test item and their 95% confidence limits were calculated by Probit Analysis (Ref. 1, 2). The 3-hour EC50 and EC80 could not be calculated since the inhibition of the respiration rate was below 50% up to and including the highest test concentration of 1000 mg/L. The 3-hour EC50-value of the reference item 3,5-dichlorophenol and its 95% confidence limits was calculated by Probit Analysis (Ref. 1, 2).

All test results were based on the nominal concentrations of the test item.

2.6.3 Water Quality Criteria

The pH-values and dissolved oxygen concentrations were determined in all test media and the controls at the start and at the end of the 3-hour incubation period. The water temperature was measured in one control at the start and end of the incubation period, and was continuously monitored in all test media and the controls during measurements of the respiration rate. Before the addition of activated sludge and synthetic waste-water, the appearance of the test media was recorded.

3 RESULTS AND DISCUSSION

3.1 TOXICITY OF

The influence of on the respiration rate of activated sludge is presented in Table 1 and Figure 1.

Up to and including the concentration of 100 mg/L, the test item had no significant inhibitory effect (<15%) on the respiration rate of activated sludge after the incubation period of three hours (Table 1 and Figure 1). At the higher test item concentrations of 320 and 1000 mg/L, the respiration rate was inhibited by 22 and 41%, respectively.

The 3-hour EC20 was determined to be 264 mg/L with 95% confidence limits of 171 and 364 mg/L. The 3-hour EC50 and 3-hour EC80 could not be determined but were above the highest test concentration of 1000 mg/L.

The 3-hour NOEC (determined as the calculated 3-hour EC15) was 174 mg/L with 95% confidence limits of 96 and 249 mg/L.

The oxygen consumption rates of the two controls (run at the start or at the end of the test) differed by 8% (guideline-recommended maximum variation: 15%).

3.2 TOXICITY OF 3,5-DICHLOROPHENOL (POSITIVE CONTROL)

The results are presented in Table 2 and Figure 1.

The 3-hour EC50 of the reference item 3,5-dichlorophenol (positive control) was calculated to be 15 mg/L with 95% confidence limits of 14 and 16 mg/L. The 3-hour EC50 is within the guideline-recommended range of 5-30 mg/L, confirming the suitability of the activated sludge used.

4 TABLES

Table 1: Influence of [redacted] on oxygen consumption of activated sludge

Flask	Test chemical	Nominal concentration of test chemical (mg/L)	Oxygen consumption rate (mg O_2/L min^{-1})	Inhibition (%)	pH values start*	pH values end*	Oxygen concentration (mg O_2/L) start*	Oxygen concentration (mg O_2/L) end*
1	Control	0	1.384		8.0	8.5	8.9	9.2
10	Control	0	1.500		8.0	8.4	8.8	9.2
Mean Deviation (%)			1.442 8.3					
5	[redacted]	10	1.284	10.9	7.9	8.5	8.9	8.2
6		32	1.293	10.3	7.9	8.5	8.8	8.5
7		100	1.298	10.0	8.1	8.6	9.0	8.9
8		320	1.124	22.1	7.9	8.4	8.7	8.6
9		1000	0.845	41.4	7.9	8.6	8.8	8.0

* Start and end of the 3-hour incubation period

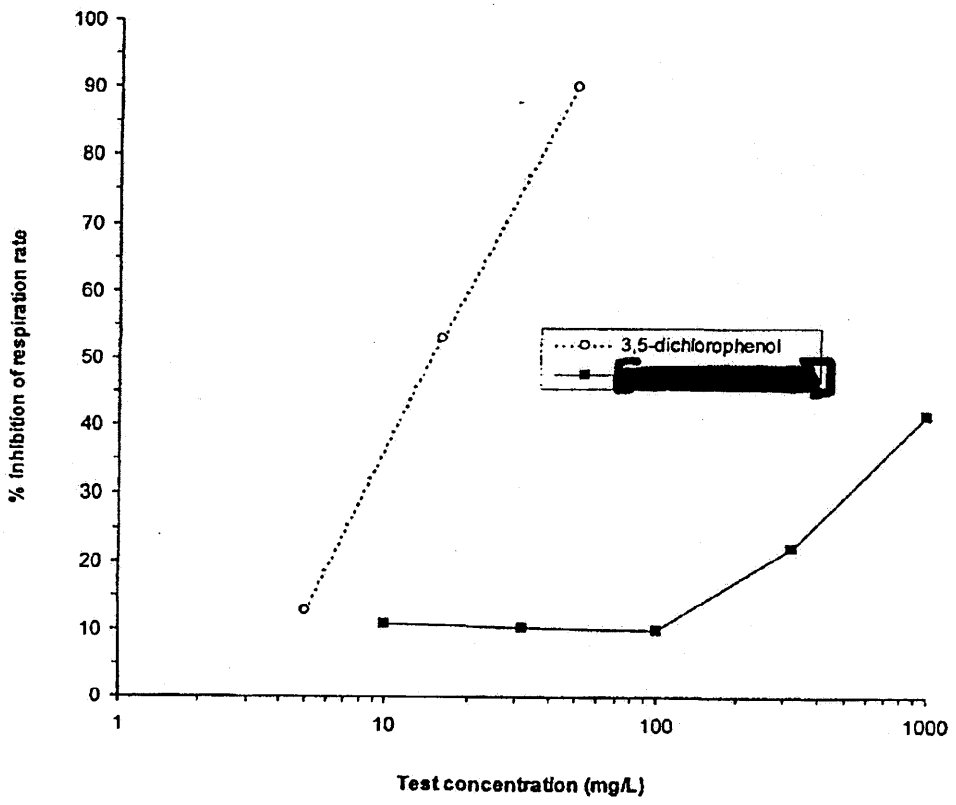
Table 2: Influence of 3,5-dichlorophenol on oxygen consumption of activated sludge

Flask	Test chemical	Nominal concentration of test chemical (mg/L)	Oxygen consumption rate (mg O_2/L min^{-1})	Inhibition (%)	pH values start*	pH values end*	Oxygen concentration (mg O_2/L) start*	Oxygen concentration (mg O_2/L) end*
1	Control	0	1.384		8.0	8.5	8.9	9.2
10	Control	0	1.500		8.0	8.4	8.8	9.2
Mean Deviation (%)			1.442 8.3					
2	3,5-dichlorophenol	5	1.258	12.8	8.0	8.5	8.2	8.4
3	3,5-dichlorophenol	16	0.678	53.0	8.0	8.6	8.7	8.4
4	3,5-dichlorophenol	50	0.143	90.1	8.0	8.6	8.1	8.3

* Start and end of the 3-hour incubation period

5 FIGURE

Figure 1: Influence of [redacted] and 3,5-dichlorophenol on the respiration rate of activated sludge



6 REFERENCES

1) FINNEY, D.J. (1971):
Probit Analysis,
3rd Edition, Cambridge University Press, London

2) DAVIES, R.G. (1971):
Computer Programming in Quantitative Biology,
Academic Press, London

STUDY REPORT

DETERMINATION OF THE ULTIMATE READY BIODEGRADABILITY OF [redacted] IN A MODIFIED STURM TEST (CO_2 EVOLUTION)

Study ECE 040125

AUTHOR : DAVE GORE (STUDY DIRECTOR)

[redacted] **Confidential - Class 2 Circulation**

This report must not be circulated further, copied, or destroyed without reference to the Reports Administrator [redacted]

[redacted]

Date : September 2008

STUDY INFORMATION

Study title	:	Determination of the ultimate ready biodegradability of [redacted] in a modified Sturm test (CO_2 evolution)
Study number	:	ECE 040125
Study location	:	[redacted]

STUDY DATES

Date protocol signed	:	22 July 2004
Experimental period	:	29 July 2004 to 8 September 2004

ARCHIVING

Data & Report	:	Datacare Business Management Systems 3012 Heyford Park Upper Heyford Bucks OX25 5HF
Test item(s)	:	[redacted]

STUDY PERSONNEL

Study Director	:	Dave Gore

MONITORING ROLES

Scientific Reviewer	:	Naheed Rehman
Quality Assurance	:	Harjit Lall, Fay Williams, Emma Butlin

STUDY INFORMATION (CONTINUED)

CROSS REFERENCES

Project number	:	221130
Q file reference	:	Q02/634

AUTHORISATION STATEMENT

Study number : ECE 040125

Study title : Determination of the ultimate ready biodegradability of [redacted] in a modified Sturm test (CO_2 evolution)

This report has been authorised for issue to the appropriate recipients.

N.R. Colson

NEIL COLSON
DEPARTMENT HEAD [redacted]

05/01/05
DATE

[redacted]

QUALITY ASSURANCE STATEMENT

Study Number : ECE040125

Study Title: Determination of the Ultimate ready Biodegradability of [redacted] in a Modified Strum Test (CO_2 Evolution)

This study was conducted at [redacted]

The following inspections and audits were conducted on the study. The dates on which they were performed and the dates on which any findings were reported to the Study Director and to Management are given below.

Audit Type	Audit Date	Report Date
Protocol Audit	22-Jul-2004	22-Jul-2003
Study Report Audit	13- Sep-2004	15-Sep-2004
Process Inspection - Strum Test	01-Jul-2004	20-Sep-2004
Facility Inspection	24-Feb-2004	16-Mar-2004

As far as can reasonably be established, this report has been accepted by Quality Assurance as being an accurate presentation of the raw data and findings of the study.

H LALL

22 Dec 2004

DATE

QUALITY ASSURANCE

[redacted]

AUTHENTICATION STATEMENT

Study number : ECE 040125

Study title : Determination of the ultimate ready biodegradability of [redacted] in a modified Sturm test (CO_2 evolution)

I, the undersigned, hereby declare that this study has been conducted under my supervision, as Study Director, in accordance with [redacted] policy on Good Laboratory Practice which is based on the UK Good Laboratory Practice Regulations 1999, Statutory Instrument No. 3106 (amended by the Good Laboratory Practice (Codification Amendments etc) Regulations 2004, Statutory Instrument No. 994) and OECD Principles on Good Laboratory Practice (as revised in 1997) ENV/MC/CHEM/ (98)17.

I also certify that this report presents a true and accurate account of the procedures used and the results obtained.

DAVE GORE
STUDY DIRECTOR

22-12-04
DATE

[redacted]

DETERMINATION OF THE ULTIMATE READY BIODEGRADABILITY OF [redacted] IN A MODIFIED STURM TEST (CO_2 EVOLUTION)

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DETERMINATION OF THE ULTIMATE READY BIODEGRADABILITY OF [redacted] IN A MODIFIED STURM TEST (CO_2 EVOLUTION)

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DETERMINATION OF THE ULTIMATE READY BIODEGRADABILITY OF [redacted] IN A MODIFIED STURM TEST (CO_2 EVOLUTION)

SUMMARY

Test Details

The purpose of this study was to determine the ultimate ready biodegradability of the test item using a modified Sturm test (CO_2 evolution).

Test Item:	[redacted]	S2539801
Reference Material:	Sodium Benzoate	S2446901
Test Duration:	28 days	
Test Temperature Range:	22.5 – 23.7°C.	
Source of inoculum:	10% vol. Final effluent – collected from Broadholme sewage works (Ditchford Lane, Wellingborough).	

Test Concentrations

Sample	Stock soln (g)/100ml	% Carbon	Volume added (ml)	Carbon Concentration in test media (mg/l)	Theoretical maximum Carbon in trap(mg/l)
[redacted]	0.9979	48.45	10	16.12	48.35
Sodium Benzoate	0.9517	57.7	10	18.30	54.90

Summary of biodegradation results

The observed % mineralisation of the test item was 2.3%. As a result the test item [redacted] cannot be classed as being readily or ultimately biodegradable. Based on the data obtained for the toxicity control vessels there is no evidence to suggest that the test item was inhibitory to microoganisms.

1. INTRODUCTION

The purpose of this study was to determine the ultimate ready biodegradability of the test item using a modified Sturm Test – CO_2 evolution. The test method is a recommended OECD [1] test method for determining ready ultimate biodegradation.

2. SUBSTANCES TESTED

The test item used in this investigation was [redacted] It is registered in compound control as sample number S2539801. The test item is a [redacted] The reference substance is Sodium Benzoate. It is registered in compound control as sample number S2446901. The reference substance is a white powder. Both the test item and reference substance are stored under ambient conditions.

2.1 Test item

Characterisation, stability and homogeneity is confirmed by analysis in study number AC 030449.

2.2 Reference substance

Characterisation, homogeneity and stability of the sample was confirmed by paperwork supplied with the sample which had been signed by the supplier. The correct identity of the sample was been confirmed by Compound Control by comparison of the sample label and appearance with the description and formulation supplied. It was not practical to analyse the sample further, the sample was not therefore characterised or stability or homogeneity addressed by **experimental work** in [redacted]

3. METHOD

The test system was comprised of a series of stirred flasks containing an non preacclimatised inocula/mineral salts media. The test item and reference substance were assessed in triplicate vessels. In addition triplicate toxicity control vessels were assessed. These contained test item and reference substance so as to assess the inhibitory potential of the test item to microorganisms. Triplicate control vessels were also included to allow the background respiration of the inoculum to be determined. The test system was aerated and the rate and extent of biodegradation/ mineralisation of the test item, reference substance and toxicity control was investigated by measuring the concentration of evolved carbon dioxide in sodium hydroxide traps compared to a control.

3.1 Carbon Analysis

The carbon content of the test material was determined by dissolving 1.0022g of the test item in 100ml of water. The stock solution was diluted ten fold and the organic carbon of the resulting solution was determined using a Shimadzu TOC-V carbon analyser.

3.2 Day -1

A suitable volume of secondary effluent from a sewage treatment plant treating predominantly domestic sewage (Broadholme Sewage Treatment Works) was collected and aerated until required. This was filtered through a Whatman 541. The effluent was used to inoculate, at a rate of 100ml to every litre, the test medium.

The test medium was prepared by adding about 12 litres of RGW in a clean 25 litre glass aspirator. Add 610ml of solution (a), 61ml of solutions (b), (c) and (d) and the appropriate volume (6100ml)of inoculum (make-up of solutions a to d are standard mineral salts stock solutions as recommended in OECD 301A [1]). Make up to 21 litres with RGW. There is a risk of precipitation so before addition of each stock the media was stirred well. The pH of the test medium was measured and adjusted if outside the range 7.2 $\pm$ 0.2.

To each clean 5 litre culture vessel 2 litres of R.G.W. was added and 1 litre of test medium. Each vessel was connected to a CO_2 scrubbing rig and allowed to aerate with CO_2 free air at 25-35 ml/minute. The test system was left aerating overnight to purge the system of carbon dioxide prior to the addition of test stock solutions.

3.3 Day 0

Sufficient test/reference stock solutions were added to the respective cultures (see Tables 1 and 2), these were mixed thoroughly so that the test concentration was between 15 and 20 mg/l as carbon. CO_2 absorbers containing 0.05M sodium hydroxide traps were added to each culture vessel. These were, labelled A which contained 250ml and B contained 100ml.

Those for connection to vessel 1 are A1 and B1; those for vessel 2 are A2 and B2 and so on for each vessel.

3.4 Analysis

Analysis was approximately every second or third day for the first 10 days then more frequent than every fifth day until day 28.

Aliqots of 5ml from each of the traps labelled A were removed and added to vials (carbon analysis) containing 15ml of reagent grade water and stopper. The vials were analysed on a Shimadzu TOC-V Carbon Analyser for inorganic carbon.

On the 28th day of the test, 50 ml of test medium was removed by pipette, from each vessel, for measurement of pH and dissolved organic carbon. 1 ml of concentrated hydrochloric acid was added to each culture vessel. The vessels were aerated for a further 24 hours then all traps were analysed as described above.

3.5 Calculation of Results

The percentage carbon for both the test item and reference substance was used to calculate the carbon concentration in the test media.

$$\textit{Mass of sample (g/l)} \times \frac{\%carbon}{100} = \textit{g/l as Carbon or mg/ml as Carbon}$$

Take into account the volume of the stock solution added to the test vessel this gave the theoretical carbon in media. Inorganic carbon analysis of each cumulative trap is expressed as mg/l. Since 5 ml of the trap (total volume 250ml) is diluted to 20 ml the theoretical carbon in the media is the same as the theoretical maximum carbon in the trap.

$$\textit{\% cumulative biodegradation} = \frac{\textit{Difference between treatment and control}}{\textit{Theoretical maximum carbon in the trap}} \times 100$$

The % biodegradation was plotted on a graph against time.

3.6 Study dates

The study was conducted from 29 July 2004 to 8 September 2004.

3.7 Storage and retention of data and test item

The protocol, any amendments, the raw data and final report will be placed in Datacare Business Management Systems, 3012 Heyford Park, Upper Heyford, Bucks, OX25 5HF.

Datacare is not a member of the UK GLP compliance programme. The test item(s) will be archived in [redacted]

4. RESULTS AND DISCUSSIONS

The carbon concentration of a ten-fold dilution of a stock solution 1.0022g/100ml was 485.6mg/l. Therefore, the percentage carbon of the test item was calculated as 48.45%.

The temperature range throughout the study was 22.5°C to 23.7°C which was within the range recommended 22±2°C

The concentration of inorganic carbon at each sampling occasion and subsequent cumulative extent of % mineralisation is presented in Tables 3 to 10. The % mineralisation is summarised in Figure 1.

Figure 1 shows a decrease in the % mineralisation on day 14 compared to day 9. This decrease is explained by the fact that the inorganic carbon concentration of control vessel number 1 was slightly lower than the other two controls on days 3, 6 and 9. On day 14 similar concentration for all controls were observed. Figure 1 also shows a greater than expected increase on day 24.

Table 11 summarises the inorganic data obtained for traps labelled B. Negligible concentrations of inorganic carbon were observed in the traps thereby confirming the trapping efficiency of the first trap, as a result the cumulative mineralisation has not been adjusted.

Table 12 summarises the organic carbon data obtained for the media of each vessel. This clearly demonstrates that the test item was still present as organic carbon. The data for the reference substance vessels further supported the inorganic carbon data in that the reference material was no longer present as organic carbon.

Table 13 summarises the pH of each vessel on day 28. The pH's were within the range that supports growth of micro-organisms.

5. CONCLUSION

The observed % mineralisation of the test item was 2.3%. As a result the test item [redacted] cannot be classed as being readily or ultimately biodegradable. Based on the data obtained for the toxicity control vessels there is no evidence to suggest that the test item was inhibitory to microoganisms.

6. REFERENCES

[1] OECD Guidelines For The Testing of Chemicals. 301B - Ready Biodegradability (CO_2 Evolution Test). ISBN 92-64-12221-4

TABLE 1. SUMMARY TEST CONCENTRATIONS

Sample	Stock soln (g)/100ml	% Carbon	Volume added (ml)	Carbon Concentration in test media (mg/l)	Theoretical maximum Carbon in trap(mg/l)
[redacted]	0.9979	48.45	10	16.12	48.35
Sodium Benzoate	0.9517	57.7	10	18.30	54.90

TABLE 2. SUMMARY TEST ITEM ADDITION

Vessel no	Vessel type	Sample code	Vol of stock solution (ml)	Vol of media (ml)	Final concentration in Media (mg/l as Carbon)
1,2,3	Control	Water	10	3000	0.0
4,5,6	Test	S2539801	10	3000	16.12
7,8,9	Reference	S2446901	10	3000	18.30
10,11,12	Toxicity control	S2539801 and S2446901	10 and 10	3000	16.12 and 18.30

TABLE 3. SUMMARY DAY 3

Vessel	Inorganic Carbon [mg/l]	Difference IC[mg/l]	Cumulative % mineralisation
1	6.613		
2	10.010		
3	11.390		
Mean control	9.338		
4	12.230		
5	11.790		
6	9.381		
Mean test	11.134	1.796	3.715
7	28.870		
8	33.240		
9	33.940		
Mean reference	32.017	22.679	41.300
10	32.970		
11	30.150		
12	34.940		
Mean toxicity control	32.687	23.349	42.520

TABLE 4. SUMMARY DAY 6

Vessel	Inorganic Carbon [mg/l]	Difference IC[mg/l]	Cumulative % mineralisation
1	8.911		
2	14.020		
3	13.500		
Mean control	12.144		
4	17.420		
5	17.060		
6	12.760		
Mean test	15.747	3.603	7.452
7	38.900		
8	42.330		
9	42.640		
Mean reference	41.290	29.146	53.077
10	43.570		
11	39.770		
12	44.970		
Mean toxicity control	42.770	30.626	55.772

TABLE 5. SUMMARY DAY 9

Vessel	Inorganic Carbon [mg/l]	Difference IC[mg/l]	Cumulative % mineralisation
1	11.630		
2	20.130		
3	24.310		
Mean control	18.690		
4	23.610		
5	22.960		
6	19.240		
Mean test	21.937	3.247	6.715
7	48.760		
8	53.500		
9	54.670		
Mean reference	52.310	33.620	61.224
10	52.730		
11	52.150		
12	56.010		
Mean toxicity control	53.630	34.940	63.628

TABLE 6. SUMMARY DAY 14

Vessel	Inorganic Carbon [mg/l]	Difference IC[mg/l]	Cumulative % mineralisation
1	19.530		
2	19.790		
3	19.970		
Mean control	19.763		
4	20.010		
5	20.000		
6	19.890		
Mean test	19.967	0.204	0.422
7	59.890		
8	59.870		
9	60.010		
Mean reference	59.923	40.160	73.151
10	59.790		
11	60.220		
12	60.040		
Mean toxicity control	60.017	40.254	73.321

TABLE 7. SUMMARY DAY 17

Vessel	Inorganic Carbon [mg/l]	Difference IC[mg/l]	Cumulative % mineralisation
1	20.070		
2	20.240		
3	20.280		
Mean control	20.197		
4	20.700		
5	20.850		
6	20.280		
Mean test	20.610	0.413	0.855
7	61.100		
8	61.020		
9	61.020		
Mean reference	61.047	40.850	74.390
10	60.800		
11	61.300		
12	60.570		
Mean toxicity control	60.890	40.693	74.105

TABLE 8. SUMMARY DAY 21

Vessel	Inorganic Carbon [mg/l]	Difference IC[mg/l]	Cumulative % mineralisation
1	20.140		
2	20.270		
3	20.340		
Mean control	20.250		
4	20.710		
5	20.810		
6	20.640		
Mean test	20.720	0.470	0.972
7	62.290		
8	62.240		
9	62.420		
Mean reference	62.317	42.067	76.606
10	62.050		
11	62.290		
12	62.340		
Mean toxicity control	62.227	41.977	76.442

TABLE 9. SUMMARY DAY 24

Vessel	Inorganic Carbon [mg/l]	Difference IC[mg/l]	Cumulative % mineralisation
1	8.855		
2	7.281		
3	6.854		
Mean control	7.663		
4	13.310		
5	13.810		
6	15.580		
Mean test	14.233	6.570	13.588
7	62.780		
8	62.630		
9	62.580		
Mean reference	62.663	55.000	100.158
10	62.500		
11	62.710		
12	62.800		
Mean toxicity control	62.670	55.007	100.171

TABLE 10. SUMMARY DAY 29

Vessel	Inorganic Carbon [mg/l]	Difference IC[mg/l]	Cumulative % mineralisation
1	12.970		
2	13.070		
3	11.980		
Mean control	12.673		
4	12.090		
5	13.600		
6	15.680		
Mean test	13.790	1.117	2.310
7	64.350		
8	64.150		
9	64.250		
Mean reference	64.250	51.577	93.924
10	64.170		
11	64.060		
12	64.090		
Mean toxicity control	64.107	51.433	93.663

TABLE 11. SUMMARY TRAP B DAY 29

Vessel	Inorganic Carbon [mg/l]
1	0.3956
2	0.3391
3	0.3195
4	0.3208
5	0.3253
6	0.3297
7	0.3392
8	0.3563
9	0.3346
10	0.3383
11	0.3275
12	0.3295

TABLE 12. SUMMARY ORGANIC CARBON DATA DAY 28

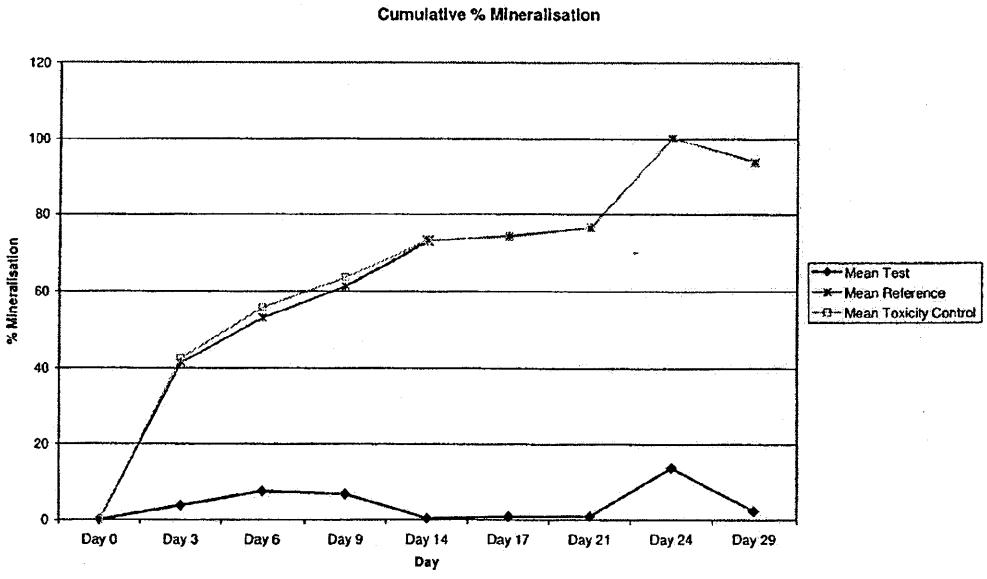
Vessel	Dissolved Organic Carbon [DOC] [mg/l]	% of Initial Dissolved Organic Carbon
1	0.6674	
2	0.7804	
3	0.6681	
4	17.00	105.5
5	16.91	104.9
6	16.94	105.1
7	0.6701	3.7
8	0.7147	3.9
9	1.013	5.5
10	17.28	107.2 *
11	16.97	105.3 *
12	16.97	105.3 *

* Figure calculated based on initial dissolved organic carbon contribution from test item.

TABLE 13. SUMMARY OF PH DAY 28

Vessel	pH
1	7.23
2	7.21
3	7.25
4	7.24
5	7.29
6	7.21
7	7.15
8	7.12
9	7.14
10	7.19
11	7.15
12	7.13

FIGURE 1. SUMMARY OF % MINERALISATION DATA



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DETERMINATION OF ABIOTIC DEGRADATION, - HYDROLYSIS AS A FUNCTION OF pH

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1736-023.doc/MSOffice

[redacted] Study Reference Number: KDD030428

QUALITY ASSURANCE REPORT

Inspection of the routine and repetitive procedures that constitute the study is process based (as defined by OECD) and is 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:

§	21 April 2004	Protocol Compliance Audit
	04, 07, 16 June 2004	Abiotic Degradation, Hydrolysis as a Function of pH
§	20 July 2004	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: 24 SEP 2004

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

[redacted]tudy Reference Number: KDD030428

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 2004/9/EC and 2004/10/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, South Africa, 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 SEP 2004

S M Woolley AMRSC
Study Director

[redacted] Study Reference Number: KDD030428

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[redacted]

DETERMINATION OF ABIOTIC DEGRADATION, HYDROLYSIS AS A FUNCTION OF pH

SUMMARY

Abiotic Degradation, Hydrolysis as a Function of pH. Assessment of hydrolytic stability was carried out using Method C7 of Commission Directive 92/69/EEC. The results are as follows:

pH	Rate constant (s^{-1})	Estimated half-life at 25°C
4	-	>1 year
7	1.18×10^{-6}	6.79 days
9	-	<1 day

[redacted]

DETERMINATION OF ABIOTIC DEGRADATION, HYDROLYSIS AS A FUNCTION OF pH

1. INTRODUCTION

The abiotic degradation, hydrolysis as a function of pH of the test material has been determined.

The method employed complied with that specified in Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

Testing was conducted between 01 June 2004 and 23 June 2004.

2. TEST MATERIAL

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	[redacted]
[redacted] Sample Number	:	S2539801
Description	:	[redacted]
Date received	:	06 April 2004
Storage conditions	:	room temperature, in the dark

The identity, purity and stability of the test material (test item) is being addressed by the sponsor in a GLP compliant study [redacted] Study AC030449).

3. 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. The remaining test material (test item) will be returned to the Sponsor. Archiving of test materials (test items) is the responsibility of the Sponsor.

4. ABIOTIC DEGRADATION, HYDROLYSIS AS A FUNCTION OF pH

4.1 Method

The determination was carried out using Method C7 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

4.1.1 Procedure

4.1.1.1 Specification of buffer solutions

Table 4.1

Buffer solution	Components	Concentration (mol dm^{-3})
4	Potassium hydrogen phthalate	0.05
7	Disodium hydrogen orthophosphate (anhydrous)	0.03
	Potassium dihydrogen orthophosphate	0.02
	Sodium chloride	0.02
9	Disodium tetraborate	0.01
	Sodium chloride	0.02

The buffer solutions were filtered through a 0.2 μm membrane filter to ensure they were sterile before commencement of the test. Also these solutions were subjected to ultrasonication and degassing with nitrogen to minimise dissolved oxygen content.

4.1.1.2 Preparation of samples

Sample solutions were prepared in stoppered glass flasks at a nominal concentration of 1.0 g/l in the three buffer solutions.

The solutions were shielded from light whilst maintained at the test temperature.

4.1.1.3 Preliminary test

Sample solutions at pH 4, 7 and 9 were maintained at 50.0 ± 0.5°C for a maximum period of 5 days.

4.1.1.4 Test 3

Results from the Preliminary test showed it was necessary to undertake further testing at pH 7, with a solution being maintained at 40.0 ± 0.5°C for a period of 2 days.

4.1.1.5 Analysis of sample solutions

Aliquots of the sample solutions were taken from the flasks at various times and the pH of each solution recorded.

The concentration of the sample solution was determined by high performance liquid chromatography (HPLC).

Samples

Duplicate aliquots (A and B) of sample solution at pH 4 were diluted by a factor of 10 using water.

Duplicate aliquots (A and B) of sample solution at pH 7 and pH 9 were diluted by a factor of 10 using pH 4 buffer solution*.

Standards

Duplicate standard solutions of test material were prepared in water:pH 4 buffer solution* (90:10 v/v) at a nominal concentration of 100 mg/l for the pH 4 sample solutions, and in pH 4 buffer solution:pH 7/pH 9 buffer solution* (90:10 v/v) at a nominal concentration of 100 mg/l, to ensure the matrices used matched that of the relevant pH 7 and pH 9 sample solutions.

Analysis

The standard and sample solutions were analysed by HPLC using the following conditions:

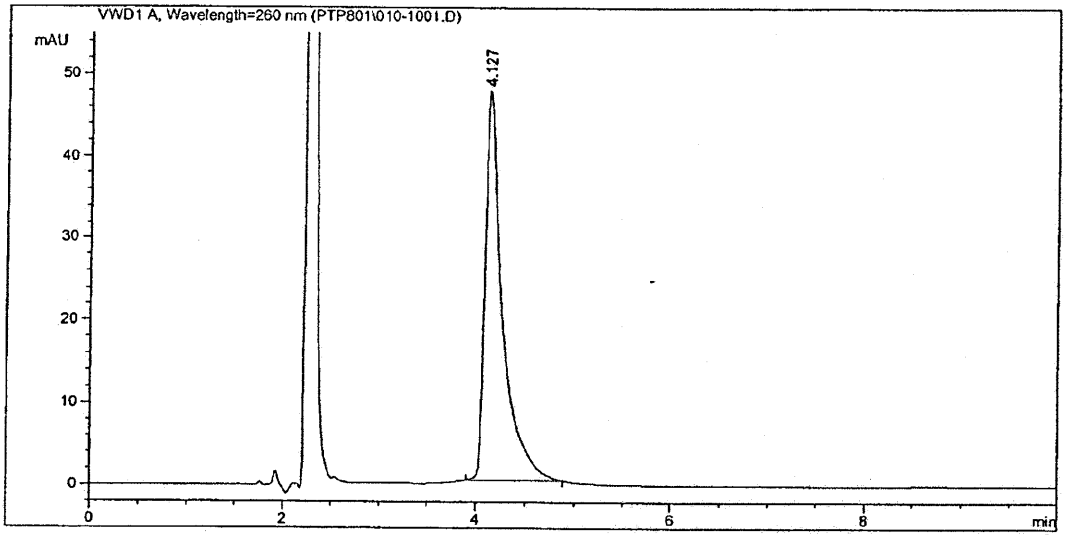
HPLC System	:	Agilent Technologies 1100, incorporating autosampler and workstation
Column	:	Spherisorb S5 C_6 (250 x 4.6 mm id)
Column temperature	:	30°C
Mobile phase	:	buffer#:acetonitrile (65:35 v/v)
Flow-rate	:	1.5 ml/min
UV detector wavelength	:	260 nm
Injection volume	:	10 µl
Retention time	:	~ 4.1 mins

* see Table 4.1 for specification

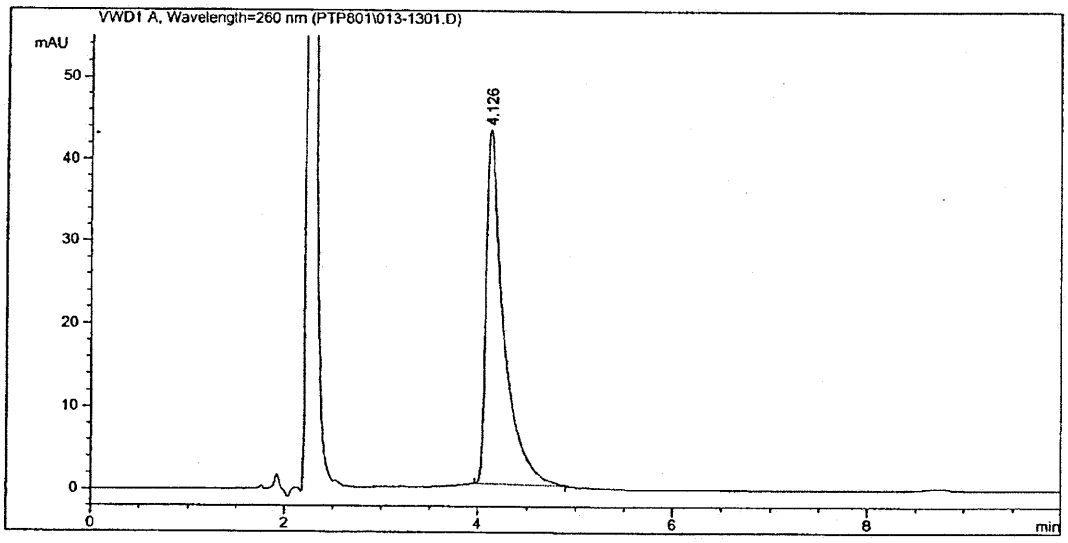
buffer preparation – approximately 2.1628 g of 1-octane sulfonic acid sodium salt dissolved in 650 ml reverse osmosis water. Approximately 1.012 g of triethylamine added and the pH adjusted to 2.5 using orthophosphoric acid.

Typical Chromatography

Standard 103 mg/l



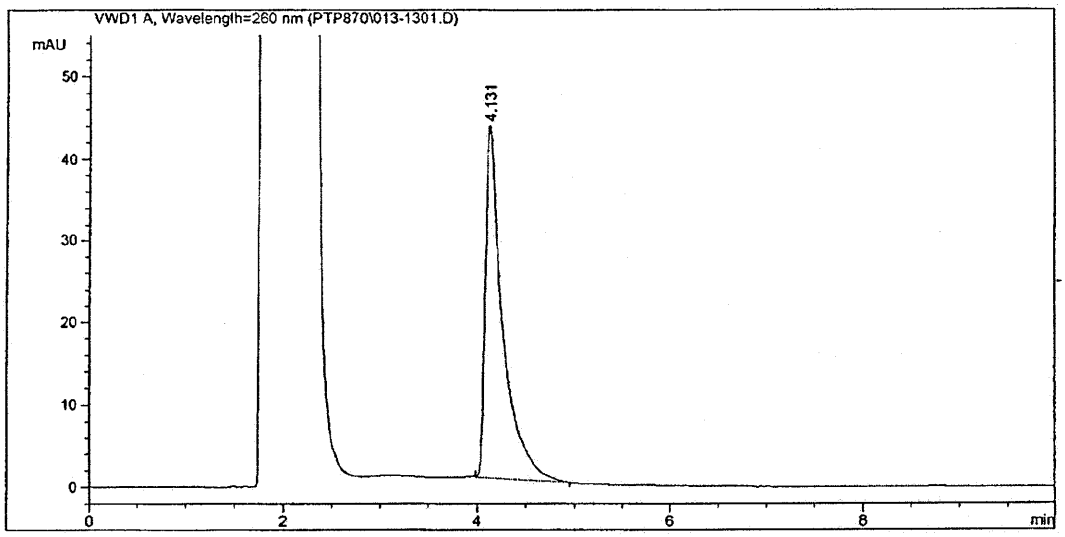
120 Hour Sample A; pH 4, 50°C



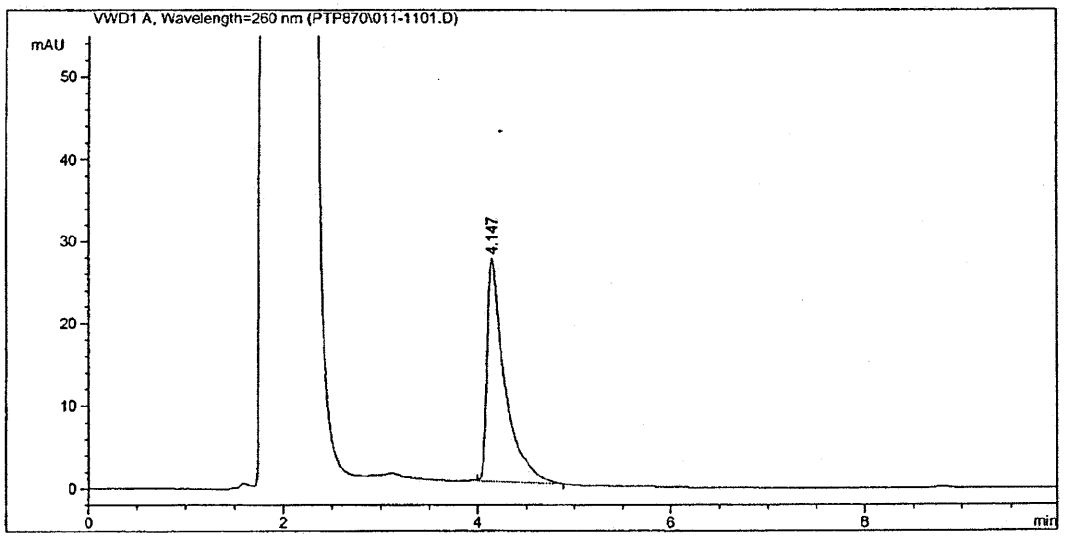
[redacted]Study Reference Number: KDD030428

Typical Chromatography

Standard 101 mg/l



24 Hour Sample B; pH 7, 40°C



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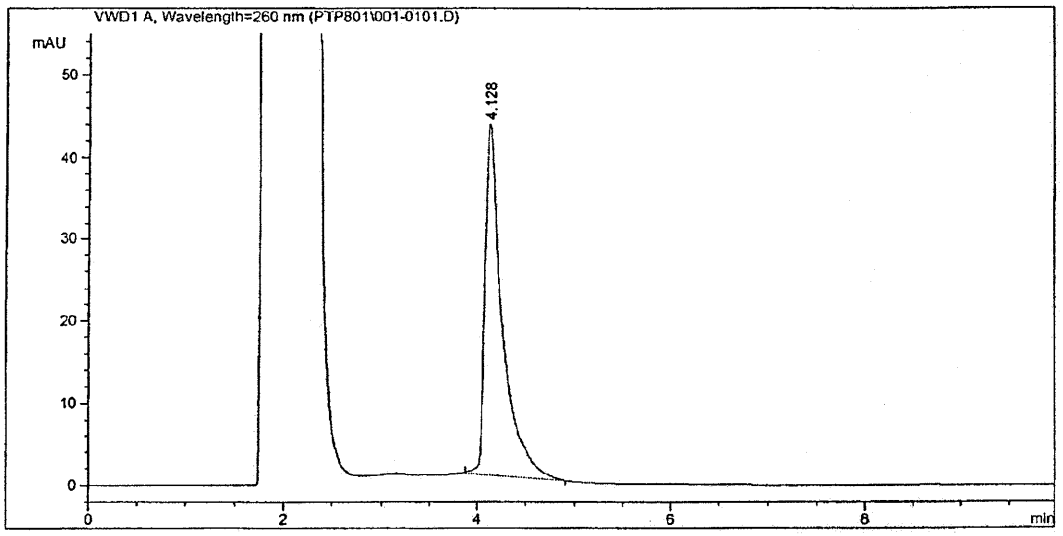
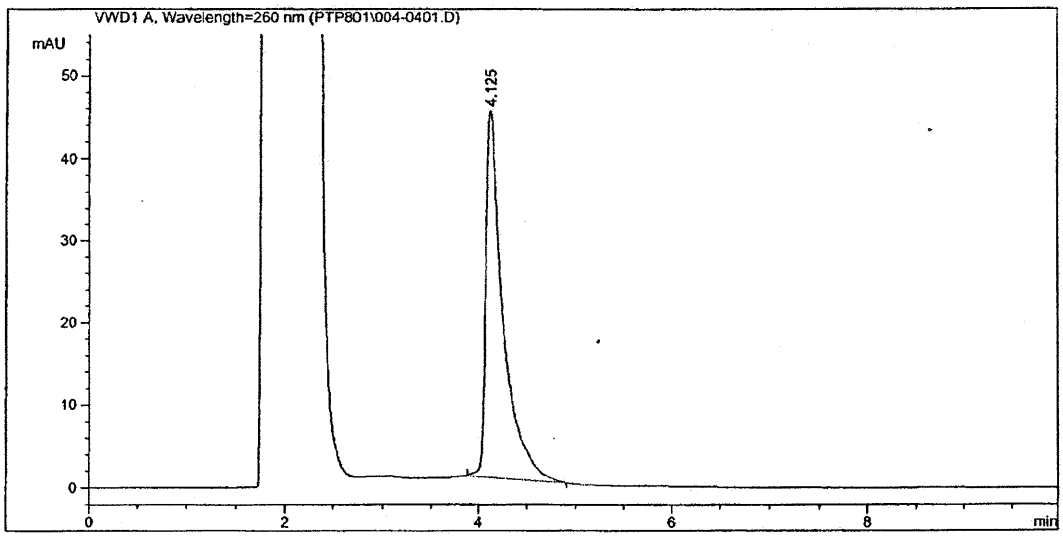
Typical Chromatography

Standard 101 mg/l



Initial Sample A; pH 9, 50°C



[redacted] Study Reference Number: KDD030428

4.1.1.6 Treatment of results

Graphs of the common logarithm of the concentration (g/l) versus time (hours) were plotted for pH 7 at 40 and 50°C (see Figures 4.1 and 4.2) and the rate constant and half-life calculated using Equation 4.2 and Equation 4.3 respectively.

By plotting the natural logarithm of the rate constants against the reciprocal of the temperature (K), the rate constant and half-lives at 25°C were obtained by extrapolation (see Figure 4.3).

4.1.2 Calculation

4.1.2.1 Sample solution concentration

The mean peak area of each standard was corrected to a nominal concentration of 100 mg/l and the mean value taken.

The concentration of the sample solutions (g/l) was calculated using Equation 4.1.

Equation 4.1

$$C_{spl} = \frac{P_{spl}}{P_{std}} \times C_{std} \times D \times \frac{1}{1000}$$

where:

C_{spl}	=	sample concentration (g/l)
P_{spl}	=	mean peak area of sample solution
P_{std}	=	mean peak area of standard solution, corrected to nominal standard concentration
C_{std}	=	nominal standard concentration (100 mg/l)
D	=	sample dilution factor (10)

4.1.2.2 Estimation of half-life

From the graph of $\log_{10}$ concentration (g/l) versus time, the rate constant was calculated using Equation 4.2.

Equation 4.2

$$k_{obs} = -\text{slope} \times 2.303$$

and the half-life was obtained from the rate constant using Equation 4.3.

Equation 4.3

$$t_{½} = \frac{0.693}{k_{obs}}$$

where:

k_{obs}	=	rate constant ($hours^{-1}$)
$t_{½}$	=	half-life (hours)

4.2 Results

4.2.1 Preliminary test/Test 1

The mean peak areas relating to the standard and sample solutions are shown in the following table:

Table 4.2

Solution	Mean Peak Area
Standard 103 mg/l	567.874
Standard 101 mg/l	562.089
Initial Sample A, pH 4	571.269
Initial Sample B, pH 4	573.851
2.4 Hour Sample A, pH 4	544.589
2.4 Hour Sample B, pH 4	571.609
Standard 100 mg/l	564.182
Standard 100 mg/l	567.720
24 Hour Sample A, pH 4	470.594*
24 Hour Sample B, pH 4	534.699
Standard 107 mg/l	606.643
Standard 102 mg/l	575.028
72 Hour Sample A, pH 4	552.589
72 Hour Sample B, pH 4	552.208
Standard 103 mg/l	575.520
Standard 101 mg/l	553.876
120 Hour Sample A, pH 4	521.172
120 Hour Sample B, pH 4	522.747
Standard 101 mg/l	547.577
Standard 101 mg/l	548.330
Initial Sample A, pH 7	513.084
Initial Sample B, pH 7	539.599
3.0 Hour Sample A, pH 7	461.295
3.0 Hour Sample B, pH 7	446.678
4.0 Hour Sample A, pH 7	456.196
4.0 Hour Sample B, pH 7	457.178
5.0 Hour Sample A, pH 7	430.939
5.0 Hour Sample B, pH 7	432.132

* Not used in further calculations due to extraneous result obtained.

Table 4.2 - continued

Solution	Mean Peak Area
Standard 101 mg/l	546.898
Standard 101 mg/l	549.360
6.0 Hour Sample A, pH 7	405.580
6.0 Hour Sample B, pH 7	410.558
Standard 100 mg/l	525.211
Standard 101 mg/l	551.111
23 Hour Sample A, pH 7	131.339
23 Hour Sample B, pH 7	126.559
24 Hour Sample A, pH 7	122.868
24 Hour Sample B, pH 7	121.467
26 Hour Sample A, pH 7	104.866
26 Hour Sample B, pH 7	106.930
Standard 101 mg/l	548.277
Standard 101 mg/l	542.077
Initial Sample A, pH 9	546.110
Initial Sample B, pH 9	553.236
2.4 Hour Sample A, pH 9	<2.210*
2.4 Hour Sample B, pH 9	<2.210*

The test material concentrations at the given time points are shown in the following tables:

Table 4.3 pH 4 at 50.0 ± 0.5°C

	Time (Hours)				
	0	2.4	24	72	120
Concentration (g/l)	1.03	1.00	0.946	0.973	0.944
% of initial	-	97.5	91.9	94.5	91.7

Result: Less than 10% hydrolysis after 5 days at 50°C, equivalent to a half-life greater than 1 year at 25°C.

* Limit value used, taken from small peak present in sample A at the retention time of the test material.

Table 4.4 pH 7 at 50.0 ± 0.5°C

	Time (Hours)							
	0	3.0	4.0	5.0	6.0	23	24	26
Concentration (g/l)	0.968	0.835	0.840	0.794	0.750	0.242	0.229	0.198
Log_{10} [concentration (g/l)]	-1.40×10^{-2}	-7.82×10^{-2}	-7.57×10^{-2}	-0.100	-0.125	-0.617	-0.640	-0.703
% of initial	-	86.3	86.8	82.0	77.5	24.9	23.6	20.5

Result: Slope = -2.75×10^{-2} $hour^{-1}$ (see Figure 4.1)

k_{obs} = 6.33×10^{-2} $hour^{-1}$

= 1.76×10^{-5} $second^{-1}$

$t_{½}$ = 11.0 hours

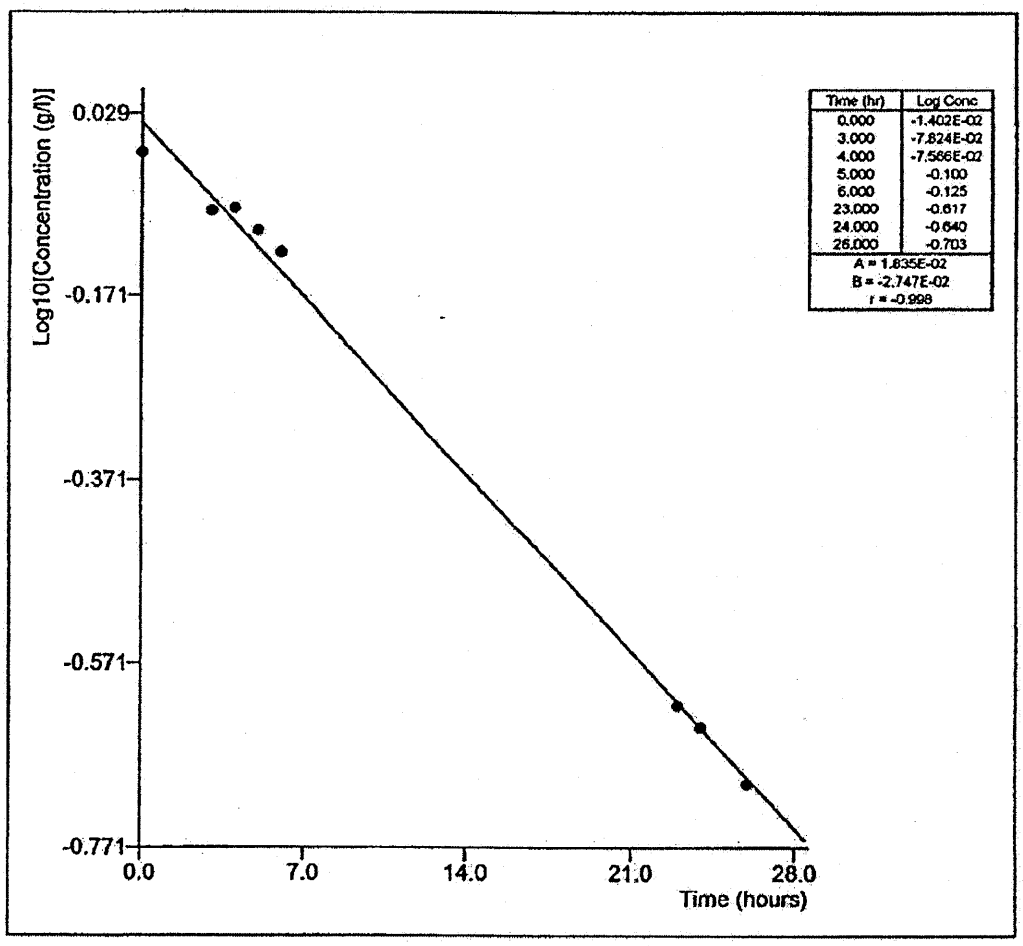
The extent of hydrolysis after 26 hours indicated that a further test (Test 3), conducted at 40°C, was required to estimate the rate constant and half-life.

Table 4.5 pH 9 at 50.0 ± 0.5°C

	Time (Hours)	
	0	2.4
Concentration (g/l)	1.01	$<4.08 \times 10^{-3}$
% of initial	-	<0.402

Result: Greater than 50% hydrolysis after 2.4 hours at 50°C, equivalent to a half-life of less than 1 day at 25°C.

Figure 4.1 pH 7 at 50.0 ± 0.5°C
Graph of $\log_{10}$ Concentration (g/l) versus Time (Hours)



[redacted] Study Reference Number: KDD030428

4.2.2 Test 3

The mean peak areas relating to the standard and sample solutions are shown in the following table:

Table 4.6

Solution	Mean Peak Area
Standard 101 mg/l	538.443
Standard 100 mg/l	518.036
Initial Sample A, pH 7, 40°C	533.671
Initial Sample B, pH 7, 40°C	532.706
Standard 100 mg/l	520.661
Standard 101 mg/l	530.246
18 Hour Sample A, pH 7, 40°C	393.593
18 Hour Sample B, pH 7, 40°C	392.332
20 Hour Sample A, pH 7, 40°C	377.320
20 Hour Sample B, pH 7, 40°C	379.879
22 Hour Sample A, pH 7, 40°C	363.763
22 Hour Sample B, pH 7, 40°C	363.367
24 Hour Sample A, pH 7, 40°C	347.696
24 Hour Sample B, pH 7, 40°C	336.915
Standard 102 mg/l	541.954
Standard 103 mg/l	530.238
42 Hour Sample A, pH 7, 40°C	219.347
42 Hour Sample B, pH 7, 40°C	219.881
48 Hour Sample A, pH 7, 40°C	184.209
48 Hour Sample B, pH 7, 40°C	185.402

The test material concentrations at the given time points are shown in the following table:

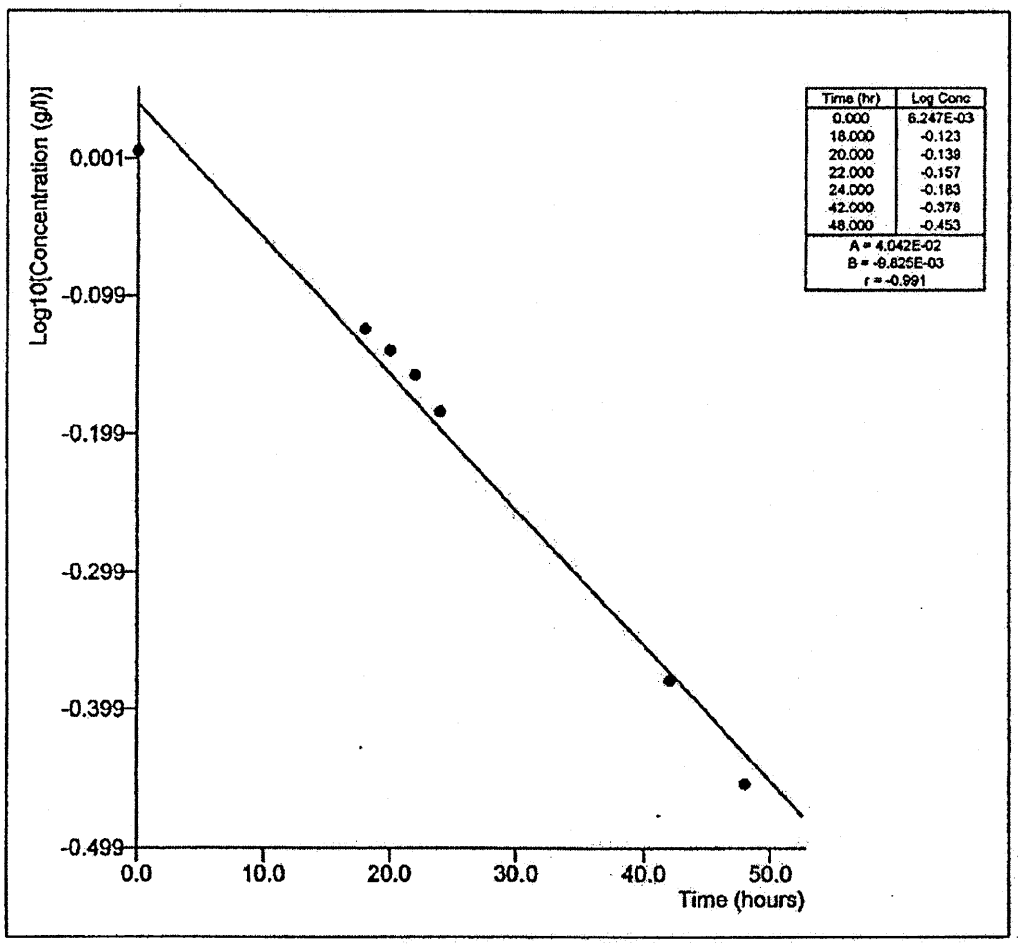
Table 4.7 pH 7 at 40.0 ± 0.5°C

	Time (Hours)						
	0	18	20	22	24	42	48
Concentration (g/l)	1.01	0.753	0.726	0.697	0.656	0.419	0.352
Log_{10} [concentration (g/l)]	6.25×10^{-3}	-0.123	-0.139	-0.157	-0.183	-0.378	-0.453
% of initial	-	74.3	71.6	68.7	64.7	41.3	34.7

Result: Slope = -9.83×10^{-3} hour^{-1} (see Figure 4.2)
k_{obs} = 2.26×10^{-2} hour^{-1}
= 6.28×10^{-6} second^{-1}
$t_{½}$ = 30.6 hours

[redacted] Study Reference Number: KDD030428

Figure 4.2 pH 7 at 40.0 ± 0.5°C
Graph of $\log_{10}$ Concentration (g/l) versus Time (Hours)



[redacted] Study Reference Number: KDD030428

The Arrhenius plot was constructed using the data shown in the following table:

Table 4.8 pH 7 Arrhenius Data

T (°C)	T (K)	$\frac{1}{T(K)}$	k_{obs} (hr^{-1})	Ln k_{obs}
40.0	313	3.19×10^{-3}	2.26×10^{-2}	-3.79
50.0	323	3.10×10^{-3}	6.33×10^{-2}	-2.76

From the graph (Figure 4.3) of the above data, the rate constant and half-life at 25°C have been estimated to be as follows:

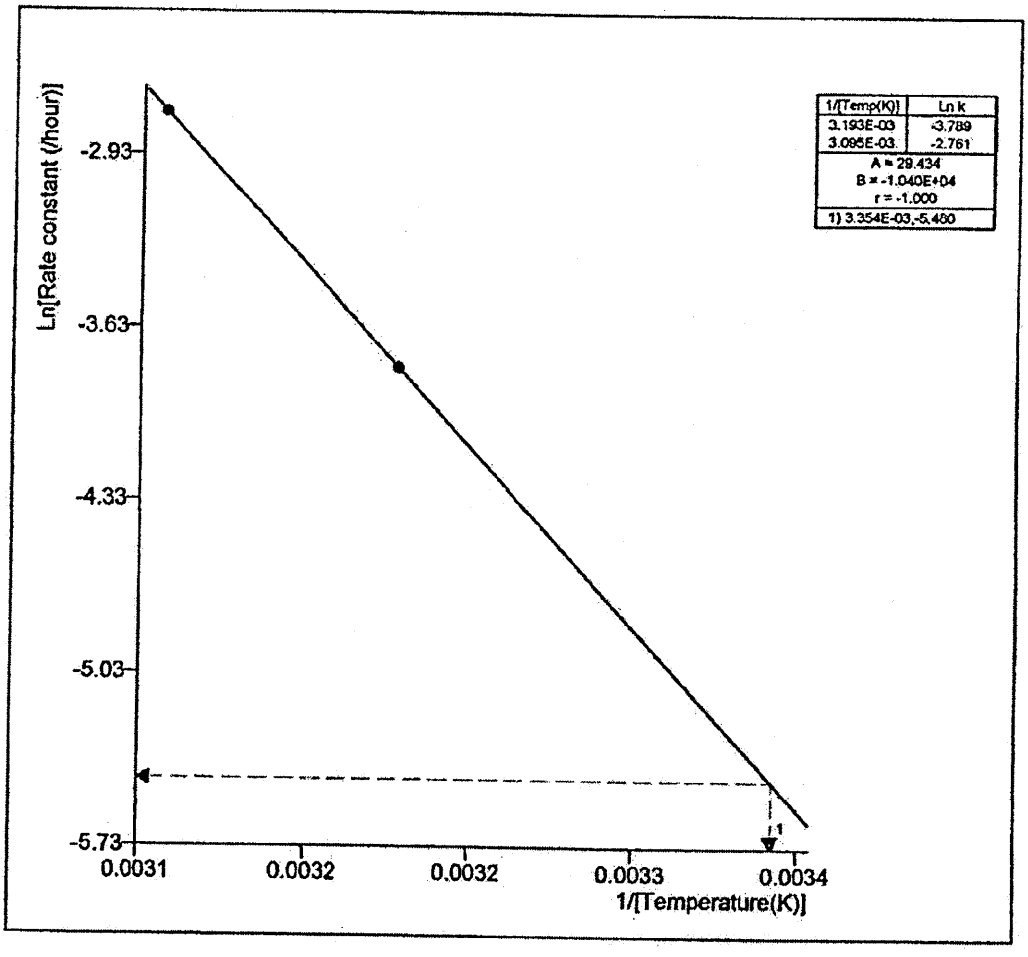
k_{obs} = 4.25×10^{-3} $hour^{-1}$

= 1.18×10^{-6} $second^{-1}$

$t_{½}$ = 163 hours

= 6.79 days

Figure 4.3 pH 7
Graph of $\ln.k_{obs}$ versus 1/T (K)



[redacted] Study Reference Number: KDD030428

4.3 Validation

The linearity of the detector response with respect to concentration was assessed over the nominal concentration range of 0 to 200 mg/l. This was satisfactory with a correlation coefficient of 1.000 being obtained.

4.4 Discussion

The kinetics of the study have been determined to be consistent with that of a pseudo-first order reaction as the graphs of $\log_{10}$ concentration versus time are straight lines.

It has been observed that the rate of hydrolysis increases with an increase in pH.

4.5 Conclusion

The rate constant and estimated half-life at 25°C of the test material are shown in the following table:

Table 4.9

pH	Rate constant (s^{-1})	Estimated half-life at 25°C
4	-	>1 year
7	1.18×10^{-6}	6.79 days
9	-	< 1day

Appendix 1 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	TEST TYPE
SafePharm Limited **Shardlow Business Park,** **London Road,** **Shardlow,** **Derbyshire,** **DE72 2GD**	**Analytical/Clinical Chemistry** **Environmental tox.** **Environmental fate** **Mutagenicity** **Phys./Chem. tests** **Toxicology**

DATE OF INSPECTION

2nd December 2002

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.

13/2/03

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

[redacted] Study Reference Number: KDD030428

REPORT

Study Title:

ADSORPTION/DESORPTION OF
[redacted]ON ACTIVATED SEWAGE SLUDGE

Data Requirement/Test Guidelines:

OECD Guideline 106 adopted January 21, 2000:
Adsorption/Desorption using a batch equilibrium method

Under consideration of:

Commission Directive 95/36/EC, July 14, 1995 amending
Council Directive 91/414/EEC; Annex II, 7.1.2, 1991

Study Director:

Dr. Wolfgang Völkel

Study Completion Date:

September 16, 2004

Test Facility:

RCC Ltd
Environmental Chemistry &
Pharmanalytics
CH-4452 Itingen/Switzerland

Sponsor:

[redacted]

Study Numbers:

RCC Study No.: 851849
[redacted] Study Reference No.: KAS030427

GLP CERTIFICATE

The Swiss GLP Monitoring Authorities

Swiss Federal Office of Public Health | Swiss Agency for the Environment, Forests and Landscape | swissmedic Swissmedic Swiss Agency for Therapeutic Products

Statement of GLP Compliance

It is hereby confirmed that

during the period of November 18 – 22, 2002

the following Test Facilities of

RCC Ltd
4452 Itingen
Switzerland

were inspected by the Federal Office of Public Health, the Swiss Agency for Therapeutic Products and the Swiss Agency for the Environment, Forests and Landscape with respect to the compliance with the Swiss legislation on Good Laboratory Practice.

Test Facilities	**Areas of expertise ***
- Toxicology	TOX, ACC, MUT, OTH (Safety Pharmacology)
- Environmental Chemistry and Pharmanalytics	ACC, ECT, ENF, EMN, PCT, RES, OTH (Animal metabolism)

The inspections were performed in agreement with the OECD Guidelines for National GLP Inspections and Audits. It was found that the aforementioned test facilities were operating in compliance with the Swiss Ordinance relating to Good Laboratory Practice [RS 813.016.5] at the time they were inspected.

Federal Office of Public Health
The Director

Bern, March 2003

Prof. Th. Zeltner

* TOX = Toxicology ; ACC = Analytical and Clinical Chemistry ; ECT = Environmental toxicity on aquatic and terrestrial organisms ; ENF = Behaviour in water, soil and air. Bioaccumulation ; EMN = Studies on effects on mesocosms and natural ecosystems; MUT = Mutagenicity; PCT = Physical-chemical testing ; RES = Residue studies ; OTH = Other, to be specified.

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE

RCC Study Number: 851849

Test Item: [redacted]

Study Director: Dr. W. Völkel

Study Title: Adsorption/Desorption of [redacted] on Activated Sewage Sludge

This study was conducted in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted February 2nd, 2000 [RS 813.016.5]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C(97)186/Final].

There were no circumstances that may have affected the quality or integrity of the data.

Study Director: Dr. W. Völkel

W. Völkel

Date: September 2004

SIGNATURES

Study Director: Dr. W. Völkel

Date: September 16, 2004

Management: Dr. U. Morgenroth

Date: September 16, 2004

QUALITY ASSURANCE

RCC Ltd, Environmental Chemistry & Pharmanalytics, CH-4452 Itingen / Switzerland

STATEMENT

RCC Study Number: 851849

Test Item: [redacted]

Study Director: Dr. W. Völkel

Study Title: Adsorption/Desorption of [redacted] n Activated Sewage Sludge

The general facilities and activities are inspected periodically and the results are reported to the responsible person and the management.

Study procedures were periodically inspected. The study plan and this report were audited by the Quality Assurance. The dates are given below:

Dates and Types of QA Inspections		Dates of Reports to the Study Director and to the Management
December 17, 2003	Study Plan	December 17, 2003
December 22, 2003	Test system	December 22, 2003
April 26 – May 02, 2004	Draft Report	May 02, 2004
September 13, 2004	Report	September 13, 2004

This statement also confirms that this final report reflects the raw data.

Quality Assurance: Ms. U. Memmert

Date: September 16, 2004

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PREFACE

GENERAL

Study Title:	Adsorption/Desorption of [redacted] on Activated Sewage Sludge
Sponsor:	[redacted]
Scientific Representative:	Mr. D. Gore
[redacted] Contacts:	Ms. C. Talbot Claire.Talbot@unilever.com
Test Facility:	RCC Ltd Environmental Chemistry & Pharmanalytics Zelgliweg 1 CH-4452 Itingen / Switzerland

RESPONSIBILITIES

Study Director:	Dr. W. Völkel
Deputy Study Director:	Dr. A. Mamouni
Technical Coordinator:	Mr. K. Schwob
Head of RCC Quality Assurance:	Mrs. Iris Wüthrich

SCHEDULE

Experimental Starting Date:	December 22, 2003
Experimental Completion Date:	February 19, 2004
Study Completion Date:	September 16, 2004

ARCHIVING

RCC Ltd, CH-4452 Itingen/Switzerland will retain the study plan, raw data, a sample of test item and the final report of the present study for at least ten years. No data will be discarded without the sponsor's consent. The remaining test item will be returned to the Sponsor. Archiving of the test items is the responsibility of the Sponsor.

DATA REQUIREMENT / TEST GUIDELINES

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

- OECD Guideline 106: Adsorption/Desorption using a Batch Equilibrium Method; January 21, 2000.

And under consideration of

- Commission Directive 95/36/EC; July 14, 1995; amending Council Directive 91/414/EEC concerning plant protection products. Annex II, Part A: Chemical Substances, Section 7.1.2; Adsorption and Desorption, 1991.

SUMMARY OF STUDY PLAN AMENDMENTS

There were no amendments to the study plan.

SUMMARY

Adsorption and desorption of [redacted] were determined in the following three sludges using the batch equilibrium method according to OECD guideline no. 106: Pratteln (industrial, sludge I), Füllinsdorf (municipal, sludge II) and Sissach (municipal, sludge III).

In the preliminary test, the adsorption behaviour of [redacted] was tested using three different sludge dry weight to aqueous phase ratios. For this, sludge samples of 0.5 g, 1 g and 2 g, respectively, were shaken each with 25 mL of 0.01 M $CaCl_2$ solution, at a test concentration of ca. 2.0 mg [redacted] The sludge/aqueous phase suspensions were incubated in sealed Teflon® centrifuge tubes on a shaker at about 150 strokes per minute, in the dark at 20 °C. After 2, 5, 24 and 48 hours of agitation, aliquots of the aqueous phase were analysed by LSC and after 48 hours, additionally by HPLC/RAD.

The percentage of the applied amount that was adsorbed after 48 hours depended on the relative amount of sludge and increased with increasing sludge-to-aqueous phase ratio. Sludge I (Pratteln) showed 45.7%, 53.1% and 59.2% adsorption at the ratios 1/50, 1/25 and 2/25, respectively. For sludge II (Füllinsdorf), the corresponding values were 59.2%, 70.3% and 94.2%, and for sludge III (Sissach) 66.4%, 74.5% and 81.6% .

The control samples showed no adsorption of the test item on the tube walls. Blank samples showed only background radioactivity.

The pH of the aqueous phase was measured before and after contact with the sludge. This measurement resulted in a pH of 6.38 for the aqueous phase before, and a pH of 6.14, 6.28 and 6.33 after contact with sludges I, II and III, respectively.

The resulting Koc values for sludges I (Pratteln), II (Füllinsdorf) and III (Sissach) at the sludge-to-aqueous phase ratio of 1/25, were 77 mL/g, 200 mL/g and 261 mL/g, respectively. The variation of the Koc values shows that the adsorption of [redacted] did not depend on the organic carbon content of the sludge.

The recovery of the radioactivity applied was investigated in selected mass balance samples at a sludge/solution ratio of 1/25 and a test concentration of 2.0 mg/L. It was found to be 94.4%, 95.5% and 98.0% for sludges I, II and III, respectively. In the supernatant, amounts of 48.1%, 31.2% and 27.1% of the radioactivity applied were detected, respectively. Only low amounts of radioactivity adsorbed to the sludge could be extracted using acetonitrile/water (4:1; v/v). For sludge I 3.8%, for sludge II 2.7% and for sludge III below 0.1% of the radioactivity applied was recovered by extraction. However, 42.6%, 61.6% and 70.9% of the amount of radioactivity applied remained non-extracted.

Analysis of the extractables by HPLC showed 86% to 95% [redacted]

Adsorption and desorption isotherms were determined in a succeeding advanced test at five concentrations (2.077, 1.011, 0.200, 0.103 and 0.020 mg/L) covering two orders of magnitude in all three sludges. The sludge/solution ratio of 1/25 was employed and the agitation time used was 24 hours for adsorption, as well as for desorption.

The following results for the adsorption and the desorption were obtained:

Sludge	Sludge I (Pratteln)	Sludge II (Füllinsdorf)	Sludge III (Sissach)
K_F	21.6	65.6	62.8
K_{oc}	55	201	202
1/n	0.93	0.95	0.95
$K_{des,F}$	42.9	111.4	148.1
$K_{des,oc}$	108	342	475
1/n	0.94	0.95	0.98

The Koc values for adsorption ranged from 55 to 202, with a mean of 153, and the Koc values for desorption ranged from 108 to 475, with a mean of 308.

The 1/n values were in the range of 0.93 to 0.98 showing almost linearity involved in the adsorption and desorption process.

The calculated K_{des}oc values were much higher than those obtained for the adsorption isotherms, indicating the partial irreversibility of the adsorption step.

The adsorption kinetics showed that equilibrium was reached after about 24 hours of agitation, but desorption kinetics showed that equilibrium was achieved already after about 5 hours.

The mass balance showed that no losses of radioactivity occurred.

1 PURPOSE

Elimination of organic substances in a sewage-purification plant occurs mainly via biological degradation. For substances with low solubility, sorption on the sludge can contribute to the elimination of material from the liquid phase. By a possible later agricultural usage of the sludge, a desorption process may arise causing the release of these substances. The availability of the test item in the sludge-water environment and finally its degradability are significantly influenced by its adsorption/desorption characteristics.

The distribution of a chemical between solid sludge and aqueous phases is a complex process depending on a number of different factors such as the chemical nature of the substance and the characteristics of the sludge. The batch equilibrium method attempts to define the numerous phenomena and mechanisms involved in the process of adsorption of a chemical by sludge, and more importantly, it provides valuable information on the environmental relevance of the adsorption of a chemical.

The objective of the study was to determine the sorption of the test item to various sludge types of differing organic carbon content.

2 MATERIAL AND METHODS

2.1 CHEMICALS

All data of test items as provided by the sponsor, except where otherwise stated.

2.1.1 Test Item

Characterisation of the sample is confirmed by paperwork from a fine chemicals supplier (Amersham) indicating purity and identity. Stability has been assured by reference to the expiry date provided by the supplier. Homogeneity is assured since this is a pure sample (>96%). The correct identity of the sample has been confirmed by the sponsor by comparison of the sample label, lot/batch number with the Certificate of Analysis provided by the supplier. The sample will not therefore be characterised or stability or homogeneity addressed by **experimental** work by the sponsor.

Identity:	
Lot Number:	CFQ 13410 sample number S2512901 (RCC 144774/A)
Spec. Radioactivity:	27 mCi/mmol, 40.8 µCi/mg, 1.51 MBq/mg
Radiochemical Purity:	96.2%
Appearance:	
Amount Supplied:	10 MBq
Storage:	Freezer; at RCC at about –20 °C
Stability of the Test Substance in Vehicle:	Stable under normal conditions. It will be checked any way by appropriate analytical methods using an aliquot of the application solution before and after application.
Safety Precautions:	All work with the test item should be conducted under reduced level of light. Routine hygienic procedures according to the Swiss Legislation on Radiological Protection (Switzerland, ordinance of June 22, 1994).

2.1.2 Unlabelled Test Item

Characterisation, stability and homogeneity are being addressed by the Sponsor in a GLP compliant study, [redacted] Study Reference Number AC030449.

The unlabelled test item was used for co-chromatography.

Identity:	[redacted]
Empirical Formula:	[redacted]
[redacted] Sample No.:	S2539801 (RCC 144207/B)
Molecular Weight:	660.3 g/mol
Purity:	97.89% (HPLC)
Appearance:	[redacted]
Solubility in Water:	approx. 5 percent by weight
Expiry Date:	January 01, 2005
Storage:	At ambient temperature in the dark
Stability:	In water: 7 days in the refrigerator
Safety Precautions:	All work with the test item should be conducted under reduced level of light. Routine hygienic procedures.

2.2 TEST SYSTEM

Rationale for the Selection of the Test System

The sludges chosen from different treatment plants represented a range of different organic carbon contents, that are believed most important for adsorption.

2.2.1 Sludges

The sewage sludges chosen in this study represent sludges from two municipal and an industrial purification plant, differing in organic matter. The specifications of the sludges are given in Table 1.

2.2.2 Tubes and Equilibration

The study was performed in sealed Teflon centrifuge tubes. All experiments including controls and blanks were performed in duplicate. They were carried out at a constant temperature of 20 ± 2 °C. Tubes were shaken in the dark at about 150 strokes per minute. The agitation device kept the sludge in suspension during shaking.

2.2.3 Sludge Preparation and Conditioning

After sampling, the sewage sludge settled down in the container. The supernatant water was decanted. The remaining sludge was suspended in tap water and then centrifuged for 10 minutes at 2800 rpm. The supernatant liquid was decanted and the solid material re-suspended in tap water and again centrifuged. This procedure was repeated twice. The dry weight of the sludges was then determined. The washed sludge was kept at about 10 °C until usage.

The sludge samples were equilibrated by shaking with 0.01M $CaCl_2$ solution in tared test tubes. The sludge samples were equilibrated with about 90% of the targeted volume of the aqueous phase by shaking overnight (about 18 to 21 hours) before the application of the test item.

The pH of the aqueous phase was measured before and after contact with the sludge in the preliminary test.

2.3 STUDY CONDUCT

The study consisted of two parts:

1. The **preliminary test** was performed with three sludges in order to:
   - select the optimal sludge/solution ratio
   - determine the equilibration time for adsorption and the amount of test item adsorbed at equilibrium
   - determine the adsorption of the test item on the surfaces of the test vessels and the stability of the test item during the shaking period.
   - determine the mass balance of in three sludges, for one sludge/solution ratio per sludge where aqueous phases and sludges are worked up directly after the adsorption step.

2. **Advanced test:** determination of the Freundlich adsorption/desorption isotherms to show the influence of the concentration on the extent of adsorption/desorption on three sludges.

2.3.1 Preparation of Application Solutions

Preliminary Test

Stock solution I was prepared by dissolving an aliquot (about 2.4 mg) of the radiolabelled test item in 1 mL acetone. The solution was treated for about 5 minutes in an ultrasonic bath. Thereafter, an aliquot of 450 µL stock solution I was transferred to a 50-mL volumetric flask. The organic solvent was evaporated in a stream of nitrogen and the flask was filled up to the mark with 0.01 M calcium chloride ($CaCl_2$) solution. This application solution was treated for 15 minutes in an ultrasonic bath.
The amount of [redacted] was determined by Liquid Scintillation Counter (LSC). Three subsamples of 100 µL were measured by LSC counting. Based on the total radioactivity measured (115652000 dpm) and the specific activity (1.51 MBq/mg) the amount of [redacted] was calculated to be 1.28 mg /50 mL application solution (= 0.026 mg/mL).

Aliquots of 2000 µL of the application solution were added to each. The exact amount added to each tube was determined in the control solution of the 0-hour interval to be 51.8 µg test item equivalents per 25 mL 0.01 M $CaCl_2$. Considering the purity of 97.9% in the application solution, per tube 50.8 µg [redacted] were applied, resulting in a test concentration of ca. 2.0 mg/L.

Advanced Test

Five different test concentrations were prepared. For the highest concentration, a stock solution II was prepared by dissolving an aliquot of the radiolabelled test item (about 2 mg) in 1.5 mL acetone. An aliquot of 1150 µL of stock solution II was transferred to a 50 mL volumetric flask and the solvent was evaporated under a stream of nitrogen. The residue was dissolved in 50 mL of 0.01 M calcium chloride solution. The solution was treated in an ultrasonic bath for ten minutes. The amount of [redacted] in the present solution was determined by measuring three aliquots of 10 µL by LSC. Based on the total radioactivity measured (156820000 dpm) and the specific activity (1.51 MBq/mg) the amount of [redacted] was calculated to be 1.73 mg /50 mL application solution (= Solution A).

The lower concentrations were prepared by diluting with 0.01 M $CaCl_2$ solution as follows:

Solution B: 10 mL of solution A diluted to 20 mL
Solution C: 2 mL of solution A diluted to 20 mL
Solution D: 2 mL of solution B diluted to 20 mL
Solution E: 2 mL of solution C diluted to 20 mL

An aliquot of 1500 µL of the appropriate solution was added to the samples after equilibration of 1 g sludge with 21.5 mL 0.01 M $CaCl_2$. The exact amount applied was determined by LSC measurements of subsamples of the control solutions. The following test concentrations were obtained:

Samples treated with solution A: 2.077 mg/L (= 51.93 µg / 25 mL)
Samples treated with solution B: 1.011 mg/L (= 25.27 µg / 25 mL)
Samples treated with solution C: 0.200 mg/L (= 5.01 µg / 25 mL)
Samples treated with solution D: 0.103 mg/L (= 2.58 µg / 25 mL)

Samples treated with solution E: 0.020 mg/L (= 0.49 µg / 25 mL)

2.3.1.1 Test Concentrations

The following table summarises the test concentrations of the different parts of the adsorption/desorption test.

Part	Test concentration [mg/L]				
Preliminary test	2.01				
Advanced test	2.077	1.011	0.200	0.103	0.020

2.3.2 Application

After equilibration of the sludge with 0.01 M $CaCl_2$ solution, the defined volumes of the corresponding application solutions were added to the surface of the supernatant (tubes were centrifuged before application during two minutes at about 3300 rpm). Thereafter, the volume of the aqueous solution was adjusted with 0.01 M $CaCl_2$ solution to the target volume for the corresponding sludge to aqueous solution ratio.

The test tubes were briefly shaken by hand, and then mechanically shaken at about 20°C on a rotary shaker in a temperature-controlled room. The following table summarises the amounts of sludge and the volumes of the corresponding 0.01 M $CaCl_2$ solution used.

Part	Amount of sludge [g]	Volume of aqueous phase for equilibration [mL]	Volume of application solution [mL]	Volume of adsorption solution [mL]	sludge dry weight / aqueous phase ratio
Preliminary test	0.5	22.5*	2.000	25**	1/50
	1	22.5*	2.000	25**	1/25
	2	22.5*	2.000	25**	2/25
Advanced test	1	22.5*	1.500	25**	1/25

* Includes residual water of the sludge (up to 2 mL)
** Missing volume was added using 0.01 M $CaCl_2$ solution

Control samples (duplicate) containing the test item only in 0.01 M $CaCl_2$ solution (i.e. without sludge) were subjected to precisely the same steps as the test samples in order to check the stability of the test item in $CaCl_2$ solution and its possible adsorption to the surface of the test vessels.

A blank run for each sludge type with the same amount of sludge and the same total aqueous phase volume (without test item) was subjected to the same test procedure. This served as a background control during the analysis to detect any contamination.

2.3.3 Performance of the Adsorption/Desorption Tests

Adsorption of the Test Item to Sludge

Following application, the test tubes were shaken horizontally at about 215 strokes per minute at 20 °C. After shaking, the sludges were centrifuged (10 minutes at about 3300 rpm at room temperature). In the preliminary test, subsamples (80 µL for intervals 2 to 24 hours and 200 µL at 48 hours) were taken for LSC measurements at different time intervals (after centrifugation) and the samples were incubated again. At the end of the adsorption, the samples were centrifuged and the aqueous phases decanted. The volumes of the supernatants were determined gravimetrically. The supernatants were analysed by LSC and HPLC.

The equilibrium concentration of the test item and its total amount in the aqueous phase were calculated based on the results of the radioassays. The amount of test item adsorbed onto sludge particles was obtained from the difference between the initial and final amount of test item in the aqueous phase. Only for the mass balance tubes the amount o[redacted] adsorbed to sludge was additionally quantitatively analysed by extraction, HPLC/RAD analysis and combustion of the extracted sludge.

Desorption of the Test Item

After separation of the sludge from the adsorption solution, the same volume of 0.01 M $CaCl_2$ as used for the adsorption was added. Thereafter, these mixtures were agitated similarly as for the adsorption step. They were then centrifuged and the supernatant analysed by LSC.

2.3.4 Preliminary Test

2.3.4.1 Selection of Solid to Liquid Ratio

Three sludges and three sludge/solution ratios (nine experiments) were used. The sludge-to-solution ratios 1/50, 1/25 and 2/25 were tested.

The pH of the aqueous phase (0.01 M $CaCl_2$ solution) was measured before and after contact with the sludge.

2.3.4.2 Determination of the Equilibration Time

Samples at the above-mentioned ratios were set up for one test item concentration. After 2, 5, 24 and 48 hours of shaking, duplicate aliquots of the supernatants were measured by LSC.

The volume of these aliquots did not exceed 1% of the total aqueous solution volume in order to avoid significantly changing the sludge/solution ratio or decreasing the mass of solute available for adsorption during the test. The stability of the test item in the aqueous phase was determined by HPLC/RAD after 48 hours of adsorption.

2.3.4.3 Mass Balance

The mass balance was carried out for all sludges in the course of the preliminary test, at a sludge/solution ratio 1/25, at a test concentration of 2.0 mg/L.

After 48 hours of adsorption the supernatant was removed and the sludge was extracted three times using acetonitrile/water (4:1; (v/v)), in order to show the extractability of the test item. The radioactivity remaining in sludge was determined by combustion of sludge aliquots. The extracts resulting from the same tube were combined and quantified by LSC. The aqueous supernatant and the combined extracts of each tube were submitted to HPLC/RAD analysis.

2.3.5 Advanced Test (Adsorption Isotherms and Desorption Kinetics/ Desorption Isotherms)

With respect to the results obtained in the preliminary test, the study of the adsorptive behaviour of [redacted] in sludge and its potential mobility was further followed by determining the Freundlich adsorption isotherms.

2.3.5.1 Adsorption Isotherms

The procedure for this test was similar to that used in the preliminary test, except that five different concentrations were used.

- The sludge/solution ratio of 1/25 was applied, i.e. 1 g sludge/25 mL 0.01 M $CaCl_2$.
- Three sludge types were used.
- The agitation time used was 24 hours.
- Five concentrations (2.077, 1.011, 0.200, 0.103 and 0.020 µg/mL, see Section 2.3.1.1) were selected.
- Duplicate tubes for each sludge and concentration, as well as for control (no sludge) except the blank (no test item, single tube was taken), were set up in this test.

2.3.5.2 Desorption Kinetics and Isotherms

The procedure to determine the desorption equilibration time and the desorption isotherms was similar to that used as in the preliminary test and as in the advanced test for the adsorption isotherms. The purpose was to investigate whether [redacted] reversibly or irreversibly adsorbed to sludge.

The desorption experiment was carried out on each sample used in the test for the determination of the adsorption isotherms. After the adsorption, the mixture was centrifuged and the aqueous phase was removed. The amount of remaining sludge water was determined by weighing and the mass of the test item left over from the adsorption equilibrium due to incomplete volume replacement was calculated.

Duplicate samples were analysed by LSC after 2, 5 and 24 hours of desorption. Aliquots of up to 3.0 mL were taken for the LSC measurements. For each interval individual tubes were set-up. Therefore, a higher volume for LSC measurement was available and the sludge/solution ratio was not changed.

2.4 ANALYTICAL METHODS

2.4.1 Measurement of Radioactivity

The aqueous samples obtained from the sludge after adsorption and desorption were analysed by LSC (Liquid Scintillation Counting) using Packard liquid scintillation counters equipped with DPM and luminescence options (TRI-CARB 2500TR/2700TR). Quench curves are prepared twice a year in this facility and the measurements are automatically corrected. One set of vials from Packard each containing a different amount of radioactivity was measured every two weeks. The recovery was greater than 95% of their radioactivity content. Furthermore, the efficiency of the counters was checked once a day by measurement of a Packard-vial containing a known amount of radioactivity and comparison to its measured radioactivity content. All measurements were performed at least in duplicate, corrected for background, and counted for a time interval allowing a counting error below 5%.

The scintillation mixture INSTA-GEL II Plus (Packard Instrument) was used. Aqueous and organic (extracts) samples (up to 3 mL) were mixed with 10 mL of scintillation mixture and their radioactivity measured in the scintillation counter.

Quantification of Non-Extractable Radioactivity

After all extractions (mass balance tubes), aliquots of sludge samples (ca. 0.5 g) were placed into ceramic sample boats and was combusted by an OX 500 sample oxidiser (Zinsser Analytic; Frankfurt/Germany) in a stream of oxygen gas. The combustion temperature was 900 °C. The combustion products were passed through a series of catalysts at 600 °C. All determinations were done in triplicate. Evolved $^{14}CO_2$ was absorbed in 9 mL of a 1:3 (v/v) mixture of ethanolamine/methoxyethanol. The resulting absorption solution was mixed with 10 mL of the scintillation mixture mentioned above and the radioactivity was quantified by LSC. Aliquots of calibration standard solutions were combusted at the beginning of each series. The yield of combustion of calibration standards solution was approximately 100%.

2.4.2 High-Performance Liquid Chromatography (HPLC)

HPLC was used to analyse [redacted] in the supernatant solutions from the adsorption and desorption tubes for each sludge. The following conditions were used for the study:

Instruments

Pump: Merck-Hitachi L-7100
Autosampler: Merck-Hitachi AS-2000A
UV-detector: Merck-Hitachi L-4000 (260 nm)
[redacted]

Operating Conditions

Precolumn: 4 mm x 4 mm, 5 µm Li-Chrospher C18 (Merck)
Column: 125 mm x 4 mm, 3 µm Spherisorb C6
Temperature: Room temperature
Mobile Phase: Solvent A: 10 mM octanesulfonic acid (sodium salt)+ 1% triethyl amine, adjusted to pH 2.5 with phosphoric acid
Solvent B: acetonitrile

Gradient:

Time (min.):	0	3	30	35	35.1	40
Solvent A (%):	95	95	30	30	95	95
Solvent B (%):	5	5	70	70	5	5

Flow: 1 mL/min
Sample: A volume of up to 350 µL was injected.
[redacted]

Chromatograms are shown in Figures 8 to 12.

2.5 CALCULATIONS

Calculations were performed with a commercially available computer program (Excel 97). The results given in the tables are rounded up to the last digit given. For further calculation, the unrounded values were used. Thus, hand calculations may differ slightly from those presented due to rounding.

2.5.1 Preliminary Test

Adsorption

Percentage of substance adsorbed:

$$A_{ti} = \frac{m_s^{ads}(t_i) \cdot 100\%}{m_0}$$

Where:

A_{ti}	=	Adsorption percentage at the time point t_i (%)
$m_s^{ads}(t_i)$	=	Mass of the test item adsorbed on the sludge at the time t_i (µg)
m_0	=	Mass of the test item in the test tube at the beginning of the test (µg)

Distribution coefficient:

$$K_d = \frac{m_s^{ads}(eq)}{m_{aq}^{ads}(eq)} \cdot \frac{V_0}{m_{soil}} \qquad (mL \cdot g^{-1})$$

Where:

$m_s^{ads}(eq)$	=	Mass of test item adsorbed on the sludge at adsorption equilibrium (µg)
$m_{aq}^{ads}(eq)$	=	Mass of test item in the solution at adsorption equilibrium (µg)
m_{sludge}	=	Quantity of the sludge phase expressed in dry mass of sludge (g)
V_0	=	Initial volume of the aqueous phase in contact with the sludge (mL)

The adsorption coefficient normalised to the organic carbon content of the sludge, Koc is derived from K_d via the relationship:

K_d $= f_{oc} \times Koc$, where f_{oc} = fraction of the organic carbon content in sludge.

Mass balance:

$$MB = \frac{(V_{rec} \cdot C_{aq}^{ads}(eq) + m_E) \cdot 100}{V_0 \cdot C_0} \qquad (\%)$$

Where:

MB	=	Mass balance (%)
m_E	=	Total mass of test item extracted from the sludge (µg)
C_0	=	Initial mass concentration of the test item in solution (µg cm^{-3})
V_{rec}	=	Volume of the supernatant recovered after the adsorption equilibrium (mL).
$C_{aq}^{ads}(eq)$	=	Mass concentration of the substance in the aqueous phase at adsorption equilibrium (µg cm^{-3})

2.5.2 Advanced Test

Adsorption isotherms:

$$C_s^{ads}(eq) = \frac{m_s^{ads}(eq)}{m_{sludge}} = \frac{[C_0 - C_{aq}^{ads}(eq)] \cdot V_0}{m_{sludge}} \quad (\mu g \cdot g^{-1})$$

The nonlinear Freundlich adsorption equation is given by:

$$C_s^{ads}(eq) = K_F^{ads} \cdot C_{aq}^{ads}(eq)^{1/n} \quad (\mu g \cdot g^{-1})$$

Where:

K_F^{ads} = Freundlich adsorption coefficient; its dimension is $mL \cdot g^{-1}$ only if 1/n = 1; in all other cases, the slope 1/n is introduced in the dimension of K_F^{ads} $(\mu g^{1-1/n} (mL)^{1/n} g^{-1}$

1/n = Freundlich exponent; 1/n generally ranges between 0.7-1.0, indicating that sorption data is frequently slightly non-linear.

The linear form of the above Freundlich equation is given by:

$$\log C_s^{ads}(eq) = \log K_F + 1/n \cdot \log C_{aq}^{ads}(eq)$$

which is used to evaluate K_F (at $C_{aq}^{ads}(eq)$ = 1µg/mL). K_F equals K_D for 1/n = 1.

The adsorption coefficient normalised to the organic carbon content, K_{OC}, is obtained according to:

$K_D \approx K_F$ = $f_{oc} \cdot K_{OC}$, where f_{oc} = fraction of the organic carbon content in sludge.

The adsorption coefficient normalised to the organic matter content, K_{om}, is then obtained according to:

$K_{OC}=1.724 \cdot K_{om}$

Desorption

$$D_{ti} = \frac{m_{aq}^{des}(t_i)}{m_s^{ads}(eq)} \cdot 100$$

Where:

D_{ti} = Desorption percentage at a time point t_i (%)

$m_{aq}^{des}(t_i)$ = Mass of the test item desorbed from sludge at a time point t_i (µg)

$m_s^{ads}(eq)$ = Mass of the test item adsorbed on sludge at adsorption equilibrium (µg)

Desorption isotherms:

$$C_s^{des}(eq) = \frac{m_s^{ads}(eq) - m_{aq}^{des}(eq)}{m_{sludge}} = \frac{m_s^{des}(eq)}{m_{sludge}} \quad (\mu g \cdot g^{-1})$$

where $m_{aq}^{des}(eq)$ is defined as:

$$m_{aq}^{des}(eq) = m_m^{des}(eq) \cdot \frac{V_0}{V_r^F} - m_{aq}^A \quad (\mu g)$$

$C_s^{des}(eq)$ = Amount of the test item remaining adsorbed on the sludge at desorption equilibrium ($\mu g \cdot g^{-1}$)

$m_m^{des}(eq)$ = Mass of the test item determined analytically in the aqueous phase at desorption equilibrium (µg)

m_{aq}^A = Mass of the test item left over in solution from the adsorption equilibrium due to incomplete volume replacement (µg)

$m_{aq}^{ads}(eq)$ = Mass of the substance in the solution at adsorption equilibrium (µg)

$$m_{aq}^A = m_{aq}^{ads}(eq) \cdot \left(\frac{V_0 - V_R}{V_0}\right)$$

V_r^F = Volume of the solution taken from the tube for the measurement of the test item at desorption equilibrium (mL)

V_R = Volume of the supernatant removed from the tube after the attainment of adsorption equilibrium and replaced by the same volume of a 0.01M $CaCl_2$ solution (mL)

m_s^{des} = mass of test item remaining adsorbed on the sludge at desorption equilibrium (µg).

Nonlinear Freundlich desorption equation:

$$C_s^{des}(eq) = K_F^{des} \cdot C_{aq}^{des}(eq)^{1/n} \quad (\mu g \cdot g^{-1})$$

Where:

K_F^{des} = Freundlich desorption coefficient

1/n = Freundlich exponent

$C_{aq}^{des}(eq)$ = Mass concentration of the test item in the aqueous phase at desorption equilibrium ($\mu g \cdot cm^{-3}$)

The linear form of the above Freundlich equation is given by:

$$\log C_s^{des}(eq) = \log K_F^{des} + 1/n \cdot \log C_{aq}^{des}(eq)$$

This formula will be applied to evaluate K_F for the desorption process, and from this, K_{OC} and K_{om} can be determined according to the formula given under "adsorption isotherms" above.

2.5.3 Estimation of the Limit of Detection and Limit of Quantitation

After sampling, aqueous phase and sludge were separated and aliquots of the water phase were measured by LSC. Estimation of the limit of quantitation based on the radioassays is shown for a representative example of a mass balance sample from the preliminary test:

Liquid Scintillation Counter (LSC, Mass balance sludge I, Sample A, Table 5)

The limit of detection of the LSC counter was set to two fold the background radioactivity. Background radioactivity was determined to be about 20 dpm. This value was subtracted automatically from each sample after measurement. The limit of quantitation was set to three times the background radioactivity.

The value of 100% applied is the total radioactivity applied without correction for the purity of the test item in the application solution.

1) Aqueous Phase

- test item applied: 51.8 µg/25 mL = 4696875 dpm/25 mL
- Sample size measured: 0.2 mL
- Volume of water phase: 25 mL

	dpm	Total dpm	% applied	Parent equivalents	mg/kg water
	(0.2 mL)	(for 25 mL)	(of 4696875 dpm)	(µg)	(ppm)
Background LSC (BG LSC)	20	2500	0.06	0.03	0.001
Detection limit (2xBG LSC)	40	5000	0.11	0.06	0.002
Limit of quantification (3xBG LSC)	60	7500	0.17	0.08	0.003

2) Sludge Extracts

- test item applied: 51.8 µg/25 mL = 4696875 dpm/25 mL
- Sample size measured: 0.1 mL
- Volume first extract: 8.6 mL
- Dry sludge: 10.0 g

	dpm	Total dpm	% applied	Parent equivalents	mg/kg dry sludge
	(0.05 mL)	(for 2.2 mL)	(of 4696875 dpm)	(µg)	(ppm)
Background LSC (BG LSC)	20	880	0.02	0.01	0.010
Detection limit (2xBG LSC)	40	1760	0.04	0.02	0.019
Limit of quantification (3xBG LSC)	60	2640	0.06	0.03	0.029

3) Combustion Non-Extractables

- test item applied: 51.8 µg/25 mL = 4696875 dpm/1 g sludge
- Sample size combusted (mean of triplicate): 0.058 g
- Weight of the wet sludge after extraction: 1.15 g
- Weight of the air-dried sludge: 1.0 g

	dpm per aliquot	Total dpm	% applied	Parent equivalents	mg/kg dry sludge
	(0.058 g)	(for 1.15 g)	(of 4696875 dpm)	(µg)	(ppb)
Background LSC (BG LSC)	20	397	0.01	0.004	0.004
Detection limit (2xBG LSC)	40	793	0.02	0.009	0.009
Limit of quantification (3xBG LSC)	60	1190	0.03	0.013	0.013

3 RESULTS AND DISCUSSION

3.1 RADIOCHEMICAL PURITY AND STABILITY OF THE TEST ITEM

The purity of [redacted] in the application solution of the preliminary test was 97.9% and 95.8% in the advanced test (Fig. 8 top). The stability in 0.01 M $CaCl_2$ solution was tested by HPLC/RAD in the control samples after the treatments in the preliminary and advanced test. [redacted] was determined to be stable in 0.01 M $CaCl_2$ solution, showing a purity of about 96% after 48 hours of shaking in the preliminary test and of 95% (mean value) in the control in the advanced test (Fig. 8 middle).

However, in the supernatant solutions after contact with sludge, the test item showed a lower purity, i.e. 90% to 91% in the preliminary test and 91.6% , 85.1% and 93.0% in the advanced test for sludges I through III. The radioactivity found in the supernatants was corrected for the corresponding purity. This correction takes into account the in part observed degradation of [redacted] after contact with sludge but finally, did not significantly change the resulting Freundlich adsorption constants.

3.2 PRELIMINARY TEST

All values reported in Sections 3.2 and 3.3 represent the mean of duplicate sample analysis. The preliminary test was performed using three sludge types, sludge I (Pratteln, industrial), sludge II (Füllinsdorf, municipal) and sludge III (Sissach, municipal). Three different sludge-to-solution ratios were tested: 1/50, 1/25 and 2/25 at a test concentration of 2.0 mg/L each.

The equilibration time for the adsorption was determined for the three sludges at the three sludge to aqueous solution ratios. In Table 2, it can be seen that equilibration needs a maximum of 24 hours of adsorption.

The percentage of the applied amount that was adsorbed after 48 hours depended on the relative amount of sludge, and increased with the increasing sludge-to-aqueous phase ratio. Sludge I (Pratteln) showed 45.7%, 53.1% and 59.2% adsorption at the ratios 1/50, 1/25 and 2/25, respectively (Table 2, Fig. 1). For sludge II (Füllinsdorf), the corresponding values were 59.2%, 70.3% and 94.2%, and for sludge III (Sissach) 66.4%, 74.5% and 81.6% .

Blank samples showed only background radioactivity. Radioactivity in the control samples remained constant throughout the incubation period of 48 hours (Table 3 and Fig. 2). The pH of the aqueous phase was measured before and after contact with the sludge. This measurement resulted in a pH of 6.38 for the aqueous phase before, and a pH of 6.14, 6.28 and 6.33 after contact with sludges I, II and III, respectively.

Koc values were calculated for the three sludges at all sludge-to-aqueous phase ratios (Table 4).

The resulting Koc values for sludges I (Pratteln), II (Füllinsdorf) and III (Sissach) at the sludge-to-aqueous phase ratio of 1/25, were 77 mL/g, 200 mL/g and 261 mL/g, respectively (Table 4).

The variation of the Koc values shows that the adsorption of [redacted] did not depend on the organic carbon content of the sludge.

For the advanced test, the ratio 1/25 was selected. The ratio 2/25 was not used, since for sludge II already 94.2% of the test item was adsorbed and at a lower test concentration a higher percentage of adsorption could be expected. Higher adsorption means less amount to be measured in the supernatant, which risked to be less precise. The ration 1/50 was not selected due to the value "kd*ratio" which was 0.9 for sludge I (Table 4). This value is close to the value 0.3, where the indirect method, i.e. determination of the adsorption by measurement of the test item concentration in the supernatant solution, is not any longer recommended.

The mass balance was performed for all three sludges at the sludge-to-solution ratio of 1/25.

The balance of the radioactivity applied was 94.4%, 95.5% and 98.0% for sludges I, II and III, respectively (Table 5). In the supernatant, amounts of 48.1%, 31.2% and 27.1% of the radioactivity applied were detected, respectively. Only low amounts of radioactivity adsorbed to the sludge could be extracted using acetonitrile/water (4:1; v/v). For sludge I 3.8%, for sludge II 2.7% and for sludge III below 0.1% of the radioactivity (Table 5) applied was recovered by extraction. However, 42.6%, 61.6% and 70.9% of the amount of radioactivity applied remained non-extracted.

Analysis of the extractables by HPLC showed 86% to 95% [redacted] (Figure 12).

3.3 ADVANCED TEST

3.3.1 Adsorption Isotherms

Five concentrations (2.077, 1.011, 0.200, 0.103 and 0.020 mg/L) covering two orders of magnitude were tested. The sludge/solution ratio of 1/25 and an agitation time of 24 hours were used.

After adsorption, the amounts of test item adsorbed were determined (Table 6, Fig. 3) for the three sludges and five test concentrations. The results obtained were evaluated by applying the linear Freundlich equation (Figure 4). The resulting constants K_F, 1/n and Koc are presented below, and in more detail in Table 8. Representative HPLC chromatograms are presented in Figures 9 to 11.

Sludge	Sludge I (Pratteln)	Sludge II (Füllinsdorf)	Sludge III (Sissach)
K_F	21.6	65.6	62.8
K_{oc}	55	201	202
1/n	0.93	0.95	0.95
r^2	0.9995	0.9999	0.9994

The K_{oc} values range from 55 to 202 with a mean of 153.

The coefficients of determination (r^2) for the linear regression of the linear form of the Freundlich equation were not lower than 0.9994.

The 1/n values being in the range from 0.93 to 0.95 show near linearity involved in the adsorption step.

Blank samples showed only background radioactivity. Radioactivity in the control samples was entirely recovered and was constant for all concentrations (Table 7 bottom).

3.3.2 Desorption Kinetics

The time to reach desorption equilibration was determined for the three sludges at the highest test concentration (2.0 mg/L) at a sludge/solution ratio of 1/25. Supernatants were analysed after 2, 5 and 24 hours of desorption (Table 10, Fig. 6). The mass of test item left over from the adsorption equilibrium due to incomplete volume replacement is presented in Table 9. Desorption equilibrium was approximately reached after 5 hours, but the evaluation according to Freundlich was finally performed with the results obtained after 24 hours.

The percentage of the amount remaining adsorbed after the adsorption step, i.e. after 24 hours, was calculated for all sludges at the highest test concentration (Table 10).

For sludge I (Pratteln), the amount desorbed after 24 hours of desorption amounted to 24.8% of the amount remaining adsorbed after the adsorption step. For sludges II (Füllinsdorf) and III (Sissach), the corresponding values were 15.0% and 10.3%, respectively (Table 10). It is remarkable, that more test substance could be desorbed by 0.01 M $CaCl_2$ solution than by extraction with acetonitrile/water (Section 3.2).

3.3.3 Desorption Isotherms

The determination of the desorption isotherms was performed for all five test concentrations (2.077, 1.011, 0.200, 0.103 and 0.020 mg/L at a sludge/solution ratio of 1/25 (Table 11)) for all three sludges. The nonlinear desorption isotherms are presented in Fig. 5 and the linear desorption isotherms in Fig. 6. The (linear and nonlinear) isotherms were established from the values obtained after 24 hours of desorption (Table 11, Fig. 6).

The results obtained for the three sludges tested were evaluated by applying the linear Freundlich equation (Figures 5 and 6). The resulting constants $K_{des,F}$, 1/n and $K_{des,oc}$ are presented below, and in more detail in Table 12. Representative HPLC chromatograms after 48 hours of desorption are presented in Figs. 9 to 11.

Sludge	Sludge I (Pratteln)	Sludge II (Füllinsdorf)	Sludge III (Sissach)
$K_{des,F}$	42.9	111.4	148.1
$K_{des,oc}$	108	342	475
1/n	0.94	0.95	0.98
r^2	0.9997	0.9968	0.9990

The K_{oc} values for desorption range from 108 to 475 with a mean of 308.

The $K_{des,oc}$ values calculated were much higher than those obtained for the adsorption isotherms (Section 3.3.1), indicating partial irreversibility of the adsorption process. The coefficients of determination (r^2) for the linear regression of the linear form of the Freundlich equation were not lower than 0.9968.

The 1/n values of the desorption step, being very close to those of the adsorption isotherms, are in the range of 0.94 to 0.98, reflecting a similar sorption behaviour as found for the adsorption process.

4 CONCLUSIONS

The adsorption/desorption behaviour of [redacted] was investigated on three sludge types: Pratteln (industrial), Füllinsdorf (municipal) and Sissach (municipal). Five test concentrations, namely 2.077, 1.011, 0.200, 0.103 and 0.020 mg/L were selected to establish the Freundlich adsorption and desorption isotherms at a sludge/solution ratio of 1/25.

The following results were obtained:

Sludge	Sludge I (Pratteln)	Sludge II (Füllinsdorf)	Sludge III (Sissach)
K_F	21.6	65.6	62.8
K_{oc}	55	201	202
1/n	0.93	0.95	0.95
$K_{des,F}$	42.9	111.4	148.1
$K_{des,oc}$	108	342	475
1/n	0.94	0.95	0.98

A mean adsorption K_{oc} of 153 and a mean desorption K_{oc} of 308 were obtained. However, the adsorption of [redacted] did not depend on the organic carbon content of the sludge.

The calculated $K_{des,oc}$ values were much higher than those obtained for the adsorption process, indicating a partial irreversibility of the adsorption. The 1/n values obtained for the adsorption and desorption isotherms are very similar, all ranging from 0.93 to 0.98.

The adsorption kinetics showed that equilibrium was reached after about 24 hours of agitation, but desorption kinetics showed that equilibrium was achieved already after about 5 hours

The mass balance showed that no losses of radioactivity occurred.

5 REFERENCES

[1] Soil Taxonomy. A basic system of sludge classification for making and interpreting sludge surveys. Soil Survey Staff. Soil Conservation Service. U.S. Department of Agriculture. Agriculture Handbook No. 436, 1975.

[2] Lyman, W.J., et al. (eds.) 1990. Handbook of Chemical Property Estimation Methods, page 4-3 American Chemical Society, Washington, DC.

6 TABLES

Table 1: Sludge types and their characteristics

Parameter	Sewage Sludge		
	Pratteln (industrial)	Füllinsdorf (municipal)	Sissach (municipal)
Organic C [%]	39.68	32.62	31.16
Dry mass [g/100 g wet sludge]	8.7	15.0	8.5
pH [in 0.01 M $CaCl_2$]	6.05	6.24	6.25

Organic carbon content as determined by AgroLab AG, CH-6031 Ebikon / Switzerland

All three sludges were freshly collected in December 2003 from the following sites:

Industrial Sewage Sludge: ARA Rhein (Pratteln, Switzerland)
Municipal Sewage Sludge: ARA Füllinsdorf (Switzerland)
Municipal Sewage Sludge: ARA Sissach (Switzerland)

Table 2: Preliminary test: Radioactivity adsorbed to sludges I (Pratteln), II (Füllinsdorf) and III (Sissach), using three different sludge-to-solution ratios at an initial concentration of 2.0 mg/L. Values given in percent of applied.

Sludge I (Pratteln) % of initial	Adsorption time (hours)			
	2	5	24	48
Ratio 1/50				
Sample A	37.9	38.7	42.6	45.6
Sample B	37.5	36.5	41.4	45.9
Mean	37.7	37.6	42.0	45.7
Ratio 1/25				
Sample A	46.9	47.0	50.5	53.4
Sample B	45.7	46.7	47.9	52.7
Mean	46.3	46.9	49.2	53.1
Ratio 2/25				
Sample A	56.9	55.4	58.2	58.0
Sample B	59.9	56.5	58.8	60.4
Mean	58.4	56.0	58.5	59.2

Sludge II (Füllinsdorf) % of initial	Adsorption time (hours)			
	2	5	24	48
Ratio 1/50				
Sample A	59.5	55.4	56.5	58.1
Sample B	60.5	58.4	58.8	60.2
Mean	60.0	56.9	57.7	59.2
Ratio 1/25				
Sample A	76.2	76.4	72.6	70.6
Sample B	76.3	75.5	70.1	69.9
Mean	76.3	75.9	71.4	70.3
Ratio 2/25				
Sample A	56.4	57.3	93.8	94.1
Sample B	56.5	56.4	94.0	94.3
Mean	56.4	56.8	93.9	94.2

Sludge III (Sissach) % of initial	Adsorption time (hours)			
	2	5	24	48
Ratio 1/50				
Sample A	57.9	60.7	65.4	66.3
Sample B	56.7	64.0	65.1	66.5
Mean	57.3	62.3	65.2	66.4
Ratio 1/25				
Sample A	67.6	70.8	74.4	74.7
Sample B	69.2	71.4	74.6	74.3
Mean	68.4	71.1	74.5	74.5
Ratio 2/25				
Sample A	75.7	78.4	81.4	80.7
Sample B	77.9	80.0	83.4	82.5
Mean	76.8	79.2	82.4	81.6

Table 3: Preliminary test: Radioactivity remaining in solution of the control samples at an initial concentration of 2.0 mg/L. Values given in percent of applied.

Control	Adsorption time (hours)			
	2	5	24	48
Sample A	97.0	99.1	99.0	98.7
Sample B	97.9	97.0	96.2	98.3
Mean	97.5	98.0	97.6	98.5

Table 4: Results of the preliminary test. Distribution of [redacted] between aqueous phase and sludge (I: Pratteln, II: Füllinsdorf, III: Sissach) after adsorption for 48 hours at three different sludge-to-aqueous phase ratios.

Sample	Ratio	m [g]	Vo [mL]	m(ads)s [µg]	m(ads)aq [µg]	Kd [mL/g]	Kd*ratio [mL/g]	Koc [mL/g]
Sludge I (Pratteln)								
Sample A	1 : 50	0.5	25	23.12	25.43	45.5	0.9	115
Sample B		0.5	25	23.31	25.26	46.1	0.9	116
Mean				23.21	25.35	45.8	0.9	115
Sludge I (Pratteln)								
Sample A	1 : 25	1.0	25	27.09	21.79	31.1	1.2	78
Sample B		1.0	25	26.76	22.08	30.3	1.2	76
Mean				26.93	21.93	30.7	1.2	77
Sludge I (Pratteln)								
Sample A	2 : 25	2.0	25	29.42	19.64	18.7	1.5	47
Sample B		2.0	25	30.64	18.51	20.7	1.7	52
Mean				30.03	19.07	19.7	1.6	50
Sludge II (Füllinsdorf)								
Sample A	1 : 50	0.5	25	29.49	19.28	76.5	1.5	234
Sample B		0.5	25	30.56	18.31	83.4	1.7	256
Mean				30.02	18.79	80.0	1.6	245
Sludge II (Füllinsdorf)								
Sample A	1 : 25	1.0	25	35.83	13.53	66.2	2.6	203
Sample B		1.0	25	35.49	13.84	64.1	2.6	197
Mean				35.66	13.68	65.2	2.6	200
Sludge II (Füllinsdorf)								
Sample A	2 : 25	2.0	25	47.74	2.74	218.1	17.4	669
Sample B		2.0	25	47.85	2.63	227.4	18.2	697
Mean				47.79	2.68	222.8	17.8	683
Sludge III (Sissach)								
Sample A	1 : 50	0.5	25	33.66	15.35	109.6	2.2	352
Sample B		0.5	25	33.74	15.28	110.4	2.2	354
Mean				33.70	15.32	110.0	2.2	353
Sludge III (Sissach)								
Sample A	1 : 25	1.0	25	37.92	11.52	82.3	3.3	264
Sample B		1.0	25	37.70	11.72	80.4	3.2	258
Mean				37.81	11.62	81.4	3.3	261
Sludge III (Sissach)								
Sample A	2 : 25	2.0	25	40.96	8.79	58.2	4.7	187
Sample B		2.0	25	41.87	7.98	65.6	5.2	210
Mean				41.41	8.39	61.9	5.0	199

m = mass of sludge (g)
Vo =Initial volume of the aqueous phase in contact with the sludge (mL)
m(ads)s =Mass of the test item adsorbed on the sludge at adsorption equilibrium (µg)
m(ads)aq =Mass of test item in the solution at adsorption equilibrium (µg)
Kd =Distribution coefficient (mL/g)
Koc =Adsorption coefficient related to the organic carbon content of the sludge (mL/g)

Table 5: Preliminary test: mass balance in percent of radioactivity applied for sludge I (Pratteln), sludge II (Füllinsdorf) and sludge III (Sissach) after 48 hours of adsorption at an initial concentration of 2.0 mg/L.

Sample	Ratio	mass applied [µg]	(ads)aq [%]	(ads)extract [%]	(ads)residue [%]	recovery [%]
Sludge I (Pratteln)						
Sample A	1 : 25	51.8	47.8	4.2	45.0	96.9
Sample B		51.8	48.4	3.3	40.2	91.8
Mean		51.8	48.1	3.8	42.6	94.4
Sludge II (Füllinsdorf)						
Sample A	1 : 25	51.8	30.9	3.6	61.7	96.2
Sample B		51.8	31.5	1.8	61.5	94.9
Mean		51.8	31.2	2.7	61.6	95.5
Sludge III (Sissach)						
Sample A	1 : 25	51.8	26.9	<0.1	70.3	97.1
Sample B		51.8	27.3	<0.1	71.5	98.8
Mean		51.8	27.1	<0.1	70.9	98.0

(ads)aq: percent of the amount of radioactivity applied remaining in the aqueous phase
(ads)extract percent of the amount of radioactivity applied extracted by acetonitrile/water (4:1; v/v)
(ads)residue: percent of the amount of radioactivity applied remaining in the sludge

Table 6: Advanced test: distribution of [redacted] between aqueous phase and sludge after 24 hours of adsorption. Evaluation according to the linear Freundlich isotherms.

	Sludge I (Pratteln)				
Concentration Ci (µg/mL)	2.077	1.011	0.200	0.103	0.020
m(ads)s (µg)					
Sample A	23.50	11.34	2.38	1.25	0.26
Sample B	23.61	11.15	2.39	1.25	0.26
Mean (µg)	23.55	11.24	2.39	1.25	0.26
Mean (% of applied)	45.4	44.5	47.6	48.4	53.0
m(ads)s / m(sludge) (µg/g)	23.555	11.244	2.386	1.248	0.262
log(m(ads)s / m(sludge)	1.372	1.051	0.378	0.096	-0.581
c(ads)aq (µg/mL)	1.039	0.514	0.096	0.049	0.009
log(c(ads)aq)	0.017	-0.289	-1.017	-1.313	-2.070

	Sludge II (Füllinsdorf)				
Concentration Ci (µg/mL)	2.077	1.011	0.200	0.103	0.020
m(ads)s (µg)					
Sample A	36.51	17.88	2.14	1.92	0.37
Sample B	35.54	17.87	2.15	1.88	0.36
Mean (µg)	36.02	17.87	2.15	1.90	0.37
Mean (% of applied)	69.4	70.7	42.8	73.6	74.3
m(ads)s / m(sludge) (µg/g)	36.023	17.872	2.146	1.897	0.368
log(m(ads)s / m(sludge))	1.557	1.252	0.332	0.278	-0.435
c(ads)aq (µg/mL)	0.541	0.252	0.097	0.023	0.004
log(c(ads)aq)	-0.267	-0.599	-1.011	-1.636	-2.365

Ci = Initial concentration in the supernatant before adsorption
m(ads)s = Mass of the test item adsorbed on the sludge at adsorption equilibrium (µg)
m(sludge) = mass of sludge (1 g)
C(ads)aq = Concentration of test item in the solution at adsorption equilibrium (µg)

Table 6 continued:

	Sludge III (Sissach)				
Concentration Ci (µg/mL)	2.077	1.011	0.200	0.103	0.020
m(ads)s (µg)					
Sample A	37.45	17.70	3.61	1.90	0.38
Sample B	37.17	17.81	3.61	1.88	0.37
Mean (µg)	37.31	17.75	3.61	1.89	0.37
Mean (% of applied)	71.8	70.3	72.0	73.4	75.4
m(ads)s / m(sludge) (µg/g)	37.307	17.752	3.607	1.891	0.373
log(m(ads)s / m(sludge))	1.572	1.249	0.557	0.277	-0.428
c(ads)aq (µg/mL)	0.544	0.279	0.052	0.026	0.005
log(c(ads)aq)	-0.265	-0.554	-1.283	-1.593	-2.345

Ci = Initial concentration in the supernatant before adsorption
m(ads)s = Mass of the test item adsorbed on the sludge at adsorption equilibrium (µg)
m(sludge) = mass of sludge (1 g)
C(ads)aq = Concentration of test item in the solution at adsorption equilibrium (µg)

Table 7: Controls

	Controls				
Concentration Ci (µg/cm^3)	2.077	1.011	0.200	0.103	0.020
m(ads)aq (%)					
Sample A	98.4	98.2	99.8	100.8	99.2
Sample B	100.3	100.9	99.9	99.2	98.7
Mean	99.3	99.5	99.8	100.0	98.9

Table 8: Freundlich parameters after adsorption of [redacted]

Parameter	Sludge I (Pratteln)	Sludge II (Füllinsdorf)	Sludge III (Sissach)
$\log(K_F)$	1.34	1.82	1.80
K_F	21.6	65.6	62.8
Koc	55	201	202
Kom	32	117	117
1/n	0.93	0.95	0.95
r^2	0.9995	0.9999	0.9994

K_F = Freundlich adsorption constant
Koc = Freundlich adsorption constant related to sludge organic carbon content
Kom = Freundlich adsorption constant related to sludge organic matter content
1/n = Freundlich exponent
r^2 = Coefficient of determination

Table 9: Amount of test substance remaining in the sludge pore water after 24 hours of adsorption in the advanced test (sludge/solution ratio 1/25) at the highest test concentration of 2.0 mg/L.

	Sample	Sludge I (Pratteln)	Sludge II (Füllinsdorf)	Sludge III (Sissach)
Mass adsorbed on equlibrium (µg)	A	23.50	36.51	37.45
	B	23.61	35.54	37.17
	Mean	23.55	36.02	37.31
Concentration in aqueous phase (µg/mL)	A	1.041	0.525	0.539
	B	1.037	0.558	0.549
	Mean	1.039	0.541	0.544
Pore water remaining in sludge (mL)	A	3.57	3.88	2.76
	B	4.87	4.54	2.63
	Mean	4.22	4.21	2.70
Mass remaining in the sludge pore water (µg)	A	3.72	2.04	1.49
	B	5.05	2.53	1.44
	Mean	4.38	2.28	1.47

First and second row taken from Table 6 (test concentration 2.0 mg/L)

Table 10: Desorption kinetics for sludges I (Pratteln), II (Füllinsdorf) and III (Sissach). The mass desorbed is given in µg and in percent of the amount adsorbed.

	Sample	Sludge I (Pratteln)	Sludge II (Füllinsdorf)	Sludge III (Sissach)
Mass adsorbed on equlibrium (µg)	A	23.50	36.51	37.45
	B	23.61	35.54	37.17
	Mean	23.55	36.02	37.31
Mass desorbed from sludge after 2 hours (µg)	A	5.81	5.80	2.96
	B	5.08	5.53	3.30
Desorption after 2 hours (% of adsorbed)	A	24.7	15.9	7.9
	B	21.5	15.6	8.9
	Mean	23.1	15.7	8.4
Mass desorbed from sludge after 5 hours (µg)	A	6.47	5.11	3.25
	B	5.25	7.50	3.26
Desorption after 5 hours (% of adsorbed)	A	27.5	14.0	8.7
	B	22.2	21.1	8.8
	Mean	24.9	17.6	8.7
Mass desorbed from sludge after 24 hours (µg)	A	5.90	5.25	3.82
	B	5.77	5.52	3.89
Desorption after 24 hours (% of adsorbed)	A	25.1	14.4	10.2
	B	24.4	15.5	10.5
	Mean	24.8	15.0	10.3

Table 11: Advanced test: distribution of [redacted] between aqueous phase and sludge after 24 hours of desorption. Evaluation according to the linear Freundlich desorption isotherms

	Sludge I (Pratteln)				
Initial concentration Ci before adsorption (µg/mL)	2.077	1.011	0.200	0.103	0.020
m(des)s (µg)					
Sample A	17.602	8.111	1.792	0.940	0.199
Sample B	17.838	7.802	1.772	0.950	0.208
Mean (µg)	17.720	7.956	1.782	0.945	0.203
Mean (% of applied)	34.1	31.5	35.6	36.7	41.1
m(des)s / m(sludge) (µg/g)	17.720	7.956	1.782	0.945	0.203
log(m(des)s / m(sludge))	1.248	0.901	0.251	-0.025	-0.692
c(des)aq (µg/mL)	0.375	0.173	0.035	0.017	0.003
log(c(des)aq)	-0.425	-0.762	-1.451	-1.765	-2.475

	Sludge II (Füllinsdorf)				
Initial concentration Ci before adsorption (µg/mL)	2.077	1.011	0.200	0.103	0.020
m(des)s (µg)					
Sample A	31.259	14.767	2.030	1.673	0.330
Sample B	30.013	16.860	1.977	1.601	0.325
Mean (µg)	30.636	15.813	2.004	1.637	0.328
Mean (% of applied)	58.99	62.58	40.00	63.52	66.22
m(des)s / m(sludge) (µg/g)	30.636	15.813	2.004	1.637	0.328
log(m(des)s / m(sludge))	1.486	1.199	0.302	0.214	-0.485
c(des)aq (µg/mL)	0.286	0.108	0.015	0.013	0.002
log(c(des)aq)	-0.543	-0.966	-1.814	-1.895	-2.667

Ci =Initial concentration in the supernatant before adsorption (µg/mL)
m(des)s = Mass of the test item remaining adsorbed on the sludge at desorption equilibrium (µg)
m (sludge) = Mass of sludge (1 g)
C(des)aq =Concentration of test item in the solution at desorption equilibrium (µg/mL)

Table 11 continued:

	Sludge III (Sissach)				
Initial concentration Ci before adsorption (µg/mL)	2.077	1.011	0.200	0.103	0.020
m(des)s (µg)					
Sample A	33.627	15.578	3.257	1.715	0.337
Sample B	33.275	15.632	3.210	1.663	0.330
Mean (µg)	33.451	15.605	3.234	1.689	0.333
Mean (% of applied)	64.41	61.76	64.55	65.54	67.42
m(des)s / m(sludge) (µg/g)	33.451	15.605	3.234	1.689	0.333
log(m(des)s / m(sludge))	1.524	1.193	0.510	0.228	-0.477
c(des)aq (µg/mL)	0.203	0.109	0.019	0.010	0.002
log(c(des)aq)	-0.693	-0.961	-1.712	-1.991	-2.715

Ci =Initial concentration in the supernatant before adsorption (µg/mL)
m(des)s = Mass of the test item remaining adsorbed on the sludge at desorption equilibrium (µg)
m (sludge) = Mass of sludge (1 g)
C(des)aq =Concentration of test item in the solution at desorption equilibrium (µg/mL)

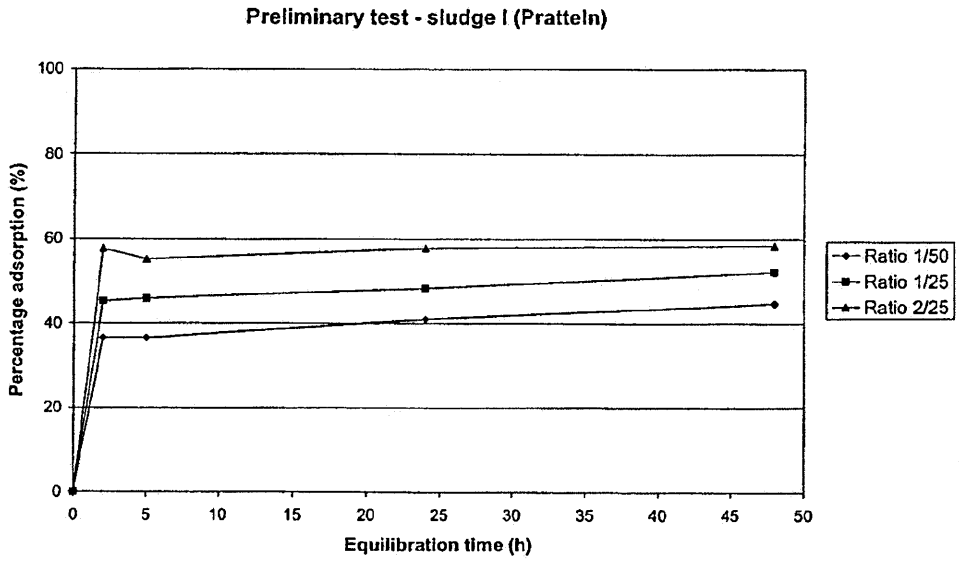
Table 12: Freundlich parameters after 24 hours of desorption of [redacted]

Parameter	Sludge I (Pratteln)	Sludge II (Füllinsdorf)	Sludge III (Sissach)
$\log(K_{des,F})$	1.63	2.05	2.17
$K_{des,F}$	42.9	111.4	148.1
$K_{des}oc$	108	342	475
$K_{des}om$	63	198	276
1/n	0.94	0.95	0.98
r^2	0.9997	0.9968	0.9990

$K_{des,F}$ = Freundlich desorption constant
$K_{des}oc$ = Desorption constant related to sludge organic carbon content
$K_{des}om$ = Desorption constant related to sludge organic matter content
1/n = Freundlich exponent
r^2 = Coefficient of determination

7 FIGURES

Figure 1: Determination of the adsorption plateau of [redacted] in sludges I (Pratteln), II (Füllinsdorf) and III (Sissach) in the preliminary test at different sludge-to-aqueous phase.



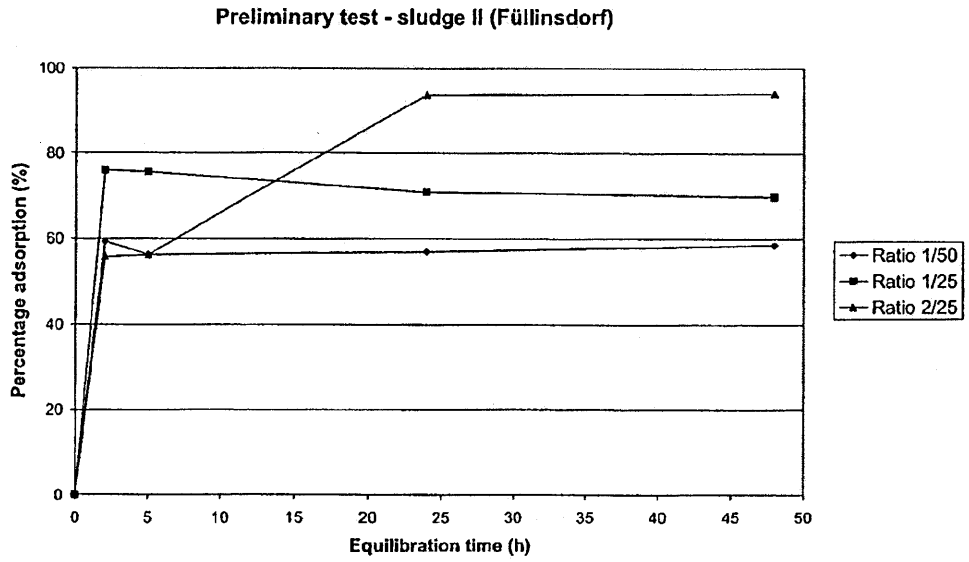


Figure 1 continued :

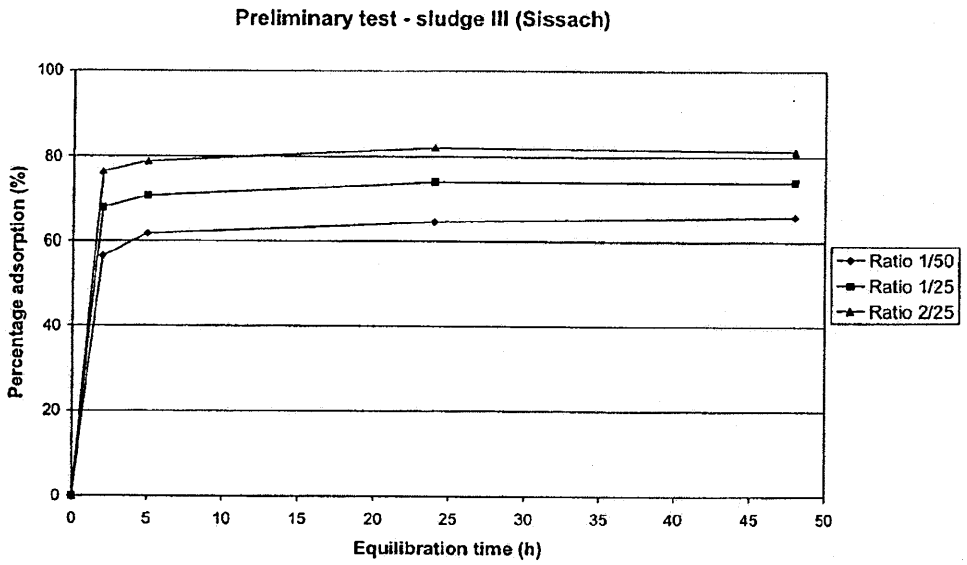


Figure 2: Percent radioactivity of [redacted]emaining in the sludge-less solution of the controls during the preliminary test.

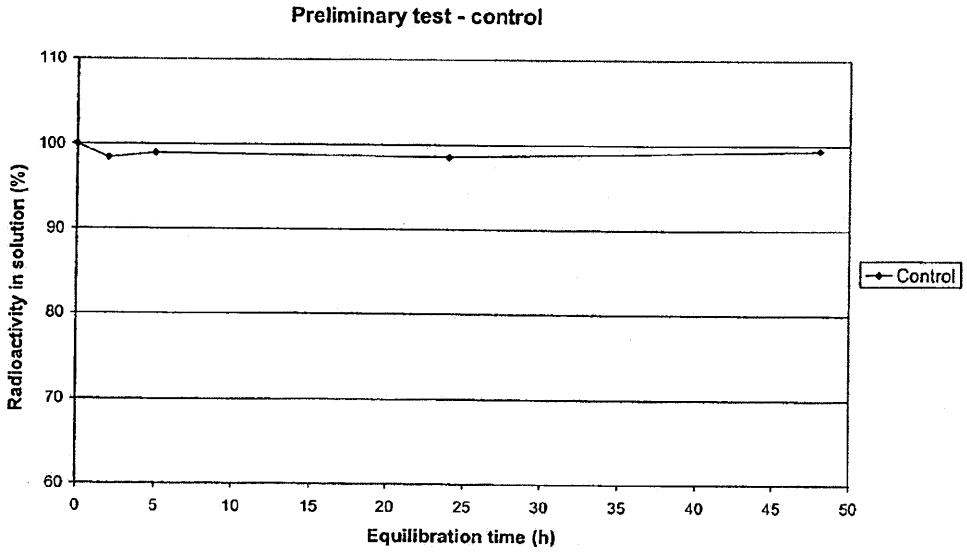


Figure 3: Nonlinear Freundlich adsorption isotherms of in the advanced test.

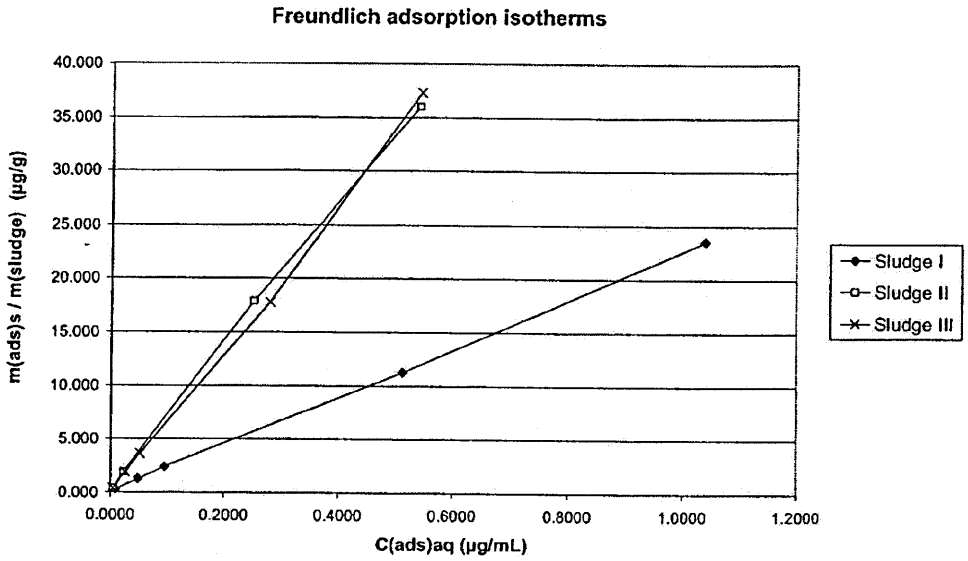
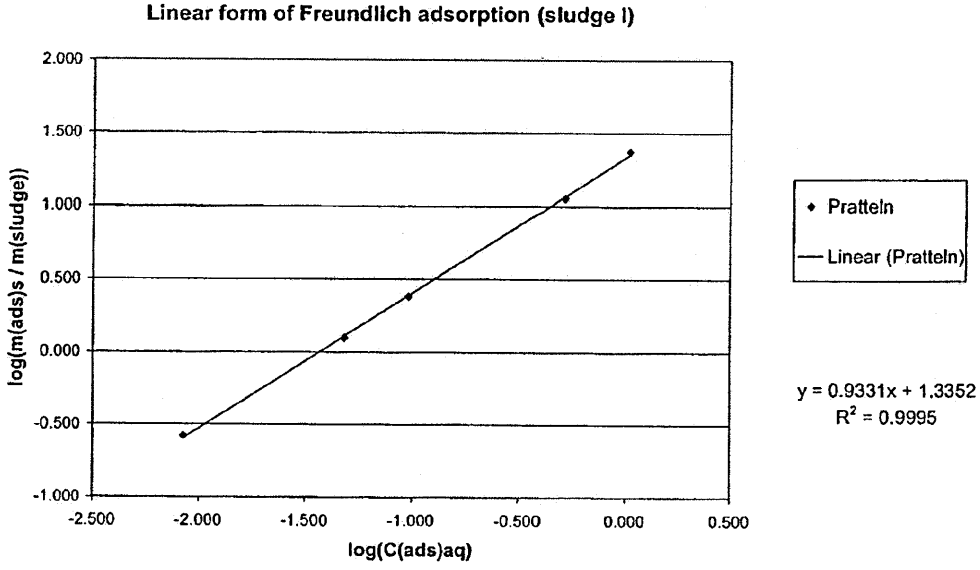
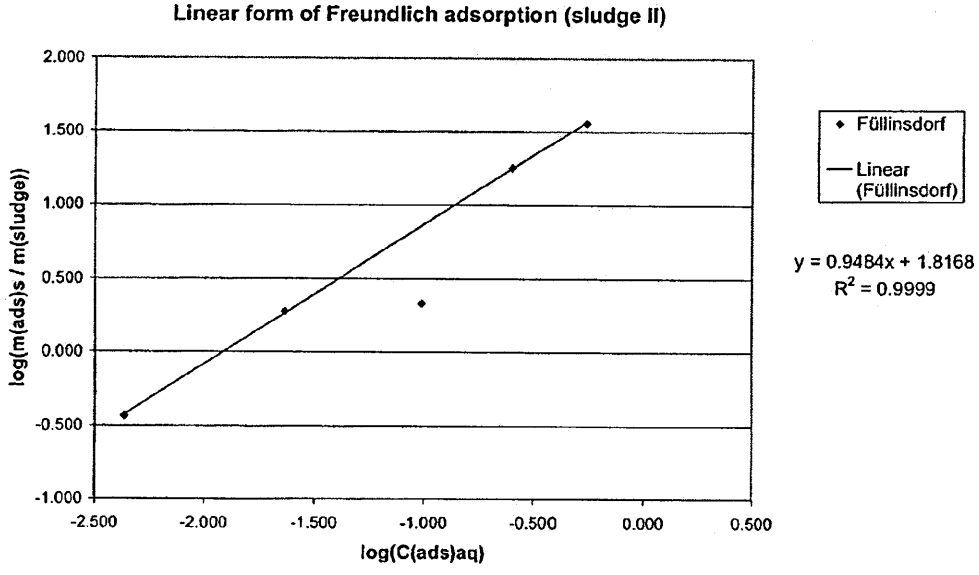


Figure 4: Linear form of the Freundlich adsorption isotherms of [redacted] in the advanced test for sludges I (Pratteln), II (Füllinsdorf) and III (Sissach).





Note: The point at the test concentration 0.20 µg/mL was not used for the Freundlich evaluation.

Figure 4 continued:

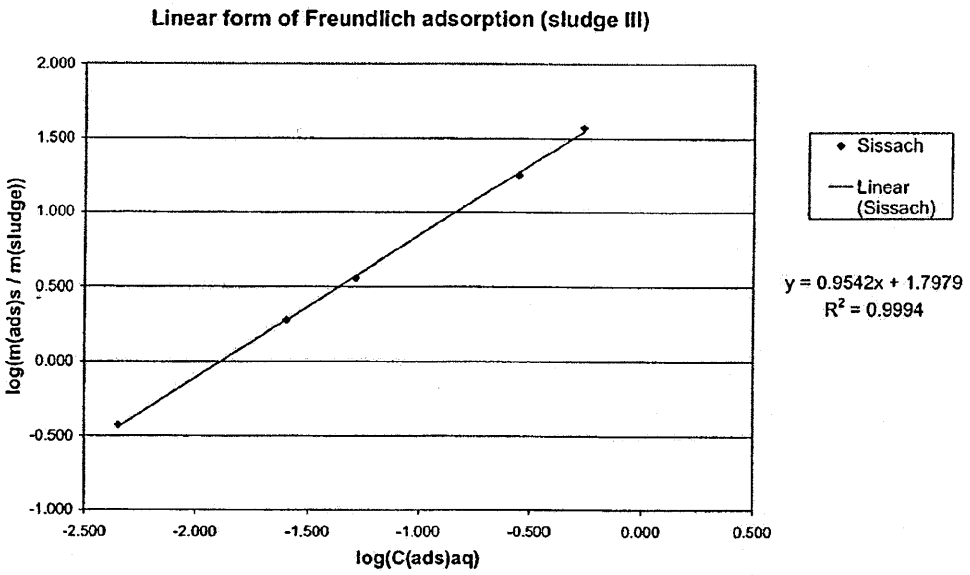


Figure 5: Determination of the desorption plateau of [redacted] adsorbed to sludges I (Pratteln), II (Füllinsdorf) and III (Sissach) at an initial concentration of 2.0 µg/mL.

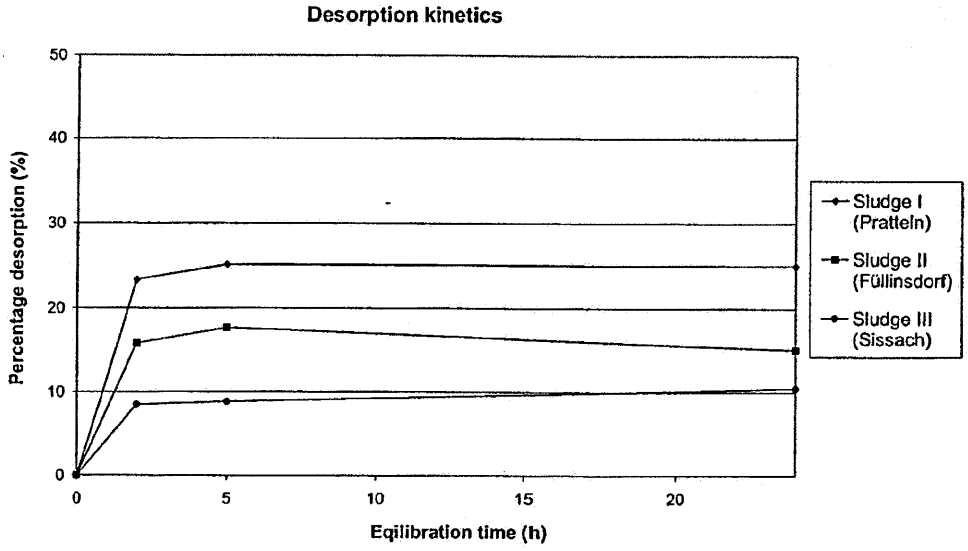


Figure 6: Nonlinear Freundlich desorption isotherms of [redacted] in the advanced test after 24 hours of desorption.

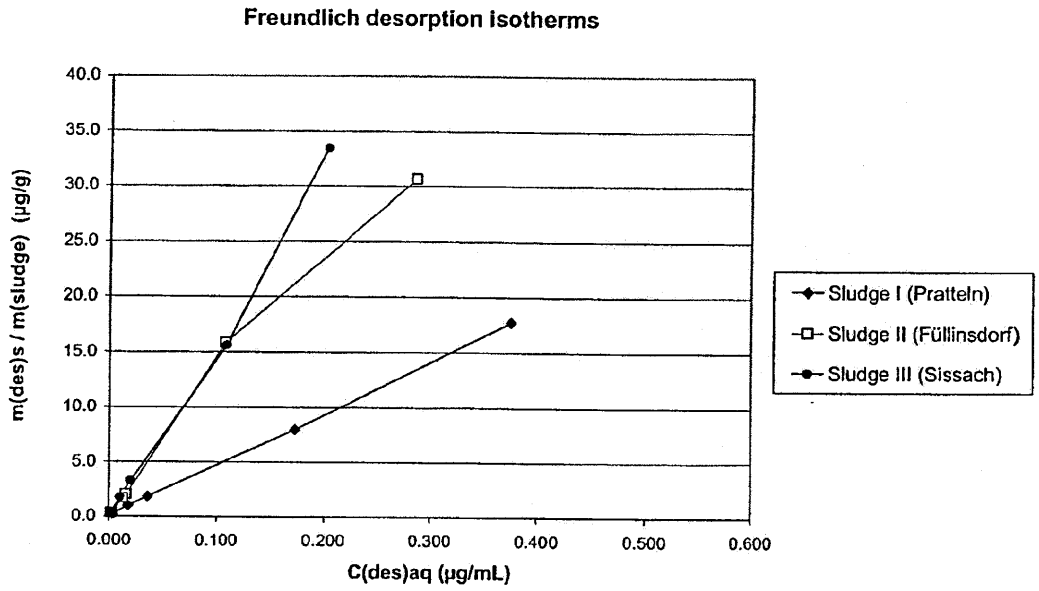
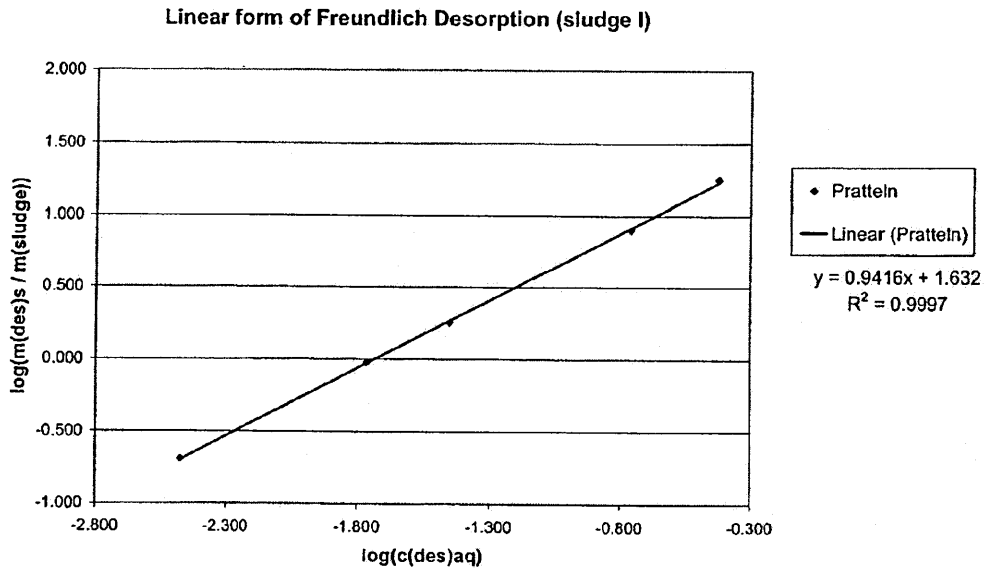


Figure 7: Linear form of the Freundlich desorption isotherms of [redacted] in the advanced test for Sludge I (Pratteln), II (Füllinsdorf) and III (Sissach).



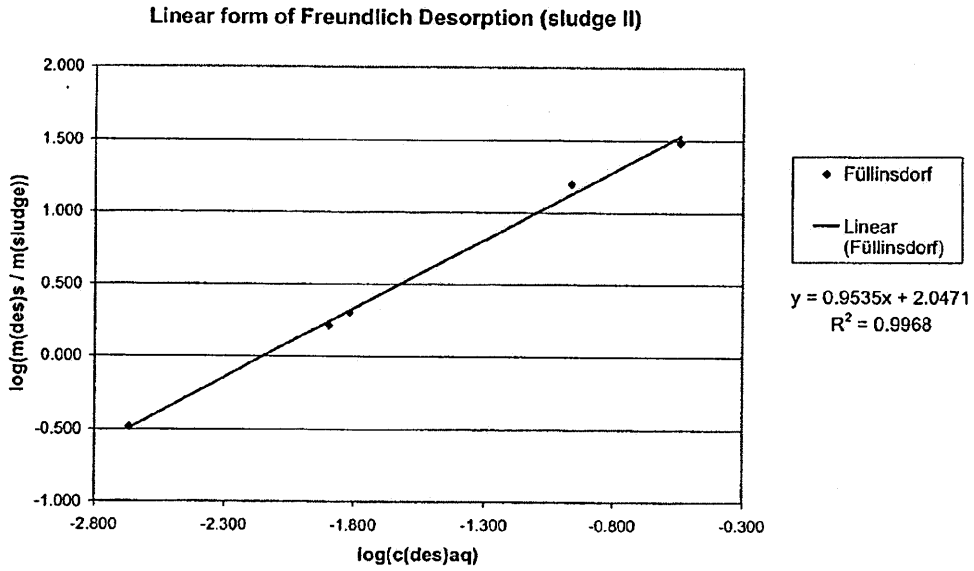


Figure 7 continued:

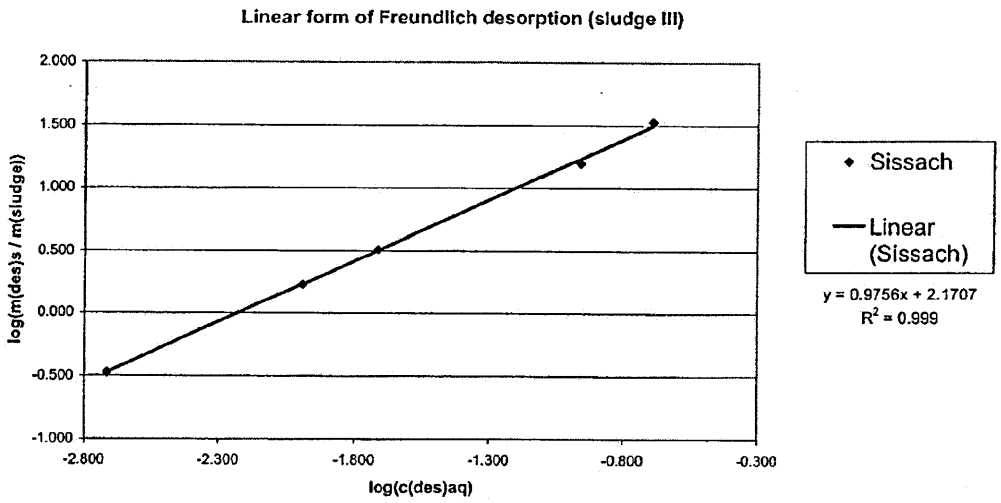
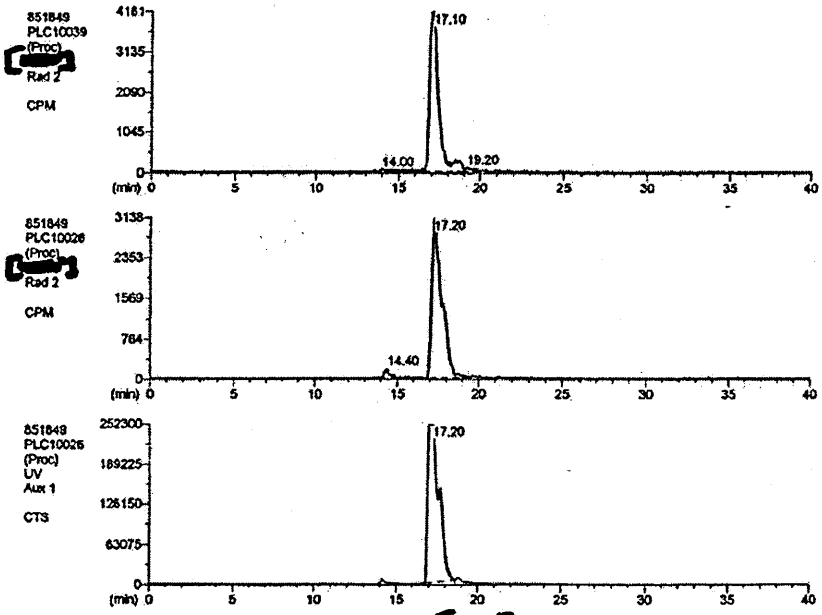


Figure 8: HPLC chromatograms for the determination of the radiochemical purity of the test item of the application solution for the advanced test (top) and of the control sample solution (middle) after 24 hours of adsorption. Co-chromatography with unlabelled test item (bottom).



Peak Report: PLC10039 Channel 2[redacted](CPM)

Pk #	Name	Ret. Time Mins	Net Area CPM	% Peaks	Height CPM
1		14.00	695	2.43	52
2		17.10	27463	95.84	4196
3		19.20	497	1.73	104
			28655		

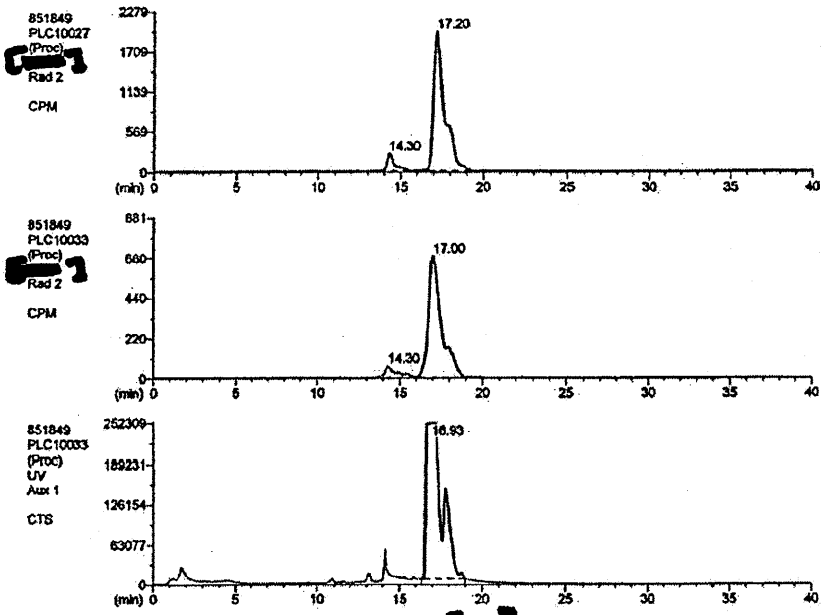
Total Run Area: 29689

Peak Report: PLC10026 Channel 2[redacted](CPM)

Pk #	Name	Ret. Time Mins	Net Area CPM	% Peaks	Height CPM
1		14.40	666	2.88	178
2		17.20	22439	97.12	3138
			23105		

Total Run Area: 23673

Figure 9: HPLC chromatograms of the supernatants of sludge I after 24 hours of adsorption (top) and 24 hours of desorption (middle) in the advanced test. Co-chromatography with reference item (bottom).



Peak Report: PLC10027 Channel 2[redacted] (CPM)

Pk #	Name	Ret. Time Mins	Net Area CPM	% Peaks	Height CPM
1		14.30	1185	7.20	236
2		17.20	15264	92.80	2000
			16449		

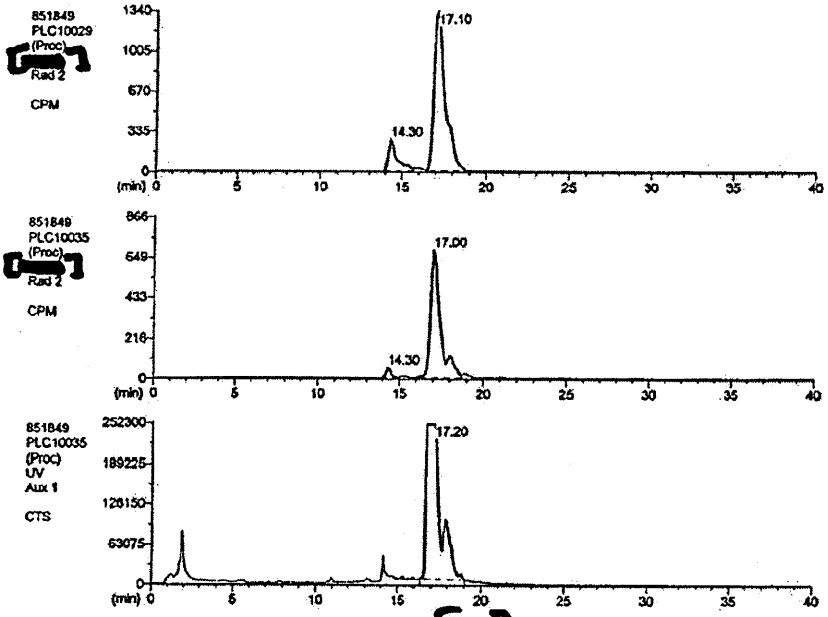
Total Run Area: 16557

Peak Report: PLC10033 Channel 2[redacted] (CPM)

Pk #	Name	Ret. Time Mins	Net Area CPM	% Peaks	Height CPM
1		14.30	460	6.89	61
2		17.00	6220	93.11	673
			6680		

Total Run Area: 6736

Figure 10: HPLC chromatograms of the supernatants of sludge II after 24 hours of adsorption (top) and 24 hours of desorption (middle) in the advanced test. Co-chromatography with reference item (bottom).



Peak Report: PLC10029 Channel 2 [redacted] (CPM)

Pk #	Name	Ret. Time Mins	Net Area CPM	% Peaks	Height CPM
1		14.30	1618	13.60	261
2		17.10	10275	86.40	1340
			11893		

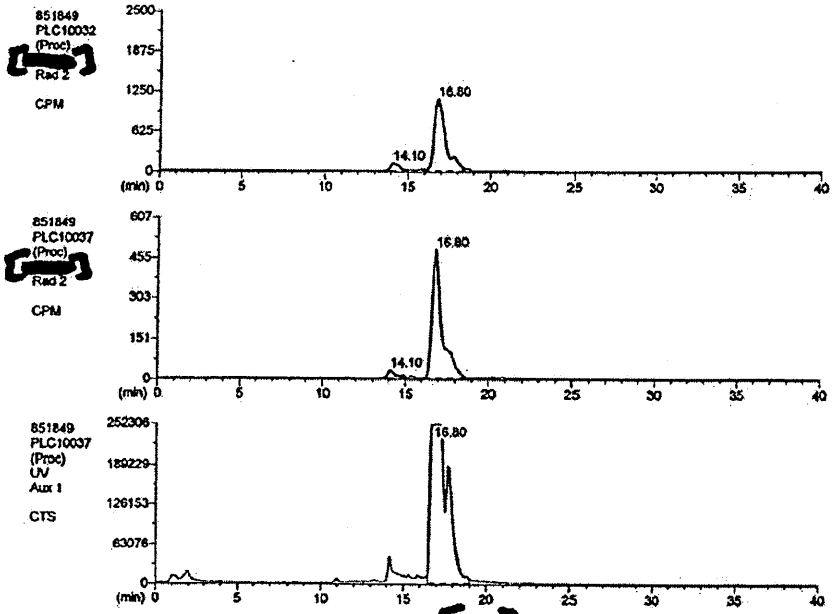
Total Run Area: 12026

Peak Report: PLC10035 Channel 2 [redacted] (CPM)

Pk #	Name	Ret. Time Mins	Net Area CPM	% Peaks	Height CPM
1		14.30	186	3.70	56
2		17.00	4842	96.30	694
			5028		

Total Run Area: 5234

Figure 11: HPLC chromatograms of the supernatants of sludge III after 24 hours of adsorption (top) and 24 hours of desorption (middle) in the advanced test. Co-chromatography with reference item (bottom).



Peak Report: PLC10032 Channel 2 [redacted] (CPM)

Pk #	Name	Ret. Time Mins	Net Area CPM	% Peaks	Height CPM
1		14.10	659	6.93	113
2		16.80	8852	93.07	1127
			9511		

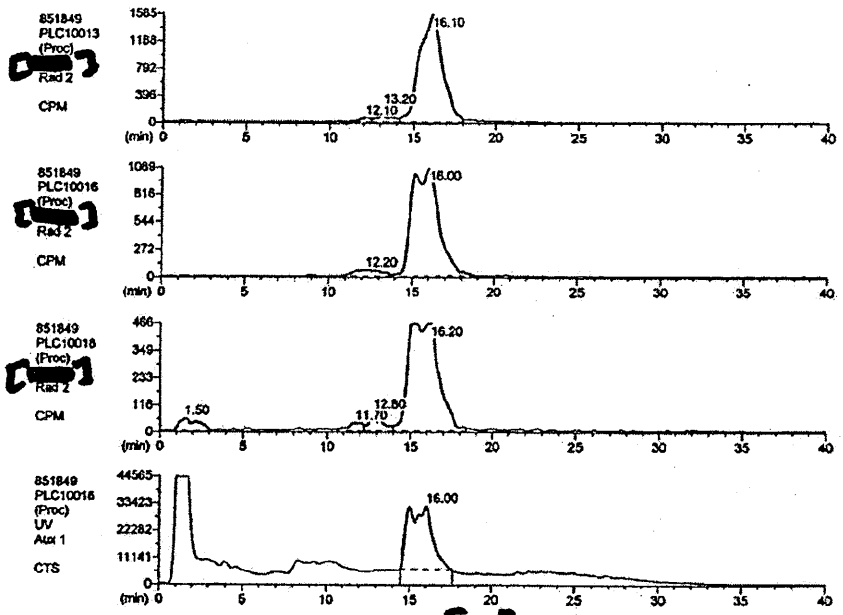
Total Run Area: 9817

Peak Report: PLC10037 Channel 2 [redacted] (CPM)

Pk #	Name	Ret. Time Mins	Net Area CPM	% Peaks	Height CPM
1		14.10	177	4.95	30
2		16.80	3400	95.05	488
			3577		

Total Run Area: 3646

Figure 12: HPLC chromatogram of the organic solvent extracts of Sludge I (top), Sludge II (upper middle) and Sludge III (lower middle) in the preliminary test. Co-chromatography with reference item (bottom).



Peak Report: PLC10013 Channel 2 [redacted] (CPM)

Pk #	Name	Ret. Time Mins	Net Area CPM	% Peaks	Height CPM
1		12.10	525	1.90	64
2		13.20	1099	3.98	88
3		16.10	25995	94.12	1601
			27619		

Total Run Area: 29378

Peak Report: PLC10016 Channel 2 [redacted] (CPM)

Pk #	Name	Ret. Time Mins	Net Area CPM	% Peaks	Height CPM
1		12.20	1130	5.20	60
2		16.00	20605	94.80	1098
			21735		

Total Run Area: 23117

Peak Report: PLC10018 Channel 2 [redacted] (CPM)

Pk #	Name	Ret. Time Mins	Net Area CPM	% Peaks	Height CPM
1		1.50	713	5.95	56
2		11.70	384	3.21	36
3		12.80	617	5.15	48
4		16.20	10260	85.69	482
			11974		

Total Run Area: 12946

U.S. Customers

[redacted]