SafePharm Laboratories

PFHA:

ACUTE TOXICITY TO *DAPHNIA MAGNA*

SPL PROJECT NUMBER: 1742/019

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QUALITY ASSURANCE REPORT

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

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

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

	14 May 2003	Standard Test Method Compliance Audit
	26 July 2004	Test Material Preparation
	27 July 2004	Test System Preparation
	28 July 2004	Exposure
	22 July 2004	Assessment of Response
	15, 19 July 2004	Chemical Analysis
§	03 September 2004	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: 16 DEC 2004

For Safepharm Quality Assurance Unit*

***Authorised QA Signatures:**

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Senior Audit Staff:	JV Johnson BSc MRQA; G Wren ONC MRQA

GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). 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: 16 DEC 2004

T J Goodband BSc
Study Director

The analytical data presented in this report were compiled by me or under my supervision and accurately reflect the data obtained.

..... Date: 16 DEC 2004

Dr J McKenzie CChem MRSC
Head of Analytical Services

CONTENTS

PFHA:

ACUTE TOXICITY TO *DAPHNIA MAGNA*

SUMMARY

Introduction. A study was performed to assess the acute toxicity of the test material to *Daphnia magna*. The method followed that described in the OECD Guidelines for Testing of Chemicals (1984) No 202, "*Daphnia* sp, Acute Immobilisation Test and Reproduction Test" referenced as Method C.2 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

Methods. Following a preliminary range-finding test, twenty daphnids (2 replicates of 10 animals) were exposed to an aqueous solution of the test material at concentrations of 1.0, 1.8, 3.2, 5.6, 10, 18, 32, 56 and 100 mg/l for 48 hours at a temperature of 21.3°C to 22.3°C under static test conditions. The number of immobilised *Daphnia* were recorded after 24 and 48 hours.

Results. The 48-Hour EC_{50} for the test material to *Daphnia magna* based on nominal test concentrations was greater than 100 mg/l and correspondingly the No Observed Effect Concentration was 100 mg/l.

Analysis of the test preparations at 0 and 48 hours showed measured test concentrations to range from 94% to 105% of nominal value and so the results are based on nominal test concentrations only.

PFHA:

ACUTE TOXICITY TO *DAPHNIA MAGNA*

1. INTRODUCTION

This report contains a description of the methods used and results obtained during a study to investigate the acute toxicity of the test material to *Daphnia magna*. The method followed the recommendations of the OECD Guidelines for Testing of Chemicals (1984) No 202 "*Daphnia* sp, Acute Immobilisation Test and Reproduction Test" referenced as Method C.2 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

Daphnia magna is a freshwater invertebrate representative of a wide variety of natural habitats, and can therefore be considered as an important non-target organism in freshwater ecosystems.

The initial range-finding test was conducted between 28 April 2004 and 30 April 2004, the definitive test between 26 July 2004 and 28 July 2004 and the post test range-finding test between 29 July 2004 and 31 July 2004.

2. TEST MATERIAL AND EXPERIMENTAL PREPARATION

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	PFHA
Description	:	colourless liquid
Chemical name	:	perfluorohexanoic acid
Purity	:	99%
Lot number	:	C15009601
Date received	:	12 January 2004
Storage conditions	:	room temperature in the dark

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor.

2.2 Experimental Preparation

For the purpose of the definitive test the test material was dissolved directly in reconstituted water.

An amount of test material (200 mg) was dissolved in reconstituted water and the volume adjusted to 2 litres to give the 100 mg/l test concentration. Aliquots (10, 18, 32, 56, 100, 180, 320 and 560 ml) of the 100 mg/l test concentration were each separately dispersed in a final volume of 1 litre of reconstituted water to give the remainder of the test concentrations of 1.0, 1.8, 3.2, 5.6, 10, 18, 32 and 56 mg/l respectively.

Each prepared concentration was inverted several times to ensure adequate mixing and homogeneity.

The concentration and stability of the test material in the test preparations were verified by chemical analysis at 0 and 48 hours (see Appendix 1).

3. METHODS

3.1 Test Species

The test was carried out using 1st instar *Daphnia magna* derived from in-house laboratory cultures.

Adult *Daphnia* were maintained in polypropylene vessels containing approximately 2 litres of reconstituted water in a temperature controlled room at approximately 21°C. The lighting cycle was controlled to give a 16 hours light and 8 hours darkness cycle with 20 minute dawn and dusk transition periods. Each culture was fed daily with a suspension of algae (*Chlorella* sp.). Culture conditions ensured that reproduction was by parthenogenesis. Gravid adults were isolated the day before initiation of the test, such that the young daphnids produced overnight were less than 24 hours old. These young were removed from the cultures and used for testing. The diet and diluent water are considered not to contain any contaminant that would affect the integrity or outcome of the study.

3.2 Test Water

The reconstituted water used for both the range-finding and definitive tests was the same as that used to maintain the stock animals.

The reconstituted water is defined in Appendix 2.

3.3 Procedure

3.3.1 Initial Range-finding test

The test concentrations to be used in the definitive test were determined by a preliminary range-finding test.

In the initial range-finding test *Daphnia magna* were exposed to a series of nominal test concentrations of 0.010, 0.10, 1.0, 10 and 100 mg/l. The test material was dissolved directly in water.

An amount of test material (100 mg) was dissolved in reconstituted water with the aid of shaking by hand for approximately 1 minute and the volume adjusted to 1 litre to give the 100 mg/l test concentration from which serial dilutions were made to give the remainder of the test series of 10, 1.0, 0.10 and 0.010 mg/l.

Each prepared concentration was inverted several times to ensure adequate mixing and homogeneity.

In the range-finding test 10 daphnids were placed in each test and control vessel and maintained in a temperature controlled room at approximately 21°C with a photoperiod of 16 hours light and 8 hours darkness for a period of 48 hours with 20 minute dawn and dusk transition periods. Each 250 ml test and control vessel contained 200 ml of test media and was covered to reduce evaporation. After 24 and 48 hours the number of immobilised *Daphnia magna* were recorded.

The control group was maintained under identical conditions but not exposed to the test material.

3.3.2 Definitive test

Based on the results of the range-finding test the following test concentrations were assigned to the definitive test: 1.0, 1.8, 3.2, 5.6, 10, 18, 32, 56 and 100 mg/l.

3.3.2.1 Preparation of the test material

For the purpose of the definitive test the required amount of test material was added to each test vessel using the method described in Section 2.2.

3.3.2.2 Exposure conditions

As in the range-finding test 250 ml glass jars containing approximately 200 ml of test preparation were used. At the start of the study 10 daphnids were placed in each test and control vessel at random, in the test preparations. Duplicate test vessels were used for each test and control group. The test vessels were then covered to reduce evaporation and maintained in a temperature controlled room at 21.3°C to 22.3°C with a photoperiod of 16 hours light and 8 hours darkness with 20 minute dawn and dusk transition periods. The daphnids were not individually identified, received no food during exposure and the test vessels were not aerated.

The control group was maintained under identical conditions but not exposed to the test material.

The test preparations were not renewed during the exposure period. Any immobilisation or adverse reactions to exposure were recorded at 24 and 48 hours after the start of exposure. The criterion of effect used was that *Daphnia* were considered to be immobilised if they were unable to swim for approximately 15 seconds after gentle agitation.

3.3.2.3 Physico-chemical measurements

Water temperature was recorded daily throughout the test. Dissolved oxygen concentrations and pH were recorded at the start and termination of the test. The pH and dissolved oxygen concentration were measured using a WTW pH/Oxi 340I pH and dissolved oxygen meter and the temperature was measured using a Hanna Instruments HI 93510 digital thermometer.

3.3.2.4 Verification of test concentrations

Water samples were taken from the control, 1.0, 3.2, 10, 32 and 100 mg/l test groups (replicates $R_1 - R_2$ pooled) at 0 and 48 hours for quantitative analysis.

Duplicate samples and samples of the 1.8, 5.6, 18 and 56 mg/l test groups were taken and stored at approximately -20°C for further analysis if necessary.

The method of analysis, stability, recovery and test preparation analyses are described in Appendix 1.

3.3.2.5 Evaluation of data

An estimate of the EC_{50} values was given by inspection of the immobilisation data.

3.3.3 Post Test Range-finding test

As no immobilisation was observed in the definitive test a final range-finding test was conducted to confirm the results of the definitive test.

In the final range-finding test *Daphnia magna* were exposed to a series of nominal test concentrations of 1.0, 10 and 100 mg/l. The test material was dissolved directly in water.

An amount of test material (100 mg) was dissolved in reconstituted water and the volume adjusted to 1 litre to give the 100 mg/l test concentration from which serial dilutions were made to give the remainder of the test series of 10 and 1.0 mg/l.

Each prepared concentration was inverted several times to ensure adequate mixing and homogeneity.

In the final range-finding test 10 daphnids were placed in each test and control vessel and maintained in a temperature controlled room at 21.2°C to 22.9°C with a photoperiod of 16 hours light and 8 hours darkness for a period of 48 hours with 20 minute dawn and dusk transition periods. Some of the water temperatures at 0 hours were shown to be outside of the temperature range specified in the protocol of 21 ± 1°C. This slight deviation was considered not to have adversely affected the outcome of the test as no immobilisation or sub-lethal effects of exposure were observed in the control animals.

Each 250 ml test and control vessel contained 200 ml of test medium and was covered to reduce evaporation. After 24 and 48 hours the number of immobilised *Daphnia magna* were recorded.

The control group was maintained under identical conditions but not exposed to the test material.

4. ARCHIVES

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

5. RESULTS

5.1 Initial Range-finding Test

Cumulative immobilisation data from the exposure of *Daphnia magna* to the test material during the initial range-finding test are given in Table 1.

No immobilisation was observed at the test concentrations of 0.010, 0.10 and 1.0 mg/l. However, immobilisation was observed at 10 and 100 mg/l.

Based on this information test concentrations of 1.0, 1.8, 3.2, 5.6, 10, 18, 32, 56 and 100 mg/l were selected for the definitive test.

5.2 Definitive Test

5.2.1 Immobilisation data

Cumulative immobilisation data from the exposure of *Daphnia magna* to the test material during the definitive test are given in Table 2.

Inspection of the immobilisation data gave the following results:

Time (h)	EC_{50} (mg/l)	95% Confidence limits (mg/l)
24	> 100	-
48	> 100	-

The No Observed Effect Concentration after 24 and 48 hours exposure was 100 mg/l. The No Observed Effect Concentration is based upon zero immobilisation at this concentration.

The results observed in the definitive test differed from those of the initial range-finding test in that no immobilisation was observed in any test concentration, whilst 60% and 100% immobilisation was observed after 24 and 48 hours at 10 and 100 mg/l respectively in the initial range-finding test. To confirm the results of the definitive test a final range-finding test was conducted which showed no immobilisation at 100 mg/l. Therefore, the immobilisation in the initial range-finding test was considered to be due to possible contamination of the test vessels or an incorrect preparation of the test media. Since the solutions used in the definitive test were analysed and confirmed the correct test media preparation (see Section 5.2.5) and no immobilisation was observed these data are considered valid.

5.2.2 Observations on test material solubility

The test preparations were observed to be clear, colourless solutions throughout the duration of the test.

5.2.3 Physico-chemical measurements

The results of the physico-chemical measurements are given in Appendix 3. Temperature was maintained at 21.3°C to 22.3°C throughout the test. While there were no treatment related differences for oxygen concentration, concentration dependent differences in pH were observed throughout the test.

Some of the water temperatures at 48 hours during the definitive test were shown to be outside of the temperature range specified in the protocol of 21 ± 1°C. This slight deviation was considered not to have adversely affected the outcome of the test as no immobilisation or sub-lethal effects of exposure were observed in the control animals and that the deviation was only 0.3°C.

5.2.4 Verification of test concentrations

Analysis of the test preparations at 0 and 48 hours (see Appendix 1) showed measured test concentrations to range from 94% to 105% of nominal value and so it was considered justifiable to estimate the EC_{50} values in terms of the nominal test concentrations only.

5.3 Post Test Range-finding Test

Cumulative immobilisation data from the exposure of *Daphnia magna* to the test material during the final range-finding test are given in Table 3.

No immobilisation was observed at the test concentrations of 1.0, 10 and 100 mg/l.

These results confirmed the results of the definitive test.

6. CONCLUSION

The acute toxicity of the test material to the freshwater invertebrate *Daphnia magna* has been investigated and gave a 48-Hour EC_{50} of greater than 100 mg/l. Correspondingly the No Observed Effect Concentration was 100 mg/l.

PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Table 1 Cumulative Immobilisation Data in the Initial Range-finding Test

Nominal Concentration (mg/l)	Cumulative Immobilised *Daphnia* (Initial Population: 10 Per Replicate)	
	24 Hours	48 Hours
Control	0	0
0.010	0	0
0.10	0	0
1.0	0	0
10	6	6
100	10	10

PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Table 2 Cumulative Immobilisation Data in the Definitive Test

Nominal Concentration (mg/l)	Cumulative Immobilised *Daphnia* (Initial Population: 10 Per Replicate)							
	24 Hours				48 Hours			
	R_1	R_2	Total	%	R_1	R_2	Total	%
Control	0	0	0	0	0	0	0	0
1.0	0	0	0	0	0	0	0	0
1.8	0	0	0	0	0	0	0	0
3.2	0	0	0	0	0	0	0	0
5.6	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0

$R_1 - R_2$ = Replicates 1 and 2

PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Table 3 Cumulative Immobilisation Data in the Post Test Range-finding Test

Nominal Concentration (mg/l)	Cumulative Immobilised *Daphnia* (Initial Population: 10 Per Replicate)	
	24 Hours	48 Hours
Control	0	0
1.0	0	0
10	0	0
100	0	0

PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 Verification of Test Concentrations

1. METHOD OF ANALYSIS

1.1 Introduction

The test material concentration in the test samples was determined by high performance liquid chromatography (HPLC) using an external standard. The test material gave a chromatographic profile consisting of a single peak.

The method was developed by the Department of Analytical Services, Safepharm Laboratories Limited.

1.2 Sample Preparation

A volume of test sample was diluted with methanol to give a final theoretical concentration of between 0.5 and 1.0 mg/l.

1.3 Standards

Standard solutions of test material were prepared in methanol at a nominal concentration of 1.0 mg/l.

1.4 Procedure

The standards and samples were analysed by HPLC using the following conditions:

HPLC System	:	Agilent Technologies 1100 MSD, incorporating autosampler and workstation
Mass selective detector		
Source	:	electrospray
Fragmentation energy	:	50 volts
Polarity	:	negative
Mode	:	single ion mode with 269 amu, 313 amu and 314 amu
Gas temperature	:	275°C

Appendix 1 (continued) Verification of Test Concentrations

Drying gas	:	11 litre/minute
Nebuliser pressure	:	40 psi
Capillary voltage	:	2000 volts
Gain	:	1
Column	:	Luna C18, 5 µ, (250 x 4.6 mm id)
Column temperature	:	30°C
Mobile phase	:	methanol:0.1% ammonium carbonate (90:10, v/v)
Flow rate	:	0.5 ml/min
Injection volume	:	5 µl
Retention time	:	approximately 5 minutes

2. VALIDATION

2.1 Linearity

A range of standard solutions covering 0.1 to 2 mg/l (10% to 200% of the standard concentration) was analysed.

Linearity was confirmed (correlation factor, $R^2 = 0.9967$) ranging from 0 to 2 mg/l.

The results are presented graphically on page 18.

2.2 Recoveries

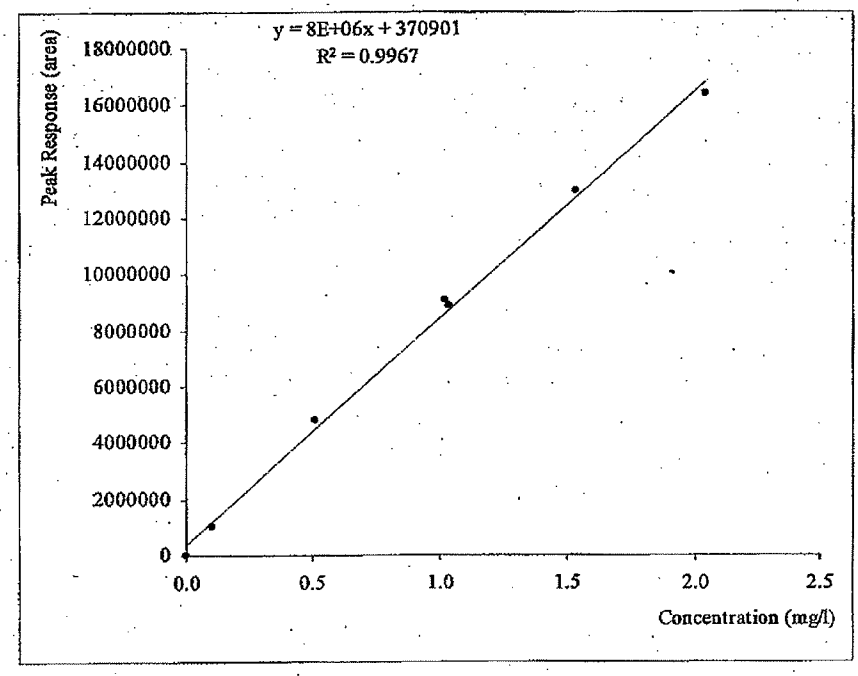
A range of preliminary test samples, accurately fortified at known concentrations of test material, was prepared and analysed.

The recovery samples were prepared by direct addition of the test material to a sample of test medium.

PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations

Linearity of Detector Response



PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations

Fortification (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
1.47	1.41	96	93
1.47	1.40	96	
14.7	13.4	92	
14.7	13.2	90	
147	134	91	
147	134	92	

The method has been considered to be sufficiently accurate for the purposes of this test. The test sample results have not been corrected for recovery.

2.3 Limit of Quantitation

The limit of quantitation has been assessed down to 0.011 mg/l.

3. STABILITY

A range of preliminary test samples was prepared, analysed initially and then after storage in sealed glass vessels at ambient temperature in light and dark conditions for approximately 48 hours (equivalent to the test exposure period). In addition a test sample was tested for stability without prior mixing (sonication) of the test sample bottle to assess for losses due to adsorption and/or insolubility.

Nominal concentration (mg/l)	1.0	10	100
Concentration found initially (mg/l)	1.40	13.3	134
Concentration found after storage in light conditions (mg/l)	1.44	12.9	133
Expressed as a percent of the initial concentration	102	97	99
Concentration found after storage in dark conditions (mg/l)	1.43	12.8	130
Expressed as a percent of the initial concentration	102	96	97
Concentration found after storage in dark conditions (mg/l) – unsonicated sample	1.36	NA	133
Expressed as a percent of the initial concentration	97	-	99

NA = Not applicable

PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations

The test samples have been shown to be stable in the test medium.

The unsonicated stability vessel showed no evidence of insolubility or adherence to glass.

4. RESULTS

Sample	Nominal Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percent of the Nominal Concentration (%)
0 Hours	Control	<LOQ	-
	1.0	0.997	100
	3.2	3.11	97
	10	10.5	105
	32	32.9	103
	100	102	102
48 Hours	Control	<LOQ	-
	1.0	0.942	94
	3.2	3.04	95
	10	9.97	100
	32	31.5	98
	100	97.6	98

5. DISCUSSION

The detection system was found to have acceptable linearity. The analytical procedure had acceptable recoveries of test material in test medium. A method of analysis was validated and proven to be suitable for use.

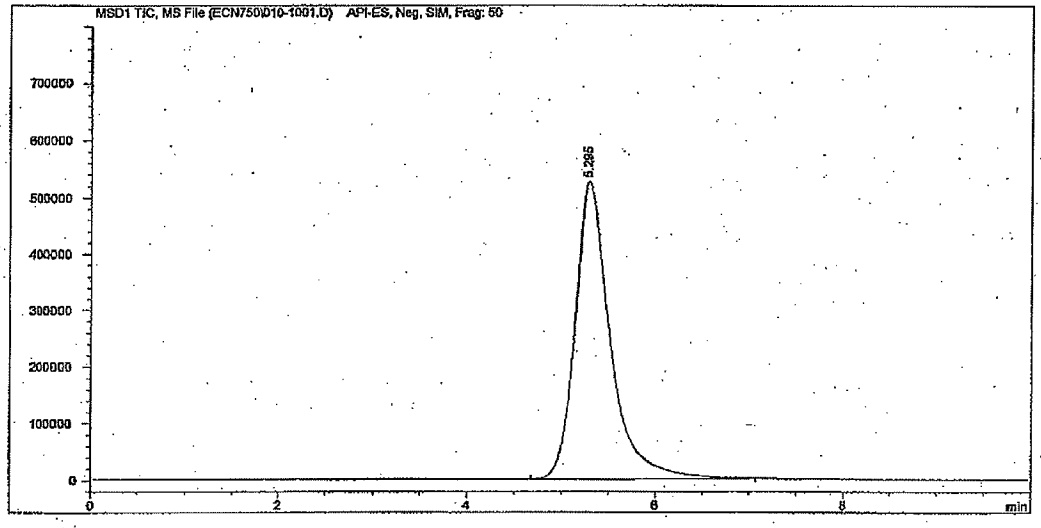
The test material was stable in the test medium for the duration of the test.

LOQ = Limit of quantitation

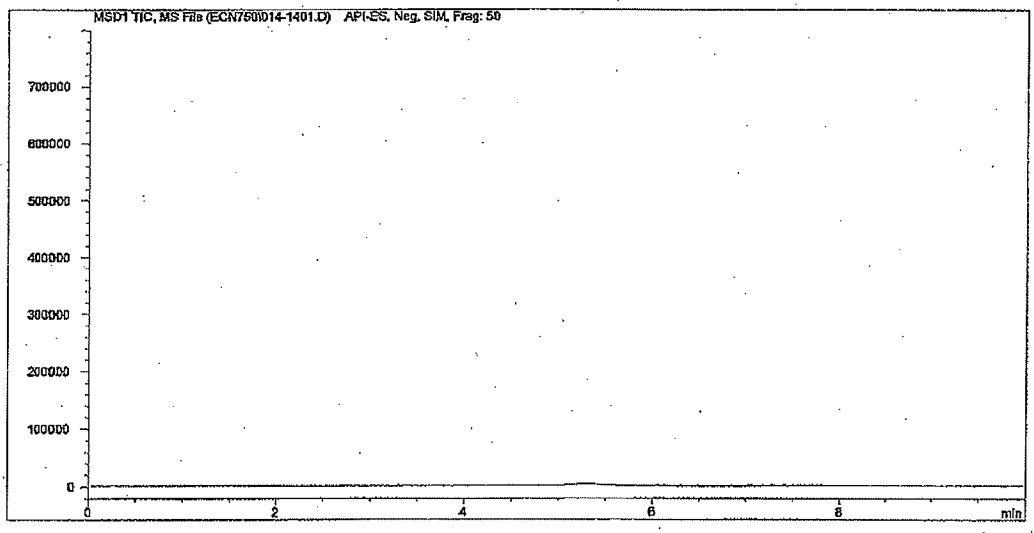
PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations

6. TYPICAL CHROMATOGRAPHY



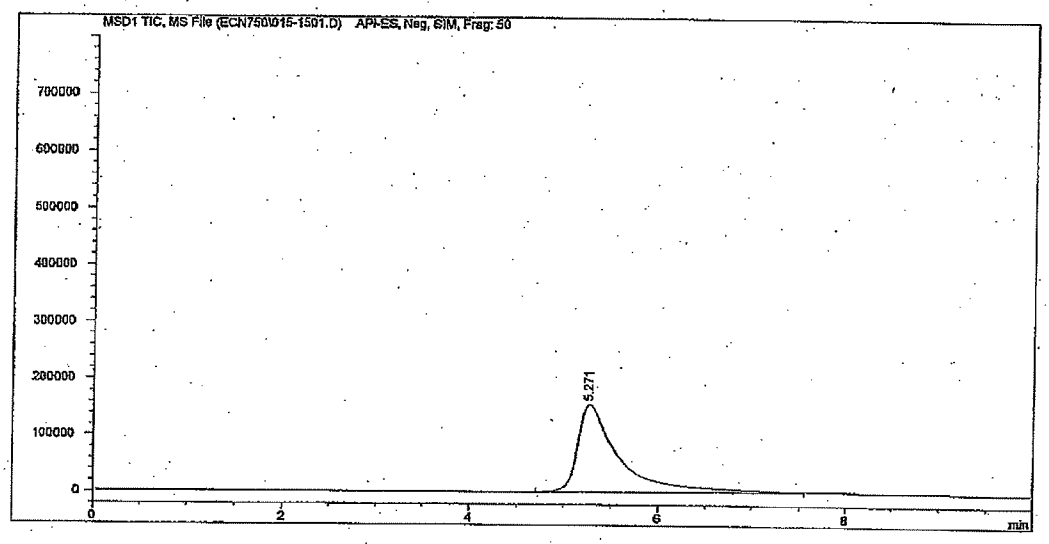
Standard 1.0 mg/l



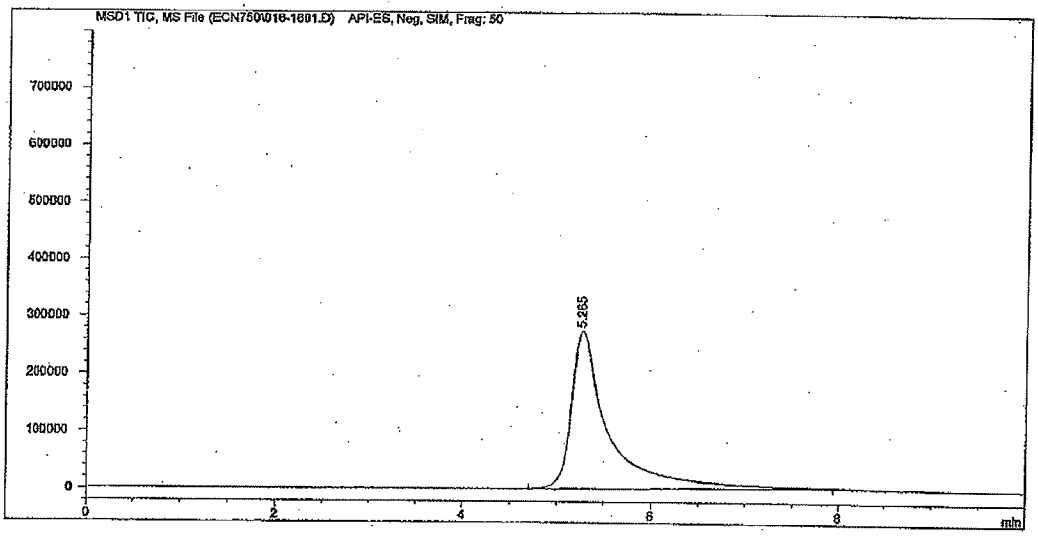
Control Sample

PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations



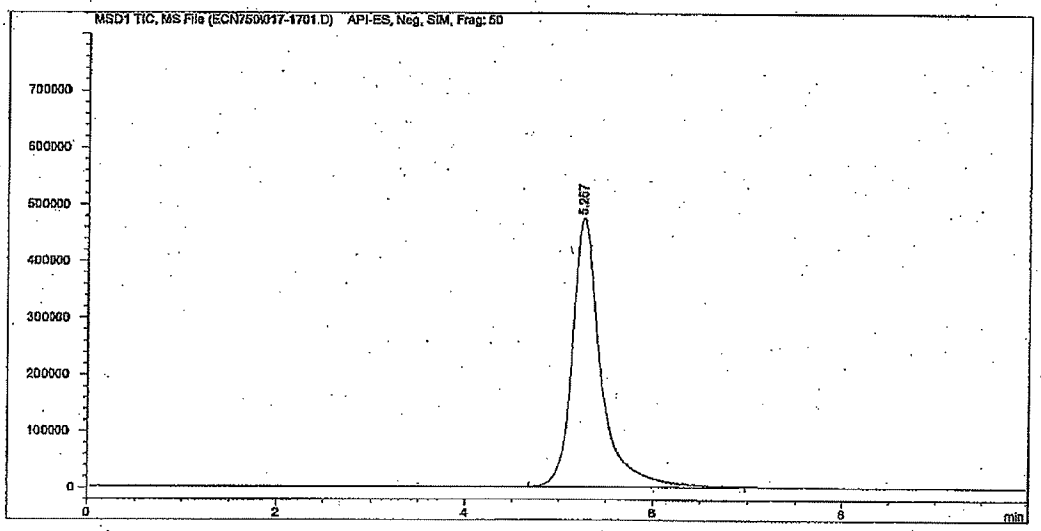
Test Sample 1.0 mg/l



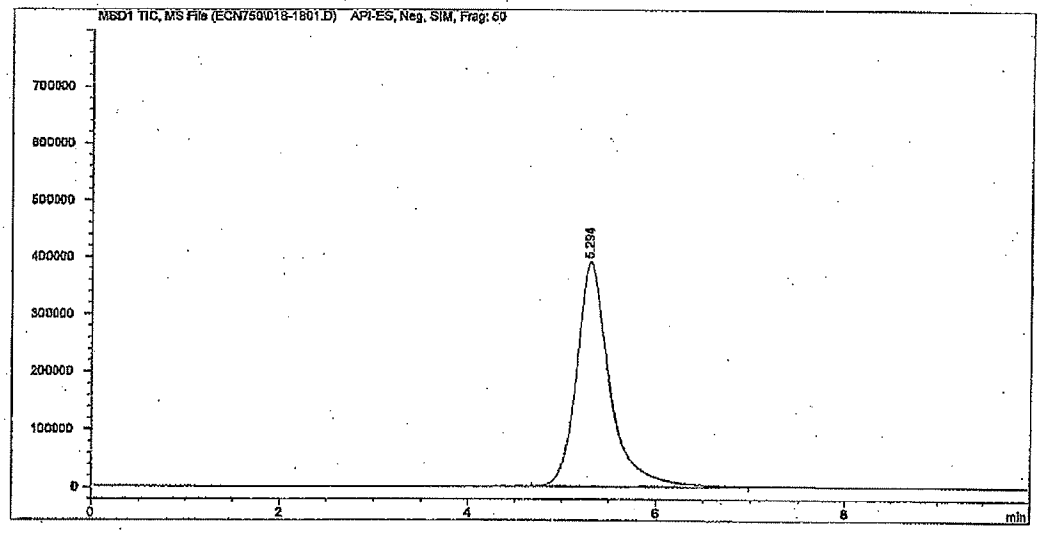
Test Sample 3.2 mg/l

PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations



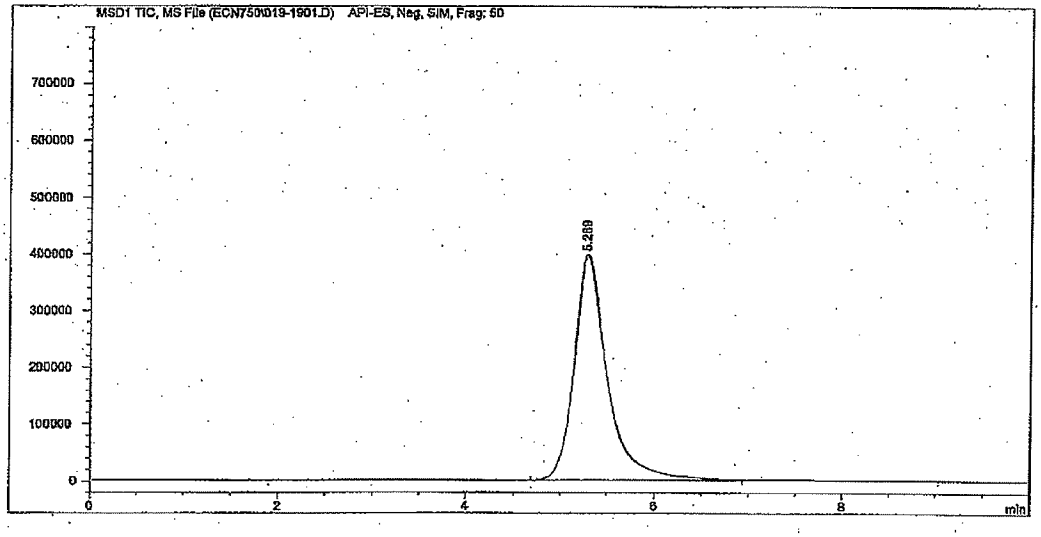
Test Sample 10 mg/l



Test Sample 32 mg/l

PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations



Test Sample 100 mg/l

PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 2 Reconstituted Water

i) Stock Solutions

a) $CaCl_2.2H_2O$	11.76 g/l
b) $MgSO_4.7H_2O$	4.93 g/l
c) $NaHCO_3$	2.59 g/l
d) KCl	0.23 g/l

ii) Preparation

An aliquot (25 ml) of each of solutions a-d was added to each litre (final volume) of deionised water with a conductivity of <5 $\mu S\ cm^{-1}$. The reconstituted water had a pH of 7.8 ± 0.2 adjusted (if necessary) with NaOH or HCl and was aerated until the dissolved oxygen concentration was approximately air-saturation value.

The reconstituted water had an approximate theoretical total hardness of 250 mg/l as $CaCO_3$.

PFHA: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 3 Physico-Chemical Measurements

Nominal Concentration (mg/l)		0 Hours				24 Hours	48 Hours			
		pH	mg O_2/l	%ASV*	T°C	T°C	pH	mg O_2/l	%ASV*	T°C
Control	R_1	7.8	8.4	94	21.3	21.8	8.0	8.6	99	22.3
	R_2	7.8	8.4	94	21.4	21.8	8.0	8.6	99	22.2
1.0	R_1	8.0	8.5	98	22.0	21.7	8.0	8.7	100	22.3
	R_2	8.0	8.5	98	22.0	21.7	8.0	8.7	100	22.3
1.8	R_1	8.0	8.5	98	22.0	21.7	8.0	8.6	99	22.2
	R_2	7.9	8.5	98	22.0	21.7	8.0	8.6	99	22.1
3.2	R_1	7.9	8.5	98	22.0	21.6	8.0	8.7	100	22.1
	R_2	7.9	8.5	98	22.0	21.6	8.0	8.6	99	22.1
5.6	R_1	7.8	8.5	98	22.0	21.5	8.0	8.7	100	21.9
	R_2	7.8	8.5	98	22.0	21.5	8.0	8.7	100	22.0
10	R_1	7.8	8.5	98	22.0	21.7	7.9	8.7	100	22.3
	R_2	7.7	8.5	98	22.0	21.7	7.9	8.7	100	22.3
18	R_1	7.7	8.5	98	21.9	21.6	7.9	8.7	100	22.1
	R_2	7.7	8.5	98	21.9	21.6	7.9	8.6	99	22.1
32	R_1	7.6	8.5	98	21.9	21.6	7.8	8.6	99	22.1
	R_2	7.6	8.5	98	22.0	21.6	7.8	8.6	99	22.0
56	R_1	7.4	8.5	98	22.0	21.5	7.8	8.7	100	21.9
	R_2	7.4	8.5	98	22.0	21.5	7.7	8.7	100	22.0
100	R_1	7.1	8.5	98	22.0	21.6	7.6	8.6	99	21.9
	R_2	7.1	8.5	98	22.0	21.6	7.7	8.6	99	22.0

*ASV = Dissolved oxygen concentration expressed as a percentage of Air Saturation Value
R_1 - R_2 = Replicates 1 and 2

Appendix 4 Statement of GLP Compliance in Accordance with Directive 88/320/EEC

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY
SafePharm Limited
Shardlow Business Park,
London Road,
Shardlow,
Derbyshire,
DE72 2GD

TEST TYPE
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