SafePharm Laboratories

C6-2AL:

ACUTE TOXICITY TO *DAPHNIA MAGNA*

SPL PROJECT NUMBER: 1742/022

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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:

13 May 2003	Standard Test Method Compliance Audit
11 October 2004	Test Material Preparation
11 October 2004	Test System Preparation
20 October 2004	Exposure
14 October 2004	Assessment of Response
26, 29 October 2004	Chemical Analysis
§ 04 January 2005	Draft Report Audit
§ Date of QA Signature	Final Report Audit

§ Evaluation specific to this study

..... DATE: 25 JAN 2005

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: 24 JAN 2005

T J Goodband
Study Director

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

..... Date: 24 JAN 2005

Dr J McKenzie CChem MRSC
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CONTAINS CONFIDENTIAL BUSINESS INFORMATION

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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 solutions of the test material at nominal concentrations of 0.14, 0.25, 0.45, 0.78, 1.4, 2.5, 4.5, 7.8 and 14 mg/l for 48 hours at a temperature of 21°C under static test conditions. The test material solutions were prepared by stirring an excess (50 mg/l) of test material in reconstituted water for a period of time and then removing any undissolved test material by filtration. This "saturated" solution, with nominal test concentration of 14 mg/l, was then further diluted, as necessary, to provide the remaining test groups. 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 8.3 mg/l with 95% confidence limits of 7.1 - 9.8 mg/l. The No Observed Effect Concentration was 2.5 mg/l.

Analysis of the test preparations at 0 hours showed measured test concentrations to range from 81% to 109% of nominal with the exception of the 1.4 and 4.5 mg/l test concentrations which showed measured test concentrations of 121% and 78% respectively although at 48 hours these test concentrations showed measured concentrations of 98% and 88% of nominal. Analysis of the test preparations at 48 hours showed measured test concentrations to range from 82% to 101% of nominal with the exception of the 0.14 mg/l test concentration which showed a measured test concentration of 76% of nominal. As the 0.14 mg/l test concentration was below the No Observed Effect Concentration and close to the 80% acceptance level, it was considered that reanalysis was unnecessary. Given that the majority of the measured test concentrations were within the 80% to 120% acceptance limits and that no significant decline in measured test concentrations was observed over the 48-Hour test period, it was considered justifiable to base the results on nominal test concentrations only.

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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.

Preliminary work was conducted from the 4 May 2004. The range-finding test was conducted between 20 September 2004 and 23 September 2004 and the definitive test between 12 October 2004 and 15 October 2004.

In view of the difficulties associated with the evaluation of aquatic toxicity of poorly water soluble test materials, a modification of the standard method for the preparation of aqueous media was performed. An approach endorsed by several important regulatory authorities in the EU and elsewhere (ECETOC 1996, and OECD 2000), is to expose organisms to a saturated solution of the test material in cases where the test material is of high purity and is poorly soluble in water and in the permitted auxiliary solvents and surfactants. Using this approach, a saturated solution was prepared by stirring an excess (50 mg/l) of test material with reconstituted water for 24 hours and then removing the undissolved test material by filtration through a pre-conditioned filter (0.2 µm) to give a saturated solution with a nominal concentration of 14 mg/l.

2. TEST MATERIAL AND EXPERIMENTAL PREPARATION

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	C6-2AL
Description	:	clear colourless liquid
Chemical name	:	1H, 1H, 2H, 2H-tridecafluoro-1-octanol
Batch number	:	001002
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

Due to the low aqueous solubility and high purity of the test material the test concentration used in the definitive test was a saturated solution prepared from an initial test material dispersion at a concentration of 50 mg/l.

An amount of test material (550 mg) was dispersed in 11 litres of reconstituted water with the aid of propeller stirring at approximately 1500 rpm for a period of 24 hours. After 24 hours the stirring was stopped and the undissolved test material removed by filtration (0.2 µm Gelman SuporCap filter, first approximate 1 litre discarded in order to pre-condition the filter) to give a saturated solution with a nominal concentration of 14 mg/l. Aliquots (10, 18, 32, 56, 100, 179, 321 and 557 ml) of the 14 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 series of nominal test concentrations of 0.14, 0.25, 0.45, 0.78, 1.4, 2.5, 4.5 and 7.8 mg/l.

Preliminary dosing work (see Section 3.3.1) conducted indicated that no significant loss of dissolved test material occurred when filtering a 50 mg/l test material dispersion through a pre-conditioned filter. It was therefore considered justifiable to use filtration as a method of removing dispersed test material to produce a saturated solution of the test material.

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 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* spp.). 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 Media Preparation Trials

Media preparation trials were conducted using a solvent spike method of preparation for the test material. However the results of the media preparation trials indicated that using a solvent spike method of preparation was not suitable for this test material as low and inconsistent measured concentrations were obtained.

Therefore media preparation trials were conducted to determine if the production of a saturated solution would be the most appropriate method of preparation for the test material. An amount of test material (550 mg) was dispersed in 11 litres of reconstituted water with the aid of propeller stirring at approximately 1500 rpm to give an initial test material dispersion of 50 mg/l. This was stirred for a period of 24 hours. Samples were taken from the saturated solution for chemical analysis after centrifugation (at either 10000 *g* or 40000 *g*) and after filtration through a 0.2 µm

Gelman SuporCap filter (after either the initial 1 or 2 litres discarded to pre-condition the filters). The results of the chemical analyses indicated that a test concentration of approximately 14 mg/l was obtained following all methods of preparation.

Given that the results indicated that filtration through a pre-conditioned filter (after either the initial 1 or 2 litres discarded) did not result in a dissolved test material concentration significantly lower than that obtained via centrifugation, it was considered that dissolved test material did not adsorb to the filters used.

Based on these results it was considered justifiable to prepare the test media using a 24-Hour preparation period followed by filtration (0.2 µm), with the first approximately 1 litre discarded, to remove excess dispersed test material and produce a saturated solution.

3.3.2 Range-finding test

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

In the range-finding test *Daphnia magna* were exposed to a series of nominal test concentrations of 0.014, 0.14, 1.4 and 14* mg/l. Due to the low aqueous solubility and high purity of the test material the test concentrations used in the range-finding test were prepared by diluting a saturated solution prepared from an initial test material dispersion at a concentration of 50 mg/l.

An amount of test material (550 mg) was dispersed in 11 litres of reconstituted water with the aid of propeller stirring at approximately 1500 rpm at a temperature of 21°C for 24 hours. After 24 hours the stirring was stopped prior to removal of the undissolved test material by filtration (0.2 µm Gelman SuporCap filter, first approximate 1 litre discarded in order to pre-condition the filter) to give a saturated solution with a nominal concentration of 14* mg/l. Serial dilutions were prepared from this nominal 14 mg/l test concentration to give the remainder of the test series of 0.014, 0.14 and 1.4 mg/l.

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

* Concentration based on the results of preliminary analysis of a saturated solution of the test material

In the range-finding test 10 daphnids were placed in each test and control vessel and maintained in a temperature controlled room at 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 glass stoppered conical flask was completely filled with minimal headspace due to the suspected volatile nature of the test material. 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.

3.3.3 Definitive test

Based on the results of the range-finding test conducted, the test material solutions for the definitive test were prepared by stirring an excess (50 mg/l) of test material in reconstituted water at approximately 1500 rpm for 24 hours. After 24 hours undissolved test material was removed by filtration (0.2 µm Gelman SuporCap Filter, first approximate 1 litre discarded in order to pre-condition the filter) to give a saturated solution of the test material with a nominal concentration of 14* mg/l which was then further diluted, as necessary, to produce the remainder of the test series of nominal test concentrations of 0.14, 0.25, 0.45, 0.78, 1.4, 2.5, 4.5 and 7.8 mg/l.

3.3.3.1 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.

3.3.3.2 Exposure conditions

As in the range-finding test 250 ml glass stoppered conical flasks completely filled with minimal headspace were used due to the suspected volatile nature of the test material. At the start of the test 10 daphnids were placed in each test and control vessel at random, in the prepared test solutions. Duplicate test vessels were used for each test and control group. The test vessels were then maintained in a temperature controlled room at 21°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.

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* Concentration based on the results of preliminary analysis of a saturated solution of the test material

The test solutions 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.

3.3.3.3 Physico-chemical measurements

Water temperature was recorded daily throughout the test. Dissolved oxygen concentration and pH were recorded at the start and termination of the test. The pH and the dissolved oxygen concentration was measured using a WTW pH/Oxi 340I pH and dissolved oxygen meter and the temperature was measured using a Hanna Instruments HI93510 digital thermometer.

3.3.3.4 Verification of test concentrations

Water samples were taken from the control (replicates $R_1 - R_2$ pooled) and all test groups (replicates $R_1 - R_2$ pooled) at 0 and 48 hours for quantitative analysis.

Duplicate samples were taken and stored at approximately -20°C for further analysis if necessary.

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

3.3.3.5 Evaluation of data

The EC_{50} values and associated confidence limits at 48 hours were calculated by the maximum-likelihood probit method (Finney 1971) using the ToxCalc computer software package (ToxCalc 1999) and at 24 hours the EC_{50} value was calculated using the geometric mean method as follows:

EC_{50} value = $\sqrt{C_1 \times C_2}$

Where:

C_1 = concentration showing 0% immobilisation
C_2 = concentration showing 100% immobilisation

Probit analysis is used where two or more partial responses to exposure are shown.

If there is no immobilisation between 0% and 100% immobilisation, then the geometric mean of the highest test concentration showing no lethality and the lowest test concentration showing

100% lethality is calculated. The concentrations resulting in 0% and 100% immobilisation will be the 95% confidence limits.

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 Media Preparation Trials

Media preparation trials were conducted using a solvent spike method of preparation for the test material. However the results of the media preparation trials indicated that using a solvent spike method of preparation was not suitable for this test material as low and inconsistent measured concentrations were obtained.

A further media preparation trial was conducted using a saturated solution method of preparation. Filtration of the test material dispersions through a 0.2 µm Gelman SuporCap filter showed measured concentrations of 14 and 15 mg/l, discarding the first approximate 1 litre or 2 litres respectively in order to pre-condition the filter. Centrifugation of the test material dispersions at either 10000 *g* or 40000 *g* showed measured test concentrations of 15 and 16 mg/l respectively.

Based on these results it was considered justifiable to prepare the test media using a 24-Hour preparation period followed by filtration (0.2 µm), with the first approximately 1 litre discarded, to remove excess dispersed test material and produce a saturated solution

5.2 Range-finding Test

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

The results showed no immobilisation at the nominal test concentrations of 0.014, 0.14 and 1.4 mg/l. However, immobilisation was observed at 14 mg/l.

Based on this information the test material solutions for the definitive test were prepared by stirring an excess (50 mg/l) of the test material in reconstituted water for a period of time and then removing any undissolved test material by filtration to give a saturated solution with a nominal concentration of 14 mg/l. This saturated solution was then further diluted, as necessary, to produce the remaining test concentrations of 0.14, 0.25, 0.45, 0.78, 1.4, 2.5, 4.5 and 7.8 mg/l.

5.3 Definitive Test

5.3.1 Immobilisation data

Cumulative immobilisation data from the exposure of *Daphnia magna* to the test material during the definitive test are given in Table 2 and the relationship between percentage immobilisation and concentration at 24 and 48 hours is given in Figures 1 and 2.

Analysis of the immobilisation data by the geometric mean method at 24 hours and the trimmed Spearman-Karber method (Hamilton *et al* 1977) at 48 hours based on the nominal test concentrations gave the following results:

Time (h)	EC_{50} (mg/l)	95% Confidence limits (mg/l)
24	10	7.8 - 14
48	8.3	7.1 - 9.8

The No Observed Effect Concentrations after 24 and 48 hours exposure were 7.8 and 2.5 mg/l respectively. The No Observed Effect Concentration is based upon zero immobilisation at this concentration.

5.3.2 Physico-chemical measurements

The results of the physico-chemical measurements are given in Appendix 3. Temperature was maintained at approximately 21°C throughout the test, while there were no treatment related differences for oxygen concentration or pH.

5.3.3 Observations on test material solubility

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

5.3.4 Verification of test concentrations

Due to the low aqueous solubility and high purity of the test material, the test material solution was prepared by stirring an excess (50 mg/l) of test material in reconstituted water for a period of time and then removing any undissolved test material by filtration. This saturated solution was then further diluted, as necessary, to produce the remaining test groups.

Analysis of the test preparations at 0 hours (see Appendix 1) showed measured test concentrations to range from 81% to 109% of nominal with the exception of the 1.4 and 4.5 mg/l test concentrations which showed measured test concentrations of 121% and 78% respectively although at 48 hours these test concentrations showed measured concentrations of 98% and 88% of nominal.

Analysis of the test preparations at 48 hours (see Appendix 1) showed measured test concentrations to range from 82% to 101% of nominal with the exception of the 0.14 mg/l test concentration which showed a measured test concentration of 76% of nominal. As the 0.14 mg/l test concentration was below the No Observed Effect Concentration and close to the 80% acceptance level, it was considered that re-analysis was unnecessary.

Given that the majority of the measured test concentrations were within the 80% to 120% acceptance limits and that no significant decline in measured test concentrations was observed over the 48-Hour test period, it was considered justifiable to base the results on nominal test concentrations only.

6. CONCLUSION

The acute toxicity of the test material to the freshwater invertebrate *Daphnia magna* has been investigated and based on nominal test concentrations gave a 48-Hour EC_{50} value of 8.3 mg/l with 95% confidence limits of 7.1 – 9.8 mg/l. The No Observed Effect Concentration at 48 hours was 2.5 mg/l.

7. REFERENCES

Environment Directorate, Organisation for Economic Co-operation and Development (OECD) (2000) Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures.

European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Monograph No. 26 (1996) Aquatic Toxicity Testing of Sparingly Soluble, Volatile and Unstable Substances.

Finney, D J (1971) Statistical Method in Biological Assay. London : Griffin and Company Ltd.

ToxCalc Version 5.0.23C (1999), Tidepool Scientific Software, McKinleyville, CA 95519, USA.

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Table 1 Cumulative Immobilisation Data in the Range-finding Test

Initial Test Material Dispersion (mg/l)*	Cumulative Immobilised *Daphnia* (Initial Population: 10 Per Replicate)	
	24 Hours	48 Hours
Control	0	0
0.014	0	0
0.14	0	0
1.4	0	0
14	10	10

* Concentration based on the results of preliminary analysis of a saturated solution of the test material

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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
0.14	0	0	0	0	0	0	0	0
0.25	0	0	0	0	0	0	0	0
0.45	0	0	0	0	0	0	0	0
0.78	0	0	0	0	0	0	0	0
1.4	0	0	0	0	0	0	0	0
2.5	0	0	0	0	0	0	0	0
4.5	0	0	0	0	1	1	2	10
7.8	0	0	0	0	3	2	5	25
14	10	10	20	100	10	10	20	100

* Concentration based on the results of preliminary analysis of a saturated solution of the test material

$R_1 - R_2$ = Replicates 1 and 2

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Figure 1 **Concentration-Response Curve After 24 Hours**

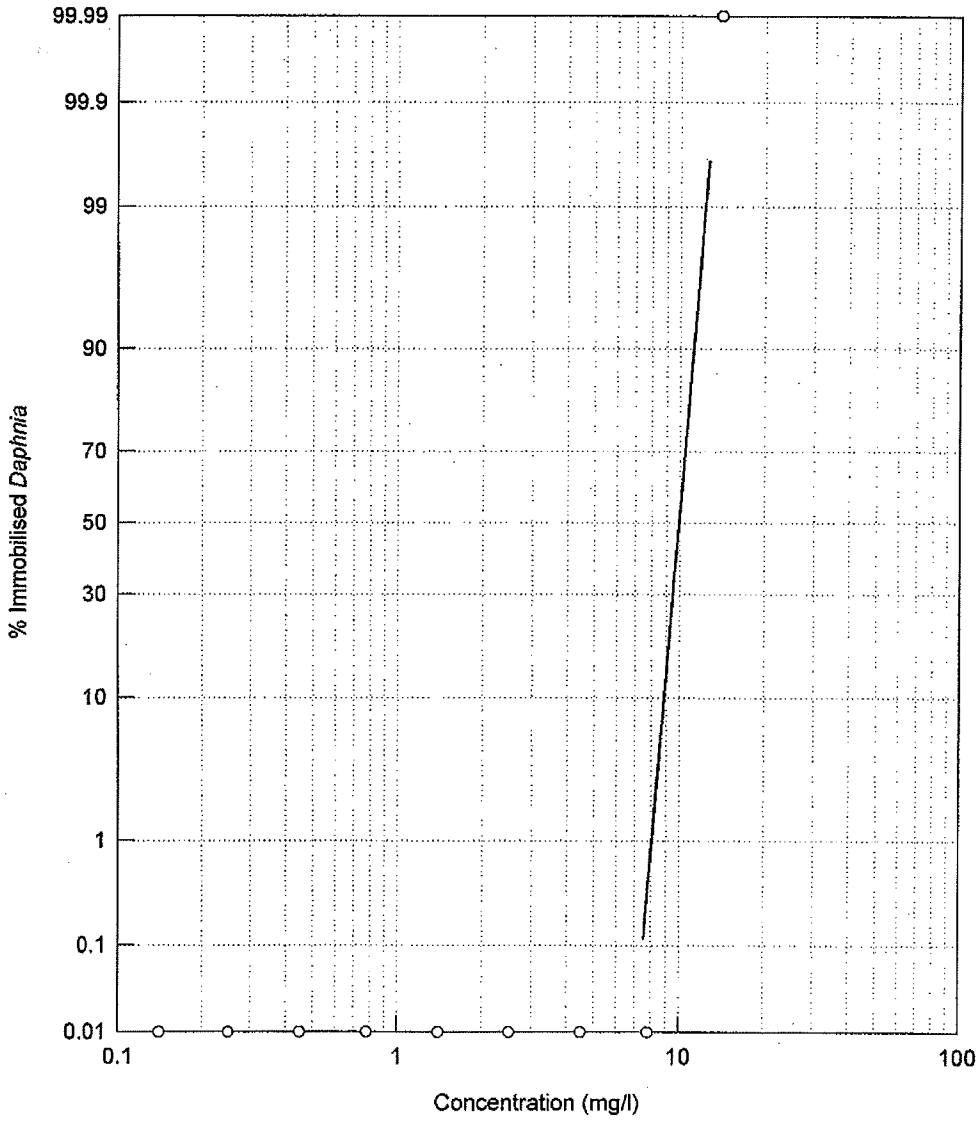

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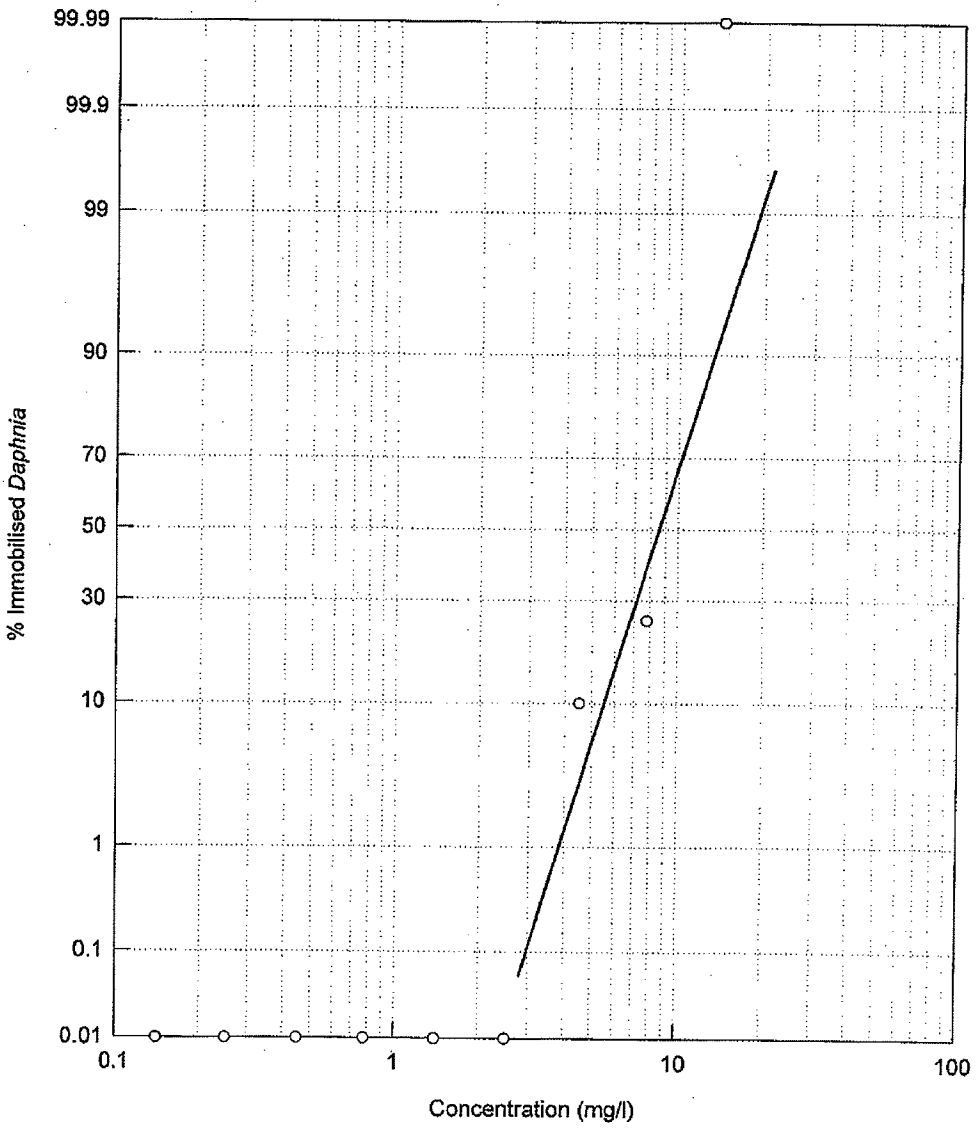
Figure 2 **Concentration-Response Curve After 48 Hours**



Appendix 1 Verification of Test Concentrations

1. METHOD OF ANALYSIS

1.1 Introduction

The test material concentration in the test samples was determined by gas chromatography (GC) 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 Strata X solid phase extraction (SPE) cartridge was sequentially pre-conditioned with methanol and water. A volume of test sample was eluted through the cartridge and the cartridge kept wet. The test material was eluted from the cartridge with methanol and made to volume to give final theoretical concentrations of approximately 7 to 14 mg/l.

1.3 Standards

Standard solutions of test material were prepared in methanol at a nominal concentration of 10 mg/l.

1.4 Procedure

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

GC System	:	Agilent Technologies 5890, incorporating autosampler and workstation
Column	:	ZB-5(30 m x 0.53 mm id, 5 µm film)
Oven temperature program	:	initial 60 °C for 2 minutes rate 10 °C/minute final 150°C

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Appendix 1 (continued) Verification of Test Concentrations

Injector temperature	:	200°C
Detector temperature	:	250°C
Carrier gas and pressure	:	nitrogen at 5 psi
Injection volume	:	1 µl
Injection mode	:	splitless purge on at 1 minutes
Detector	:	flame ionisation detector (FID)
Retention time	:	approximately 7 minutes

2. PRELIMINARY SATURATED SOLUTION

The preliminary samples were prepared by direct addition of the test material to a sample of the test media. The dispersion was stirred using a propeller stirrer at approximately 1500 rpm for 24 hours at approximately 21°C. After the stirring period the samples were analysed after centrifugation at either 10000 *g* or 40000 *g* and after filtration through a 0.2 µm Gelman SuporCap filter (after either the initial 1 or 2 litres discarded to pre-condition the filter).

Saturated solution samples	mg/l
After centrifugation at 10000 *g*	15.0
After centrifugation at 40000 *g*	15.6
After filtration (initial 1 litre discarded)	14.0
After filtration (initial 2 litres discarded)	15.5

3. VALIDATION

3.1 Linearity

A range of standard solutions covering 1.0 to 98 mg/l (10% to 980% of the working concentration) was analysed.

Linearity was confirmed (percentage fit =1.000) ranging from 1.0 to 98 mg/l.

The results are presented graphically on page 23.

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Appendix 1 (continued) Verification of Test Concentrations

3.2 Fortified Recoveries

The recovery samples were prepared by addition of a standard solution of test material to a sample of test medium. A standard solution was accurately prepared by dissolving the test material in methanol. An accurate volume of the standard solution was added to a known volume of test medium to achieve the required concentration of test material in water.

Fortification (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
0.149	0.101	68	68
0.149	0.100	67	
1.49	1.07	72	69
1.49	0.982	66	
14.9	12.4	83	79
14.9	11.1	74	

The recovery results are outside the accepted limits of 80 to 120 %. Procedural recoveries were run alongside the test samples. The method has been considered to be sufficiently accurate for the purposes of this test. All test sample results have been corrected for recovery.

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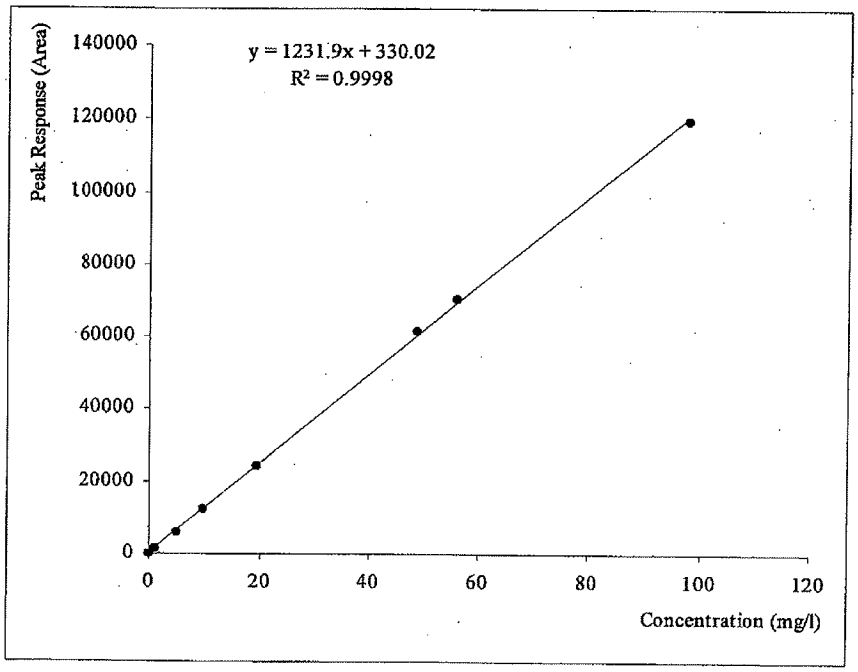
Appendix 1 (continued) Verification of Test Concentrations

Linearity of Detector Response



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Appendix 1 (continued) Verification of Test Concentrations

3.3 Saturated Solution Recoveries

The recovery samples were prepared by direct addition of the test material to a sample of test medium.The dispersion was stirred using a propeller set at 1500 rpm for 24 hours at 21°C. After the stirring period, undissolved test material was removed by filtration through a pre-conditioned 0.2 µm Gelman Acrocap filter.

Nominal Concentration (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
0.140	0.220	157	140
0.140	0.173	124	
1.40	1.81	130	122
1.40	1.61	115	
14.0	14.3	102	100
14.0	13.6	97	

The recovery results are outside the accepted limits of 80 to 120 %. Procedural recoveries were run alongside the test samples. All test sample results have been corrected for recovery.

3.4 Limit of Quantitation

The limit of quantitation has been assessed down to 0.0067 mg/l.

4 STABILITY

4.1 Fortified Recovery 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) the sample bottle to assess for losses due to adsorption and/or insolubility.

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Appendix 1 (continued) Verification of Test Concentrations

Nominal concentration (mg/l)	0.140	1.40	14.0
Concentration found initially (mg/l)	0.101	1.03	11.7
Concentration found after storage in light conditions (mg/l)	0.0819	0.603	10.5
Expressed as a percent of the initial concentration	81	59	90
Concentration found after storage in dark conditions (mg/l)	0.0773	0.755	11.9
Expressed as a percent of the initial concentration	77	74	102
Concentration found after storage in dark conditions (mg/l) – unsonicated sample	0.0874	NA	11.0
Expressed as a percent of the initial concentration	87	-	94

The middle level was below the acceptance limit of 80 to 120 %, otherwise the test samples have shown to be stable in the test medium in light and dark conditions.

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

4.2 Saturated Solution Stability

A range of preliminary test samples was prepared, as in Section 3.3, 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) the test sample bottle to assess for losses due to adsorption and/or insolubility.

NA = Not applicable

C6-2AL: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations

Nominal concentration (mg/l)	0.140	1.40	14.0
Concentration found initially (mg/l)	0.197	1.71	14.0
Concentration found after storage in light conditions (mg/l)	0.119	1.40	8.20
Expressed as a percent of the initial concentration	60	81	59
Concentration found after storage in dark conditions (mg/l)	0.177	0.837	10.5
Expressed as a percent of the initial concentration	90	49	75
Concentration found after storage in dark conditions (mg/l) – unsonicated sample	0.167	NA	8.49
Expressed as a percent of the initial concentration	85	-	61

Some variability was observed, there was no conclusive result. However, based on the structure and information supplied by the Sponsor, the test material was considered to be stable. The observed variability was likely due to the analytical procedure.

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

NA = Not applicable

C6-2AL: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations

5. RESULTS

Sample	Nominal Concentration (mg/l)	Concentration Found (mg/l)*	Expressed as a Percent of the Nominal Concentration (%)
0 Hours	Control	<LOQ	-
	0.14	0.144	103
	0.25	0.273	109
	0.45	0.459	102
	0.78	0.833	107
	1.4	1.70	121
	2.5	2.16	86
	4.5	3.50	78
	7.8	6.32	81
	14	11.4	81
48 Hours	Control	<LOQ	-
	0.14	0.107	76
	0.25	0.205	82
	0.45	0.382	85
	0.78	0.746	96
	1.4	1.37	98
	2.5	2.21	88
	4.5	3.96	88
	7.8	7.16	92
	14	14.1	101

6. 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.

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* Corrected for recovery values from validation as the procedural recovery results alongside test samples were considered erroneous
LOQ = Limit of quantitation

C6-2AL: ACUTE TOXICITY TO *DAPHNIA MAGNA*

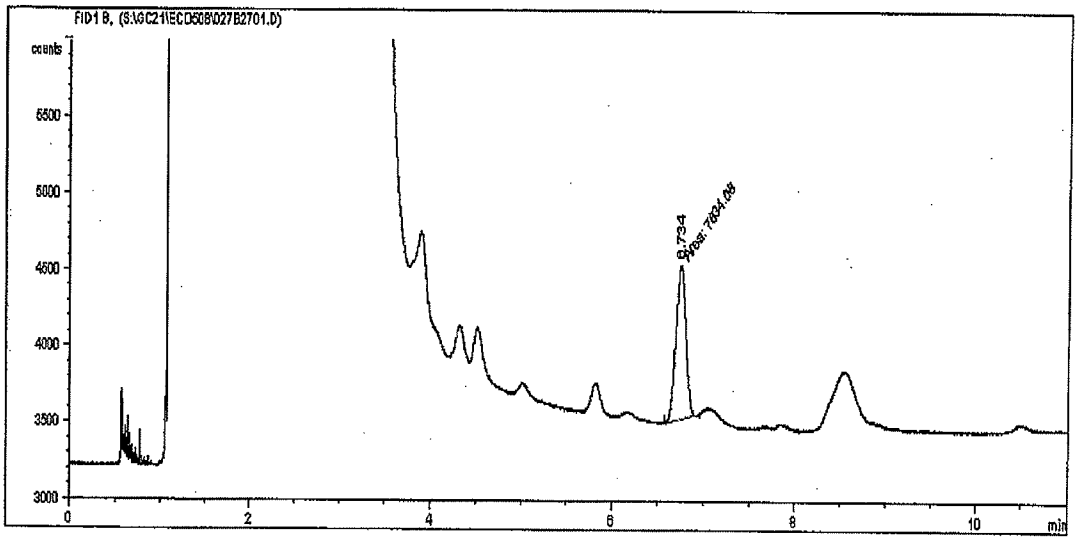
Appendix 1 (continued) Verification of Test Concentrations

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

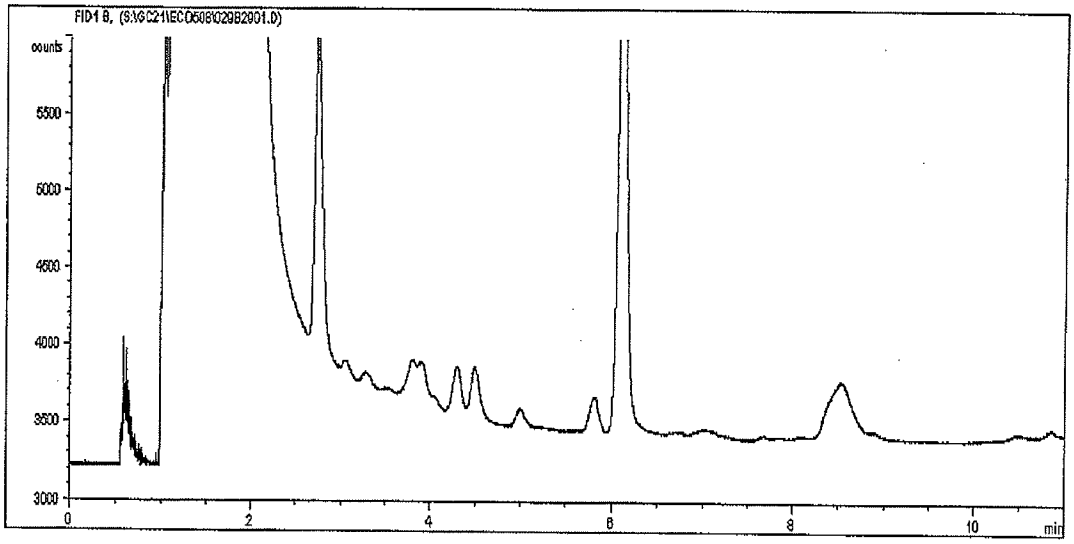
C6-2AL: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations

7. TYPICAL CHROMATOGRAPHY



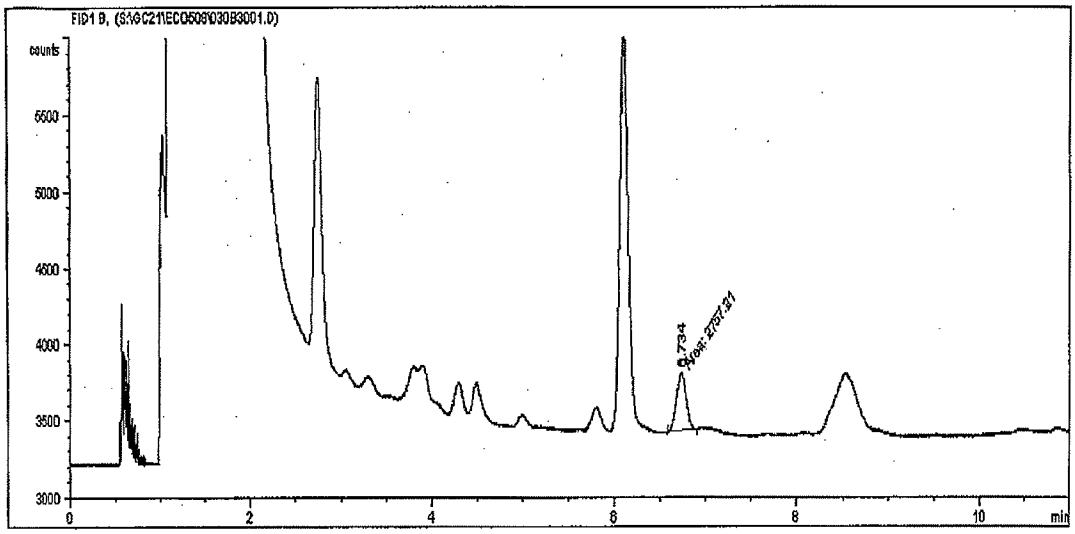
Standard 10 mg/l



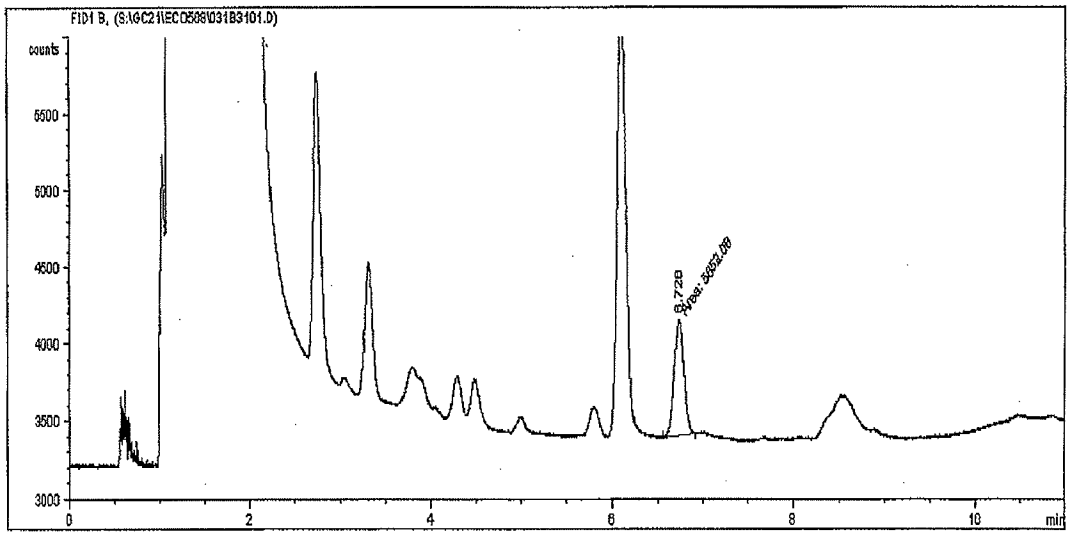
Control Sample

C6-2AL: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations



Test Sample 0 .14mg/l

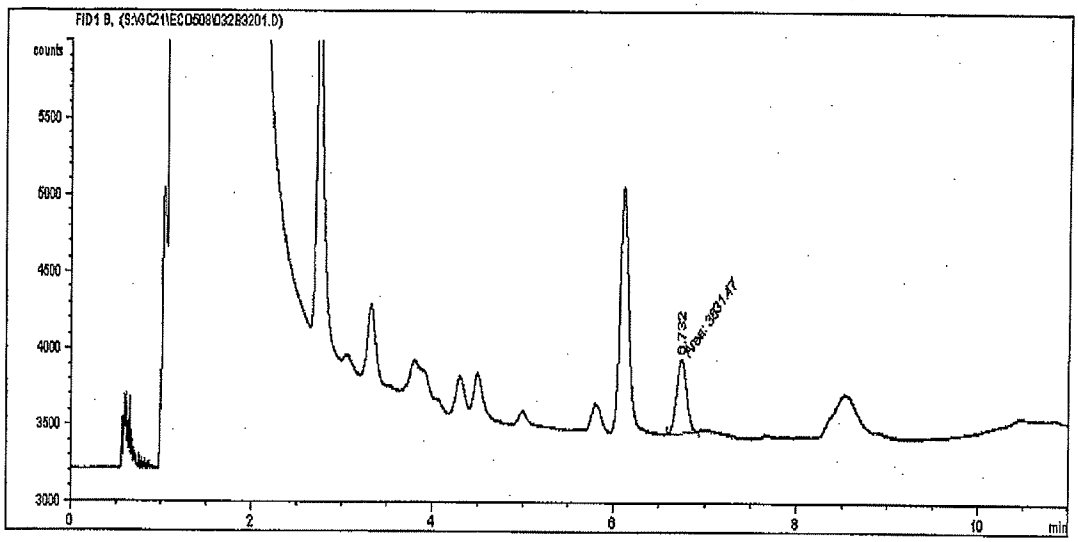


Test Sample 0.25mg/l

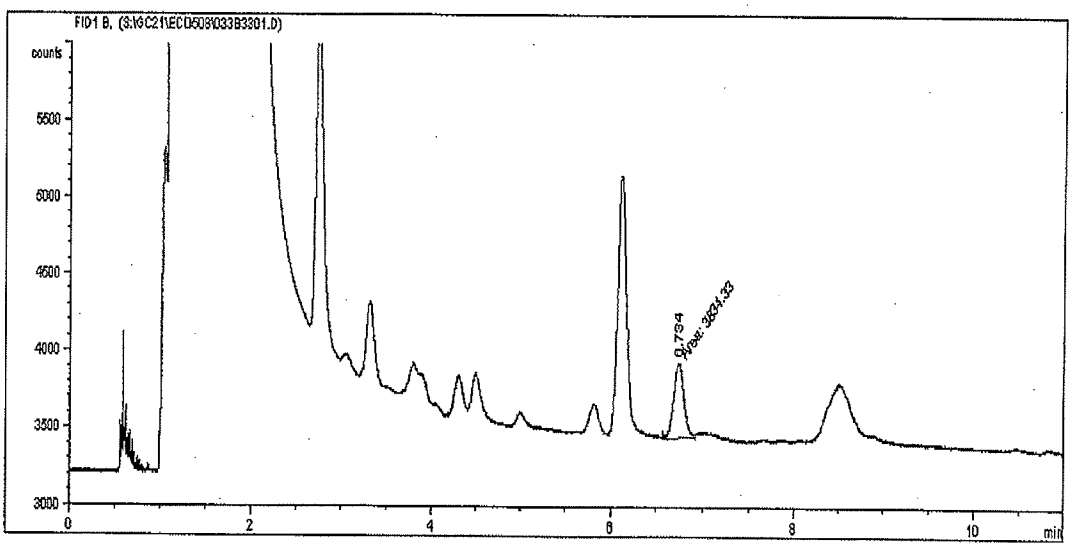
CONTAINS CONFIDENTIAL BUSINESS INFORMATION

C6-2AL: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations



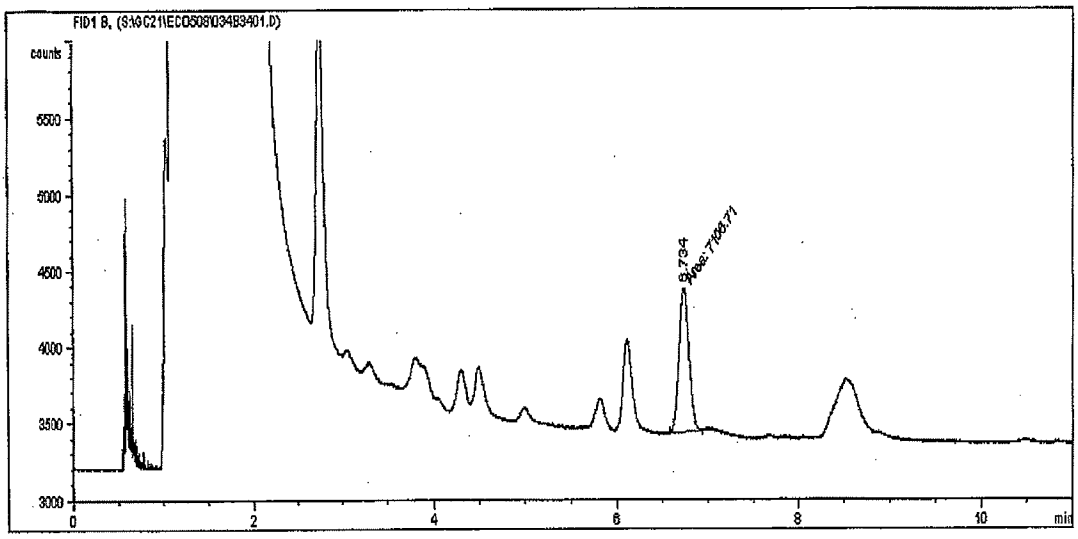
Test Sample 0.45mg/l



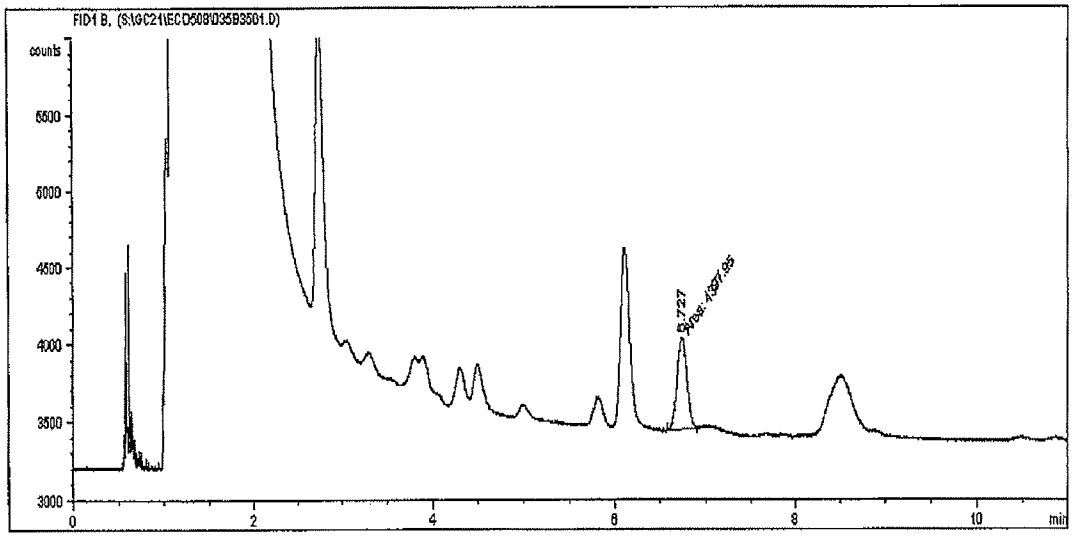
Test Sample 0.78mg/l

C6-2AL: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations



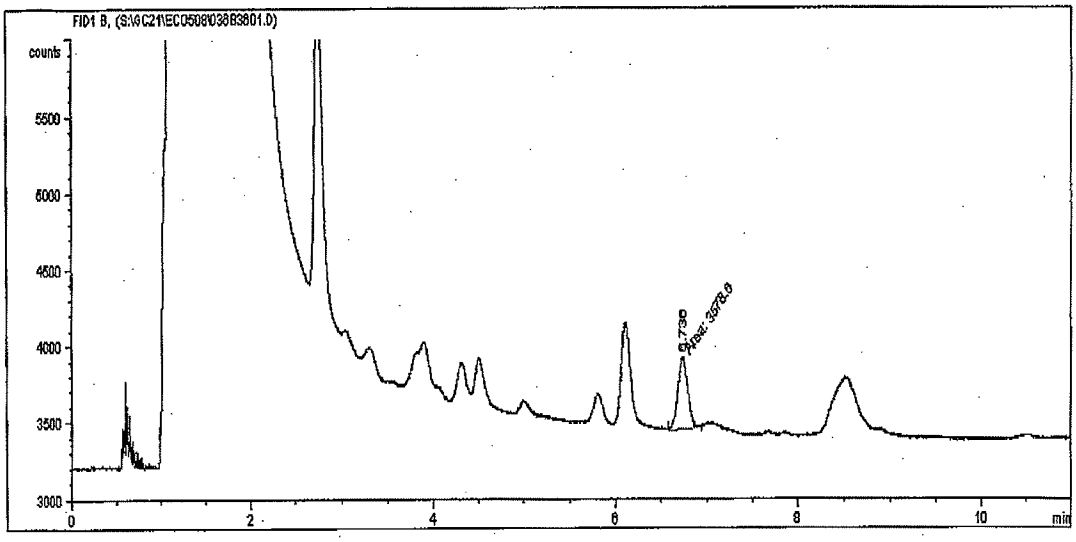
Test Sample 1.4mg/l



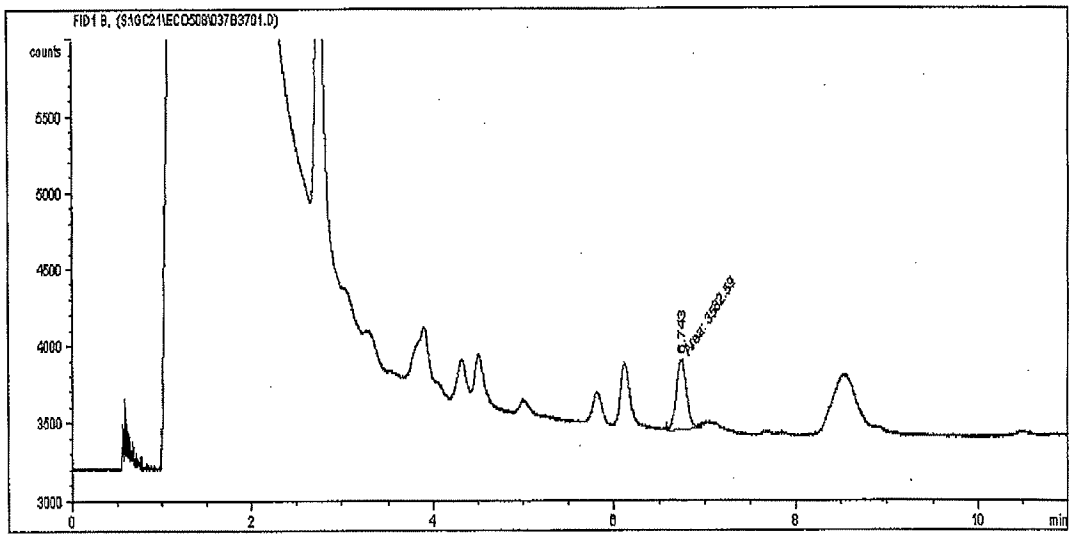
Test Sample 2.5 mg/l

C6-2AL: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations



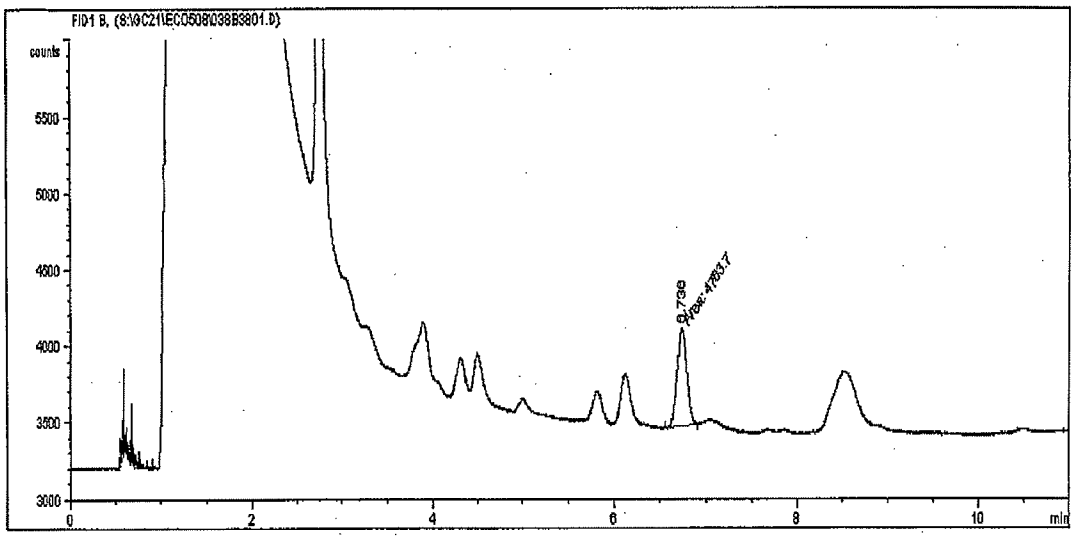
Test Sample 4.5 mg/l



Test Sample 7.8 mg/l

C6-2AL: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 1 (continued) Verification of Test Concentrations



Test Sample 14 mg/l

C6-2AL: 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 $\mu S\ cm^{-1}$ and pH equal to 7.8 ± 0.2, adjusted (if necessary) with NaOH or HCl. The reconstituted water 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 $CaCO_3$.

C6-2AL: ACUTE TOXICITY TO *DAPHNIA MAGNA*

Appendix 3 Physico-Chemical Measurements

Nominal Concentration (mg/l)		0 Hours				24 Hours	48 Hours			
		pH	mg O_2/1	%ASV*	T°C	T°C	pH	mg O_2/1	%ASV*	T°C
Control	R_1	8.0	8.3	93	21.2	21.2	8.0	8.3	93	21.1
	R_2	8.0	8.3	93	21.2	21.2	8.0	8.3	93	21.2
0.14	R_1	8.0	8.3	93	21.3	21.1	8.0	8.3	93	21.2
	R_2	8.0	8.2	92	21.3	21.1	8.0	8.2	92	21.2
0.25	R_1	8.0	8.2	92	21.3	21.2	8.0	8.2	92	21.2
	R_2	8.0	8.2	92	21.2	21.1	8.0	8.2	92	21.2
0.45	R_1	7.9	8.3	93	21.3	21.1	8.0	8.3	93	21.1
	R_2	8.0	8.2	92	21.2	21.2	8.0	8.2	92	21.1
0.78	R_1	8.0	8.2	92	21.2	21.2	8.0	8.2	92	21.1
	R_2	8.0	8.3	93	21.3	21.1	8.0	8.2	92	21.1
1.4	R_1	8.0	8.2	94	21.5	21.1	8.0	8.2	92	21.2
	R_2	8.0	8.3	93	21.4	21.1	8.0	8.2	92	21.2
2.5	R_1	8.0	8.2	94	21.5	21.2	8.0	8.2	92	21.2
	R_2	8.0	8.2	92	21.4	21.2	8.0	8.2	92	21.2
4.5	R_1	7.9	8.2	92	21.3	21.2	8.0	8.2	92	21.2
	R_2	8.0	8.3	93	21.4	21.2	8.0	8.2	92	21.1
7.8	R_1	8.0	8.2	92	21.4	21.1	8.0	8.2	92	21.1
	R_2	8.0	8.2	92	21.3	21.1	8.0	8.2	92	21.2
14	R_1	8.0	8.2	92	21.4	21.2	8.0	8.2	92	21.1
	R_2	8.0	8.3	93	21.3	21.2	8.0	8.3	93	21.1

*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