Appendix 7

ISTITUTO DI RICERCHE BIOMEDICHE "ANTOINE MARXER" RBM S.p.A.
Sede Legale e Laborat.: Via Ribes, 1 - 10010 Colleretto Giacosa (TO) - ITALY
Telef.: 0125/53746 (5 linee) - Telefax: 0125/53585 - C.P. 226 - 10015 Ivrea (TO)

RBM Exp. 950053

Asta file 0012 1,3,4,

Haematococcus pluvialis,
unicellular green algae

"ACUTE TOXICITY STUDY IN RATS TREATED BY ORAL ROUTE"

RBM EXP. No. 950053

Issued on September 7, 1995

SPONSOR

KINETICON AB
Kungsangsvagen 31
S-753 23 UPPSALA
Sweden

PERFORMING LABORATORY

Istituto di Ricerche Biomediche
"Antoine Marxer" RBM S.p.A.
Via Ribes, 1
10010 - COLLERETTO GIACOSA (Torino)
Italy

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Telef.: 0125/53746 (5 linee) - Telefax: 0125/53585 - C.P. 226 - 10015 Ivrea (TO)

TITLE OF THE STUDY

Haematococcus pluvialis, unicellular green algae: "Acute toxicity study in rats treated by oral route".

PURPOSE OF THE STUDY

The purpose of the study was to evaluate the acute oral toxicity of the test article **Haematococcus pluvialis, unicellular green algae** and to calculate the LD_{50}, slope values and 95% confidence limits.

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This report consists of 32 pages.

Ivrea, September 7, 1995

Mr. Enrico Gillio Tos
RBM Study Director

ISTITUTO DI RICERCHE BIOMEDICHE "ANTOINE MARXER" RBM S.p.A.
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FOREWORD

On behalf of **KINETICON AB - Kungsangsvagen 31 - S-753 23 UPPSALA - Sweden**, Istituto di Ricerche Biomediche "Antoine Marxer" RBM S.p.A., authorized by the Italian Health Authorities (1-2) to conduct safety studies, has performed an acute toxicity study by oral route in Crl: CD(SD) BR rats (RBM- Experiment no. 950053), with the test article:

Haematococcus pluvialis, unicellular green algae

A sample of the substance used, along with pertinent documentation, is held in sufficient quantity in the RBM archives and is at the disposal of the Ministero della Sanità.

The undersigned declare that the experiment was conducted using the same batch of substance as that of the sample held on file.

For verification by the Ministero della Sanità, the undersigned moreover guarantee the identification and classification of all those materials, documents and recordings used in conducting the experiment, held on file for a period of at least 10 years from the date of this report. Following this time, they will be placed at the disposal of the Sponsor.

Dr. Roberto Maraschin

Scientific Director Recognized by the Italian Health Authorities as Responsible for General Toxicology Experimentation

Dr. Angelo Conz

General Manager of the Istituto di Ricerche Biomediche "Antoine Marxer", RBM S.p.A.

Ivrea, September 7, 1995

(1): **Pharmaceuticals:**
Authorization dated March 12, 1976 in accordance with "Circolare 73", May 16, 1974

(2): **Chemicals:**
Authorization in accordance with DPR 927/81 (D.M. dated January 7, 1988 published in G.U. No. 12, dated January 16, 1988).

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RBM Exp. 950053

QUALITY ASSURANCE STATEMENT

RBM Experiment number: 950053

Study title:

Haematococcus pluvialis, unicellular green algae: "Acute toxicity study in rats treated by oral route".

Studies of the type described in this report are conducted in a manner which involves frequent repetition of identical or similar procedures.

In compliance with the Principles of Good Laboratory Practice, at the time of this study, procedure-based inspections were made by the Q.A.U. of critical phases and procedures relevant to this type of study. For the inspection of any given procedure, studies were selected at random. All such inspections were reported promptly to the study director and to facility management.

Dates of inspection/audit	Dates of report to Study Director and Management
May 22, 1995	May 22, 1995
May 24 and 25, 1995	May 26, 1995
June 8, 1995	June 9, 1995
July 6, 1995	July 6, 1995

This report has been audited by the Q.A.U. and was found to be an accurate description of such methods and procedures as were used during the conduct of the study and an accurate reflection of the raw data.

Date of final report audit: September 18, 1995

Enrico Invernizzi
Head of Quality Assurance Unit

Date: September 18, 1995

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RBM Exp. 950053

RBM MANAGEMENT DECLARATION OF GLP COMPLIANCE

Study No. 950053 entitled :

Haematococcus pluvialis, unicellular green algae: "Acute toxicity study in rats treated by oral route"

was performed in compliance with the OECD-GLP in the testing of chemicals, [C(81) 30 (final)], regulations also enforced by the Italian Health Authority [D.M. dated June 26, 1986 as published in G.U. No. 198, dated August 27, 1986 and D.L. January 27, 1992, No. 120 as published in G.U. (Supplement) No. 40, February 18, 1992].

Mr. Enrico Gillio Tos

RBM Study Director

Dr. Angelo Conz

General Manager of the Istituto di Ricerche Biomediche "Antoine Marxer", RBM S.p.A.

Ivrea, September 19, '95

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SCIENTISTS INVOLVED IN THE STUDY

STUDY No. 950053

Haematococcus pluvialis, unicellular green algae: "Acute toxicity study in rats treated by oral route".

RBM Study Director	Mr Enrico Gillio Tos	
Scientific Director Toxicology	Dr. Roberto Maraschin	
Head of General Toxicology Unit	Dr. Luciana Orlando	
Head of Pharmacy Unit	Mr. Pietro Zaninelli	
Formulate preparation	for Dr. Bruna Piccioli	

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MATERIALS AND METHODS

EXPERIMENTAL DESIGN

RBM Experiment No.:	950053
Test article:	**Haematococcus pluvialis, unicellular green algae** batch 950123
Vehicle:	Intralipid® solution 20% manufactured by PHARMACIA AB
Sponsor:	**KINETICON AB** **Kungsangsvagen 31** **S-753 23 UPPSALA** **Sweden**
Study Monitor:	**Mr. T. Lundqvist** **KINETICON AB** **Kungsangsvagen 31** **S-753 23 UPPSALA** **Sweden**
Dosages administered:	12000 mg/kg
Administration volume:	40 ml/kg b.w. (2 administration of 20+20 ml/kg spaced about 2 hours)
Concentration of the test article in the vehicle:	300 mg/ml (as a suspension)
Administration route:	oral
Reason for selection of the administration route:	possible accidental ingestion by humans
Dosing regimen :	single dose
Duration of post-treatment observation period:	14 days
Number and sex of animals:	32 rats (16M+16F): 10 (5M+5F) for part I; 10 (5M+5F) for part II and 12 (6M+6F) for part III of the study.

Species, strain and substrain of the test system :	CD (SD) BR rat
Justification for selection of the test system :	the Sprague Dawley rat was chosen as rodent species since it is an appropriate experimental model widely accepted by Health Authorities, with documented susceptibility to a wide range of toxic substances

DESCRIPTION OF THE EXPERIMENTAL DESIGN

Part I: Preliminary acute study

Initially, a group of 5 animals/sex, randomly selected, was administered 20 ml/kg of the vehicle Intralipid® solution 20%. Since no animals died or showed toxic effects, this group was administered the same volume (20 ml/kg) after about 2 hours from the first administration. There were no effects even after this administration for the whole day.

Part II: Acute toxicity study

On the basis of the results obtained in Part I, a group of 5 animals/sex randomly selected, was administered 40 ml/kg (two administrations of 20 ml/kg spaced about two hours) of the test article Haematococcus pluvialis in order to obtain the dose of 12 g/kg, the maximum test article concentration in the vehicle being 300 mg/ml.

Part III: Toxicokinetics study

A group of 12 animals (6 males and 6 females) was administered the same dose used in Part II of the study (12 g/kg).
Sampling times: 0, 1, 2, 4, 8 and 12 hours.
Two sampling times/animal.
Two males + two females/point curve.
The animal numbers and the blood sampling schedule are given in the following scheme:

Sampling time (hour)	0	1	2	4	8	12
Animal no. and sex	25M: 26M 31F: 32F	21M: 22M 27F: 28F	23M: 24M 29F: 30F	25M: 26M 31F: 32F	21M: 22M 27F: 28F	23M: 24M 29F: 30F

The plasma samples were kept at -20 °C and will be sent to the Sponsor for analysis.
The data analysis will not be included in this report.

STUDY DATES

Par I: May 24, 1995

Part II: May 25 - June 8, 1995

Part III: June 8, 1995

TEST ARTICLE CHARACTERIZATION

Identification:	Haematococcus pluvialis, unicellular green algae
Batch:	950123
Characteristics:	red powder
Manufacturing date:	January 23, 1995
Expiry date:	12 months from the preparation
Storage:	frozen at -20°C, in the dark

VEHICLE CHARACTERIZATION

Identification:	Intralipid® 20% solution
Batch:	66727-51
Manufacturing date:	February, 1995
Expiry date:	July, 1996
Producer:	Pharmacia AB

ANIMAL HUSBANDRY

SUPPLY, ACCEPTANCE OF THE ANIMALS AND SELECTION FOR THE EXPERIMENT

The 20 (10M + 10F) CD (SD) BR rats, selected for parts I and II this study from a larger group (22M + 22F) than that required, were purchased from Charles River Italia S.p.A., Via Indipendenza 11 - 22050 CALCO (Como) - (received on May 19, 1995 - shipping slip no. 04177, dated May 19, 1995). The 12 (6M + 6F) CD:(SD) BR rats, selected for part III of this study from a larger group (13M + 13F) than that required, were purchased from Charles River Italia S.p.A., Via Indipendenza, 11 - 22050 CALCO (Como) - (received on June 2, 1995 - shipping slip no. 04593, dated June 2, 1995).

When received, the rats were about 7/9 weeks old and weighed about 200-225 g (females) or 225-250 g (males).

On arrival at RBM, all animals were clinically observed and 20% of them were weighed: their weight conformed to that required. The animals were housed in the same room in which the treatment was performed.

During the pre-treatment acclimatization period the animals were clinically observed every day.

In view of the normal health of all animals of the batch received, the entire group was deemed fit for the experiment.

ACCOMMODATION OF THE ANIMALS

The animals were housed in room T11C.

Animal room controls were set to maintain temperature and relative humidity at 22°C ± 2 and 55% ± 10, respectively. There were approximately 20 air changes per hour (filtered on HEPA 99.97%). The rooms were illuminated by artificial lighting with a 12-hour circadian cycle (7 a.m. - 7 p.m.).

For the entire duration of the study the rats were kept in wire cages measuring 40.5x38.5x18h cm, with stainless steel feeders. The waste that dropped through the wire bottom on to a removable paper was periodically disposed of.

Five animals/sex were housed in each cage (in parts I and II) and two animals/sex in part III.

DIET AND WATER SUPPLY

The rats were fed a diet coded "4 RF 21 GLP Top Certificate" produced by the Charles River Italia's feed licensee Mucedola S.r.l., Settimo Milanese.

On the label, the contents, as declared by the Producer, were:

Moisture	12.00 %
Crude protein	18.50 %
Crude fat	3.00 %
Crude fiber	6.00 %
Ash	7.00 %

The Producer supplemented the diet with vitamins and trace elements.

According to the analytical certificates provided by the Supplier, the contents of the batches of diet used in this study were within ± 5% of the declared values and the presence and the levels of contaminants were within the limits proposed by EPA-TSCA (44FR : 44053-44093, July 26, 1979).

Animal feed, in compliance with RBM SOP's, is analyzed twice a year for bacterial contamination.

The diet was available "ad libitum" to the animals.

Filtered water was distributed by means of an automatic watering valve system.

The drinking water offered to the animals came from the municipal water main.

The water is periodically analyzed for microbiological count, for the presence of heavy metals, other contaminants (e.g. solvents, pesticides) and other physical and chemical properties.

The acceptance limits for the quality of drinking water are those defined in EEC Directive 80/778.

Contaminants that might interfere with the objectives of the study are not expected to be present either in the diet or in the water.

The analytical certificates of the animals' feed and water are filed at RBM premises.

ALLOCATION TO GROUPS AND IDENTIFICATION SYSTEM

The rats used in this study were selected by a randomization program from those purchased. Numbering of animals went from 1 to 10 for part I, from 11 to 20 for part II and from 21 to 32 for part III.

Each rat was numbered and individually identified by appropriately coloring different areas of limbs.

Each single cage bore a tag bearing indelible indications of experiment number, route of administration, dosage group, sex and date of administration.

The animals and the cages were numbered as follows:

Dosage administered	12000 mg/kg	Haematococcus pluvialis. unicellular green algae
Part I:		
Males:	No.s 1 - 5	cages 1
Females:	No.s 6 - 10	cages 2
Part II:		
Males:	No.s 11 - 15	cages 3
Females:	No.s 16 - 20	cages 4
Part III:		
Males:	No.s 21 - 22	cages 5
	No.s 23 - 24	cage 6
	No.s 25 - 26	cage 7
Females:	No.s 27 - 28	cages 8
	No.s 29 - 30	cage 9
	No.s 31 - 32	cage 10

PREPARATION OF THE TEST ARTICLE FORMULATES

On each treatment day, an exact amount of test article was weighed, ground into mortar with vehicle, transferred into a suitable graduated container and made up to final volume with vehicle to obtain the concentration requested.

The suspension was prepared just prior to the administration and was kept magnetically stirred until the end of administration.

METHOD OF ADMINISTRATION

The oral administration was done by gavage with appropriately gauged plastic syringes.

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TYPE AND FREQUENCY OF OBSERVATIONS AND EXAMINATIONS

CLINICAL OBSERVATIONS

MORTALITY AND CLINICAL SIGNS

Part I

All signs of ill health, together with any behavioral change or reaction to treatment were recorded frequently during the observation period (day of treatment).

Part II

Inspections for mortality were made twice a day.
All signs of ill health, together with any behavioral change or reaction to treatment were recorded twice a day during the 14-day observation period.
In particular, on the day of treatment, the animals were observed at 30 minutes and at 2, 4 and 6 hours from the administration.

BODY WEIGHT

The animals were weighed twice pre-trial (at randomization and on day 1, just before treatment) and afterwards on days 3, 8 and 14.

Administration volumes were based on the day 1 individual fasting (about 16 hours) body weight; feed was returned to animals about three hours after treatment.

POST-MORTEM EXAMINATIONS

GROSS PATHOLOGY

A thorough autopsy was performed on all rats of parts I and II of the study (after a fasting period of about 16 hours) killed at the end of the post-treatment observation period by excision of the femoral arteries after having been completely anesthetized with an i.p. injection of an overdosage of 5% sodium pentobarbital.

LD_{50} CALCULATION

The LD_{50} was not calculated.

RECORD FILING

The protocol, its errata-corrige, a reserve sample of the batch of the test article used, the raw data bound in registers numbered 950053/0 and 1, the final report and all other documents pertinent to the conduct of this study, including records and reports of maintenance, cleaning, calibration and inspection of equipment, will be filed at RBM premises for ten years from the issue date of this report and then sent to the Sponsor.

PROCEDURAL DETAILS

The study was conducted in accordance with the procedures described in the RBM Standard Operating Procedures (SOP's) collection.

Protection of animals used in the experiment is in accordance with Directive 86/609/EEC, enforced by the Italian D. L. No. 116 of January 27, 1992.

Physical facilities and equipment for accommodation and care of animals are in accordance with the provisions of EEC Council Directive 86/609.

The Institute is fully authorized by Competent Veterinary Health Authorities.

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RESULTS

CLINICAL OBSERVATIONS

MORTALITY CALCULATION *(TABLE 1)*

No animals died during the study. The LD_{50} was not calculated and it is higher than 12 g/kg.

CLINICAL SIGNS *(TABLE 2 AND APPENDIX 1)*

No clinical signs or behavioral alterations were noted during the observed period in any animal.

BODY WEIGHT *(APPENDIX 2)*

Body weight gain of all treated animals resulted unaffected by treatment.

POST-MORTEM EXAMINATION

GROSS PATHOLOGY *(TABLE 3 AND APPENDIX 3)*

Animals killed at the end of the study

No appreciable macroscopic findings were observed at the autopsy of the animals killed at the end of the study

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SUMMARY AND CONCLUSIONS

This study refers to the results obtained in an acute toxicity study in which CD (SD) BR rats were treated by oral route with the test article **Haematococcus pluvialis**.

The test article was given as a suspension in Intralipid® solution 20% at the maximum administrable dose 12 g/kg. The formulate was prepared just before treatment and administered at the volume of 40 ml/kg b.w. (two administrations of 20 ml/kg spaced at about 2 hours; concentration: 300 mg/ml).

The experimental group consisted of 5 males and 5 females.

Clinical observations, including clinical signs and body weight measurements, were carried out. A detailed gross pathology examination was performed on all the treated animals killed at the end of the 14-day post-treatment observation period.

No rats died during the study.

The LD_{50} was higher than 12 g/kg.

No clinical signs or behavioral alterations were noted during the observation period in any animal.

Body weight gain resulted unaffected by treatment.

At the autopsy performed at the end of the study there were no gross change.

In conclusion, the LD_{50} of **Haematococcus pluvialis** is higher than 12 g/kg.
No pathological changes were noted.

SEPT. 7, 1995

Mr. Enrico Gillio Tos
RBM Study Director

Dr. Roberto Maraschin
Scientific Director Recognized by the Italian Health Authorities as Responsible for General Toxicology Experimentation