EVALUATION OF TEST DEVICE PROTECTION IN PILOCARPINE INDUCED RAT MODEL OF EPILEPSY

Study Number: BRM-001-EF

Proposal Number: EFF2056B

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Regulatory status: Non-GLP

Notebook Reference: Notebooks # 1, 2

Study dates:

Study Initiation: 30.08.15

Study Termination: 03.09.15

Test Facility: Pharmaseed Ltd.

Hamazmera St. # 9

Ness-Ziona, 74047, Israel

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1. ABBREVIATIONS

AAALAC Association for Assessment and Accreditation of Laboratory Animal Care

BW Body weight

GLP Good Laboratory Practice

HEPA High Efficiency Particulate Air

IP Intraperitoneal

mg/kg milligram per kilogram

NA Not Applicable

OECD Organization for Economic Co-operation and Development

QA Quality Assurance

SOP Standard Operating Procedure

SE Status Epilepticus

TLE Temporal Lobe Epilepsy

2. STUDY INFORMATION

Study Title:	**Evaluation of Test Device Protection in Pilocarpine Induced Rat Model of Epilepsy**
Study Number:	BRM-001-EF
Key Personnel **Study Director:**	Michael Krakovsky MD, PhD. michael@Pharmaseedltd.com Tel : 08-9302771 Fax: 08-9302773
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Test Facility	Pharmaseed Ltd. Hamazmera # 9, Ness Ziona, 74047 Israel
Sponsor:	Alfa Rhythm Ltd Tel: (+972) 54-770-9405 www.alfarhythm.com Israel

3. QUALITY ASSURANCE

Pharmaseed's Quality Assurance periodically inspected the study, ensuring adherence to Pharmaseed's SOPs and the study protocol. Inspections included, but were not limited to, the protocol, study procedures, raw data, and the final report. Findings of all inspections were reported to the Study Director.

4. STUDY COMPLIANCE

The study was based on the OECD principles of Good Laboratory Practice ENV/MC/CHEM (98)17, however this study did not follow the complete GLP regulations and was thus considered a non-GLP study. The study follows the mutually agreed protocol and Pharmaseed's SOPs.

5. AMENDMENT/ DEVIATION PROCEDURES

This study was not ammeded and no deviation occurred from the original study plan

6. DISTRIBUTION

Hard Copies of Study Report are distributed to:

6.1 Sponsor (Controlled Copy)

6.2 Study Director (Original)

6.3 Study QA (Scanned electronic copy)

7. SAFETY PRECAUTIONS

Safety precautions regarding handling the animals consisted of: Gloves, lab coat, face mask shoe cover and goggles.

8. BACKGROUND

Epilepsy is the fourth most common severe neurological condition affecting approximately 65 million people worldwide. In the US, about 100,000 new cases of epilepsy are diagnosed annually. In the UK, between 1 in 140 to 1 in 200 people are currently being treated for epilepsy. Epidemiological studies suggest that between 70 and 80% of people developing epilepsy will go into remission, while the remaining patients continue to have seizures and are refractory to treatment with the currently available therapies. Epilepsies are characterized by spontaneous recurrent seizures, caused by focal or generalized paroxysmal changes in neurological functions triggered by abnormal electrical activity in the cortex. Approximately 50% of patients with epilepsy have partial epilepsy, which is often of temporal lobe origin. Temporal lobe epilepsy (TLE) is thus the most common form of partial epilepsy, probably affecting at least 20% of all patients with epilepsy. It is the most common form of drug-refractory epilepsy (Cavalheiro et al 2006). Atrophy of mesial temporal structures is well-known to be associated with TLE and hippocampal sclerosis, which is the most frequent histological abnormality in this form of epilepsy (Cavalheiro 1996). Pilocarpine-treated rats have become an important model of temporal lobe epilepsy. In its most common application, the model involves intraperitonealy administering a peripherally acting muscarinic acetylcholine receptor antagonist, such as methyl scopolamine, before a single high-dose of pilocarpine. Pilocarpine's actions include activation of M1 muscarinic receptors, which evokes status epilepticus. After surviving status epilepticus, rats begin displaying spontaneous seizures days later, and their epileptic condition is permanent. In the current study the pilocarpine model of epilepsy in rats was applied to assess Test Device protection.

9. RATIONALE FOR EXPERIMENT DESIGN

9.1 Principle of the test

Pilocarpine-treated rats are an important model of temporal lobe epilepsy. In its most common application, the model involves intraperitonealy administratio of a peripherally acting muscarinic acetylcholine receptor antagonist, such as methyl scopolamine, before a single high-dose of pilocarpine. Using this rat model of pilocarpine epilepsy, the Test Device was applied to assess its protection efficacy.

9.2 Rationale for test groups size:

A total of 10 male rats were utilized and divided into two groups: one control and one treated group, including five rats in each. The number of animals was the minimal number per group that might be sufficient to obtain indicative information.

9.3 Justification for the selection of test system

The species selected for the present study was the Sprague Dawley (SD) rat. Healthy adult male animals (about 290-325 g at study initiation) of this commonly used laboratory strain were used. The rat was the chosen species for the study, and has historically been used for for the defined objective by Pharmaseed and others.

10. OBJECTIVE

This study objective was to evaluate a possible therapeutic effect of Test Device in a pilocarpine model of epilepsy

10.1 Study end points

- Body weight
- Morbidity and mortality
- Severity of seizures score (at model establishment)
- Number and duration of spontaneous recurrent seizures
- Brains harvesting for histological evaluation (**optional**)

11. MATERIALS AND METHODS

11.1 Model Induction Material

Name:	Pilocarpine
Manufacturer:	Sigma
Batch/lot No.:	SLBH2677V
Batch Supplied by:	Sigma
Storage conditions:	RT
Expiry Date:	02.2019
Name to be used in the report:	Pilocarpine
Date of receipt at test facility:	04.08.15

11.2 Sponsor responsibility

The tested Device was supplied by the Sponsor and under his responsibility.

The Sponsor was responsible for supplying all Test Device's documentation, its safety instructions and operation manual.

11.3 Disposal of Materials

No materials requiring disposal.

12. EXPERIMENTAL MODEL

12.1 Animals

12.1.2 Species/Strain:

Rat/Sprague Dawley (SD)

12.1.3 Gender /Number /Age:

Male /10 /3 months

12.1.4 Source:

Harlan Laboratories, Israel

12.1.5 Body weight:

The average body weight was 332.9 g at study initiation in the two study groups (on Day 1). The minimal and maximal weight recorded in each group was within the range of ± 20 % of the group mean.

12.1.6 Acclimation period:

Not less than five days.

12.1.7 Identification

Three position ear notching and cage cards.

12.2 Animal Management

12.2.1 Housing

Animal handling was performed according to guidelines of the National Institute of Health (NIH) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals were housed in polyethylene cages (3/cage) measuring 35×30×15 cm, with stainless steel top grill facilitating pelleted food and drinking water in plastic bottle; bedding: steam sterilized clean paddy husk (Harlan, Sani-chip, Cat#: 7090C) were used and bedding material was changed along with the cage at least twice a week.

12.2.2 Diet

Animals were fed *ad libitum* a commercial rodent diet (Teklad Certified Global 18% Protein Diet cat #: 2018SC). Animals had free access to autoclaved and acidified drinking water (pH between 2.5 and 3.5) obtained from the municipality supply.

12.2.3 Contaminants

Food and water supplies were free of contaminants that could affect the study.

12.2.4 Environment conditions

Animals were housed under standard laboratory conditions, air conditioned and filtered (HEPA F6/6) with adequate fresh air supply (Minimum 15 air changes/hour). Animals were kept in a climate controlled environment. Temperatures range was 20–24°C and relative humidity range was 30–70% with controlled 12 hours light and 12 hours dark cycle.

12.3 Facility

Pharmaseed conducts its preclinical studies in Ness–Ziona Israel. The Pharmaseed facility includes a vivarium with a surgery suite and several procedure rooms.

12.4 Veterinary care

Animals were inspected upon arrival, and were found to be healthy and fit for the study.

12.5 Ethics Committee

This study was performed after approval by "The Israel Board for Animal Experiments" and in compliance with "The Israel Animal Welfare Act" , Approval Number **IL-15-07-239**.

13. EXPERIMENTAL DESIGN AND CONDITIONS

13.1 Animals Welfare

13.1.1 Pain reducing methods

The pilocarpine-induced model of epilepsy did not induce pain, therefore no analgesic treatment was needed. Animals were monitored daily.

13.1.2 Humane Endpoints:

None of the animals was found in a moribund condition or showed severe pain or enduring signs of severe distress.

13.2 Study Initiation and Termination Definitions:

Treatment day was defined as "DAY 1" of the study. Rats from all groups were monitored until Day 5. Upon completion of the last stimulation, rats were sacrificed.

See Table 1 for Group Allocation.

13.3 Allocation to Treatment Groups

Rats were allocated into one of the two study groups (n=5 in each) according seizures severity by Racine modified scale so that the groups were contain approximately similar scores.

Table 1: Group allocation

Group	Treatment
1M; N=5 (1, 2, 3, 4, 9)	Tested device
2M; N=5 (5, 6, 7, 8, 10)	Controls

13.4 Application of Test Devices

All animals were treated with the tested device attached to the head of restrained rats on 12 noon for 30 minutes daily during four consecutive days started one day post epilepsy induction. All rats were similarly restrained between 12 noon to 12:30 AM. .

13.5 Pilocarpine administration

To minimize the peripheral cholinergic side effects of pilocarpine, rats were injected with methyl scopolamine 30 min prior to pilocarpine (1 mg/kg in 0.9% NaCl; IP). Rats were then injected with pilocarpine (350 mg/kg; IP).

13.6 Tests and evaluation

13.6.1 Body weight

Body weight was monitored prior to the treatment and before study termination.

13.6.2 Mortality and morbidity

Mortality and morbidity were monitored daily until study termination.

13.6.3 Severity and duration of the seizures:

Animals were monitored for four consecutive days to assess the number and duration of the recurrent seizures.

13.6.4 Animals Sacrifice, tissue fixation and histological evaluation

On Study Day 6 animals were sacrificed. Brains were harvested and fixed in 4% PFA for future histological evaluation, only according Sponsor’s instructions.

13.6.5 Statistical analysis:

Numerical results were given as means ± standard errors. If applicable, statistical analysis was carried out using the two-way or one-way ANOVA followed by Bonferroni post-hoc test. A probability of 5 % ($p \leq 0.05$) was regarded as significant. In the figures, the degree of statistically significant differences between groups.

14. RESULTS

Altogether 10 rats were treated by pilocarpine.

14.1 Mortality and animals exlusion

One rat # 9 died one day after pilocarpine administration. Two (2) rats died durind the study. Rat # 3 from the group 1M and rat # 6 from the group 2M. Total mortality of 30% (3/10), which is common in these pilocarpine studies.

14.2 Body Weight

During the study no differences in body weight were observed between the two animal groups. (Figure 1 and Post Text Table 1).

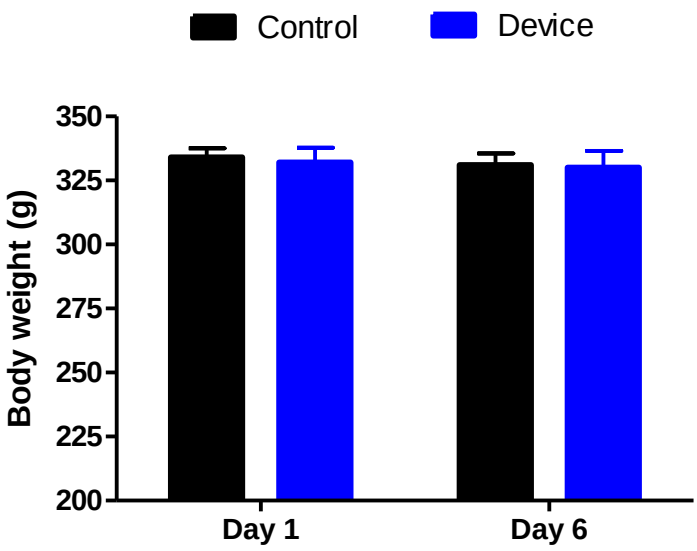

Figure 1 Body weight in grams during the study

No statistically significant differences between the groups were observed.

14.3 Severity and duration of the seizures:

Most of the recorded seizures during the the study were graded at the two milder Stages of Racine scale, namely Stages 1 and 2. However, the effect of the device was mostly observed on the two more sever Stages 3 and 4. Following the tested device application, a decrease in facial clonus (Stage 1 by Racine scale) compared to non-treated controls was observed on Days 1 and 3 but not on Days 2 and 4. (Figure 2 and Post Text Table 2 in Appendix I). A decrease in facial clonus, rhythmical head nodding (Stage 2 by Racine scale) was observed only on Day 1 following the tested device application (Figure 2a and Post Text Table 2 in Appendix I). A decrease in facial clonus, rhythmical head nodding front limb clonus (Stage 3 by Racine scale) was observed on all study days following the tested device application (Figure 2b and Post Text Table 2 in Appendix I). A decrease in facial clonus, rhythmical head nodding front limb clonus hind limb standing up, jerks (Stage 4 by Racine scale) was observed on Days 1, 2 and 4 following the tested device application (Figure 2c and Post Text Table 2 in Appendix I), since on Day 3 no Stage 4 seizures were recorded in both groups.

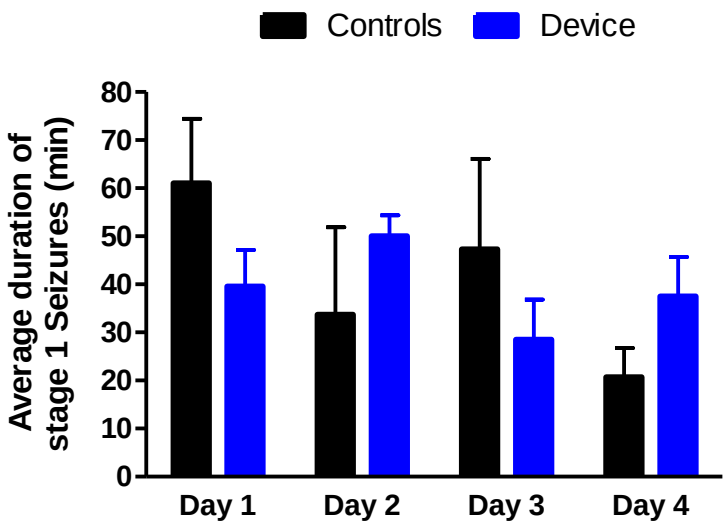

Figure 2 Duration of Stage 1 seizures (in minutes) by days

Two-way ANOVA followed by Bonferroni post-hoc comparisons revealed no statistically significant difference between the groups.

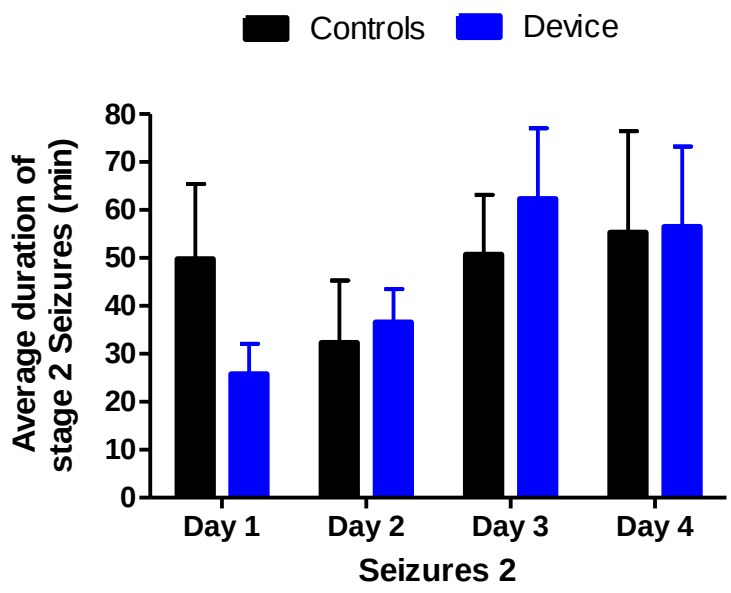

Figure 2a Duration of Stage 2 seizures (in minutes) by days

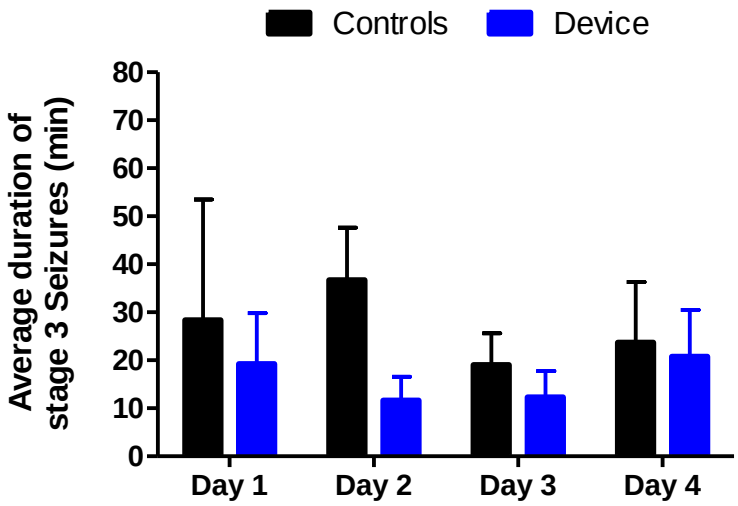

Figure 2b Duration of Stage 3 seizures (in minutes) by days

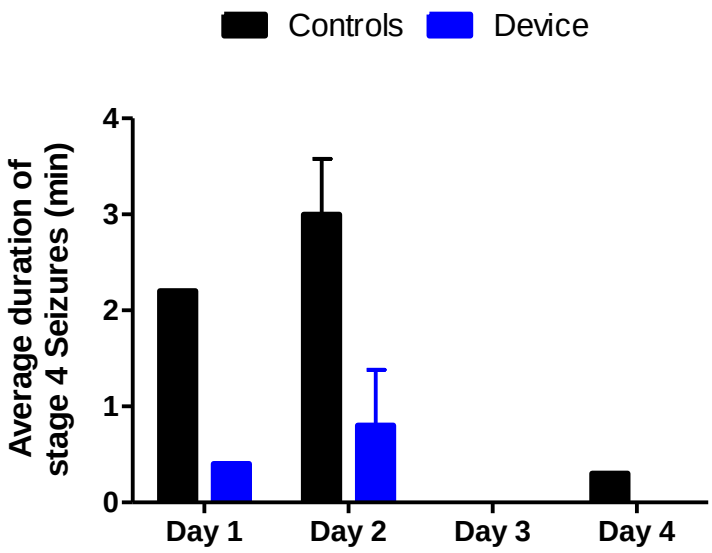

Figure 2c Duration of Stage 4 seizures (in minutes) by days

In all the above figures, two-way ANOVA followed by Bonferroni post-hoc comparisons revealed no statistically significant difference between the groups.

In order to examine the individual behavior of the rats in the study, the response to Stage 3 and 4 seizures were depicted by study days on Figure 3 and Figure 3a respectively.

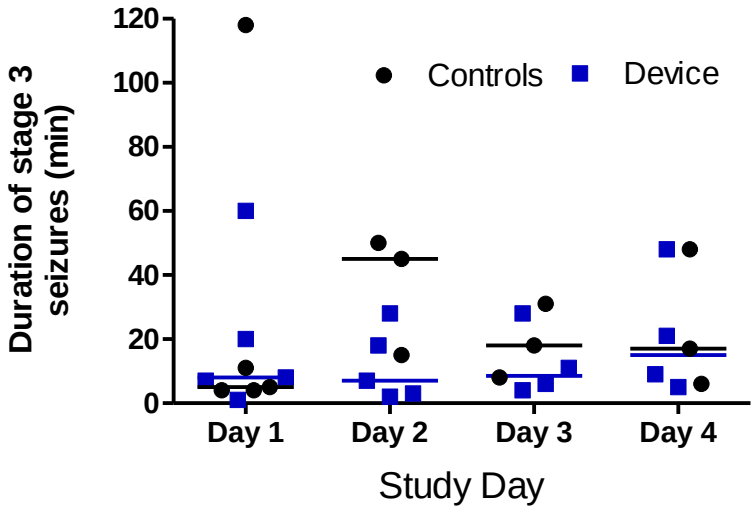

Figure 3 Individual duration of Stage 3 seizures in minutes and median values by days

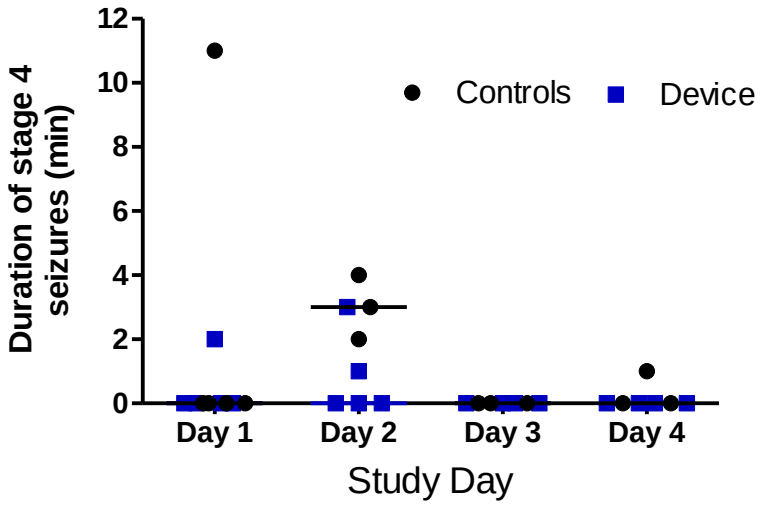

Figure 3a Individual duration of Stage 4 seizures (in minutes) by days

The small sample of animals in the study imposed a great impediment on reaching conclusions. It seems however that the largest effect was recorded on Day 2 of the study.

15. DISCUSSION AND CONCLUSIONS

The pilocarpine model of epilepsy in rats was applied in order to evaluate the Test Device protection potential against temporal lobe pilocarpine-induced seizures.

Stimulation by the tested device decreased the duration of the seizures on all study days mostly for the more sever seizures (Stages 3 and 4 on Racine scale). Since the seizures develop with time after pilocarpine exposure, the most pronounced effect was recorded on Day 2. Nevertheless, this therapeutic effect did not reach statistical significance, mostly due to the small number of animals in this study groups.

CONCLUSIONS:

Stimulation by the tested device indicated a possible therapeutic potential of the device for the treatment of epilepsy.

16. REFERENCES

1. Borges K, Gearing M, McDermott DL, Smith AB, Almonte AG, Wainer BH, Dingledine R. Neuronal and glial pathological changes during epileptogenesis in the mouse Pilocarpine model. Exp Neurol. 2003; 182:21–34.
2. Buckmaster PS, Lew FH. Rapamycin suppresses mossy fiber sprouting but not seizure frequency in a mouse model of temporal lobe epilepsy. J Neurosci. 2011; 31:2337–2347.
3. Buckmaster PS, Wen X. Rapamycin suppresses axon sprouting by somatostatin interneurons in a mouse model of temporal lobe epilepsy. Epilepsia. 2011; 52:2057–2064.
4. Cavalheiro EA, Naffah-Mazzacoratti MG, Mello LE, Leite JP. The Pilocarpine model of seizures. In: Pitkänen A, Schwartzkroin PA, Moshé SL, editors. Models of Seizures and Epilepsy. New York: Elsevier; 2006. pp. 433–448.
5. Cavalheiro EA, Santos NF, Priel MR. The Pilocarpine model of epilepsy in mice. Epilepsia. 1996; 37:1015–1019.
6. Cavalheiro EA, Silva DF, Turski WA, Calderazzo-Filho LS, Bortolotto ZA, Turski L. The susceptibility of rats to Pilocarpine-induced seizures is age-dependent. Dev Brain Res. 1987; 37:43–58.
7. OECD principles of Good Laboratory Practice ENV/MC/CHEM (98)17.
8. Pharmaseed SOPs
9. Racine R.J., Gartner J.G., Burnham W.M. Epileptiform activity and neural plasticity in limbic structures. Brain Res. 1972; 47(1):262-268.

17. ARCHIVING

The following items will be retained in the Document archive for a period of one year (For non-GLP studies). At the end of that time period the Sponsor will be contacted and requested to either receive the archived material, agree in writing to its disposal or left it in Pharmaseed archive for an annual fee.

The following documentation will be archived:

- Study plan and study plan amendments/deviations.
- Final report.
- Study Book.
- Original Test Results (if applicable).
- Miscellaneous study data (if applicable).
- Relevant correspondence

- QA inspections.
- Cage cards
- Slides (if applicable)
- Paraffin Blocks (if applicable)

Archive samples/specimens will be stored in the Samples Archive cupboard at Pharmaseed's laboratory until one month after study termination. At the end of this period the Sponsor will be contacted to determine by joint agreement continued storage, submission to the Sponsor or destruction of samples.

APPENDICES

Appendix I

Raw Data Tables

Post Text Table 1: Body Weight (in gr) by Study Day and by Study Group

Group #	Rat #	D -5	D 1	D 6
1M	1	315	335	336
1M	2	312	334	335
1M	3	313	339	NA
1M	4	307	320	322
1M	9	314	340	NA
	AVE	**312.2**	**333.6**	**331.0**
	SD	**3.1**	**8.0**	**7.8**
2M	5	285	309	312
2M	6	319	340	NA
2M	7	315	337	329
2M	8	311	334	337
2M	10	316	341	342
	AVE	**309.2**	**332.2**	**330.0**
	SD	**13.8**	**13.3**	**13.1**

Marked in yellow are rats that died during the study.

Post Text Table 2: Severity and duration in min of the seizures by study days

Group #	Rat #	Day 1				Day 2			
Grade		1	2	3	4	1	2	3	4
1M	9	90	16	4	0	NA	NA	NA	NA
1M	1	45	65	5	0	15	50	45	4
1M	2	35	75	11	0	16	40	50	3
1M	4	45	77	118	11	70	7	15	2
1M	3	90	16	4	0	NA	NA	NA	NA
	AVE	61.0	49.8	28.4	2.2	33.7	32.3	36.7	3.0
	SD	26.8	31.2	50.2	0.0	31.5	22.5	18.9	1.0
2M	5	52	25	8	0	54	46	7	0
2M	6	56	41	7	0	40	45	28	3
2M	7	45	4	1	2	45	52	18	0
2M	8	15	24	60	0	46	21	3	0
2M	10	30	35	20	0	65	19	2	1
	AVE	39.6	25.8	19.2	0.4	50.0	36.6	11.6	0.8
	SD	16.9	14.1	23.8	0.0	9.8	15.4	11.1	1.3

Post Text Table 2: Severity and duration of the seizures by days (Continue)

Group #	Rat #	Day 3				Day 4			
Grade		1	2	3	4	1	2	3	4
1M	9	NA	NA	NA	NA	NA	NA	NA	NA
1M	1	16	71	31	0	10	95	48	0
1M	2	81	28	8	0	21	23	17	0
1M	4	45	53	18	0	31	48	6	1
1M	3	NA	NA	NA	NA	NA	NA	NA	NA
	AVE	47.3	50.7	19.0	0	20.7	55.3	23.7	0.3
	SD	32.6	21.6	11.5	0	10.5	36.6	21.8	0.0
2M	5	30	21	11	0	47	12	48	0
2M	6	NA	NA	NA	NA	NA	NA	NA	NA
2M	7	12	76	28	0	34	63	21	0
2M	8	21	89	6	0	16	93	9	0
2M	10	51	63	4	0	53	58	5	0
	AVE	28.5	62.3	12.3	0	37.5	56.5	20.8	0
	SD	16.7	29.5	10.9	0	16.4	33.5	19.4	0