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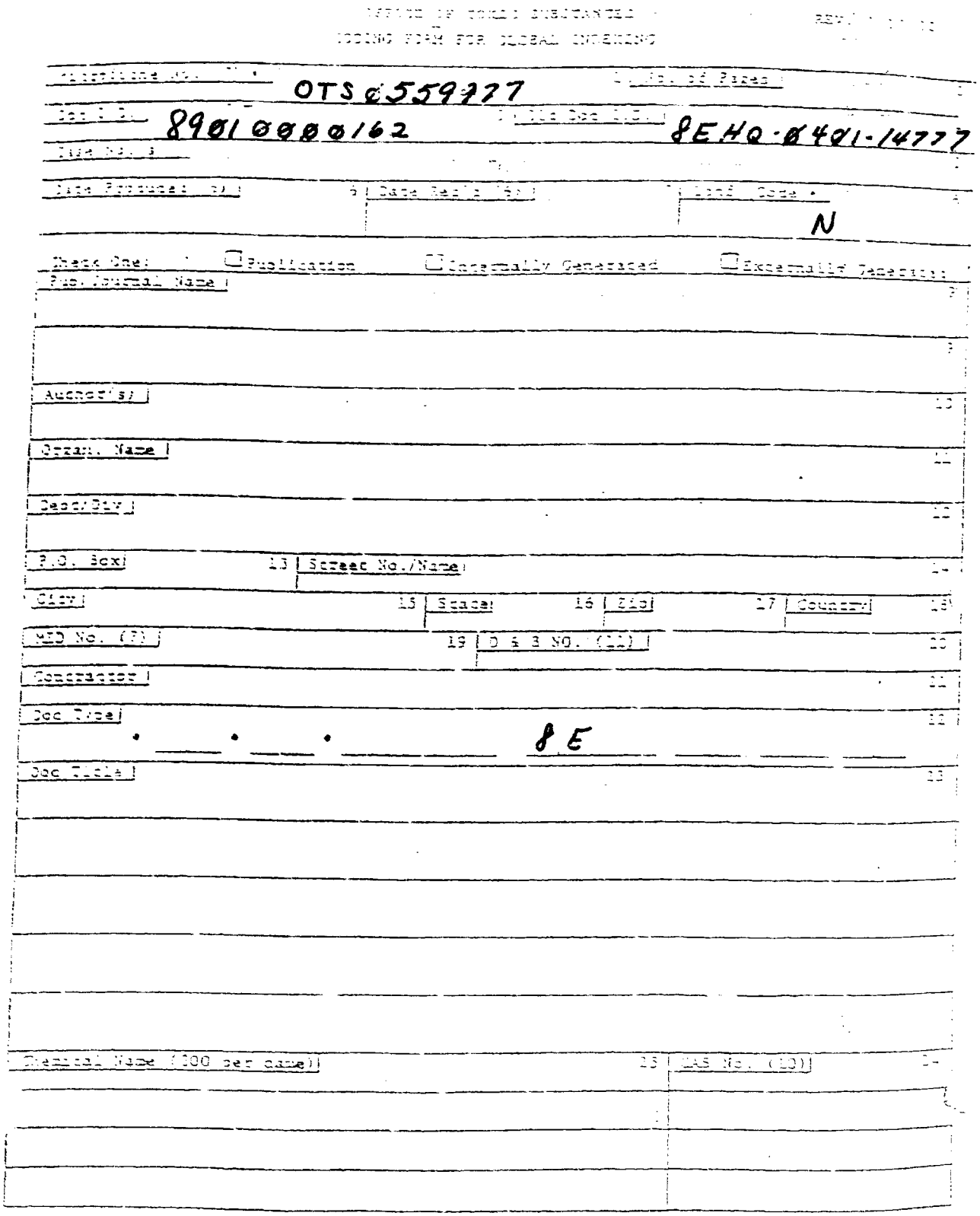

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April 27, 2001

TSCA Document Control Officer (7408)
Office of Pollution Prevention and Toxics
US Environmental Protection Agency
1200 Pennsylvania Ave NW
Washington, DC 20460

Attn: TSCA Section 8(e) Coordinator

RE: **Propylene Glycol Tertiary Butyl Ether (PTB) Study of Reproductive Function and Fertility in the CD Rat by Oral Gavage Administration**
8EHQ Number: 8EHQ-00-14777

Dear Sir or Madam:

Enclosed, on behalf of Lyondell Chemical Company, please find a final report entitled **Propylene Glycol Tertiary Butyl Ether (PTB) Study of Reproductive Function and Fertility in the CD Rat by Oral Gavage Administration**. Lyondell is submitting this report as follow-up to a TSCA Section 8(e) notification (8EHQ Number: 8EHQ-00-14777) based on preliminary data from the study.

Should you have any questions or require additional details please do not hesitate to call me at 713/309-2136. I may also be reached by facsimile at 713/951-1574 or by e-mail at patrick.gibson@lyondell.com.

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Sincerely,

Patrick L. Gibson
Product Safety Specialist - Regulatory
Corporate TSCA Coordinator
Lyondell Chemical Company/Equistar Chemicals, LP

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8EHQ-00-14777

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Huntingdon
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PTB

STUDY OF REPRODUCTIVE FUNCTION AND FERTILITY

IN THE CD RAT BY ORAL GAVAGE ADMINISTRATION

Volume 1

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PTB

STUDY OF REPRODUCTIVE FUNCTION AND FERTILITY IN THE CD RAT BY ORAL GAVAGE ADMINISTRATION

Volume 1

Sponsor

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Final: 23 February 2001

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COMPLIANCE WITH GOOD LABORATORY PRACTICE

PTB

STUDY OF REPRODUCTIVE FUNCTION AND FERTILITY

IN THE CD RAT BY ORAL GAVAGE ADMINISTRATION

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

The United Kingdom Good Laboratory Practice Regulations, 1997 (Statutory Instrument No 654) and, from 14 December 1999, the United Kingdom Good Laboratory Practice Regulations, 1999 (Statutory Instrument No 3106).

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No L 77/8).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

S Fulcher

23 February 2001

S.M. Fulcher, B.A., F.I.A.T
Study Director,
Huntingdon Life Sciences Ltd.

Date

A 10

QUALITY ASSURANCE STATEMENT

PTB

STUDY OF REPRODUCTIVE FUNCTION AND FERTILITY

IN THE CD RAT BY ORAL GAVAGE ADMINISTRATION

The following inspections and audits have been carried out in relation to this study

Study Phase	**Date of Inspection**	**Date of Reporting**
Protocol Audit	29 November 1999	29 November 1999
Study Based Inspections		
Dose Preparation	16 March 2000	16 March 2000
Dosing / Post dose observations	20 March 2000	20 March 2000
Necropsy - Adults	17 July 2000	17 July 2000
Report Audit	21 February 2001	21 February 2001

Protocol Audit: An audit of the protocol for this study was conducted and reported to the Study Director and Company Management as indicated above.

Study based inspections: Inspections of phases of this study were conducted and reported to the Study Director and Company Management as indicated above.

Process based inspections: At or about the time this study was in progress inspections of other routine and repetitive procedures employed on this type of study were carried out. These were promptly reported to appropriate Company Management.

Report Audit: This report has been audited by the Quality Assurance Department. This audit was conducted and reported to the Study Director and Company Management as indicated above.

The methods, procedures and observations were found to be accurately described and the reported results to reflect the raw data.

N Jolly

23 FEBRUARY 2001

N. Jolly, B.Sc.
Group Manager
Department of Quality Assurance
Huntingdon Life Sciences Ltd.

Date

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CONTRIBUTING SCIENTISTS

PTB

STUDY OF REPRODUCTIVE FUNCTION AND FERTILITY

IN THE CD RAT BY ORAL GAVAGE ADMINISTRATION

STUDY MANAGEMENT

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Study Director

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Study Supervisor

MATING

M.J. Collier, B.Sc.
Senior Scientist, Neurobehavioural Studies

FORMULATION CHEMISTRY

A.D. Clemson, B.Sc., M.Sc., Ph.D., C.Chem., F.R.S.C.
Scientific Manager

A 12

SUMMARY

This report describes a study performed to assess the influence of PTB, an industrial chemical, on gonadal function, mating behaviour and fertility in sexually mature male and female rats of the CD strain, and on growth and survival of their offspring up to weaning (Day 21 of age).

For this purpose, PTB was administered by oral gavage at dosages of 100, 300 and 1000 mg/kg/day to groups of 24 male and 24 female rats. Males were treated for 71 days before pairing until termination and female animals from 15 days before pairing until termination, including gestation and lactation phases. A Control group of 48 males and 48 female rats were administered the vehicle, water obtained by reverse osmosis, over the same treatment period.

The parental males were killed after the majority of females had littered and were subjected to a macroscopic necropsy, including weighing of the reproductive organs and, for Control and high dosage animals, assessment of sperm motility. Parental females were killed on Day 27 after birth and subjected to macroscopic necropsy, including weighing of the reproductive organs and implantation count. Offspring reared to weaning were killed on Day 21 of age and subjected to macroscopic necropsy.

Exposure of the offspring to the test material was indirect; in *utero* and via the milk.

Results

Adults

There were no deaths on the study that were considered to be related to treatment. Clinical signs attributed to treatment were restricted to transient post-dosing salivation in both sexes at 1000 and 300 mg/kg/day and occasional males on isolated occasions at 100 mg/kg/day. The incidence of this finding showed a clear dosage-relationship and is considered likely to reflect distaste of the test formulation.

There was no adverse effect of treatment on bodyweight gain of males throughout the study or females during the pre-pairing, gestation or lactation phases. Bodyweight gain at 1000 mg/kg/day was marginally superior to the Control during lactation, confirming that they were in good condition during this phase of the study.

There was no clear adverse effect on food intake of either sex prior to pairing or for females during gestation and lactation.

Oestrous cycle regularity, mating performance and fertility, gestation and parturition were not obviously affected by treatment. Pregnancy rate was high in all treated groups with uneventful gestation and parturition.

Macroscopic necropsy examination of both sexes, including assessment of reproductive organ weights and for high dosage males sperm motility, did not reveal any adverse effect of treatment.

Litter data

Mean litter size at birth was similar for all groups but at 1000 mg/kg/day offspring bodyweight at birth and subsequent growth to weaning was lower than Control. A slight decrease in the live birth index (survival over 1 day post parturition) was noted in the 1000 mg/kg/day group (96.4%) when compared to the Control group value (99.3%). The viability index (survival to Day 4 post parturition) was also decreased in the 1000 mg/kg/day group (91.3%) when compared to the Control group value (96.2%); however, this difference was not statistically significant. Survival from Day 4 of age to weaning was comparable to Control.

At 300 and 100 mg/kg/day there was no adverse effect of treatment on the number of implantations, litter size at birth or subsequent survival to weaning, bodyweight at birth or bodyweight gain to weaning.

Macroscopic necropsy examination of offspring dying during the study or killed following weaning on Day 21 of age did not reveal any adverse effect of treatment at any dosage.

Conclusion

Oral gavage administration with PTB at dosages up to 1000 mg/kg/day, for 10 weeks prior to pairing in adult male rats and 2 weeks prior to pairing in females, was well tolerated by parent animals and had no adverse effect on their fertility or general reproductive performance. This dosage is therefore considered to be the no-effect-level (NOEL) for the parent females

Effects in the offspring of dams exposed to 1000 mg/kg/day included slightly lower birth weights, reduced weight gains from birth to weaning, and a slight decrease in postnatal survival. Therefore, in this study, direct treatment of the parents at 300 mg/kg/day is considered to be the no-effect-level (NOEL) for growth and development of the offspring to weaning.

INTRODUCTION

Objective

The objective of this study was to assess the influence of daily oral (gavage) administration of PTB, an industrial chemical, on gonadal function, mating behaviour and fertility in sexually mature male and female rats of the CD strain, and on growth and survival of their offspring up to weaning (Day 21 of age).

The study was conducted in compliance with Good Laboratory Practice principles, and in accordance with the following regulatory guideline:

OECD Guideline for Testing of Chemicals 415, One-Generation Reproduction Toxicity Study adopted 26 May 1983.

Justification for the test system

The rat was chosen because it is widely accepted as a predictor of reproductive change in man and satisfies the requirement for a rodent species by regulatory agencies. The CD strain was used because of the background control data available in this laboratory.

Justification for the treatment regimen

PTB was administered by the oral gavage route to simulate a potential route of human exposure.

The dosages of 100, 300 or 1000 mg/kg/day were selected in collaboration with the Sponsor following a preliminary investigation of reproductive function and fertility in the rat (HLS Report No.: ACR034/000040). In this preliminary study treatment at 1000 mg/kg/day produced no indication of toxicity to parental animals and only equivocal evidence of a minimal effect on offspring growth and survival. The high dosage of 1000 mg/kg/day represents the limit dose commonly employed in studies of this design.

Because of a change in diet used in these laboratories between the conduct of the preliminary and main studies, a double sized Control group was employed to increase the power of within study comparisons.

Study organisation

Testing facilities:

The testing laboratory was:

Huntingdon Life Sciences
Eye
Suffolk
IP23 7PX
England

Study timing:

F0 males arrived	:	15 March 2000
F0 females arrived	:	10 May 2000
Treatment of F0 males commenced	:	20 March 2000
Treatment of F0 females commenced	:	15 May 2000
Pairing	:	29 May 2000
Offspring necropsy completed	:	27 July 2000
Adult necropsy completed	:	2 August 2000

B 02

MATERIALS AND METHODS

Animals

A total of 132 adult virgin male and 132 adult virgin female rats of the Crl: CD® (SD) IGS BR strain (of Sprague-Dawley origin) were obtained from Charles River (UK) Limited, Margate, Kent, England. They were allowed 5 days acclimatisation during which time they were inspected daily to check their physical condition. Before treatment animals were allocated to treatment groups, using stratified bodyweight ranges; surplus animals, at the extreme of the bodyweight ranges, were discarded. This procedure ensured that all groups contained populations of rats with similar initial mean and range of bodyweights. Individual animals were assigned a number and identified by tail tattoo.

At commencement of treatment, animals selected for the study were in the weight range 184 to 238g for males and 209 to 247g for females (approximately 6 to 7 and 10 to 11 weeks of age respectively).

Environmental control

The animals were housed inside a barriered, limited access, rodent facility.

The facility was designed to minimise the entry of external biological and chemical agents and to minimise the transference of such agents between rooms. Access was limited to authorised personnel who were required to shower and change into clean protective clothing. Where practicable, materials and equipment entered the facility through a chamber in which their external surfaces were disinfected. Before the study commenced the room was cleaned and disinfected with a bactericide.

The animal room was kept at positive pressure with respect to the outside by its own supply of filtered fresh air which was passed to the atmosphere and not recirculated, providing approximately 15 room air changes per hour. The temperature and relative humidity (RH) in the animal room were controlled and target ranges were 19-25°C and 40-70% RH respectively; the achieved range being 19.5-23°C and 41-63% RH respectively. Periodic checks were made on the number of air changes in the animal rooms and temperature and humidity were monitored daily. Since these data show that there were no significant deviations from target values they are not presented.

Lighting was controlled to provide a 12-hour light: 12-hour dark cycle with the lights on at 6.00 GMT. Alarms were set to activate if there was any failure of the ventilation system, or if temperature limits were exceeded. A stand-by electricity supply was available which was automatically brought into operation in the event of public supply failure.

Caging

Depending upon the phase of the study, rats were housed in TR18 cages from Arrowmight Biosciences, Hereford, England and RB3 or RB3 modified cages from North Kent Plastic Cages Limited, Erith, Kent, England. The cages consisted of stainless steel (TR18) or high-density polypropylene (RB3 and RB3 modified) bodies with lids of stainless steel grid. TR18 and RB3 modified cages had stainless steel floors and were suspended in batteries over trays covered with absorbent paper which was replaced twice weekly or daily during pairing. RB3 cages had solid polypropylene floors and autoclaved wood shavings were provided as bedding, renewed at least twice weekly.

The cages were distributed on the racking to equalise, as far as possible, environmental influences amongst the groups. Colour coded cage labels identified the occupants by experiment, treatment group, sex and animal number.

At various stages of the study the maximum number of rats per cage was:

Stage	Number of rats M	Number of rats F	Cage type
Acclimatisation	Up to 4	Up to 4	TR18
Pre-mating (F0)	Up to 4	Up to 4	TR18
Mating	1	1	RB3 modified
Males after mating to termination	Up to 4	-	TR18
Females during gestation (up to Day 17 after mating)	-	1	RB3 modified
Females littering (from Day 17 after mating to Day 14-18 of lactation)	-	1 + litter	RB3
Females during lactation (Day 14-18 of lactation until weaning)	-	1 + litter	TR18

Diet, bedding and water supply

The animals were allowed free access to a commercially available pelleted laboratory animal diet (VRF-1C) from Charles River UK, Margate, Kent, England. Each batch of diet was routinely analysed by the supplier (who provided a Certificate of Analysis with every batch) for various nutritional components and chemical and microbiological contaminants. Supplier's analytical certificates were scrutinised and approved before any batch of diet was released for use. The diet contained no added antibiotic or other chemotherapeutic agent.

Water was taken from the public supply (Essex and Suffolk Water plc, Chelmsford, Essex, England) was freely available, via polythene or polycarbonate bottles fitted with sipper tubes. The quality of the water supply is governed by regulations published by the Department of the Environment. Certificates of analysis were routinely received from the Supplier.

Autoclaved wood shavings (Lignocel type 3/4 wood flakes) were provided as bedding for the females and their litters. The supplier (RS. Biotech, Finedon, Northants, England) provided a Certificate of Analysis with every batch.

Since the results of these analyses did not provide evidence of contamination that might have prejudiced the study they are not presented. No other specific contaminants that were likely to be present in the diet, water or bedding were analysed, as none that may have interfered with or prejudiced the outcome of the study was known.

Copies of the relevant analyses have been filed in the archives of Huntingdon Life Sciences.

Test material

Approximately 4.75kg of a 20 litres supply of PTB (also known as Arcosolv ® PTB Propylene Glycol t-Butyl Ether), an industrial chemical, (Lot No. SC980216JO6/218047) received at Huntingdon Life Sciences, Huntingdon, on 23 November 1999 (transferred to ERC Pharmacy on 24 November 1999) was used on the study. The test material, a colourless liquid, was stored at 4°C under Nitrogen in the dark. The stated purity of the test material was 99.7 % (see Sponsor's certificate of analysis:

Before use, the identity, strength, purity, composition, or other characteristics, which appropriately defined the batch from which the test material for this study was drawn, were determined by the Sponsor. Stability of the test material and methods of synthesis, fabrication or derivation were documented by the Sponsor.

Before the consignment of the test material was used, a representative 1g sample was taken. This was stored in the archives at Huntingdon Life Sciences Ltd., Eye under the conditions specified for the bulk supply of the test material.

All dosages and concentrations are expressed in terms of the material as supplied.

Formulation

Formulations were prepared on a weekly basis as an assumed solution in purified water (obtained by reverse osmosis). For the highest concentration the required quantity (by weight) of PTB was mixed in an appropriate volume of water. For the intermediate concentration an appropriate volume of this formulation was taken and diluted with water to give the required concentration. Similarly, for the low concentration, an appropriate volume of the intermediate dose formulation was diluted to give the required concentration. The final concentrations achieved were chosen to give a constant dosage volume of 10ml/kg bodyweight for all groups. Following preparation, these bulk formulations were divided into daily aliquots and stored at 4°C until use. All were clear and colourless.

Group	Treatment	Dosage (mg/kg/day)	Concentration (mg/ml)	Dose volume (ml/kg)
1	Control	0	0	10
2	PTB	100	10	10
3	PTB	300	30	10
4	PTB	1000	100	10

Stability of PTB at concentrations of 1 and 100 mg/ml in aqueous formulations during storage at 4°C was confirmed for up to 15 days during this study. The experimental details and the results obtained for these analytical investigations are presented in the Formulation Chemistry Report (Addendum 2). Samples of each formulation prepared for administration on one occasion during Weeks 1 (commencement of treatment for males), 11 (pairing/early gestation) and 14 of treatment (late gestation/lactation) were analysed for achieved concentration of the test material. Results of these analyses are also presented in the Formulation Chemistry Report.

Treatment

Male animals were dosed from 71 days before pairing until termination and female animals from 15 days before pairing until termination by the oral gavage route. The volume administered to each animal was calculated by the most recently recorded scheduled bodyweight, except that for females the dose volume was fixed from Day 17 after mating until parturition had occurred. For a number of females the volume was adjusted in error on Day 20 after mating. This deviation from the protocol resulted in 16/48, 12/24, 9/24 and 11/23 females in Groups 1 to 4 receiving slightly more than the intended dose; this had no detrimental impact on the outcome of the study. Females found to be in parturition at the time of dosing were not dosed for that day.

Control animals all received the vehicle at the same dosage over the same treatment period.

Formulations were stirred using magnetic stirrer before and throughout the dosing procedure. A daily record of the usage of formulation was maintained based on weight used. This balance was compared with the expected usage (based on volume) as a check of correct administration. No significant discrepancy was found.

Constitution of the Groups

The 240 allocated animals were assigned to four groups as follows:

Group	Treatment	Dosage (mg/kg/day)	Number of Animals		Animal identity numbers	
			M	F	M	F
1	Control	0	48	48	1-48	121-168
2	PTB	100	24	24	49-72	169-192
3	PTB	300	24	24	73-96	193-216
4	PTB	1000	24	24	97-120	217-240

SERIAL OBSERVATIONS (ADULTS)

Mortality

One animal (No. 236) was killed for reasons of animal welfare during the study; it was subjected to a thorough macroscopic examination of the visceral organs and specimens of abnormal tissues were retained. As the decision to sacrifice the animal was taken outside normal working hours the animal was killed and then stored overnight at 4°C prior to necropsy.

Clinical signs

All animals were observed at least twice daily throughout the study and any visible signs of reaction to treatment or ill health were recorded, with details of type, severity, time of onset and duration. A more detailed weekly physical examination was also performed throughout the treatment period.

In addition, detailed observations were made in association with dosing according to the following schedule:

Schedule: Daily during first week of treatment.
Twice a week (middle and end of each week) - Weeks 2-4 of treatment.
Once a week - Weeks 5 onwards.

Bodyweight

Males were weighed at twice weekly intervals throughout the study and before necropsy.

Females were weighed at twice weekly intervals until evidence of mating was detected, and then on Days 0, 3, 6, 9, 13, 17 and 20 after mating and then on Days 1, 4, 7, 10, 14, 17 and 21 of the lactation period, and before necropsy. Five Control females were not weighed on Day 0 of gestation in error and were subsequently weighed on Day 1 of gestation when this error became apparent. Day 1 data for these animals are presented but have not been used in the calculation of group values. It is not considered that this deviation from protocol has had a detrimental impact on the outcome of the study.

Food consumption

Food consumption was recorded on a cage basis (four animals per cage unless reduced by mortality) for males and females weekly before being paired for mating. Food consumption for females after mating was recorded on an individual basis for Days 0-5, 6-12, 13-19 after mating and Days 1-3, 4-6, 7-13, and 14-20 of lactation.

As similarly described for bodyweights, food consumption for a small number of Control females was not commenced on Day 0 of gestation in error and was subsequently commenced on Day 1 of gestation when this error became apparent. Data collected for the period 1-5 has been presented and used in the calculation of group values. It is not considered that this deviation from protocol has had a detrimental impact on the outcome of the study.

Oestrous cycles

Dry vaginal smears were taken from all females fifteen days prior to pairing in order to assess the regularity and duration of oestrous cycles.

Wet vaginal smears were taken from all females following pairing until evidence of mating was confirmed.

ASSESSMENT OF REPRODUCTIVE PERFORMANCE

Mating procedure

After their appropriate treatment periods, males and females were paired on a one-to-one basis within treatment groups. The male whose designated female partner had died was not paired. Each morning following the start of the pairing phase, the trays beneath the cages were checked for ejected copulation plugs and a vaginal smear was prepared for each female and examined for the presence of spermatozoa. The day on which evidence of mating was detected was designated as Day 0 of gestation. Once mating had been confirmed, the females were separated from the males and vaginal smearing was discontinued. The one female which failed to mate after two weeks of being paired with it's original partner was paired with another male from that group which had already mated.

The pre-coital interval was calculated for each female as the time between initial pairing and evidence of mating.

Parturition and duration of gestation

All females were permitted to deliver and raise their young naturally. From Day 20 after mating, the females were inspected three times daily for the onset, progress and completion of parturition. Individual gestation lengths were calculated from the day mating occurred and the day parturition commenced.

POST-NATAL OBSERVATIONS (OFFSPRING)

Observations at Day 1 of age

All offspring were examined at approximately 24 hours after birth (Day 1) and the number of offspring born (live and dead) was recorded. Live offspring were individually identified within each litter by toe tattoo and individual bodyweight, sex and any clinical observations were recorded.

Clinical signs

Litters were observed daily for evidence of abnormal appearance or behaviour.

Mortality and litter size

Daily records were maintained of mortality and consequent changes in litter size. Wherever possible, any offspring found dead were preserved in industrial methylated spirit prior to an external and internal necropsy examination.

On Day 4 of age, litters containing eleven or more offspring were reduced to ten by random culling, leaving wherever possible, five male and five female pups in each litter. Necropsy examination of culled offspring was restricted to those showing external abnormalities.

Sex ratio

The offspring were sexed at weighing on Day 1 of age and sexes were confirmed on Days 4 (before and after culling) and 21 of age.

Bodyweight

The F1 offspring were weighed individually on Days 1, 4 (before culling), 7, 14 and 21 of age. Mean values for each sex are presented for all litters.

TERMINAL STUDIES

Adult males

When the majority of F0 females had littered, the males were killed by inhaled carbon dioxide and subject to detailed macroscopic examination. Reproductive organs (testes, epididymides, prostate and seminal vesicles with coagulating gland) were weighed and retained; the pituitary gland was also retained, but not weighed, for males with suspect fertility. For one animal (No 98) receiving 1000 mg/kg/day the pituitary was not retained in error. This deviation from protocol had no effect on the scientific integrity of the study. Paired organs were identified as left and right for weighing and retention. Specimens of any abnormal tissues were also retained in an appropriate fixative. Right testes and right epididymides were fixed initially in Bouin's solution followed by industrial methylated spirit. Other retained tissues were stored in 10% neutral buffered formaldehyde.

For each male a fresh sperm sample was taken from the left vas deferens for assessment of sperm motility using a computer assisted sperm analyser (CASA). Data were collected for all groups but only analysed for Groups 1 and 4. A sample was retained in 10% neutral buffered formaldehyde for possible assessment of sperm morphology. In addition, for all males, the left cauda epididymis was retained frozen (-20°C) for possible assessment of sperm count and the left testis was retained frozen (-20°C) for possible assessment of homogenisation-resistant spermatid count. In the absence of any adverse effect on fertility or sperm motility at the highest dosage, no further assessment of the retained samples was performed.

Adult females

The four females that failed to litter were killed on Day 25 after mating. The two females which lost their entire litter *post partum* were killed on the day of litter loss. Remaining females which reared young to weaning were killed on Day 27 after birth.

All females were killed by inhaled carbon dioxide and examined externally and internally for macroscopic abnormalities. The uterus was examined and the numbers of implantation sites were recorded; for non-littering females a Salewski staining technique (Salewski, 1964) was used to detect the presence of implantation sites. For animals showing total litter loss *post partum* the activity of the mammary tissue was assessed. Reproductive organs (ovaries, and uterus with oviducts and cervix) were weighed and retained; the vagina was also retained but not weighed. For females with suspect fertility the pituitary was retained. Paired organs were identified as left and right for weighing and retention. Specimens of any abnormal tissues were also retained in appropriate fixative. Tissues were preserved in 10% neutral buffered formaldehyde.

Offspring

Offspring culled on Day 4 of age were killed by intraperitoneal injection of sodium pentobarbitone. Examination of culled offspring was restricted to those showing external abnormalities.

Offspring that died prior to weaning were retained in Industrial Methylated Spirit, and later examined for abnormalities and eviscerated. Offspring which were killed for humane considerations were sent for necropsy on that day.

Offspring surviving until weaning were killed on Day 21 of lactation by inhaled carbon dioxide and examined externally and internally for macroscopic abnormalities. Abnormal tissues were preserved in appropriate fixative.

TREATMENT OF DATA

Group values

For presentation purposes, values in appendices are frequently displayed as rounded values. Group mean values and standard deviations presented in tables were calculated from unrounded values, and therefore it is not always possible to derive the exact values presented in tables from the data presented in appendices. Due to an idiosyncrasy of some of the software used for data handling, in some circumstances where the calculated value is exactly half a unit different to that presented, values may be rounded down.

Data have generally been expressed as group means with standard deviations (SD), where appropriate, calculated according to the formula:

$$SD = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$$

where x = individual or litter mean values
$\bar{x}$ = group mean value
n = sample size

Bodyweight

In view of the longer treatment period prior to pairing in the males group mean values and SD were only presented on a weekly basis. For females values were presented on a twice-weekly basis for the pre-pairing treatment period.

Group mean values and SD for females during gestation were presented for the scheduled days of bodyweight recording, based on animals observed to have given birth to a litter. A small number of Control females were not weighed on Day 0 of gestation in error and were subsequently weighed on Day 1 of gestation when this error became apparent. Day 1 data for these animals were presented but have not been used in the calculation of group values.

Group mean values and SD for females during lactation were presented for the scheduled days of bodyweight recording, based on animals rearing young to weaning.

Weight changes were calculated and plotted graphically with respect to the first day of each relevant period.

Food consumption

Group mean food consumption of parental males and females before pairing was calculated on a weekly basis from cage mean values.

Mean daily intakes for females after mating were calculated individually for Days 0-5, 6-12 and 13-19. Group mean values (± SD) during gestation were based on animals observed to give birth to a live litter. Food consumption for a small number of Control females was not commenced on Day 0 of gestation in error and was subsequently commenced on Day 1 of gestation when this error became apparent. Data collected based on the reduced period 1-5 have been presented and used in the calculation of group values.

Mean daily intakes for females during lactation were calculated individually for Days 1-3, 4-6, 7-13 and 14-20. Group mean values (± SD) during lactation were based on animals which reared young to weaning. It is considered that food consumed between Days 14 and 20 of lactation may include diet consumed directly by the offspring.

Oestrous cycles and pre-coital interval

Oestrus cycles were calculated as the percentage showing the following cycles:

Regular:	Cycles of 4-5 days
Irregular:	Cycles of 2, 3 or 6-10 days
Acyclic:	At least 10 days without oestrus

Pre-coital intervals were calculated as the percentage of females in the categories 1-4, 5-8, 9-12, 13-16 or 17-21 days, the time elapsing between initial pairing and mating.

Mating performance and fertility

For each group and sex the following were calculated:

$$\text{Percentage mating} = \frac{\text{Animals with evidence of mating}}{\text{Animals paired}} \times 100$$

$$\text{Conception rate} = \frac{\text{Animals achieving a pregnancy}}{\text{Animals with evidence of mating}} \times 100$$

$$\text{Fertility index} = \frac{\text{Animals achieving a pregnancy}}{\text{Animals paired}} \times 100$$

B 10

Gestation length

Gestation length was calculated as the number of gestation days up to and including the day on which offspring were first observed, with Day 1 = day of mating for calculation purposes. Where parturition had started overnight, this value was adjusted by subtracting half of one day. Gestation index was calculated as:

$$\text{Gestation index} = \frac{\text{Number of live litters born}}{\text{Number pregnant}} \times 100$$

Litter parameters

The standard unit of assessment was the litter, therefore group mean values are calculated from individual litter values. Individual values are presented for all litters, group mean values presented only include those litters successfully reared to weaning.

Survival indices

Mean survival indices for each group were calculated based on individual litter values as follows:

$$\text{Post-implantation survival index} = \frac{\text{Total number of offspring born}}{\text{Total number of implantation sites}} \times 100$$

Where total litter size exceeded the number of implantations post-implantation loss was assumed to be zero.

$$\text{Live birth index} = \frac{\text{Total number of offspring on Day 1 after littering}}{\text{Total number of offspring born}} \times 100$$

$$\text{Viability index} = \frac{\text{Number of live offspring on Day 4 (pre-cull) after littering}}{\text{Number of live offspring on Day 1 after littering}} \times 100$$

$$\text{Lactation index} = \frac{\text{Number of live offspring on Day of examination}}{\text{Number of live offspring at Day 4 (post-cull) after littering}} \times 100$$

In view of the high survival of offspring after Day 4 the lactation index was only presented for Day 21.

Group mean litter size

Group mean litter size and SD were calculated from individual litter values and data presented for Days 1, 4 (pre- and post-cull), 7, 14 and 21.

Group mean offspring bodyweight

Group mean offspring bodyweight and SD was calculated separately for male and female offspring for Days 1, 4 (pre- and post-cull), 7 14 and 21. Bodyweight changes presented were calculated with respect to the first day of the relevant period. Bodyweight changes on Day 4 are calculated from post-cull values.

Sex ratio

For each group the ratio of male to female offspring was calculated for all offspring at Day 1 and for live offspring on Days 1, 4 (pre and post cull) and 21 of age. The ratio was expressed as the percentage of males using the following formulae:

$$\frac{\text{Number of males in litter}}{\text{Total number of offspring in litter}} \times 100$$

Necropsy observations

For adults, individual necropsy findings and a group incidence summary are presented for each sex.

For offspring, findings are presented on an individual litter basis and an overall group incidence summary was not considered necessary.

Organ weight evaluation

The weight of paired organs were summed for the presentation of group mean values. Group mean values and SD were calculated for absolute weights and also for weights expressed relative to bodyweight (%) for organs weighed at necropsy. Group mean values for females include only those which reared young to weaning.

LT	Left
RT	Right
EPIDID	Epididymides
PROSTATE V	Ventral prostate
SEM V + COAG	Seminal vesicles (including coagulating gland)
UTRUS, ODUC UTRUS, ODUCT, CVIX	Uterus (with cervix and oviducts)

Statistical evaluation

Statistical analyses were performed on the majority of data presented and results of these tests, whether significant or non-significant, are presented on the relevant tables. For some parameters, such as oestrous cycles and mating performance, the similarity of the data was such that analyses were not considered to be necessary.

For bodyweight and bodyweight change for the pre-pairing treatment phase, absolute organ weights and bodyweight-relative organ weights, homogeneity of variance was assessed using Bartlett's test (Bartlett, 1937). Whenever this was found to be statistically significant a Behrens-Fisher test (Cochran and Cox, 1957) was used to perform pairwise comparison, otherwise a Dunnett's (Dunnett, 1955/64) test was used.

The distribution of gestation lengths was assessed using a linear by linear association test (Agresti, 1990, Agresti, Mehta and Patel, 1990).

For all other data where statistical analyses were performed, including bodyweights and bodyweight changes during gestation and lactation, food consumption, litter data and offspring bodyweights, analyses were conducted using the following methodology. Dependent on the heterogeneity of variance between treatment groups, parametric tests (analysis of variance, Snedecor and Cochran 1967) followed by Williams' test (Williams' 1971/2) or non-parametric tests, (Kruskal-Wallis, Hollander and Wolfe 1973) followed by Shirley's test (Shirley 1977) were used to analyse these data, as appropriate. Where 75% or more of the values for a given variable are the same, a Fisher's exact test (Fisher, 1950) was used. All significant (i.e. $p<0.05$) inter-group differences from the control are reported only where supported by a significant analysis of variance (i.e. $p<0.05$).

For litter data the basic sample unit was generally the litter and, due to preponderance of non-normal distributions, non-parametric analyses were routinely used.

Archives

All experimental data arising from the study (including documentary raw data, specimens, records, and other materials; collectively defined as the "materials") will remain the property of the Sponsor.

Samples and materials which are unsuitable, by reasons of instability, for long term retention and archiving may be disposed of after the periods defined in Huntingdon Life Sciences Standard Operating Procedures have been completed.

All other samples, specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of 5 years from the date of issue of the final report. After such time, the Sponsor will be contacted and their advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

RESULTS

Formulation analysis (Addendum 2)

The homogeneity and stability of PTB in aqueous formulations was assessed and considered to be satisfactory following ambient storage for two days or refrigerated storage for 15 days. Full details of the methodology and results of these investigations are presented the Formulation Chemistry Report (Addendum 2).

Samples of dosing formulations prepared for administration during selected weeks of the study, pertaining to the commencement of treatment, pairing and lactation, were analysed for achieved concentration and were found to be 92.3-104% of nominal concentration. Full details of the methodology and results of these investigations are also presented the Formulation Chemistry Report.

ADULT RESPONSES

Mortality (Table 1, Appendix 1)

There was a single mortality on the study, occurring at 1000 mg/kg/day. Animal number 236 was killed for reasons of animal welfare after showing piloerection, hunched posture, underactive behaviour and slow and shallow respiration on Day 6 of treatment. Necropsy examination (conducted on the following day) revealed fluid in the lungs. This observation, in conjunction with the type of signs observed prior to death, is consistent with an intubation error during dosing and in the absence of any similar findings amongst other animals at this dosage, this death is not considered to be related to treatment.

Clinical signs (Appendices 2-5)

Clinical signs associated with treatment were restricted to transient post-dosing salivation in both sexes. The incidence of salivation showed a clear dosage relationship with all animals at 1000 mg/kg/day being affected; it generally became apparent during the first week of treatment and occurred regularly throughout the study, sometimes being noted prior to dosing in males. The majority of animals at 300 mg/kg/day also showed salivation but the frequency of this sign was less than that observed at the highest dosage. At 100 mg/kg/day salivation was restricted to occasional animals (8 in total) on isolated occasions. Post-dose salivation is frequently observed where animals are dosed orally and is generally regarded as being related to distaste of the formulation.

There were no other clinical signs considered to be related to treatment at any dosage.

Bodyweight and bodyweight change of males (Figure 1, Tables 2-3, Appendix 6)

There were no obvious adverse effects of treatment on bodyweights or bodyweight gain of males during the 10 weeks prior to pairing or to the end of the study 8 weeks later.

Bodyweight and bodyweight change of females (Figure 2-3, Tables 4-9, Appendices 7-9)

Inter-group differences in bodyweight and bodyweight gain during the 2 week period prior to pairing did not indicate any adverse effect of treatment.

There were no adverse effects of treatment on bodyweights or bodyweight gain of females during gestation or lactation.

At 1000 mg/kg/day it was noted that the pattern of bodyweight change during late lactation was marginally superior to the Control and other treated groups. This indicates that impairment of maternal condition was not the cause of the slower growth of the offspring at this dosage.

Food consumption of males (Table 10, Appendix 10)

Food consumption at 300 and 1000 mg/kg/day were frequently higher than Control during the pre-pairing treatment period, attaining statistical significance on two occasions at the highest dosage. However, these differences in food consumption did not exceed 10% of the concurrent Control value and were considered unlikely to be of any toxicological significance.

Food consumption of females (Tables 11-12, Appendices 11-13)

The pattern of food intake for females prior to pairing, during gestation and during lactation did not indicate any adverse effect of treatment.

Oestrus cycles, pre-coital interval and mating performance (Tables 13,14,16, Appendix 14)

There was no obvious adverse effect of treatment on oestrous cycles or pre-coital interval. The majority of females showed a regular 4 or 5 day cycle and mated, as expected, within the first four days of pairing.

Mating performance and fertility of the males was unaffected by treatment; only one male (N0. 98 at 1000 mg/kg/day) failed to show evidence of mating within 14 days of cohabitation but all other paired males at this dosage successfully induced pregnancy in their partner.

Gestation length and parturition (Tables 15, Appendix 14)

Gestation length was unaffected by treatment and there were no evident problems during parturition at any dosage.

LITTER RESPONSES

There were two occurrences of total litter loss *post partum* in the study, one in the Control group and one at 1000 mg/kg/day. In addition one Control female showed evidence of implantations but was not observed to give birth to a litter. The following assessment is based on the 44, 24, 23 and 22 dams rearing live young to weaning in the Control group, 100, 300 and 1000 mg/kg/day respectively.

Litter size and offspring viability (Tables 17,18, Appendices 15-16)

The mean number of implantations (recorded at necropsy) was very similar for all groups and there was no obvious adverse effect of treatment on litter size (total and live) on Day 1 of age. A slight decrease in the live birth index (survival over 1 day post parturition) was noted in the 1000 mg/kg/day group (96.4%) when compared to the Control Group value (99.3%).

The viability index (survival to Day 4 post parturition) was also decreased in the 1000 mg/kg/day group (91.3%) when compared to the Control group value (96.2%); however, this difference was not statistically significant.

There was no adverse effect of treatment on pup survival to Day 4 at lower dosages. Following standardisation of litter size on Day 4, pup survival to weaning was unaffected by treatment at any dosage.

Sex ratio (Tables 19, Appendix 17)

The ratio of male to female offspring at birth was similar in all groups and there was no evidence of any selective effect of treatment on the survival of either sex.

Offspring bodyweight and bodyweight gain (Figure 4, Tables 20-21, Appendices 18-21)

For both sexes at 1000 mg/kg/day bodyweight at birth and subsequent bodyweight gain to weaning was lower than the concurrent Control; the pattern established prior to standardisation of litter size persisted beyond the main period of lactation, with differences in both sexes by 14 and 21 days of age attaining statistical significance.

At 300 and 100 mg/kg/day bodyweight at birth and subsequent bodyweight gain to weaning was comparable to Control for both sexes.

TERMINAL EXAMINATIONS

Necropsy of adults (Tables 22-23, Appendices 23-24)

The frequency and distribution of macroscopic changes observed during necropsy of the adult animals did not indicate any obvious adverse effect of treatment.

Necropsy of offspring (Appendix 22)

The macroscopic changes observed during necropsy of offspring which failed to survive to weaning or offspring killed on Day 21 of age were unremarkable and did not indicate any adverse effect of treatment.

Organ weights of adults (Tables 24-27, Appendices 25-28)

There was no obvious adverse effect of treatment on absolute or bodyweight-relative organ weights of the male or female reproductive system.

Absolute epididymal weight was statistically higher than Control at 1000 mg/kg/day. However, this is not considered to reflect a biologically significant effect because:

- bodyweight – relative epididymal weights showed no such difference
- there was no effect on fertility parameters (including sperm motility) for males dosed at 1000 mg/kg/day.

Sperm Motility (Tables 28, Appendix 29)

Assessment of sperm motility in males receiving 1000 mg/kg/day did not indicate any obvious adverse effect of treatment; values being essentially similar to Control.

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CONCLUSION

Oral gavage administration with PTB at dosages up to 1000 g/kg/day, for 10 weeks prior to pairing in adult male rats and 2 weeks prior to pairing in females, was well tolerated by parent animals and had no adverse effect on their fertility or general reproductive performance. This dosage is therefore considered to be the no-effect-level (NOEL) for the parent females

Effects in the offspring of dams exposed to 1000 mg/kg/day included slightly lower birth weights, reduced weight gains from birth to weaning, and a slight decrease in postnatal survival. Therefore, in this study, direct treatment of the parents at 300 mg/kg/day is considered to be the no-effect-level (NOEL) for growth and development of the offspring to weaning.

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ARCO Chemical Company
3801 West Chester Pike
Newtown Square, Pennsylvania 19073-2387

SC980216J06 / 2I8047

CERTIFICATE OF ANALYSIS

Consignee:

Chemical Mktg.
New Milford, CT

Specification No. 0426819
Customer Order No. Sample
Vessel/Container No.
~~Tank Truck~~ B/L No. N 26202
Quantity Shipped 1
Date Shipped. 1-5-99
Code No.

ARCOSOLV® PTB, Propylene Glycol t-Butyl Ether

Property	Lot # SC980216J06 / 2I8047 Limits	Analysis
Specific/Gravity @ 25/25°C	0.870-0.874	0.872
Distillation @ 760 mmHg		
IBP, min.	145°C	149.7
DP, max.	153°C	151.6
Acidity, wt. % as acetic acid, max.	0.03	0.001
Water, wt. %, max.	0.25	0.03
Color, APHA, max.	15	4
Purity, wt. % by G C	99.0	99.7

Technician

Laboratory Supervisor

The information in this certificate of analysis is believed to be accurate but is made without warranty. ARCO Chemical disclaims any liability in connection with the use of the information, and does not warrant against infringement by reason of the use of its products in combination with other material or in any process.

04

ACR035/003252

ADDENDUM 2

Formulation chemistry report

05

ACR035/003252

PTB

FORMULATION CHEMISTRY

Author:

Dr A D Clemson

06

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INTRODUCTION

This report details the analytical procedure used and the results obtained for:

The determination of the homogeneity and re-suspendibility of PTB in water formulations.

The determination of the concentrations of PTB in formulations prepared for dosing during Weeks 1, 11 and 14 of treatment.

The validation of the analytical procedure for the determination of PTB in water formulations.

EXPERIMENTAL PROCEDURE

PTB (Batch No. SC980216J06/218047) was dissolved in water for oral administration to the study animals. The homogeneity and re-suspendibility of PTB in water were assessed analytically in trial formulations, at concentrations of 1 and 100 mg/ml, prepared before the commencement of treatment. The concentrations of PTB in water were also determined in all formulations prepared for dosing during Weeks 1, 11 and 14 of treatment.

HOMOGENEITY AND RE-SUSPENDIBILITY IN WATER FORMULATIONS

Trial formulations (400 ml), containing PTB in water, at nominal concentrations of 1 mg/ml and 100 mg/ml, were prepared and sub-divided into amber screw-top jars (4 x 100 ml). On receipt, one jar of each formulation was retained for immediate analysis (Day 0) and then stored at ambient temperature (+21°C) for 2 days, the remainder were refrigerated (nominally +4°C) for 2, 8 and 15 days.

On each day of sampling, the appropriate formulations were mixed by inversion followed by magnetic stirring. One sample (1 ml) was removed for analysis from approximately one-quarter, one-half and three-quarters the depth (representing the top, middle and bottom) of the continuously stirred formulation at each time-point as detailed below. Each 1 ml sample was accurately weighed.

- Day 0, a jar of each formulation was mixed on receipt and sampled after 5 minutes, 1 hour and 2 hours continuous stirring.
- Day 2, a jar of each formulation was removed from storage:
  - at ambient temperature, mixed and sampled after 5 minutes continuous stirring.
  - under refrigeration, mixed and sampled after 5 minutes continuous stirring.
- Day 8 and Day 15, a jar of each formulation was removed from refrigerated storage, mixed and sampled after 5 minutes continuous stirring.

At each time-point, the three sub-samples from each formulation were analysed in accordance with the analytical procedure.

ACHIEVED CONCENTRATIONS IN DOSE FORMULATIONS

Samples (1 ml) were taken for analysis from all formulations prepared for dosing during Weeks 1, 11 and 14 of treatment.

PROCEDURAL RECOVERIES

As the formulations were true solutions, procedural recoveries were not performed with the analysis of study samples.

RESULTS

The results obtained for the homogeneity and re-suspendibility of PTB formulated in water are presented in Table 1.

The achieved concentrations of PTB in dose formulations analysed and the mean found concentrations as a percentage of nominal values, are summarised in Table 2.

The analytical method used and the results for validation of the analytical procedure determined with respect to linearity of detector response, system precision, method accuracy and precision are presented in Appendices 1A - E.

Typical chromatograms of study samples are presented in Figures 1A – D.

DISCUSSION AND CONCLUSION

The homogeneity and re-suspendibility of PTB in water were assessed analytically in trial formulations, at concentrations of 1 and 100 mg/ml, prepared before the commencement of treatment. The results show that PTB produced an homogeneous formulation in water, which could be maintained for up to 2 hours while magnetically stirred and which could also be successfully remixed, following ambient temperature storage for 2 days and refrigerated storage for 15 days. The mean achieved concentrations in both formulations were within 5% of the time 0 value at each time point.

The mean concentrations of PTB in formulations, prepared for dosing during Weeks 1, 11 and 14 of treatment, ranged from 92.3% to 104% of nominal concentrations and were considered satisfactory.

The analytical procedure was validated for PTB in water with respect to the specificity of the chromatographic analysis, linearity of detector response, precision of injection, method accuracy and precision.

TABLE 1

Homogeneity and re-suspendibility of PTB in water formulations

Nominal concentration (mg/ml)	Jar No	Storage conditions			Found concentration (mg/ml)			Mean	CV (%)
		Hours	Days	Temp	Bottom	Middle	Top		
1	1	0	0	+21°C	0.964	0.953	0.993	0.970	2.13
		1		+21°C	0.981	0.981	0.964	0.975	1.01
		2		+21°C	0.983	0.981	0.982	0.982	0.102
		48		+21°C	1.02	1.02	0.992	1.01	1.60
	2		2	+4°C	0.957	1.01	1.01	0.992	3.08
	3		8	+4°C	0.979	0.974	0.974	0.976	0.296
	4		15	+4°C	0.996	1.03	1.02	1.02	1.72
100	1	0	0	+21°C	105	100	102	102	2.46
		1		+21°C	106	98.8	101	102	3.62
		2		+21°C	104	99.8	98.5	101	2.85
		48		+21°C	96.4	97.0	98.2	97.2	0.943
	2		2	+4°C	96.7	98.7	104	99.8	3.78
	3		8	+4°C	101	95.7	101	99.2	3.08
	4		15	+4°C	92.6	98.8	99.4	96.9	3.88

CV = Coefficient of variation

Analysed concentrations were calculated using unrounded figures.

TABLE 2

Achieved concentrations of PTB in dose formulations

Occasion of Dosing	Group	Intended concentration (mg/ml)	Achieved concentration (mg/ml)		Mean achieved Concentration (mg/ml)	Achieved Concentration as % of intended
Week 1	1	0	ND	ND	ND	-
	2	10	9.87	9.91	9.89	98.9
	3	30	30.4	30.3	30.4	101
	4	100	104	104	104	104
Week 11	1	0	ND	ND	ND	-
	2	10	10.0	9.52	9.76	97.6
	3	30	27.3	28.0	27.7	92.3
	4	100	92.9	101	97.0	97.0
Week 14	1	0	ND	ND	ND	-
	2	10	10.1	10.0	10.1	101
	3	30	30.2	30.3	30.3	101
	4	100	99.7	99.5	99.6	99.6

ND Not detected

Analysed concentrations were calculated using unrounded figures

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FIGURES

Figure 1A
Typical sample chromatogram - Group 1 Control

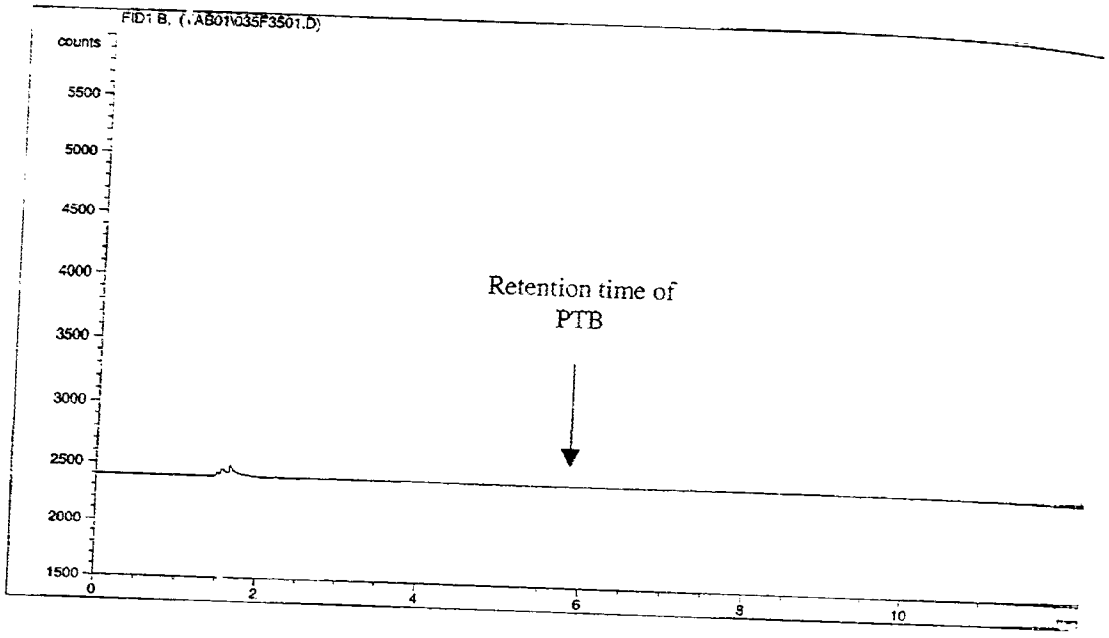
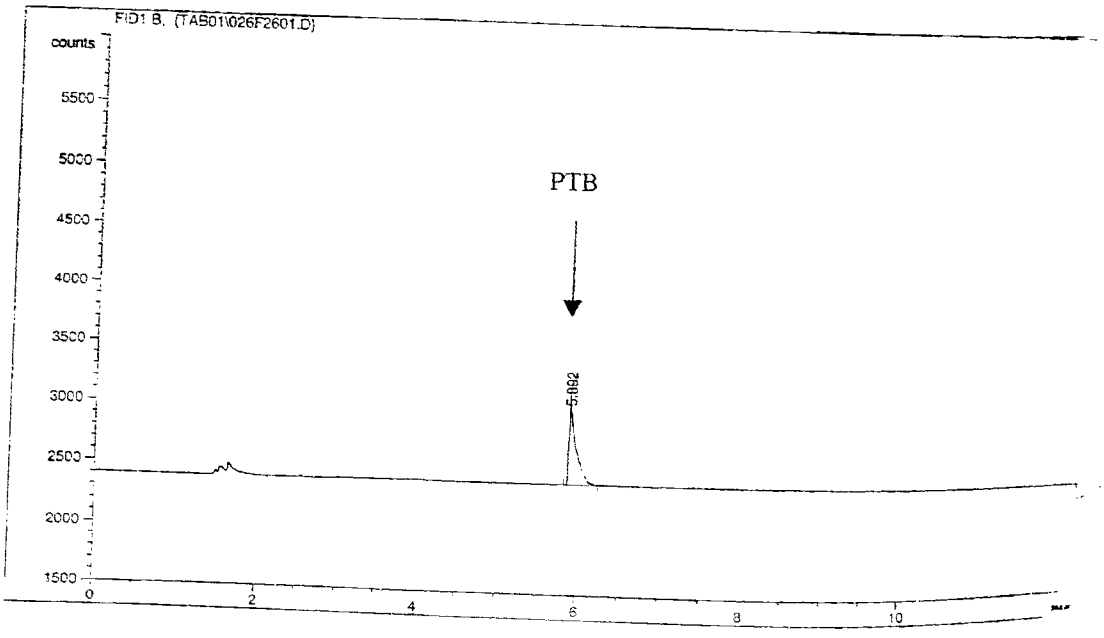


Figure 1B
Typical sample chromatogram - Group 2 10 mg/ml



Formulation Chemistry

FIGURES (continued)

Figure 1C
Typical sample chromatogram - Group 3 30 mg/ml

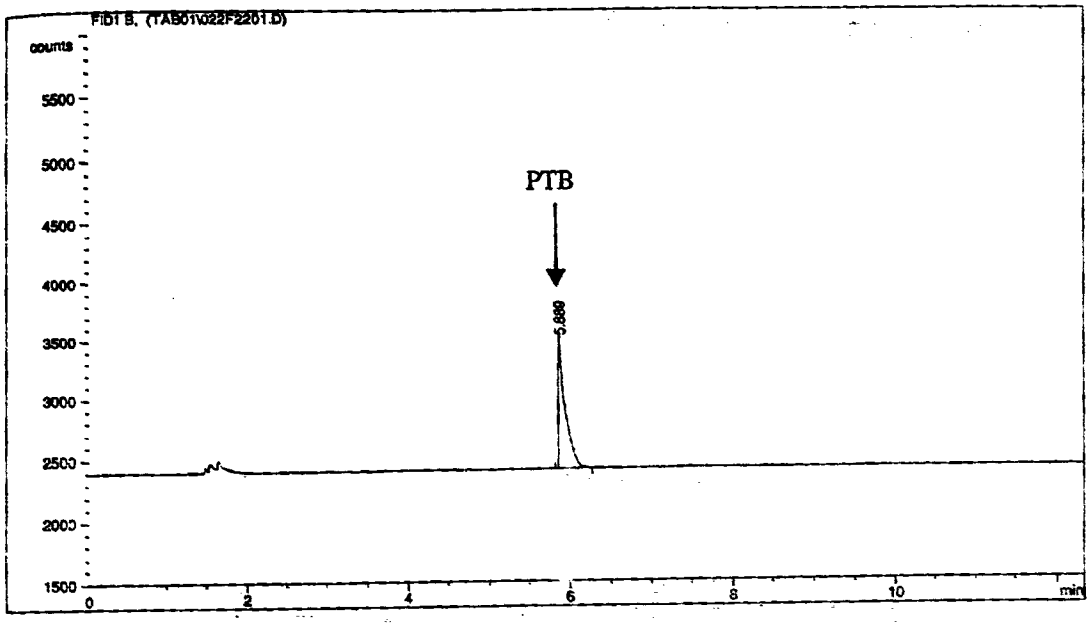
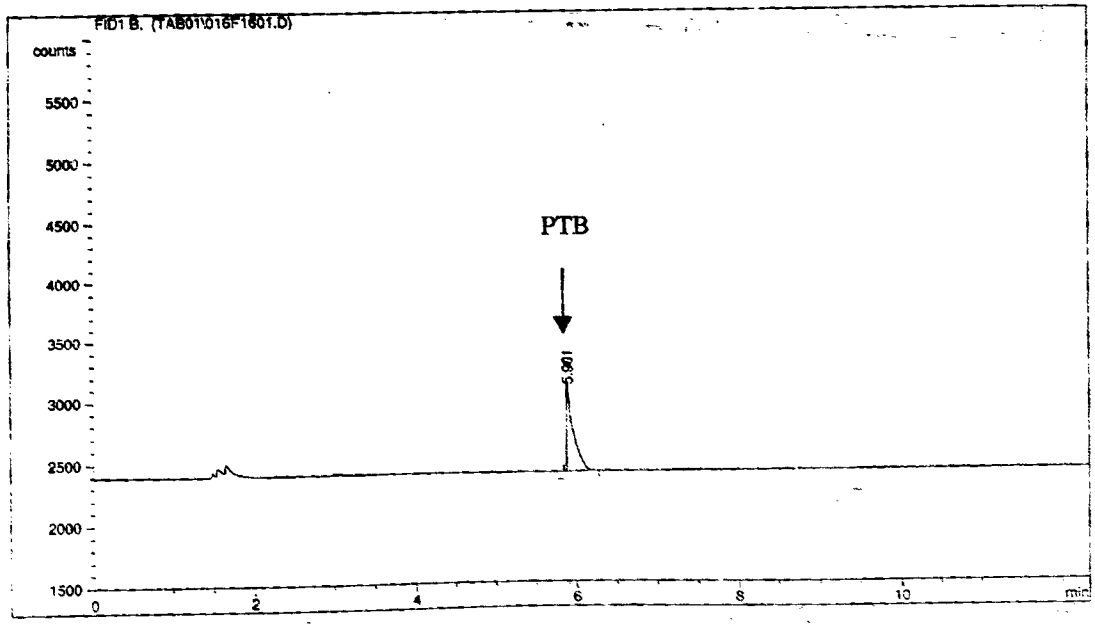


Figure 1D
Typical sample chromatogram - Group 4 100 mg/ml



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APPENDIX 1A

ANALYSIS OF PTB IN WATER FORMULATIONS

This method details the procedure used for the Gas Chromatography (GC) assay of PTB in water formulations at concentrations in the range 1 mg/ml - 200 mg/ml. The method (Ref 1) was modified to comply with current Huntingdon Life Sciences standard procedures and instrumentation.

1. TEST SUBSTANCE

Name:	PTB
Supplier:	Sponsor
Appearance:	Clear colourless liquid
Batch :	
Analytical:	Not supplied.
Technical:	Same batch as used in the formulation.
Storage:	
Test substance:	Refrigerator (+4°C).
Test formulations:	Freezer (-20°C).
Sample extracts:	Refrigerator (+4°C).
Hazard classification:	Huntingdon Life Sciences CLASS 2

Ref 1: Adapted from method contained in HRC formulation chemistry report ACR 027/985236

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2. APPARATUS

GC system:	As detailed in the chromatographic section
Balances:	Analytical capable of weighing to 0.01 mg.
Autodiluter:	Hamilton Ltd, Microlab 1000.

General laboratory apparatus.

3. REAGENTS

Control vehicle:	Reverse osmosis water.
Water:	Riedel-de Haën Chromasolv grade or Fisher HPLC grade.

4. SAMPLE PROCESS

The formulations were analysed as soon as possible after preparation. If it was not possible to analyse the samples on the day of receipt, they were stored in a freezer at -20°C.

4.1 Dilution

Each sample was quantitatively transferred to the volumetric flask, dissolved in a suitable volume of water with the aid of swirling and diluted (Table 1, Dilution A) to volume using water.

Using the Hamilton Microlab 1000 autodiluter, the extract was diluted (Table 1, Dilution B) using water to provide a solution containing the test substance in the range 20 – 40 µg/ml.

Table 1: Summary of sample preparation

Nominal inclusion (mg/ml)	Sample Volume (ml)	Dilution (ml)		Dilution (ml/dose)
		A#	B#	
0*	1*	*	*	*
1	1	50	Not required	50
10	1	50	1 → 10	500
30	1	50	1 → 20	1000
100	1	100	1 → 50	5000
200	1	100	1 → 50	5000

Diluent: #water

*0 mg/ml (Control) samples are diluted identically to the lowest concentration being analysed.

5. STANDARD PREPARATION

5.1 Calibration

The test substance (about 25 mg) was weighed (to an accuracy of 0.01 mg) into a volumetric flask (50 ml), dissolved in water and diluted to volume (50 ml) using water to provide primary standard A (approximately 500 µg/ml). A second standard was prepared as above to provide primary standard B.

The secondary standards were sequentially diluted using water, to provide calibration standards for the nominal concentration range 10 - 50 µg/ml as detailed in Table 2.

Table 2: Summary of standard preparation

Standard reference	Nominal concentration	Standard preparation			
Standard A,	500 µg/ml	Test substance,	approx. 25 mg	→	50 ml *
Standard B,	500 µg/ml	Test substance,	approx. 25 mg	→	50 ml *
Standard1A,	50 µg/ml	Dilute standard A,	1	→	10 *
Standard1B,	50 µg/ml	Dilute standard B,	1	→	10 *
Standard 2,	40 µg/ml	Dilute standard A,	1	→	13 *
Standard 3,	30 µg/ml	Dilute standard A,	1	→	17 *
Standard 4,	20 µg/ml	Dilute standard A,	1	→	25 *
Standard 5,	10 µg/ml	Dilute standard A,	1	→	50 *

Diluent: * water

Standards 1A and 1B were injected in duplicate to assess standard reproducibility and each analytical run calibrated by injecting the five point calibration prepared from standard A and a reagent blank at the beginning of the run. Standard 3 was injected, as a running standard, after a maximum of every four sample injections.

5.2 Procedural Recoveries

Procedural recovery samples were prepared and analysed at the highest and lowest concentrations planned for the toxicology studies, 1 mg/ml and 200 mg/ml, as part of the method validation. They were prepared by fortifying control vehicle either with standard solution or with the neat test substance, as detailed in Table 3 and analysed in accordance with the sample extraction procedure. As the samples were deemed to be true solutions, no procedural recovery analyses were performed concurrently with study samples.

Table 3: Summary of procedural recovery fortification

Nominal inclusion (mg/ml)	Fortification		
1	2 ml of primary Standard A	+	1 ml control
200	approx. 200 mg	+	1 ml control

6. TYPICAL CHROMATOGRAPHIC CONDITIONS

6.1 Instrumentation

GC system:	HP 5890 with flame ionisation detector and HP ChemStation
Analytical column:	J & W Scientific DB-5, 30 m x 0.32 mm, Film thickness 0.25 µm

6.2 Operating conditions

Column temperature:	Initial:	50°C held for 5 min
	Rate:	30°C/min
	Final:	120°C held for 5 min
Carrier gas:	Helium:	2.0 ml/min at 50°C
Injector temperature:	200°C	
Injection volume:	1 µl, split	
Septum Purge:	4.6 ml/min	
Split vent purge:	15 ml/min	
Detector temperature:	275°C	
	Hydrogen:	40 ml/min
	Air:	450 ml/min
	Auxiliary (helium):	30 ml/min
Retention time:	Approximately 6 min	

Slight variations of the gas flow rates may have been carried out on occasion in order to provide a suitable retention time for the test substance and to optimise resolution.

7. CALCULATION

From the peak area response of the test compound in each calibration standard chromatogram, calibration curves were constructed by linear regression of standard response versus standard concentration to demonstrate linearity. The concentration of the test compound in each sample and procedural recovery was calculated, from the area response observed at the characteristic retention time for the test compound, by reference to the bracketing calibration standards. The calculations were based upon the following equation.

$$\text{Analysed concentration, mg/g} = Y \times \frac{C_c}{R_c} \times P \times \frac{V}{W} \times 10^{-3}$$

Concentration of test samples were corrected for density using the following equation:

$$\text{Corrected concentration, mg/ml} = \text{Analysed concentration, mg/g} \times D$$

Where

- Y = Peak area response for the test substance in test chromatogram
- C_c = Concentration (µg/ml) of the single point bracketing calibration standard
- R_c = Mean peak area response of bracketing calibration standards
- V = Dilution volume of sample (ml)
- W = Weight of sample (g)
- D = Density (g/ml), a value of 1 was used if all formulation densities were within the range 0.99 - 1.01 g/ml
- P = Purity factor for bracketing calibration standard (= % purity / 100), if applicable

8.

Typical calibration graph

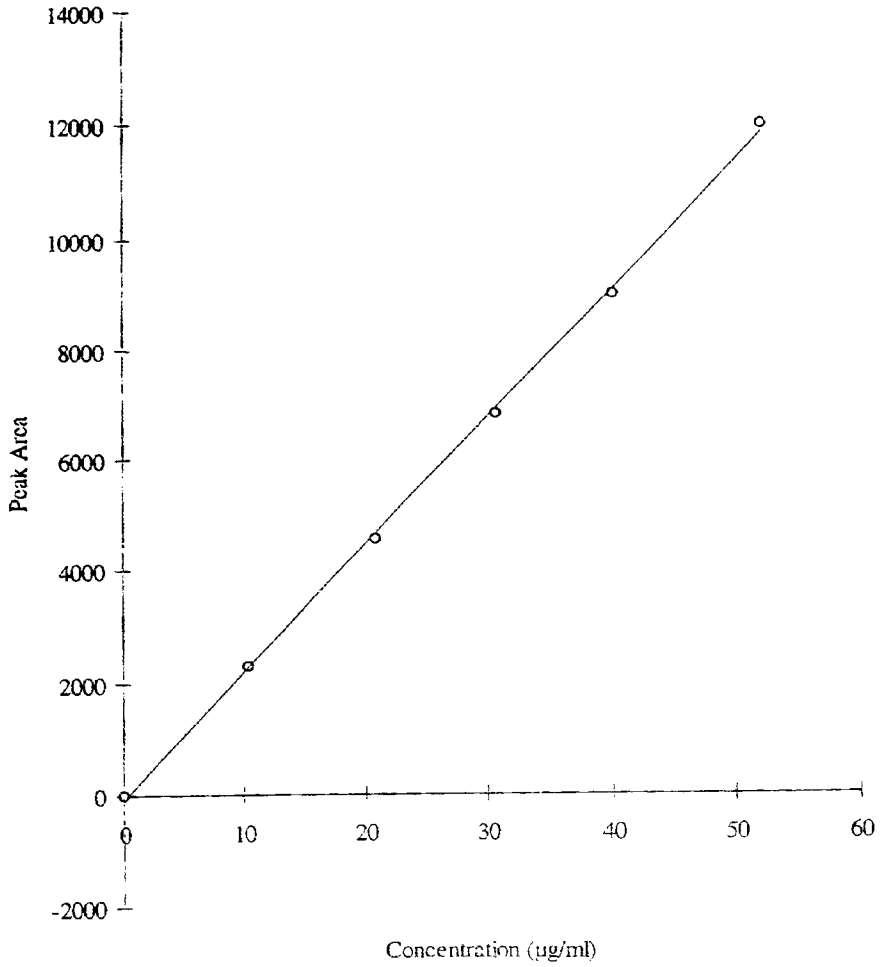
Conc. (µg/ml)	Peak Area
0	0
10.4	2299.522
20.8	4580.646
30.6	6824.593
40.0	8978.739
52.0	11949.838

Linear Regression

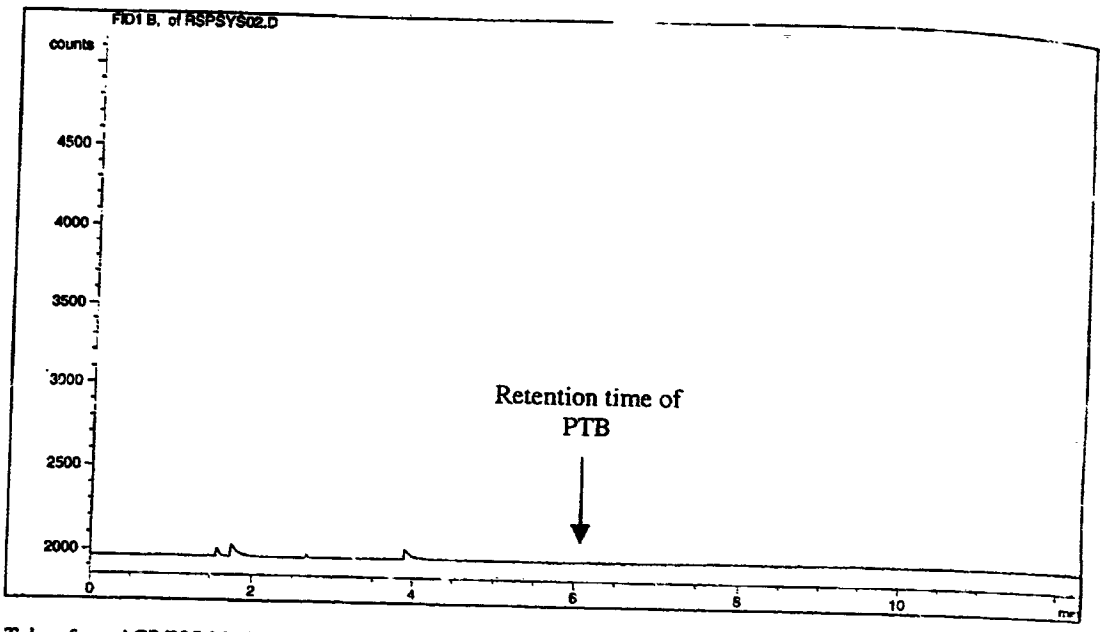
y = 229 x Conc. -94.0

r = 0.9997

Data taken from ACR/035 Method development. RSP Worksheet 2CD00L\tab01

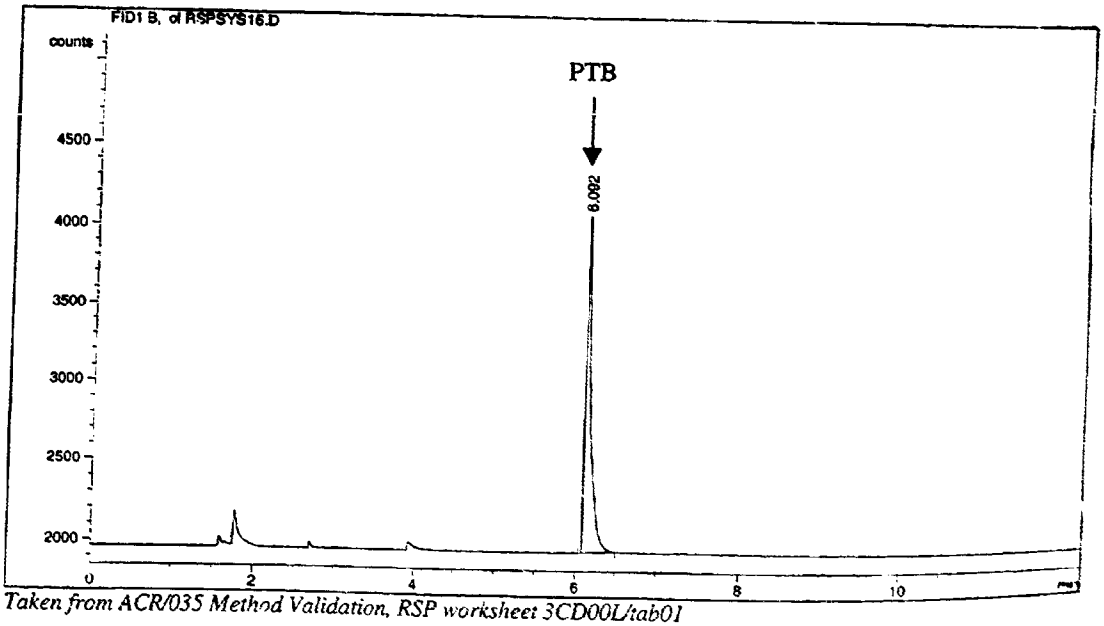


9. **Typical chromatogram of control vehicle. (Dilution 1:50)**



Taken from ACR/035 Method Validation, RSP worksheet 3CDOOL/tab02

10. **Typical chromatogram of procedural recovery containing PTB at 201 mg/ml. (Dilution 1:5000)**



Taken from ACR/035 Method Validation, RSP worksheet 3CD00L/tab01

APPENDIX 1B

Validation of the analytical method

The analytical method was validated by determining the linearity of detector response, specificity of the chromatographic analysis, precision of injection, method accuracy and precision.

Linearity

The linearity of the standard calibration was examined by preparing a series of standard solutions (10 µg/ml - 50 µg/ml) to encompass the working range of the assay. The peak area response for each standard was plotted against the concentration using least square regression analysis to provide information on the slope, intercept and correlation coefficient. A fresh set of standards was prepared on the next day and chromatographed alongside the original set that had been left on the autosampler overnight. The linearity of detector response was confirmed for PTB over the concentration range 10 µg/ml - 50 µg/ml (Appendix 1C) and there was no indication of any loss of PTB from solutions left at ambient temperature for at least 16 hours.

Specificity

Chromatograms obtained for control extracts were examined for peaks that might interfere with the quantitation of PTB. The specificity of the GC assay was demonstrated by the absence of a peak at the characteristic retention time for PTB in the control sample chromatograms.

System precision

The repeatability of injection was evaluated by determining the precision of six replicate injections of both the extract of the lowest procedural recovery solution (1.0 mg/ml) and an intermediate standard solution (30.6 µg/ml). The precision of injection was confirmed with the coefficients of variation for both series being less than 2.5% (Appendix 1D).

Method accuracy and precision

The analytical procedure was validated by determining duplicate procedural recoveries at nominal concentrations of 1.0 mg/ml and 200 mg/ml.

Procedural recoveries were prepared by fortifying samples (1 ml) of control water with known amounts of PTB, added either as a solution in water (inclusion levels <20 mg/ml) or as the neat PTB (inclusion levels ≥20 mg/ml). The prepared procedural recoveries were analysed in accordance with the analytical procedure.

The procedural recovery data showed that the mean recovery at 1.0 mg/ml was 100.4% (CV = 0.775%, n = 2) and at 200 mg/ml was 99.8% (CV = 3.61%, n = 2). The overall recovery data showed that the mean recovery was 100.1% (CV = 2.16%, n = 4, Appendix 1E).

APPENDIX 1C

Linearity determination of PTB standard solutions

Day 1

Concentration (µg/ml)	Peak area
53.0	11949.838
40.0	8978.739
30.6	6824.593
20.8	4580.646
10.4	2299.522
0	0.0
r	.9997
Gradient	229
Intercept	94.0

Day 2

Concentration (µg/ml)	Peak area
53.0	11924.716
40.8	9218.888
31.2	7141.840
21.2	4825.958
10.6	2334.453
0	0
r	0.9999
Gradient	226
Intercept	4.55

r = Correlation Coefficient

APPENDIX 1D

System precision of PTB standard solutions

Concentration	1.0 mg/ml spike	30.6 µg/ml standard
Peak area	5026.320	7054.381
	4874.401	6735.716
	4942.709	6578.863
	4900.884	6734.868
	4906.744	6802.354
	4866.487	6940.062
Mean	4919.591	6807.707
CV (%)	1.20	2.47
n	6	6

CV = Coefficient of variation
n = Number of determinations

APPENDIX 1E

Validation procedural recovery data for PTB in water formulation

Nominal fortification (mg/ml)	Validation Occasion	Recovery (%)	Accuracy and precision data			
			Mean	CV	Overall	
					Mean	CV
200	Day 1	102.3	99.8	3.61	100.1	2.16
200		97.2				
1.00		99.8	100.4	0.775	-	-
1.00		100.9				

CV Coefficient of variation

A 12

Study Number : ACR/035

Huntingdon Life Sciences

CONFIDENTIAL

PROTOCOL

PTB

STUDY OF REPRODUCTIVE FUNCTION AND FERTILITY IN THE CD RAT BY ORAL GAVAGE ADMINISTRATION

Sponsor

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Research Laboratory

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ENGLAND

Total number of pages: 22

Final Protocol

Huntingdon Life Sciences Ltd, registered in England, 1815730

A 13

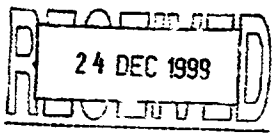
Huntingdon Life Sciences

CONTACT DETAILS

Sponsor's Monitoring Scientist	: J. Bootman
Sponsor's Contact	: J. McCuen

A 14

Study Number : 3CR/035



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PROTOCOL APPROVAL

PTS

STUDY OF REPRODUCTIVE FUNCTION AND

FERTILITY IN THE CD RAT BY

ORAL GAVAGE ADMINISTRATION

S Fulcher 25 November 1999

S.M. Fulcher, B.A., F.I.A.T.
Study Director,
Huntingdon Life Sciences Ltd.

Date

The signature of the Study Director confirms this protocol as the working document for the study. Any changes made subsequent to the date of the Study Director's signature will be documented in formal amendments.

P Aughton 25 November '99

P. Aughton, B.Sc., D.A.B.T., Dip.R.C.Path., C.Biol., M.I.Biol.
Management,
Huntingdon Life Sciences Ltd.

Date

James L McCann December 21, 1999

Sponsor,
Lyondell Chemical Worldwide, Inc.

Date

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

B 01

Study Number : **ACR/035**

Huntingdon Life Sciences

PTB

STUDY OF REPRODUCTIVE FUNCTION AND

FERTILITY IN THE CD RAT BY

ORAL GAVAGE ADMINISTRATION

Enquiry Number: 20280A

Number of pages for internal distribution: 19

This working document is approved for circulation and use:

Study Director

25 November 1999
Date

Primary location of study

Eye Research Centre
Eye
Suffolk
IP23 7PX

Building Number: 30

All procedures to be performed at the above site.

Final Protocol

Study Number : ACR/035

Huntingdon Life Sciences

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Huntingdon Life Sciences

1. INTRODUCTION

Management of study

Study Director	:	S.M. Fulcher
Monitoring Toxicologist	:	A.M. Bottomley
In the temporary absence of the Study Director, the scientific responsibilities will be taken over by the Monitoring Toxicologist; other items of routine study management should be referred to the following person in the first instance.	:	S.C.J. Reader

Objective

Assessment of the influence of oral gavage administration of the test substance on gonadal function, mating behaviour and fertility in treated rats of the F_0 generation and the survival of offspring up to Day 21 of age.

Regulatory compliance

The study will be performed in accordance with the following regulations or guidelines:

OECD (415), adopted 26 May 1983.

Good Laboratory Practice

The study will be conducted in compliance with principles of Good Laboratory Practice Standards as set forth in:

The UK Good Laboratory Practice Regulations 1997 (Statutory Instrument No 654).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No L 77/8).

Animals (Scientific Procedures) Act 1986 compliance

The in-life experimental procedures to be undertaken during the course of this study are subject to the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986 (the Act). The Act, administered by the UK Home Office, regulates all scientific procedures in living animals which may cause pain, suffering, distress or lasting harm and provides for the designation of establishments where procedures may be undertaken, the licensing of trained individuals who perform the practical techniques and the issue of project licences for specified programmes of work.

This study will comply with all applicable sections of the Act and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures and the Humane Killing of Animals under Schedule 1 to the Act, issued under section 21 of the Act.

Animal model	:	CD rat, accepted as a predictor of reproductive change in man; requirement for a rodent species by regulatory agencies.
Route	:	Oral gavage, to simulate a potential condition of human exposure.

Study Number : ACR/035

Huntingdon Life Sciences

Treatment groups and dosages

Group	:	1	2	3	4
Compound	:	Control		PTB	
Dosage (mg/kg/day)†	:	0	Low	Intermediate	High

† Dosage, to be selected following completion of a preliminary investigation of reproductive function and fertility in the rat (ACR/034) and added by protocol amendment.

2. STUDY SCHEDULE AND STRUCTURE

2.1. Duration of treatment

F_0 males	:	For 71 days before pairing until necropsy, until the majority of the F_0 females have been killed.
F_0 females	:	For 15 days before pairing until necropsy after weaning at Day 21 of lactation.
F_1 animals	:	From about Day 14 of age until necropsy at Day 21 of age.

2.2. Scheduled time plan

(to be up-dated as required in an amendment to protocol)

Sample of PTB to arrive	:	23 November 1999
F_0 males to arrive	:	+
F_0 females to arrive	:	+
Treatment of F_0 males to commence	:	+
Treatment of F_0 females to commence	:	+
Draft report to be issued	:	+ (estimated)

+ Dates to be incorporated by protocol amendment.

Study Number : ACR/035

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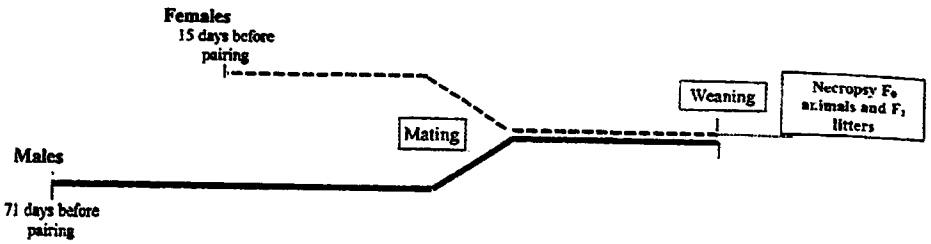
2.3. Study design



Male treatment period

Female treatment period

Only F_0 animals treated.

2.4. Identity of treatment groups

(F_0 to be selected from 216 animals ordered)

F_0 generation

Group	Treatment	Dosage (mg/kg/day)@ #	Number of animals		Animal numbers	
			Male	Female	Male	Female
1	Control	0	24	24	1-24	97-120
2	PTB	Low	24	24	25-48	121-144
3	PTB	Intermediate	24	24	49-72	145-168
4	PTB	High	24	24	73-96	169-192

\# Expressed in terms of the test substance as supplied. A conversion factor for alternative forms (*e.g.* active ingredient, base weight) will be used, if requested by the Sponsor and documented in an amendment to the protocol.

@ Dosages to be selected following completion of a preliminary investigation of reproductive function and fertility in the rat (ACR/034), and added by protocol amendment.

B 06

3. TEST SUBSTANCE AND FORMULATION

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work etc. Act 1974, and the Control of Substances Hazardous to Health Regulations 1994, it is a condition of undertaking the study that the Sponsor shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to Huntingdon Life Sciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

Information received will be used to set the Huntingdon Life Sciences Hazard Class, which determines safety precautions taken in the workplace.

Huntingdon Life Sciences Hazard Class: 2

3.1. Test substance

Sponsor's identification	:	PTB
Storage conditions	:	In a refrigerator (approximately 4°C) in the dark.
Sponsor's responsibilities	:	Documentation of methods of synthesis, fabrication or derivation. Stability data. Certificate of analysis.
Certificate of analysis details	:	Test substance identity. Batch number. Purity. Composition. Other appropriate characteristics. Current expiry date.

3.2. Formulation

Treatment		
Group 1, Control	:	Vehicle.
Group 2	:	PTB; low mg/ml.
Group 3	:	PTB; intermediate mg/ml.
Group 4	:	PTB; high mg/ml.
Conversion factor	:	The test substance will be used as supplied unless otherwise advised by the Sponsor. Any such requirement will be documented in an amendment to protocol.
Vehicle	:	Water for formulation.
Method of preparation	:	Will be documented in the study data and included in the final report.
Frequency of preparation	:	Will depend upon the availability of supporting stability data.

3.3. Quality control of dosage form

Liquid formulation	:	Before commencement of treatment, the suitability of the proposed mixing procedures will be determined and specimen formulations at 1 and 200 mg/ml will be analysed to assess the homogeneity and stability of the test substance in the liquid matrix. At specified intervals during treatment, the test formulations will be analysed for achieved concentration of the test substance.
Analysis	:	The formulated samples will be analysed using a method validated with respect to the determination of the specificity of analysis, limits of quantitation and/or detection, linearity of detector response, reproducibility, method accuracy and precision.

Homogeneity and stability

Preparation	:	Specimen formulations (nominally 400 ml) will be prepared at 1 and 200 mg/ml and equally distributed between four screw-capped amber bottles.
Sampling and determination	:	Before the initial sampling, each formulation will be mixed by inversion and magnetic stirring for 5 minutes. At each time-point, samples (nominally 1 ml) will be taken for assay from the top, middle and bottom of the formulation and accurately weighed.
Storage conditions		Ambient temperature (nominally 21°C) for 0, 1 and 2 hours while magnetically stirred and 48 hours (Bottle 1). Refrigeration (nominally 4°C) for 2, 8 and 15 days (Bottles 2, 3 and 4).

Achieved concentration

Content check	:	All groups at commencement of treatment of the F_0 males, F_0 mating and F_0 lactation. Four samples (nominally 1 ml accurately weighed) from all groups; 2 assays from each group. The remainder will be retained frozen (nominally -20°C) as contingency for analysis if any result requires confirmation.

4. ANIMAL MANAGEMENT

4.1. Animals - supply, acclimatisation and allocation

4.1.1. Animals

Species	:	Rat.
Strain	:	Crl:CD® BR.
Age ordered	:	F_0 males 5 - 6 weeks. F_0 females 9 - 10 weeks.
Weight range ordered	:	F_0 males 150 - 170 g. F_0 females 200 - 220 g.
Supplier	:	Charles River (UK) Limited.

4.1.2. Acclimatisation

Duration	:	Approximately 1 week.
Husbandry conditions	:	Refer to Section 4.2.

4.1.3. Allocation to treatment groups (F_0)

Allocation	:	After a period of acclimatisation.
Method	:	Randomised allocation; exclusion of animals showing signs of ill-health, or at the extremes of the bodyweight range.
Cage distribution	:	Arrangement designed to minimise environmental variables.

4.1.4. Identification

Numbering	:	Unique for each adult within study (F_0 animals).
Method	:	Tail tattoo (F_0 animals), toe tattoo (F_1 animals).
Cage labels	:	Uniquely identifying the occupants.

4.1.5. Animal replacement

12 male and 12 female spare animals will be ordered to replace any individuals rejected, following randomisation or during the first week of treatment.

Replacement before treatment : Ill-health.

Replacement during Week 1 of treatment : Accidental causes.

Replacement during treatment : None scheduled.

4.2. Animals - housing, diet and water supply

4.2.1. Environmental control

Rodent facility : Full barrier - to minimise entry of external biological and chemical agents.

Air supply : Filtered, not recirculated.

Temperature : Maintained within the range of 19-25°C.

Relative humidity : Maintained within the range of 40-70%.

Monitored continuously or daily. Excursions outside these ranges documented in the study data.

Lighting : 12 hours light : 12 hours dark.

Alarm systems : Activated on ventilation failure and when temperature/humidity limits exceeded.

Electricity supply : Public supply with automatic stand-by generators.

4.2.2. Animal accommodation

Study period	Number of animals per cage		Cage material	Cage flooring
	Male	Female		
Pre-mating	Up to 4	Up to 4	Stainless steel	Stainless steel grid
Mating	1	1	Polypropylene	Stainless steel grid
Males after mating	Up to 4	-	Stainless steel	Stainless steel grid
Gestation (up to Day 17 after mating)	-	1	Polypropylene	Stainless steel grid
Littering (from Day 17 after mating to Day 14-18 of lactation)	-	1 + litter	Polypropylene	Solid polypropylene
Lactation (day 14-18 of lactation until weaning)	-	1 + litter	Stainless steel	Stainless steel grid

Grid cages will be suspended above absorbent paper which will be changed at least twice each week or daily during mating. Solid bottomed cages will have wood flakes bedding changed at least twice each week. Cages, cage-trays, food hoppers and water bottles will be changed at appropriate intervals. Precise details of caging will be included in the final report.

4.2.3. Bedding, diet and water supply

Copies of all certificates of analysis are stored in the archives.

Bedding supply (for solid bottomed cages)

Bedding name	:	Lignocel 3/4 wood flakes.
Certification	:	Certificates are routinely received from the supplier.

Diet supply

Diet name	:	Rodent Diet VFR - 1C.
Diet type	:	Powdered diet.
Availability	:	Non-restricted.
Certification	:	Before delivery each batch of diet is analysed by the supplier for various nutritional components and chemical and microbiological contaminants. Supplier's analytical certificates are scrutinised and approved before any batch of diet is released for use.

This diet contains no added antibiotic or other chemotherapeutic or prophylactic agent.

Water supply

Supply	:	Public drinking water.
Regulatory agency	:	U.K. Department of the Environment.
Availability	:	Non-restricted via polyethylene or polycarbonate bottles with sipper tubes.
Certification	:	Certificates are routinely received from the supplier.

Copies of all certificates of analysis are stored in the archives.

4.2.4. Contaminants assay

It is the Sponsor's responsibility to advise Huntingdon Life Sciences of any specific contaminants likely to prejudice the outcome of the study. Analyses for such contaminants may be performed if requested by the Sponsor.

4.3. Animals - procedures

4.3.1. Administration

Route	:	Oral gavage.
Treated at	:	Constant dosages in mg/kg/day.
Volume dosage	:	10 ml/kg/day.
Individual dose volume	:	Calculated from the most recently recorded scheduled bodyweight up to and including Day 17 after mating; thereafter the dosage volume will remain constant to Day 1 of lactation. From Day 1 of lactation dose volume will be calculated, once again, from the most recently recorded bodyweight.
Controls (Group 1)	:	Vehicle at the same volume dosage as treated groups.
Frequency	:	Once daily at approximately the same time each day.
Sequence	:	By group.
Formulation	:	A daily record of the usage of formulation will be maintained based on weights. This balance is compared with the expected usage as a check of correct administration. Formulations are stirred using a magnetic stirrer before and throughout the dosing procedure, unless otherwise directed by the Sponsor.

4.3.2. Clinical observations

Animals and their cages	:	Visually inspected at least twice daily for evidence of reaction to treatment or ill-health.
Deviations from normal recorded at the time in respect of	:	Nature and severity. Date and time of onset. Duration and progress of the observed condition.

In addition detailed observations will be performed to establish and confirm a pattern of signs according to the following minimum schedule:

Minimum schedule	:	1. Week 1 - Daily. 2. Weeks 2 to 4 - twice weekly (middle and end of the week) 3. Week 5 onward - once each week.

Detailed post-dosing observations will be performed daily, in the treatment period, at the following times during the day:

Dose observation	:	1. Pre-dose observation. 2. As each animal is returned to its home cage. 3. At the end of dosing each group 4. Between 1 and 2 hours after completion of dosing all groups or, when the duration of dosing is protracted, 1 to 2 hours after completion of each group. 5. As late as possible in the working day.

The above schedule will be amended, as necessary, in light of signs observed.

4.3.3. Mortality

Premature sacrifice	:	Animals may be killed on humane grounds or if considered *in extremis*.
Animals found dead, killed *in extremis* or on humane grounds	:	A necropsy is performed as soon as possible. Animals found outside the normal workday will be preserved in a refrigerator (approximately 4°C) provided for this purpose.

4.3.4. Bodyweight

F_0 males	:	Twice weekly until mating. Before necropsy.
F_0 females	:	Twice weekly. After mating: Days 0, 3, 6, 9, 13, 17, 20. During lactation: Days 1, 4, 7, 10, 14, 17, 21. Before necropsy.

More frequent weighing may be performed to aid monitoring of condition of animals displaying ill-health. These data will be retained in the archives.

4.3.5. Food consumption

(F_0 animals only)

Until paired for mating	:	Weekly.
Females after mating	:	Days 0-5, 6-12, 13-19.
Females after parturition	:	Days 1-3, 4-6, 7-13, 14-20.

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4.3.6. Oestrous cycles

Dry smears	:	For 15 days before pairing.
Wet smears	:	After pairing until evidence of mating confirmed.

4.3.7. Mating

F_0 Pairing commences	:	After 71 days of treatment for males / 15 days of treatment for females.
Male:female ratio	:	1:1
Duration of pairing	:	Up to 3 weeks. If there has been no positive evidence of mating after 14 days, the male partner will be replaced by a proven male within the same group.
Daily checks for evidence of mating	:	Ejected copulation plugs. Sperm within vaginal smear.
Day 0 of gestation	:	When positive evidence of mating detected.
Male/female separation	:	Day when mating evidence confirmed (smearing may be continued up to 5 days after inconclusive mating to confirm positive mating).
Pre-coital interval	:	Calculated for each female as time between first pairing and evidence of mating.

4.3.8. Parturition observations and gestation length

Duration of gestation	:	Time elapsing between detection of mating and commencement of parturition.
Parturition observations	:	From Day 20 after mating, animals checked 3 times daily for evidence of parturition. If difficulties observed, progress of parturition process monitored. Approximate numbers of live and dead offspring recorded.

4.3.9. Records made during littering phase

Litter size	:	Days 1, 4, 7, 11, 14, 18, 21. Litters culled to 10 (where possible, 5M + 5F) on Day 4 of age.
Sex ratio	:	Recorded Days 1, 4 (before and after culling) and 21.
Offspring identification	:	By toe tattoo on Day 1 of age.
Individual offspring bodyweight	:	Days 1, 4, 7, 14, 21.

4.4. Animals - termination

All animals will be subject to terminal investigations (Section 5).

5. NECROPSY AND HISTOLOGY

5.1. Time of necropsy

F_0 males	:	When majority of F_0 females have been killed.
F_0 females	:	i) After litters weaned. ii) On day of litter loss. iii) Day 25 after mating or pairing if no young born.
F_1 animals	:	Day 21 of age.

5.2. Method of kill

Adult animals and offspring after weaning	:	Inhaled carbon dioxide.
Culled offspring	:	Intraperitoneal sodium pentobarbitone injection discarded without necropsy.

5.3. Macroscopic pathology

All animals (except those culled) will be subject to a detailed macroscopic examination for evidence of disease or adverse reaction to treatment and abnormal tissues retained. Special attention will be paid to the organs of the reproductive system.

5.3.1. Examination of males

The following procedures will be performed:

F_0 males: Gross necropsy.

Organs will be weighed and retained as detailed in Table 1 - Pathology procedures (Section 6).

5.3.2. Sperm Analysis

Vas deferens (from left side)	:	Sperm sample assessed for motility using a computer assisted sperm analyser (CASA). Groups 1 and 4

5.3.3. Examination of females

The following procedures will be performed:

F_0 Females: After litter weaned/died, if failure to mate (Day 25 after last oestrus) or 25 days after mating if no litter born.	:	Gross necropsy. Number of implantation sites recorded. Organs will be weighed and retained as detailed in Table 1 - (section 5.5).
Females with total litter loss	:	Mammary tissue also retained.

5.3.4. Examination of offspring

Dead offspring†	:	Fix in Industrial Methylated Spirits. Examined. Eviscerated. Retained in Industrial Methylated Spirits.
Grossly abnormal offspring at Day 4 of age	:	Gross examination. Eviscerated. Retained in Industrial Methylated Spirits.

† Offspring older than 14 days may not be fixed prior to examination.

Photographs may be prepared showing representative treatment-related macroscopic abnormalities.

5.4. Organ weights

Recording	:	As detailed in Table 1 - (section 5.5).

5.5. Fixation

Standard	:	4% neutral buffered formaldehyde. Testes and epididymides - Initially in Bouin's fluid.

TABLE 1 - Pathology procedures

Tissue	Weigh	Fix
Abnormalities		*
Epididymides	L+R	R
Mammary glands - caudal		a
Ovaries	L+R	L+R
Pituitary		c
Prostate	*	*
Seminal vesicles and coagulation gland	*	*
Testes	L+R	R
Uterus, oviduct, cervix	*	*
Vagina		*
Target organs d	if poss	*

a For adult females with total litter loss.
c For parental animals with suspect fertility.
d Where these are identified during the course of the study they will be specified in an amendment to protocol and a supplementary cost will be issued to cover additional work not foreseen at the start of the study
* Organs to be weighed and/or tissue samples to be fixed.

6. DATA TREATMENT

6.1. Data processing

Summary data presented as mean with standard deviation (SD) as appropriate.

Bodyweight

- **Adults** : Group mean values and SD calculated from individual data.

- **Offspring** : Mean and SD calculated for each litter. Group mean and SD calculated from individual litter values.

Food consumption : Group mean values and SD calculated for the periods specified in Section 4.3.

Oestrous cycles : Percentage of females showing the following cycle types calculated:

Regular : All observed cycles of 4 or 5 days (may be divided into cycles of 4, 4 and 5, and 5 days, if inter-group differences are apparent).

Irregular : At least one cycle of 2, 3 or 6 to 10 days.

Acyclic : At least 10 days without oestrus (beginning before pairing).

Additional classification may be included if necessary.

C 03

Pre-coital interval : Percentage of females with pre-coital intervals calculated for durations of 1-4, 5-8, 9-12, 13-16 or 17-21 days.

Percentage mating * : $\frac{\text{Number animals mating}}{\text{Animals paired}} \times 100$

Conception rate * : $\frac{\text{Number animals achieving pregnancy}}{\text{Animals mated}} \times 100$

Fertility index * : $\frac{\text{Number animals achieving pregnancy}}{\text{Animals paired}} \times 100$

Gestation index : $\frac{\text{Number of live litters born}}{\text{Number pregnant}} \times 100$

Post-implantation survival index : $\frac{\text{Total number offspring born}}{\text{Total number uterine implantation sites}} \times 100$

Live birth index : $\frac{\text{Number live offspring on Day 1 after littering}}{\text{Total number of offspring born}} \times 100$

* Calculated separately for males and females

Viability index : $\frac{\text{Number live offspring on Day 4 after littering}}{\text{Number live offspring on Day 1 after littering}} \times 100$

Lactation index : $\frac{\text{Number live offspring on day of examination}}{\text{Number live offspring on Day 4 after littering}} \times 100$

Sex ratio : Calculated f. Day 1, live offspring Days 1, 4 (before and after culling) and at Day 21 of age.

Organ weights : Absolute and relative to bodyweight. Group mean values and SD calculated.

Sperm analysis

Vas deferens : The percentages of motile and progressively motile sperm will be reported. Additional motility data will be recorded but no assessment of this data will be made unless there is evidence of an effect on the percentage motile sperm.

6.2. Statistical analysis

Appropriate tests will be performed as required.

Details of statistical tests used will be included in the final report.

7. REPORTING

Study progress : Periodic verbal and written updates on study progress will be provided by the Study Director.

Draft final report : For review by the Sponsor.

Authorised final report : After approval from the Sponsor.

Routinely reports are supplied on A4 paper. The following numbers of reports are supplied:

Type of report	Printing	Number of copies	
		Bound	Unbound
Draft report	Single-sided	0	2
Authorised final	Double-sided	1	0
	Single-sided	0	1
Photographic report (if any)	Single-sided	1	0

Any additions or corrections to an authorised final report will be documented as a formal addendum/amendment to the final report.

8. QUALITY ASSURANCE AND ARCHIVING PROCEDURES

8.1. Quality Assurance

The following will be inspected or audited in relation to this study:

Protocol Audit : Authorised protocol and any amendments.

Study based inspections : Critical phases of this study will be inspected.

Process based inspections : Routine and repetitive procedures will be inspected on representative studies, not necessarily on this study.

Report Audit : The draft report and study data will be audited after issue of the draft report to the Sponsor.

QA findings will be reported to the Study Director and Company Management promptly on completion of each action, except for process based inspections, which will be reported to appropriate Company Management only.

8.2. Archiving

All raw data, samples and specimens arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

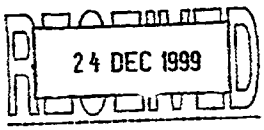
All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

06

Study Number : ACR/035
Protocol Amendment Number : 1

Huntingdon Life Sciences



PTB

STUDY OF REPRODUCTIVE FUNCTION AND

FERTILITY IN THE CD RAT BY ORAL GAVAGE

ADMINISTRATION

Total number of pages: 2

Number of pages for internal distribution: 2

Study Director : S.M. Fulcher, B.A., F.I.A.T.

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

FIRST AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: S Fulcher Date: 6 December 1999
(Study Director)

For the Sponsor

Approved by: Jean L. McCune Date: December 21, 1999

C 07

Study Number : **ACR/035**
Protocol Amendment Number : **1**

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PTB

STUDY OF REPRODUCTIVE FUNCTION AND

FERTILITY IN THE CD RAT BY ORAL GAVAGE

ADMINISTRATION

Reason for amendment : Section 3.3: Due to problems with the preparation of a 200 mg/ml formulation, a high concentration of 100 mg/ml will used for assessment of homogeneity and stability.

Amendments

3. TEST SUBSTANCE AND FORMULATION

3.3 Quality control of dosage form

Liquid formulation : Before commencement of treatment, the suitability of the proposed mixing procedures will be determined and specimen formulations at 1 and ~~200~~ 100 mg/ml will be analysed to assess the homogeneity and stability of the test substance in the liquid matrix.

At specified intervals during treatment, the test formulations will be analysed for achieved concentration of the test substance.

Analysis : The formulated samples will be analysed using a method validated with respect to the determination of the specificity of analysis, limits of quantitation and/or detection, linearity of detector response, reproducibility, method accuracy and precision.

Homogeneity and stability

Preparation : Specimen formulations (nominally 400 ml) will be prepared at 1 and ~~200~~ 100 mg/ml and equally distributed between four screw-capped amber bottles.

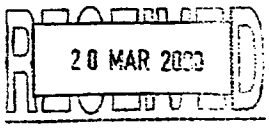
Sampling and determination : Before the initial sampling, each formulation will be mixed by inversion and magnetic stirring for 5 minutes. At each time-point, samples (nominally 1 ml) will be taken for assay from the top, middle and bottom of the formulation and accurately weighed.

Storage conditions : Ambient temperature (nominally 21°C) for 0, 1 and 2 hours while magnetically stirred and
48 hours (Bottle 1). Refrigeration (nominally 4°C) for 2, 8 and 15 days (Bottles 2, 3 and 4).

C 08

Study Number : ACR/035
Protocol Amendment Number : 2

Huntingdon Life Sciences



PTB

STUDY OF REPRODUCTIVE FUNCTION AND

FERTILITY IN THE CD RAT BY ORAL GAVAGE

ADMINISTRATION

Total number of pages: 8

Number of pages for internal distribution: 8

Study Director : S.M. Fulcher, B.A., F.I.A.T.

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

SECOND AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: S Fulcher Date: 8 March 2000
(Study Director)

For the Sponsor

Approved by: Joan L. McCann Date: 15 March 2000

C 09

Study Number : ACR/035
Protocol Amendment Number : 2

Huntingdon Life Sciences

PTB

STUDY OF REPRODUCTIVE FUNCTION AND FERTILITY IN THE CD RAT BY ORAL GAVAGE ADMINISTRATION

Reason for amendment : Section 1: Updating of UK GLP regulation.
Section 1, 2.4: Addition of dosages.
Section 2.1: Correction to duration of treatment - F_1 animals are untreated.
Section 2.2: Completion of time plan.
Section 2.4: Addition of further animals to the Control group.
Section 3.1: Addition of Nitrogen to storage conditions.
Section 3.2: Addition of dose concentrations and frequency of preparation of dose formulations.
Section 4.3.1: Clarification of dosing procedure immediately following parturition.
Section 4.3.2: Clarification of procedures for clinical signs and post-dosing observations.
Section 4.3.4: Correction to bodyweight schedule.
Section 4.3.9: Correction to frequency of litter size recordings.
Section 5.3.2: Clarification of procedures for sperm analysis.

Amendments

1. INTRODUCTION

Management of study

Study Director	: S.M. Fulcher
Monitoring Toxicologist	: A.M. Bottomley
In the temporary absence of the Study Director, the scientific responsibilities will be taken over by the Monitoring Toxicologist; other items of routine study management should be referred to the following person in the first instance.	: S.C.J. Reader

Objective

Study Number : ACR/035
Protocol Amendment Number : 2

Huntingdon Life Sciences

Assessment of the influence of oral gavage administration of the test substance on gonadal function, mating behaviour and fertility in treated rats of the F_0 generation and the survival of offspring up to Day 21 of age.

Regulatory compliance

The study will be performed in accordance with the following regulations or guidelines:

OECD (415), adopted 26 May 1983.

Good Laboratory Practice

The study will be conducted in compliance with principles of Good Laboratory Practice Standards as set forth in:

The UK Good Laboratory Practice Regulations 1997 (Statutory Instrument No 654 and, from 14 December 1999, The UK Good Laboratory Practice Regulations 1999 (Statutory Instrument No 3106).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17.

EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No L 77/8).

Animals (Scientific Procedures) Act 1986 compliance

The in-life experimental procedures to be undertaken during the course of this study are subject to the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986 (the Act). The Act, administered by the UK Home Office, regulates all scientific procedures in living animals which may cause pain, suffering, distress or lasting harm and provides for the designation of establishments where procedures may be undertaken, the licensing of trained individuals who perform the practical techniques and the issue of project licences for specified programmes of work.

This study will comply with all applicable sections of the Act and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures and the Humane Killing of Animals under Schedule 1 to the Act, issued under section 21 of the Act.

Animal model : CD rat, accepted as a predictor of reproductive change in man; requirement for a rodent species by regulatory agencies.

Route : Oral gavage, to simulate a potential condition of human exposure.

Treatment groups and dosages

Group	:	1	2	3	4
Compound	:	Control		PTB	
Dosage (mg/kg/day)†	:	0	~~Low~~ 100	~~Intermediate~~ 300	~~High~~ 1000

† Dosages~~, to be~~ selected following completion of a preliminary investigation of reproductive function and fertility in the rat (ACR/034)~~ and added by protocol amendment~~.

2.1 Duration of treatment

F_0 males	:	For 71 days before pairing until necropsy, until the majority of the F_0 females have been killed.
F_0 females	:	For 15 days before pairing until necropsy after weaning at Day 21 of lactation.
~~F_1 animals~~	:	~~From about Day 14 of age until necropsy at Day 21 of age.~~

2.2 Scheduled time plan

(to be up-dated as required in an amendment to protocol)

Sample of PTB to arrived	:	23 November 1999
F_0 males to arrive	:	~~+~~15 March 2000
F_0 females to arrive	:	~~+~~10 May 2000
Treatment of F_0 males to commence	:	~~+~~20 March 2000
Treatment of F_0 females to commence	:	~~+~~15 May 2000
Draft report to be issued	:	~~+~~End of September 2000 (estimated)

~~+ Dates to be incorporated by protocol amendment.~~

2.4 Identity of treatment groups

(F_0 to be selected from ~~216~~ 264 animals ordered)

F_0 generation

Group	Treatment	Dosage (mg/kg/day)@ #	Number of animals		Animal numbers	
			Male	Female	Male	Female
1	Control	0	~~24~~ 48	~~24~~ 48	~~1-24~~ 1-48	~~97-120~~ 121-168
2	PTB	~~Low~~ 100	24	24	~~25-48~~ 49-72	~~121-144~~ 269-192
3	PTB	~~Intermediate~~ 300	24	24	~~49-72~~ 73-96	~~145-168~~ 193-216
4	PTB	~~High~~ 1000	24	24	~~73-96~~ 97-120	~~169-192~~ 217-240

\# Expressed in terms of the test substance as supplied. A conversion factor for alternative forms (*e.g.* active ingredient, base weight) will be used, if requested by the Sponsor and documented in an amendment to the protocol.

@ Dosages ~~to be~~ selected following completion of a preliminary investigation of reproductive function and fertility in the rat (ACR/034)~~, and added by protocol amendment~~.

3.1 Test substance

Sponsor's identification	:	PTB
Storage conditions	:	In a refrigerator (approximately 4°C) in the dark under Nitrogen.
Sponsor's responsibilities	:	Documentation of methods of synthesis, fabrication or derivation. Stability data. Certificate of analysis.
Certificate of analysis details	:	Test substance identity. Batch number. Purity. Composition. Other appropriate characteristics. Current expiry date.

Study Number : ACR/035
Protocol Amendment Number : 2

Huntingdon Life Sciences

3.2 Formulation

Treatment		
Group 1, Control	:	Vehicle.
Group 2	:	PTB; ~~low~~ 10 mg/ml.
Group 3	:	PTB; ~~intermediate~~ 30 mg/ml.
Group 4	:	PTB; ~~high~~ 100 mg/ml.
Conversion factor	:	The test substance will be used as supplied unless otherwise advised by the Sponsor. Any such requirement will be documented in an amendment to protocol.
Vehicle	:	Water for formulation.
Method of preparation	:	Will be documented in the study data and included in the final report.
Frequency of preparation	:	~~Will depend upon the availability of supporting stability data.~~ Formulations will be prepared in batches of up to one week, and may be prepared up to 3 days in advance of first use.

4.3.1 Administration

Route	:	Oral gavage.
Treated at	:	Constant dosages in mg/kg/day.
Volume dosage	:	10 ml/kg/day.
Individual dose volume	:	Calculated from the most recently recorded scheduled bodyweight up to and including Day 17 after mating; thereafter the dosage volume will remain constant to Day 1 of lactation. From Day 1 of lactation dose volume will be calculated, once again, from the most recently recorded bodyweight.
Controls (Group 1)	:	Vehicle at the same volume dosage as treated groups.

Frequency	:	Once daily† at approximately the same time each day.
Sequence	:	By group.
Formulation	:	A daily record of the usage of formulation will be maintained based on weights. This balance is compared with the expected usage as a check of correct administration. Formulations are stirred using a magnetic stirrer before and throughout the dosing procedure, unless otherwise directed by the Sponsor.

† Animals that are in parturition will not be dosed. Animals that have completed parturition and can be dosed (prior to Day 1) will be weighed and dose volumes adjusted accordingly. These data will be recorded. Where Day 1 weighing is scheduled for later on the same day this earlier weight will be accepted as the Day 1 weight.

4.3.2 Clinical observations

Animals and their cages	:	Visually inspected at least twice daily for evidence of reaction to treatment or ill-health.
Deviations from normal recorded at the time in respect of	:	Nature and severity. Date and time of onset. Duration and progress of the observed condition.
Clinical signs	:	Recorded on a weekly basis.

Post dosing observations

In addition detailed observations will be performed to establish and confirm a pattern of signs according to the following minimum schedule:

Minimum schedule	:	1. Week 1 - Daily. 2. Weeks 2 to 4 - twice weekly (middle and end of the week) 3. Week 5 onward - once each week.

Detailed post-dosing observations will be performed daily, in the treatment period, at the following times during the day:

Dose observation	:	1. Pre-dose observation. 2. As each animal is returned to its home cage. 3. At the end of dosing each group. 4. Between 1 and 2 hours after completion of dosing all groups or, when the duration of dosing is protracted, 1 to 2 hours after completion of each group. 5. As late as possible in the working day.

The above schedule will be amended, as necessary, in light of signs observed.

Study Number : ACR/035
Protocol Amendment Number : 2

Huntingdon Life Sciences

4.3.4 Bodyweight

F_0 males	:	Twice weekly ~~until mating~~. Before necropsy.
F_0 females	:	Twice weekly until mating. After mating: Days 0, 3, 6, 9, 13, 17, 20. During lactation: Days 1, 4, 7, 10, 14, 17, 21. Before necropsy.

More frequent weighing may be performed to aid monitoring of condition of animals displaying ill-health. These data will be retained in the archives.

4.3.9 Records made during littering phase

Litter size	:	~~Days 1, 4, 7, 11, 14, 18, 21.~~ Daily. Litters culled to 10 (where possible, 5M + 5F) on Day 4 of age.
Sex ratio	:	Recorded Days 1, 4 (before and after culling) and 21.
Offspring identification	:	By toe tattoo on Day 1 of age.
Individual offspring bodyweight	:	Days 1, 4, 7, 14, 21.

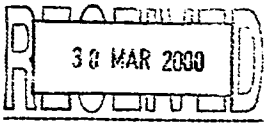
5.3.2 Sperm Analysis

Vas deferens (from left side)	:	Sperm sample assessed for motility using a computer assisted sperm analyser (CASA). ~~Groups 1 and 4.~~ Data are automatically collected for all animals but will only be reported for Groups 1 and 4 (data will be presented for Groups 2 and 3 if a treatment related response is suspected in Group 4).
	:	Sample retained for possible manual assessment of sperm morphology.
Cauda epididymis	:	The left cauda epididymis will be weighed and retained for possible assessment of sperm count using a computer assisted sperm analyser. All groups.
Testis	:	The left testis will be weighed and retained for possible assessment of homogenisation-resistant spermatid count using a computer assisted sperm analyser (CASA). All groups.

D 02

Study Number : ACR/035
Protocol Amendment Number : 3

Huntingdon Life Sciences



PTB

STUDY OF REPRODUCTIVE FUNCTION AND FERTILITY IN THE CD RAT BY ORAL GAVAGE ADMINISTRATION

Total number of pages: 3

Number of pages for internal distribution: 3

Study Director : S.M. Fulcher, B.A., F.I.A.T.

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

SF 6/5/00

THIRD
~~SECOND~~ AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: _____ Date: 20 March 2000
(Study Director)

For the Sponsor

Approved by: Jean L. McEwen Date: 24 March 2000

Study Number : **ACR/035**
Protocol Amendment Number : **3**

Huntingdon Life Sciences

PTB

STUDY OF REPRODUCTIVE FUNCTION AND FERTILITY IN THE CD RAT BY ORAL GAVAGE ADMINISTRATION

Reason for amendment : Section 2.4: Correction to typographical error.
Section 4.2.3: Correction of diet type.
Section 5.3.1: Correction to typographical error.

Amendments

2.4 Identity of treatment groups

(F_0 to be selected from 264 animals ordered)

F_0 generation

Group	Treatment	Dosage (mg/kg/day)@ #	Number of animals		Animal numbers	
			Male	Female	Male	Female
1	Control	0	48	48	1-48	121-168
2	PTB	100	24	24	49-72	~~269~~ 169-192
3	PTB	300	24	24	73-96	193-216
4	PTB	1000	24	24	97-120	217-240

Expressed in terms of the test substance as supplied. A conversion factor for alternative forms (*e.g.* active ingredient, base weight) will be used, if requested by the Sponsor and documented in an amendment to the protocol.

@ Dosages selected following completion of a preliminary investigation of reproductive function and fertility in the rat (ACR/034).

D 04

4.2.3 Bedding, diet and water supply

Copies of all certificates of analysis are stored in the archives.

Bedding supply (for solid bottomed cages)

Bedding name	:	Lignocel 3/4 wood flakes.
Certification	:	Certificates are routinely received from the supplier.

Diet supply

Diet name	:	Rodent Diet VFR - 1C.
Diet type	:	~~Powdered diet.~~ Pelleted diet.
Availability	:	Non-restricted.
Certification	:	Before delivery each batch of diet is analysed by the supplier for various nutritional components and chemical and microbiological contaminants. Supplier's analytical certificates are scrutinised and approved before any batch of diet is released for use.

This diet contains no added antibiotic or other chemotherapeutic or prophylactic agent.

Water supply

Supply	:	Public drinking water.
Regulatory agency	:	U.K. Department of the Environment.
Availability	:	Non-restricted via polyethylene or polycarbonate bottles with sipper tubes.
Certification	:	Certificates are routinely received from the supplier.

Copies of all certificates of analysis are stored in the archives.

5.3.1 Examination of males

The following procedures will be performed:

F_0 males: Gross necropsy.

Organs will be weighed and retained as detailed in Table 1 - Pathology procedures (Section ~~6~~ 5.5).

D 05

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