US EPA ARCHIVE DOCUMENT

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF PREVENTION, PESTICIDES AND TOXIC SUBSTANCES

June 9, 2000

Memorandum

Subject: Secondary Review of Data Evaluation Reports on Four Studies (MRIDs 45045201-03, and 45056701; D264411) with Oil of Lemon Eucalyptus Technical (PC 011550:WPC Brands; Case No. 062646; S565022).

From: Roger Gardner, Toxicologist Roger Gardner 6/9/00
Biochemical Pesticides Branch
Biopesticides and Pollution Prevention Division (7511C)

Thru: Sheryl Reilly, Ph.D., Acting Chief
Biochemical Pesticides Branch
Biopesticides and Pollution Prevention Division (7511C)

To: Jim Downing, Regulatory Action Leader
Biochemical Pesticides Branch
Biopesticides and Pollution Prevention Division (7511C)

Action Requested

Secondary review of the Data Evaluation Reports (DER) on the following studies:

28-Day dermal toxicity study (MRID 45045203)
Dermal developmental toxicity study (MRID 45056701)
In vitro mammalian cell point mutation assay (MRID 45045201)
In vivo mouse micronucleus assay (MRID 45045202)

Recommendations and Conclusions

The attached DERs are acceptable reviews of the dermal toxicity, developmental toxicity and mutagenicity studies with Oil of Lemon Eucalyptus Technical. However, it should be noted that the developmental toxicity study is reclassified as supplementary for reasons described below.

Discussion

The attached DERs were prepared by members of the Chemical Hazard Evaluation Group, Toxicology and Risk Analysis Section, Life Sciences Division, Oak Ridge National Laboratory.

A. Dermal toxicity study

The DER summarized the results of the study as follows:

> In a 28-day dermal toxicity study (MRID 45045203), groups of 10 male and 10 female Crl:CD®(SD)/GS BR VAF/Plus® rats were treated with citriodiol (100%, Lot No. 0299088R-B) at the limit dose of 1000 mg/kg/day in a dosage volume of 1 mL/kg/day. Two identical vehicle control groups of 10 rats/sex/group (VC1 and VC2) were treated with 1 mL/kg/day of white mineral oil. Animals were treated by dermal occlusion for 6 hours/day for 28 consecutive days. A functional observational battery (FOB) followed by a motor activity evaluation were conducted on day 29 prior to sacrifice.
>
> There were no treatment-related deaths or signs of systemic toxicity. Final body weights were non-statistically significantly reduced for male rats (97 and 97% of control values) and statistically significantly reduced for female rats compared with VC2 (95%, $p<0.05$) but not VC1 (98%). These reductions in body weight were not considered biologically significant. There were no treatment-related effects on food consumption, hematology, clinical chemistry, or organ weights. There were no effects on FOB parameters or motor activity. Skin irritation including flaking, erythema, and edema, was observed at the site of application and the severity was greater in the test substance group than in the control groups. Incidences of flaking and erythema were significantly increased in both sexes compared with both control groups ($p<0.01$). Microscopic examination of these and adjacent skin sites revealed hyperplasia of the sebaceous glands, hyperplasia/ hyperkeratosis of the epidermis, and multifocal dermal inflammation.
>
> **The dermal LOAEL is 1000 mg/kg/day based on slight skin irritation and microscopic changes in the skin at the site of application. A dermal NOAEL was not identified. The NOAEL for systemic toxicity is 1000 mg/kg/day based on a lack of effects on body weight, food consumption, organ weights, tissue/organ histology (exclusive of the skin), functional observational battery parameters and motor activity. A LOAEL for systemic effects was not identified.**
>
> This study is classified as **Acceptable/Guideline** and satisfies the guideline requirements for a subchronic dermal study [OPPTS 870.3200 (§82-2)] in the rat.

B. Developmental toxicity study

1. Primary review conclusions

Results of the study were summarized in the DER as follows:

> In a developmental toxicity study (MRID 45056701), 25 presumed pregnant Crl:CD®(SD)IGS BR VAF/Plus rats per group were given either mineral oil or 1000 mg/kg/day of oil of lemon eucalyptus (100% a.i.; Lot No.: 0299088R-B) on gestation days (GD) 6-20, inclusive. The test or control article was applied neat by dermal semi-occlusion for 6 hr/day. On GD 21, all surviving dams were sacrificed and all fetuses were weighed and examined for external malformations/variations. Approximately one-half of the fetuses were examined viscerally; the

heads of these fetuses were placed in Bouin's fixative and subsequently examined by free-hand cross-sectioning. The remaining one-half of the fetuses were fixed in alcohol and processed for skeletal examination.

All animals survived until scheduled sacrifice and maternal necropsy was unremarkable. Significantly ($p \leq 0.01$) increased incidences of grade 1 erythema (24/25), grade 1 edema (11/25), grade 1 flaking (25/25), and grade 2 flaking (9/25) were observed in treated animals compared with only one control animal with grade 1 flaking. No other clinical signs of toxicity were observed. No differences in absolute body weights were observed between the treated and control groups throughout the study. Adjusted body weights were similar between the treated and control groups. Body weight gains by the treated dams were significantly ($p \leq 0.05$ or 0.01) reduced for GD 6-9, 9-12, and 6-21. The most pronounced effect on body weight gains was after the initiation of treatment for the interval of GD 6-9 when weight gain by the treated animals was 66.4% of the control group level. Weight gains by the treated group were 85.4-102.2% of controls for the remainder of the dosing interval and 92.4% of controls for the entire dosing interval.

Therefore, the maternal toxicity LOAEL is 1000 mg/kg/day based on skin irritation and transient reductions in body weight gains. The maternal toxicity NOAEL was not identified.

The number of corpora lutea/dam, implantations/dam, gravid uterine weights, pre- or post-implantation loss, resorptions/dam, fetal body weights, and fetal sex ratios were not affected by treatment with the test article.

No treatment-related external, visceral, or skeletal malformations/variations were observed in the fetuses following maternal treatment with the test article.

Therefore, the developmental toxicity NOAEL is ≥1000 mg/kg/day and the developmental toxicity LOAEL was not identified.

This study is classified as **Acceptable/Guideline** and satisfies the guideline requirements for a developmental toxicity study [870.3700 (§83-3a)] in rats. It should be noted that only one dose was tested. However, this dose was the limit dose for developmental toxicity studies, maternal effects were mild and transient, and no developmental toxicity was observed. Testing of additional dose levels would not provide any more useful information. Therefore, this study is considered adequate for assessing the developmental toxicity potential of oil of lemon eucalyptus in the rat.

2. Additional considerations

There were no significant differences in group mean body weights between the control and treated group, and there were no clinical signs of toxicity other than dermal irritation to suggest that oil of lemon eucalyptus caused systemic maternal toxicity. However, there are additional considerations described below that indicate that the study should be reclassified as supplementary.

Although dermal absorption of an OLE component (*p*-menthane-3,8-diol [PMD]) was observed in the human study, an absorption rate was not determined. Dr. John Caldwell, a co-investigator for a rat metabolism study with menthol, which is metabolized to PMD, noted, "Our unpublished data with radiolabeled menthol (in a separate *in vitro* experiment) showed that less than 1% of the total radioactivity applied to the surface penetrated through fresh full thickness human skin, due to marked loss of applied menthol from the skin surface." This loss was attributed to the volatility of menthol, but no information was provided on the proportions of applied radioactivity recovered in

the treated skin. Nevertheless, the submitted information on PMD and menthol suggests that dermal absorption of substances similar to those found in oil of lemon eucalyptus is probably low.

Since dermal absorption of OLE is probably low, the capacity of the developmental toxicity study to evaluate OLE, particularly with regard to events that occur soon after implantation and during early organogenesis may be reduced. Typically, the Agency recommends that dermal developmental toxicity studies begin topical dosing on gestation day 0 when the studies are initiated because the kinetics of dermal absorption in cases such as OLE generally require a few days of treatment before a maximum daily absorbed dose can be maintained, and since dermal treatment was not initiated until near the time of implantation in the OLE study (gestation day 6), the dermal developmental toxicity study with OLE is of limited value in the assessment of developmental toxicity (e.g., evaluation of events such as early and late resorptions). This test protocol modification has been used in testing of other active ingredients used as insect repellents (e.g., PMD). Therefore, the submitted study is classified as supplementary.

C. Mutagenicity studies

1. Mammalian cell point mutation assay

The results from this mutagenicity assay were sumarized in the DER as follows:

> In a mammalian cell gene mutation assay (MRID 45045201), mouse lymphoma L5178Y cells cultured in vitro were exposed to oil of lemon eucalyptus (100%) in dimethylsulfoxide (DMSO) at concentrations of 50, 75, 100, 150, 175, 200, or 210 µg/mL (initial assay) and 50, 75, 100, 150, or 225 µg/mL (confirmatory assay) in the absence of mammalian metabolic activation (S9-mix). Cells were exposed to concentrations of 50, 75, 100, 150, 200, 250, 275, 300, or 325 µg/mL (initial assay) and 250, 275, 300, 310, 320, 330, 340, or 350 µg/mL (confirmatory assay) in the presence of S9-mix. The S9-fraction was obtained from Aroclor 1254 induced male Sprague-Dawley rat liver.
>
> Oil of lemon eucalyptus was tested up to concentrations limited by cytotoxicity. A preliminary cytotoxicity (range-finding) study without S9 at concentrations ranging from 3.93 to 2000 µg/mL showed that cell survival was 31.7% of the solvent control value at 250 µg/mL. In the presence of S9-mix, cell survival was <1% of the solvent control value at 500 µg/mL. In the main mutagenicity tests, in the absence of S9-mix, no increase in mutant frequency compared to solvent controls or dose-response was observed at any concentration tested in either duplicate culture, reaching a maximum of 62.8 mutants per 10^6 surviving cells at 210 µg/mL in replicate 1 and 80.6 mutants per 10^6 surviving cells at 225 µg/mL in replicate 2. In the presence of S9-mix, no increase in mutant frequency or dose-response was observed at any concentration tested in both duplicate cultures, reaching a maximum of 48.4 mutants per 10^6 surviving cells at 100 µg/mL in replicate 1 and 87.5 mutants per 10^6 surviving cells at 300 µg/mL in replicate 2. The positive and solvent controls induced the appropriate responses. **There was no evidence of induced mutant colonies over background.**
>
> This study is classified as **Acceptable/Guideline**. It satisfies the requirement for FIFRA Test Guideline [OPPTS 870.5300 (§84-2)] for in vitro mutagenicity (mammalian forward gene mutation) data.

2. Mouse micronucleus assay

The mouse micronucleus assay was summarized in the DER as follows:

In a mouse bone marrow micronucleus assay (MRID 45045202), six male mice/sex/dose/ harvest time were treated once by intraperitoneal injection with oil of lemon eucalyptus (100%) in corn oil at doses of 250, 500, or 1000 mg/kg. Bone marrow cells were harvested from five vehicle control and high-dose animals at 24 and 48 hours post-treatment and from five low- and mid-dose animals at 24 hours post-treatment.

Mortality was observed in 7/12 high-dose animals; animals in the mid- and high-dose groups exhibited prostration, labored breathing, ataxia, and slight hypoactivity. No clinical signs were noted in low-dose animals. (In a range-finding study, clinical signs were observed at 500, 800, and 1200 mg/kg and deaths were observed at 1200 mg/kg. No clinical signs were noted at 200 mg/kg in the range-finding study). There was no significant increase in the number of micronucleated polychromatic erythrocytes at any concentration at any of the three sampling times. The positive (cyclophosphamide) and solvent controls induced the appropriate response. **There was no significant increase in the frequency of micronucleated polychromatic erythrocytes in bone marrow after any dose or treatment time.**

This study is classified as **Acceptable/Guideline.** It does satisfy the requirement for FIFRA Test Guideline [OPPTS 870.5395 (§84-2)] for *in vivo* cytogenetic mutagenicity data.

Attachments

Data Evaluation Reports on the Following Citations:

Parker, R.M. (2000) 28-Day dermal toxicity study of oil of lemon eucalyptus in rats. Argus Research Laboratories, Inc., 905 Sheehy Drive, Building A, Horsham, PA 19044. Argus Research Laboratories, Inc., Protocol Number 720-004. February 9, 2000. MRID 45045203. Unpublished.

Parker, R.M. (2000) Dermal developmental toxicity study of oil of lemon eucalyptus in rats. Argus Research Laboratories, Inc., 905 Sheehy Drive, Building A, Horsham, PA 19044. Laboratory Project ID. 720-005. February 8, 2000. MRID 45056701. Unpublished.

Cifone, M.A. (2000) L5178Y $TK^{+/-}$ Mouse Lymphoma Forward Mutation Assay with a Confirmatory Assay with Oil of Lemon Eucalyptus. Covance Laboratories Inc., 9200 Leesburg Pike, Vienna, Virginia 22182. Laboratory Project Identification number: Covance 20901-0-431 OECD, February 4, 2000. MRID 45045201. Unpublished

Myhr, B.C. (2000) *In vivo* Mouse Micronucleus Assay with Oil of Lemon Eucalyptus. Covance Laboratories Inc., 9200 Leesburg Pike, Vienna, Virginia 22182. Laboratory Project Identification number Covance 20901-0-455OECD, February 4, 2000. MRID 45045202. Unpublished

DATA EVALUATION REPORT

CITRIODIOL
(OIL OF LEMON EUCALYPTUS)

STUDY TYPE: REPEATED DOSE DERMAL TOXICITY – RAT
(OPPTS 870.3200 [82-2])

MRID 45045203

Prepared for

Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Chemical Hazard Evaluation Group
Toxicology and Risk Analysis Section
Life Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37831
Task Order No. 33

Primary Reviewer: Sylvia S. Talmage, Ph.D., D.A.B.T.	Signature:	Sylvia S. Talmage
	Date:	APR 14 2000
Secondary Reviewers: Carol Forsyth, Ph.D., D.A.B.T.	Signature:	
	Date:	APR 14 2000
Robert H. Ross, M.S., Group Leader	Signature:	Robert H. Ross
	Date:	APR 14 2000
Quality Assurance: Lee Ann Wilson, M.A.	Signature:	L. A. Wilson
	Date:	APR 14 2000

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the U.S. Department of Energy under Contract No. DE-AC05-00OR22725.

7 21

EPA Reviewer: Freshteh Toghrol, Ph.D. Date: 4/19/00
EPA Work Assignment Manager: Sheryl Reilly Date: 4/26/00
Biopesticides and Pollution Prevention Division (7511W)

DATA EVALUATION RECORD

STUDY TYPE: 28 Day Dermal Toxicity, Rat [OPPTS 870.3200 (§82-2)]

DP BARCODE: D264411
P.C.CODE.: 011550 (p-Menthane-3,8-diol)

SUBMISSION CODE: S565022
CASE: 062646

TEST MATERIAL (PURITY): Citriodiol; active ingredient: extract of lemon eucalyptus (100%); extract of lemon eucalyptus is composed of 68.6 *p*-menthane-3,8-diol (66% *cis- p*-menthane 3,8-diol and 34% *trans-p*-menthane-3,8-diol). Citronellol (~5%), citronellal (~0.1%), mixed isomers of isopulegois (~12%), and other compounds related to citronellol and citronellal (~17%) are present as coextractives.

SYNONYMS: *p*-menthane 3,8-diol

CITATION: Parker, R.M. (2000) 28-Day dermal toxicity study of oil of lemon eucalyptus in rats. Argus Research Laboratories, Inc., 905 Sheehy Drive, Building A, Horsham, PA 19044. Argus Research Laboratories, Inc., Protocol Number 720-004. February 9, 2000. MRID 45045203. Unpublished.

SPONSOR: WPC Brands, Inc., 1 Repel Road, P.O. 198, Jackson, WI 53037.

EXECUTIVE SUMMARY: In a 28-day dermal toxicity study (MRID 45045203), groups of 10 male and 10 female Crl:CD®(SD)/GS BR VAF/Plus® rats were treated with citriodiol (100%, Lot No. 0299088R-B) at the limit dose of 1000 mg/kg/day in a dosage volume of 1 mL/kg/day. Two identical vehicle control groups of 10 rats/sex/group (VC1 and VC2) were treated with 1 mL/kg/day of white mineral oil. Animals were treated by dermal occlusion for 6 hours/day for 28 consecutive days. A functional observational battery (FOB) followed by a motor activity evaluation were conducted on day 29 prior to sacrifice.

There were no treatment-related deaths or signs of systemic toxicity. Final body weights were non-statistically significantly reduced for male rats (97 and 97% of control values) and statistically significantly reduced for female rats compared with VC2 (95%, $p<0.05$) but not VC1 (98%). These reductions in body weight were not considered biologically significant. There were no treatment-related effects on food consumption, hematology, clinical chemistry, or organ weights. There were no effects on FOB parameters or motor activity. Skin irritation including flaking, erythema, and edema, was observed at the site of application and the severity was greater in the test substance group than in the control groups. Incidences of flaking and erythema were significantly increased in both sexes compared with both control groups ($p<0.01$). Microscopic

examination of these and adjacent skin sites revealed hyperplasia of the sebaceous glands, hyperplasia/ hyperkeratosis of the epidermis, and multifocal dermal inflammation.

The dermal LOAEL is 1000 mg/kg/day based on slight skin irritation and microscopic changes in the skin at the site of application. A dermal NOAEL was not identified. The NOAEL for systemic toxicity is 1000 mg/kg/day based on a lack of effects on body weight, food consumption, organ weights, tissue/organ histology (exclusive of the skin), functional observational battery parameters and motor activity. A LOAEL for systemic effects was not identified.

This study is classified as **Acceptable/Guideline** and satisfies the guideline requirements for a subchronic dermal study [OPPTS 870.3200 (§82-2)] in the rat.

COMPLIANCE: Signed and dated Quality Assurance, Data Confidentiality, Good Laboratory Practice, were present.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Citriodiol (100% oil of lemon eucalyptus)

   Description: slightly viscous amber liquid
   Lot/Batch #: 0299088R-B
   Purity/Stability: Purity: 100% oil of lemon eucalyptus composed of 68.6% *p*-menthane-3,8-diol (66% cis-*p*-menthane-3,8-diol and 34% *trans-p*-menthane-3,8-diol) with coextractives present at ≤17%. Stability information was not provided.
   Structure: Not Available

2. Vehicle and/or positive control

   The test material was administered undiluted. White mineral oil, (USP; lot number N06704) was administered to both control groups. There was no positive control.

3. Test animals

   Species: rat
   Strain: Crl:CD®(SD)/GS BR VAF/Plus®
   Age and weight at study initiation: males: 8 weeks, 298-343; females: 8 weeks, 220-242 g
   Source: Charles River Laboratories, Inc., Raleigh, NC
   Housing: 1/stainless steel, wire-mesh-bottomed cage
   Diet: Certified Rodent Diet® #5002 (PMI Nutrition International, St. Louis, MO), *ad libitum*
   Water: reverse osmosis water with chlorine added as bacteriostat, *ad libitum*
   Environmental conditions:

Temperature: 70.4 ± 0.9°F
Humidity: 52.1 ± 9.2%
Air changes: 10 changes/hour
Photoperiod: 12 hr light/dark
Acclimation period: 7 days

B. STUDY DESIGN

1. In life dates

Start: October 4, 1999 end: November 4, 1999

2. Animal assignment

Rats were assigned to the test groups in Table 1 by means of a computer-generated (weight-ordered) randomization procedure.

TABLE 1. Study design

Dose Group	Test Material	Dose	No. of Animals	
			Male	Female
1 (VC1)[a]	white mineral oil	1 mL/kg/day	10	10
2 (VC2)[b]	white mineral oil	1 mL/kg/day	10	10
3 (TS)[c]	oil of lemon eucalyptus	1000 mg/kg/day[d]	10	10

Data taken from MRID 45045203, p. 24.
[a]Vehicle control 1.
[b]Vehicle control 2.
[c]Test substance.
[d]The dosage volume was 1 mL/kg/day.

3. Dose selection rationale

The single limit dose of 1000 mg/kg/day was selected by the Sponsor on the basis of information known about the test substance; no further information was provided.

4. Test substance preparation and analysis

The test substance was applied undiluted over a clipped area of the backs of the rats as a single daily application for 28 consecutive days. The test substance was assumed to be 100% pure and no analyses were performed. No homogeneity or stability analyses were provided. The concentration analysis was provided in Appendix F of the study report.

10 24

5. Dose application

The dorsal surface of each rat was clipped free of hair with an electric clipper approximately 24-hours prior to initial dosing and as necessary, thereafter. The vehicle control or test substance was applied at a dose volume of 1 mL/kg evenly to the clipped area with a syringe and distributed on the skin with a glass rod. The test substance was warmed to approximately 48.9°C and stirred continuously during the treatment period.

The treated area was semi-occluded with a porous gauze patch held in place with non-irritating tape. An Elizabethan collar prevented oral ingestion of the test substance. After 6 hours, the dressings and collars were removed and the treated sites were washed with a mild liquid soap, rinsed with water and gently patted dry. Animals were treated for 28 consecutive days and sacrificed one day later.

6. Statistics

The treated (group 3) and the two control groups were compared separately (groups 1 and 3 and groups 2 and 3). Variables with interval or ratio scales of measurement such as body weight, food consumption, hematologic parameters, clinical chemistry parameters, and absolute and relative organ weights were analyzed for homogeneity of variance with Bartlett's test. If variances were homogeneous (nonsignificant), the groups were compared using an Analysis of Variance (ANOVA) test with significant results between the two groups compared with Dunnett's test. Hematology and clinical chemistry data were tabulated, summarized and statistically analyzed using the LABCAT System version HE4.41C. If Bartlett's test was significant (nonparametric results) and 75% or fewer of the scores in all groups were tied, the Kruskal-Wallis test was used to analyze the data; if the results were significant, Dunn's test was used to compare the test substance and control groups. When fewer than 75% of the scores in any dosage group were tied, Fisher's Exact test was used to compare the proportion of ties in the dosage groups. Variables having graded or count scores were analyzed according to the nonparametric procedures above. Clinical observation incidence data and other proportion data were analyzed as contingency tables using the Variance Test for Homogeneity of the Binomial Distribution.

C. METHODS

1. Observations

Animals were examined for mortality twice daily. Prior to each application, dosing sites were examined for skin irritation (erythema, edema, or flaking) which was scored using a modified Draize system. Animals were also examined for clinical signs approximately 60 minutes after removal of the test substance. A detailed clinical examination that included many of the observations of a functional observational battery was conducted once prior to the test period and once weekly

during the treatment period. These examinations were conducted outside of the home cage.

2. Body weight

Animals were weighed prior to initial dosing, weekly thereafter, and immediately before terminal sacrifice.

3. Food consumption and food efficiency

Food consumption was measured weekly throughout the acclimation and study period. Food efficiency was not calculated by the study authors.

4. Ophthalmoscopic examination

Ophthalmoscopic examinations were performed prior to the test period and during the fourth week of the study.

5. Neurotoxicity evaluations

Functional observation battery (FOB). All animals were subjected to a FOB prior to sacrifice on day 29. Rats were tested in a random manner and the observer was blind to the treatment group. Home cage observations included the following: the state of arousal; mobility; posture; unusual, stereotypy or bizarre behavior; limb or whole body tremors, and seizures. During handling, reactions to removal and handling such as resistance, vocalization, muscle tone, and throat rattles were scored. In the open field, defecation, urination, level of arousal, stereotyped or bizarre behavior, tremors, seizures, gait patterns and abnormalities, palpebral closure, prominence of the eye, lacrimation, salivation, piloerection, and appearance were scored. Also in the open field, sensorimotor and neuromuscular measurements including responses to approach, touch, noise, and tail pinch; righting reflex; pupil response to light; forelimb and hindlimb grip strength; and landing foot splay were scored.

Motor activity. Following the FOB, motor activity was measured in stainless steel, wire-bottomed cages equipped with infrared sensors. Up to 32 cages and sensors could be monitored during each session. The total monitoring time was 1.5 hours with the number of movements and time spent in movement tabulated after each 5-minute interval.

6. Blood samples

Blood samples were obtained from the inferior vena cava at the time of sacrifice. It was not stated if animals were fasted prior to blood collection. The CHECKED (X) parameters were examined.

12 26

a. Hematology

X		X	
X	Hematocrit (HCT)*	X	Leukocyte differential count*
X	Hemoglobin (HGB)*	X	Mean corpuscular HGB (MCH)
X	Leukocyte count (WBC)*	X	Mean corpuscular HGB conc.(MCHC)
X	Erythrocyte count (RBC)*	X	Mean corpuscular volume (MCV)
X	Platelet count*		Reticulocyte count
	Blood clotting measurements*		
X	(Thromboplastin time)		
	(Clotting time)		
X	(Prothrombin time)		
	(Kaolin-cephalin time)		
X	Erythrocyte morphology		

*Recommended for dermal toxicity studies based on OPPTS 870.3200 Guidelines.

b. Clinical chemistry

X	ELECTROLYTES	X	OTHER
X	Calcium*	X	Albumin*
X	Chloride*	X	Blood creatinine*
	Magnesium*	X	Blood urea nitrogen*
X	Phosphorus*	X	Total Cholesterol*
X	Potassium*	X	Globulin*
X	Sodium*	X	Glucose*
		X	Total bilirubin*
	ENZYMES	X	Total serum protein (TP)*
X	Alkaline phosphatase (ALK)*	X	Triglycerides
	Cholinesterase (ChE)		Serum protein electrophoresis
	Creatine kinase		
	Lactic acid dehydrogenase (LDH)		
X	Serum alanine amino-transferase (also SGPT)*		
X	Serum aspartate amino-transferase (also SGOT)*		
	Gamma glutamyl transferase (GGT)*		
	Glutamate dehydrogenase		
	Sorbitol dehydrogenase*		

*Recommended for dermal toxicity studies based on OPPTS 870.3200 Guidelines.

7. Urinalysis

Urinalysis was not required and was not performed.

8. Sacrifice and pathology

At the end of the study, animals were sacrificed by carbon dioxide asphyxiation and necropsies were performed on all animals. The CHECKED (X) tissues from all groups were preserved in formalin and the tissues from Control Vehicle I (Group 1) and the Test Substance (Group 3) groups were examined histologically. The (XX) organs, in addition, were weighed.

X	DIGESTIVE SYSTEM	X	CARDIOVASCULAR/ HEMATOLOGICAL	X	NEUROLOGIC
	Tongue	X	Aorta*	XX	Brain*
X	Salivary glands*	XX	Heart*	X	Peripheral nerve*
X	Esophagus*	X	Bone marrow*	X	Spinal cord (3 levels)*
X	Stomach*	X	Lymph nodes*	X	Pituitary*
X	Duodenum*	XX	Spleen*	X	Eyes (optic nerve)*
X	Jejunum*	XX	Thymus*		
X	Ileum*				**GLANDULAR**
X	Cecum*		**UROGENITAL**	XX	Adrenal gland*
X	Colon*	XX	Kidneys*		Lacrimal gland
X	Rectum*	X	Urinary bladder*	X	Mammary gland
XX	Liver*	XX	Testes*	X	Parathyroids*
	Gall bladder (dogs)*	XX	Epididymides*	X	Thyroids*
X	Pancreas*	X	Prostate*		
		X	Seminal vesicle*		**OTHER**
	RESPIRATORY	XX	Ovaries*		Bone*
X	Trachea*	XX	Uterus*		Skeletal muscle
X	Lung*		Vagina	X	Skin (treated and untreated)*
X	Nose*			X	All gross lesions and masses*
X	Pharynx*				
X	Larynx*				

*Required for dermal toxicity studies based on OPPTS 870.3200 Guidelines.

II. RESULTS

A. OBSERVATIONS

All animals survived to the scheduled termination. No treatment-related signs of systemic toxicity were observed. Skin irritation including flaking, erythema, and edema, was observed in the vehicle control and Citriodiol test groups. Incidences of flaking (grades 1 and 2) and slight erythema (grade 1) were statistically significant ($p \leq 0.01$) when compared with both vehicle control groups (Table 2). In females, the incidence of grade 1 (slight) edema was also statistically significantly increased compared to VC1 ($p<0.01$).

TABLE 2. Incidences of skin irritation in rats topically treated with citriodiol for 28 days

Type of irritation	Vehicle control 1	Vehicle control 2	Citriodiol (1000 mg/kg/day)
Males			
Flaking			
grade 1	5/2[a]	0/0	56/10**++
grade 2	0/0	0/0	7/4**++
Erythema (grade 1)	0/0	1/1	7/4**++
Edema (grade 1)	5/2	0/0	3/2
Females			
Flaking			
grade 1	0/0	3/1	153/10**++
grade 2	0/0	0/0	41/7**++
Erythema			
grade 1	4/2	6/3	64/10**++
grade 2	0/0	0/0	3/1
Edema (grade 1)	0/0	3/1	22/5**

Data from Tables B1 and B2, pp. 50 and 51, MRID 45045203.
[a] Total number of observations/number of rats with observation.
** Significantly different from Control Group 1, $p \leq 0.01$.
++ Significantly different from Control Group 2, $p \leq 0.01$.

B. BODY WEIGHT

Mean body weight of male and female rats for selected days are reported in Table 3. Topical treatment of male rats with citriodiol at a dose of 1000 mg/kg/day had little effect on body weight as the final mean body weight was 97% of both vehicle control values. A slight lag in body weight gain was present by day 8 (significantly different from the VC2 [$p \leq 0.05$]) but did not increase substantially thereafter and was not statistically significantly less at later time periods (body weight gains are not shown in Table 3). For males, the mean total body weight gains for the VC1, VC2, and citriodiol groups were 99.2, 101.4, and 84.3 g, respectively. The mean body weight gain of the citriodiol treatment group was 85 and 83% of the respective control groups (not statistically significant).

For female rats, mean group body weights were significantly reduced compared with the VC2 at days 22 and 29 ($p<0.05$), but the reductions were all $\leq 5\%$. Body weight gains in the VC1, VC2, and citriodiol treatment groups were 31.4, 38.1, and 25.4 g. The body weight gain of the citriodiol treatment group was 81 and 67% of the respective control groups. The mean weekly weight gain was significantly different from both the VC1 and VC2 ($p \leq 0.01$) only during the 0-8 day interval and not thereafter.

TABLE 3. Group mean body weights (g) and mean total body weight gain (g) of rats topically treated with citriodiol for 28 days

Day of study	Vehicle control 1	Vehicle control 2	Citriodiol
Males			
1	335.0	330.0	334.7
8	367.7	366.0	359.8
15	390.0	389.6	376.1
22	416.4	410.3	402.5
29	434.2	431.4	419.0 (97, 97)
Total body weight gain, days 1-29	99.2	101.4	84.3 (85, 83)
Females			
1	234.8	235.0	235.4
8	246.1	247.1	240.4
15	251.5	255.1	246.7
22	259.5	265.1	255.0 (98, 96*)
29	266.2	273.1	260.8 (98, 95*)
Total body weight gain, days 1-29	31.4	38.1	25.4 (81, 67)

Data from Tables B3-B6, pp. 52-55, MRID 45045203.
Percent of Vehicle Control Group 1 and 2 values, respectively, in parenthesis (calculated by reviewer).
*Significantly different from Vehicle Control Group 2, $p \leq 0.05$.

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

Food consumption values did not differ among the three groups for either sex. For males, mean food consumption values for the VC1, VC2, and the test substance groups over the 29-day period were 30.7, 30.1, and 29.4 g/day, respectively. For females, mean food consumption values for the VC1, VC2, and test substance groups over the 29-day period were 21.0, 20.8, and 20.4 g/day, respectively. Food efficiency was not calculated because there were no treatment-related effects on food consumption or body weights.

D. OPHTHALMOSCOPIC EXAMINATION

No treatment-related ophthalmoscopic effects were observed.

E. NEUROTOXICITY

There were no significant differences among groups for either sex in any parameter in the FOB. Motor activity is reported in Table 4. For male rats, there were no significant differences among groups in the average total number of movements or total time spent in

movement over the 1.5 hour period, although the total time spent in movement was slightly less in the citriodiol treatment group than in either control group. Females were more active than males. For treated females, both the number of movements and the total time spent in movement were significantly increased compared with the VC2 value ($p \leq 0.05$) but not compared with the VC1 value.

When the 18 five-minute intervals were considered, the number of movements of male rats was significantly less than the VC1 only during the 3rd interval ($p \leq 0.05$). The time spent in movement was significantly less than that of the rats in the VC2 during both the 3rd and 14th interval ($p \leq 0.05$). Compared with the VC2, the number of movements for females was significantly increased during the 7th through 11th five-minute intervals ($p \leq 0.05$ or $p \leq 0.01$). Values were also increased compared with the VC1, but the differences were not significant. A similar trend was observed in the time spent in movement with significant increases compared with one or both control groups during the 7th, 8th, and 9th intervals (data not shown).

TABLE 4. Number of movements and time spent in movement (seconds) of rats topically treated with citriodiol for 28 days

Parameter	Treatment group		
	VC1	VC2	Citriodiol
Males			
Number of movements	574.6 ± 135.7	544.3 ± 118.3	554.2 ± 120.2
Time spent in movement	1190.4 ± 320.4	1184.3 ± 182.4	1074.1 ± 246.0
Females			
Number of movements	738.1 ± 173.5	670.3 ±134.5	802.3 ± 132.7*
Time spent in movement	1466.5 ± 443.5	1323.8 ± 196.7	1623.3 ± 258.2*

Data taken from Tables B13-B14, pages 70-73, MRID 45045203.
*Significantly different from VC2, $p \leq 0.05$.

F. BLOODWORK

For treated male rats, no hematology values were affected. The clinical chemistry parameters of total bilirubin and triglycerides in the test substance group were significantly increased ($p<0.01$ or $p<0.05$) compared with one of the control groups but not with the other control group. For treated females, statistically significant increases ($p<0.05$ or $p<0.01$) were observed for some hematology and clinical parameters compared with one control group (leukocyte count, mean platelet volume, lymphocyte count, and creatinine) but not with the other control group. Because all of these values were within the normal range and comparable to one of the control groups, they were considered incidental to treatment.

G. URINALYSIS

Urinalysis was not required and was not performed.

H. SACRIFICE AND PATHOLOGY

1. Organ weight

For treated male rats, the absolute thymus and heart weights were statistically significantly reduced ($p<0.05$ or $p<0.01$) compared to the VC1. Relative to body weight, liver weight was statistically significantly increased ($p<0.05$) compared to the VC2 and heart weight was statistically significantly reduced ($p<0.01$) compared to the VC1. Relative to brain weight, the thymus and heart weights were statistically significantly reduced ($p<0.05$ or $p<0.01$) compared to the VC1.

For female rats, the terminal body weight was statistically significantly reduced ($p<0.05$) compared with the VC2 and the absolute and relative fixed uterus with cervix weights were statistically significantly reduced compared with the VC1 ($p<0.01$ or $p<0.05$). There were no changes relative to brain weight.

All of the weight differences were small and, according to the study author, were considered to be within the range of values at the test facility. Furthermore, no changes were statistically significant compared to both control groups.

2. Gross pathology

No treatment-related systemic effects were noted. Gross pathological observations of small epididymides and testes (one male rat), constricted median lobe of the liver (one male rat), and slight hydrometra of the uterus (two female rats) occurred only in animals in the control groups.

3. Microscopic pathology

All microscopic lesions were confined primarily to the site of application, although some lesions were also observed in the adjacent skin. Minimal to moderate hyperplasia and hyperkeratosis of the epidermis, minimal to moderate hyperplasia of the sebaceous glands, and scattered dermal inflammation were observed in the VC1 and test substance groups, with the incidences and severity of effects increased in the male and female test substance groups compared with the VC1 (Table 5). Although total incidences were similar for hyperplasia of the sebaceous glands and hyperplasia and hyperkeratosis of the epidermis in the VC1 and treated groups, the severity of the lesions was increased in the male and female test substance groups. Both the incidences and severity of multifocal dermal inflammation were clearly increased for both males and females in the test substance group. The skin of the VC2 rats was not examined microscopically.

TABLE 5. Incidences of histological alterations at the site of application in rats topically treated with citriodiol for 28 days

Parameter	Treatment group		
	VC1	VC2	Citriodiol
Males			
Hyperplasia, sebaceous glands			
minimal	3	—	0
mild	7	—	8
moderate	0	—	2
Hyperplasia/hyperkeratosis, epidermal			
minimal	2	—	0
mild	8	—	3
moderate	0	—	7
Inflammation, dermal, multifocal			
minimal	0	—	4
mild	0	—	1
Females			
Hyperplasia, sebaceous glands			
minimal	5	—	1
mild	0	—	6
moderate	0	—	3
Hyperplasia/hyperkeratosis, epidermal			
minimal	6	—	0
mild	4	—	5
moderate	0	—	5
Inflammation, dermal, multifocal			
minimal	1	—	6
mild	0	—	0

Data taken from Table 1, page 244, MRID 45045203.

III. DISCUSSION

A. There was no test substance-related mortality and no treatment-related signs of systemic toxicity. There were no effects on final body weight, food consumption, hematology or clinical chemistry parameters, organ weight, or histopathology of major organs. A treatment-related effect on body weight gain was observed during the first week of the study, but by study termination, mean body weights were 97% of control values for males and 95-98% of control values for females. These body weight differences were not considered biologically significant.

There were no effects on FOB observations or parameters. Motor activity, both number of movements and time spent in movement was increased in female rats compared with the VC2 ($p<0.05$) but was not statistically significantly different than the VC1. Furthermore, there was no pattern to the increased motor activity in females as it occurred

in the middle of the test session (observed during intervals 7-11) and the activity of males was not increased compared with control values at any interval. Therefore, the reviewer did not consider the increased activity in female rats a treatment-related effect.

All treatment-related effects were confined to the site of test substance application. Statistically significant increases in skin irritation including flaking, erythema, and edema, were grossly observed in males and females in the test substance group and were statistically significant ($p \leq 0.01$) when compared with one or both Vehicle Control groups. Most of these irritant effects were graded as slight. Microscopically, these lesions were present as hyperplasia of the sebaceous glands, hyperplasia/hyperkeratosis of the epidermis, and multifocal dermal inflammation. Therefore, a NOAEL for dermal irritation was not attained. However, because the test substance was applied at the limit dose for all 28 days (rather than 5 days/week) and because the resulting dermal irritation was generally characterized as slight and the microscopic effects as minimal, the study should not be rejected for not attaining a NOAEL for dermal irritation (attainment of a NOAEL is supplemental according to Subdivision F 82-2 guidelines).

The test substance was tested at the limit dose of 1000 mg/kg/day. Based on the irritant effects and microscopic changes in the skin at the site of application, the limit dose of 1000 mg/kg/day is a LOAEL for dermal irritation. A dermal NOAEL was not identified. Based on the absence of systemic effects including effects on the FOB and motor activity, the limit dose of 1000 mg/kg/day is a NOAEL for systemic toxicity.

This study is classified as **Acceptable/Guideline** and satisfies the guideline requirements for a subchronic dermal toxicity study [OPPTS 870.3200 (§82-2)] in rats.

B. STUDY DEFICIENCIES

The limit dose of 1000 mg/kg/day was a LOAEL for irritant effects to the skin; a NOAEL for skin irritation was not established.

It is not clear if the study was intended to fulfill the guideline for a neurotoxicity screening battery (OPPTS 870.6200). The study does not fulfill this guideline as positive control data providing evidence of the ability of the observational methods used to detect major neurotoxic endpoints and to exhibit central and peripheral neuropathology were not provided to the reviewer. Animals were not observed/tested prior to study initiation in order to establish a baseline. If the study is considered acceptable by EPA as a screening or range-finding study, then the NOAEL for neurotoxicity is 1000 mg/kg/day based on a lack of effects on the FOB and motor activity. A LOAEL for neurotoxicity was not identified.

controls for the remainder of the dosing interval and 92.4% of controls for the entire dosing interval.

Therefore, the maternal toxicity LOAEL is 1000 mg/kg/day based on skin irritation and transient reductions in body weight gains. The maternal toxicity NOAEL was not identified.

The number of corpora lutea/dam, implantations/dam, gravid uterine weights, pre- or post-implantation loss, resorptions/dam, fetal body weights, and fetal sex ratios were not affected by treatment with the test article.

No treatment-related external, visceral, or skeletal malformations/variations were observed in the fetuses following maternal treatment with the test article.

Therefore, the developmental toxicity NOAEL is ≥1000 mg/kg/day and the developmental toxicity LOAEL was not identified.

This study is classified as **Acceptable/Guideline** and satisfies the guideline requirements for a developmental toxicity study [870.3700 (§83-3a)] in rats. It should be noted that only one dose was tested. However, this dose was the limit dose for developmental toxicity studies, maternal effects were mild and transient, and no developmental toxicity was observed. Testing of additional dose levels would not provide any more useful information. Therefore, this study is considered adequate for assessing the developmental toxicity potential of oil of lemon eucalyptus in the rat.

COMPLIANCE: Signed and dated Quality Assurance, Good Laboratory Practice, Data Confidentiality, and Flagging statements were included.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Oil of lemon eucalyptus

   Description: amber, slightly viscous liquid
   Lot No.: 0299088R-B
   Purity: 100% a.i.
   Stability of compound: not stated; expiration date 9/1/00
   CAS No.: 129828-24-6
   Structure: Not available

2. Vehicle and/or positive control

   Mineral oil, USP, from J.T. Baker, Phillipsburg, New Jersey (Lot No. N06704) was used as the negative control. No vehicle or positive control was used in this study.

21 35

3. Test animals

Species: rat
Strain: Crl:CD®(SD)IGS BR VAF/Plus
Age and weight at study initiation: approximately 80 days; 237-264 g
Source: The Charles River Laboratories, Inc., Kingston, NY
Housing: Animals were housed individually in stainless steel, wire-bottomed cages.
Diet: Certified Rodent Diet® #5002 was available *ad libitum.*
Water: R.O. water was available *ad libitum.*
Environmental conditions:
Temperature: 20-23° C
Humidity: 30-70%
Air changes: 10/hour
Photoperiod: 12 hr light/dark
Acclimation period: 2 weeks

B. PROCEDURES AND STUDY DESIGN

This study was designed to assess the developmental toxicity potential of oil of lemon eucalyptus when administered dermally to rats on gestation days 6 through 20, inclusive.

1. In life dates

Start: October 5, 1999; end: October 29, 1999

2. Mating

Females were mated to males of the same strain and source at a ratio of 1:1 for a maximum of five days. Copulation was determined by the presence of sperm in a vaginal smear or a copulatory plug *in situ*. The day evidence of mating was detected was designated as GD 0.

3. Animal assignment and dose selection are presented in Table 1. Mated females were assigned to dosage groups on the basis of computer-generated (weight-ordered) randomization procedure.

TABLE 1. Animal assignment

Group	Dose (mg/kg/day)	Number of Animals
Vehicle Control	0	25
Test Article	1000	25

Data taken from text table p. 21, MRID 45056701.

2236

4. Dose selection rationale

The dose was selected by the sponsor on the basis of information known about the test article. No further details were included with the current study.

5. Dosing

The test article was applied, as supplied, dermally by semi-occlusion. Females were acclimated to the Elizabethan collars and the wrapping procedures prior to mating. Approximately 21 hours before the first administration, the backs of the rats were clipped from the shoulders to approximately 2 cm anterior to the hip joints and the application site identified using an indelible marker. Any regrowth of hair was clipped and the application site was remarked as needed throughout the study. Dose volumes were adjusted daily on the basis of individual body weights. After a 6-hour exposure period, the collar and dressing were removed and the application site was washed, rinsed, and patted dry.

6. Dose solution preparation and analysis

The test article was applied neat; therefore, solution analyses were not conducted. During dosing, the test substance was warmed to approximately 48.9°C and stirred continuously. Aliquots removed from the stock sample were only used for one day of dosage.

C. OBSERVATIONS

1. Maternal observations and evaluations

Prior to daily application of the test article, the skin was graded for irritation. All animals were observed for clinical signs of toxicity and mortality immediately before dosing and approximately one hour after removal of the test substance. Body weights were recorded on GD 0, daily during dosing (GD 6-20), and at sacrifice on GD 21. Food consumption was recorded on GDs 0, 6, 9, 12, 15, 18, and 21. Dams were killed by carbon dioxide asphyxiation and subjected to gross necropsy. Livers were weighed and placed in neutral buffered 10% formalin. Skin from the application site and from an adjacent untreated site was excised and placed in neutral buffered 10% formalin. The uterus was removed and weighed and examined for numbers and locations of implantation sites, live and dead fetuses, and early and late resorptions; uteri were stored in neutral buffered 10% formalin. The number of corpora lutea on each ovary was also recorded. Uteri from females that appeared nongravid were pressed between glass plates to confirm the absence of implantation sites, examined, and retained in neutral buffered 10% formalin. Any gross lesions were also preserved for possible future evaluation.

2. Fetal evaluations

Live fetuses were sacrificed by an intraperitoneal injection of Beuthanasia®-D Special. Each fetus was weighed, sexed, and examined for external malformations/variations. Approximately one-half of the fetuses from each litter were examined viscerally using a variation of the microdissection technique of Staples. The heads of these fetuses were placed in Bouin's fixative and subsequently examined by free-hand cross-sectioning. The remaining one-half of the fetuses from each litter were fixed in alcohol and processed for skeletal examination.

D. DATA ANALYSIS

1. Statistical analysis

Clinical observation and other proportion data were analyzed using the variance test for homogeneity of the binomial distribution. Continuous data were analyzed using Bartlett's test for homogeneity of variances and the Analysis of Variance (ANOVA). If the ANOVA was significant, Dunnett's test was used to identify statistical significance of the individual groups. If the ANOVA was not appropriate, the Kruskal-Wallis test was used, when ≤75% ties were present, followed by Dunn's method of multiple comparisons. If there were >75% ties, Fisher's exact test was used to analyze the data. Count data obtained at cesarean section were analyzed by the Kruskal-Wallis test.

2. Historical control data were provided to allow comparison with concurrent controls.

II. RESULTS

A. MATERNAL TOXICITY

1. Mortality and clinical signs

Treatment-related clinical signs of toxicity were limited to skin reactions on the treated animals. Significantly ($p \leq 0.01$) increased incidences of grade 1 erythema (24/25), grade 1 edema (11/25), grade 1 flaking (25/25), and grade 2 flaking (9/25) were observed in treated animals compared with only one control animal with grade 1 flaking. All animals survived until scheduled sacrifice.

2. Body weight

Selected maternal body weight data are given in Table 2. No differences in absolute body weights were observed between the treated and control groups throughout the study. Adjusted body weights were similar between the treated and control groups. Body weight gains by the treated dams were significantly ($p \leq 0.05$ or 0.01) reduced for GD 6-9, 9-12, and 6-21. The most pronounced effect on body weight gains was

during GD 6-9 when weight gain by the treated animals was 66.4% of the control group level. Weight gains by the treated group were 85.4-102.2% of controls for the remainder of the dosing interval and 92.4% of controls for the entire dosing interval.

TABLE 2: Selected maternal body weights and body weight changes (g) during gestation

Gestation Day	0 mg/kg/day	1000 mg/kg/day
0	250.8	250.3
6	282.2	283.1
9	295.0	291.6
12	310.7	305.0
15	328.9	323.6
21	420.2	410.6
Adjusted[a]	317.3	309.7
Wt. Gain 0-6	31.4	32.8
Wt. Gain 6-9	12.8	8.5** (66.4)[b]
Wt. Gain 9-12	15.7	13.4* (85.4)
Wt. Gain 6-21	138.0	127.5* (92.4)
Wt. Gain 0-21	169.4	160.3

Data taken from Tables 3 and 4, pp. 39-40 and 41, respectively, MRID 45056701.
[a]Adjusted body wt. = GD 21 wt. - gravid uterine wt.
[b]Numbers in parentheses are percent of control; calculated by reviewer.
Significantly different from control: *$p \leq 0.05$; **$p \leq 0.01$.

3. Food consumption

Maternal food consumption was not affected by treatment.

4. Pathology

No treatment-related abnormalities were found in any animal at necropsy. Terminal body weights and absolute and relative liver weights were similar between the treated and control groups.

5. Cesarean section data

Cesarean section data are summarized in Table 3. The number of corpora lutea/dam, implantations/dam, gravid uterine weights, pre- or post-implantation loss, resorptions/dam, fetal body weights, and fetal sex ratios were similar between the

treated and the control groups. No dam had complete litter resorption and no dead fetuses were observed.

TABLE 3. Cesarean section observations

Observation	0 mg/kg/day	1000 mg/kg/day
No. Assigned	25	25
No. Pregnant (%)	24 (96)	23 (92)
No. Died/sacrificed	0	0
No. Delivered Early	0	0
Gravid Uterine Weight (g)	102.90	100.85
Total Corpora Lutea	395	386
Corpora Lutea/Dam	16.4	16.8
Total Implantations	358	343
Implantations/Dam	14.9	14.9
Preimplantation loss (%)[a]	9.4	11.1
Postimplantation loss (%)	3.8	4.6
Total Live Fetuses	344	328
Live Fetuses/litter	14.3	14.3
Mean Fetal Weight (g)	5.26	5.21
Sex Ratio (% Male)	46.2	47.0
Total Resorptions/Dam	0.6	0.6
Early resorptions/Dam	0.6	0.6
Late resorptions/Dam	0.0	0.0
Dams with all Resorptions	0	0
Total dead fetuses	0	0

Data taken from Tables 3, 7, 8, and 19, pp. 40, 44, 45, and 67-68, respectively, MRID 45056701.
[a]Calculated by reviewer from group totals.

B. DEVELOPMENTAL TOXICITY

No treatment-related external, visceral, or skeletal malformations/variations were observed following maternal treatment with the test article. A summary of findings is given in Table 4.

1. External examination

The total number of fetuses(litters) examined in the control and treated groups was 344(24) and 328(23), respectively. No treatment-related external malformations/variations were observed. In the control group, one fetus had cleft palate and another fetus from a different litter had a small right eye. No external anomalies were observed in fetuses from treated dams.

2. Visceral examination

The total number of fetuses(litters) examined in the control and treated groups was 170(24) and 160(23), respectively. One control fetus had absent kidneys and ureter and one treated fetus had a malformation of the umbilical artery. No other visceral malformations/variations were seen in any fetus from the control or test article groups.

3. Skeletal examination

The total number of fetuses(litters) examined in the control and treated groups was 178(24) and 168(23), respectively. A 7th cervical rib was observed in 4 fetuses from 3 control litters and in 2 fetuses from 1 treated litter. Other skeletal variations, such as wavy ribs, incomplete ossification of the sternal centra, and bifid centrum of the thoracic vertebrae, occurred only in a single litter each. The mean number of ossification sites of the hyoid, sternum, and fore- and hindlimbs was similar between the treated and control groups.

TABLE 4: Summary of findings at fetal examination [fetuses (litters)]

Observation	0 mg/kg/day (vehicle)	1000 mg/kg/day
Total with malformations/variations	10 (7)	5 (4)
Cleft palate	1 (1)	0 (0)
Small right eye	1 (1)	0 (0)
Absent kidneys and ureter	1 (1)	0 (0)
7th cervical rib	4 (3)	2 (1)
Wavy ribs	2 (1)	0 (0)

Data taken from Tables 9-12, pp. 46-49, respectively, MRID 45056701.

III. DISCUSSION

A. INVESTIGATORS' CONCLUSIONS

The study author concluded that the test article caused only minor skin irritation which resulted in slight decreases in body weight gains of the treated dams. No systemic toxicity was observed. No cesarean or litter parameters were affected and no external, visceral, or skeletal fetal alterations were caused by the test article. Based on the results of this study, the maternal and developmental toxicity NOAELs were >1000 mg/kg/day.

B. REVIEWER'S DISCUSSION

1. MATERNAL TOXICITY

Evidence of slight skin irritation was observed in all treated dams. Transient reductions in body weight gains also occurred after the initiation of dosing. Because food consumption was not affected, the reduced body weight gains may have been due to stress as a secondary effect of the irritation. Similar results were seen in a 28-day dermal study (MRID 45045203) with the test article in rats. Use of only one treatment group in this study precludes a dose-response assessment. However, since the maternal effects were mild and the dose tested was the limit dose, the study is considered adequate for the purpose of assessing maternal toxicity in the rat.

Therefore, the maternal toxicity LOAEL is 1000 mg/kg/day based on skin irritation and transient reductions in body weight gains. The maternal toxicity NOAEL was not identified.

2. DEVELOPMENTAL TOXICITY

a. Deaths/resorptions

Maternal treatment with the test article did not result in an increase in either resorptions or dead fetuses.

b. Altered growth

Growth of the fetuses was not affected by maternal treatment with the test article. Fetal weights and ossification rates were similar between the treated and control groups.

c. Developmental variations

Treatment with the test article did not cause an increase in fetal variations. Variations common to the rat fetus were observed in both the treated and control groups.

d. Malformations

The test article did not induce major malformations in the fetuses.

Therefore, the developmental toxicity NOAEL is ≥1000 mg/kg/day and the developmental toxicity LOAEL was not identified.

C. STUDY DEFICIENCIES

A minor deficiency in the conduct of this study was that only one dose was tested. However, at the limit dose only mild maternal effects were noted and no developmental toxicity was seen. Testing of additional dose levels would not provide any more useful information. Therefore, this deficiency did not affect the interpretation of the study.

D. CLASSIFICATION

This study is classified as **Acceptable/Guideline** and satisfies the guideline requirements for a developmental toxicity study [870.3700 (§83-3a)] in rats.

DATA EVALUATION REPORT

CITRIODIOL
(OIL OF LEMON EUCALYPUS)

Study Type: DEVELOPMENTAL TOXICITY- RAT [870.3700 (83-3A)]
MRID 45056701

Prepared for

Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Chemical Hazard Evaluation Group
Toxicology and Risk Analysis Section
Life Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37831
Work Assignment No. 33

Primary Reviewer: Carol S. Forsyth, Ph.D., D.A.B.T.	Signature: Carol S. Forsyth Date: APR 14 2000
Secondary Reviewers: Kowetha A. Davidson, Ph.D., D.A.B.T.	Signature: Date: APR 14 2000
Robert H. Ross, M.S., Group Leader	Signature: Robert H. Ross Date: APR 14 2000
Quality Assurance: Lee Ann Wilson, M.A.	Signature: L. A. Wilson Date: APR 14 2000

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the U.S. Dept. of Energy under contract DE-AC05-00OR22725.

30 44

EPA Reviewer: F. Toghrol, Ph.D. Roger Gardner Date: 4/19/00

EPA Work Assignment Manager: Sheryl Reilly, Ph.D. _____ Date: _____
Biopesticides and Pollution Prevention Division (7511W)

DATA EVALUATION RECORD

STUDY TYPE: Developmental Toxicity - Rat [OPPTS 870.3700 (§83-3a)]

DP BARCODE: D264411/062646
P.C. CODE: 011550

SUBMISSION CODE: S565022
CASE NO.: 062646

TEST MATERIAL (PURITY): Extract of lemon eucalyptus (100% a.i.)

SYNONYMS: p-Methane-3,8-diol; Citriodiol

CITATION: Parker, R.M. (2000) Dermal developmental toxicity study of oil of lemon eucalyptus in rats. Argus Research Laboratories, Inc., 905 Sheehy Drive, Building A, Horsham, PA 19044. Laboratory Project ID. 720-005. February 8, 2000. MRID 45056701. Unpublished.

SPONSORS: WPC Brands, Inc., 1 Repel Road, P.O. Box 198, Jackson, Wisconsin 53037

EXECUTIVE SUMMARY: In a developmental toxicity study (MRID 45056701), 25 presumed pregnant Crl:CD®(SD)IGS BR VAF/Plus rats per group were given either mineral oil or 1000 mg/kg/day of oil of lemon eucalyptus (100% a.i.; Lot No.: 0299088R-B) on gestation days (GD) 6-20, inclusive. The test or control article was applied neat by dermal semi-occlusion for 6 hr/day. On GD 21, all surviving dams were sacrificed and all fetuses were weighed and examined for external malformations/variations. Approximately one-half of the fetuses were examined viscerally; the heads of these fetuses were placed in Bouin's fixative and subsequently examined by free-hand cross-sectioning. The remaining one-half of the fetuses were fixed in alcohol and processed for skeletal examination.

All animals survived until scheduled sacrifice and maternal necropsy was unremarkable. Significantly ($p \leq 0.01$) increased incidences of grade 1 erythema (24/25), grade 1 edema (11/25), grade 1 flaking (25/25), and grade 2 flaking (9/25) were observed in treated animals compared with only one control animal with grade 1 flaking. No other clinical signs of toxicity were observed. No differences in absolute body weights were observed between the treated and control groups throughout the study. Adjusted body weights were similar between the treated and control groups. Body weight gains by the treated dams were significantly ($p \leq 0.05$ or 0.01) reduced for GD 6-9, 9-12, and 6-21. The most pronounced effect on body weight gains was after the initiation of treatment for the interval of GD 6-9 when weight gain by the treated animals was 66.4% of the control group level. Weight gains by the treated group were 85.4-102.2% of

controls for the remainder of the dosing interval and 92.4% of controls for the entire dosing interval.

Therefore, the maternal toxicity LOAEL is 1000 mg/kg/day based on skin irritation and transient reductions in body weight gains. The maternal toxicity NOAEL was not identified.

The number of corpora lutea/dam, implantations/dam, gravid uterine weights, pre- or post-implantation loss, resorptions/dam, fetal body weights, and fetal sex ratios were not affected by treatment with the test article.

No treatment-related external, visceral, or skeletal malformations/variations were observed in the fetuses following maternal treatment with the test article.

Therefore, the developmental toxicity NOAEL is ≥1000 mg/kg/day and the developmental toxicity LOAEL was not identified.

This study is classified as **Supplementary/not upgradable** and does not satisfy the guideline requirements for a developmental toxicity study [870.3700 (§83-3a)] in rats. It should be noted that only one dose (a limit dose) was tested, and doses were administered from gestation day 6 instead of gestation day 0 which is preferred when the dermal route is being investigated. (The classification has been changed from the original primary review.)

COMPLIANCE: Signed and dated Quality Assurance, Good Laboratory Practice, Data Confidentiality, and Flagging statements were included.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Oil of lemon eucalyptus

   Description: amber, slightly viscous liquid
   Lot No.: 0299088R-B
   Purity: 100% a.i.
   Stability of compound: not stated; expiration date 9/1/00
   CAS No.: 129828-24-6
   Structure: Not available

2. Vehicle and/or positive control

   Mineral oil, USP, from J.T. Baker, Phillipsburg, New Jersey (Lot No. N06704) was used as the negative control. No vehicle or positive control was used in this study.

3. Test animals

32 46

Species: rat
Strain: Crl:CD®(SD)IGS BR VAF/Plus
Age and weight at study initiation: approximately 80 days; 237-264 g
Source: The Charles River Laboratories, Inc., Kingston, NY
Housing: Animals were housed individually in stainless steel, wire-bottomed cages.
Diet: Certified Rodent Diet® #5002 was available *ad libitum*.
Water: R.O. water was available *ad libitum*.
Environmental conditions:
Temperature: 20-23° C
Humidity: 30-70%
Air changes: 10/hour
Photoperiod: 12 hr light/dark
Acclimation period: 2 weeks

B. PROCEDURES AND STUDY DESIGN

This study was designed to assess the developmental toxicity potential of oil of lemon eucalyptus when administered dermally to rats on gestation days 6 through 20, inclusive.

1. In life dates

Start: October 5, 1999; end: October 29, 1999

2. Mating

Females were mated to males of the same strain and source at a ratio of 1:1 for a maximum of five days. Copulation was determined by the presence of sperm in a vaginal smear or a copulatory plug *in situ*. The day evidence of mating was detected was designated as GD 0.

3. Animal assignment and dose selection are presented in Table 1. Mated females were assigned to dosage groups on the basis of computer-generated (weight-ordered) randomization procedure.

TABLE 1. Animal assignment

Group	Dose (mg/kg/day)	Number of Animals
Vehicle Control	0	25
Test Article	1000	25

Data taken from text table p. 21, MRID 45056701.

4. Dose selection rationale

The dose was selected by the sponsor on the basis of information known about the test article. No further details were included with the current study.

5. Dosing

The test article was applied, as supplied, dermally by semi-occlusion. Females were acclimated to the Elizabethan collars and the wrapping procedures prior to mating. Approximately 21 hours before the first administration, the backs of the rats were clipped from the shoulders to approximately 2 cm anterior to the hip joints and the application site identified using an indelible marker. Any regrowth of hair was clipped and the application site was remarked as needed throughout the study. Dose volumes were adjusted daily on the basis of individual body weights. After a 6-hour exposure period, the collar and dressing were removed and the application site was washed, rinsed, and patted dry.

6. Dose solution preparation and analysis

The test article was applied neat; therefore, solution analyses were not conducted. During dosing, the test substance was warmed to approximately 48.9 °C and stirred continuously. Aliquots removed from the stock sample were only used for one day of dosage.

C. OBSERVATIONS

1. Maternal observations and evaluations

Prior to daily application of the test article, the skin was graded for irritation. All animals were observed for clinical signs of toxicity and mortality immediately before dosing and approximately one hour after removal of the test substance. Body weights were recorded on GD 0, daily during dosing (GD 6-20), and at sacrifice on GD 21. Food consumption was recorded on GDs 0, 6, 9, 12, 15, 18, and 21. Dams were killed by carbon dioxide asphyxiation and subjected to gross necropsy. Livers were weighed and placed in neutral buffered 10% formalin. Skin from the application site and from an adjacent untreated site was excised and placed in neutral buffered 10% formalin. The uterus was removed and weighed and examined for numbers and locations of implantation sites, live and dead fetuses, and early and late resorptions; uteri were stored in neutral buffered 10% formalin. The number of corpora lutea on each ovary was also recorded. Uteri from females that appeared nongravid were pressed between glass plates to confirm the absence of implantation sites, examined, and retained in neutral buffered 10% formalin. Any gross lesions were also preserved for possible future evaluation.

2. Fetal evaluations

Live fetuses were sacrificed by an intraperitoneal injection of Beuthanasia®-D Special. Each fetus was weighed, sexed, and examined for external malformations/variations. Approximately one-half of the fetuses from each litter were examined viscerally using a variation of the microdissection technique of Staples. The heads of these fetuses were placed in Bouin's fixative and subsequently examined by free-hand cross-sectioning. The remaining one-half of the fetuses from each litter were fixed in alcohol and processed for skeletal examination.

D. DATA ANALYSIS

1. Statistical analysis

Clinical observation and other proportion data were analyzed using the variance test for homogeneity of the binomial distribution. Continuous data were analyzed using Bartlett's test for homogeneity of variances and the Analysis of Variance (ANOVA). If the ANOVA was significant, Dunnett's test was used to identify statistical significance of the individual groups. If the ANOVA was not appropriate, the Kruskal-Wallis test was used, when ≤75% ties were present, followed by Dunn's method of multiple comparisons. If there were >75% ties, Fisher's exact test was used to analyze the data. Count data obtained at cesarean section were analyzed by the Kruskal-Wallis test.

2. Historical control data were provided to allow comparison with concurrent controls.

II. RESULTS

A. MATERNAL TOXICITY

1. Mortality and clinical signs

Treatment-related clinical signs of toxicity were limited to skin reactions on the treated animals. Significantly ($p \leq 0.01$) increased incidences of grade 1 erythema (24/25), grade 1 edema (11/25), grade 1 flaking (25/25), and grade 2 flaking (9/25) were observed in treated animals compared with only one control animal with grade 1 flaking. All animals survived until scheduled sacrifice.

2. Body weight

Selected maternal body weight data are given in Table 2. No differences in absolute body weights were observed between the treated and control groups throughout the study. Adjusted body weights were similar between the treated and control groups. Body weight gains by the treated dams were significantly ($p \leq 0.05$ or 0.01) reduced for GD 6-9, 9-12, and 6-21. The most pronounced effect on body weight gains was

during GD 6-9 when weight gain by the treated animals was 66.4% of the control group level. Weight gains by the treated group were 85.4-102.2% of controls for the remainder of the dosing interval and 92.4% of controls for the entire dosing interval.

TABLE 2: Selected maternal body weights and body weight changes (g) during gestation

Gestation Day	0 mg/kg/day	1000 mg/kg/day
0	250.8	250.3
6	282.2	283.1
9	295.0	291.6
12	310.7	305.0
15	328.9	323.6
21	420.2	410.6
Adjusted[a]	317.3	309.7
Wt. Gain 0-6	31.4	32.8
Wt. Gain 6-9	12.8	8.5** (66.4)[b]
Wt. Gain 9-12	15.7	13.4* (85.4)
Wt. Gain 6-21	138.0	127.5* (92.4)
Wt. Gain 0-21	169.4	160.3

Data taken from Tables 3 and 4, pp. 39-40 and 41, respectively, MRID 45056701.
[a]Adjusted body wt. = GD 21 wt. - gravid uterine wt.
[b]Numbers in parentheses are percent of control; calculated by reviewer.
Significantly different from control: *$p \leq 0.05$; **$p \leq 0.01$.

3. Food consumption

Maternal food consumption was not affected by treatment.

4. Pathology

No treatment-related abnormalities were found in any animal at necropsy. Terminal body weights and absolute and relative liver weights were similar between the treated and control groups.

5. Cesarean section data

Cesarean section data are summarized in Table 3. The number of corpora lutea/dam, implantations/dam, gravid uterine weights, pre- or post-implantation loss, resorptions/dam, fetal body weights, and fetal sex ratios were similar between the

treated and the control groups. No dam had complete litter resorption and no dead fetuses were observed.

TABLE 3. Cesarean section observations

Observation	0 mg/kg/day	1000 mg/kg/day
No. Assigned	25	25
No. Pregnant (%)	24 (96)	23 (92)
No. Died/sacrificed	0	0
No. Delivered Early	0	0
Gravid Uterine Weight (g)	102.90	100.85
Total Corpora Lutea	395	386
Corpora Lutea/Dam	16.4	16.8
Total Implantations	358	343
Implantations/Dam	14.9	14.9
Preimplantation loss (%)[a]	9.4	11.1
Postimplantation loss (%)	3.8	4.6
Total Live Fetuses	344	328
Live Fetuses/litter	14.3	14.3
Mean Fetal Weight (g)	5.26	5.21
Sex Ratio (% Male)	46.2	47.0
Total Resorptions/Dam	0.6	0.6
Early resorptions/Dam	0.6	0.6
Late resorptions/Dam	0.0	0.0
Dams with all Resorptions	0	0
Total dead fetuses	0	0

Data taken from Tables 3, 7, 8, and 19, pp. 40, 44, 45, and 67-68, respectively, MRID 45056701.
[a]Calculated by reviewer from group totals.

B. DEVELOPMENTAL TOXICITY

No treatment-related external, visceral, or skeletal malformations/variations were observed following maternal treatment with the test article. A summary of findings is given in Table 4.

1. External examination

The total number of fetuses(litters) examined in the control and treated groups was 344(24) and 328(23), respectively. No treatment-related external malformations/variations were observed. In the control group, one fetus had cleft palate and another fetus from a different litter had a small right eye. No external anomalies were observed in fetuses from treated dams.

2. Visceral examination

The total number of fetuses(litters) examined in the control and treated groups was 170(24) and 160(23), respectively. One control fetus had absent kidneys and ureter and one treated fetus had a malformation of the umbilical artery. No other visceral malformations/variations were seen in any fetus from the control or test article groups.

3. Skeletal examination

The total number of fetuses(litters) examined in the control and treated groups was 178(24) and 168(23), respectively. A 7th cervical rib was observed in 4 fetuses from 3 control litters and in 2 fetuses from 1 treated litter. Other skeletal variations, such as wavy ribs, incomplete ossification of the sternal centra, and bifid centrum of the thoracic vertebrae, occurred only in a single litter each. The mean number of ossification sites of the hyoid, sternum, and fore- and hindlimbs was similar between the treated and control groups.

TABLE 4: Summary of findings at fetal examination [fetuses (litters)]

Observation	0 mg/kg/day (vehicle)	1000 mg/kg/day
Total with malformations/variations	10 (7)	5 (4)
Cleft palate	1 (1)	0 (0)
Small right eye	1 (1)	0 (0)
Absent kidneys and ureter	1 (1)	0 (0)
7th cervical rib	4 (3)	2 (1)
Wavy ribs	2 (1)	0 (0)

Data taken from Tables 9-12, pp. 46-49, respectively, MRID 45056701.

39 52

III. DISCUSSION

A. INVESTIGATORS' CONCLUSIONS

The study author concluded that the test article caused only minor skin irritation which resulted in slight decreases in body weight gains of the treated dams. No systemic toxicity was observed. No cesarean or litter parameters were affected and no external, visceral, or skeletal fetal alterations were caused by the test article. Based on the results of this study, the maternal and developmental toxicity NOAELs were >1000 mg/kg/day.

B. REVIEWER'S DISCUSSION

1. MATERNAL TOXICITY

Evidence of slight skin irritation was observed in all treated dams. Transient reductions in body weight gains also occurred after the initiation of dosing. Because food consumption was not affected, the reduced body weight gains may have been due to stress as a secondary effect of the irritation. Similar results were seen in a 28-day dermal study (MRID 45045203) with the test article in rats. Use of only one treatment group in this study precludes a dose-response assessment. However, since the maternal effects were mild and the dose tested was the limit dose, the study is considered adequate for the purpose of assessing maternal toxicity in the rat.

Therefore, the maternal toxicity LOAEL is 1000 mg/kg/day based on skin irritation and transient reductions in body weight gains. The maternal toxicity NOAEL was not identified.

2. DEVELOPMENTAL TOXICITY

a. Deaths/resorptions

Maternal treatment with the test article did not result in an increase in either resorptions or dead fetuses.

b. Altered growth

Growth of the fetuses was not affected by maternal treatment with the test article. Fetal weights and ossification rates were similar between the treated and control groups.

c. Developmental variations

Treatment with the test article did not cause an increase in fetal variations. Variations common to the rat fetus were observed in both the treated and control groups.

d. Malformations

The test article did not induce major malformations in the fetuses.

Therefore, the developmental toxicity NOAEL is ≥1000 mg/kg/day and the developmental toxicity LOAEL was not identified.

C. STUDY DEFICIENCIES

A minor deficiency in the conduct of this study was that only one dose was tested. However, at the limit dose only mild maternal effects were noted and no developmental toxicity was seen. Testing of additional dose levels would not provide any more useful information. Therefore, this deficiency did not affect the interpretation of the study.

D. CLASSIFICATION

This study is classified as **Supplementary/not upgradable** and does not satisfy the guideline requirements for a developmental toxicity study [870.3700 (§83-3a)] in rats.

There were no significant differences in group mean body weights between the control and treated group, and there were no clinical signs of toxicity other than dermal irritation to suggest that oil of lemon eucalyptus caused systemic maternal toxicity. However, there are additional considerations described below that indicate that the study should be reclassified as supplementary.

Although dermal absorption of an OLE component (*p*-menthane-3,8-diol [PMD]) was observed in the human study, an absorption rate was not determined. Dr. John Caldwell, a co-investigator for a rat metabolism study with menthol, which is metabolized to PMD, noted, "Our unpublished data with radiolabeled menthol (in a separate *in vitro* experiment) showed that less than 1% of the total radioactivity applied to the surface penetrated through fresh full thickness human skin, due to marked loss of applied menthol from the skin surface." This loss was attributed to the volatility of menthol, but no information was provided on the proportions of applied radioactivity recovered in the treated skin. Nevertheless, the submitted information on PMD and menthol suggests that dermal absorption of substances similar to those found in oil of lemon eucalyptus is probably low.

Since dermal absorption of OLE is probably low, the capacity of the developmental toxicity study to evaluate OLE, particularly with regard to events that occur soon after implantation and during early organogenesis may be reduced. Typically, the Agency recommends that dermal developmental toxicity studies begin topical dosing on gestation day 0 when the studies are initiated because the kinetics of dermal absorption in cases such as OLE generally require a few days of treatment before a maximum daily absorbed dose can be maintained, and since dermal treatment was not

40 ~~57~~

initiated until near the time of implantation in the OLE study (gestation day 6), the dermal developmental toxicity study with OLE is of limited value in the assessment of developmental toxicity (e.g., evaluation of events such as early and late resorptions). This test protocol modification has been used in testing of other active ingredients used as insect repellents (e.g., PMD). Therefore, the submitted study is classified as supplementary.

41

DATA EVALUATION REPORT

OIL OF LEMON EUCALYPTUS

STUDY TYPE: MAMMALIAN CELLS IN CULTURE GENE MUTATION ASSAY IN MOUSE LYMPHOMA CELLS (OPPTS 870.5300 [§84-2]) MRID 45045201

Prepared for

Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Chemical Hazard Evaluation Group
Toxicology and Risk Analysis Section
Life Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37831
Task Order No. 33

Primary Reviewer: Cheryl B. Bast, Ph.D., D.A.B.T	Signature: Cheryl B Bast Date: APR 14 2000
Secondary Reviewers: B.L. Whitfield, Ph.D.	Signature: B L Whitfield Date: APR 14 2000
Robert H. Ross, M.S., Group Leader	Signature: Robert H. Ross Date: APR 14 2000
Quality Assurance: Lee Ann Wilson, M. A.	Signature: L. A. Wilson Date: APR 14 2000

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

Oak Ridge National Laboratory managed and operated by UT-Battelle, LLC., for the U.S. Department of Energy under contract number DE-AC05-00OR22725.

42

EPA Reviewer:, F. Toghrol, Ph.D
EPA Work Assignment Manager: S. Reilly, Ph.D.

Roger Gardner, Date 4/19/00
_____, Date _____

DATA EVALUATION RECORD

STUDY TYPE: Mammalian cells in culture gene mutation assay in Mouse lymphoma L5178Y TK $^{+/-}$ cells; OPPTS 870.5300 [§84-2]

DP BARCODE: D264411
P.C. CODE.: 062646

SUBMISSION CODE: S565022
TOX. CHEM. NO.: 011550

TEST MATERIAL (PURITY): Oil of lemon eucalyptus (100%; Approx. 65% *p*-methane-3,8-diol, 66% *cis*- and 34% *trans*-isomers)

SYNONYM: Citriodiol

CITATION: Cifone, M.A. (2000) L5178Y TK$^{+/-}$ Mouse Lymphoma Forward Mutation Assay with a Confirmatory Assay with Oil of Lemon Eucalyptus. Covance Laboratories Inc., 9200 Leesburg Pike, Vienna, Virginia 22182. Laboratory Project Identification number: Covance 20901-0-431 OECD, February 4, 2000. MRID 45045201. Unpublished

SPONSOR: WPC Brands, Inc., 1 Repel Road, Jackson, Wisconsin 53037

EXECUTIVE SUMMARY: In a mammalian cell gene mutation assay (MRID 45045201), mouse lymphoma L5178Y cells cultured in vitro were exposed to oil of lemon eucalyptus (100%) in dimethylsulfoxide (DMSO) at concentrations of 50, 75, 100, 150, 175, 200, or 210 µg/mL (initial assay) and 50, 75, 100, 150, or 225 µg/mL (confirmatory assay) in the absence of mammalian metabolic activation (S9-mix). Cells were exposed to concentrations of 50, 75, 100, 150, 200, 250, 275, 300, or 325 µg/mL (initial assay) and 250, 275, 300, 310, 320, 330, 340, or 350 µg/mL (confirmatory assay) in the presence of S9-mix. The S9-fraction was obtained from Aroclor 1254 induced male Sprague-Dawley rat liver.

Oil of lemon eucalyptus was tested up to concentrations limited by cytotoxicity. A preliminary cytotoxicity (range-finding) study without S9 at concentrations ranging from 3.93 to 2000 µg/mL showed that cell survival was 31.7% of the solvent control value at 250 µg/mL. In the presence of S9-mix, cell survival was <1% of the solvent control value at 500 µg/mL. In the main mutagenicity tests, in the absence of S9-mix, no increase in mutant frequency compared to solvent controls or dose-response was observed at any concentration tested in either duplicate culture, reaching a maximum of 62.8 mutants per 10^6 surviving cells at 210 µg/mL in replicate 1 and 80.6 mutants per 10^6 surviving cells at 225 µg/mL in replicate 2. In the presence of S9-mix, no increase in mutant frequency or dose-response was observed at any concentration tested in both duplicate cultures, reaching a maximum of 48.4 mutants per 10^6 surviving cells at 100 µg/mL in replicate 1 and 87.5 mutants per 10^6 surviving cells at 300 µg/mL in replicate 2. The

positive and solvent controls induced the appropriate responses. **There was no evidence of induced mutant colonies over background.**

This study is classified as **Acceptable/Guideline**. It satisfies the requirement for FIFRA Test Guideline [OPPTS 870.5300 (§84-2)] for in vitro mutagenicity (mammalian forward gene mutation) data.

COMPLIANCE: Signed and dated GLP, Quality Assurance and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Oil of lemon eucalyptus

   Description: brownish-yellow, opaque slightly viscous liquid (light yellow and transparent when heated)
   Lot/Batch #: 0299088R-B
   Purity: 100% (Approx. 65% *p*-methane-3,8-diol, 66% *cis*- and 34% *trans*-isomers)
   Stability of compound: not stated
   CAS #: 129828-24-6 and 428822-96-6
   Structure: not provided
   Solvent used: DMSO

2. Control materials

   Negative: none
   Solvent/final concentration: DMSO / 1% v/v
   Positive: (concentrations/solvent)
   Nonactivation: methyl methanesulfonate / 6.5 or 13 µg/mL
   Activation: methylcholanthrene / 2 or 4 µg/mL

3. Activation

   S9 derived from male Sprague-Dawley rats

x Aroclor 1254	x induced	x rat x liver
_ phenobarbital	_ non-induced	_ mouse
_ lung	_ none	_ hamster

   S9 mix composition:

S9 homogenate	10 µL/mL
NADP	3 mM
Isocitrate	15 mM

44 58

4. Test cells

mammalian cells in culture
_X_ mouse lymphoma L5178Y cells
__ Chinese hamster ovary (CHO-K1-BH4) cells
__ V79 cells (Chinese hamster lung fibroblasts)

Properly maintained? Y
Periodically checked for Mycoplasma contamination? Y
Periodically checked for karyotype stability? Y
Periodically "cleansed" against high spontaneous background? Y

Media: Cells were maintained in RPMI 1640 supplemented with 10% horse serum, pluronic F68, L-glutamine, sodium pyruvate, penicillin and streptomycin. During the treatment period cells were in Fischer's medium with the same supplements as the culture medium except that serum was reduced to 5%. Cloning medium was the RPMI 1640 culture medium with up to 20% horse serum, without Pleuronic F68 and with 0.24% BBL agar added to achieve a semisolid state. Selection medium consisted of cloning medium containing 3 µg/ml TFT.

5. Locus examined

_X_ thymidine kinase (TK)
Selection agent:
_____ bromodeoxyuridine (BrdU)
_____ fluorodeoxyuridine (FdU)
_3 µg/mL_ trifluorothymidine (TFT)

_ hypoxanthine-guanine-phosphoribosyl transferase (HPRT)
Selection agent:
__ 8-azaguanine (8-AG)
__ 6-thioguanine (6-TG)

__ Na^+/K^+ ATPase
Selection agent:
__ ouabain

6. Test compound concentrations used

Preliminary cytotoxicity test:
Nonactivated conditions:
3.93, 7.85, 15.7, 31.3, 62.5, 125, 250, 500, 1000, 2000 µg/mL
Activated conditions:
3.93, 7.85, 15.7, 31.3, 62.5, 125, 250, 500, 1000, 2000 µg/mL
Mutagenicity assay:

Nonactivated conditions:
50, 75, 100, 150, 175, 200, 210 µg/mL (initial assay)
50, 75, 100, 150, 225 µg/mL (confirmatory assay)
Activated conditions:
50, 75, 100, 150, 200, 250, 275, 300, 325 µg/mL (initial assay)
250, 275, 300, 310, 320, 330, 340, 350 µg/mL (confirmatory assay)

B. TEST PERFORMANCE

1. Cell treatment

a. Cells exposed to test compound, negative/solvent or positive controls for: 4 hours (nonactivated) 4 hours (activated)

b. After washing, cells cultured for 2 days (expression period) before cell selection:

c. After expression, 1×10^6 cells/dish (3 dishes/group) were cultured for 13 days in selection medium to determine numbers of mutants and 200 cells/dish (3 dishes/group) were cultured for 13 days without selective agent to determine cloning efficiency.

2. Statistical methods:

Statistical methods were not described.

3. Evaluation criteria

The mutant frequency was expressed as the number of TFT resistant mutants per 10^6 surviving cells. Criteria for a positive response were as follows:

(1) Demonstration of dose-dependent doubling in mutant frequency over the concurrent background control or a 4-fold or higher increase in mutant frequency at a single dose at or near the highest testable toxicity.

(2) Demonstration of reproducibility in any increase in mutant frequency.

II. REPORTED RESULTS

A. PRELIMINARY CYTOTOXICITY ASSAY

In the preliminary cytotoxicity study, oil of lemon eucalyptus was tested at concentrations ranging from 3.93 to 2000 µg/mL. Cell survival was 31.7% of the solvent control value at 250 µg/mL without S9 mix. In the presence of S9-mix, cell survival was <1% of the solvent control value at 500 µg/mL. Results of the preliminary cytotoxicity test are

presented in Appendix Table 1 (MRID 45045201, p. 23). Doses for the mutagenicity tests were chosen based on the results of this toxicity test.

B. MUTAGENICITY ASSAY

In the main mutagenicity tests, in the absence of S9-mix, no increase in mutant frequency compared to solvent controls or dose-response was observed at any concentration tested in either duplicate culture, reaching a maximum of 62.8 mutants per 10^6 surviving cells at 210 µg/mL in replicate 1 and 80.6 mutants per 10^6 surviving cells at 225 µg/mL in replicate 2. In the presence of S9-mix, no increase in mutant frequency or dose-response was observed at any concentration tested in either duplicate culture, reaching a maximum of 48.4 mutants per 10^6 surviving cells at 100 µg/mL in replicate 1 and 87.5 mutants per 10^6 surviving cells at 300 µg/mL in replicate 2. The mutant frequency for the MMS positive control was 288-410 mutants per 10^6 surviving cells in the absence of S9-mix. The mutant frequency for the MCA positive control was 221-386 mutants per 10^6 surviving cells in the presence of S9-mix. Results are presented in Appendix Tables 2-5 (MRID 45045201, pp. 24, 26, 28, and 30).

III. REVIEWER'S DISCUSSION/CONCLUSIONS

A. This is an acceptable study. Oil of lemon eucalyptus was tested to cytotoxic concentrations, proper experimental protocol was followed and solvent and positive control values were appropriate. Oil of lemon eucalyptus did not induce mutations at the TK locus in L5178Y cells as tested in this study.

This study is classified as **Acceptable/Guideline**. It satisfies the requirement for FIFRA Test Guideline [OPPTS 870.5300 (§84-2)] for in vitro mutagenicity (mammalian forward gene mutation) data.

B. STUDY DEFICIENCIES: No study deficiencies were identified.

47 ~~61~~

APPENDIX

(MRID 45045201)

This appendix not available electronically. See file copy.

48 ~~69~~

PC 011550 P-MENTHANE-3,8-DIOL

Page _____ is not included in this copy.

Pages 49 through 53 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

X FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

OIL OF LEMON EUCALYPTUS

STUDY TYPE: MICRONUCLEUS (OPPTS 870.5395 [§84-2])
MRID 45045202

Prepared for

Biopesticides and Pollution Protection Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Chemical Hazard Evaluation Group
Toxicology and Risk Analysis Section
Life Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37831
Task Order No. 33

Primary Reviewer: Cheryl B. Bast, Ph.D., D.A.B.T.	Signature:	Cheryl B Bast
	Date:	APR 1 4 2000
Secondary Reviewers: Bradford L. Whitfield, Ph.D.	Signature:	B L Whitfield
	Date:	APR 1 4 2000
Robert H. Ross, M.S., Group Leader	Signature:	Robert H. Ross
	Date:	APR 1 4 2000
Quality Assurance: Lee Ann Wilson, M.A.	Signature:	L. A. Wilson
	Date:	APR 1 4 2000

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

Oak Ridge National Laboratory Managed and Operated by UT-Battelle, LLC., for the U.S. Department of Energy under contract number DE-AC05-00OR22725.

EPA Reviewer:, F. Toghrol, Ph.D. Roger Gardner, Date 4/19/00

EPA Work Assignment Manager: S. Reilly, Ph.D. _____, Date _____

DATA EVALUATION RECORD

STUDY TYPE: *In vivo* mammalian cytogenetics - micronucleus assay in mice; OPPTS 870.5395 [§84-2]

DP BARCODE: D264411
P.C. CODE.: 062646

SUBMISSION CODE: S565022
TOX. CHEM. NO.: 011550

TEST MATERIAL (PURITY): Oil of lemon eucalyptus (100%; Approx. 65% *p*-methane-3,8-diol, 66% *cis*- and 34% *trans*-isomers)

SYNONYM: Citriodiol

CITATION: Myhr, B.C. (2000) *In vivo* Mouse Micronucleus Assay with Oil of Lemon Eucalyptus. Covance Laboratories Inc., 9200 Leesburg Pike, Vienna, Virginia 22182. Laboratory Project Identification number Covance 20901-0-455OECD, February 4, 2000. MRID 45045202. Unpublished

SPONSOR: WPC Brands, Inc., 1 Repel Road, Jackson, Wisconsin 53037

EXECUTIVE SUMMARY: In a mouse bone marrow micronucleus assay (MRID 45045202), six male mice/sex/dose/ harvest time were treated once by intraperitoneal injection with oil of lemon eucalyptus (100%) in corn oil at doses of 250, 500, or 1000 mg/kg. Bone marrow cells were harvested from five vehicle control and high-dose animals at 24 and 48 hours post-treatment and from five low- and mid-dose animals at 24 hours post-treatment.

Mortality was observed in 7/12 high-dose animals; animals in the mid- and high-dose groups exhibited prostration, labored breathing, ataxia, and slight hypoactivity. No clinical signs were noted in low-dose animals. (In a range-finding study, clinical signs were observed at 500, 800, and 1200 mg/kg and deaths were observed at 1200 mg/kg. No clinical signs were noted at 200 mg/kg in the range-finding study). There was no significant increase in the number of micronucleated polychromatic erythrocytes at any concentration at any of the three sampling times. The positive (cyclophosphamide) and solvent controls induced the appropriate response. **There was no significant increase in the frequency of micronucleated polychromatic erythrocytes in bone marrow after any dose or treatment time.**

This study is classified as **Acceptable/Guideline**. It does satisfy the requirement for FIFRA Test Guideline [OPPTS 870.5395 (§84-2)] for *in vivo* cytogenetic mutagenicity data.

COMPLIANCE: Signed and dated GLP, Quality Assurance and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: oil of lemon eucalyptus

   Description: brownish-yellow, opaque, slightly viscous liquid (light-yellow and transparent when heated)
   Lot/Batch #: 0299088R-B
   Purity: 100% (Approx. 65% *p*-methane-3,8-diol, 66% *cis*- and 34% *trans*-isomers)
   Stability of compound: not stated
   CAS #: 129828-24-6 and 428822-96-6
   Structure: not given
   Solvent used: corn oil

2. Control materials

   Negative/Route of administration: none

   Vehicle/Final volume/Route of administration:
   Corn oil/ 10 mL/kg /intraperitoneal injection

   Positive/Final dose(s)/Route of administration: cyclophosphamide / 80 mg/kg / gavage

3. Test compound administration

   Volume of test substance administered: 10 mL/kg

   Route of administration: intraperitoneal injection

   Dose levels used:
   Preliminary toxicity assay: 200, 500, 800, 1200 mg/kg body weight (3 mice/sex/dose)

   Micronucleus assay: 250, 500, 1000 mg/kg body weight (6 males/dose/harvest)

4. Test animals (micronucleus assay)

   a. Species mouse Strain Crl:CD-1(ICR)BR Age 8 weeks
   Weight: Males: 30.0-36.6 g
   Source:Charles River Laboratories, Raleigh, NC

b. No. animals used per dose: 6 males/dose/harvest time
c. Properly maintained? Y

B. TEST PERFORMANCE

1. Treatment and sampling times:

a. Test compound and solvent control

Dosing: _x_ once __ twice (24 hr apart)

Sampling (after last dose): __ 6 hr __ 12 hr
__ 16 hr _x_ 24 hr _x_ 48 hr (control and high-dose only), __ other (describe):

b. Positive control

Dosing: _x_ once __ twice (24 hr apart)

Sampling (after last dose): __ 6 hr __ 12 hr
_x_ 24 hr __ 48 hr __ 72 hr (mark all that are appropriate), other (describe):

2. Tissues and cells examined:

_x_ bone marrow

No. of polychromatic erythrocytes (PCE) examined per animal: 2000
No. of normochromatic erythrocytes (NCE; more mature RBCs) examined per animal: the number found in at least the first 200 PCEs

3. Details of slide preparation

The mice were killed by CO_2 inhalation followed by incision of the diaphragm. Bone marrow was harvested from the tibias and/or femurs from the first five (as determined by eartag number) surviving animals per dose group. The bone marrow was flushed into tubes containing fetal bovine serum. Suspensions were then centrifuged, supernatant aspirated, and portions of the pellets spread on slides and air dried. Smears were fixed in methanol and stained with May-Grunwald/Giemsa solution and mounted.

4. Statistical methods

ANOVA was used on untransformed proportions of cells with micronuclei per animal and on untransformed PCE:NCE ratios when variances were homogeneous. Ranked proportions were used on heterogeneous variances. If the ANOVA was significant

($p \leq 0.05$), a Dunnett's t-test was used to determine significant differences of treated groups from vehicle controls.

5. Evaluation criteria

The results were considered positive if the number of micronucleated PCEs was statistically increased over the solvent control value for at least one dose level and if a dose-response was present.

II. REPORTED RESULTS

A. PRELIMINARY TOXICITY ASSAY

In a preliminary toxicity assay, three male and three female mice were dosed once by intraperitoneal injection with 200, 500, 800, or 1200 mg/kg. Clinical signs including prostration, labored breathing, ataxia, hunched posture, and hypoactivity were observed at 500, 800, and 1200 mg/kg and deaths were observed at 1200 mg/kg. No clinical signs were noted at 200 mg/kg.

Based on these results, 1000 mg/kg was chosen as the high-dose for the micronucleus assay. Because there were no differences in clinical signs between the sexes, only males were utilized in the micronucleus study.

B. MICRONUCLEUS ASSAY

Six male mice per dose per harvest time were treated once by intraperitoneal injection with 250, 500, or 1000 mg/kg oil of lemon eucalytpus in corn oil at a volume of 10 mL/kg body weight. Harvest times were 24 and 48 hours post-dosing for the vechicle control and high-dose groups and 24 hours post-dosing for the low- and mid-dose groups. (A secondary group of six high-dose replacement males were also dosed as potential replacements for the original high-dose group. Animals not used as replacements were euthanized at the end of the study). Mortality was observed in high-dose animals; animals in the mid- and high-dose groups exhibited prostration, labored breathing, ataxia, and slight hypoactivity. No clinical signs were noted in low-dose animals. There were no significant increases in micronucleated polychromatic erythroctyes at any dose at any sampling time. At 24 hours, the mean percentage of micronucleated PCEs was 0.05 (250 mg/kg), 0.05 (500 mg/kg), and 0.07 (1000 mg/kg) compared to the solvent control value of 0.04%. At 48 hours, the mean percentage of micronucleated PCEs was 0.08 (1000 mg/kg) compared to the solvent control value of 0.05%. The solvent and positive control values were appropriate. Results of the micronucleus assay are summarized in Appendix Tables 1-3 (Tables 1-3 of MRID 45045202, pp. 22-24).

III. REVIEWER'S DISCUSSION/CONCLUSIONS

A. This is an acceptable study. Oil of lemon eucalyptus was tested to doses limited by toxicity, and acceptable experimental protocol was followed. Although only male animals were utilized in the micronucleus assay, there were no substantial diffferences in clinical signs between males and females in the range-finding assay and it is unlikely that micronuclei results would vary between the sexes. The positive and solvent control values were appropriate.

This study is classified as **Acceptable/Guideline**. It does satisfy the requirement for FIFRA Test Guideline [OPPTS 870.5395 (§84-2)] for *in vivo* cytogenetic mutagenicity data.

B. STUDY DEFICIENCIES - None identified.

APPENDIX
(MRID 45045202)

This Appendix is not available electronically. See the file copy.

PC 011550 P-MENTHANE-3,8-DIOL

Page _____ is not included in this copy.

Pages 61 through 63 are not included in this copy.

The material not included contains the following type of information:

_____ Identity of product inert ingredients.

_____ Identity of product impurities.

_____ Description of the product manufacturing process.

_____ Description of quality control procedures.

_____ Identity of the source of product ingredients.

_____ Sales or other commercial/financial information.

_____ A draft product label.

_____ The product confidential statement of formula.

_____ Information about a pending registration action.

X FIFRA registration data.

_____ The document is a duplicate of page(s) _____.

_____ The document is not responsive to the request.

The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.