DATA EVALUATION RECORD

MALATHION
STUDY TYPE: METABOLISM - HUMAN
[OPPTS: 870.7485 (§85-1)]
MRIDs 45244601, 45125602, 45125603, 45125604

Prepared for

Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1801 Bell Street
Arlington, VA 22202

Prepared by

Toxicology and Hazard Assessment Group
Life Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37831
Task Order No. 63-2004

Primary Reviewer:		
Robert A. Young, Ph.D., D.A.B.T.	Signature:	_____
	Date:	_____
Secondary Reviewers:		
H.T. Borges, Ph.D., MT (ASCP), D.A.B.T.	Signature:	_____
	Date:	_____
Robert H. Ross, M.S., Group Leader	Signature:	_____
	Date:	_____
Quality Assurance:		
LeeAnn Wilson, M.A.	Signature:	_____
	Date:	_____

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EPA Reviewer: Yung G. Yang, Ph.D. **Signature**: _____
Risk Assessment Branch VI, Health Effects Division (7509C) **Date**_____
EPA Secondary Reviewer: Evisabel Craig, Ph.D., DABT **Signature**: _____
Risk Assessment Branch VI, Health Effects Division (7509C) **Date**_____

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DATA EVALUATION RECORD

STUDY TYPE: Metabolism - Human *in vivo* and *in vitro* [non guideline].

PC CODE: 057701 **DP BARCODE:** D266499

TEST MATERIAL (PURITY): (Malathion; 95.4 %)

SYNONYMS: Diethyl (dimethoxythiophosphorylthio) succinate

CITATION:

1) Aston, L.S. (2000). Determination of residues of malathion dicarboxylic acid (DCA), malathion monocarboxylic acid (MCA), dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), and dimethyl dithiophosphate (DMDTP) in human urine. Pacific Toxicology Laboratories, 6160 Variel Avenue, Woodland Hills, CA 91367. PTL119801. October 11, 2000. MRID 45244601. Unpublished.

2) Gillies, D., Dickson, J. (2000). A randomised double blind ascending single oral dose study with malathion to determine the no effect level on plasma and RBC cholinesterase activity. Inveresk Research, Elphinstone Research Centre, Tranent, EH 32 2NE, Scotland. ICR 013177. March 20, 2000. MRID 45125602. Unpublished.

3) Scott, D.L., Larner, J., Marshall, D.E., McGuire, G.M. (1999). Establishment and validation of an analytical method for the determination of malathion and malaoxon in human plasma. Inveresk Research, Tranent, EH 33 2NE, Scotland. Laboratory Report ID 17123. October 29, 1999. MRID 45125603. Unpublished.

4) Scott, D.L., Marshall, D.E., McGuire, G.M. (1999). Establishment and validation of an analytical method for the determination of malathion and malaoxon in human plasma; Addendum 1. Inveresk Research, Tranent, EH 33 2NE, Scotland. Laboratory Report ID 17123. December 23, 1999. MRID 45125604. Unpublished.

SPONSOR: Chemonova Agro A/S, P. O. Box 9, DK-7620, Lemvig, Denmark.

EXECUTIVE SUMMARY: In a randomized double blind study (MRID 45125602, 45244601), human volunteer subjects (27 males, 7 females, aged 18-50 years) were administered a single oral dose (0.5, 1.5, 5.0, 10.0, or 15.0 mg/kg in gelatin capsules) of malathion (Lot No. 50913-01, 95.4% chemical purity) after completing a light breakfast. Control subjects (11 males, 3 females) received a placebo (lactose in gelatin capsule). The study was conducted in 7 treatment blocks with ascending single oral dose of malathion. Vital signs and electrocardiography (ECG) were measured on day -1, pre-dose (0) and at 2,4, 8, and 24 hours after dosing. Blood samples were

collected for evaluation of hematology, clinical chemistry, plasma and RBC cholinesterase (ChE) activity, and plasma levels of malathion and its metabolite malaoxon. Urine samples were collected on all volunteers from 12 hours before dosing to 48 hours after dosing at -12-0, 0-12, 12-24, and 24-48 hour intervals and analyzed to evaluate the magnitude of five malathion metabolites: malathion dicarboxylic acid (DCA), malathion monocarboxylic acid (MCA), dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), and dimethyl dithiophosphate (DMDTP).

Results of the double-blind study with human volunteer subjects showed that there were no significant treatment-related changes in vital signs, ECGs, hematology, clinical chemistry, urinalysis or physical examination in any subject during the study. There were no significant treatment-related effects on RBC and plasma ChE activities up to the highest dose tested (15 mg/kg). Although the statistical analysis on ChE activity was considered inadequate, it does not change the conclusion of the study. At this time, the plasma and RBC ChE data are not appropriate for use as a point of departure for risk assessment. Plasma levels of malathion and malaoxon were both below quantifiable levels (<102 ng/mL and 99.8 ng/mL for malathion and malaoxon, respectively) 1-12 hours after dosing in all male and female volunteers who received 15 mg/kg malathion or placebo, respectively.

Urine samples collected from the volunteers were analyzed with focus on the detection and quantitation of malathion dicarboxylic acid (DCA), malathion monocarboxylic acid (MCA), dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), and dimethyl dithiophosphate (DMDTP) in human urine. The analyses showed that excretion of malathion residues occurred primarily in the first 12 hours and generally increased with dose. MCA was the most prevalent urinary component excreted over 48 hours followed by DMTP, DCA, DMP, and DMDTP.

Two additional studies (MRID 45125603, 45125604) described and validated analytical methods for determination of malathion and malaoxon in human plasma and verified storage stability of malathion and malaoxon in plasma samples. The GC method was validated by demonstrating adequate accuracy, precision, detection limits and limits of quantitation. Limit of detection was 0.0002 ppm for DCA and MCA, and 0.0125 ppm for DMP, DMTP, and DMDTP. Limit of quantitation was 0.020 ppm for DCA and MCA, and 0.025 ppm for DMP, DMTP, and DMDTP. Based upon established criteria for column efficiency, precision, resolution, detection and quantitation limits, and intraday accuracy and precision, the method was considered valid and appropriate.

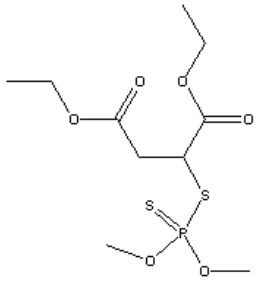
Under conditions of the study, this double blind study in humans is classified Acceptable/Non-Guideline.

COMPLIANCE: Signed GLP, Data Confidentiality Claim, and Quality Assurance statements were provided in both study reports.

I. MATERIALS AND METHODS:

A. MATERIALS:

1. Test compound:

Radiolabelled test material:	NA
Radiochemical purity	NA
Specific Activity	NA
Lot/Batch #:	NA
Non-Radiolabelled test material:	Malathion (supplied by sponsor) (MRID 45125602) Malathion (supplied by sponsor) (MRID 45125603; 45125604) Malaoxon (supplied by sponsor) (MRID 45125603; 45125604)
Description:	clear liquid
Lot/Batch #:	Malathion 50913-01 (MRID 45125602) 324-OSJ-54C (MRID 45125603; 45125604) Malaoxon 279-ABB-09-01 (MRID 45125603; 45125604)
Purity:	Malathion 95.4% w/w (MRID 45125602) 99.4% (MRID 45125603; 45125604) Malaoxon 96.9% (MRID 45125603; 45125604)
Contaminants:	none specified
CAS # of TGAI:	121-75-5
Structure:	

2. Vehicle and/or positive control: No vehicle or positive control was used.

3. Test subjects:

38 male and 10 female informed volunteers (MRID 45125602); subjects required to meet inclusion criteria and given pre-study screening which were described in detail in MRID 45125602; withdrawal from study also allowed

Age/weight at study initiation: Males: 18-48 years old, Females: 18-41 years old.
Weights: 53.9-92.9 kg (MRID 45125602)

4. Dose preparation:

For the malathion/cholinesterase study (MRID 45125602), the test material or placebo (lactose) was administered in a hard gelatin capsule with 150 mL of water approximately 5 minutes after a light breakfast. Dose was determined by subject body weight.

For the study evaluating storage stability of malathion and malaoxon in human plasma (MRID 45125604), replicate samples were prepared containing approximately 200 or 800 ng/mL of human plasma.

B. STUDY DESIGN AND METHODS:

1. **Group arrangements:** The experimental protocols for the various studies are summarized in Table 1. The study protocol was reviewed and approved by an independent Research Ethics Committee. For the malathion/cholinesterase study (MRID 45125602), detailed inclusion and exclusion criteria were provided regarding selection of the human volunteers. The study was conducted in 7 treatment blocks with ascending single oral dose of malathion (Table 1.1). A comprehensive array of physiological/clinical parameters (e.g., vital signs, ECG, hematologic indices, and clinical chemistry) were evaluated for all subjects (Table 1.2).

Table 1.1 Dose levels of malathion administration

	Placebo	0.5 mg/kg	1.5 mg/kg	5.0 mg/kg	10.0 mg/kg	15.0 mg/kg
Session 1 Subject 001-004	1	3				
Session 2 Subject 005-008	1		3			
Session 3 Subject 009-018	3			7		
Session 4 Subject 019-022	1				3	
Session 5 Subject 023-031	2				4	3
Session 6 Subject 032-038	3					4
Session 7 Subject 039-048	3					7

a= Sessions 1-6, males
b= session 7, females

TABLE 1.2. Experimental protocols for malathion and malathion residue studies.

Experiment	Subjects/Exposure/Dose	Comments
Urinalysis	48 human volunteer subjects; single oral dose: 0 (placebo), 0.5, 1.5, 5.0, 10.0, 15.0 mg malathion/kg	Urine collected over 12 hrs pre-dose and 0-12 hrs, 12-24 hrs, and 24-48 hrs post-dose and analyzed for 5 metabolites and creatinine (MRID 45244601)
Plasma & RBC ChE inhibition	11_; 3 _ placebo[a] 3 _ 0.5 mg/kg 3 _ 1.5 mg/kg 7 _ 5.0 mg/kg 7 _ 10.0 mg/kg 7 _ 15.0 mg/kg 7 _ 0.5 mg/kg	Vital signs monitored on Day -1, and at 2, 4, 8, and 24 hrs post dose; ECG at -30 min., and at 2, 4, 8, and 24 hrs post dose; hematology, clinical chemistry, urinalysis evaluated. Blood collected on Days 9, 7, 5, 2 and 1 and 30 min. pre-dose and at 1, 2, 4, 8, 12, 24, and 48 hrs post dose for plasma and RBC ChE activity analysis. (MRID 45125602)
Method Validation	6 replicate plasma samples analyzed each containing 30, 40, or 50 ng test material/mL plasma	Validation of Analytical Method No. 6674 for determining malathion and malaoxon levels in human plasma (MRID 45215603)
Storage stability	4 replicate 200 and 800 ng/mL samples of malathion and malaoxon	Samples stored at -80 °C for 2, 6, 12, 18, and 26 wks and analyzed by Analytical Method No. 6674 to evaluate storage stability (MRID 45215604)

[a] Subjects receiving placebo were distributed and matched among the treatment groups; assignment of subjects to treatment and placebo groups was via confidential randomized code
Data taken from p.18, MRID 45244601, p.17, MRID 45125602, p. 15, MRID 45215603, and p. 9, MRID 45215604.

2. Dosing and sample collection/preparation:

For the urinalysis study (MRID 45244601), urine samples were collected from the human volunteers over 12-hr intervals starting at 12 hours prior to dosing and to 48 hours post dose (Table 2). Total urine volume voided during each interval was recorded and aliquots of samples were retained at -6 to -15°C until analysis. Additional details regarding dosing and collection of samples were under a separate experimental protocol from work performed by Pacific Toxicology Laboratories (PTL) and reported in MRID 45244601.

For the study examining the dose-response for plasma and RBC ChE activities (MRID 45125602), the human volunteers were given the test article or lactose placebo in a hard gelatin capsule with water. The capsule contents were prepared by direct weighing of the test material necessary to attain the target dose for the body weight (kg) of the subject. Accuracy of capsule filling was ±5%.

For the storage stability study (MRID 45125604), samples of plasma to which malathion, malaoxon, or internal standard had been added were analyzed following storage for 2, 6, 12, 18 or 26 weeks.

3. Analytical techniques:

Reference standards: Reference standards of the following were utilized in the urinalyses report (MRID 45244601): malathion dicarboxylic acid (DCA; Lot No. 167-BSe-71c, 99.0% purity), malathion monocarboxylic acid (MCA; Lot No. 275-MJH-82-1, 91.0% purity), dimethyl phosphate (DMP; Lot No., 302-OSJ-50B, 98.4% purity), dimethyl thiophosphate (DMTP; Lot No., 267-OSJ-54B, 97.9% purity), dimethyl dithiophosphate (DMDTP, Lot No. 291-BSe-62A, 99.1% purity), diazinon (Lot No. 214-64A, 99.3% purity), and fenthion (Lot No., 208-52B, 98.0% purity).

For the plasma malathion/malaoxon method validation (MRID 45215603), bromophos-ethyl (Batch Nos. 31530 and 80080, Promochem Ltd.) was used as the internal standard. Standard solutions of malathion and malaoxon were prepared with polyethylene glycol in acetone (0.02%) to provide malathion and malaoxon equivalents of ~1-200 ng (in an 8 µl volume) on the analytical column.

Gas chromatography (GC): GC analysis was performed using a Perkin-Elmer 8500 gas chromatograph with autosampler, DB-120 column, and flame photometric detector (phosphorus mode) with a nitrogen carrier gas.

Sample processing/analysis:

Urine (MRID 45244601): Urine from subjects in the ChE study (MRID 45125602) underwent typical urinalysis (pH, protein, glucose, ketones, bilirubin, blood, urobilinogen, and specific gravity). The samples were thawed, vigorously shaken and aliquots removed

for analysis by a residue analytical method assessing malathion carboxylic acid (MCA) residue and malathion alkyl phosphate (MAP) residue. For MCA residue analysis, quality control samples using urine from laboratory personnel were fortified with 0 (blank), 30 ppb, or 300 ppb of DCA and MCA, and analyzed with the study samples. Reagent blanks and six calibration standards were also prepared for the analyses. Preparation/extraction procedures were extensively described in the published report and involved adjusting the urine to pH 1-2, extraction of the malathion acid metabolites, derivatization, and analysis by GC. For the malathion alkyl phosphate residue analysis, quality control samples were spiked with 0 (blank), 25 ppb, or 1000 ppb of DMP, DMTP, and DMDTP, and analyzed with the study samples. Samples were freeze-dried and derivatized in acetone and 10% 1-benzyl-3-p-tolyltriazine at 70°C for two hours. Twenty-four hours later, sample pH was adjusted with 6 N HCl and 10 mL of saturated NaCl was added. Fenthion (0.5 mL of 0.5 µg/mL solution) was added as an internal standard and the mixture centrifuged. The organic supernatant was removed, twice extracted with cyclohexane, and analyzed by GC after evaporation of the cyclohexane. Reagent blanks and six calibration standards were also prepared.

Creatinine: Urine creatinine analyses (MRID 45244601) was done using the standard Jaffee reaction.

Blood plasma and RBCs: Blood collected from the human subjects (MRID 45125602) in two 7 mL lithium heparin tubes was centrifuged, stored at -70 °C, and transferred in dry ice for analysis (Inveresk Analytical Method No. 6674 validated for concentrations of 100-1000 ng/mL).

Blood cholinesterase (ChE) assay (MRID 45125602): Blood collected in EDTA-tubes was used for evaluation of plasma and RBC ChE. Aliquots of plasma (0.1 mL) were spiked with malathion and malaoxon in acetonitrile and used as calibration standards and for quality control. Bromophos-ethyl in acetonitrile was used as the internal standard and was added to the samples before extraction with toluene. The extracts were evaporated and reconstituted in polyethylene glycol in acetone. Aliquots (8 µL) were analyzed by GC. Malathion concentration was determined by interpolation from the calibration curve developed by plotting peak area ratio of the standard samples vs the concentration of each compound in plasma. Least squares linear regression (1/concentration) of each standard was used.

4. Method validation:

Urinalysis of malathion metabolites (MRID 45244601): Method validation for detection/quantitation of metabolites addressed the following:

1) Analytical procedures for MCA and MAP metabolites included assay specificity, limit of detection, limit of quantitation, assay linearity, intraday variation, and concurrent recovery
2) Systems suitability included column efficiency, tailing factor, resolution, and precision

Plasma malathion/malaoxon analysis (MRID 45125603): The following criteria were evaluated to validate the method with human plasma: 1) system suitability/column efficiency/tailing/resolution/precision, 2) specificity, 3) linearity, 4) limit of detection, 5)

limit of quantitation, 6) intraday accuracy and precision, 7) absolute recovery from human plasma, and 8) absolute recovery of internal standard from human plasma.

5. **Storage stability**: In MRID 45125602, stability of urine samples was assessed using normal spiked samples containing 40 ppb and 200 ppb of DCA and MCA and pooled urine samples spiked with 50 and 250 ppb of DMP, DMPT, and DMDTP that had been stored at -6 to -15°C (same as study samples). At intervals of 10, 35, 45, 55, and 65 weeks, the samples were analyzed in triplicate.

   Results: Stability of the phosphate metabolites at 372 days, only DMDTP (67% of nominal) exhibited levels less than 80% of nominal. For the carboxylic acid metabolites, all were ≥88% of nominal after 467 days of storage.

   Storage stability of human plasma containing malathion or malaoxon was assessed following storage at -80 °C for durations ranging from 2 to 26 weeks (MRID 45125604).

6. **Calculations/ statistical analysis**: Calculations procedures/explanations for the following were provided:
   a) Concentration of residues in samples including response factor, analyte concentration, and
   b) Percent recovery from fortified samples.

   Statistical analyses (MRID 45125602) were performed using SAS (v6.09). For plasma and RBC ChE activities, change from baseline at each time point was calculated and tabulated by gender and dose. Both individual and group values were evaluated. Baseline was defined as all available pre-dose values except screening. Changes from baseline were analyzed using ANOVA and pairwise comparisons between placebo and each dose using Student's t-test at each time point. Significance was set at $p \leq 0.05$. Distributional assumptions (e.g., normality) was assessed using the Shapiro-Wilk test.

   The statistical analysis on ChE data was reviewed by the HED Chemistry and Exposure Branch (David Miller and Bayazid Sarkar). HED concluded that the registrant provided inadequate information on the statistical analysis. The statistical aspects of the study submission are judged to be inadequate (detailed description is attached in the appendix).

II. RESULTS:

A. ANALYTICAL/METHOD VALIDATION - URINALYSIS (MRID 45244601):

1. METHOD VALIDATION

Malathion Acid metabolites: Method validation data for analysis of DCA and MCA in fortified urine samples are summarized in Table 2. Accuracy was determined by "% recovery" while precision was measured by "%RSD (relative standard deviation)."

TABLE 2. Method validation for analysis of malathion dicarboxylic acid (DCA) and malathion monocarboxylic acid (MCA) in human urine.

Fortification (ppb)	DCA		MCA	
	Recovery (%)	RSD[b] (%)	Recovery (%)	RSD[a] (%)
30	103	3	96	3
300	91	4	95	9
600	106	2	106	2

Data taken from p. 38, MRID 45244601
[a] n=7 runs
[b] RSD: relative standard deviation

Results of concurrent recovery of malathion acid metabolites from fortified urine samples are shown in Table 3.

TABLE 3. Concurrent recovery of malathion dicarboxylic acid (DCA) and malathion monocarboxylic acid (MCA) in human urine.

Fortification (ppb)	DCA		MCA	
	Recovery (%)	RSD[b] (%)	Recovery (%)	RSD[a] (%)
30	108	16	102	13
300	104	19	99	11

Data taken from p. 39, p. 47, MRID 45244601
[a] n=17 runs
[b] RSD: relative standard deviation

Malathion Alkyl Phosphate metabolites: Method validation data for analysis of DMP, DMTP and DMDTP in fortified urine samples are summarized in Table 4.

TABLE 4. Method validation for analysis of dimethyl phosphate (DMP), dimethyl-thiophosphate (DMTP), and dimethyl dithiophosphate (DMDTP) in human urine.

Fortification (ppb)	DMP		DMTP		DMDTP	
	Recovery %	RSD[a] (%)	Recovery %	RSD[a] (%)	Recovery %	RSD[a] (%)
25	98	16	100	16	126	26
1000	99	11	101	12	132	18

Data taken from p. 38, MRID 45244601
[a] n=7 runs
[b] RSD: relative standard deviation

Results of concurrent recovery of malathion alkyl phosphate metabolites from fortified urine samples are shown in Table 5.

TABLE 5. Concurrent recovery of dimethyl phosphate (DMP), dimethyl- thiophosphate (DMTP), and dimethyl dithiophosphate (DMDTP) in human urine.

Fortification (ppb)	DMP		DMTP		DMDTP	
	Recovery %	RSD[a] (%)	Recovery %	RSD[a] (%)	Recovery %	RSD[a] (%)
25	112	14	103	12	106	17
1000	110	19	99	17	120	23

Data taken from p. 39, MRID 45244601
[a] n=16 runs
[b] RSD: relative standard deviation

Detection limits and limits of quantitation: Limits of detection for DCA and MCA were 0.0002 ppm, and 0.0125 ppm for DMP, DMTP, and DMDTP. Limits of quantitation were 0.020 ppm for DCA and MCA, and 0.025 ppm for DMP, DMTP, and DMDTP.

2. Urinalysis:

Malathion metabolites: Urine samples were analyzed for DCA, MCA, DMP, DMTP, and DMDTP. The metabolites were identified based on GC relative retention times (RRT) of the samples compared to internal standard RRT values. Analysis of extensive tabulated data revealed that most of the malathion metabolites appeared to be excreted within 12 hours and that MCA was quantitatively the most prevalent metabolite (Table 6). Prevalence of the remaining metabolites in decreasing order was DMTP, DCA, DMP, and DMDTP. Relative to the 0-12 hour values, excretion during the 24-48 hour period was minor. Considerable variability was observed in metabolite levels among subjects in the same dose group and for similar sampling times. Generally, metabolite levels tended to increase proportionally to the dose although exceptions were observed. Some metabolites were detected in the urine of control subjects (i.e., those receiving the placebo) although the levels were far below those detected in the urine of even low-dose treated subjects; these findings were attributed to sample contamination.

TABLE 6. Malathion metabolites (ppm) in human 48-hour urine samples[a]

Malathion dose (mg/kg)	DCA	MCA	DMP	DMTP	DMDTP
0 (Placebo)	ND	ND	ND/NQ	ND	ND
	ND	ND	ND	ND	ND
	0.440	0.553	0.051	0.175	0.055
	0.579	0.475	0.116	0.200	0.031
	ND/NQ	ND/NQ	ND	0.027	ND
	0.022	0.026	ND	ND	ND
	ND/NQ	ND/NQ	ND	ND	ND
	ND	ND	ND	ND/NQ	ND
	0.090	0.120	ND	ND	ND
	ND	ND	ND/NQ	ND	ND
	ND/NQ	ND/NQ	ND	ND	ND
	0.022	0.026	ND/NQ	ND	ND
	ND/NQ	ND/NQ	ND	ND	ND
0.5	4.25	6.64	0.967	0.640	0.052
	1.965	6.553	0.902	1.709	0.311
	3.693	9.403	1.825	3.946	0.800
1.5	6.747	29.129	8.480	17.327	0.314
	14.103	43.952	9.146	17.207	1.176
	4.334	30.724	6.448	11.747	1.613
5.0	11.916	7.889	27.934	38.627	15.628
	8.447	23.100	1.926	2.658	0.794
	42.450	6.103	14.482	21.245	6.286
	68.276	155.640	19.085	54.788	12.635
	42.515	82.278	19.647	43.725	5.601
	15.410	75.240	9.507	24.392	3.648
	21.253	34.861	10.452	18.518	1.501
10.0	83.370	276.621	73.881	128.996	9.464
	41.648	231.837	81.355	58.341	4.729
	28.475	320.819	48.748	56.750	4.407
	37.934	307.755	35.105	19.774	4.719
	410.038	187.223	33.749	80.065	5.80
	11.593	62.045	41.62	51.977	4.113
15.0	37.890	304.288	124.848	65.421	10.471
	23.784	267.696	62.722	174.171	10.200
	43.038	251.383	47.362	77.112	7.441
	98.517	506.798	49.772	147.700	10.396
	283.667	100.717	58.138	56.570	3.040
	70.935	904.332	203.635	441.947	15.272
	119.678	871.843	284.611	94.336	3.290
	20.335	181.966	58.158	18.695	2.989
	32.706	160.297	22.070	73.142	23.529
	32.634	181.964	10.092	18.206	5.294
	8.816	207.914	53.016	77.791	3.353
	43.601	64.713	133.917	187.915	5.435
	70.217	321.222	15.546	22.563	2.866
	39.209	317.600	30.287	101.65	8.691

[a] Sum total of -12-0, 0-12, 12-24, and 24-48 hrs samples; results are total 0-48 hr excretion for each subject.
ND = not detected; NQ = not quantitiated
Data derived from Table V, pp. 49-53, MRID 45244601.

Creatinine: Urine creatinine was highly variable ranging from 0.36 to 3.61 g/L although values for most subjects were between 1 and 2 g/L.

B. BLOOD CHOLINESTERASE (MRID 45125602):

Plasma cholinesterase: Results of malathion treatment on plasma ChE activity of male and female human volunteers are summarized in Tables 7 and 8, respectively. For males, there was no consistent effect on plasma ChE following malathion treatment. Trend analysis was significant only at 2 hrs and 7 days (p=0.016 and 0.004, respectively), however, pairwise comparison of all dose groups with the placebo at these time points was not indicative of a significant effect.

TABLE 7. Effects of malathion on plasma cholinesterase activity (% change from baseline) in male volunteers.

Time after dose	Placebo	0.5 mg/kg	1.5 mg/kg	5.0 mg/kg	10.0 mg/kg	15.0 mg/kg
Baseline	0	0	0	0	0	0
1 hr	-7.07	+5.92*	-14.37	-5.26	-5.57	-10.07
2 hrs	-4.34	+9.59	-11.62	-2.69	-4.64	-9.95
4 hrs	+1.51	+14.23*	-7.83	-3.58	-3.94	+13.47*
8 hrs	-7.89	-8.48	-3.45	-7.02	-7.15	-13.45
12 hrs	-7.22	-11.83	-3.26	-7.63	-7.20	-10.47
24 hrs	-5.20	-8.23	-6.74	-2.14	-1.49	-9.22
48 hrs	-4.62	-6.79	-7.65	-3.75	-2.09	-6.33
Day 4	-2.22	-0.39	-2.99	+4.90	+0.11	-0.66
Day 7	-5.69	-13.90	-8.82	-2.83	-0.64	-1.54
Day 14	-3.74	-4.62	+0.73	-0.54	+2.48	-1.22

Data taken from Table 5, p. 44, and Table M1.1.1, p. 657, MRID 45125602.
*p<0.05 relative to placebo.

For females, there was no significant difference between the 15 mg/kg group and the placebo group regarding plasma ChE activities.

TABLE 8. Effects of malathion on plasma cholinesterase activity (% change from baseline) in female volunteers.

Time after dose	Placebo	15.0 mg/kg
Baseline	0	0
1 hr	-9.28	-7.68
2 hrs	-6.76	-4.91
4 hrs	-4.79	-4.99
8 hrs	-3.68	-5.87
12 hrs	-1.73	-6.65
24 hrs	-4.21	-0.21
48 hrs	-7.21	-2.35
Day 4	-2.74	+0.92
Day 7	-4.46	-2.68
Day 14	+0.70	+2.48

Data taken from Table 6, p. 44, MRID 45125602.

RBC cholinesterase: RBC ChE activity from MRID 45125602 is shown in Tables 9 (males) and 10 (females). Analysis of data for male subjects did not reveal any significant effect of the malathion treatment at any time point or for any dose or any significant trend related to the treatment.

TABLE 9. Effects of malathion on RBC cholinesterase activity (% change from baseline) in male volunteers.

Time after dose	Placebo	0.5 mg/kg	1.5 mg/kg	5.0 mg/kg	10.0 mg/kg	15.0 mg/kg
Baseline	0	0	0	0	0	0
1 hr	-5.94	+1.55	-13.19	-5.17	+1.91	-2.86
2 hrs	-0.35	+3.56	-10.04	-6.81	+2.09	-0.61
4 hrs	-0.54	+0.97	-7.11	-2.43	-0.16	+1.17
8 hrs	-6.27	-4.99	-6.86	-7.34	-2.55	+2.52
12 hrs	-0.25	+5.54	+2.33	+0.92	+4.03	+2.84
24 hrs	-4.78	-2.83	-8.24	-7.85	-4.30	-6.97
48 hrs	-2.35	-3.73	-5.22	-6.75	+1.63	+3.22
Day 4	+3.50	-10.02	+4.07	-2.21	+5.64	+3.49
Day 7	+3.12	-4.08	-4.10	+7.66	+0.41	-4.35
Day 14	+0.44	-5.06	-9.30	+3.97	-0.39	-2.18

Data taken from Table 7, p. 51, MRID 45125602.
*$p<0.05$ relative to placebo.

For females, there was no significant difference between the 15 mg/kg group and the placebo group regarding RBC ChE activity.

TABLE 10. Effects of malathion on RBC cholinesterase activity (% change from baseline) in female volunteers.

Time after dose	Placebo	15.0 mg/kg
Baseline	0	0
1 hr	-3.23	-1.20
2 hrs	+6.13	-0.67
4 hrs	-5.98	-6.15
8 hrs	-3.75	+0.40
12 hrs	+4.48	+3.59
24 hrs	-2.19	-7.74
48 hrs	+1.70	-5.63
Day 4	-6.70	-1.91
Day 7	-0.10	-5.25
Day 14	-0.51	-9.45

Data taken from Table 8, p. 51, MRID 45244602.

Adverse Events (AES): A series of AES was recorded among the various treatment groups. A total of 40 AES was reported in 18 of the 48 subjects during the study. These events represented a wide range of effects (dizziness, headache, rhinitis, flatulence, pharyngitis, abdominal pain, and dry skin, among others) and were observed in groups receiving malathion as well as the placebo groups. Some of the effects were present prior to treatment and most were considered unrelated to the malathion treatment. Some of those considered possibly related to treatment included headache, hepatobiliary functions, gastrointestinal disorders, and dizziness.

Plasma Analysis: Since no inhibition of either plasma or RBC ChE was observed at any dose level, analysis of plasma for malathion and malaoxon was restricted to sample from volunteers who were dosed at 15 mg/kg (7 males and 7 females) and the corresponding placebos (5 males and 3 females) and were analyzed for malathion and malaoxon. Plasma samples collected pre-dose and 1, 2, 4, 8, and 12 hours post-dose were analyzed. Results showed that the plasma concentrations of both malathion and malaoxon were below quantifiable levels (<102 ng/mL and 99.8 ng/mL for malathion and malaoxon, respectively), 1-12 hour after dosing in all male and female volunteers who received 15 mg/kg malathion or placebo.

C. METHOD VALIDATION - PLASMA MALATHION/MALAOXON ANALYSIS (MRID 45125603: Based upon criteria noted in Section B.5. of this Data Evaluation Record, the system developed for analysis of plasma malathion and malaoxon were validated (Tables 11-12).

System suitability/column efficiency/tailing factor/linearity/resolution/precision/ detection limits: Analyses of malathion, malaoxon and an internal standard showed column efficiency was acceptable (Table 11). Resolution, detection, quantitation limits, and intraday accuracy/precision were also acceptable (Table 11). Linearity of the detector response for malathion and malaoxon over the range of concentrations tested was 3.986-199.3 ng for malathion and 0.9908-198.2 ng for malaoxon.

TABLE 11. Evaluation of gas chromatography critical parameters for assay of plasma malathion and malaoxon.

Parameter	Malathion	Malaoxon	Internal Std.
Column efficiency (no. theoretical plates)	302,000	265,000	480,000
Tailing factor	1.01	0.95	1.37
Precision (%)[a]	1.7	3.2	1.3
Resolution	4.71[b] 9.90[c]	–	–
Plasma limit of detection (ng/mL)	41	35	–
Plasma limit of quantification (ng/mL)	100	100	–
Intraday accuracy in human plasma (%)[d]			–
200 ng/mL	105.0	102.1	
400 ng/mL	96.8	96.3	
800 ng/mL	96.2	98.3	
Intraday precision in human plasma (%)[e]			–
200 ng/mL	3.6	4.6	
400 ng/mL	5.6	8.2	
800 ng/mL	3.7	7.7	

[a] Coef. of variation based on 10 replicate samples
[b] Resolution between malathion and malaoxon
[c] Resolution between malathion and internal standard
[d] Accuracy based on comparison of assay results with known concentrations in human plasma; 85-115% considered acceptable
[e] Expressed as coef. of variation; ±15% considered acceptable
Data taken from p. 17, p. 20, p. 22, p. 25and Tables 3 (p 29) and 6- 9 (pp. 28-32), MRID 45125603.

Absolute recovery of malathion and malaoxon in human plasma: Data showing absolute recovery of malathion, malaoxon and internal standard from human plasma are summarized in Table 12.

TABLE 12. Absolute recovery of malathion, malaoxon, and internal standard from human plasma[a].

Malathion		Malaoxon		Internal standard	
Amount on column (ng)	Mean recovery (%)	Amount on column (ng)	Mean recovery (%)	Amount on column (ng)	Mean recovery (%)
8.130	52.3	8.016	81.9	215.6	13.4
16.26	55.9	16.03	88.5		
32.52	61.3	32.06	98.8		

[a] Mean recovery values represent mean of six samples.

Data taken from Tables 10-13, pp. 32-34, MRID 45125603.

D. <u>STORAGE STABILITY IN HUMAN PLASMA (MRID 45215604)</u>: Results of the storage stability study are shown in Table 13. For malathion in plasma, all analyses revealed that stability remained within 15% of the original concentration. For malaoxon in plasma, one sample each at the 15-day, 93-day, and 196-day analysis exceeded the 15% acceptability coefficient of variation but mean values remained within 15%.

TABLE 13. Storage stability of malathion and malaoxon in human plasma at -80 °C.

Test Material (Concentration)	Storage Duration (Days)	Mean Calculated[a] Concentration (ng/mL)	Mean Percent of Actual [a] Concentration/Coef. Var (ng/mL)
Malathion (203.3 ng/mL)	0	226.4	111.3/2.5
	15	201.5	99.2/5.3
	51	197.0	97.0/4.3
	93	208.5	102.5/7.7
	196	209.3	102.8/5.6
Malathion (813.0 ng/mL)	0	889.2	109.4/3.4
	15	852.8	104.9/11.0
	51	790.0	97.2/2.6
	93	776.0	95.4/9.0
	196	821.5	101.0/5.1
Malaoxon (194.6 ng/mL)	0	191.4	98.4/7.9
	15	182.3	93.6/11.9
	51	192.3	98.8/8.6
	93	193.5	99.4/8.1
	196	198.3	101.9/3.1
Malaoxon (778.3 ng/mL)	0	733.8	94.3/7.1
	15	826.0	106.1/12.7
	51	700.0	89.9/3.4
	93	733.0	94.2/10.6
	196	821.0	105.5/10.6

[a] Values are means of three to five samples.
Data taken from Tables 1 and 2, pp. 13 and 14, MRID 45125604.

III. DISCUSSION AND CONCLUSIONS:

A. INVESTIGATORS' CONCLUSIONS: MRID 45244601 reported on the validation of analytical methods for detection and quantitation of malathion acid metabolites (DCA and MCA) and alkyl phosphate metabolites (DMP, DMTP, and DMDTP) in human urine. Results to evaluate recovery from urine samples spiked with known amounts of these metabolites validated the method by showing 91-131% recovery for both high and low levels of fortification. Adequate stability was demonstrated for metabolites stored up to 13 months. Analysis of urinary metabolites of malathion from 48 human volunteer subjects receiving single oral doses of malathion (0.5, 1.5, 5.0, 10.0 or 15.0 mg/kg or a placebo) revealed that most of the malathion was excreted as MCA (897 ppm maximum concentration). Additional metabolites were also detected with maximum concentrations of 244 ppm (DCA), 425 ppm (DMTP), 197 ppm (DMP), and 23 ppm (DMDTP). The 0-12 hour urine samples generally contained the highest concentrations of metabolites and represented most of the excretion over the 48-hour sampling period. The acid metabolites (MCA and DCA) represented 66% of the total urinary metabolites. The investigator concluded that metabolism and excretion of malathion was rapid (within 12 hrs) and is essentially complete within 24 hours regardless of gender or dose (0.5-15 mg/kg/day).

In the randomized double-blind study (MRID 45125602) of malathion effects in human volunteers, there were no statistically significant changes in plasma or RBC ChE among male subjects given a single dose of 0.5, 1.0., 1.5, 5.0, 10.0 or 15.0 mg/kg or in female subjects given a single 15 mg/kg dose. There were no treatment-related effects on vital signs, ECG, hematologic, urinalysis, or clinical chemistry parameters. The investigators concluded that 15 mg/kg was a minimum No Observed Effects Level (NOEL) for malathion given as a single oral dose to healthy male or female human volunteers.

MRID 45125603 reported on the development and validation of Inveresk Research Analytical Method No. 6674 for determination of malathion and malaoxon in human plasma. The investigators concluded that the method was satisfactory. This conclusion was based upon results of procedures demonstrating acceptable system suitability, assay specificity and linearity, intraday accuracy and precision, and absolute recovery of malathion and malaoxon in human plasma at concentrations ranging from 100 to 1000 ng/mL. Limit of quantitation was 100 ng/mL for both malathion and malaoxon, and limits of detection were 41 ng/mL and 35 ng/mL, respectively.

Storage stability of plasma samples containing malathion or malaoxon (200 or 800 ng/mL) was found to be acceptable for up to 196 days at -80 °C.

B. REVIEWER COMMENTS: Two studies (MRID 54244601, 45125602) were performed to identify a no-effect level for plasma and red blood cell (RBC) cholinesterase (ChE) inhibition in human volunteers (27 males, 7 females) following a single oral dose (0.5, 1.5, 5.0, 10., or 15.0 mg/kg) of malathion (Lot no. 50913-01, 95.4% chemical purity) and to determine malathion residues in urine samples of these subjects. Control subjects (11 males, 3 females) received a lactose placebo. Two additional studies (MRID 45125603, 45125604) described and validated an analytical method for determination of malathion and malaoxon in human plasma and to verify storage stability of malathion and malaoxon-containing plasma samples.

All of these studies accomplished their respective objectives. The double-blind study (MRID 45125602) plasma and RBC ChE inhibition in human volunteer subjects given a single oral dose of malathion suggested that 15.0 mg/kg was a no-effect level. Assessment of ChE changes was appropriately evaluated relative to individual baseline values and all variability within and among individuals was provided. Changes in plasma or RBC ChE activity were evaluated for 14 days following malathion administration. Assessment of ChE activity routinely exhibits considerable variability and results from this study were typical. The investigators also included clinical data, relevance of the findings to ChE, and detailed information regarding exception criteria. The statistical analysis methodology was considered inadequate; however, it does not change the conclusion of the study. At this time, the plasma and RBC ChE data are not appropriate for use as a point of departure for risk assessment.

Urine samples generated in the double blind study were used to develop and validate methodology for assessment of malathion residues (MRID 45244601). This study focused on the detection and quantitation of malathion dicarboxylic acid (DCA), malathion monocarboxylic acid (MCA), dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), and dimethyl dithiophosphate (DMDTP) in human urine. The GC method was validated by demonstrating adequate accuracy, precision, detection limits and limits of quantitation. Limits of detection were 0.0002 ppm for DCA and MCA, and 0.0125 ppm for DMP, DMTP, and DMDTP. Limits of quantitation were 0.020 ppm for DCA and MCA, and 0.025 ppm for DMP, DMTP, and DMDTP. Urinalysis, conducted using the previous methods along with comparison to reference standards, showed that excretion of malathion residues occurred primarily in the first 12 hours following dosing and generally increased with dose. MCA was the most prevalent urinary component excreted over 48 hours followed by DMTP, DCA, DMP, and DMDTP. There was no assessment of other components nor was it possible to ascertain total recovery of the administered dose. Absolute recovery specified in the method validation study (MRID 45125603) was acceptable for malaoxon but marginal for malathion and an internal standard; possibly a function of the extraction solvent as much as the GC analytical method.

Data validating Inveresk Research Analytical Method No. 6674 for the determination of malathion and malaoxon in human plasma was provided in MRID 45125603 and storage stability of malathion and malaoxon in human plasma samples was reported in MRID 45215604. Based upon established criteria for column efficiency, precision, resolution, detection and quantitation limits, and intraday accuracy and intraday precision, the method was considered valid and appropriate. Absolute recovery, although low for malathion (~52-61%) and the internal standard (~13%), was reproducible. Reproducibility in conjunction with acceptable accuracy and precision, was considered acceptable. Storage stability of malathion and malaoxon in human plasma at -80 °C over 196 days was demonstrated by mean percent recovery values ranging from ~90-110% with coefficients of variance from 2.5 to 13.

These reports describing and validating a methodology for analysis of malathion and malaoxon in human plasma (MRID 45244601, 45125603, 45125604, 45125602) and identification of a no-effect level for malathion-induced inhibition of plasma and RBC ChE activity in humans are classified **Acceptable/Non-Guideline**.

By design, these studies described and validated analytical methods for determination of malathion and metabolites in human plasma and urine (MRID 45244601), provided a no-effect level for plasma and RBC-ChE activity based upon a specific exposure protocol with informed human volunteer subjects (MRID 45125602), described and validated a methodology for determination of malathion and malaoxon in human plasma (MRID 45125603), and validated storage stability of these chemicals in human plasma samples (MRID 45125604).

C. STUDY DEFICIENCIES: Statistical aspects of the study are considered inadequate; however, it would not change the conclusion of the study.

Appendix

Statistical Analysis of Cholinesterase data:
Statistical method to analyze RBC and Plasma Cholinesterase data were described in section 5.5.2 of registrant's submission. The main submission indicated that the registrant used SAS V6.09 to perform all statistical analysis. The document indicated that the "*percentage changes from baseline for RBC and plasma ChE were analyzed using a repeated-measures analysis of variance (ANOVA) including terms for dose level, time point, and dose level by time point interaction. Subject was included as a random effect*". RBC and plasma cholinesterase were summarized (i.e., mean, standard deviation, minimum, maximum and n) at each time point, including changes from baseline, by gender and dose level. Additionally, the percentage from baseline at each time point was tabulated by gender and dose level. Baseline was defined as the mean of all available pre-dose values (i.e. days -9, -7, -5, -2, -1 and -30 minutes).

For the male data, percentage change from baseline for RBC cholinesterase and for plasma cholinesterase were analyzed using a repeated measures analysis of variance (R-ANOVA) including terms of dose level, time point (i.e., 1-, 2-, 4-, 8- , 12- , 24- , 48- , and 72-h, day 4, day 7 and day 14 post-dose) and dose level by time point interaction. Subject was treated as random effect. At each time point, separately, a test for linear trend with dose was performed using a linear contrast. In addition, using the error variance from the R-ANOVA pairwise comparisons between placebo and each dose level were conducted at each time point. At each time point, if the test of linear trend was significant at the 5% level then the pairwise comparisons at that time point were not adjusted for multiple comparisons. If the test for linear trend was not significant at 5% level, a Bonferroni adjustment was applied to the pairwise comparison at that time point. Treatment group adjusted means were presented together with the significance level of the 't' test and the test for linear trend. In addition, where a Bonferroni adjustment was applied, the significance level of the pairwise comparisons after adjustment was also presented.
For the female data, percentage change from baseline for RBC cholinesterase and plasma cholinesterase were similarly analyzed using a repeated measures analysis of variance (R-ANOVA) including terms of dose level, time point, and dose level by time point interaction. Subject was used as random effect. Using error variance from the R-ANOVA, a comparison between placebo and active group was carried out, at each time point, using a Student's 't' test. Adjusted means were presented together with the significance level of the 't' test.
Distributional assumptions underlying the statistical analyses were assessed as follows: Normality was examined using a Shapiro-Wilk test while homogeneity of variance was assessed by plotting the residuals against the predicted values for the model. If there was significant non-normality which could not be resolved by transforming the data, the data were analyzed excluding the outliers. However, the authors did not mention whether they used Box-Cox or some other method to determine suitable transformation to stabilize the variance. The authors stated that if the omission of outliers has no effect on the conclusions, the results of the full dataset was reported. The authors further mentioned that normality and homogeneity of variance assumptions were satisfied for the statistical analysis of female subjects and for the statistical analysis of the percent change in RBC ChE for the male subjects. However, the authors expressed some doubt over the validity of the assumptions for the statistical analysis of the percent change of ChE in plasma for the male subjects and mentioned that the plot of the residuals showed that this was possibly due to number of outliers. Note residual diagnostics plots were not included in the report.

Reviewer's comment: Although the authors mentioned that SAS software used for the statistical analysis, they did not specify which specific SAS procedure was used for this analysis to account for correlation due to repeated measurements. PROC MIXED or GLIMMIX can be used for this dataset because both procedures provide flexible and rich selection of variance-covariance matrix (i.e., autoregressive, compound symmetric, unstructured, etc.) to model error correlation in longitudinal data. Further, the authors also did not specify which variance-covariance matrix was used to model correlations between different time points, how this variance-covariance matrix was selected, or how it was determined to be best or most appropriate selection among competing options. A common covariance structure which is frequently observed in repeated measures data is an autoregressive structure, which recognizes that observations which are closer are more correlated than measures that are more distant, may be more suitable for modeling covariance structure of the ChE data. Instead of using percent change of ChE from base line as dependent variable, another potential alternative model would be using ChE as dependent variable and baseline as covariate. The authors stated that there is some doubt over the validity of the assumptions (i.e., normality and homogeneity of variance) for the statistical analysis of the percent change from the baseline in plasma ChE for the male subjects and the plots of residuals showed that this was possibly due to number of outliers. The authors did not indicate what particular transformation was used in case of the departure or deviation from those assumptions (normality and homogeneity of variance). The authors discussed detection and (possibly) dealing with outliers, saying:

As there were similar numbers of outliers in each direction and these were of similar magnitude, they should offset each other, and the results should not be altered significantly; it was therefore considered appropriate not to perform any statistical analysis on the reduced dataset and to report only the results of the full dataset. (MRID 45125602, page 53).

"Outliers" were defined as being outside the standard whisker lengths for box plots, but no mention was made if these were transformed and this represents a poor justification for determining if a given value is an outlier, particularly given that distributions are not necessarily normal. Although no "outliers" were ultimately removed from the dataset, using as justification that "the high ones offset the low ones" represents poor statistical practice. The ideal method for determining outlier in regression setting is to use standardized or studentized residual instead of using interquartile range the authors used. The authors applied correction for multiple comparison based on the result of linear trend test. Correction for multiple testing should not be dependent on the result of linear trend test. It should be also noted that Bonferroni method is more conservative than other correction methods for multiple testing such as Sidak, Tukey or Dunnett's test.

CONCLUSION:

The registrant provided inadequate information on the statistical analysis that was used to estimate the NOAEL to as to allow the Agency to judge the adequacy of the determination of a NOAEL of 15 mg/kg. Specifically, the registrant did not provide any information regarding:

- ❑ The statistical SAS code or PROC procedure that was used

- ❑ What assumptions were made regarding the structure of the variance-covariance matrix (e.g., compound symmetry, autoregressive, unstructured, etc.) to model correlations between different timepoints, or how any final determination of what

which variance-covariance matrix was most appropriate. We note that – given the time points at which measurements were made -- a number of these variance-covariance structures would be considered inappropriate *a priori*.

- What kinds of tests were performed to judge the adequacy of the final model of percent change of ChE in plasma for the male subjects? The authors express doubt about the validity of the assumptions for statistical analysis of plasma ChE in male. They discuss such things as if there were "violations or departure from normality" and issues with any "homogeneity of variance assumption" and discuss the need for "transformations in case of the departure or deviation from those assumptions", but no further information on what was -or was not – done was provided. And they discuss "discarding outliers" if such transformations failed to resolve the statistical issues – but don't discuss if those issues were resolved, nor do they adequately indicate how it was determined or what criteria was used if a measurement was such an outlier other than saying that they were located beyond the standard "mean + 1.5 x IQR" whisker boundaries and that -as so defined – there were 5 high outliers that were "offset" by 4 low outliers.

- The authors discussed applying a linear trend test for determining if -- on a given day – there was a dose response relationship between percent cholinesterase inhibition and dose, and applying -- or not -- a Bonferroni multiple comparison correction depending on the statistical significance of the dose-response relationship, but it is unclear what connection there is between the need (or not) to apply a multiple correction factor and the linear trend test. We also note that a Bonferroni correction is more conservative than other adjustment methods such as Sidak, Tukey or Dunnett's test and thus more likely to determine a relationship is not significant when compared to other available tests.

Given the above, the statistical aspects of the study submission are judged inadequate.