

RECEIVED
OPPT CBIC

96 OCT 28 PM 2:57

8EHQ-96-13785

889700000 35s

8EHQ-1096-13785s

October 23, 1996

Document Control Office (7404)
Office of Toxic Substances
U.S. Environmental Protection Agency
401 M Street, S.W.
Washington, D.C. 20460

COMPANY SANITIZED

Attn: TSCA Section 8(e) Coordinator

[

] SANITIZED

RE: TSCA Section 8(e) Notification of Tetrahydrofurfuryl Benzoate

[]

Gentlemen:

[] is submitting a TSCA Section 8(e) substantial risk notification concerning a bacterial reverse mutation assay with an independent repeat assay (Ames Assay) with tetrahydrofurfuryl benzoate. The CAS Registry Number for the chemical is 2217-32-5. The assay was performed at Microbiological Associates, Inc., Rockville, Maryland, under their Study No. []. The information summarized below was received on October 8, 1996 via the final report (copy enclosed).

Initial assay and an independent repeat assay were performed using the plate incorporation method and tester strains TA98, TA100, TA1535, and TA1537 and E. Coli (WP2uvrA). Assays were done both in the presence and absence of Aroclor-induced rat liver S9. Dimethylsulfoxide was selected as the solvent of choice.

In the initial mutagenicity assay, positive responses were observed with tester strains TA1537 and WP2uvrA in the absence of S9 activation and with tester strain WP2uvrA in the presence of S9 activation. In the independent repeat assay, positive responses were observed with tester strains TA98, TA1537, and WP2uvrA in both the presence and absence of S9 activation.

RECEIVED
GEN TOXIC
OCT 23 1996
AM 11:35

If you have any questions, please contact me at [] .

Sincerely,

[]

[]
Enclosure

STATEMENT OF COMPLIANCE

Study No. [] was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article have not been determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

The stability of the test or control article under the test conditions has not been determined by the testing facility.

Valentine O. Wagner, III
Valentine O. Wagner, III, M.S.
Study Director

10/3/96
Date

QUALITY ASSURANCE STATEMENT

Study Title: BACTERIAL REVERSE MUTATION ASSAY WITH AN INDEPENDENT REPEAT ASSAY

Study Number: []

Study Director: Valentine O. Wagner, III, M.S.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 25 JUL 96, TO STUDY DIR 25 JUL 96, TO MGMT 25 JUL 96
PHASE: Protocol Review

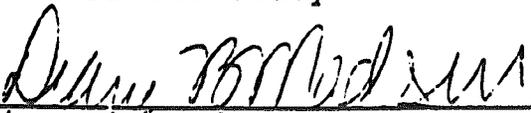
INSPECT ON 30 JUL 96, TO STUDY DIR 30 JUL 96, TO MGMT 02 AUG 96
PHASE: Dilution of test and/or control material

INSPECT ON 06 AUG 96, TO STUDY DIR 08 AUG 96, TO MGMT 08 AUG 96
PHASE: Strain characterization

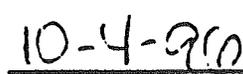
INSPECT ON 08 AUG 96, TO STUDY DIR 08 AUG 96, TO MGMT 09 AUG 96
PHASE: Strain characterization - Plate Evaluation

INSPECT ON 30 SEP 96, TO STUDY DIR 30 SEP 96, TO MGMT 04 OCT 96
PHASE: Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.



Diane B. Madsen
QUALITY ASSURANCE



DATE

**Bacterial Reverse Mutation Assay with
an Independent Repeat Assay**

FINAL REPORT

Sponsor: []

Authorized Representative: []

Performing Laboratory: **Microbiological Associates, Inc. (MA)
9630 Medical Center Drive
Rockville, Maryland 20850**

Test Article I.D.: **Tetrahydrofurfuryl benzoate**

Test Article Lot No.: **PP37-6E29-2945-111**

MA Study No.: []

Test Article Description: **clear, colorless liquid**

Storage Conditions: **room temperature; protected from exposure to
light**

Test Article Receipt: **07/19/96**

Study Initiation: **07/25/96**

Study Director: Valentine O. Wagner III 10/3/96
Valentine O. Wagner, III, M.S. Date

TABLE OF CONTENTS

	Page
Summary	6
Purpose	7
Characterization of Test and Control Articles	7
Materials and Methods	8
Results and Discussion	12
Conclusion	13
References	13
Data Tables	14
Appendix I: Historical Control Data	44
Appendix II: Study Protocol	46
Appendix III: Information for Japanese Regulatory Agencies	57

SUMMARY

The test article, Tetrahydrofurfuryl benzoate, was tested in the bacterial reverse mutation assay using *S. typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *E. coli* tester strain WP2 *uvrA* in the presence and absence of Aroclor-induced rat liver S9. The assay was performed in two phases, using the plate incorporation method. The first phase, the preliminary toxicity assay, was used to establish the dose range for the mutagenicity assay. The second phase, the mutagenicity assay (initial and independent repeat assays), was used to evaluate the mutagenic potential of the test article.

Dimethylsulfoxide was selected as the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in dimethylsulfoxide at approximately 500 mg/ml, the maximum concentration tested.

In the preliminary toxicity assay, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 100 mg/ml and a 50 µl plating aliquot. Neither precipitate nor appreciable toxicity was observed. Based on the findings of the toxicity assay, the maximum dose plated in the mutagenicity assay was 5000 µg per plate.

In the initial mutagenicity assay, positive responses were observed with tester strains TA1537 and WP2 *uvrA* in the absence of S9 activation and with tester strain WP2 *uvrA* in the presence of S9 activation. In the independent repeat assay, positive responses were observed with tester strains TA98, TA1537 and WP2 *uvrA* in the presence and absence of S9 activation. Neither precipitate nor appreciable toxicity was observed. The overall evaluation and dose ranges tested are as follows:

S9 Activation	Overall Evaluation ^a and Dose Range Tested (µg/plate)									
	TA98		TA100		TA1535		TA1537		WP2 <i>uvrA</i>	
	Low	High	Low	High	Low	High	Low	High	Low	High
None	-; +(5.7)		-		-		+(5.3;35.3)		+(16.8;10.2)	
	100	5000	100	5000	100	5000	100	5000	100	5000
Rat	-; +(2.7;2.3)		-		-		-; +(4.6)		+(3.6;10.2)	
	100	5000	100	5000	100	5000	100	5000	100	5000

^a. - = negative, + = positive (maximum fold increase)

Under the conditions of this study, test article Tetrahydrofurfuryl benzoate was concluded to be positive in the Bacterial Reverse Mutation Assay with an Independent Repeat Assay.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test article (or its metabolites) by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and one strain of *E. coli* in the presence and absence of S9 activation.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, Tetrahydrofurfuryl benzoate, was received by Microbiological Associates, Inc. on 07/19/96 and was assigned the code number 96BF85. The test article was characterized by the Sponsor as a colorless mobile liquid that should be stored in a cool, dry, well ventilated location. An expiration date was not provided. Upon receipt, the test article was described as a clear, colorless liquid and was stored at room temperature, protected from exposure to light.

The vehicle used to deliver Tetrahydrofurfuryl benzoate to the test system was dimethylsulfoxide (DMSO), (CAS# 67-68-5), obtained from Fisher Scientific.

Positive controls plated concurrently with the mutagenicity assay are listed below:

Strain	S9 Activation	Positive Control	Concentration ($\mu\text{g}/\text{plate}$)
All <i>Salmonella</i> Strains	+	2-aminoanthracene (Sigma Chemical Co.)	1.0
WP2 <i>uvrA</i>			10
TA98	-	2-nitrofluorene (Aldrich Chemical Co., Inc.)	1.0
TA100, TA1535		sodium azide (Sigma Chemical Co.)	1.0
TA1537		9-aminoacridine (Sigma Chemical Co.)	75
WP2 <i>uvrA</i>		methyl methanesulfonate (Aldrich Chemical Co., Inc.)	1,000

To determine the sterility of the test article, the highest test article dose level used in the mutagenicity assay was plated on selective agar with an aliquot volume equal to that used in the assay.

Solubility Test

A solubility test was conducted to select the vehicle. The test was conducted using one or more of the following solvents in the order of preference as listed: purified water, dimethylsulfoxide, ethanol and acetone. The test article was tested to determine the vehicle, selected in order of preference, that permitted preparation of the highest soluble or workable stock concentration, up to 500 mg/ml.

Preliminary Toxicity Assay

The preliminary toxicity assay was used to establish the dose-range over which the test article would be assayed. Ten dose levels of the test article were plated, one plate per dose, with overnight cultures of TA98, TA100, TA1535, TA1537 and WP2 *uvrA* on selective minimal agar in both the presence and absence of rat liver S9 activation.

Mutagenicity Assay

The mutagenicity assay (initial and independent repeat assays) was used to evaluate the mutagenic potential of the test article. A minimum of five dose levels of test article along with appropriate vehicle and positive controls were plated with tester strains TA98, TA100, TA1535, TA1537 and WP2 *uvrA* in the presence and absence of rat liver S9 activation. All dose levels of test article, vehicle controls and positive controls were plated in triplicate.

Plating and Scoring Procedures

The test system was exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

On the day of its use, minimal top agar, containing 0.8 % agar (w/v) and 0.5 % NaCl (w/v), was melted and supplemented with L-histidine, D-biotin and L-tryptophan solution to a final concentration of 50 μ M each. Top agar not used with S9 or Sham mix was supplemented with 25 ml of water for each 100 ml of minimal top agar. For the preparation of media and reagents, all references to water imply sterile, deionized water produced by the Milli-Q Reagent Water System. Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) containing 1.5 % (w/v) agar. Nutrient bottom agar was Vogel-Bonner minimal medium E containing 1.5 % (w/v) agar and supplemented with 2.5 % (w/v) Oxoid Nutrient Broth No. 2 (dry powder). Nutrient Broth was Vogel-Bonner salt solution supplemented with 2.5 % (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Each plate was labeled with a code system that identified the test article, test phase, dose level, tester strain, and activation, as described in detail in Microbiological Associates, Inc.'s Standard Operating Procedures.

Test article dilutions were prepared immediately before use. One-half (0.5) milliliter of S9 or Sham mix, 100 μ l of tester strain and 50 μ l of vehicle or test article were added to 2.0 ml of molten selective top agar at $45 \pm 2^\circ\text{C}$. After vortexing, the mixture was overlaid onto the surface of 25 ml of minimal bottom agar. When plating the positive controls, the test article aliquot was replaced by a 50 μ l aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at $37 \pm 2^\circ\text{C}$. Plates that were not counted immediately following the incubation period were stored at $4 \pm 2^\circ\text{C}$ until colony counting could be conducted.

The condition of the bacterial background lawn was evaluated for evidence of test article toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown below.

Code	Description	Characteristics
1	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Severely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over $\geq 90\%$ of the plate.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test article precipitate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than 10% of the revertant colony count (e.g., ≤ 3 particles on a plate with 30 revertants.)
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., > 3 particles on a plate with 30 revertants.)

Revertant colonies for a given tester strain and activation condition were counted either entirely by automated colony counter or entirely by hand unless the assay was the preliminary toxicity assay or the plate exhibited toxicity. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

Salmonella Mutagenicity Assay

Preliminary Toxicity Assay

Table 1

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : []
 Experiment No. : A1
 Date Plated : 07/30/96
 Counted by : hand
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl

Test Article Concentration µg per plate	TA98			
	With S9 Activation		Without Activation	
	Revertants per plate	Background Code ^a	Revertants per plate	Background Code ^a
Vehicle	14	1	19	1
6.7	29	1	24	1
10	23	1	20	1
33	18	1	16	1
67	15	1	11	1
100	24	1	20	1
333	13	1	11	1
667	22	1	28	1
1000	22	1	28	1
3333	18	1	40	1
5000	60	1	21	1

^aBackground bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
 4=Extremely reduced 5=Absent 6=Obscured by precipitate
 NP=Non-Interfering Precipitate IF=Interfering Precipitate

Salmonella Mutagenicity Assay

Preliminary Toxicity Assay

Table 2

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : []
 Experiment No. : A1
 Date Plated : 07/30/96
 Counted by : machine
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl

Test Article Concentration µg per plate	TA100			
	With S9 Activation		Without Activation	
	Revertants per plate	Background Code ^a	Revertants per plate	Background Code ^a
Vehicle	147	1	120	1
6.7	133	1	100	1
10	141	1	134	1
33	126	1	135	1
67	120	1	150	1
100	130	1	140	1
333	116	1	157	1
667	140	1	152	1
1000	123	1	168	1
3333	154	1	256	1
5000	175	1	238	1

^aBackground bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
 4=Extremely reduced 5=Absent 6=Obscured by precipitate
 NF=Non-Interfering Precipitate IP=Interfering Precipitate

Salmonella Mutagenicity Assay

Preliminary Toxicity Assay

Table 3

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : []
 Experiment No. : A1
 Date Plated : 07/30/96
 Counted by : hand
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl

Test Article Concentration µg per plate	TA1535			
	With S9 Activation		Without Activation	
	Revertants per plate	Background Code ^a	Revertants per plate	Background Code ^a
Vehicle	11	1	10	1
6.7	8	1	12	1
10	15	1	12	1
33	9	1	14	1
67	13	1	15	1
100	12	1	8	1
333	11	1	6	1
667	9	1	8	1
1000	9	1	8	1
3333	8	1	2	1
5000	6	1	6	1

^aBackground bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
 4=Extremely reduced 5=Absent 6=Obscured by precipitate
 NP=Non-Interfering Precipitate IF=Interfering Precipitate

Salmonella Mutagenicity Assay

Preliminary Toxicity Assay

Table 4

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : []
 Experiment No. : A1
 Date Plated : 07/30/96
 Counted by : hand
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl

Test Article Concentration µg per plate	TA1537			
	With S9 Activation		Without Activation	
	Revertants per plate	Background Code ^a	Revertants per plate	Background Code ^a
Vehicle	7	1	10	1
6.7	11	1	7	1
10	11	1	4	1
33	9	1	4	1
67	4	1	5	1
100	4	1	5	1
333	3	1	9	1
667	5	1	12	1
1000	10	1	11	1
3333	20	1	57	2
5000	47	1	28	2

^aBackground bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
 4=Extremely reduced 5=Absent 6=Obscured by precipitate
 NP=Non-Interfering Precipitate IP=Interfering Precipitate

E. coli Mutagenicity Assay

Preliminary Toxicity Assay

Table 5

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : []
 Experiment No. : A1
 Date Plated : 07/30/96
 Counted by : hand
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl

Test Article Concentration µg per plate	WP2 uvrA			
	With S9 Activation		Without Activation	
	Revertants per plate	Background Code ^a	Revertants per plate	Background Code ^a
Vehicle	20	1	13	1
6.7	12	1	9	1
10	14	1	15	1
33	12	1	17	1
67	22	1	11	1
100	12	1	7	1
333	9	1	9	1
667	20	1	11	1
1000	22	1	24	1
3333	34	1	30	1
5000	22	1	22	1

^aBackground bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
 4=Extremely reduced 5=Absent 6=Obscured by precipitate
 NP=Non-Interfering Precipitate IP=Interfering Precipitate

Salmonella Mutagenicity Assay

Table 6

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B1
 Strain : TA98 Cells Seeded : 5.2 X 10⁸
 Liver Microsomes : None Date Plated : 08/06/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	18	1		
	02	23	1		
	03	28	1	23	5
100	01	14	1		
	02	16	1		
	03	13	1	14	2
333	01	14	1		
	02	19	1		
	03	23	1	19	5
1000	01	35	1		
	02	34	1		
	03	27	1	32	4
2000	01	32	1		
	02	39	1		
	03	50	1	40	9
3333	01	30	2		
	02	33	2		
	03	39	2	34	5
4000	01	41	2		
	02	23	2		
	03	25	2	30	10
5000	01	32	1		
	02	34	1		
	03	34	1	33	1
Positive Control 2-nitrofluorene 1.0 µg per plate ^b					
	01	129	1		
	02	94	1		
	03	93	1	105	21

^aBackground bacterial evaluation code

1=Normal

2=Slightly reduced

3=Moderately reduced

4=Extremely reduced

5=Absent

6=Obscured by precipitate

NF=Non-Interfering Precipitate

IF=Interfering Precipitate

^bPositive control plates were machine counted

Salmonella Mutagenicity Assay

Table 7

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B1
 Strain : TA98 Cells Seeded : 5.2 X 10⁸
 Liver Microsomes : Rat liver S9 Date Plated : 08/06/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	13	1	33	17
	02	44	1		
	03	42	1		
100	01	25	1	24	8
	02	31	1		
	03	16	1		
333	01	42	1	35	6
	02	33	1		
	03	30	1		
1000	01	46	1	45	2
	02	43	1		
	03	46	1		
2000	01	14	1	25	10
	02	31	1		
	03	30	1		
3333	01	32	1	28	9
	02	35	1		
	03	18	1		
4000	01	45	1	38	7
	02	32	1		
	03	37	1		
5000	01	40	1	36	12
	02	45	1		
	03	23	1		
Positive Control 2-aminoanthracene 1.0 µg per plate ^b					
	01	365	1	458	151
	02	377	1		
	03	632	1		

^aBackground bacterial evaluation code

1=Normal

2=Slightly reduced

3=Moderately reduced

4=Extremely reduced

5=Absent

6=Obscured by precipitate

MF=Non-Interfering Precipitate

IF=Interfering Precipitate

^bPositive control plates were machine counted

Salmonella Mutagenicity Assay

Table 8

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B1
 Strain : TA100 Cells Seeded : 10.0 X 10⁸
 Liver Microsomes : None Date Plated : 08/06/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	132	1	141	14
	02	134	1		
	03	157	1		
100	01	134	1	136	5
	02	141	1		
	03	132	1		
333	01	77	1	122	39
	02	145	1		
	03	144	1		
1000	01	206	1	182	45
	02	210	1		
	03	130	1		
2000	01	195	1	189	11
	02	196	1		
	03	176	1		
3333	01	286	1	264	30
	02	276	1		
	03	230	1		
4000	01	245	1	257	17
	02	249	1		
	03	276	1		
5000	01	265	1	275	9
	02	282	1		
	03	279	1		
Positive Control sodium azide 1.0 µg per plate					
	01	446	1	457	47
	02	416	1		
	03	509	1		

^aBackground bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
 4=Extremely reduced 5=Absent 6=Obscured by precipitate
 NP=Non-Interfering Precipitate IP=Interfering Precipitate

Salmonella Mutagenicity Assay

Table 9

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B1
 Strain : TA100 Cells Seeded : 10.0 X 10⁸
 Liver Microsomes : Rat liver S9 Date Plated : 08/06/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	154	1	144	10
	02	145	1		
	03	134	1		
100	01	119	1	126	9
	02	124	1		
	03	136	1		
333	01	129	1	133	9
	02	144	1		
	03	127	1		
1000	01	137	1	141	15
	02	128	1		
	03	157	1		
3333	01	148	1	141	12
	02	128	1		
	03	148	1		
5000	01	177	1	176	8
	02	183	1 ^h		
	03	168	1		
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	608	1	573	64
	02	611	1		
	03	499	1		

^aBackground bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
 4=Extremely reduced 5=Absent 6=Obscured by precipitate
 NP=Non-Interfering Precipitate IP=Interfering Precipitate

Salmonella Mutagenicity Assay

Table 10

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B1
 Strain : TA1535 Cells Seeded : 13.6 X 10⁸
 Liver Microsomes : None Date Plated : 08/06/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	22	1	19	2
	02	18	1		
	03	18	1		
100	01	12	1	18	5
	02	21	1		
	03	21	1		
333	01	17	1	17	6
	02	11	1		
	03	22	1		
1000	01	18	1	19	1
	02	18	1		
	03	20	1		
3333	01	14	1	15	4
	02	12	1		
	03	19	1		
5000	01	13	1	10	3
	02	7	1		
	03	11	1		
Positive Control sodium azide 1.0 µg per plate ^b					
	01	518	1	468	53
	02	475	1		
	03	412	1		

^aBackground bacterial evaluation code

1=Normal 2=Slightly reduced

4=Extremely reduced 5=Absent

NP=Non-Interfering Precipitate

3=Moderately reduced

6=Obscured by precipitate

IP=Interfering Precipitate

^bPositive control plates were machine counted

Salmonella Mutagenicity Assay

Table 11

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B1
 Strain : TA1537 Cells Seeded : 16.5 X 10⁸
 Liver Microsomes : None Date Plated : 08/06/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	3	1		
	02	12	1		
	03	6	1	7	5
100	01	6	1		
	02	8	1		
	03	3	1	6	3
333	01	8	1		
	02	17	1		
	03	5	1	10	6
1000	01	14	1		
	02	19	1		
	03	75	1	36	34
2000	01	53	1		
	02	50	1		
	03	7	1	37	26
3333	01	14	2		
	02	3	2		
	03	2	2	6	7
4000	01	13	2		
	02	18	2		
	03	19	2	17	3
5000	01	17	2		
	02	28	2		
	03	22	2	22	6
Positive Control 9-aminoacridine 7 ^c µg per plate ^b					
	01	674	1		
	02	617	1		
	03	518	1	603	79

^aBackground bacterial evaluation code

1=Normal

2=Slightly reduced

3=Moderately reduced

4=Extremely reduced

5=Absent

6=Obscured by precipitate

NP=Non-Interfering Precipitate

IP=Interfering Precipitate

^bPositive control plates were machine counted

Salmonella Mutagenicity Assay

Table 12

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B1
 Strain : TA1537 Calls Seeded : 16.5 X 10⁸
 Liver Microsomes : Rat liver S9 Date Plated : 08/06/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	8	1		
	02	4	1		
	03	8	1	7	2
100	01	6	1		
	02	8	1		
	03	6	1	7	1
333	01	8	1		
	02	6	1		
	03	6	1	7	1
1000	01	9	1		
	02	9	1		
	03	14	1	11	3
2000	01	14	1		
	02	11	1		
	03	8	1	11	3
3333	01	40	1		
	02	32	1		
	03	25	1	32	8
4000	01	25	1		
	02	27	1		
	03	35	1	29	5
5000	01	26	1		
	02	30	1		
	03	23	1	26	4
Positive Control 2-aminoanthracene 1.0 µg per plate ^b					
	01	44	1		
	02	50	1		
	03	52	1	49	4

^aBackground bacterial evaluation code

1=Normal

2=Slightly reduced

3=Moderately reduced

4=Extremely reduced

5=Absent

6=Obscured by precipitate

NP=Non-Interfering Precipitate

IP=Interfering Precipitate

^bPositive control plates were machine counted

E. coli Mutagenicity Assay

Table 13

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B1
 Strain : WP2 uvrA Cells Seeded : 23.3 X 10⁸
 Liver Microsomes : None Date Plated : 08/06/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	16	1		
	02	10	1		
	03	14	1	13	3
100	01	21	1		
	02	12	1		
	03	14	1	16	5
333	01	21	1		
	02	14	1		
	03	16	1	17	4
1000	01	42	1		
	02	36	1		
	03	35	1	38	4
2000	01	91	1		
	02	81	1		
	03	93	1	88	6
3333	01	143	1		
	02	152	1		
	03	119	1	138	17
4000	01	160	1		
	02	133	1		
	03	189	1	161	28
5000	01	291	1		
	02	178	1		
	03	185	1	218	63
Positive Control methyl methanesulfonate 1000 µg per plate ^b					
	01	189	1		
	02	209	1		
	03	192	1	197	11

^aBackground bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
 4=Extremely reduced 5=Absent 6=Obscured by precipitate
 NP=Non-Interfering Precipitate IP=Interfering Precipitate

^bPositive control plates were machine counted

E. coli Mutagenicity Assay

Table 14

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B1
 Strain : WP2 uvrA Cells Seeded : 23.3 X 10⁸
 Liver Microsomes : Rat liver S9 Date Plated : 08/06/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	30	1	21	8
	02	17	1		
	03	15	1		
100	01	29	1	26	3
	02	27	1		
	03	23	1		
333	01	20	1	23	3
	02	26	1		
	03	23	1		
1000	01	18	1	19	2
	02	19	1		
	03	21	1		
3333	01	62	1	60	5
	02	54	1		
	03	63	1		
5000	01	61	1	75	20
	02	98	1		
	03	67	1		
Positive Control 2-aminoanthracene 10 µg per plate ^b					
	01	66	1	64	5
	02	68	1		
	03	59	1		

^aBackground bacterial evaluation code

1=Normal

2=Slightly reduced

3=Moderately reduced

4=Extremely reduced

5=Absent

6=Obscured by precipitate

NP=Non-Interfering Precipitate

IP=Interfering Precipitate

^bPositive control plates were machine counted

Salmonella Mutagenicity Assay

Table 15

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B2
 Strain : TA1535 Cells Seeded : 4.2 X 10⁸
 Liver Microsomes : Rat liver S9 Date Plated : 08/16/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	10	1	10	2
	02	8	1		
	03	12	1		
100	01	13	1	12	3
	02	9	1		
	03	14	1		
333	01	13	1	13	3
	02	10	1		
	03	16	1		
1000	01	8	1	9	2
	02	8	1		
	03	11	1		
3333	01	18	1	18	1
	02	19	1		
	03	17	1		
5000	01	12	1	12	1
	02	12	1		
	03	13	1		
Positive Control 2-aminoanthracene 1.0 µg per plate ^b					
	01	57	1	46	10
	02	44	1		
	03	38	1		

^aBackground bacterial evaluation code

1=Normal

2=Slightly reduced

3=Moderately reduced

4=Extremely reduced

5=Absent

6=Obscured by precipitate

NP=Non-Interfering Precipitate

IF=Interfering Precipitate

^bPositive control plates were machine counted

Salmonella Mutagenicity Assay

Table 16

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B3
 Strain : TA98 Cells Seeded : 1.4 X 10⁸
 Liver Microsomes : Rat liver S9 Date Plated : 08/16/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	14	1	15	3
	02	13	1		
	03	18	1		
100	01	25	1	23	3
	02	25	1		
	03	20	1		
333	01	25	1	23	6
	02	16	1		
	03	28	1		
1000	01	25	1	20	6
	02	23	1		
	03	13	1		
2000	01	35	1	32	3
	02	30	1		
	03	30	1		
3333	01	46	1	41	4
	02	39	1		
	03	38	1		
4000	01	40	1	36	4
	02	32	1		
	03	35	1		
5000	01	47	1	37	10
	02	27	1		
	03	36	1		
Positive Control 2-aminoanthracene 1.0 µg per plate ^b					
	01	78	1	74	5
	02	69	1		
	03	74	1		

^aBackground bacterial evaluation code

1=Normal

2=Slightly reduced

3=Moderately reduced

4=Extremely reduced

5=Absent

6=Obscured by precipitate

NP=Non-Interfering Precipitate

IF=Interfering Precipitate

^bPositive control plates were machine counted

Salmonella Mutagenicity Assay

Table 17

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B3
 Strain : TA1535 Cells Seeded : 3.2 X 10⁸
 Liver Microsomes : Rat liver S9 Date Plated : 08/16/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	16	1	14	2
	02	13	1		
	03	13	1		
100	01	5	1	10	5
	02	14	1		
	03	10	1		
333	01	13	1	15	5
	02	11	1		
	03	21	1		
1000	01	9	1	10	2
	02	12	1		
	03	9	1		
3333	01	10	1	11	3
	02	9	1		
	03	14	1		
5000	01	11	1	12	2
	02	12	1		
	03	14	1		
Positive Control 2-aminoanthracene 1.0 µg per plate ^b					
	01	247	1	211	33
	02	184	1		
	03	201	1		

^aBackground bacterial evaluation code
 1=Normal 2=Slightly reduced 3=Moderately reduced
 4=Extremely reduced 5=Absent 6=Obscured by precipitate
 NP=Non-Interfering Precipitate IF=Interfering Precipitate

^bPositive control plates were machine counted

Salmonella Mutagenicity Assay

Table 18

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B3
 Strain : TA1537 Cells Seeded : 0.7 X 10⁸
 Liver Microsomes : Rat liver S9 Date Plated : 08/16/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	4	1		
	02	8	1		
	03	8	1	7	2
100	01	2	1		
	02	5	1		
	03	6	1	4	2
333	01	7	1		
	02	7	1		
	03	4	1	6	2
1000	01	14	1		
	02	11	1		
	03	5	1	10	5
2000	01	15	1		
	02	13	1		
	03	14	1	14	1
3333	01	21	1		
	02	35	1		
	03	39	1	32	9
4000	01	26	1		
	02	32	1		
	03	36	1	31	5
5000	01	34	1		
	02	20	1		
	03	29	1	28	7
Positive Control 2-aminoanthracene 1.0 µg per plate ^b					
	01	826	1		
	02	503	1		
	03	309	1	546	261

^aBackground bacterial evaluation code

1=Normal

2=Slightly reduced

3=Moderately reduced

4=Extremely reduced

5=Absent

6=Obscured by precipitate

NP=Non-Interfering Precipitate

IP=Interfering Precipitate

^bPositive control plates were machine counted

E. coli Mutagenicity Assay

Table 19

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B3
 Strain : WP2 uvrA Cells Seeded : 2.5 X 10⁸
 Liver Microsomes : Rat liver S9 Date Plated : 08/16/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	12	1		
	02	14	1		
	03	14	1	13	1
100	01	16	1		
	02	12	1		
	03	12	1	13	2
333	01	15	1		
	02	16	1		
	03	20	1	17	3
1000	01	20	1		
	02	16	1		
	03	14	1	17	3
3333	01	39	1		
	02	55	1		
	03	48	1	47	8
5000	01	46	1		
	02	37	1		
	03	39	1	41	5
Positive Control 2-aminoanthracene 10 µg per plate ^b					
	01	126	1		
	02	167	1		
	03	146	1	146	21

^aBackground bacterial evaluation code

1=Normal

2=Slightly reduced

3=Moderately reduced

4=Extremely reduced

5=Absent

6=Obscured by precipitate

NP=Non-Interfering Precipitate

IP=Interfering Precipitate

^bPositive control plates were machine counted

Salmonella Mutagenicity Assay

Table 20

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B4
 Strain : TA98 Cells Seeded : 5.4 X 10⁸
 Liver Microsomes : None Date Plated : 08/30/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	8	1		
	02	16	1		
	03	13	1	12	4
100	01	14	1		
	02	15	1		
	03	19	1	16	3
333	01	20	1		
	02	19	1		
	03	10	1	16	6
1000	01	36	1		
	02	21	1		
	03	27	1	28	8
2000	01	71	1		
	02	78	1		
	03	54	1	68	12
3333	01	57	1		
	02	42	1		
	03	45	1	48	8
4000	01	49	1		
	02	52	1		
	03	52	1	51	2
5000	01	41	1		
	02	34	1		
	03	40	1	38	4
Positive Control 2-nitrofluorene 1.0 µg per plate ^b					
	01	145	1		
	02	111	1		
	03	132	1	129	17

^aBackground bacterial evaluation code

1-Normal

2-Slightly reduced

3-Moderately reduced

4-Extremely reduced

5-Absent

6-Obscured by precipitate

MF-Non-Interfering Precipitate

IF-Interfering Precipitate

^bPositive control plates were machine counted

Salmonella Mutagenicity Assay

Table 21

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B4
 Strain : TA98 Cells Seeded : 5.4 X 10⁸
 Liver Microsomes : Rat liver S9 Date Plated : 08/30/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	18	1	22	6
	02	20	1		
	03	29	1		
100	01	19	1	20	4
	02	17	1		
	03	25	1		
333	01	31	1	25	6
	02	20	1		
	03	24	1		
1000	01	16	1	30	12
	02	36	1		
	03	38	1		
2000	01	41	1	37	4
	02	36	1		
	03	34	1		
3333	01	34	1	50	16
	02	51	1		
	03	65	1		
4000	01	37	1	46	13
	02	61	1		
	03	41	1		
5000	01	44	1	43	5
	02	38	1		
	03	48	1		
Positive Control 2-aminoanthracene 1.0 µg per plate ^b					
	01	539	1	547	12
	02	561	1		
	03	542	1		

^aBackground bacterial evaluation code

1=Normal

4=Extremely reduced

NP=Non-Interfering Precipitate

2=Slightly reduced

5=Absent

3=Moderately reduced

6=Obscured by precipitate

IP=Interfering Precipitate

^bPositive control plates were machine counted

Salmonella Mutagenicity Assay

Table 22

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : []
 Strain : TA100
 Liver Microsomes : None
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl
 Experiment No : B4
 Cells Seeded : 5.5 X 10⁸
 Date Plated : 08/30/96
 Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	125	1	133	16
	02	123	1		
	03	151	1		
100	01	128	1	131	9
	02	124	1		
	03	141	1		
333	01	136	1	126	23
	02	142	1		
	03	99	1		
1000	01	167	1	173	6
	02	178	1		
	03	174	1		
2000	01	212	1	232	30
	02	266	1		
	03	218	1		
3333	01	166	1	205	35
	02	214	1		
	03	235	1		
4000	01	144	2	160	14
	02	168	2		
	03	169	2		
5000	01	113	2	126	11
	02	131	2		
	03	133	2		
Positive Control sodium azide 1.0 µg per plate ^b					
	01	415	1	413	33
	02	445	1		
	03	380	1		

^aBackground bacterial evaluation code

1=Normal

2=Slightly reduced

3=Moderately reduced

4=Extremely reduced

5=Absent

6=Obscured by precipitate

NP=Non-Interfering Precipitate

IP=Interfering Precipitate

^bPlates were hand counted

Salmonella Mutagenicity Assay

Table 23

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B4
 Strain : TA100 Cells Seeded : 5.5 X 10⁸
 Liver Microsomes : Rat liver S9 Date Plated : 08/30/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : machine

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	149	1	149	1
	02	149	1		
	03	148	1		
100	01	158	1	152	11
	02	158	1		
	03	139	1		
333	01	166	1	159	12
	02	146	1		
	03	166	1		
1000	01	169	1	165	12
	02	151	1		
	03	174	1		
3333	01	197	1	200	17
	02	218	1		
	03	185	1		
5000	01	245	1	208	42
	02	163	1		
	03	216	1		
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	783	1	794	26
	02	824	1		
	03	775	1		

^aBackground bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced
 4=Extremely reduced 5=Absent 6=Obscured by precipitate
 NP=Non-Interfering Precipitate IP=Interfering Precipitate

Salmonella Mutagenicity Assay

Table 24

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number :
 Strain : TA1535
 Liver Microsomes : None
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl
 Experiment No : B4
 Cells Seeded : 12.9 X 10⁸
 Date Plated : 08/30/96
 Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	11	1		
	02	12	1		
	03	11	1	11	1
100	01	4	1		
	02	15	1		
	03	2	1	7	7
333	01	11	1		
	02	14	1		
	03	13	1	13	2
1000	01	17	1		
	02	14	1		
	03	13	1	15	2
3333	01	11	2		
	02	7	2		
	03	8	2	9	2
5000	01	8	2		
	02	7	2		
	03	3	2	6	3
Positive Control sodium azide 1.0 µg per plate ^b					
	01	300	1		
	02	271	1		
	03	345	1	305	37

^aBackground bacterial evaluation code

1=Normal

4=Extremely reduced

NP=Non-Interfering Precipitate

2=Slightly reduced

5=Absent

3=Moderately reduced

6=Obscured by precipitate

IP=Interfering Precipitate

^bPositive control plates were machine counted

Salmonella Mutagenicity Assay

Table 25

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B4
 Strain : TA1537 Cells Seeded : 2.2 X 10⁸
 Liver Microsomes : None Date Plated : 08/30/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	2	1		
	02	2	1		
	03	6	1	3	2
100	01	3	1		
	02	9	1		
	03	5	1	6	3
333	01	10	1		
	02	11	1		
	03	8	1	10	2
1000	01	23	1		
	02	29	1		
	03	19	1	24	5
2000	01	111	1		
	02	127	1		
	03	81	1	106	23
3333	01	21	2		
	02	46	2		
	03	32	2	33	13
4000	01	7	3		
	02	1	4		
	03	8	3	5	4
5000	01	3	4		
	02	1	4		
	03	1	4	2	1
Positive Control 9-aminoacridine 75 µg per plate ^b					
	01	969	1		
	02	436	1		
	03	916	1	774	294

^aBackground bacterial evaluation code

1=Normal

4=Extremely reduced

NP=Non-Interfering Precipitate

2=Slightly reduced

5=Absent

3=Moderately reduced

6=Obscured by precipitate

IP=Interfering Precipitate

^bPositive control plates were machine counted

E. coli Mutagenicity Assay

Table 26

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B4
 Strain : WP2 uvrA Cells Seeded : 4.8 X 10⁸
 Liver Microsomes : None Date Plated : 08/30/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	5	1		
	02	13	1		
	03	15	1	11	5
100	01	6	1		
	02	9	1		
	03	12	1	9	3
333	01	7	1		
	02	15	1		
	03	16	1	13	5
1000	01	22	1		
	02	44	1		
	03	50	1	39	15
2000	01	46	1		
	02	59	1		
	03	44	1	50	8
3333	01	46	1		
	02	54	1		
	03	52	1	51	4
4000	01	100	1		
	02	88	1		
	03	72	1	87	14
5000	01	123	1		
	02	104	1		
	03	108	1	112	10
Positive Control methyl methanesulfonate 1000 µg per plate ^b					
	01	160	1		
	02	113	1		
	03	151	1	141	25

^aBackground bacterial evaluation code

1=Normal

2=Slightly reduced

3=Moderately reduced

4=Extremely reduced

5=Absent

6=Obscured by precipitate

NP=Non-Interfering Precipitate

IP=Interfering Precipitate

^bPositive control plates were machine counted

E. coli Mutagenicity Assay

Table 27

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : [] Experiment No : B4
 Strain : WP2 uvrA Cells Seeded : 4.8 X 10⁸
 Liver Microsomes : Rat liver S9 Date Plated : 08/30/96
 Vehicle : dimethylsulfoxide (DMSO)
 Plating Aliquot : 50 µl Counted by : hand

Concentration µg per plate	Plate Number	Revertants per plate	Background Code ^a	Average Revertants	Standard Deviation
Vehicle	01	10	1	13	2
	02	14	1		
	03	14	1		
100	01	13	1	16	7
	02	24	1		
	03	10	1		
333	01	18	1	15	4
	02	10	1		
	03	17	1		
1000	01	31	1	26	5
	02	22	1		
	03	25	1		
2000	01	62	1	74	28
	02	106	1		
	03	53	1		
3333	01	74	1	84	10
	02	84	1		
	03	93	1		
4000	01	49	1	100	46
	02	139	1		
	03	113	1		
5000	01	218	1	132	74
	02	95	1		
	03	84	1		
Positive Control 2-aminoanthracene 10 µg per plate ^b					
	01	86	1	137	65
	02	210	1		
	03	116	1		

^aBackground bacterial evaluation code

1=Normal 2=Slightly reduced 3=Moderately reduced

4=Extremely reduced 5=Absent 6=Obscured by precipitate

NP=Non-Interfering Precipitate IP=Interfering Precipitate

^bPositive control plates were machine counted

**Salmonella/E. coli Mutagenicity Assay
Summary of Results**

Table 28

Test Article Id : Tetrahydrofurfuryl benzoate
 Study Number : Experiment No : B1/B2

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (µg)	TA98		TA100		TA1535		TA1537		WP2 uvrA	
0.0	23 ±	5	141 ±	14	19 ±	2	7 ±	5	13 ±	3
100	14 ±	2	136 ±	5	18 ±	5	6 ±	3	16 ±	5
333	19 ±	5	122 ±	39	17 ±	6	10 ±	6	17 ±	4
1000	32 ±	4	182 ±	45	19 ±	1	36 ±	34	38 ±	4
2000	40 ±	9	189 ±	11			37 ±	26	88 ±	6
3333	34 ±	5	264 ±	30	15 ±	4	6 ±	7	138 ±	17
4000	30 ±	10	257 ±	17			17 ±	3	61 ±	28
5000	33 ±	1	275 ±	9	10 ±	3	22 ±	6	218 ±	63
Pos	105 ±	21	457 ±	47	468 ±	53	603 ±	79	197 ±	11

Liver Microsomes: Rat liver S9

Dose (µg)	TA98		TA100		TA1535 ^a		TA1537		WP2 uvrA	
0.0	33 ±	17	144 ±	10	10 ±	2	7 ±	2	21 ±	8
100	24 ±	8	126 ±	9	12 ±	3	7 ±	1	26 ±	3
333	35 ±	6	133 ±	9	13 ±	3	7 ±	1	23 ±	3
1000	45 ±	2	141 ±	15	9 ±	2	11 ±	3	19 ±	2
2000	25 ±	10					11 ±	3		
3333	28 ±	9	141 ±	12	18 ±	1	32 ±	8	60 ±	5
4000	38 ±	7					29 ±	5		
5000	36 ±	12	176 ±	8	12 ±	1	26 ±	4	75 ±	20
Pos	458 ±	151	573 ±	64	46 ±	10	49 ±	4	64 ±	5

0.0 = Vehicle plating aliquot of 50 µl

Pos = Positive Control concentrations as specified in Materials and Methods section.

a = Data from Experiment B2

Salmonella/E. coli Mutagenicity Assay
Summary of Results

Table 29

Test Article Id : Tetrahydrofurfuryl benzoate
Study Number [] Experiment No : B3

Average Revertants Per Plate \pm Standard Deviation

Liver Microsomes: Rat liver S9

Dose (μ g)	TA98		TA1535		TA1537		WP2 uvrA	
0.0	15 \pm	3	14 \pm	2	7 \pm	2	13 \pm	1
100	23 \pm	3	10 \pm	5	4 \pm	2	13 \pm	2
333	23 \pm	6	15 \pm	5	6 \pm	2	17 \pm	3
1000	20 \pm	6	10 \pm	2	10 \pm	5	17 \pm	3
2000	32 \pm	3			14 \pm	1		
3333	41 \pm	4	11 \pm	3	32 \pm	9	47 \pm	8
4000	36 \pm	4			31 \pm	5		
5000	37 \pm	10	12 \pm	2	28 \pm	7	41 \pm	5
Pos	74 \pm	5	211 \pm	33	546 \pm	261	146 \pm	21

0.0 = Vehicle plating aliquot of 50 μ l

Pos = Positive Control concentrations as specified in Materials and Methods section.

Salmonella/E. coli Mutagenicity Assay
Summary of Results

Table 30

Test Article Id : Tetrahydrofurfuryl benzoate
Study Number : [] Experiment No : B4

Average Revertants Per Plate \pm Standard Deviation

Liver Microsomes: None

Dose (μ g)	TA98		TA100		TA1535		TA1537		WP2 uvrA	
0.0	12 \pm	4	133 \pm	16	11 \pm	1	3 \pm	2	11 \pm	5
100	16 \pm	3	131 \pm	9	7 \pm	7	6 \pm	3	9 \pm	3
333	16 \pm	6	126 \pm	23	13 \pm	2	10 \pm	2	13 \pm	5
1000	28 \pm	8	173 \pm	6	15 \pm	2	24 \pm	5	39 \pm	15
2000	68 \pm	12	232 \pm	30			106 \pm	23	50 \pm	8
3333	48 \pm	8	205 \pm	35	9 \pm	2	33 \pm	13	51 \pm	4
4000	51 \pm	2	160 \pm	14			5 \pm	4	87 \pm	14
5000	38 \pm	4	126 \pm	11	6 \pm	3	2 \pm	1	112 \pm	10
Pos	129 \pm	17	413 \pm	33	305 \pm	37	774 \pm	294	141 \pm	25

Liver Microsomes: Rat liver S9

Dose (μ g)	TA98		TA100		WP2 uvrA	
0.0	22 \pm	6	149 \pm	1	13 \pm	2
100	20 \pm	4	152 \pm	11	16 \pm	7
333	25 \pm	6	159 \pm	12	15 \pm	4
1000	30 \pm	12	165 \pm	12	26 \pm	5
2000	37 \pm	4			74 \pm	28
3333	50 \pm	16	200 \pm	17	84 \pm	10
4000	46 \pm	13			100 \pm	46
5000	43 \pm	5	208 \pm	42	132 \pm	74
Pos	547 \pm	12	794 \pm	26	137 \pm	65

0.0 = Vehicle plating aliquot of 50 μ l

Pos = Positive Control concentrations as specified in Materials and Methods section.

APPENDIX I
Historical Control Data

APPENDIX II

Study Protocol

APPROVED

Study Number: []

Bacterial Reverse Mutation Assay with an Independent Repeat Assay

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvrA* in the presence and absence of S9 activation.

2.0 SPONSOR

2.1 Name: []

2.2 Address: []

2.3 Representative: []

2.4 Sponsor Project #: Not Applicable

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: Tetrahydrofurfuryl benzoate

3.2 Controls: Negative: Test article vehicle

Positive: 9-aminoacridine
2-aminoanthracene
methyl methanesulfonate
2-nitrofluorene
sodium azide

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

3.4 Test Article Retention Sample

The retention of a reserve sample of the test article will be the responsibility of the Sponsor.

Protocol [] 04/03/96

1 of 10



MA Study No. []

47

4.0 TESTING FACILITY AND KEY PERSONNEL

- 4.1 Name: Toxicology Testing Facility
Microbiological Associates, Inc.
- 4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850
- 4.3 Study Director: Valentine O. Wagner III, M.S.

5.0 TEST SCHEDULE

- 5.1 Proposed Experimental Initiation Date: 07/30/96
- 5.2 Proposed Experimental Completion Date: 09/10/96
- 5.3 Proposed Report Date: 04/24/96

6.0 TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvrA* as described by Green and Muriel (1976).

Genotype of the Strains Used for Mutagen Testing

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
<i>hisG46</i>	<i>hisC3076</i>	<i>hisD3052</i>	<i>trpE</i>	LPS	Repair	R-factor
TA1535	TA1537	-	-	<i>rfa</i>	Δ <i>uvrB</i>	-
TA100	-	TA98	-	<i>rfa</i>	Δ <i>uvrB</i>	+R
-	-	-	WP2 <i>uvrA</i>	-	Δ <i>uvrA</i>	-

Each *S. typhimurium* tester strain contains, in addition to a mutation in the histidine operon, additional mutations that enhance sensitivity to some mutagens. The *rfa* mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The deletion in the *uvrB* gene results in a deficient DNA excision-repair system. Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor). It has been suggested that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA100 is reverted by both frameshift and base substitution mutagens and TA1535 is reverted only by mutagens that cause base substitutions.

Protocol [] 04/03/96

2 of 10

 MICROBIOLOGICAL ASSOCIATES, INC.

MA Study No. []

48

The *E. coli* tester strain has an AT base pair at the critical mutation site within the *trpE* gene (Wilcox *et al.*, 1990). Tester strain WP2 *uvrA* has a deletion in the *uvrA* gene resulting in a deficient DNA excision-repair system. Tryptophan revertants can arise due to a base change at the originally mutated site or by a base change elsewhere in the chromosome causing the original mutation to be suppressed. Thus, the specificity of the reversion mechanism is sensitive to base-pair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

The *S. typhimurium* tester strains were received directly from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was received from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The test article will be tested at a minimum of five dose levels along with appropriate negative and positive controls with tester strains TA98, TA100, TA1535, TA1537 and WP2 *uvrA* with and without S9 activation. All dose levels of test article, negative controls and positive controls will be plated in triplicate.

7.1 Solubility Determination

Unless the Sponsor has indicated the test article vehicle, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension up to a maximum of 500 mg/ml. Vehicles compatible with this test system, in order of preference, include but are not limited to: deionized water (CAS 7732-18-5), dimethylsulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice will be the solvent, selected in order of preference, that permits preparation of the highest workable/soluble stock concentration, up to 500 mg/ml.

7.2 Preliminary Toxicity Assay to Select Dose Levels

Selection of dose levels for the mutagenicity assay will be based upon the toxicity and precipitation profile of the test article assessed in a preliminary toxicity assay. This preliminary assay will be conducted by exposing TA98, TA100, TA1535, TA1537 and WP2 *uvrA* to negative controls and to at least eight concentrations of test article, one plate per dose level, in both the presence and absence of S9 activation. Unless indicated otherwise by the Sponsor, the highest dose will be the highest workable concentration in the vehicle of choice but not to exceed 5 mg/plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated following the incubation period. In the event that the test article cannot be delivered at a high enough concentration in an appropriate vehicle to be toxic or if test article precipitate is present on the plates after incubation, the Sponsor will be consulted prior to selection of dose levels for the mutagenicity assay. In selecting dose levels for the mutagenicity assay the following guidelines will be employed. Whenever possible, the highest dose for the mutagenicity assay will be selected to give some indication of toxicity without exceeding 5 mg/plate. For freely

soluble, nontoxic test articles, the highest dose level will be 5 mg/plate. For precipitating, nontoxic test articles, the highest dose level will be selected in an attempt to yield precipitate at only the top one or two dose levels. The precipitate will be evaluated after the incubation period by visual examination without magnification. Doses will be selected such that precipitate does not interfere with scoring.

7.3 Frequency and Route of Administration

The test system will be exposed to the test article via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

After the data generated in the first assay have been evaluated, the mutagenicity assay will be repeated. The dose levels used in the second assay will be the same as those used in the first assay unless the Study Director determines that the dose range should be changed due to such parameters as excessive cytotoxicity or precipitate.

7.4 Controls

7.4.1 Positive Controls

All combinations of positive controls and tester strains plated concurrently with the assay are listed below:

Positive Controls

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
<i>Salmonella</i> Strains	+	2-aminoanthracene	1.0
WP2 <i>uvrA</i>			10
TA98	-	2-nitrofluorene	1.0
TA100, TA1535	-	sodium azide	1.0
TA1537	-	9-aminoacridine	75
WP2 <i>uvrA</i>	-	methyl methanesulfonate	1,000

7.4.2 Negative Controls

Appropriate negative controls will be plated for each tester strain with and without S9 activation. The negative control will be the vehicle alone, unless there is no historical basis for use of the selected vehicle. In the latter case, both untreated and vehicle controls will be used.

7.4.3 Sterility Controls

The most concentrated test article dilution and the Sham and S9 mixes will be checked for sterility.

7.5 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 homogenate will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used. Each batch of S9 homogenate will be assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenzanthracene to forms mutagenic to *S. typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 10% S9 homogenate, 5 mM glucose-6-phosphate, 4 mM β -nicotinamide-adenine dinucleotide phosphate, 8 mM MgCl₂ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. This mixture is referred to as S9 mix. Sham mix will be 100 mM phosphate buffer at pH 7.4.

7.6 Preparation of Tester Strain

Overnight cultures will be inoculated from the appropriate master plate or from the appropriate frozen stock. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. At the end of the working day, each inoculated flask will be placed in a resting shaker/incubator at room temperature. The shaker/incubator will be programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10⁹ cells/ml.

7.7 Test System Identification

Each plate will be labeled with a code system that identifies the test article, test phase, dose level, tester strain and activation type as described in Microbiological Associates' Microbial Mutagenesis Standard Operating Procedures.

7.8 Test Article Preparation

Unless specified otherwise, test article dilutions will be prepared immediately prior to use. All test article dosing will be at room temperature under yellow light.

7.9 Treatment of Test System

One half milliliter (0.5 ml) of S9 mix or Sham mix, 100 μ l of tester strain and 50 μ l of vehicle, test article dilution or positive control will be added to 2.0 ml of molten selective top agar at $45\pm 2^\circ\text{C}$. When necessary to achieve the target concentration or eliminate toxic vehicle effects, aliquots of other than 50 μ l of test article/vehicle/positive control will be plated. The mixture will be vortex mixed and overlaid onto the surface of 25 ml of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 to 72 hours at $37\pm 2^\circ\text{C}$. Plates that are not counted immediately following the incubation period will be stored at $4\pm 2^\circ\text{C}$.

7.10 Colony Counting

The condition of the bacterial background lawn will be evaluated for evidence of test article toxicity and precipitate. Evidence of toxicity will be scored relative to the negative control plate and recorded along with the revertant count for that plate.

7.11 Tester Strain Verification

On the day of use in the mutagenicity assay, all *S. typhimurium* tester strain cultures will be checked for the following genetic markers:

The presence of the *rfa* wall mutation will be confirmed for all tester strains by demonstrating sensitivity to crystal violet. The presence of the *uvrB* mutation will be confirmed for tester strains TA98, TA100, TA1535 and TA1537 by demonstrating sensitivity to ultraviolet light. The presence of the pKM101 plasmid will be confirmed for tester strains TA98 and TA100 by demonstrating resistance to ampicillin.

On the day of use in the mutagenicity assay, the *E. coli* tester strain cultures will be checked for the presence of the *uvrA* mutation by demonstrating sensitivity to ultraviolet light.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the mutagenicity assay to be considered valid:

8.1 Tester Strain Integrity

To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvrB* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvrA* mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Protocol [] 04/03/96

6 of 10

MA Study No. []

52

 **MICROBIOLOGICAL
ASSOCIATES, INC.**

8.2 Spontaneous Revertant Background Frequency

Based on historical control data, all tester strain cultures must exhibit characteristic number of spontaneous revertants per plate in the negative controls (vehicle). The mean revertants per plate must be within the following ranges (inclusive): TA98, 10 - 50; TA100, 80 - 240; TA1535, 5 - 45; TA1537, 3 - 21; WP2 *uvrA*, 10 - 60.

8.3 Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

8.4 Positive Control Values

Each mean positive control value must exhibit at least a three fold increase over the respective mean negative control value (vehicle) for each tester strain.

8.5 Toxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean negative control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that fewer than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

9.0 EVALUATION OF TEST RESULTS

For a test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test article as specified below:

9.1 Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than three times the mean negative control value (vehicle).

9.2 Strains TA98, TA100 and WP2 *uvrA*

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean negative control value (vehicle).

In consultation with the Sponsor, negative results may be confirmed as needed and equivocal results may be clarified by further testing using modified experimental conditions.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. The report will include:

- Test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test article, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test article in solvent/vehicle, if known.
- Strains: strains used; number of cells/ml per culture; strain characteristics.
- Test conditions: amount of test substance per plate with rationale for dose selection and number of plates per concentration; media used; type and composition of metabolic activation system, including acceptability criteria; treatment procedures.
- Results: signs of toxicity; signs of precipitation; individual plate counts; the mean number of revertant colonies per plate and standard deviation; dose-response relationship, where possible; statistical analysis, if any; concurrent negative and positive control data means and standard deviations; historical negative and positive control data with ranges, means and standard deviation.
- Discussion of results.
- Conclusion.

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Quality Assurance Unit of Microbiological Associates, Rockville, MD in accordance with the relevant Good Laboratory Practices Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guidelines 471 and 472 (Genetic Toxicology: Bacterial Reverse Mutation Assay), Revised Draft Document, September 1995 and with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, Step 4 Final Draft, July 18, 1995.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Protocol [] 04/03/96

8 of 10

 **MICROBIOLOGICAL
ASSOCIATES, INC.**

MA Study No. []

54

Will this study be submitted to a regulatory agency? Yes If so, to which agency or agencies? EPA (TSCA), EU, Japan

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutation Research* 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using *trp*⁺ reversion in *Escherichia coli*. *Mutation Research* 38:3-32.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, Step 4 Final Draft, July 18, 1995.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. *Proc. Natl. Acad. Sci. USA* 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. *Proc. Natl. Acad. Sci. USA* 72:5135-5139.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. *Mutation Research* 113:173-215.

OECD Guidelines 471 and 472 (Genetic Toxicology: Bacterial Reverse Mutation Assay), Revised Draft Document, September 1995.

Wilcox, P., Naidoo, A., Wedd, D.J. and Gatehouse, D.G. (1990). Comparison of *Salmonella typhimurium* TA102 with *Escherichia coli* WP2 tester strains. *Mutagenesis* 5:285-291.

Protocol [] 04/03/96

9 of 10

MA Study No. []

55

 MICROBIOLOGICAL ASSOCIATES, INC.

14.0 APPROVAL

[_____]

(Print or Type Name)

Valentine C. Wagner, III
STUDY DIRECTOR

7/25/96
DATE

Protocol [] 04/03/96

10 of 10

MA Study No. []

56

APPENDIX III

Information for Japanese Regulatory Agencies

3. S9 Mix

(1) Source of S9 (Encircle the applicable number, and fill in the relevant entries)

① Made in-house	Prepared on	04/24/96 and 07/03/96	(date)
2. Purchase	Supplier		
	Prepared on		(date)
	Purchased on		(date)
	Lot. No.		

(2) Storage Temperature, etc. of S9

Storage temperature	≤-70°C	Name and model of storage apparatus	So-Low, Model PR27-120
---------------------	--------	-------------------------------------	------------------------

(3) Preparation of S9 (If purchased material, fill in spaces to extent possible)

Animal used		Inducing substance	
Species, Strain	Rattus norvegicus, Sprague Dawley	Name	Aroclor 1254
Sex	Male	Administration method	intraperitoneal
Age (in weeks)	7;9	Administration period and amount (g/kg-weight)	5 days 0.5 gm/kg body weight
Weight	181 to 202 g 209 to 244 g		

(4) Composition of S9 mix

Constituents	Amount in 1 ml S9 Mix	Constituents	Amount in 1 ml S9 Mix
S9	0.1 ml	NADPH	not applicable
MgCl ₂	8 μmol	NADH	not applicable
KCl	33 μmol	Na-phosphate buffer (pH 7.4)	100 μmol
Glucose-6-phosphate	5 μmol	Others (NADP)	4 μmol
Glucose-6-phosphate dehydrogenase	not applicable		

4. Positive Control Substance

(1) Positive control

Name	Manufacture	Lot No.	Grade	Purity (%)	Solvent used
9-Aminoacridine (9AAD)	Sigma Chemical Company	096F05641		98 %	DMSO
2-Aminoanthracene (2AA)	Sigma Chemical Company	35H2507	Practical		DMSO
Methyl methanesulfonate (MMS)	Aldrich Chemical Co., Inc.	PF051812PF		99 %	DMSO
2-Nitrofluorene (2NF)	Aldrich Chemical Co., Inc.	10815HG		98 %	DMSO
Sodium azide (SA)	Sigma Chemical Company	85H0476	Practical		water

(2) Solvent

Name	Manufacture	Grade	Purity (%)
Dimethylsulfoxide (DMSO)	Fisher Scientific	Certified ACS	
Water	Life Technologies, Inc.	distilled	

(3) Preparation and storage of positive control solution (Encircle the applicable number)

Prepare or Store	1. Prepare just before test
	② Store subdivided solutions (Storage temp. $-20 \pm 5^{\circ}\text{C}$)
	3. Others ()

5. Preparation of Test Substance Solution (Encircle the applicable number, and fill in the relevant entries)

Solvent used	Name	Manufacture	Lot No.	Grade	Purity (%)
	Dimethylsulfoxide (DMSO)	Fisher Scientific	952812	Certified ACS	
Stability of test substance in the solvent	unknown				
Reason to choose the solvent:					
Method of suspension when test substance is difficult to dissolve					
Storage time and temp. from preparation to use for test	0 hours	<30 min.	ambient	$^{\circ}\text{C}$...
Conversion by purity	1. Yes		② No		

6. Conditions of Pre-culture (Encircle the applicable number, and fill in the relevant entries)

(1) Conditions

Nutrient broth	Name	Manufacturer	Lot No.
	Oxoid Nutrient Broth No. 2	Oxoid Ltd.	CH.-8, = 3185540
Period of pre-culture	12 hours ± 1 hour		
* Storage time and temp. from inoculation to beginning of shaking culture	2 to 5 hours	0 min.	ambient °C
* Storage time and temp. from end of culture to use for test	<7 hours	30 min.	4 ± 2 °C
* Model and manufacturer of shaker	New Brunswick Scientific, model G-24		
* Method of shaking (shaking type, speed, etc.)	1. Reciprocal	② Rotary	3. Other
	125 rev/min.		
* Culture vessel (shape, capacity)	shape: cylinder, 200 ml		
* Culture volume	50 ml		
* Volume of inoculum	1 colony		

(2) Fresh cell

		Base-pair substitution type				Frameshift type		
		TA100	TA1535	WP2 <i>uvrA</i>		TA98	TA1537	TA1538
Number of fresh cells (X10 ⁸ /ml)	Dose-finding study	3.9	4.4	2.8		1.8	2.1	
	Main study	10.0	13.6;4.2	23.3		5.2	16.5	
	Confirmatory study	5.5	3.2;12.9	2.5;4.8		1.4;5.4	0.7;2.2	
Measurement Method		1. Conversion by OD value ② Dilution method 3. Other ()						

7. Agar Plate Medium

(1) Top agar

Agar	Name	BBL Select
	Manufacturer	Becton Dickinson
	Lot No.	H7DEAJ

(2) Minimum Glucose Agar (Encircle the applicable number, and fill in the relevant entries)

① Made in-house	Agar	Name	BBL Select
		Manufacturer	Becton Dickinson
		Lot No.	H7DEAJ
	Volume of agar plate medium		25 ml
2. Purchased	Manufacturer		
	Prepared on	(date)	
	Purchased on	(date)	
	Lot No.		

8. Sterility test (Encircle the applicable number)

	Bacterial growth other than those used for test	
Test substance solution	1. Yes	② No
S9 Mix	1. Yes	② No

11. Test Results

(1) Test results should be reported on the attached form.

(2) Judgement of the results

Judgement (Encircle one)	<u>positive</u>	negative
Reason for judgement and referential matters:		
Positive response was observed with tester strains TA98, TA1537 and WP2 <i>uvrA</i> in the presence and absence of Aroclor-induced rat liver S9. No other positive responses were observed with any of the remaining tester strain/activation conditions.		

(3) Referential matters

The vehicle and positive control values indicate that all tester strains were functioning correctly and were capable of detecting a mutagen.
--

[Remark] "Referential matters" - Fill in the view etc. of the Study Director on the test results.

12. Others

Testing Facility	Name	Microbiological Associates, Inc.	
	Address	9630 Medical Center Drive Rockville, Maryland 20850, U.S.A. Tel. (301) 738-1000	
Testing Facility Manager	Name	David Jacobson-Kram, Ph.D.	<i>David Jacobson-Kram</i> Signature
	Title	Vice President, Toxicology Group	
Filing and Storage	Name	Diane Gray	<i>Diane Gray</i> Signature
	Title	Secretary	
Manager of QAU	Name	Claire L. Courtemanche, B.S.	<i>Claire L. Courtemanche</i> Signature
	Title	Quality Assurance Manager - Toxicology	
Study Director	Name	Valentine O. Wagner, III, M.S.	<i>Valentine O. Wagner, III</i> Signature
	Title	Study Director, Bacterial Mutagenesis Studies	Experience: 15 years
Responsible Scientist	Name	Michelle L. Klug, B.S.	<i>Michelle L. Klug</i> Signature
	Title	Laboratory Team Leader, Bacterial Mutagenesis Studies	Experience: 8 years
Test dates	from	07/30/96	to 09/11/96
Study number	[]		

Table of Test Results

Name of Test Substance: Tetrahydrofurfuryl benzoate

With (+) or without (-) SB Mix	Test substance concentration (µg/plate)	Number of revertants (number of colonies/plate)						
		Base-pair substitution type				Frameshift type		
		TA100	TA1636	WP2 <i>uvrA</i>		TA98	TA1637	TA1638
SB Mix (-)	Solvent control	132 (141) 134 157	22 (19) 18 18	16 (13) 10 14		18 (23) 23 28	3 (7) 12 6	
	100	134 (136) 141 132	12 (18) 21 21	21 (16) 12 14		14 (14) 16 13	6 (6) 6 3	
	333	77 (122) 145 144	17 (17) 11 22	21 (17) 14 16		14 (19) 19 23	8 (10) 17 6	
	1000	206 (182) 210 130	18 (19) 18 20	42 (38) 36 35		35 (32) 34 27	14 (36) 19 75	
	2000	195 (189) 196 176		81 (88) 81 93		32 (40) 39 60	53 (37) 60 7	
	3333	286 (264) 276 230	14 (16) 12 19	143 (138) 152 119		30 (34) 33 39	14 (6) 3 2	
	4000	245 (267) 249 276		160 (161) 133 189		41 (30) 23 26	13 (17) 18 19	
	6000	265 (276) 282 279	13 (10) 7 11	291 (218) 178 185		32 (33) 34 34	17 (22) 28 22	
SB Mix (+)	Solvent control	154 (144) 145 134	10 (10) 8 12	30 (21) 17 15		13 (23) 44 42	8 (7) 4 8	
	100	119 (126) 124 136	13 (12) 9 14	29 (26) 27 23		25 (24) 31 16	6 (7) 8 6	
	333	129 (133) 144 127	13 (13) 10 16	20 (23) 26 23		42 (35) 33 30	8 (7) 6 6	
	1000	137 (141) 128 157	8 (9) 8 11	18 (19) 19 21		46 (45) 43 46	9 (11) 9 14	
	2000					14 (25) 31 30	14 (11) 11 8	
	3333	148 (141) 128 148	18 (18) 19 17	62 (60) 64 63		32 (28) 35 18	40 (32) 32 26	
	4000					45 (38) 32 37	25 (28) 27 32	
	6000	177 (176) 183 168	12 (12) 12 13	61 (76) 98 67		40 (38) 46 23	28 (26) 30 23	
Positive control net requiring SB Mix	Name	SA	SA	MMS		2NF	SAAD	2NF
	Concentration (µg/plate)	1.0	1.0	1000		1.0	75	1.0
	Number of colonies/plate	446 (457) 416 609	618 (468) 475 412	189 (197) 208 192		129 (102) 84 63	674 (693) 617 518	0
Positive control requiring SB Mix	Name	2AA	2AA	2AA		2AA	2AA	2AA
	Concentration (µg/plate)	1.0	1.0	10		1.0	1.0	1.0
	Number of colonies/plate	609 (573) 5*1 488	67 (46) 44 38	66 (64) 68 58		365 (458) 377 632	44 (48) 60 63	0

Notes:

1. When inhibition is found against growth of the bacteria, mark the applicable value with an asterisk.
2. Fill the average number of colonies in each concentration in the ().
3. "Number of revertants" - Fill in the observed value and average value in order beginning with the low concentrations of the test substance.

Table of Test Results

Name of Test Substance: Tetrahydrofurfuryl benzoate

With (+) or without (-) SB Mix	Test substance concentration (µg/plate)	Number of revertants (number of colonies/plate)						
		Base-pair substitution type				Frameshift type		
		TA100	TA1635	WP2 <i>uvrA</i>		TA98	TA1637	TA1638
SB Mix (-)	Solvent control	125 (133) 123 161	11 (11) 12 11	6 (11) 13 16		8 (12) 16 13	2 (3) 2 6	
	100	128 (131) 124 141	4 (7) 6 2	6 (9) 9 12		14 (16) 15 19	3 (6) 9 6	
	333	138 (128) 142 98	11 (13) 14 13	7 (13) 15 18		20 (16) 19 10	10 (10) 11 8	
	1000	187 (173) 178 174	17 (16) 14 13	22 (29) 44 60		38 (28) 21 27	23 (24) 29 19	
	2000	212 (232) 268 218		46 (60) 59 44		71 (68) 78 64	111 (106) 127 81	
	3333	188 (206) 214 236	11 (8) 7 8	46 (51) 64 62		67 (48) 42 46	21 (33) 46 32	
	4000	144 (160) 188 169		100 (87) 88 72		49 (51) 52 52	7 (6) 1 8	
	6000	113 (128) 131 133	8 (8) 7 3	123 (112) 104 108		41 (38) 34 40	3 (2) 1 1	
SB Mix (+)	Solvent control	149 (148) 149 148	18 (14) 13 13	10 (13) 14 14		18 (22) 20 29	4 (7) 8 8	
	100	158 (152) 158 138	6 (10) 14 10	13 (16) 24 10		18 (20) 17 26	2 (4) 6 6	
	333	166 (159) 148 166	13 (16) 11 21	18 (16) 10 17		31 (26) 20 24	7 (8) 7 4	
	1000	169 (166) 161 174	9 (10) 9 9	31 (28) 22 26		16 (30) 38 38	14 (10) 11 6	
	2000			62 (74) 106 63		41 (37) 38 34	16 (14) 13 14	
	3333	197 (200) 218 186	10 (11) 9 14	74 (84) 84 83		34 (60) 61 66	21 (32) 36 39	
	4000			49 (100) 139 113		37 (46) 61 41	28 (31) 32 36	
	6000	246 (208) 163 218	11 (12) 12 14	218 (132) 86 84		44 (43) 38 48	34 (28) 20 28	
Positive control not requiring SB Mix	Name	SA	SA	MMS		2NF	9AAD	2NF
	Concentration (µg/plate)	1.0	1.0	1000		1.0	75	1.0
	Number of colonies/plate	416 (413) 446 380	300 (306) 271 346	160 (141) 113 161		146 (129) 111 132	869 (774) 438 916	0
Positive control requiring SB Mix	Name	2AA	2AA	2AA		2AA	2AA	2AA
	Concentration (µg/plate)	1.0	1.0	10		1.0	1.0	1.0
	Number of colonies/plate	783 (784) 824 776	247 (211) 184 201	88 (137) 210 118		639 (647) 661 642	828 (646) 603 308	0

Notes:

- When inhibition is found against growth of the bacteria, mark the applicable value with an asterisk.
- Fill the average number of colonies in each concentration in the [].
- "Number of revertants" - Fill in the observed value and average value in order beginning with the low concentrations of the test substance.

Table of Test Results (Potency)

Name of Test Substance: Tetrahydrofurfuryl benzoate

	Test Strain	-S9 mix		+S9 mix	
		Potency	Dose	Potency	Dose
		(revertants/mg)	(μ g/plate)	(revertants/mg)	(μ g/plate)
Dose-finding study	TA100	41	3333	6	5000
	TA1535	75	67	400	10
	WP2 <i>uvrA</i>	5	3333	4	3333
	TA98	6	3333	9	5000
	TA1537	14	3333	8	5000
	TA1538				
Main study	TA100	27	5000	6	5000
	TA1535	0	5000	2	3333
	WP2 <i>uvrA</i>	41	5000	11	5000
	TA98	9	2000	12	1000
	TA1537	15	2000	8	3333
	TA1538				
Confirmatory study	TA100	50	2000	12	5000
	TA1535	4	1000	3	333
	WP2 <i>uvrA</i>	20	5000	24	5000
	TA98	28	2000	8	3333
	TA1537	52	2000	8	3333
	TA1538				

Potency (revertants/mg) = maximum net revertants/concentration (mg)

If the maximum revertant count is observed at more than one dose level, the higher concentration (or highest concentration, if applicable) is used in the potency computation.

If the maximum revertant count is equal to or less than the vehicle control value, the potency is indicated as zero at the highest dose level tested.

Best Available Copy

MA Study No. []