

**SafePharm
Laboratories**

**N-ACETYL-L-HYDROXYPROLINE:
REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM*
AND *ESCHERICHIA COLI***

SPL PROJECT NUMBER: 732/091

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QUALITY ASSURANCE REPORT

This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress.

This report has been audited by Safeparm Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

02 February 2000	Standard Test Method Compliance Audit
29 May 2001	Test Material Preparation
21 May 2001	Test System Preparation
29 May 2001	Exposure
21 May 2001	Assessment of Response
§ 25 June 2001	Draft Report Audit
§ Date of QA Signature	Final Report Audit
§ Evaluation specific to this study	



DATE: 30 AUG 2001

For Safeparm Quality Assurance Unit*

***Authorised QA Signatures:**

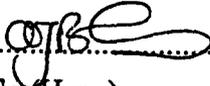
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GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 87/18/EEC (as amended by Directive 1999/11/EC) and 88/320/EEC (as amended by Directive 1999/12/EC).

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

This report fully and accurately reflects the procedures used and data generated.

.....  DATE: 29 AUG 2001

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Study Director

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**N-ACETYL-L-HYDROXYPROLINE:
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*ESCHERICHIA COLI***

SUMMARY

Introduction. The method was designed to conform to the guidelines for bacterial mutagenicity testing published by the major Japanese Regulatory Authorities including MITI, MHW, MOL and MAFF. It also meets the requirements of the OECD Guidelines for Testing of Chemicals No. 471 "Reverse Mutation Study", Method B14 of Commission Directive 92/69/EEC and the USA, EPA (TSCA) OPPTS harmonised guidelines.

Methods. *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 and *Escherichia coli* strain WP2uvrA- were treated with the test material using the Ames plate incorporation method at five dose levels, in triplicate, both with and without the addition of a rat liver homogenate metabolising system (10% liver S9 in standard co-factors). The dose range was determined in a preliminary toxicity assay and was 50 to 5000 µg/plate in the range-finding study. The experiment was repeated on a separate day using the same dose range as the range-finding study, fresh cultures of the bacterial strains and fresh test material formulations.

Results. The vehicle (sterile distilled water) control plates gave counts of revertant colonies within the normal range. All of the positive control chemicals used in the test induced marked increases in the frequency of revertant colonies, both with and without metabolic activation. Thus, the sensitivity of the assay and the efficacy of the S9-mix were validated.

The test material caused no visible reduction in the growth of the bacterial background lawn at any dose level. The test material was, therefore, tested up to the maximum recommended dose level of 5000 µg/plate. No test material precipitate was observed on the plates at any of the doses tested in either the presence or absence of S9-mix.

No significant increases in the frequency of revertant colonies were recorded for any of the bacterial strains, with any dose of the test material, either with or without metabolic activation.

Conclusion. The test material was considered to be non-mutagenic under the conditions of this test.

**N-ACETYL-L-HYDROXYPROLINE:
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1. INTRODUCTION

This study was designed to assess the mutagenic potential of the test material using a bacterial/microsome test system. The study was based on the in vitro technique described by Ames and his co-workers (1, 2, 3) and Garner *et al* (4) in which mutagenic activity is assessed by exposing histidine auxotrophs of *Salmonella typhimurium* and tryptophan auxotrophs of *Escherichia coli* to various concentrations of the test material. The method conforms to the guidelines for bacterial mutagenicity testing published by the major Japanese Regulatory Authorities including MITI, MHW, MOL and MAFF. This method also conforms with the OECD Guidelines for the Testing of Chemicals, Protocol No. 471, Method B14 in EC Commission Directive 92/69/EEC and the USA, EPA (TSCA) OPPTS harmonised guidelines. A copy of the Certificate of Compliance with GLP, issued by the UK Department of Health, is included as Appendix 3.

The mutant strains of *Salmonella* are incapable of synthesising histidine and are, therefore, dependent for growth on an external source of this particular amino acid. When exposed to a mutagenic agent these bacteria may undergo a reverse mutation to histidine independent forms which are detected by their ability to grow on a histidine deficient medium. Using various strains of this organism, revertants produced after exposure to a chemical mutagen may arise as a result of base-pair substitution in the genetic material (miscoding) or frame-shift mutation in which genetic material is either added or deleted. In order to make the bacteria more sensitive to mutation by chemical and physical agents, several additional traits have been introduced. These include a deletion through the excision repair gene (*uvrB*⁻ *Salmonella* strains) which renders the organism incapable of DNA excision repair and deep rough mutation (*rfa*) which increases the permeability of the cell wall. A mutant strain of *Escherichia coli* (WP2*uvrA*⁻), which requires tryptophan and which can be reverse mutated by base substitution to tryptophan independence, was used to complement the *Salmonella* strains. This strain also has a deletion in an excision repair gene (*uvrA*⁻).

Since many compounds do not exert a mutagenic effect until they have been metabolised by enzyme systems not available in the bacterial cell, the test material and the bacteria are also

incubated in the presence of a liver microsomal preparation (S9-mix) prepared from rats pre-treated with a compound known to induce an elevated level of these enzymes.

The study was performed between 16 May 2001 and 11 June 2001.

2. TEST MATERIAL AND EXPERIMENTAL PREPARATION

Sponsor's identification : N-ACETYL-L-HYDROXYPROLINE
Description : white solid
Lot number : 000703
Date received : 23 April 2001
Storage conditions : room temperature in the dark

Data relating to the identity, purity and stability of the test material are the responsibility of the Sponsor.

3. METHODS

3.1 Tester Strains

Salmonella typhimurium TA1535, TA1537, TA98 and TA100
Escherichia coli WP2uvrA⁻

The *Salmonella typhimurium* strains were obtained from the University of California at Berkeley on culture discs on 4 August 1995 whilst *Escherichia coli* strain WP2uvrA⁻ was obtained from the British Industrial Biological Research Association on 17 August 1987. All of the strains were stored at -196°C in a Statebourne liquid nitrogen freezer, model SXR 34. Prior to the master strains being used, characterisation checks were carried out to confirm the amino-acid requirement, presence of rfa, R factors, uvrB or uvrA mutation and the spontaneous reversion rate.

In this assay, overnight sub-cultures of the appropriate coded stock cultures were prepared in nutrient broth and incubated at 37°C for approximately 10 hours. Each culture was monitored spectrophotometrically for turbidity with titres determined by viable count analysis on nutrient agar plates.

3.2 Preparation of Test and Control Materials

The test material was accurately weighed and approximate half-log dilutions prepared in sterile distilled water by mixing on a vortex mixer on the day of each experiment. Analysis for concentration, homogeneity and stability of the test material formulations is not a requirement of the test guidelines and was, therefore, not determined.

Vehicle and positive controls were used in parallel with the test material. A solvent treatment group was used as the vehicle control and the positive control materials were as follows:

N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG): 2 µg/plate for WP2uvrA, 3 µg/plate for TA100
and 5 µg/plate for TA1535
9-Aminoacridine (9AA): 80 µg/plate for TA1537
4-Nitroquinoline-1-oxide (4NQO): 0.2 µg/plate for TA98

In addition, 2-Aminoanthracene (2AA) and Benzo(a)pyrene (BP), which are non-mutagenic in the absence of metabolising enzymes, were used in the series of plates with S9-mix at the following concentrations:

2AA at 1 µg/plate for TA100
2AA at 2 µg/plate for TA1535 and TA1537
2AA at 10 µg/plate for WP2uvrA
BP at 5 µg/plate for TA98

3.3 Microsomal Enzyme Fraction

S9 was prepared in-house on 20 January 2001 (Preliminary Toxicity Test only) and 17 March 2001 from the livers of male Sprague-Dawley rats weighing ~ 250g. These had each orally received three consecutive daily doses of phenobarbitone/β-naphthoflavone (80/100 mg per kg per day) prior to S9 preparation. Before use, each batch of S9 was assayed for its ability to metabolize the indirect mutagens 2-Aminoanthracene and Benzo(a)pyrene. The S9 was stored at -196°C.

3.4 S9-Mix and Agar

The S9-mix was prepared immediately before use using sterilised co-factors and maintained on ice for the duration of the test.

S9	5.0 ml
1.65 M KCl/0.4 M MgCl ₂	1.0 ml
0.1 M Glucose-6-phosphate	2.5 ml
0.1 M NADPH	2.0 ml
0.1 M NADH	2.0 ml
0.2 M Sodium phosphate buffer (pH 7.4)	25.0 ml
Sterile distilled water	12.5 ml

A 0.5 ml aliquot of S9-mix and 2 ml of molten, trace histidine or tryptophan supplemented, top agar was overlaid onto a sterile Vogel-Bonner Minimal agar plate in order to assess the sterility of the S9-mix. This procedure was repeated, in triplicate, on the day of each experiment.

Top agar was prepared using 0.6% Difco Bacto agar and 0.5% sodium chloride with 5 ml of 1.0 mM histidine and 1.0 mM biotin or 1.0 mM tryptophan solution added to each 100 ml of top agar. Vogel-Bonner Minimal agar plates were purchased from Becton Dickinson GmbH (Preliminary Toxicity Assay only) and Fred Baker Scientific.

3.5 Test Procedure

3.5.1 Preliminary Toxicity Study

In order to select appropriate dose levels for use in the main study, a preliminary test was carried out to determine the toxicity of the test material. The dose range of the test material was 0, 0.15, 0.5, 1.5, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate. The study was performed by mixing 0.1 ml of bacterial culture (TA100 or WP2uvrA), 0.1 ml of test material formulation, 0.5 ml of S9-mix or phosphate buffer and 2 ml of molten, trace histidine or tryptophan supplemented, top agar and overlaying onto sterile plates of Vogel-Bonner Minimal agar (30 ml/plate). Ten concentrations of the test material and a vehicle control (sterile distilled water) were tested. In addition, 0.1 ml of the maximum concentration of the test material and 2 ml of molten, trace histidine or tryptophan supplemented, top agar was overlaid onto a sterile Nutrient agar plate in order to assess the sterility of the test material. After approximately 48 hours incubation at 37°C the plates were assessed for numbers of revertant colonies using a Domino colony counter and examined for effects on the growth of the bacterial background lawn.

3.5.2 Mutation Study – Experiment 1 (Range-finding Study)

Five concentrations of the test material (50, 150, 500, 1500 and 5000 µg/plate) were assayed in triplicate against each tester strain, using the direct plate incorporation method.

Measured aliquots (0.1 ml) of one of the bacterial cultures were dispensed into sets of test tubes followed by 2.0 ml of molten, trace histidine or tryptophan supplemented, top agar, 0.1 ml of the test material formulation, vehicle or positive control and either 0.5 ml of S9-mix or phosphate buffer. The contents of each test tube were mixed and equally distributed onto the surface of Vogel-Bonner Minimal agar plates (one tube per plate). This procedure was repeated, in triplicate, for each bacterial strain and for each concentration of test material both with and without S9-mix.

All of the plates were incubated at 37°C for approximately 48 hours and the frequency of revertant colonies assessed using a Domino colony counter.

3.5.3 Mutation Study – Experiment 2 (Main Study)

The second experiment was performed using methodology as described for the range-finding study, using fresh bacterial cultures, test material and control solutions. The test material dose range was the same as the range-finding study (50 to 5000 µg/plate).

3.6 Acceptance Criteria

The reverse mutation assay may be considered valid if the following criteria are met:

All tester strain cultures exhibit a characteristic number of spontaneous revertants per plate in the vehicle and untreated controls. Acceptable ranges are presented in the standard test method section 3.2 with historical control ranges for 1999 and 2000 presented in Appendix 2.

The appropriate characteristics for each tester strain have been confirmed, eg *rfa* cell-wall mutation and *pkM101* plasmid R-factor etc.

All tester strain cultures should be in the approximate range of 1 to 9.9×10^9 bacteria per ml.

Each mean positive control value should be at least two times the respective vehicle control value for each strain, thus demonstrating both the intrinsic sensitivity of the tester strains to mutagenic exposure and the integrity of the S9-mix. The positive control historical ranges for 1999 and 2000 are presented in Appendix 2.

There should be a minimum of four non-toxic test material dose levels.

There should be no evidence of excessive contamination.

3.7 Evaluation Criteria

The test material may be considered positive in this test system if the following criteria are met:

The test material should have induced a reproducible, dose-related and statistically (Dunnett's method of linear regression(5)) significant increase in the revertant count in at least one strain of bacteria.

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safeparm archives for five years, after which instructions will be sought as to further retention or disposal.

5. RESULTS

5.1 Preliminary Toxicity Study

The test material was non-toxic to the strains of bacteria used (TA100 and WP2uvrA). The test material formulation and S9-mix used in this experiment were both shown to be sterile.

The number of revertant colonies for the toxicity assay were:

With (+) or without (-) S9-mix	Strain	Dose ($\mu\text{g}/\text{plate}$)										
		0	0.15	0.5	1.5	5	15	50	150	500	1500	5000
-	TA100	123	152	162	153	157	177	134	136	92	93	105
+	TA100	148	95	96	121	126	126	119	114	108	101	130
-	WP2uvrA	24	34	26	34	35	23	24	25	36	25	23
+	WP2uvrA	36	30	36	30	29	17	28	40	29	38	32

5.2 Mutation Study

Prior to use, the master strains were checked for characteristics, viability and spontaneous reversion rate (all were found to be satisfactory). These data are not given in the report. The S9-mix used in both experiments of the main study was shown to be sterile.

Results for the negative controls (spontaneous mutation rates) are presented in Table 1 and were considered to be acceptable. These data are for concurrent untreated control plates performed on the same day as the Mutation Study.

The individual plate counts, the mean number of revertant colonies and the standard deviations for the test material, vehicle and positive controls both with and without metabolic activation, are presented in Table 2 to Table 5 with the results also expressed graphically in Figure 1 to Figure 4.

Information regarding the equipment and methods used in these experiments as required by the Japanese Ministry of Labour, Japanese Ministry of International Trade and Industry and Japanese Ministry of Health and Welfare are presented in Appendix 1.

A history profile of vehicle and positive control values is presented in Appendix 2.

The test material caused no visible reduction in the growth of the bacterial background lawn at any dose level. The test material was, therefore, tested up to the maximum recommended dose level of 5000 µg/plate. No test material precipitate was observed on the plates at any of the doses tested in either the presence or absence of S9-mix.

No significant increases in the frequency of revertant colonies were recorded for any of the bacterial strains, with any dose of the test material, either with or without metabolic activation.

All of the positive control chemicals used in the test induced marked increases in the frequency of revertant colonies thus confirming the activity of the S9-mix and the sensitivity of the bacterial strains.

6. CONCLUSION

The test material was considered to be non-mutagenic under the conditions of this test.

7. REFERENCES

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Table 1 Spontaneous Mutation Rates (Concurrent Negative Controls)

Range-finding Study

Number of revertants (mean number of colonies per plate)				
Base-pair substitution type			Frameshift type	
TA100	TA1535	WP2uvrA ⁻	TA98	TA1537
87	15	15	23	6
81 (85)	16 (14)	19 (16)	21 (22)	12 (8)
86	10	13	23	7

Main Study

Number of revertants (mean number of colonies per plate)				
Base-pair substitution type			Frameshift type	
TA100	TA1535	WP2uvrA ⁻	TA98	TA1537
119	15	31	22	15
129 (127)	14 (15)	16 (22)	13 (16)	6 (10)
133	16	19	14	10

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Table 2 Test Results: Range-Finding Study – Without Metabolic Activation

With or without S9-Mix	Test substance concentration (µg/plate)	Number of revertants (mean number of colonies per plate)									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2uvrA-		TA98		TA1537	
-	0	90 83 89	(87) 3.8#	10 18 16	(15) 4.2	25 22 25	(24) 1.7	28 21 31	(27) 5.1	13 20 9	(14) 5.6
-	50	80 73 93	(82) 10.1	16 19 20	(18) 2.1	36 28 35	(33) 4.4	21 74 31	(42) 28.2	18 3 6	(9) 7.9
-	150	91 89 99	(93) 5.3	18 18 11	(16) 4.0	25 23 25	(24) 1.2	19 28 16	(21) 6.2	6 6 10	(7) 2.3
-	500	84 71 79	(78) 6.6	10 15 17	(14) 3.6	27 14 20	(20) 6.5	23 41 33	(32) 9.0	4 4 10	(6) 3.5
-	1500	92 79 88	(86) 6.7	15 10 16	(14) 3.2	23 15 25	(21) 5.3	11 35 50	(32) 19.7	6 10 13	(10) 3.5
-	5000	90 78 62	(77) 14.0	16 12 16	(15) 2.3	16 15 18	(16) 1.5	18 30 31	(26) 7.2	8 8 7	(8) 0.6
Positive controls	Name	ENNG		ENNG		ENNG		4NQO		9AA	
S9-Mix	Concentration (µg/plate)	3		5		2		0.2		80	
-	No. colonies per plate	464 527 568	(520) 52.4	630 513 521	(555) 65.4	695 701 629	(675) 39.9	120 139 150	(136) 15.2	860 827 852	(846) 17.2

ENNG N-ethyl-N'-nitro-N-nitrosoguanidine
 4NQO 4-Nitroquinoline-1-oxide
 9AA 9-Aminoacridine
 # Standard deviation

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Table 3 Test Results: Range-Finding Study – With Metabolic Activation

With or without S9-Mix	Test substance concentration (µg/plate)	Number of revertants (mean number of colonies per plate)									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2uvrA-		TA98		TA1537	
+	0	92		16		34		35		13	
		93	(95)	23	(17)	27	(29)	49	(38)	9	(11)
		99	3.8#	13	5.1	26	4.4	30	9.8	10	2.1
+	50	82		16		11		40		15	
		89	(86)	16	(16)	35	(23)	38	(37)	7	(12)
		88	3.8	15	0.6	23	12.0	33	3.6	13	4.2
+	150	102		13		29		23		8	
		85	(92)	13	(15)	27	(26)	24	(26)	11	(8)
		89	8.9	19	3.5	23	3.1	30	3.8	6	2.5
+	500	97		14		18		27		4	
		89	(88)	14	(16)	22	(23)	39	(30)	9	(6)
		79	9.0	19	2.9	29	5.6	24	7.9	6	2.5
+	1500	89		11		35		53		9	
		87	(92)	10	(11)	42	(36)	37	(45)	9	(9)
		100	7.0	13	1.5	30	6.0	46	8.0	9	0.0
+	5000	80		12		28		31		10	
		108	(91)	12	(14)	40	(32)	48	(35)	8	(10)
		85	14.9	17	2.9	28	6.9	26	11.5	12	2.0
Positive controls S9-Mix +	Name Concentration (µg/plate) No. colonies per plate	2AA		2AA		2AA		BP		2AA	
		1		2		10		5		2	
		2278	(2142)	224	(234)	405	(417)	195	(188)	446	(433)
		2021	129.2	251	14.6	425	10.4	162	22.9	458	34.0
		2127		228		420		206		394	

BP Benzo(a)pyrene
 2AA 2-Aminoanthracene
 # Standard deviation

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Table 4 Test Results: Main Study – Without Metabolic Activation

With or without S9-Mix	Test substance concentration (µg/plate)	Number of revertants (mean number of colonies per plate)									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2uvrA-		TA98		TA1537	
-	0	97		12		25		25		9	
		89	(92)	11	(11)	20	(21)	24	(23)	11	(9)
		90	4.4#	10	1.0	18	3.6	21	2.1	6	2.5
-	50	101		19		30		30		9	
		102	(99)	14	(15)	16	(21)	39	(31)	6	(7)
		93	4.9	11	4.0	18	7.6	24	7.5	6	1.7
-	150	97		10		19		23		9	
		86	(88)	16	(12)	20	(19)	25	(21)	7	(8)
		80	8.6	11	3.2	19	0.6	16	4.7	7	1.2
-	500	101		10		20		27		12	
		94	(100)	13	(14)	20	(19)	24	(22)	12	(11)
		104	5.1	20	5.1	17	1.7	16	5.7	9	1.7
-	1500	83		21		16		26		10	
		111	(94)	10	(17)	16	(14)	18	(23)	4	(6)
		87	15.1	19	5.9	10	3.5	24	4.2	5	3.2
-	5000	89		13		26		22		14	
		96	(89)	13	(13)	22	(26)	28	(26)	12	(11)
		82	7.0	12	0.6	31	4.5	27	3.2	7	3.6
Positive controls S9-Mix	Name	ENNG		ENNG		ENNG		4NQO		9AA	
	Concentration (µg/plate)	3		5		2		0.2		80	
	No. colonies per plate	722	(712)	937	(839)	985	(975)	167	(194)	512	(447)
-		698	12.3	794	84.7	877	93.4	218	25.6	384	64.0
-		715		787		1063		197		446	

ENNG N-ethyl-N'-nitro-N-nitrosoguanidine
 4NQO 4-Nitroquinoline-1-oxide
 9AA 9-Aminoacridine
 # Standard deviation

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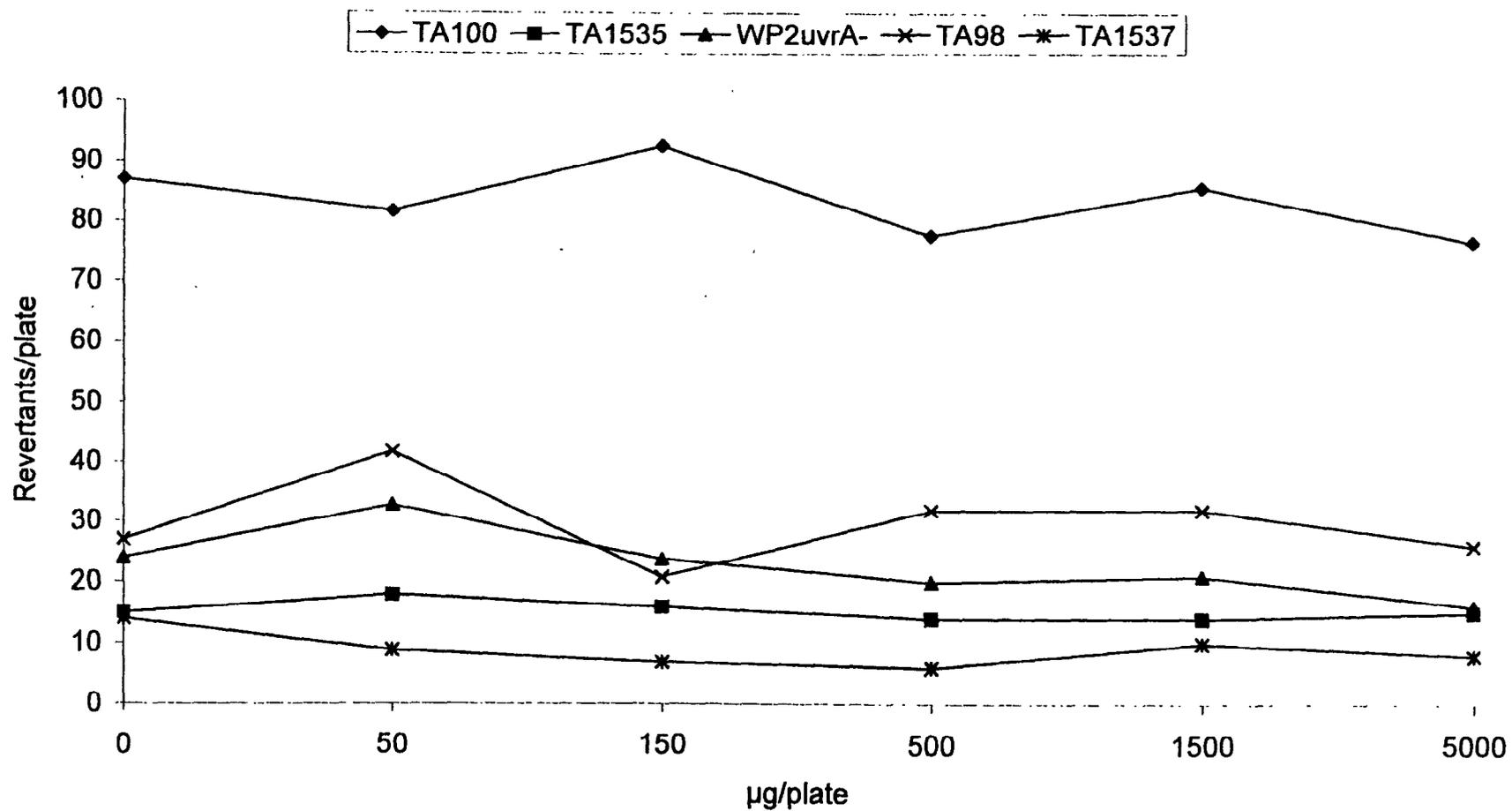
Table 5 Test Results: Main Study – With Metabolic Activation

With or without S9-Mix	Test substance concentration (µg/plate)	Number of revertants (mean number of colonies per plate)									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2uvrA-		TA98		TA1537	
+	0	92		14		21		32		14	
		102	(101)	11	(14)	24	(23)	26	(28)	7	(9)
		109	8.5#	16	2.5	23	1.5	27	3.2	7	4.0
+	50	100	(101)	18	(17)	14	(19)	27	(30)	14	(11)
		102	1.0	22	5.0	18	5.6	30	3.0	11	2.5
		101		12		25		33		9	
+	150	111	(105)	11	(13)	19	(20)	27	(28)	10	(7)
		102	5.2	14	2.1	17	3.6	31	3.1	4	3.1
		102		15		24		25		8	
+	500	104	(109)	11	(15)	25	(21)	31	(32)	7	(9)
		126	14.7	18	3.6	18	3.6	39	6.6	12	2.5
		98		16		20		26		9	
+	1500	106	(101)	12	(14)	28	(25)	28	(34)	6	(8)
		100	4.6	14	1.5	24	2.6	31	7.4	9	1.7
		97		15		23		42		9	
+	5000	114	(110)	17	(17)	23	(22)	26	(35)	9	(8)
		106	4.0	17	0.6	18	3.2	43	8.5	9	2.3
		111		16		24		35		5	
Positive controls S9-Mix +	Name	2AA		2AA		2AA		BP		2AA	
	Concentration (µg/plate)	1		2		10		5		2	
	No. colonies per plate	1780	(1766)	161	(194)	365	(417)	125	(157)	494	(439)
		1779	24.0	186	37.6	437	45.8	175	28.0	402	48.6
		1738		235		450		172		421	

BP Benzo(a)pyrene
2AA 2-Aminoanthracene
Standard deviation

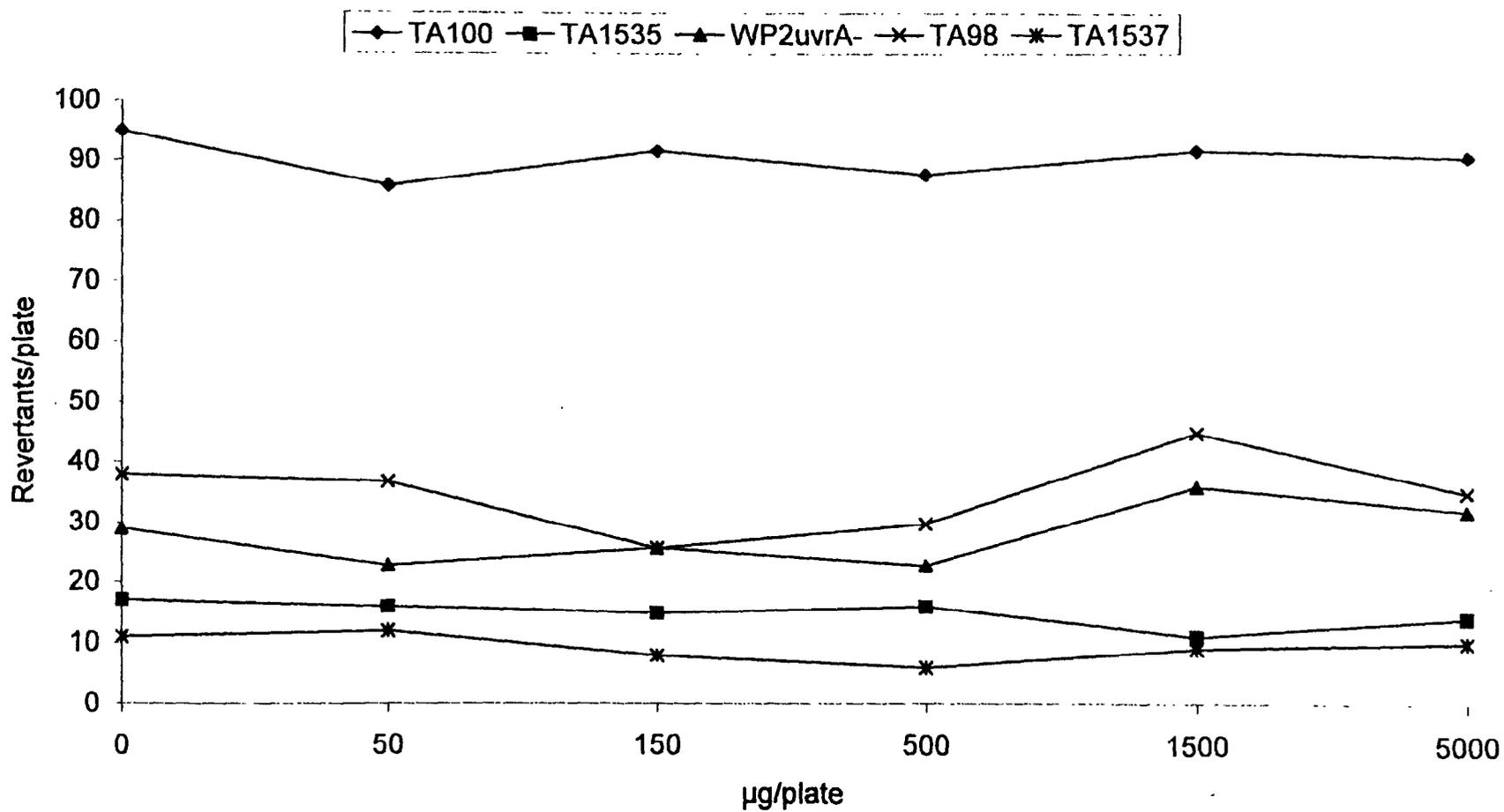
N-ACETYL-L-HYDROXYPROLINE: REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

Figure 1 Dose Response Curve: Range-Finding Study – Without Metabolic Activation



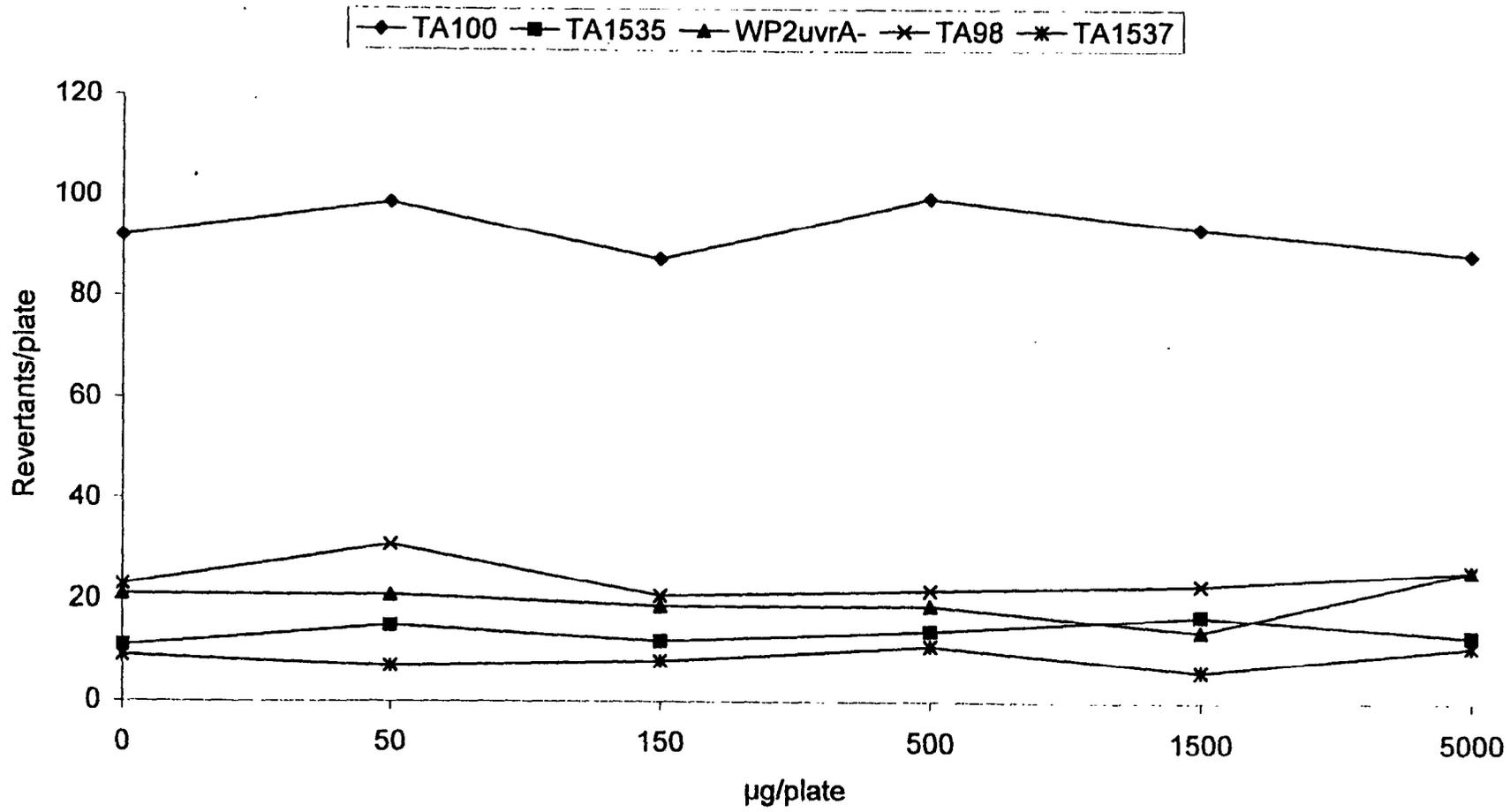
N-ACETYL-L-HYDROXYPROLINE: REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

Figure 2 Dose Response Curve: Range-Finding Study – With Metabolic Activation



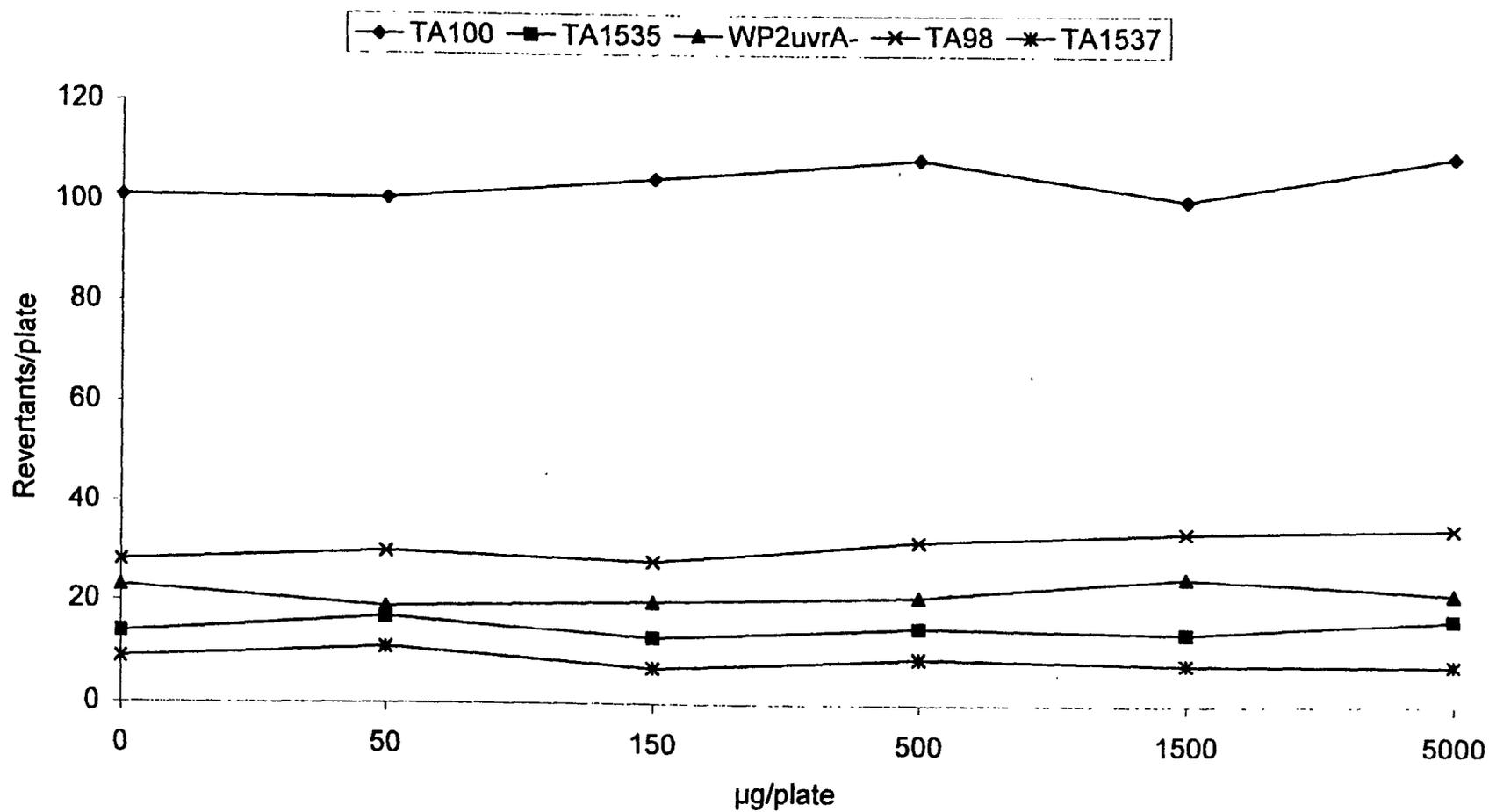
N-ACETYL-L-HYDROXYPROLINE: REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURJUM* AND *ESCHERICHIA COLI*

Figure 3 Dose Response Curve: Main Study – Without Metabolic Activation



N-ACETYL-L-HYDROXYPROLINE: REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

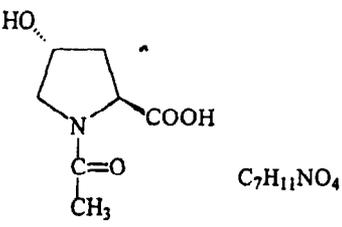
Figure 4 Dose Response Curve: Main Study – With Metabolic Activation



**N-ACETYL-L-HYDROXYPROLINE: REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI***

Appendix 1 Report of Results in Mutagenicity Test using Micro-organisms

1. GENERAL ITEMS (to be completed by the sponsor)

Name of the new chemical substance (IUPAC nomenclature)	N-ACETYL-L-HYDROXYPROLINE					
Other name	N-ACETYL-L-HYDROXYPROLINE					
Structural formula or rational formula (or outline of manufacturing method, in case both are unknown)						
Purity of the new chemical substance tested	100%	Lot No. of the new chemical substance tested	000703			
Name of impurities and concentration	-					
CAS No.	33996-33-7	Vapour pressure	-			
Molecular weight	173.17	Partition coefficient	-			
Melting point (°C)	-	Appearance at ordinary temperature	White crystalline powder			
Boiling point (°C)	-					
Stability	Stable under normal temperatures and pressure					
Degree of solubility in solvent	Solvent	Degree of solubility at 50 mg/ml	Stability in solvent	Solvent	Degree of solubility at 50 mg/ml	Stability in solvent
	Water	Soluble	*	DMSO	-	-
	Acetone	-	-	-	-	-

Solubility data generated by the testing facility

* No visible change in test material formulations throughout each experiment, therefore, assumed stable

**N-ACETYL-L-HYDROXYPROLINE: REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI***

Appendix 1 (continued)

2. TESTER STRAINS

Tester strain	Obtained from	Date obtained
TA100	UCB*	4 August 1995
TA1535	UCB*	4 August 1995
WP2uvrA	BIBRA**	17 August 1987
TA98	UCB*	4 August 1995
TA1537	UCB*	4 August 1995

3. S-9 MIX

(1) Source of S9

(encircle the applicable number and fill in the relevant entries)

Made in-house or purchase	<input checked="" type="radio"/> 1. Made in-house	2. Purchase (Supplier)
Prepared on	17 March 2001	
Lot No. (in case of purchase)	-	
Storage temperature	-196°C	

(2) Preparation of S9

Animal used		Inducing substance	
Species strain	Rat Sprague-Dawley	Name	Phenobarbitone/ β -naphthoflavone
Sex	Male	Administration method	Oral
Age (in weeks)	7 weeks	Administration period and amount (mg/kg bodyweight/day)	3 days
Weight	~ 250g		

(3) Composition of S9-Mix

Constituents	Amount in 1 ml S9-Mix	Constituents	Amount in 1 ml S9-Mix
S9	0.1 ml	NADPH	4.0 μ mol
MgCl ₂	8.0 μ mol	NADH	4.0 μ mol
KCl	33.0 μ mol	Na-phosphate buffer	100.0 μ mol
Glucose-6-phosphate	5.0 μ mol	Others ()	
Glucose-6-phosphate dehydrogenase			

* UCB: University of California at Berkeley
** BIBRA: British Industrial Biological Research Association

N-ACETYL-L-HYDROXYPROLINE: REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

Appendix 1 (continued)

4. PREPARATION OF THE TEST MATERIAL IN SOLUTION

(encircle the applicable response)

	Name	Supplier	Lot No.	Grade	Purity (%)
Solvent used	Sterile distilled water	Norton Healthcare	A0169	-	-
Reason for selection of solvent	Soluble at 50 mg/ml				
Appearance of the test material preparation	<u>Solution</u>	Suspension	Others ()		
Suspension and other methods when test substance difficult to dissolve	Solution achieved by action on an autovortex mixer				
Storage time and temperature of solution from preparation until use	40 minutes room temperature (range-finding study) 45 minutes room temperature (main study)				
Conversion of purity	Yes			<u>No</u>	

5. CONDITIONS OF PRE-CULTURE ETC.

(1) Conditions

Nutrient Broth	Name	Manufacturer	Lot No.
	Oxoid	Oxoid Ltd.	241116 11/05
Period of pre-culture	10 hr		
Culture flask (form, size)	Costar 75 cm ² tissue culture flasks		
Amount of culture medium	5 ml	Amount of strain inoculated	20µl

(2) Cell viability at the end of pre-culture

		Base-pair substitution type				Frameshift type		
		TA100	TA1535	WP2uvrA ⁻		TA98	TA1537	
Cell number (x 10 ⁹ /ml)	Range-finding study	1.2	3.5	3.2		1.4	1.2	
	Main study	1.7	1.9	2.8		1.1	1.4	
Count method (encircle the applicable number)		1. Conversion from O.D. value <u>2.</u> Stepwise dilution method 3. Others ()						

**N-ACETYL-L-HYDROXYPROLINE: REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI***

Appendix 1 (continued)

6. MINIMUM GLUCOSE AGAR PLATE MEDIUM

(encircle the applicable number and fill in the relevant entries)

Made in-house or purchase	1. Made in-house 2 Purchase (Fred Baker Scientific)
Prepared on	09 April 2001 (range-finding study) 23 April 2001 (main study)
Lot No. in case of purchase	215118 10/01 217135 10/01
Name of supplier and lot no. of the used agar	-

7. TEST METHOD (encircle the applicable number and fill in the relevant entries)

(1) Test Method and the reason why it was selected

Test method used	1. Pre-incubation method 2 Plate incorporation method 3. Others ()
Reason in case of others	-

(2) Test Conditions

		Pre-incubation	Plate Method
Composition	Bacterial suspension	ml	0.1 ml
	Test substance solution	ml	0.1 ml
	Na-phosphate buffer	ml	0.5 ml
	S9-mix (in case of metabolic activation method)	ml	0.5 ml
	Top agar solution	ml	2.0 ml
	Others ()	ml	-
Pre-incubation	Temperature	°C	-
	Time	min	-
Incubation	Temperature	°C	37°C
	Time	hours	48 hours

**N-ACETYL-L-HYDROXYPROLINE: REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI***

Appendix 1 (continued)

8. COUNT METHOD OF COLONIES (Encircle 1 and 2 if both methods used)

Count method	1. By hand	② Colony counter
Conduction of correction	1. No	② Yes (Correction method: Area and miscount correction)

9. TEST RESULTS

(1) The results should be reported on the attached form

(2) Judgement of the results

Judgement (encircle one)	Positive	Negative
Reason for judgement: No significant increase in the frequency of revertant colonies was recorded for any bacterial strain used with any dose of the test material in two separate experiments either with or without metabolic activation.		

(when judged as positive, attach a table for relative activity)

(3) Referential matters

Sterile distilled water was selected as the vehicle for this study because the test material was soluble at 50 mg/ml (the most concentrated stock dilution) in solubility checks performed in this laboratory.

For a substance to be considered positive in this test system, it should have induced a reproducible, dose-related and statistically (Dunnett's method of linear regression(5)) significant increase in the revertant count in at least one strain of bacteria.

Phenobarbitone/ β -naphthoflavone was used to induce higher enzyme levels in the rat liver S9. The S9-mix used in each experiment of this study was shown to be sterile. The test material formulations showed no evidence of contamination when tested for sterility during the preliminary toxicity test. The use of approximate half-log dilutions instead of twofold dilutions was agreed with the JMOL in January 1995.

[REMARKS] "Referential matters" - (fill in the view etc of the Study Director on the test results)

10. OTHERS

	Name	Safepharm Laboratories Ltd.		
	Address	P.O. Box 45, Derby, United Kingdom	Tel No: 0044 1332 792896 Fax No: 0044 1332 799018	
Study Director	Title	Genetic Toxicologist	Name	A J Bowles BSc (Hons)
	Years of experience: 6			
Study number	732/091			
Test dates	23 April 2001		29 AUG 2001	
	Protocol authorised by Study Director		Final report authorised by Study Director	

**N-ACETYL-L-HYDROXYPROLINE: REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI***

Appendix 2 History Profile of Vehicle and Positive Control Values

COMBINED VEHICLE AND UNTREATED CONTROL VALUES 1999

Strain S9-Mix	TA100		TA98		TA1535		TA1538		TA1537		WP2uvrA*		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Mean	102	107	24	31	22	16	13	19	11	17	23	27	263	298
SD	19	20	6	7	6	4	5	5	3	4	5	6	42	32
Min	57	63	10	12	10	7	5	8	3	5	10	11	188	222
Max	179	186	46	56	40	35	32	31	25	26	52	46	352	363
Values	751	592	646	510	645	488	57	31	634	481	641	495	89	37

POSITIVE CONTROL VALUES 1999

Strain S9-Mix	TA100		TA98		TA1535		TA1538		TA1537		WP2uvrA*		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Mean	549	1019	146	312	368	245	327	446	1017	345	787	598	882	737
SD	176	378	32	129	237	60	157	184	307	152	251	232	186	117
Min	238	406	95	113	104	123	113	215	350	112	200	208	583	509
Max	1170	2060	290	717	1229	467	653	837	2031	870	1760	1294	1673	1261
Values	161	160	162	160	158	157	27	27	159	158	153	152	52	51

COMBINED VEHICLE AND UNTREATED CONTROL VALUES 2000

Strain S9-Mix	TA100		TA1535		WP2uvrA*		TA102		TA98		TA1537		TA1538		WP2uvrA* pkm101		TA97a	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Mean	115	118	22	16	22	24	284	312	23	32	12	16	14	31	222	198	102	125
SD	22	22	5	4	5	6	41	35	6	6	4	4	6	3	99	42	14	4
Min	63	63	8	9	8	10	193	224	11	13	4	8	8	29	113	168	82	122
Max	198	181	40	31	45	47	381	377	55	63	25	27	21	33	377	228	123	127
Values	801	621	761	588	706	540	149	81	764	594	749	575	6	2	5	2	6	2

POSITIVE CONTROL VALUES 2000

Strain S9-Mix	TA100		TA1535		WP2uvrA*		TA102		TA98		TA1537		TA1538		WP2uvrA* pkm101		TA97a	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Mean	488	1620	382	278	663	664	877	794	138	259	1071	466	225	607	2089	1389	261	685
SD	137	490	260	89	243	212	137	123	34	70	327	121	28	40	175	967	1	141
Min	234	443	125	128	203	185	522	388	89	129	290	191	193	568	1965	705	260	585
Max	1190	3079	1843	630	1520	1534	1161	1090	262	506	2160	768	245	648	2213	2073	261	785
Values	172	172	172	170	166	166	69	70	173	172	171	170	3	3	2	2	2	2

SD = Standard deviation
Min = Minimal value
Max = Maximum value

Appendix 3 Statement of GLP Compliance in Accordance with Directive 88/320/EEC

**THE DEPARTMENT OF HEALTH OF THE GOVERNMENT
OF THE UNITED KINGDOM****GOOD LABORATORY PRACTICE****STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC****LABORATORY**

**SafePharm Laboratories Ltd
Shardlow Business Park
London Road
Shardlow
Derbyshire
DE72 2GD**

TEST TYPE

**Analytical Chemistry
Environmental Fate
Environmental Toxicity
Mutagenicity
Phys/Chem Tests
Toxicology**

DATE OF INSPECTION

28 February 2000

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander
26/4/00

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority

SAFEPHARM LABORATORIES LIMITED

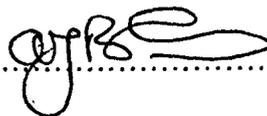
N-ACETYL-L-HYDROXYPROLINE:

**REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI***

SPL PROJECT NUMBER: 732/091

I verify that this is an exact copy of the original report which is located in the Archives of SafePharm Laboratories Limited, Derby, UK.

A J Bowles BSc (Hons)
Study Director



DATE

31 AUG 2001