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IN-VITRO MUTAGENIC EVALUATION OF THE SULFASALAZINE IMPURITY (2-HYDROXY-5-[N-(4-{[4 -PYRIDINE- 2 -YLSULFAMOYL PHENYL] SULFAMOYL}PHENYL)DIAZEN-1-YL]) BENZOIC ACID BY BACTERIAL REVERSE MUTATION TEST

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Abstract

Sulfasalazine is anti-inflammatory drug that was used in Rheumatoid arthritis and Ulcerative colitis. Pharmaceutical impurities are existing along with drug substances. Hence it is mandatory requirement to do safety studies of each impurity. The present study was performed to evaluate the mutagenic potential of Sulfasalazine Impurity (2-hydroxy-5-[N-(4-{[4-pyridine-2-ylsulfamoyl) phenyl]sulfamoyl}phenyl)diazen-1-yl]) benzoic acid in the Bacterial Reverse Mutation Assay using five tester strains. In this experiment there was no significance results were reported to establish the impurity as Mutagenic nature.

Key Words : Bacterial Reverse Mutation Assay, Reverent Colonies, Mutation, Toxicology, Sulfasalazine, and (2-hydroxy-5-[N-(4-{[4-pyridine-2-ylsulfamoyl) phenyl]sulfamoyl}phenyl)diazen-1-yl]) benzoic acid.

Introduction:

Sulfasalazine (SSZ) was devised by Dr. Nana Svartz, a Scandinavian rheumatologist and a professor of medicine at the Karolinska Institute in Stockholm, with the cooperation of Swedish pharmaceutical company Pharmacia in 1941 (Sabha Mushtaq and Rashmi Sarkar,2020).

An attempt was made to treat rheumatoid arthritis, which was assumed to be originated from bacteria. SSZ resulted due to failure in concomitant results produced by sulphonamides and Aspirin. The research was further continued by combining these two drugs resulting from SSZ which shows effective results on rheumatoid arthritis by producing antibiotic nature along with a binding affinity for connective tissue. The impressive results extended to ulcerative colitis (Dover,1971; svartz,1948; Watkinson,1986)along with dermatological application in 1971 in scleroderma (Dover,1971).

Sulfasalazine resulted due to a covalently linked azo bond formed between Mesalamine/Mesalazine 5-amino salicylic acid and a sulphonamide (sulfapyridine). Based on the oral bioavailability studies approximately 10% to 30% of the SSZ is absorbed in the small intestine and its derivative, sulfapyridine,produced in large intestine by azo reduction of bacterial azo reductases is absorb almost all [i.e >90%] where as another derivative mesalazine is absorbed only 20% to 30%, the remaining is eliminated through faeces (Sabha Mushtaq and Rashmi Sarkar,2020).

SSZ is not only distributed throughout the body but also crosses the placenta and even enters into breast milk, but it fails to cross the blood-brain barrier. SSZ concentrations in breast milk were negligible when it compared with plasma concentrations,

sulfapyridine concentration was 40%in comparison to plasma. It is unlikely to find mesalazine in breast milk due to its very low availability. SSZ sulfapyridine concentrations formed low in synovial fluid than those with plasma. (Rains CP and Noble S, 1995).

The administered SSZ was metabolized to some extent (Reference ID: 3229177), in the liver, and some proportion is eliminated through urine as unchanged (Rains CP and Noble S, 1995). In the case of sulfapyridine, some of which are eliminated through urine, some of which are metabolized to N-acetyl-sulfapyridine (inactive)in the liver. Further, sulfapyridine and its metabolite undergo hydroxylation and glucuronidation, leading to the formation of glucuronide conjugates and hydroxylated metabolites; these are eliminated through urine (Rains CP and Noble S, 1995) (Reference ID: 3229177). Mesalazine is mainly [70% - 80%] eliminated unchanged in feaces and its metabolite N-Acetyl mesalazine eliminated through urine. Sulfapyridine metabolism was affected by changing its elimination half-time between 5 to 18 hours by the acetylation phenotype (Tett SE, 1993).

SSZ is associated with two moieties that exhibit a different mechanism of action. Sulfamoiety has antimicrobial activity, whereas salicylate moiety is known for its anti-inflammatory activity (Tett SE, 1993). SSZ shows anti-inflammatory or immunomodulatory actions with different mechanisms, which include 1. Inhibition of cytokine release, 2. Inhibition of monocyte-macrophage cytokines IL-1,6,12, & TNF, 3. Inhibition of 5-lipooxygenase pathway,4. Inhibition of expression of adhesion molecules, 5. Reduction of chemotoxis and random migration of inflammatory secretory, 6. Reduction of prostaglandin E2 synthetase, 7. Apoptosis of Neutrophils, and 8. Reduction of the extracellular release of pro-inflammatory secretory phospholipase A2; SSZ also produces antiproliferative actions including 1. competitive inhibitor of brush folate conjugate enzyme, 2. Decrease of polyglutamyl folate hydrolysis, 3. Decrease in the absorption of both polyglutamyl and monoglutamyl folate (Plosker GL and Croom KF, 2005). In addition to the above antibacterial activity of SSZ includes competitive inhibition of bacterial enzyme dihydropteroate synthetase, and folic acid synthesis (Rains CP and Noble S, 1995) (Tett SE, 1993).

Bacterial Reverse Mutation Test:

Mutagenicity identification of a substance is a significant procedure in safety evaluation. Substances that are mutagenic can potentially alter the germ line, there by remains severe problems in future generations along with fertility issues and induction of cancer (OECD test guideline no: 471, 1997) (Kristien M and Errol Z, 2000).

Mutagenicity testing program can be used for the identification of point and /or deletion or insertion of small/large fragment of DNA and / or a large part of DNA might be altered or rearranged (Dorothy M *et al*, 1981).

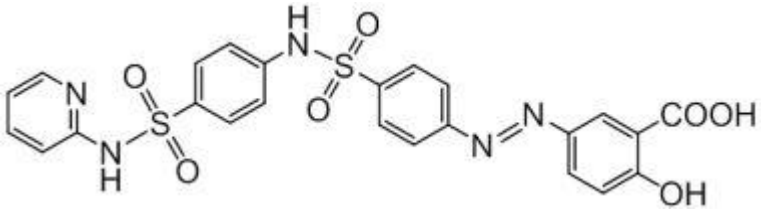
Identification of gene mutation or chromosomal damage in mammalian cells is very difficult when compared to bacterial and/or other cellular organisms. AMES Test / Salmonella test / BRMT is a well characterized and established procedure for the identification of genetic damage that leads to mutation by chemical substances(D M Maron and B N Ames, 1983). Different salmonella strains that are unable to synthesize particular amino acid, histidine, because of different pre-existing mutations in histidine operon one used on the Ames test. Any substance which has mutagenic ability will reverse the mutation and results in the growth of the strain in medium lacking histidine. The permutated salmonella strains used in this experiment are designed and differentiated from each other by their position of mutation, and it's nature of the mutation (D M Maron and B N Ames, 1983) (S Venitt and J M Parry, 1984). In addition to this, the mutation is engineered to make strains more sensitive so that a wide range of substances can be tested for their mutagenic nature. This BRMT was specifically designed to identify the chemical-induced mutagenic nature of the substances (D M Maron and B N Ames, 1983) (OECD Guideline No:473). The genotype and mutation nature of the salmonella tester strains that are used in this experiment are given below in Table 1:

Mutated Strain	Target DNA	Reversion	Operon
TA100	-G-G-G-	Base pair Substitution	hisG46
TA1535			
TA98	-C-G-C-G-C-G-C-G-	Frameshift	hisD3052
TA1538			
TA1537	+1 frameshift (near-C-C-C-run)	Frameshift	hisC3076
TA97	-C-C-C-C-C-C- (+1 cytosine at a run of C's)	Frameshift	hisD6610
TA102	TAA	Transition/Transversions	hisG428

SSZ - In house impurity : (2-hydroxy-5-[N-(4-{[4 -pyridine- 2 -ylsulfamoyl) phenyl] sulfamoyl}phenyl)diazen-1-yl]) benzoic acid .

To date, several impurities were reported for SSZ, and in those, some are well characterized and were reported in Pharmacopeia also. (2-hydroxy-5-[N-(4-{[4 -pyridine- 2 -ylsulfamoyl) phenyl] sulfamoyl}phenyl)diazen-1-yl]) benzoic acid is a novel impurity identified recently, was our interest in this experiment. It is still unclear whether this impurity is generated or evolved as a process impurity or degradative impurity. Chemical Abstract Number (CAS) was not allotted this impurity so far. Based on the available information from various sources such as regulatory agencies, research articles, and other sources, the structure and other detilas

of the impurity given in Picture -1., the molecular formula recorded as $C_{24}H_{19}N_5O_7S_2$, and the molecular weight reported as 553.36.



Molecular Formula: $C_{24}H_{19}N_5O_7S_2$
Molecular Weight : 553.36

Picture -1 – Structure , Molecular formula and weight of (2-hydroxy-5-[N-(4-{[4 -pyridine- 2 -ylsulfamoyl) phenyl] sulfamoyl}phenyl)diazen-1-yl]) benzoic acid .

Materials and Methods:

Materials:

SSZ impurity was supplied by Prathira Technologies LLP, India, with a purity of 94.32%, where it was synthesized and characterized; provided with a characterization report.

Selective strains considered as a test system, which are obtained by using specific mutagens at different genes of Histidine operon, with site specific mutated strains of ***S.typhimurium***, were used in this experiment. The selective strains were identified based on the type of mutation used and with and without the metabolic activation. DMSO was used as a negative control, and different chemicals, which are 9- Aminoacridine (TA1537), Sodium Azide (TA1535 & TA100), 2-Nitroflourene (TA98), Mitomycine-C (TA 102) for without metabolic activation with 50 µg/ml, 5 µg/ml, 7.5 µg/ml, and 0.5 µg/ml respectively and 2 Amino anthracene for with metabolic activation for all stains were used as positive controls; these were considered the test controls. Along with the test system and controls, metabolic activation was used, and ingredients were given as S9 fraction and Co Factor Mix (D Glucose-6- Phosphate, β NADP, $MgCl_2$, KCl, Na_2HPO_4, $NaH_2PO_4.H_2O$). Different media including Oxoid nutrient broth No;2, Top Agar medium, and Minimal glucose agar medium were used in this experiment.

Methodology:

Overnight cultures of each strain of frozen cultures in Oxoid nutrient broth no:2 with count not less than 1-2 X 10^9 CFU/ml based on the optical density of the cultures checked at 660nm wavelength, were used as test system in this experiment.

A pre-test was conducted to understand the toxicity of the test substance. Based on the solubility and precipitation DMSO identified as solvent and highest concentration for pre-test was selected as5.0 mg/Plate (i.e 50 mg/ml). A total of eight concentrations of test substances were prepared by serial dilutions with highest concentration as given above. The concentrations were recorded as 0.039, 0.078,.0.156,0.312,0.625,1.25,2.5, and 5.0 mg/plate respectively. TA98 and TA100 strains were used both in the presence and absence of (5 %) metabolic activation in pre -test. Doses were prepared by dissolving test substances in DMSO by serial dilution and subsequently lowest concentration was obtained. For the pre-test, 50 mg/ml stock was obtained by dissolving 200.01 mg of the test item in 4 ml DMSO. From the stock solutions by serial dilutions, eight concentrations of 12.5 mg/ml, 6.25 mg/ml, 3.12 mg/ml, 1.56 mg/ml, 0.78 mg/ml, and 0.39 mg/ml, along with the stock solution, were obtained.

The experiment executed in two parts, part one considered as plate incorporation method. Based on the results obtained in part -1 , execution of part-2 will decide. Part -2 is called pre incubation method.

For both parts in the experiment, a stock solution of 50 mg /ml was obtained by dissolving 700 mg of the test substance in 14 ml of DMSO. The stock solution of 50 mg/ml, 25 mg. ml, 12.5 mg/ml, 6.25 mg/ml, and 3.12 mg/ml concentrated solutions were obtained by serial dilutions respectively. Desired final testing solutions were obtained by adding 100µl of the above concentration solutions to top agar media for the treatment of the cell both in the presence and absence of the metabolic activation. Simultaneously positive control was added to the top agar and Negative control was added to the top agar.

For part-2test substance, the S9 mixture was kept for incubation at 37^0C ,200 RPM FOR 20 MIN. After completion of incubation, this mixture was mixed with top agar and used for treatment. Hence it is identified as pre incubation method.

For controls previously mentioned controls with specified concentrations were added to solutions. These controls solutions are not included with test substances. these controls were used both part-1 & 2

The final prepared solution of both the parts including controls were poured on minimal glucose agar medium plates. These plates were incubated at 37^{O}C for 48 to 72 hours. After incubation colony count was calculated for each plate and recorded. Further to colony count statistically significant also check for better understanding the results.

In this experiment there was no significance identified in part-1 hence part-2 was executed.

Results:

Experiment observations were recorded as colony count for pre-test, parts 1 and 2 of the experiment. Hand counting method was used to count the reverent colonies in this experiment. Back ground lawn for each plate was checked for toxicity of the test item. There is no significant lawn thinning was observed in each plate. The pre-test was performed for *Salmonella typhimurium* strains of TA100, and TA98 at concentrations of 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, and 5.0 mg/plate in both presence and absence of 5 % V/V metabolic activation system. All the doses were tested in duplication. Observations in pre-test recorded as reduction or clearing of background lawn or diminution of bacterial & reduction in revertant colony count. In this pre-test, no significant observation for any above-mentioned reduction was recorded hence the highest concentration of 50 mg/ml i.e 5 mg/plate was considered the highest dose for the main experiment. The results were recorded and reported in table -2.

TABLE-2

REVERTANT COLONY COUNT - PRETEST

Dose (mg/plate)	TA 98		TA 100	
	(-S9)	(+S9)	(-S9)	(+S9)
NC (0)	NI (20)	NI (20)	NI (117)	NI (108)
	NI (21)	NI (22)	NI (111)	NI (101)
T1 (0.039)	NI (22)	NI (22)	NI (116)	NI (98)
	NI (20)	NI (23)	NI (119)	NI (109)
T2 (0.078)	NI (21)	NI (22)	NI (99)	NI (96)
	NI (22)	NI (20)	NI (109)	NI (101)
T3 (0.156)	NI (20)	NI (22)	NI (122)	NI (123)
	NI (28)	NI (19)	NI (127)	NI (145)
T4 (0.312)	NI (21)	NI (21)	NI (112)	NI (111)
	NI (19)	NI (22)	NI (123)	NI (96)
T5 (0.625)	NI (19)	NI (19)	NI (131)	NI (133)
	NI (21)	NI (21)	NI (125)	NI (121)
T6 (1.25)	NI (18)	NI (21)	NI (157)	NI (123)
	NI (22)	NI (23)	NI (142)	NI (112)
T7 (2.5)	NI (21)	NI (20)	NI (149)	NI (125)
	NI (22)	NI (22)	NI (163)	NI (136)
T8(5)	NI (21)	NI (20)	NI (129)	NI (137)
	NI (22)	NI (23)	NI (151)	NI (144)

NI: No inhibition; NC: Negative Control; T: Test concentration

The reported results are accepted in this experiment since the control results were within the acceptance limit. The negative control recorded no significant growth in plates simultaneously significant growth was observed in positive control plates. As mentioned above in methods, this experiment was carried out in two parts: Part-1 and Part -2 since there were no significant observations recorded in Part-1

The part I executed with TA1535, TA1537, TA98, TA100, & TA102tester strains for five concentrations of 0.312, 0.625, 1.25, 2.5, and 5.0 mg/plate both in the presence and absence of metabolic activation with treatment and incubation carried for 48 hours at 37 ± 2^0C. Manually counting colonies is a method of recording results in this experiment, and results were reported.

The above-reported results were analyzed statistically for significance by means, standard deviation (SD), and Fold increase (FI). The mean is calculated for triplets of each concentration & each strain. SD was calculated for the same. Fold increase was calculated with several times increase in average colony number of each strain of each concentration to its respective negative control. Details of mean, SD, and FI fortrail-1 both in the absence and presence of metabolic activation (S9 mix) are reported in table-3 and 4.

Table-3

MEAN, STANDARD DEVIATION, AND FOLD INCREASE OF REVERTANT COLONY COUNT – MAIN STUDY (TRIAL I)

In the absence of Metabolic Activation(-S9)

		NC	T1(0.312)	T2(0.625)	T3(1.25)	T4(2.5)	T5 (5)	PC
TA 1537	**MEAN**	6.67	5.67	7.00	7.00	7.00	5.33	147.00
	SD	1.53	2.08	1.73	1.73	1.00	1.53	12.53
	FI	NA	0.85	1.05	1.05	1.05	0.80	22.05
TA 1535	**MEAN**	10.67	11.00	12.33	12.67	10.33	10.33	600.67
	SD	2.52	2.00	2.08	0.58	1.15	3.21	43.52
	FI	NA	1.03	1.16	1.19	0.97	0.97	56.29
TA 98	**MEAN**	20.67	19.33	21.00	20.33	22.33	19.00	610.00
	SD	2.52	1.53	3.00	3.21	1.15	1.73	20.81
	FI	NA	0.94	1.02	0.98	1.08	0.92	29.51
TA 100	**MEAN**	113.67	102.33	107.67	106.67	103.67	110.33	1039.7
	SD	5.03	5.13	9.50	7.09	3.79	8.08	52.04
	FI	NA	0.90	0.95	0.94	0.91	0.97	9.15
TA 102	**MEAN**	352.67	388.00	380.00	383.00	397.00	373.00	1146.0
	SD	4.73	9.54	16.52	12.17	8.00	16.37	50.57
	FI	NA	1.10	1.08	1.09	1.13	1.06	3.25

NC: Negative Control**; T:** Test concentration; **PC**: Positive control, **SD**: Standard Deviation, **NA**: Not Applicable, FI: Fold Increase.

Fold Increase = Mean of Colony Test concentration / Mean of Negative control colony

Table-4

MEAN, STANDARD DEVIATION, AND FOLD INCREASE OF REVERTANT COLONY COUNT – MAIN STUDY (TRIAL I)

In the Presence of Metabolic Activation(+S9)

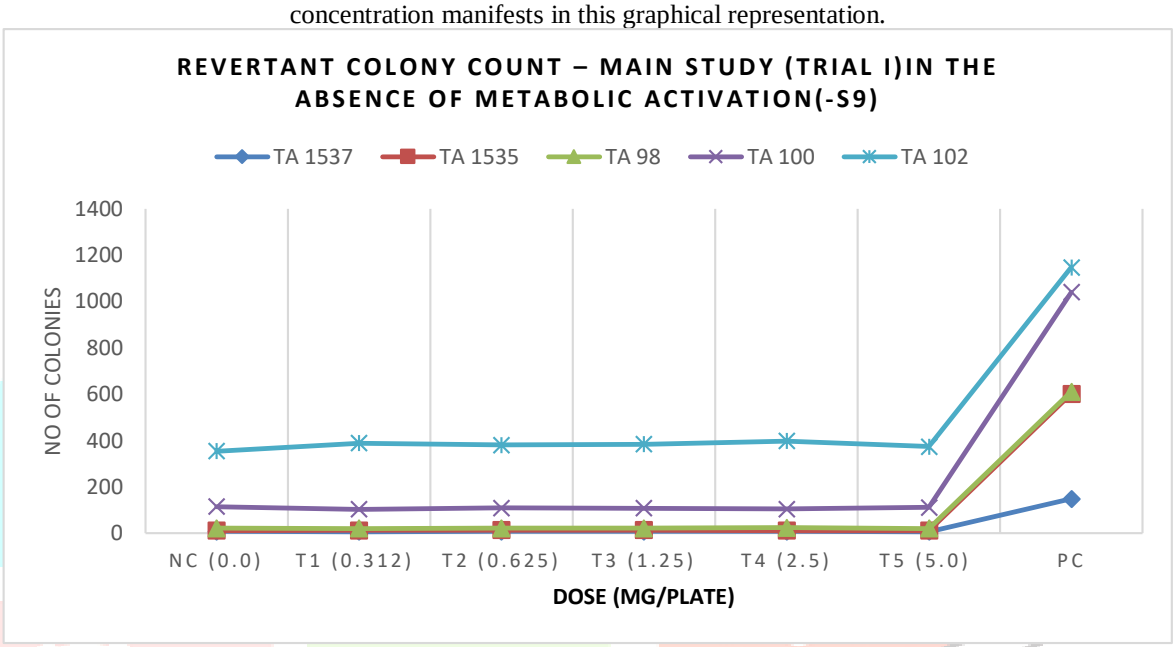
		NC	T1(0.312)	T2(0.625)	T3(1.25)	T4(2.5)	T5 (5)	PC
TA 1537	**MEAN**	6.67	8.00	6.67	8.33	7.67	6.33	144.67
	SD	1.53	1.00	2.08	0.58	1.53	1.53	8.14
	FI	NA	1.20	1.00	1.25	1.15	0.95	21.70
TA 1535	**MEAN**	6.67	11.00	9.33	9.67	9.67	9.67	526.00
	SD	1.53	1.73	1.53	2.08	2.31	1.15	30.81
	FI	NA	1.65	1.40	1.45	1.45	1.45	78.90
TA 98	**MEAN**	21.33	20.33	19.67	18.00	18.00	16.33	689.33
	SD	3.51	1.53	1.53	1.00	2.65	1.53	15.63
	FI	NA	0.95	0.92	0.84	0.84	0.77	32.32
TA 100	**MEAN**	109.33	106.33	105.67	101.67	117.00	117.00	1092.3

	SD	3.51	11.15	3.51	10.02	4.58	2.00	33.25
	FI	NA	0.97	0.97	0.93	1.07	1.07	9.99
TA 102	**MEAN**	377.67	392.33	478.67	395.33	445.67	454.33	1207.3
	SD	19.55	9.61	20.01	45.06	55.41	38.42	60.14
	FI	NA	1.04	1.27	1.05	1.18	1.20	3.20

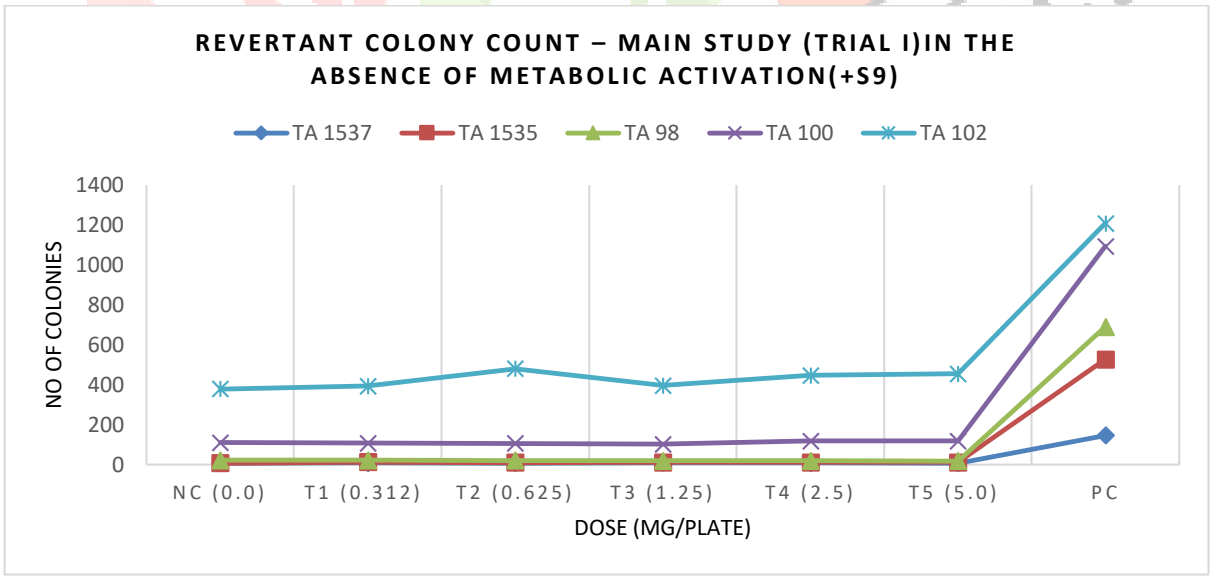
NC: Negative Control**; T:** Test concentration; **PC**: Positive control, **SD**: Standard Deviation, **NA**: Not Applicable, FI: Fold Increase.

Fold Increase = Mean of Colony Test concentration / Mean of Negative control colony

Further statistical analysis was carried out for the mean of the tester strain concentrations by plotting line graphs. The results of graphical data for trail-1 in the absence of S9 were represented in graph-1 and in the presence of S9 were represented in graph-2. The fold increase of reverent colonies for each tester strain and concentration manifests in this graphical representation.



Graph -1: Representation of the number of reverent colonies of each strain of five concentrations along with positive and negative controls – Absence of S9.



Graph -2: Representation of the number of reverent colonies of each strain of five concentrations along with positive and negative controls – Presence of S9.

Experiment part-2 was similar to experimental I except for the addition of the pre-incubation period with the S9 mix before treatment. The remaining procedure is the same and results were recorded and reported. There was no significant increase in reverent colonies observed in both parts of experiments with compare to the positive control. Further to the colony counting method experiment was continued with statistical analysis by Mean, Standard Deviation (SD), and fold increase calculation. Manually counting colonies is a method of recording results in this experiment, and results were reported.

Part-2 results were analysed statistically for significance by calculating means, standard deviation (SD), and Fold increase (FI); the same as in trail-1. The mean is calculated for triplets of each concentration & each strain. SD was calculated for the same. Fold increase was calculated with the number of times increase in average colony number of each strain of each concentration to its respective negative control. Details of mean, SD, and FI for trail-1 both in the absence and presence of metabolic activation (S9 mix) are reported in table-9 and 10.

Table-9

MEAN, STANDARD DEVIATION, AND FOLD INCREASE OF REVERTANT COLONY COUNT – MAIN STUDY (TRIAL II)

In the absence of Metabolic Activation(-S9)

		NC	T1(0.312)	T2(0.625)	T3(1.25)	T4(2.5)	T5 (5)	PC
TA 1537	**MEAN**	6.33	6.00	8.00	7.67	6.00	7.00	150.67
	SD	0.58	2.00	1.00	1.53	2.00	1.00	15.57
	FI		0.95	1.26	1.21	0.95	1.11	23.80
TA 1535	**MEAN**	10.33	9.67	9.33	8.00	9.00	8.67	565.33
	SD	1.53	1.15	1.53	2.65	2.00	1.53	32.53
	FI		0.94	0.90	0.77	0.87	0.84	54.73
TA 98	**MEAN**	20.00	19.00	20.67	20.33	19.33	20.00	617.67
	SD	1.00	1.00	1.53	2.08	2.52	2.65	27.15
	FI		0.95	1.03	1.02	0.97	1.00	30.88
TA 100	**MEAN**	111.00	120.00	103.67	109.33	112.33	103.33	1075.33
	SD	9.17	1.00	7.02	11.93	13.20	12.50	70.78
	FI		1.08	0.93	0.98	1.01	0.93	9.69
TA 102	**MEAN**	247.67	343.33	366.33	332.33	361.00	297.67	1077.33
	SD	103.84	28.92	28.71	32.32	41.04	40.08	45.00
	FI		1.39	1.48	1.34	1.46	1.20	4.35

NC: Negative Control**; T:** Test concentration; **PC**: Positive control, **SD**: Standard Deviation, **NA**: Not Applicable, **FI**: Fold Increase.

Fold increase = Test concentration colony count mean / Negative control colony count mean

Table-10

MEAN, STANDARD DEVIATION, AND FOLD INCREASE OF REVERTANT COLONY COUNT – MAIN STUDY (TRIAL II)

In the Presence of Metabolic Activation(+S9)

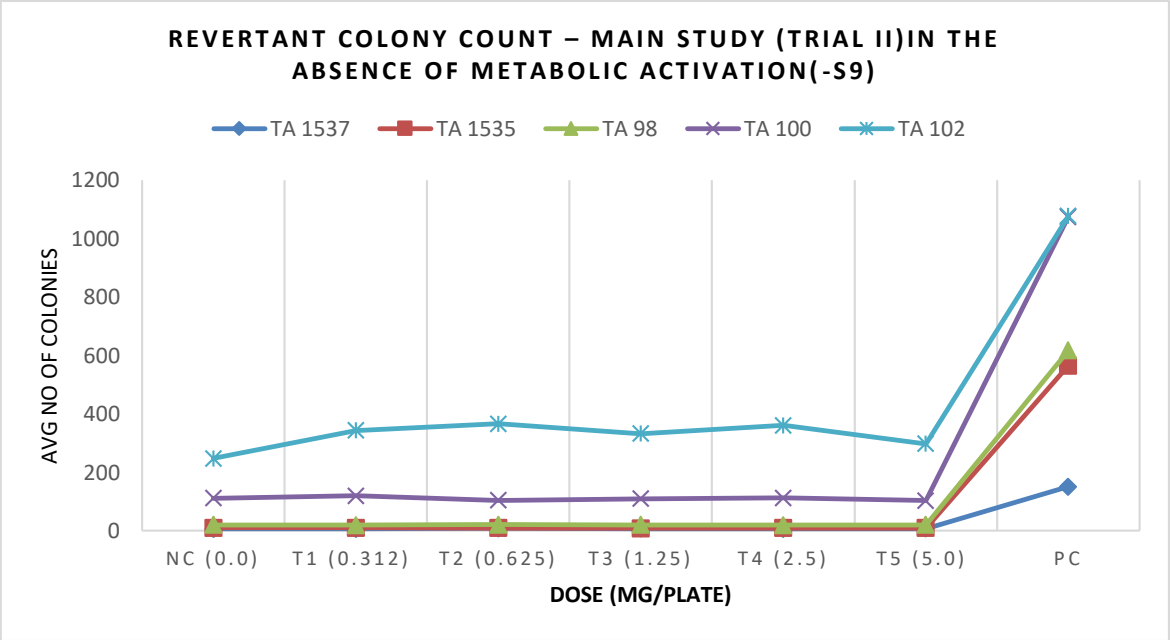
Strain		NC	T1(0.312)	T2(0.625)	T3(1.25)	T4(2.5)	T5 (5)	PC
TA 1537	**MEAN**	7.67	7.00	7.33	7.00	7.33	7.67	137.33
	SD	1.53	1.00	2.08	1.00	1.53	1.53	5.69
	FI		0.91	0.96	0.91	0.96	1.00	17.91
TA 1535	**MEAN**	8.00	7.33	9.33	9.00	8.33	7.00	549.67
	SD	1.00	1.15	2.08	2.00	1.15	1.00	12.90
	FI		0.92	1.17	1.13	1.04	0.88	68.71
TA 98	**MEAN**	20.00	19.67	20.00	18.67	18.33	19.00	598.67
	SD	3.00	1.15	2.65	2.08	2.52	2.00	28.18
	FI		0.98	1.00	0.93	0.92	0.95	29.93
TA 100	**MEAN**	121.00	110.00	103.33	114.00	120.67	102.67	1127.67
	SD	2.00	4.36	7.77	7.81	4.04	4.16	63.72
	FI		0.91	0.85	0.94	1.00	0.85	9.32
	MEAN	341.33	333.33	363.67	366.33	372.00	373.00	1121.33

TA 102	**SD**	26.63	18.01	15.70	19.04	15.39	34.39	55.75
	FI		0.98	1.07	1.07	1.09	1.09	3.29

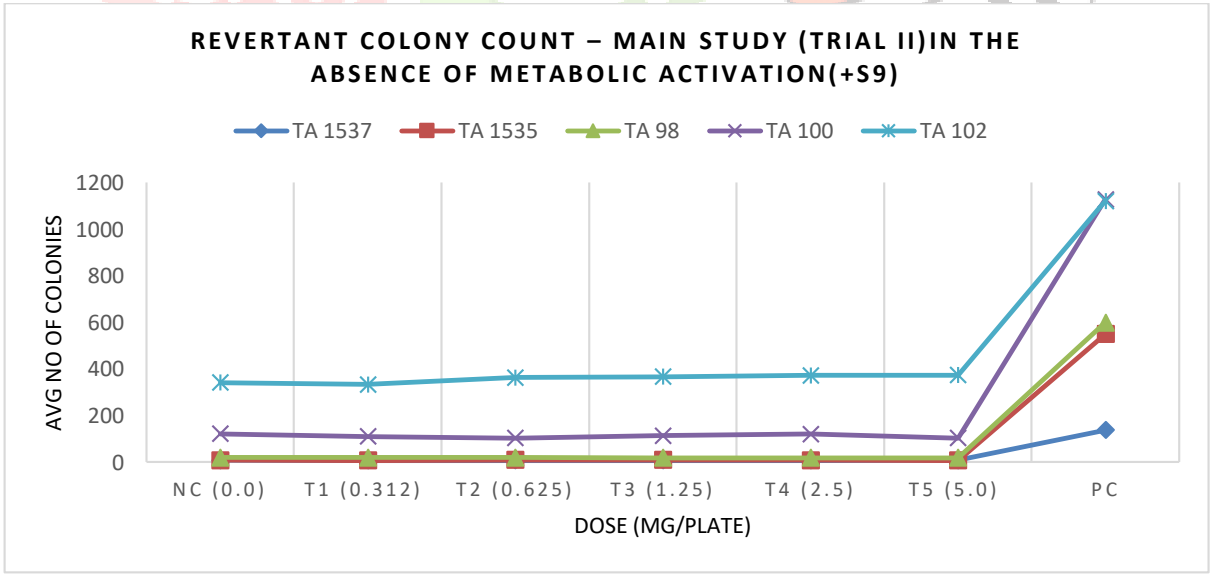
NC: Negative Control**; T:** Test concentration; **PC**: Positive control, **SD**: Standard Deviation, **NA**: Not Applicable.

Fold increase = Test concentration colony count mean / Negative control colony count mean

Further statistical analysis was carried out for the mean of the tester strain concentrations by plotting line graphs. The results of graphical data for trail-1 in the absence of S9 were represented in graph-3 and in the presence of S9 were represented in graph-4. The fold increase of reverent colonies for each tester strain and concentration manifests in this graphical representation.



Graph -3: Representation of the number of reverent colonies of each strain of five concentrations along with positive and negative controls – Absence of S9



Graph -4: Representation of the number of reverent colonies of each strain of five concentrations along with positive and negative controls – Presence of S9.

Criteria for positive result determination reported as a record of increase of revertant colonies per plate with or without metabolic activation by a dose-dependent increase or an increase in at least one test concentration in at least any one test strain. Two fold or more than two-fold increase in TA98, TA100, and TA102; three-fold or more than three-fold increase in TA1535 and TA1537 at one or more either in concentration or strains corresponds to negative control results was considered positive results thus test substance is considered as mutagenic at particular concentrations.

In this experiment, no fold increase was observed in any tester strain at any concentration both in the presence and absence of the metabolic activation in both parts of experiment (part 1 and 2).

Discussion:

Results in this experiment given as colony count number. These number were further analysed. There several statistical methods were reported in which fold increase wide accepted method for identifying the mutagenicity of test substance (L.S. Bernstein *et al*, 1982) (G.A.T. Mahon *et al*, 1989) . based on the sensitivity of the *Salmonella* tester strains to the frequency of the reverent colonies differential fold increase was calculated based on the type of test strains (N.F. Cariello and W.W. Piegorsch, 1996).Non-statistical procedures also reported to identify the mutagenicity of the test substances, where substance considered as positive when dose related increase in number reverent colonies observed either in one or more strains, no dose related increased observed it considered as negative and if no clear observation was recorded it is considered as inconclusive (E. Zeiger et al, 1992).

Biological relevance along with statistical analysis were used to considered and evaluating test results (G.A.T. Mahon et al, 1989). Results of this experiment must include but not limited to : Toxicity, Precipitation, Colony count of each plate, mean and standard deviation of each strain of each concentration, statistical information, concurrent positive and negative data, and dose dependent relationship (Redbook,2000) .

Data in this experiment was record and reported as mean of reverent colony number per concentration for each strain. The concentration of each plate reported in mg/plate. Also results in this experiment expressed as fold increase of reverent colonies with negative control. Addition to this Standard deviation was recorded for each concentration for each strain.

Criteria for determining a positive result is defined as a dose-dependent increase or a reproducible increase at one or more concentrations, in the number of revertant colonies per plate in at least one strain with or without a metabolic activation system. Two-fold/more than two-fold (TA 98, TA 100, and TA 102) or three-fold/more than threefold (TA 1537, TA 1535) dose-dependent increase in revertant colonies at one or more concentrations corresponding to negative (solvent/Vehicle) control was considered as a positive result.

In this study, a fold increase was not observed in any of the tester strains in this experiment (Part 1& 2). Furthermore, there were no significance observed in the test concentrations in this experiment. From the results of this study, it is considered that test item 2-hydroxy-5-[N-(4-{[4-pyridin-2-ylsulfamoyl)phenyl]sulfamoyl}phenyl)diazen-1-yl]benzoic acid is nonmutagenic at given concentrations in this experiment. The biological relevance of the results was considered for results evaluation.

This work is part of toxicological study of SSZ impurity - 2-hydroxy-5-[N-(4-{[4-pyridin-2-ylsulfamoyl) phenyl]sulfamoyl}phenyl)diazen-1-yl]benzoic acid. In this experiment mutagenicity nature of this substances was evaluated. After careful consideration and analysis of the results of BRMT, it is concluded that the test substance 2-hydroxy-5-[N-(4-{[4-pyridin-2-ylsulfamoyl) phenyl]sulfamoyl}phenyl)diazen-1-yl] benzoic acid is not showing any mutagenic effect.

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