In-Vitro Genotoxic Evaluation of the Sulfaslazine Impurity - (2-Hydroxy-5-[N-(4-{[4 -Pyridin- 2 -Ylsulfamoyl Phenyl] Sulfamoyl}Phenyl)Diazen-1-Yl]) Benzoic Acid by CHO Chromosomal Aberration Test

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Abstract : **(2-hydroxy-5-[N-(4-{[4 -pyridin- 2 -ylsulfamoyl phenyl] sulfamoyl}phenyl)diazen-1-yl]) benzoic acid is identified as impurity in Sulfasalazine drug. This impurity considered as in-house impurity since regulatory agencies not reported completely. It is mandatory to test toxicology effects of this impurity for regulatory compliance. The chromosome aberration test is one of the sensitive methods to identify or evaluate the mutagenicity of environmental mutagens and/or carcinogens using cultured mammalian cells. This study the clastogenic activity was tested by chromosomal aberration test by using CHO cell lines. Several chromosomal abnormalities were tested in this experiment. This experiment result was insignificant pertaining to Chromosomal aberrations. Chromosomes of Cell lines does not show any abnormality in any given concentration of the impurity.**

Key words: **Chromosome aberration, Impurity, Mutagen, Mammalian cells.**

I. INTRODUCTION

SSZ was introduced as a human drug several decades ago as anti-rheumatoid Arthritis later its effects spreads to various parts of the body. Even though SSZ has several medicinal significances, for few of them drug interactions were understood. (Helen K. H and Lauren K., 2018). Pharmacological effects of SSZ attributed to its breakdown products 5-Aminosalysilic acid (5-ASA) and Sulfapyridine (SP); precisely mechanism of action was not completely known. The antibacterial activity of SP and the anti-inflammatory activity of 5-ASA were reported but the mode of action was unclear (Namali Corea, 2007). Gastrointestinal effects of SSZ were reported in various experiments as mild to moderate medicinal applications for Ulcerative Colitis (UC) and mild application for Crohn's disease in pregnant women. But if SSZ was supplemented with folic acid, in some rare cases, reversible infant diarrhea was reported. Even though SSZ was compatible with breastfeeding, poorly excreted medication was found in breast milk.(Namali Corea, 2007).

The effectiveness of SSZ in the treatment of reactive arthritis is another significant medical application. SSZ therapy reported its improvement in histologic enterocolitis comes with reactive arthritis along with Ankylosing Spondylitis. Although the chemical response is unknown the interaction between spondyloarthropathies and the gut was demonstrated. (Nirupama B and Melissa S W,2020). Invitro studies show immunomodulatory effects by suppressing both B&T lymphocyte proliferation. This effect was also proved in invivo by a reduction in activated lymphocytes in SSZ-treated patients. Adding to this SSZ also shows inhibition of activation of transcription factor NF-KB (Tracy M.Frech and Daniel O.Clegg, 2007).

The mechanism of action of SSZ was not fully understood in both anti-inflammatory and immunomodulatory activity. However, it was understood that SSZ cleaved in the large intestine as 5-ASA and Sulfapyridine. SSZ was not recommended for patients with severe liver diseases, acute viral hepatitis, thrombocytopenia, and allergic conditions. SSZ reduces digoxin and cyclosporin levels leading to

an increase in the effects of warfarin (Ivan Padjen, 2018).

SSZ was metabolized as 5-ASA and SP, absorbed intactly in the small intestine, and deliver to the large intestine as enterohepatic circulation. The action of gut flora resulted from the division of SSZ where SP was absorbed and 5-ASA remains in Lumen. The absorbed SP moiety is metabolized by hydroxylation and acetylation. Based on the duration of acetylation toxicity of the compound depends (Kevin D.Deane and Sterling G.West, 2009).

SSZ medication also causes side effects in human studies, but the level was not significant. Patients with allergic to sulfa-containing compounds should avoid the SSZ medication (Kevin D.Deane and Sterling G.West, 2009). In addition to sympathetic and inflammation benefits in RA by SSZ, it can also slow the erosive disease progression. But it is still unknown what the mode of action of SSZ is in the treatment of RA in monotherapy. More severe reactions can occur by SSZ, mainly allergic but not limited to, including rashes, fever, hepatitis, pneumonitis, and aplastic anemia (Kevin D.Deane and Sterling G.West, 2009).

CHROMOSOMAL ABERRATIONS TEST

The chromosomal Aberrations (CA) test is to detect the effects of the test substance on the mammalian cells in the culture with or without exogenous metabolic activation system i. S9 mix. The effect of the substance in this experiment is particularly in the chromosome either with direct effect i.e damage of double-stranded DNA or indirectly resulting in errors in DNA replication or repair of DNA lesions which leads to double-strand breaks (DSB). Metaphase is the most significant stage in a cell to study the induced aberration since most of them are either lethal or broke the subsequent cell division sequence (M. Ishidate Jr. a *et al,* 1998).

Alteration of karyotype can be executed in several ways which include the change in the number of chromosomes i.e either hyperploidy or hypoploidy, and structural alternation of a chromosome where some of the regions in chromosome altered by exchange, deletion, inversion, and duplication. These different classes of change in chromosomes are generally defined as aberrations. these aberrations resulted due to errors in normal cellular procedures such as DNA transcription, replication, repair, and while in cell division (M. Ishidate Jr. a *et al,* 1998).

Apart from these natural chromosomal aberrations, they can be induced by external factors such as environmental factors, and natural and synthetic chemicals. Structural aberrations involved in both chromosomal types: both chromatids at identical places are aberrated and chromatid type: only one chromatid involves an aberration (M. Ishidate Jr. a *et al,* 1998).

Studying chromosomes via histological staining of DNA is a classic method for detecting CA and observing chromatids at the metaphase stage during cell division is the best time to study CA since chromosomes are contracted and clearly visible at the metaphase stage (8). Identification of CA can be executed through different chromosomal banding techniques. The banding pattern of chromosomes is obtained by staining partially denatured DNA with Giemsa; Giemsa gives a specific banding pattern to the chromosomes via G-banding (B.J. Kilbey *et al,* 1984). CA can be detected by a specific banding pattern with a normal banding pattern of a chromosome. More recently by using the fluorescence *in situ* hybridization [FISH] technique chromosomal regions are painted with specific labeled probes. This fish technique is used to detect a particular class of CA which are very difficult to identify in the classical banding technique (S Venitt & J M Parry,1984).

Some of the specific CA will act as Biomarkers that deviate the cell cycle from normal to, forms a transformed cell that will proctor the cancer cell; other aberrations are lethal to the cell cycle that leads to death of the cell. The finding of CA in chromosomes is a significant step in the diagnosis, treatment, and even prevention of adverse effects (R. Ian Freshney, 2005).

This study particularly concentrated on the external agents (Pharmaceutical Impurities) that induced the aberrations in a chromosome, which are formed while manufacturing the Active Pharmacopeia Ingredients (API) and/or degradation of API while in storage. So, it is very important to characterize the impurity for the release of the API or regulatory submission. Performing toxicological evolution is one of the significant parameters for the characterization of an Unknown impurity (Sheila M. Galloway and Marilyn J. Aardema, 1994).

Chinese Hamster Ovary-K1 Cells (CHO-K1 Cells) are well characterized for testing chromosomal aberrations obtained by external agents. Its stable

nature, short generation time, a smaller number of chromosomes, and hassle-free nature of analysis made these cells more reliable for the experiment. Comprehensive validation of these cells in various cytogenic experiments and its compliance with various regulatory agencies make this system more suitable for aberration test (M. Ishidate *et al*, 1998).

These cells were identical to epithelial and adherent in nature with 20 chromosomes in number. CHO-K1 cells show a normal cell cycle with a cycle time of 12-14 hours. In this experiment, reconstructed cells from frozen stock culture were used (R. Ian Freshney, 2005). These cells should be from mycoplasma contamination. Fetal Bovine Serum (FBS) of 10% solution was added to Ham's F12K medium for culturing of CHO-K1 cells. This medium is spiked with antibiotics such as Penicillin, Streptomycin, and Amphotericin B cells cultured at 370C in a 5% CO2 incubator (M. Ishidate *et al*, 1998).

Active electrophilic metabolites in mammalian cells are acts on promutagens and convert to active mutagens (T. Kawachi *et al*, 1980). Absence of Active electrophilic metabolites in *invitro* cell clines

$$\text{RICC \%} = \frac{\text{Increase in No. of cells in treated}}{\text{Increase in No. of cells in controls}} \text{ X } 100$$

promutagens failed to convert into active mutagens in some circumstances (S Venitt & J M Parry,1984). Due to this significant future of Active electrophilic metabolites, it is necessary to add these electrophilic metabolites externally to the test systems (Asakura, M. et al. (2008). In this experiment we use post mitochondrial fraction i.e S9 fraction was used as external electrophilic metabolites, also its efficacy was tested by using 2-Amino anthracene and Benzo (a) pyrene. The S9 mixture was prepared by adding S9 buffer which contains cofactor like βNADP, KCl, and Glucose -6-Phosphate to the S9 factor. 1% of the S9 factor was used in this experiment (OECD Guideline No: 473, 2016)).

II. MATERIAL AND METHODS

A. Procedure

Before the experiment, it is necessary to understand the properties of the test substance. Hence, we need to perform some of the experiments such as solubility, precipitation test, and toxicity tests.

Solubility and precipitation tests were carried out to test substance SSZ impurity. 200 mg of test substance dissolved in 1 ml of RO water and DMSO. Since the substance is completely dissolved in DMSO, a precipitation test was conducted based on serial dilutions at 100mg/ml, 50mg/ml, and 25mg/ml. pH was calculated at concentrations 2.0mg/ml, 1.0 mg/ml, o.5 mg/ml, and 0.25 mg/ml.

Cytotoxicity was performed for the test substance to identify the dose concentrations. Based on solubility the dose concentration of cytotoxicity was identified as 0.25,0.5,1.0, & 2.0 mg/ml. Overnight cultures were prepared by adding 5 X 10^5 cultured cells to 5 ml of complete medium in a 25 cm^2 culture flask at 37^0C in presence of a 5% CO_2 incubator. These culture procedures were used throughout the experiment.

Overnight culture cells were treated with test item concentrations in duplication for 4.0 hours which was considered a short-term treatment. The treated cells were cultured further for 18-21 hours which was equivalent to a 1.5-cell cycle. The cytotoxicity was performed both with and without metabolic activation for short-term treatment. Cytotoxicity was performed as a long-term treatment method in which cells are treated for more than 4 hours. But in this long-term treatment method, we perform only without metabolic activation. The cytotoxicity was estimated based on a relative increase in cell count (RICC) calculation.

$$\text{Cytotoxicity} = 100 - \% \text{ RICC}$$

RICC is for calculating cytotoxicity. The highest concentration for the experiment was identified based on less cytotoxicity observed i.e approx. 50-60 % of cytotoxicity. Results % RICC and % Cytotoxicity was reported in table 1.

Based on the cytotoxicity test results treated doses were identified as 0.5 to 2.0 mg/ml for both with and without metabolic activation. Doses were prepared; an appropriate amount of test substances was dissolved in DMSO and lower concentrations were obtained by serial dilution. (The preparation and highest dose, serial dilution). The stock concentration of 200 mg/ml was prepared to obtain the required concentration 100 mg/ml and 50 mg/ml). required test concentration was obtained by adding 10µl of respective stock solution to 1 ml of culture media both in the presence and absence of metabolic activation. 10µl of DMSO in 1.0 ml media was used as Negative control in this experiment. This experiment was executed in the

presence and absence of 1 % V/V metabolic activation i.e S9 mixture with short-period treatment. Further to the short period, this experiment continues to longer period treatment duration if negative results are obtained in short period treatment experiment.

Prior to treatment, the culture was checked under an inverted microscope for its healthy and contamination-free nature. in a short period of treatment, an experiment was carried out with medium not having serum whereas in a longer period of treatment 10% FBS was used with the medium. Both short and long-period treatment experiments were conducted in duplication for every concentration in each group. Along with controls. Dose concentrations of 0.5, 1.0, and 2.0 mg/ml were used in this experiment with/without S9 mix in both short and long-duration treatment experiments.

Overnight grown cultures were treated with the test item and kept for 4 hours incubation at 37OC and 5 % CO2 humid air. After completion of the treatment period, the medium was removed and treated cells were washed with Dulbecco's Phosphate Buffer Saline (DPBS). After washing twice with DPBS 5 ml of complete medium was added to each flask and continued with incubation for 1.5 times the cell cycle length at standard conditions.

After incubation culture was centrifuged. centrifugate was discarded and the pellet was suspended in 0.2µg/ml concentrated colchicine solution. Later the solution was centrifuged and the centrifugate was discarded. Then pellet was suspended with 3 ml of 0.56% KCL solution and incubated for 35 minutes. After incubation centrifugation was carried and the pellet was suspended in a 1:3 ratio of Acetic Acid & Methanol. Centrifugation was performed and the pellet retained then added 1:3 ratio of Acetic Acid & Methanol. This step is repeated 3 times. for the final pellet of 3 ml, a 1:3 ratio of Acetic Acid & Methanol was added for final preparation. This final solution is dropped on prechilled glass slides from two feet high. For each solution, five slides were prepared along with controls. From each concentration, two slides must be studied for aberrations by an inverted microscope.

III. RESULTS

A. Pre-test/cytotoxicity

Cytotoxicity (55 ± 5%) was observed at the test concentration of 2.0 mg/mL in the absence and presence of a metabolic activation system in the short & long-duration treatments. Cytotoxicity (55 ± 5%) was not observed at the test concentrations 0.25, 0.5, and 1.0 mg/mL in the absence and presence of a metabolic activation system in both short-term and long-term.

The observed cytotoxicity for short-term and long-term treatment with and without metabolic activation results were reported in Table -1.

TABLE-1: Pretest results - % RICC calculation

Dose (mg/mL)	Cell count/mL – initial	Cell count/mL – Harvesting	Mean cell count/ mL	% RICC	% Cyto - toxicity
Short-term treatment – in the absence of metabolic activation system (-S9)					
NCR1 (0.0)	101000	515000	517500	100	0
NCR2 (0.0)	101000	520000			
T1R1 (0.25)	101000	455000	442500	81.99	18.01
T1R2 (0.25)	101000	430000			
T2R1 (0.5)	101000	360000	367500	63.99	36.01
T2R2 (0.5)	101000	375000			
T3R1 (1.0)	101000	350000	340000	57.38	42.62
T3R2 (1.0)	101000	330000			
T4R1 (2.0)	101000	310000	300000	47.78	52.22
T4R2 (2.0)	101000	290000			
Short-term treatment – in the Presence of metabolic activation system (+S9)					
NCR1 (0.0)	101000	525000	517500	100	0
NCR2 (0.0)	101000	510000			
T1R1 (0.25)	101000	445000	437500	80.79	19.21
T1R2 (0.25)	101000	430000			
T2R1 (0.5)	101000	335000	347500	59.18	40.82
T2R2 (0.5)	101000	360000			
T3R1 (1.0)	101000	325000	335000	56.18	43.82
T3R2 (1.0)	101000	345000			
T4R1 (2.0)	101000	290000	295000	46.58	53.42
T4R2 (2.0)	101000	300000			

Long-term treatment – in the absence of metabolic activation system (-S9)					
NCR1 (0.0)	101000	510000	505000	100	0
NCR2 (0.0)	101000	500000			
T1R1 (0.25)	101000	405000	410000	76.49	23.51
T1R2 (0.25)	101000	415000			
T2R1 (0.5)	101000	385000	362500	64.73	35.27
T2R2 (0.5)	101000	340000			
T3R1 (1.0)	101000	325000	342500	59.78	40.22
T3R2 (1.0)	101000	360000			
T4R1 (2.0)	101000	285000	285000	45.54	54.46
T4R2 (2.0)	101000	285000			

B. Main study

1. Short term treatment

Cultures were exposed to 2-hydroxy-5-[N-(4-{[4-pyridin-2-ylsulfamoyl) phenyl] sulfamoyl}phenyl)diazen-1-yl] benzoic acid for a short period (4 hours) both in the absence and in the presence of metabolic activation system (1% v/v S9 mix). The results did not show any statistical significance in percent aberrant cells at the dose levels of 0.25, 0.5, and 2.0 mg of 2-hydroxy-5-[N-(4-{[4-pyridin-2-ylsulfamoyl) phenyl]sulfamoyl}phenyl)diazen-1-yl] benzoic acid /mL of culture medium, in the absence and the presence of the metabolic activation system(1%v/v), when compared with the negative control group. Details of RICC and cytotoxicity for short-duration treatment were reported in table no:2.

TABLE-2: %RICC calculation Main study								
Dose (mg /mL)	Cell count/ mL – initial	Cell count/ mL – Harvesting	Mean cell count/ mL	% RICC	% Cyto-toxicity	Mean cell count/ mL	% RICC	% Cyto-toxicity
Short term treatment			In the absence of a metabolic activation system (-S9)			In the Presence of a metabolic activation system (+S9)		
NCR1 (0.0)	102000	515000	525000	100	0	535000	100	0
NCR2 (0.0)	102000	535000						
T1R1 (0.5)	102000	405000	430000	77.54	22.46	425000	74.6	25.4
T1R2 (0.5)	102000	455000						
T2R1 (1.0)	102000	330000	347500	58.04	41.96	367500	61.32	38.68
T2R2 (1.0)	102000	365000						
T3R1 (2.0)	102000	305000	312500	49.76	50.24	300000	45.73	54.27
T3R2 (2.0)	102000	320000						
PCR1 (0.3*)	102000	270000	297500	46.22	53.78	295000	44.57	55.43
PCR2 (0.3*)	102000	325000						

Details of chromosomal aberrations, their frequencies, and % aberrations were properly recorded. Details were given below in Tables 3 & 4.

Table – 3				
	Absence of Metabolic Activation system (-S9)		Presence of Metabolic Activation system (+S9)	
Dose (mg /mL)	Culture No.	Frequencies of aberration	Culture No.	Frequencies of aberration
Negative control (0.0)	1	CLg -2, Cb-1	1	CLg - 1, CLb - 1
	2	CLg - 1, Cb - 2	2	CLg - 3, Cb - 1
0.5 (T1)	1	Cg - 1, CLg -1, Cb -2	1	Cg - 1, CLg - 4, Cb - 1
	2	CLg - 2, Cb - 2	2	Cg - 1, CLg -2, Cb -1, CLb - 1
1.0 (T2)	1	CLg - 1, Cb - 2, CLb - 1	1	Cg - 1, CLg - 1, Cb - 1
	2	Cg - 1, CLg - 2, Cb - 1	2	Cg - 2, CLg - 1, Cb - 1
2.0 (T3)	1	CLg - 1, Cb - 1, CLb -1	1	CLg - 2, Cb - 1
	2	CLg - 2, Cb - 3	2	CLg - 1, Cb - 1, CLb - 1
Positive control *, **	1	CLg -3, Cb -7, D -1, E -4	1	CLg - 4, Cb - 8, CLb - 4, E - 4
	2	Cg -2, Cb - 13, CLb - 2	2	Cg - 2, CLg - 2, Cb - 11, E - 1
*0.3µg/mL of mitomycin C was used as a positive control.			** 5.0µg/mL of Benzo(a)pyrene was used as positive control.	
Key: Cg=Chromatid gap, CLg=Chromosomal gap, Cb=Chromatid break, CLb=Chromosomal break, E=Exchange, D= Deletion.				

TABLE: 4							
% Aberrated cells - Short-term treatment Absence of Metabolic Activation system (-S9)							
Dose (mg /mL)	Culture No.	Including gaps	Total	% Aberrant cells	Excluding gaps	Total	% Aberrant cells
Negative control (0.0)	1	3	6	2	1	3	1
	2	3			2		
0.5 (T1)	1	4	8	2.67	2	4	1.33
	2	4			2		
1.0 (T2)	1	4	7	2.33	3	4	1.33
	2	3			1		
2.0 (T3)	1	3	6	2	2	5	1.67
	2	3			3		
Positive control (0.3*)	1	11	25	8.33	9	23	7.67
	2	14			14		
*0.3 µg/mL mitomycin C was used as a positive control.							
Presence of Metabolic Activation system (+S9)							
Dose (mg /mL)	Culture No.	Including gaps	Total	% Aberrant cells	Excluding gaps	Total	% Aberrant cells
Negative control (0.0)	1	2	5	1.67	1	2	0.67
	2	3			1		
0.5 (T1)	1	4	8	2.67	1	3	1
	2	4			2		
1.0 (T2)	1	4	8	2.67	1	2	0.67
	2	4			1		
2.0 (T3)	1	3	6	2	1	3	1
	2	3			2		
Positive control (5.0)	1	13	25	8.33	12	22	7.33
	2	12			10		
*5.0 µg/mL Benzo(a)pyrene was used as positive control.							

2. Long term treatment

Cultures were exposed to 2-hydroxy-5-[N-(4-{[4-pyridin-2-ylsulfamoyl) phenyl]sulfamoyl}phenyl)diazen-1-yl] benzoic acid for a Long period (Approx. 21 hours) in the absence of metabolic activation system. The results did not show any statistical significance in percent aberrant cells at the dose levels of 0.25, 0.5, 1.0 mg of 2-hydroxy-5-[N-

(4-{[4-pyridin-2-ylsulfamoyl) phenyl] sulfamoyl}phenyl)diazen-1-yl] benzoic acid/mL of culture medium, in the absence of the metabolic activation system, when compared with the negative control group. Long-period treatment results were detailed in table:5 (% of RICC), 6 (Chromosome Aberration Frequencies), and 7(% Aberrated cells).

TABLE -5: % RICC calculation Long-Term Treatment - Main study

Dose (mg /mL)	Cell count/mL – initial	Cell count/mL – Harvesting	Mean cell count/mL	% RICC	% Cytotoxicity
Long-term treatment – in the absence of metabolic activation system (-S9)					
NCR1 (0.0)	102000	530000	520000	100	0
NCR2 (0.0)	102000	510000			
T1R1 (0.5)	102000	395000	402500	71.89	28.11
T1R2 (0.5)	102000	410000			
T2R1 (1.0)	102000	390000	372500	64.71	35.29
T2R2 (1.0)	102000	355000			
T3R1 (2.0)	102000	320000	302500	47.97	52.03
T3R2 (2.0)	102000	285000			
PCR1 (0.3*)	102000	280000	290000	44.98	55.02
PCR2 (0.3*)	102000	300000			
*0.3µg/mL of mitomycin C was used as a positive control #5.0 µg/mL of Benzo(a)pyrene was used as positive control.					
% RICC: Increase in No. of cells in treated cultures (final-starting)/ Increase in No. of cells in control cultures (final-starting) x100.					
Cytotoxicity (%): 100 - % RICC					

TABLE-6: Chromosome Aberration Frequencies – Long-term treatment

Dose (µg /mL)	Culture No.	Frequencies of aberration
Absence of Metabolic Activation system (-S9)		
Negative control (0.0)	1	Cg - 1, Cb – 2
	2	CLg - 2, Cb – 1
0.5 (T1)	1	Cg - 2, CLg - 1, Cb – 1
	2	Cg - 1, Cb – 1
1.0 (T2)	1	CLg - 2
	2	Cg - 1, Cb - 2
2.0 (T3)	1	CLg - 1, Cb - 2
	2	CLg - 2, Cb - 2
Positive control(0.3*)	1	Cg - 1, Cb - 4, CLb - 6, E - 4,R - 1
	2	Cg -1, CLg - 2, Cb - 2, CLb - 2, E - 8, R - 1
*0.3 µg/mL mitomycin C was used as a positive control.		
Key: Cb=Chromatid break, Cg=Chromatid gap, CLg=Chromosomal gap, CLb=Chromosomal break, E=Exchange, R= Ring		

TABLE-7: % Aberrated cells - Long-term treatment

Dose (µg /mL)	Culture No.	Including gaps	Total	% Aberrant cells	Excluding gaps	Total	% Aberrant cells
Absence of Metabolic Activation system (-S9)							
Negative control (0.0)	1	3	6	2	2	3	1
	2	3			1		
0.5 (T1)	1	3	5	1.67	1	2	0.67
	2	2			1		
1.0 (T2)	1	1	4	1.33	0	2	0.67
	2	3			2		
2.0 (T3)	1	3	5	1.67	2	4	1.33
	2	2			2		
Positive control (0.3)	1	11	26	8.67	11	23	7.67
	2	15			12		
*0.3 µg/mL mitomycin C was used as a positive control.							

IV. CONCLUSION

The chromosomal aberration test was an efficient technology in modern toxicology for the evaluation clastogenicity of chemical compounds, natural products, and plant extracts (Elliot, B.M. et al.1992). Previously CA test was executed on isolated human lymphocytes. But other alternatives were identified in which CHOK1 was the best. CHOK1 cells were well characterized and regulatory accepted cell lines. The significant aspect is these cells are easily isolated and maintained (Galloway, S. et al. 2011).

This experiment mainly depends on the meta phase arresting by the chemical substance. If the metaphases are not arrested properly means before or after the metaphase, the sensitivity of experiment may loose. Hence it is significant use of substance that arrest metaphase, time of use, concentration of the substance and exposure period (Galloway, S.M. et al. 1994).

SSZ impurity was process impurity and it was recently identified in due course of process development in finding alternative synthetic routes. Hence it is mandatory to study detailed impurity safety and other effects. Even though the total quantity of impurities is within the limits of regulatory specifications it is important to study this impurity (Honma, M. 2011). Apart from this several other factors which might be affect the sensitivity and integrity of this experiment which includes incubation temperature, time of incubation, treatment duration, harvest time, and cell density for seeding (if applicable). Criteria of accepting results includes preparation of slides, staining techniques, aberration scoring, number of meta phases analyzed, and consideration of criteria for

identifying positive, or Negative, or equivocal results (Brookmire, L. *et al*, 2013).

In this experiment several parameters for results were considered in which some are experiment conclusive, some alters the sensitivity of the experiment, some effects the integrity of the experiment. These parameters are reported as treated cell number, harvested cell count for each culture, cell cycle length, proliferation time, cytotoxicity measurement, precipitation, number of cells scored, and concurrent positive and negative control results (Hayashi, M. et al, 2011). Supplementary to the above others information also can considered for experiments that includes historical date of positive and negative control, measurement of means and standard deviations and statistical analyses, p-values at 95% control limits for the distribution (Richardson, C. et al. 1989).

A positive sample is defined as at least one of the test concentration shows a statistically significant increase when compare with concurrent negative control. Both statistical and biological relevance were considered for evaluation of results. The results of the test substance in this experiment do not meet significance difference is consider negative results hence substance is non mutagenic. Gaps were recorded and tabulated but not considered for results evaluation. Ploidy was not observed. For additional significance statistical analysis of Chi-square test was performed (p value <0.05) between negative control and all treatment groups. Fisher's exact test was followed to check the statistical significant (p value <0.05) difference between negative and positive control (Fleiss, J. L *et al*,2003).

In this experiment, we study the clastogenic effects of SSZ impurity by CA test by CHO K1 cell lines. This experiment was executed in two parts. Before this experiment pre-study was conducted to evaluate the toxicity of impurity of cell lines. Based on solubility and precipitation tests the maximum concentration of impurity for pre-study was identified as 5mg/kg body weight. Based on pre-study results 2.0 mg/kg, 1.0mg/kg, and 0.5 mg/kg body weight concentrations of impurity were considered for the experiment. Based on the results in part-1 experiment was proceed further for part-2. Part-1 & 2 differentiated by pre-treatment with S9 mix which included in Part-2.

In Part-1 the results were recorded in this experiment, not less than 300 metaphases were observed for each concentration along with positive and negative control. the significant number of aberrations in positive control proves the integrity and efficiency of the experiment. Aberrations were recorded as chromatid break, chromatid deletion, chromosomal break, and chromosomal gap.

From the results of this study, it is concluded that 2-hydroxy-5-[N-(4-{[4-pyridin-2-ylsulfamoyl) phenyl]sulfamoyl}phenyl)diazen-1-yl] benzoic acid is negative hence it is non mutagenic (non clastogenic) in chromosome aberration test using CHO-K1 cell line both in the absence and presence (1% v/vS9 mix) of metabolic activation system for short term treatment duration and in the absence of metabolic activation system for long term treatment duration.

To bring a conclusion in this experiment test concentration results were compared with vehicle control results. The results in Part-1 show no biological or statistical significance. Similarly, trial-2 also shows no significance in either. By considering no significant results the experiment concludes the test substance showed no clastogenic activity in the given concentration. Based on the pretest studies we considered the maximum allowable concentration as 5.0mg per one kg body weight. Therefore, test substances don't show any clastogenic activity at maximum concentration.

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