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In vivo and *In vitro* Effect of Sulfamerazine on Hepatic Mixed Function Oxidase in Rats

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Abstract: Sulfamerazine (SMR) administration (i.p., 3 days) of different doses to adult male rats showed significant decrease in electron transport components and drug metabolizing enzymes. In longer duration study, 100 mg SMR kg^{-1} caused significant decreases in cytochrome P-450 and activities of aminopyrine N-demethylase and aniline hydroxylase. In inhibitory dose of 100 mg SMR kg^{-1} was selected for dosing young male, old male and adult female rats. Sulfamerazine administration to young, old male and female rats resulted in a significant decrease in electron transport component and drug metabolizing activities at 100 mg SMR kg^{-1} dose level. All other parameters were unchanged. Sulfamerazine resulted in uncompetitive type of inhibition (Ki = 3.0 mM) of aminopyrine N-demethylase *in vitro*. Sulfamerazine destructed the spectral and catalytic activity of cytochrome P-450. The studies suggest that SMR is a substrate of the mixed function oxidase system and inhibition is dependent on dosage, age and sex of the animals.

Key words: Aminopyrine N-demethylase, aniline hydroxylase, cytochrome P-450, mixed function oxidase, sodium, sulfamerazine

INTRODUCTION

Sulfamerazine (SMR) is an antibiotic compound used for the treatment of many infectious diseases of animal, widely used in human and veterinary medicine (Stolman and Stewart, 1960; Trepanier, 2004; Kao *et al*., 2001). It is also used for the treatment of furanuculosis in the salmonid fishes used as food additives and antibacterial agent (Ferguson, 1997; Renew and Huang, 2004; Martinez *et al*., 2003; Cribb *et al*., 1996; Trepanier, 1999). Sulfamerazine enter the human by food chain through milk and meat products. In addition to these clinical applications, SMR is frequently utilized as a compound for the determination of the N-acetylation activity in human and other species *in vivo* and *in vitro* (Vree *et al*., 1986).

The formation of two hydroxylation products 6-hydroxylmethyl and 4-hyrdoxylmethyl sulfamerazine is quantitatively important in dogs and ruminants (cattle and goat) whereas humans, pigs and rabbits mainly acetylates the drug. These hydroxylation products of SMR also have been observed when incubated with rat liver microsomes (Whitkamp *et al*., 1993).

The Mixed Function Oxidase system (MFO) plays a key important role in metabolism of many compounds including fatty acids sterols, alkenes, polycyclic carcinogens, drugs and environmental pollutants (Omura *et al*., 1965).

MATERIALS AND METHODS

Animals: Male Wistar rats (200-220 g, 3 months old) young growing (90-110 g, 4-5 weeks old) (350-400 g, 5 months) and females (200-220 g, 3 months) were obtained from Hoffkine Institute, Mumbai. The animals were housed in plastic cages and were fed on appropriate standard laboratory diet (Lipton India Ltd., Mumbai, India) and tap water *ad libitum*.

Chemicals: Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Nicotinamide Adenine Dinucleotide Phosphate (NADP), glucose 6-phosphate dehydrogenase, glucose 6-phosphate disodium salt, aminopyrine, aniline hydrochloride, N-[2-hydroxyethyl] Piperazine-n-[2 Ethane Sulfonicacid] (HEPES), sodium salt of sulfamerazine were obtained from Sigma Chemical Company (St. Louis , MO, USA). Phenobarbital was obtained from E Merck (Mumbai, India).

Sucrose, phenol, trichloroaceticacid, sodium chloride, potassium chloride, calcium chloride, ethylenediaminetetra acetic acid disodium salt and other chemicals were of analytical grade obtained from Qualigens Fine Chemicals (Mumbai, India).

Treatment: Adult male rats 200-220 g were divided into 4 groups each of 4 animals each and were injected i.p., daily between 7.30-8.30 a.m. Group 1 received an

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equivalent amount of saline and served as a control in every set of experiment. The volume injected into rat having body weight 200 g was 1 mL either as saline group 1 or SMR solution group 2-4 prepared in saline. Animals in group 2-4 were injected with SMR for 3 days at dose levels of 100 mg kg^{-1} body weight, respectively and were killed 24 h after the last treatment. In the second set of experiment adult, male rats were divided into 4 groups. Animals from groups 2-4 were injected i.p. with saline for 5, 3, 1 and 0 successive days, respectivel followed by 100 mg SMR kg^{-1} body weight daily for 1, 3 and 5 days. Animals from group 1 received an equivalent amount of saline as control.

In another experiment, adult female rats (200-220 g), young (90-110 g) and old (350-400 g) male rats were used for this experiment. In each case, animals were divided into 2 groups of 4 animals. Group 2 of each case received 100 mg SMR kg^{-1} i.p., body weight for 3 successive days, respectively. Group 1 of each case received an equivalent.

Preparation of hepatic microsomes: The rats used in this study were killed by cervical dislocation 24 h after the last dose. Their livers were purfused with ice cold 1.15% KCl solution containing 0.05 mM EDTA in situ and microsome were prepared following the procedure of Cinti *et al.* (1972).

The livers were rapidly excised, blotted dry, weighted, minced and homogenized with 2 volumes of ice-cold 0.25 M sucrose solution in Potter-Elvehjem type homogenizer.

The homogenate was centrifuged at 10,000 rpm for 20 min in a refrigerated centrifuge (REMI C-24). The microsomes from the supernatant fraction were isolated by the procedure of Cinti *et al.* (1972). The microsomal pellet was washed with 1.15% KCl solution containing 0.05 mM EDTA resuspended in phosphate buffer (0.1 M, pH 7.4) and the suspension used for microsomal enzyme assays. The microsomal protein was estimated by Biuret method (Gornall *et al.*, 1949) using bovine serum albumin as a standard.

Enzyme assays: The microsomal electron transport components (cytochrome P-450 and cytochrome b5) were determined using a Hitachi UV-visible recording spectrophotometer (UV-330) and the procedure of Omura and Sato (1964).

Aminopyrine N-demethylase activity was assayed according to the procedure of Schenkman *et al.* (1967). Formaldehyde liberated by the procedure of Nash (1953). Aniline hydroxylase assay was performed using the procedure reported by Govindwar and Dalvi (1990).

Effect of sulfamerazine on kinetic properties of aminopyrine N-demethylase: Inhibition of aminopyrine N-demethylase activity by SMR was performed adding different concentrations of SMR (1-5 mM). Sulfamerazine was preincubated with the reaction mixture for 30 sec and reaction was initiated by the addition of aminopyrine substrate. The inhibition data of sulfamerazine was plotted according to Dixon (1953) for the determination of apparent Ki value.

K_m determination was performed with 2 mg microsomal protein/mL incubated with concentrations of aminopyrine 2-10 mM for 5 min. Sulfamerazine (3.0 mM) was added *in vitro* to incubation mixture for the inhibition of aminopyrine N-demethylase. Sulfamerazine dissolved in glass-distilled water was added to the incubated mixture at 3.0 mM concentration and activities of aminopyrine N-demethylase and aniline hydroxylase were determined.

Determination of sleeping time: A single dose of phenobarbital 80 mg kg^{-1} in saline was injected i.p. to control and SMR pretreated (single dose of 100 mg kg^{-1} in saline, i.p., the previous day) rats to observe the effect SMR on sleeping time. About 2 mL of partially purified cytochrome P-450 (3 nmole mg^{-1} protein, 1 mg protein mL^{-1}) from untreated rat liver microsome using ω-amino-n-octyl sepharose 4B (Schenkman *et al.*, 1967), 0.5 mL of cytochrome P-450 reductase (2 μmole of cytochrome c reduced/min/mg protein, containing 1 mg protein/min/mg protein, activity) purified using the procedure reported earlier (Imai and Sato, 1974) and 150 μg of 1-phosphatidyl choline were mixed and allowed to stand for 15 min at 37°C. Formed reconstituted membrane was used to determine the aminopyrine N-demethylase activity (Schenkman *et al.*, 1967; Nash, 1953) in absence and the presence of SMR (3.0 mM).

Analysis of data: Statistical analysis was done by one way Analysis of Variance (ANOVA) with the Turkey-Kramer multiple comparison tests. $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Administration of SMR at different dose levels resulted in a marked decrease in hepatic microsomal electron transport components and drug metabolizing enzymes. No any significant change in body weight and relative liver weight (g/100 g body weight) was observed. No significant change was observed in microsomal

Table 1: Effect of different doses of sulfamerazine (i.p. for 3 days) on microsomal protein and electron transport components of the adult male rats (200-210 g)

Dose (mg kg^{-1})	Microsomal protein[a]	Cytochrome[b] b5	Cytochrome[b] P-450
Control	13.80±0.94	0.23±0.01	0.46±0.02
25	13.21±0.92	0.25±0.02	0.42±0.03
50	13.07±0.82	0.19±0.01	0.35±0.02
100	13.82±0.71	0.10±0.01***	0.20±0.04***

Values are mean of three experiments±SEM, four animals in each group; [a]mg protein/g liver; [b]nmol mg^{-1} microsomal protein. Significantly different from control at ***p<0.001 by one way analysis of variance and Tukey-Kramer multiple comparisons test

Table 2: Effect of different doses of sulfamerazine (i.p. for 3 days) on drug metabolizing enzymes in adult male rats (200-210 g)

Dose (mg kg^{-1})	Aminopyrine N-demethylase[a]	Aniline hydroxylase[b]
Control	5.29±0.16	1.13±0.21
25	4.39±0.14*	0.94±0.09
50	3.45±0.20***	0.67±0.11
100	2.48±0.21***	0.36±0.15*

Values are mean of three experiments±SEM, four animals in each group; [a]nmole of formaldehyde liberated/min/mg microsomal protein; [b]nmole of p-aminophenol formed/min/mg microsomal protein. Significantly different from control at *p<0.05; **p<0.01; ***p<0.001 by one way analysis of variance and Tukey-Kramer multiple comparisons test

protein (g/liver) at all dose levels. The significant decrease in the level of cytochrome b5 and cytochrome P-450 was observed with 100 mg kg^{-1} dose level. However, little increase in cytochrome b5 content was observed at 25 mg kg^{-1} of dose level. Marginal decrease in the level of cytochrome P-450 and cytochrome b5 was observed at 25 and 50 mg kg^{-1} dose level, respectively.

A significant decrease was observed in the activity of aminopyrine N-demethylase and aniline hydroxylase at 100 mg SMR kg^{-1} dose level while marginal decrease was observed at 25 and 50 mg SMR kg^{-1} dose level. All other parameters were unchanged (Table 1 and 2).

To study the inhibitory effect of SMR, the animals were dosed with 100 mg SMR kg^{-1} for different duration. A significant decrease was observed in the levels of cytochrome b5, cytochrome P-450 and activity of aminopyrine N-demethylase and aniline hydroxylase at all duration studied. However, a single day exposure showed little decrease when compared with 3 and 5 days exposure which showed significant decrease in the levels of cytochrome b5, cytochrome P-450, aminopyrine N-demethylase and aniline hydroxylase (Table 3).

We also studied the effect of SMR at 100 mg kg^{-1} body weight in adult female rats (200-220 g), young (90-110 g) and old (350-400 g) male rats. Sulfamerazine dosing caused significant decrease in the level of microsomal protein, cytochrome b5, cytochrome P-450, aminopyrine N-demethylase and aniline hydroxylase at 100 mg SMR kg^{-1} dose level while marginal change was observed in cytochrome b5 and no change in aminopyrine N-demethylase when compared with control. However, significant decrease was observed in aminopyrine

Table 3: Effect of duration of sulfamerazine treatment (100 mg kg^{-1} i.p. for up to 5 days), on microsomal protein and drug metabolizing enzymes in adult male rats

	Days of treatment			
Parameters	0	1	3	5
Microsomal protein (mg g^{-1} liver)	12.60±1.04	10.50±0.22	11.33±0.68	11.90±1.07
Cytochrome b5 (nmole/mg of protein)	0.26±0.02	0.24±0.02	0.13±0.01*	0.10±0.01***
Cytochrome P-450 (nmole/mg of protein)	0.39±0.01	0.28±0.01***	0.23±0.01***	0.21±.01***
Aminopyrine N-demethylase[a]	4.83±0.15	2.89±0.26***	1.69±0.12***	0.95±0.05***
Aniline hydroxylase[b]	1.08±0.20	0.62±0.15	0.36±0.06*	0.10±0.01**

Values are mean of three experiments±SEM, four animals in each group; [a]nmole of formaldehyde liberated/min/mg microsomal protein; [b]nmole of p-aminophenol formed/min/mg microsomal protein. Significantly different from control at *p<0.05; **p<0.01; ***p<0.001 by one way analysis of variance and Tukey-Kramer multiple comparisons test

Table 4: Effect of sulfamerazine 100 mg kg^{-1} (i.p. for 3 days), on microsomal protein, electron transport components and drug metabolizing enzymes in adult female rats

Parameters	Control	Sulfamerazine (100 mg kg^{-1})
Microsomal protein (mg g^{-1} liver)	12.56±1.40	10.68±1.25
Cytochrome b5 (nmole mg^{-1} protein)	0.21±0.01	0.18±0.01
Cytochrome P-450 (nmole mg^{-1} protein)	0.34±0.01	0.32±0.01
Aminopyrine N-demethylase[a]	4.70±0.19	3.27±0.06**
Aniline hydroxylase[b]	0.92±0.16	0.70±0.09

Table 5: Effect of sulfamerazine 100 mg/kg (i.p. for 3 days), on microsomal protein, electron transport components and drug metabolizing enzymes of the young male rats (90-100 g)

Parameters	Control	Sulfamerazine (100 mg kg^{-1})
Microsomal protein (mg g^{-1} liver)	10.39±0.26	7.85±0.16**
Cytochrome b5 (nmole mg^{-1} protein)	0.21±0.01	0.18±0.01
Cytochrome P-450 (nmole mg^{-1} protein)	0.47±0.01	0.22±0.01***
Aminopyrine N-demethylase[a]	4.63±0.14	4.59±0.29
Aniline hydroxylase[b]	1.24±0.12	0.76±0.06*

Values are mean of three experiments±SEM, four animals in each group; [a]nmole of formaldehyde liberated/min/mg microsomal protein; [b]nmole of p-aminophenol formed/min/mg microsomal protein. Significantly different from control at *p<0.05; **p<0.01; ***p<0.001 by one way analysis of variance and two tail p-value test

N-demethylase at 100 mg SMR kg^{-1} dose level. All other parameters were unchanged in adult female rats. A significant decrease was observed in aminopyrine N-demethylase and aniline hydroxylase at 100 mg SMR kg^{-1} dosed in old male rats. However, all other parameters viz. microsomal protein, cytochrome b5, cytochrome P-450 remains unchanged at 100 mg SMR kg^{-1} dose level (Table 4-6). Phenobarbital (PB) sleeping time was used to assess the effect on *in vivo* metabolism. A marginal decrease in sleeping time was noticed when phenobarbital injected to sulfamerazine-pretreated male rats as well as female rats (Table 7).

To extend the observations of microsomal MFO inhibition experiments were performed *in vitro*. The effect of SMR on the kinetics of aminopyrine N-demethylase was determined. An increase in K_m and decrease in V_{max} was observed in presence of SMR which is an indicative of uncompetitive type of inhibition the apparent Ki value calculated from Dixon plots was 3.0 mM (Table 8, Fig. 1). The metabolism of SMR by microsomes resulted in pronounced loss of spectrally detectable P-450-CO complex, the catalytic activity of aminopyrine N-demethylase and microsomal protein. However, this loss was not observed in presence of NADPH-generating system by the end of 10 min incubation (10 mM) *in vitro* (Table 9) SMR 3.0 mM.

The objective of these studies was to examine the effect of SMR on the microsomal MFO system. N_4-acetylation of SMZ widely used as a marker in man as a genetically determined capacity of N_4-acetyltransferase system to metabolize a variety of important therapeutic substances (Govindwar *et al*., 1983; Evan, 1969; Peters *et al*., 1975) pathways of other than acetylation can also contribute to SMR metabolizes viz. glucuronidation, sulfation and oxidation to hydroxylase which varies with species and condition. The sulfamerazine administration to adult male rats produced significant inhibition of electron transport components and drug metabolizing enzymes at 100 mg SMR kg^{-1} dose level. While in dose duration studies showed significant decrease in electron transport component and drug metabolizing enzymes at 3 and 5th day (White and Evans, 1967; Witkamp *et al*., 1992; Winter and Unadkat, 2005). These results indicate the inhibitory effect of sulfamerazine with respect to mixed function oxidase system. Uncompetitive type of inhibition of aminopyrine N-demethylase due to *in vitro* addition of SMR indicates the nature of SMR as a substrate. Considerable difference were noted in different age group and sex, variation in the percent inhibition in electron transport component and drug metabolizing enzyme activities in case of young and old male rats due to SMR treatment indicates age dependence effect of SMR and only decrease in aminopyrine N-demethylase at 100 mg SMR kg^{-1} treatment suggest that resistance of in female

Table 6: Effect of sulfamerazine 100 mg kg^{-1} (i.p. for 3 days) on microsomal protein, electron transport components and drug metabolizing enzymes of the old male rats (300-400 g)

Parameters	Control	Sulfamerazine (100 mg kg^{-1})
Microsomal protein (mg g^{-1} liver)	10.98±0.13	9.42±0.15**
Cytochrome b5 (nmolemg^{-1} protein)	0.22±0.01	0.19±0.01
Cytochrome P-450 (nmole mg^{-1} protein)	0.33±0.12	0.31±0.01
Aminopyrine N-demethylase[a]	4.21±0.12	3.15±0.15*
Aniline hydroxylase[b]	0.90±0.20	0.36±0.15*

Values are mean of three experiments±SEM, four animals in each group; [a]nmole of formaldehyde liberated/min/mg microsomal protein; [b]nmole of p-aminophenol formed/min/mg microsomal protein. Significantly different from control at *p<0.05; **p<0.01; ***p<0.001 by one way analysis of variance and two tail p-value test

Table 7: Effect of sulfamerazine pretreatment (100 mg kg^{-1}, i.p. single dose) on phenobarbital (80 mg kg^{-1}, i.p., single dose) sleeping time

	Sleeping time (h)	
Group	Male	Female
Control	2.03±0.3	3.42±0.70
Sulfamerazine	1.70±0.1	2.84±0.77

Values are mean of three experiments±SEM, five animals in each group. Analysis of variance and two tail p-value test

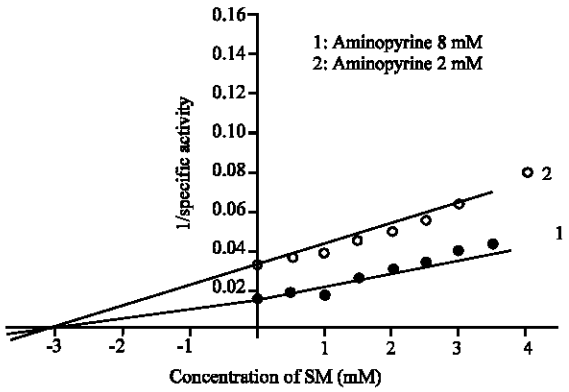

Fig. 1: Dixon plots for the inhibition of aminopyrine N-demethylase due to sulfamerazine. Aminopyrine N-demethylase activity was determined using two concentrations of substrate (aminopyrine, 2 and 8 mM) in the presence of different concentrations of sulfamerazine from 2-10 mM for 5 min incubation. Results are plotted as the mean of triplicate determinations

Table 8: *In vitro* effect of sulfamerazine (SMR, 10 mM) on cytochrome P-450 spectral and catalytic activity

Groups	Microsomal protein[a]	Aminopyrine N-demethylase[c]	Cytochrome P-450[b]
Microsomes	6.21±1.35	6.93±0.18	0.42±0.01
Microsomes+Sulfamerazine	4.72±0.64	3.44±0.91*	0.31±0.01***
Microsomes+NADPH-gen.sys.	6.11±1.04	6.81±0.69*	0.41±0.01
Microsomes+Sulfamerazine + NADPH-gen. sys.	5.37±1.10	6.72±1.50$	0.39±0.01

Values are mean of three experiments±SEM; [a]mg protein g^{-1} liver; [b]nmol mg^{-1} microsomal protein; [c]nmol of formaldehydeliberate/min/mg protein. Significantly different from control at *p<0.05; **p<0.01; ***p<0.001. Significantly different from control value at $ p<0.05 by one way analysis of variance and Tukey-Kramer multiple comparisons test

Table 9: Inhibition of aminopyrine N-demethylase activity in reconstituted system due to sulfamerazine (SMR, 3.0 mM)

	Aminopyrine N-demethylase	
	In absence of SMR	In presence of SMR
Reconstituted system #	7.85±0.02	3.91±0.01*

#Contains 2 mL of partially purified cytochrome P-450 from control rat liver microsomes (containing 1 mg protein, 3 nmole of cytochrome P-450 mg^{-1} protein), 0.5 mL cytochrome^c reductase (containing 1 mg protein having 2 μmol of cytochrome^c reduced/min/mg protein, activity) and 150 μg of l-phosphatidyl choline; values are mean of three experiments±SEM, six animals in each group; ^{a}nmole of formaldehyde liberated/min/mg microsomal protein. *Significantly different from control at *p<0.05; **p<0.01; ***p<0.001 by one way analysis of variance and Two tail p-value test. Analysis of variance and Two tail p-value test

rats with respect to drug and it is might because sex difference. Decrease in cytochrome P-450 and aminopyrine N-demethylase activity at100 mg SMR kg^{-1} can be explained on the basis of the *in vitro* inhibition of aminopyrine N-demethylase. *In vitro* addition of SMR to microsomal incubates produced loss of spectrally detectable cytochrome P-450 when incubated for 10 min. However, no further loss observed in presence of NADH-generating system indicates the destruction is due to SMR and not due to its metabolites (Kodam and Govindwar, 1995; Kobliakov *et al*., 1991; Dalvi *et al*., 2004). This investigation identified that SMR is substrate of cytochrome P-450. SMR showed significant inhibition of MFOS at 100 mg SMR kg^{-1} body weight. Effect found to be dependent on dose duration, age and sex of the animal.

The decreased cytochrome: P-450 level and aminopyrine N-demethylase due to the inhibitory effect of SMR conforms that SMR is a strong inhibitor of MFOS and itself working as an inhibitor and not its metabolites.

CONCLUSION

The aim of this study was to assess the effects of SMR administration on MFOS and its effect *in vitro* on mixed function oxidase system of hepatic microsomes in rats.

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