

IN VIVO AND IN VITRO EFFECT OF SULFAMERAZINE ON HEPATIC MIXED FUNCTION OXIDASE IN RATS

P B Dandge and S P Govindwar

Department of Biochemistry, Shivaji University, Kolhapur- 416 004, India.
padmadandge@gmail.com

ABSTRACT

Sulfamerazine (SMR) administration 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 caused significant decreases in cytochrome P-450 and activities of aminopyrine N-demethylase and aniline hydroxylase. In inhibitory dose of 100 mg SMR/kg 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 dose level. All other parameters were unchanged. Sulfamerazine resulted in uncompetitive type of inhibition ($K_i = 3.0$ mM) of aminopyrine N-demethylase *in vitro*. Sulfamerazine destructed the spectral and catalytic activity of cytochrome P-450. Our 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.

Keywords: Aminopyrine N-demethylase, aniline hydroxylase, cytochrome P-450, Sulfamerazine.

INTRODUCTION

Sulfamerazine is (SMR) an antibiotic compound used for the treatment of many infectious diseases of animal, widely used in human and veterinary medicine (Stolman *et al.* 1960, Trepanier 2004, Kao *et al.* 2001). It is also used for the treatment of furunculosis in the salmonid fishes used as food additives and antibacterial agent (Ferguson 1997, Renew *et al.* 2004, Martínez *et al.* 2003, Cribb *et al.* 1996, Trepanier *et al.* 1999). Sulfamerazine enter the human by food chain through milk and meat products. In addition to this 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* (Nouws *et al.* 1986). The formation of two hydroxylation products 6-hydroxymethyl and 4-hydroxymethyl 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 (Vree *et al.* 1986). 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). 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.

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 sulfonic acid] (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, trichloroacetic acid, 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 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, 3 and 4 prepared in saline. Animals in group 2, 3 and 4 were injected with SMR for 3 days at dose levels of 100 mg/kg 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, 3, and 4 were injected i.p with saline for 5, 3, 1 and 0 successive days, respectively, followed by 100 mg SMR/kg 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 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 perfused 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.*, (Cinti *et al.* 1972). The microsomal pellet was washed with 1.15% KCl solution containing 0.05 mM EDTA resuspended in phosphate buffer (0.1M, 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 (Omura, Sato 1964). Aminopyrine N-demethylase activity was assayed according to the procedure of Schenkman *et al.* (Schenkman *et al.* 1967). Formaldehyde liberated by the procedure of Nash (Nash 1953). Aniline hydroxylase assay was performed using the procedure reported by Govindwar and Dalvi (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 s and reaction was initiated by the addition of aminopyrine substrate. The inhibition data of

sulfamerazine was plotted according to Dixon (Dixon 1953) for the determination of apparent k_i value.

K_m determination was performed with 2 mg microsomal protein/ ml incubated with concentrations of aminopyrine 2 to 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.

Loss of spectrally detectable and catalytic activity of cytochrome P-450

In another experiment to study the *in vitro* effect of SMR on the spectrally detectable and catalytic activity of cytochrome P-450 the control rat liver microsomes were divided into 4 groups equal groups 2,3, and 4 were incubated with 10 mM SMR, NADPH-generating system or 10 mM SMR + NADPH-generating system, respectively for 10 min at 37 °C. Group 1 was incubated with phosphate buffer (0.1 M, pH 7.4) for 10 min. at 37 °C and served as control. The microsomes were isolated by centrifugation at 10,000 rpm for 30 min. after *in vitro* treatment. The pellet was resuspended in phosphate buffer (0.1 M, pH 7.4). Microsomal cytochrome P-450 level and the activity of aminopyrine N-demethylase were determined.

Determination of sleeping time

A single dose of phenobarbital 80 mg/kg in saline was injected ip to control and SMR pretreated (single dose of 100 mg/kg in saline, ip the previous day) rats to observe the effect SMR on sleeping time.

Reconstitution

Two ml of partially purified cytochrome P-450 (3 nmole/mg protein, 1mg protein /ml) 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 μ mole 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 μ g 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 values<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 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 dose level. However,

little increase in cytochrome b5 content was observed at 25 mg/kg of dose level. Marginal decrease in the level of cytochrome P-450 and cytochrome b5 was observed at 25 and 50 mg/kg dose level, respectively. A Significant decrease was observed in the activity of aminopyrine N-demethylase and aniline hydroxylase at 100 mg SMR/kg dose level, while marginal decrease was observed at 25 and 50 mg SMR/kg dose level. All other parameters were unchanged (Table 1and 2).

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)	Microsomal protein ^a	Cytochrome ^b	Cytochrome ^b
		b5	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 microsomal protein.

Significantly different from control at ***P< 0.001, by One way analysis of variance and Tukey-Kramer multiple comparisons test.

Table2: 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)	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

To study the inhibitory effect of SMR, the animals were dosed with 100 mg SMR/kg 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 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 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 N-

demethylase at 100 mg SMR/kg 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 dosed in old male rats. However all other parameters viz. microsomal protein, cytochrome b5, cytochrome P-450 remains unchanged at 100 mg SMR/kg dose level (Table 4, 5 and 6).

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

Parameters	Days of treatment			
	0	1	3	5
Microsomal protein(mg/g 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 (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
Microsomal protein (mg/g liver)	12.56 ± 1.40	10.68 ± 1.25
Cytochrome b5 (nmole/mg protein)	0.21 ± 0.01	0.18 ± 0.01
Cytochrome P-450 (nmole/mg 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

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.

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 our 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 K_i value calculated from Dixon plots was 3.0 mM. 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 8). SMR 3.0 mM addition *in vitro* in the reconstituted system containing affinity purified cytochrome P-450 from untreated adult rat liver microsomes showed a significant decrease (49%) in aminopyrine N-demethylase activity (Table 9).

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)
Microsomal protein (mg /g liver)	10.39 ± 0.26	7.85 ± 0.16**
Cytochrome b5 (nmole/mg protein)	0.21 ± 0.01	0.18 ± 0.01
CytochromeP-450 (nmole/mg 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.

Table 6: Effect of sulfamerazine 100 mg/kg (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)
Microsomal protein (mg /g liver)	10.98 ± 0.13	9.42 ± 0.15**
Cytochrome b5 (nmole/mg protein)	0.22 ± 0.01	0.19 ± 0.01
CytochromeP-450 (nmole/mg 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, i.p., single dose), on phenobarbital (80 mg/kg, i.p., single dose) sleeping time.

Group	Sleeping time (h)	
	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 $\pm$ SEM, five animals in each group. Analysis of variance and Two-tail p value test.

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

Groups	Microsomal protein ^a	Aminopyrine demethylase ^c	N- Cytochrome P-450 ^b
Microsomes	6.21 $\pm$ 1.35	6.93 $\pm$ 0.18	0.42 $\pm$ 0.01
Microsomes + Sulfamerazine	4.72 $\pm$ 0.64	3.44 $\pm$ 0.91*	0.31 $\pm$.01***
Microsomes + NADPH- gen.sys.	6.11 $\pm$ 1.04	6.81 $\pm$ 0.69*	0.41 $\pm$ 0.01
Microsomes + Sulfamerazine + NADPH- gen.sys.	5.37 $\pm$ 1.10	6.72 $\pm$ 1.50 ^{\$}	0.39 $\pm$ 0.01

Values are mean of three experiments $\pm$ SEM.

^a mg protein/ g liver.

^b nmol/mg microsomal protein.

^c nmol of formaldehydeliberate/min/mgprotein

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).

Reconstituted System #	Aminopyrine N-demethylase	
	In absence of SMR	In presence of SMR
	7.85 $\pm$ 0.02	3.91 $\pm$ 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 per mg protein), 0.5 ml cytochrome c reductase (containing 1 mg protein, having 2 μ mol of cytochrome c reduced /min/mg protein, activity) and 150 μ g of l – phosphatidyl choline.

Values are mean of three experiments $\pm$ 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.

The objective of these studies was to examine the effect of SMR on the microsomal MFO system. N₄-acetylation of SMZ widely used as a marker in man as a genetically determined capacity of N₄-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 mgSMR/kg dose level.

While in dose duration studies showed significant decrease in electron transport component and drug metabolizing enzymes at 3 and 5 days (White *et al.* 1967, Whitkamp *et al.* 1992, Winter *et al.* 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 treatment suggest that resistance of in female rats with respect to drug and it is might because sex difference. Decrease in cytochrome P-450 and aminopyrine N-demethylase activity at 100 mg SMR/kg 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 1995, Kobliakov *et al.* 1991, Dalvi *et al.* 2004)

PB sleeping time was used to assess the effect on the *in vivo* metabolism. No significant decrease

was observed in sleeping time when PB injected to SMR-pretreated male as well as female rats, which indicates no interaction of SMR in the inducing effect of PB (Achari *et al.* 1997, Kodam *et al.* 1996, Zidek *et al.* 1977).

May have higher acetylation rate of SMR and non availability of CYP 2C11 (Waxman *et al.* 1985, Adav *et al.* 2003) in that sex.

This investigation identified that SMR is substrate of cytochrome P-450. SMR showed significant inhibition of MFOS at 100 mg SMR/kg 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.

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