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Full Length Research Paper

A gender wise study of arylamine N-acetyltransferase 2 (NAT2) acetylation phenotyping using sulphamethazine by high-pressure liquid chromatography (HPLC) assay

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The acetylation polymorphism is one of the most common inherited variations in the biotransformation of drugs and chemicals and large number of studies has been done to determine the distribution of acetylator phenotypes among populations of different geographic origin. The aim of this study was to investigate the acetylator phenotypes of the Pakistani population and compare it between male and female healthy volunteers. The polymorphic acetylation of sulphamethazine has been investigated in male and female volunteers (50 each) of Pakistani population. Sulphamethazine was administered orally in Capsule form as 500 mg to each healthy male and female volunteer. Sulphamethazine and acetylsulphamethazine were determined in the six hour plasma samples by high-pressure liquid chromatography (HPLC). Acetylator phenotype was determined from the metabolic ratio of acetylsulphamethazine to sulphamethazine in the plasma samples. The acetylation of sulphamethazine by arylamine N-acetyltransferase 2 (NAT2) showed bimodal population frequency distribution. 60% of the female volunteers were found to be fast and 40% to be slow acetylators while that of male volunteers were 62% fast and 38% slow acetylators.

Key words: NAT2 acetylation phenotype, sulphamethazine, male & female volunteers, HPLC assay.

INTRODUCTION

Arylamine N-acetyltransferase 2 (NAT2, EC 2.3.1.5) is a drug metabolizing enzyme that shows common type of polymorphisms which lead to impaired drug metabolism and adverse drugs effects (Agundez et al., 2008). Polymorphisms of drug metabolizing enzymes significantly may affect the treatment outcomes and are reported to be associated with risks of developing various types of cancer and neurodegenerative disease (Bandmann et al., 2000; Wikman et al., 2001; Hein, 2006; Lilla et al., 2006). An early example of drug metabolizing enzymes polymorphism is the NAT2 enzyme, which metabolizes several drugs as isoniazid, sulphamethazine and dapsone, as well as other chemicals and carcinogens (Gross et al., 1999; Butcher et al., 2002). The human acetylation polymorphism was discovered from more than five decades during the metabolic investigation of the antituberculousis drug isoniazid (Weber, 1990) and can influence both activation and deactivation pathways of arylamine metabolism (Hein, 1988). A genetic polymorphism of the human liver NAT2 enzyme activity divides populations into two distinguishable phenotypes "slow acetylator" and "fast acetylator" phenotypes (Grant et al., 1991). A number of common sequence variants in the NAT2 gene have been described that are responsible for ‘‘slow acetylator’’ phenotype and the frequency of these sequences across populations vary markedly (Hein, 2002). The N-acetylation capacity has been investigated in different

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Abbreviations: HPLC, High-pressure liquid chromatography; **NAT2,** arylamine N-acetyltransferase 2; **SDZ,** sulfadiazine internal standard; **AcSMZ,** N4-acetylsulfamethazine; **SMZ,** sulphamethazine.

Table 1. Demographic data (Mean ± SD) for healthy male and female volunteers.

Volunteer no.		Age (years)	Weight (kg)	Height (cm)	Body temperature (˚F)	Blood pressure (mmHg)	
						Systolic	Diastolic
Mean	Male	22.26	61.66	5.617	98.174	115.24	78.2
	Female	22. 78	55.3	5.26	98.15	114.6	76.36
± SD	Male	2.29	9.039	0.28	0.839	9.527	7.669
	Female	1.951	7.28	0.23	0.89	8.51	8.05
Maximum	Male	28	90	6.1	100	130	100
	Female	27	73	5.5	99	130	90
Minimum	Male	22	45	5.3	96	90	90
	Female	18	42	5.1	96	80	80

populations and has been classified in bimodal (rapid or slow) and trimodal (rapid, intermediate or slow) allocations (Kilbane et al., 1990; Parkin et al., 1997; Kinzig-Schippers et al., 2005) and the individual differences in the NAT2 acetylation capacity results in slow or fast NAT2 acetylator phenotypes (Sillanpaa et al., 2005). The proportions of slow and fast phenotypes show a discrepancy in different ethnic groups as Caucasians individuals have 40–70% of the slow acetylator phenotype whereas the Asian populations have only 10–30% slow acetylators (Meyer and Zanger, 1997; Brans et al., 2004). Inherited interindividual variation for the drugs such as isoniazid and sulphamethazine is due to the NAT2 acetylation polymorphism (Smith et al., 1995; Hivonen, 1999). Individuals can be classified as slow and/or fast acetylators, according to the activity of this enzyme and these drugs can be used as probes to determine NAT2 acetylation phenotype (Johns and Houlston, 2000).

AT2 acetylation phenotype can be determined by dosing the subjects with specific probe drug such as sulphamethazine and then measuring the acetyl metabolites in urine or plasma to categorized the subject as ‘‘slow’’ or ‘‘fast’’ acetylator (Ognjanovic et al., 2006).

The NAT2 acetylation polymorphism is very central in clinical pharmacology and toxicology as its primary role in the activation and/or deactivation of a large number of aromatic amine and hydrazine drugs used in clinical medicine. Epidemiological studies showed that adverse drug reaction incidences increases with age (Philip et al., 1984) while the number of factors as age, sex, disease, diet and environment modulate the activity of the hepatic enzyme (Vesell, 1982) in the body. A little is known about the influence of these factors on NAT activity and several studies have shown the contradictory results on the influence of age and gender on the NAT2 phenotype (Gachalyi et al., 1984; Pontiroli et al., 1985; Paulsen and Nilsson, 1985). The present study is an investigation into the influence of gender on acetylation phenotype using sulphamethazine.

MATERIALS AND METHODS

The research work was conducted in the Pharmaceutical Research Lab. and Molecular Biochemistry Lab, Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan.

Subjects

A total of one hundred subjects took part in this study belong to different areas of Pakistan. Fifty healthy male individuals (mean age, 22 years; range 19–28 years and mean body weight, 61 kg; range 45–90 kg) and fifty healthy female individuals (mean age, 22 years; range 20–27 years and mean body weight, 55 kg; range 42–73 kg) were included in this study (Table 1). The study was approved by the Ethical Committee of Institute and written informed consent signed by all subjects was obtained. All the subjects were investigated to see any type of abnormality in their history; physical examination and laboratory study which included the complete blood picture (Table 2) and plasma biochemistry (Figures 1 and 2) and all were non alcoholics and non smokers and were not allowed to take any caffeinated drinks. All the subjects were on normal diets and none was on any medication which is known to interact with NAT2 from the week before until the end of the study and all individuals were kept in the study center from before 12 h to till sampling of the studied drug completed.

Reagents and chemicals

The reference standards of sulphamethazine and acetylsulphamethazine were purchased from reputable sources. Sulphamethazine, sulfadiazine and monohydrogen phosphate, sodium dihydrogen phosphate, were purchased from sigma, deionized distilled water was obtained from Adventec (GS-590, distillery and CPW-200), Japan from Central High Tech Lab, University of Agriculture, Faisalabad (UAF). Drug free plasma was obtained from Cheniot Dialysis Center and from Allied Hospital, Faisalabad. Acetonitril (Merk), methanol (Lab Scan), acetone purchased from Panreac. All chemicals and solvents were of high purity and HPLC grade (Sigma/ Labscan/ Panreac).

Dosing regimen and blood collection

Prior to the sampling of the probe drug all volunteers were asked not to take any caffeinated drinks before 12 h of sampling. A 10 mL of venous blank blood sample was collected from each volunteer used for complete blood picture and biochemical parameters after

Table 2. Complete blood picture (Mean ± SD) for male and female healthy subjects.

Volunteer no.		Hb (g %)	ESR (mm/1st h)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosiniphils (%)
Mean	Male	15.2	7.92	63.26	33.64	2.04	1.46
	Female	14.33	14.44	56.4	39.12	2.94	1.68
± SD	Male	0.351	3.205	8.804	8.5	1.08	1.10
	Female	1.454	9.567	10.78	10.13	1.31	0.49

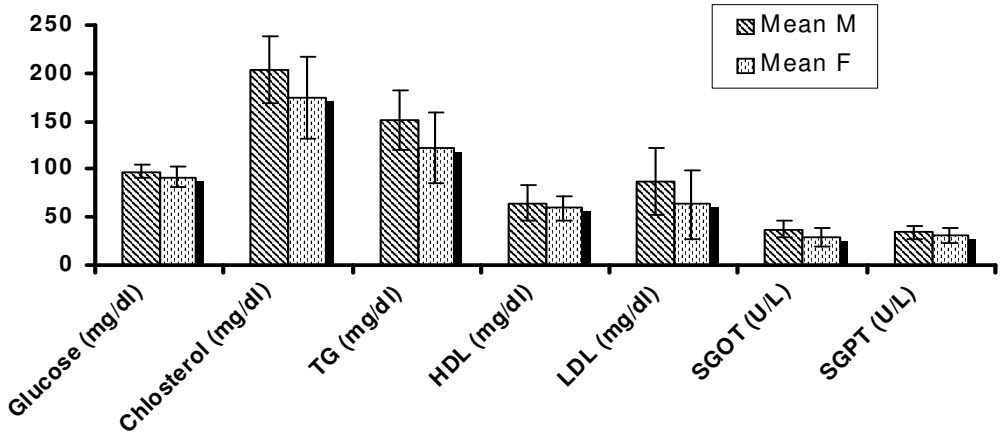

Figure 1. Mean ± SD of biochemical parameters (glucose, cholesterol, TG, HDL, LDL, SGOT and SGPT) for healthy male and female volunteers.

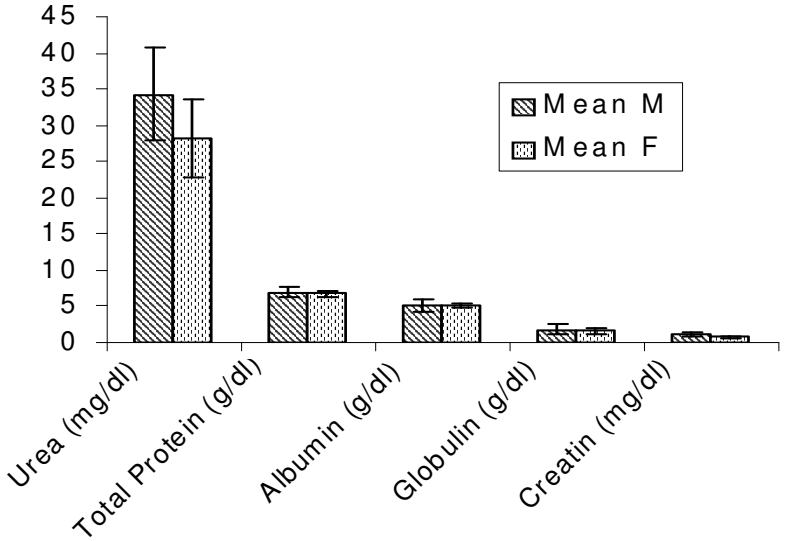

Figure 2. Mean ± SD of biochemical parameters (urea, total protein, albumin, globulin and creatiine) for healthy male and female volunteers.

an overnight fasting. All volunteers were provided an oral dose of 500 mg sulphamethazine capsule with a glass of water. Then all were provided similar breakfast and single blood sample (5 ml) was collected in heparinized centrifuge tubes at 6 h after dosing. Blood was centrifuged and plasma separated immediately to be stored at -20℃ until analysis.

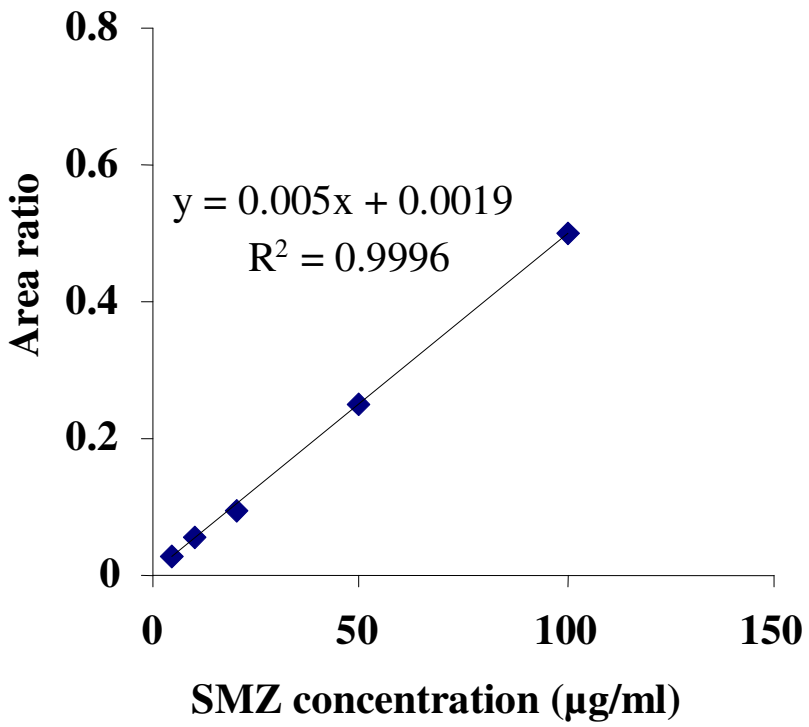

Figure 3. Standard curve for sulphamethazine.

HPLC instrumentation

The HPLC system consisted of a Shimadzu SCL-10A system controller, UV-Visible SPD-10 AV detector and LC-10 AT pump with FCU-10AL VP flow controller valve. Separation was achieved at ambient temperature with a Shim-pack CLC-ODS column (4.6 × 15 mm), pore size 5 µm. The chromatographic data was collected and analyzed using CSW 32 software.

Chromatographic conditions

Isocratic mode was used for the analysis of sulphamethazine and acetylsulphamethazine with flow rate of 1 ml/min and retention time was 5.9 and 8.5 minutes for N4-acetylsulphamethazine (AcSMZ) and sulphamethazine (SMZ), respectively. SMZ and AcSMZ were detected at 254 nm.

Standard curves

Standard curves were constructed using 5 standard concentrations in plasma and run in duplicate daily for three consecutive days. Stock solutions 1.0 mg/ml of SMZ and AcSMZ we reprepared in distilled water and sulfadiazine (SDZ) as internal standard was prepared in aqueous acetonitrile (200 ml of acetonitrile per liter of water). Standard concentrations of SMZ and AcSMZ were prepared in the drug free plasma in the concentration range of 5, 10, 20, 50 and 100 µg/ml, and quality control of 8, 40 and 80 µg/mL and internal standard was used as 100 µg/mL for plasma. Calibration curves were generated using regression analysis and obtained over the respective standard concentration range for sulphamethazine and acetylsulphamethazine and shown in Figures 3 and 4, respectively.

Determination of SMZ and AcSMZ in plasma/phenotyping assay for NAT2

Plasma samples were quickly thawed and processed (Whelpton et al., 1981). Plasma (500 µl) was transferred to a plastic 2 mL microcentrifuge tube and 500 µl of internal standard (Sulfadiazine in aqueous acetonitrile) was added. Samples were vortex mixed for one minute and then centrifuged at 13,000 rpm for 10 min. The clear supernatant was taken and filter through syringe filter (0.45 µ), injected 20 µl directly into the column.

The mobile phase consisted of phosphate buffer (0.067 molar, pH 5.9), acetonitrile and methanol (20: 7: 3) supplied at a flow rate of 1 ml/min at ambient temperature (27). The inter assay and intra assay coefficient of variation (Table: 3) were less than 10%. Drug concentrations were reported as the ratios of peak height for SMZ and AcSMZ to the internal standard. Subjects with acetylation ratio of acetylsulphamethazine to sulphamethazine less than 0.45 were assigned as slow whereas greater than 0.45 as fast acetylators. The Figure 5 showed the representative chromatogram for the sulphamethazine, acetylsulphamethazine and sulfadiazine.

Statistical analysis

Results are presented as means ± SD while simple regression was used to find the concentration of AcSMZ and SMZ. To find the comparison between male and female healthy subjects chi square test was used. A level of significance was assumed to be 5%.

RESULTS AND DISCUSSION

In this study total of 100 individuals including both male and female (50 each) were NAT2 phenotyped by SMZ.

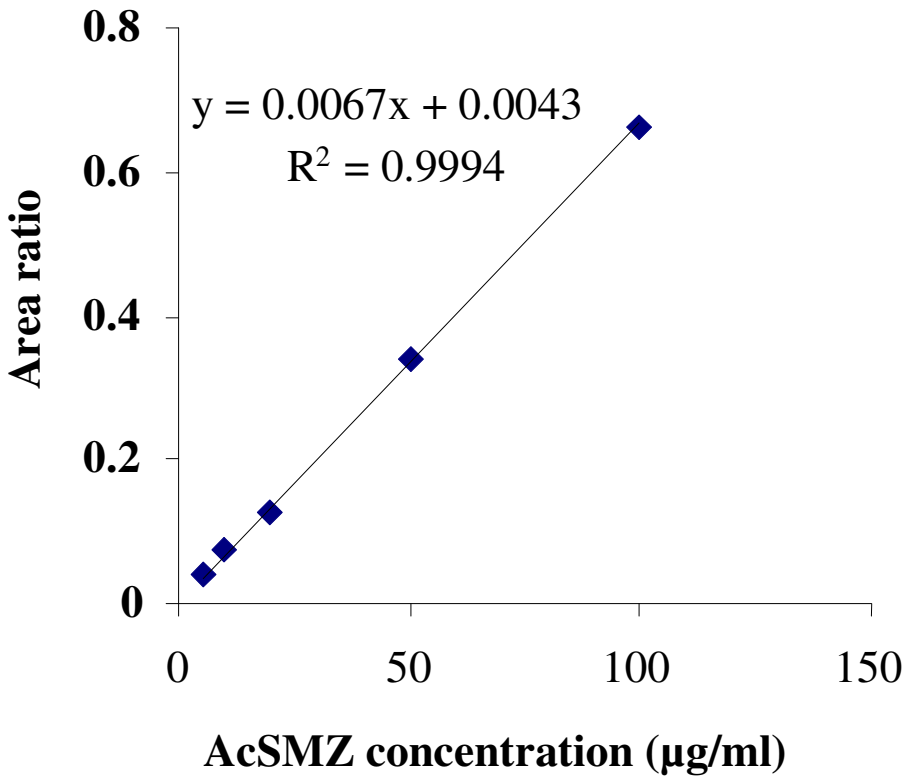

Figure 4. Standard curve for acetylsulphamethazine.

Table 3. Intra-day and inter-day precision and accuracy for sulphamethazine (SMZ) and acetylsulphamethazine (AcSMZ).

Reproducibility	Concentration (µg/mL)	Sulphamethazine			Acetylsulphamethazine		
	Added	Found (Mean ± SD)	CV (%)	Difference (found vs. added)	Found (Mean ± SD)	CV (%)	Difference (found vs. added)
Intra-assay[a]							
Quality controls	8	8.55 ± 0.60	6.98	6.84	8.69 ± 0.73	8.41	8.67
	40	41.6 ± 2.52	6.06	4.06	39.4 ± 2.44	6.13	-0.39
	80	86.9 ± 3.99	4.59	8.69	82.3 ± 3.2	3.87	2.87
Inter-assay[b]							
Quality controls	8	8.41 ± 0.7	8.3	5.13	8.68 ± 0.621	7.15	8.48
	40	38.7 ± 1.89	4.89	0.9	39.6 ±1.83	4.63	-1.01
	80	84.0 ± 3.48	4.14	5.01	80.9 ± 2.631	3.25	1.145
Standards	5	5.19 ± 0.45	8.64	3.75	5.133 ± 0.42	8.23	2.66
	10	9.37 ± 0.41	4.24	-2.7	10.49 ± 0.76	7.29	4.85
	20	20.6 ± 0.95	4.60	3.09	21.65 ± 0.62	2.88	8.27
	50	52.1 ± 2.15	4.12	4.21	48.74 ± 1.73	3.55	-2.5
	100	102.4 ± 2.15	2.10	2.41	103.2 ± 2.36	2.29	3.25

Acetylation of sulphamethazine was determined after oral administration of 500 mg sulphamethazine in healthy male and female volunteers. The blood sample was collected at 6 hour after drug administration and concentration of sulphamethazine and acetylsulphamethazine were determined by previously validated high performance liquid chromatography (HPLC) assay (Irshaid et al., 1991). At LOQ in plasma the signal to noise ratio was greater than 5:1. The inter assay and intra assay coefficients of variation were less than 10%. The molar ratio of AcSMZ/SMZ was used to find the acetylation status of all volunteers and the acetylation

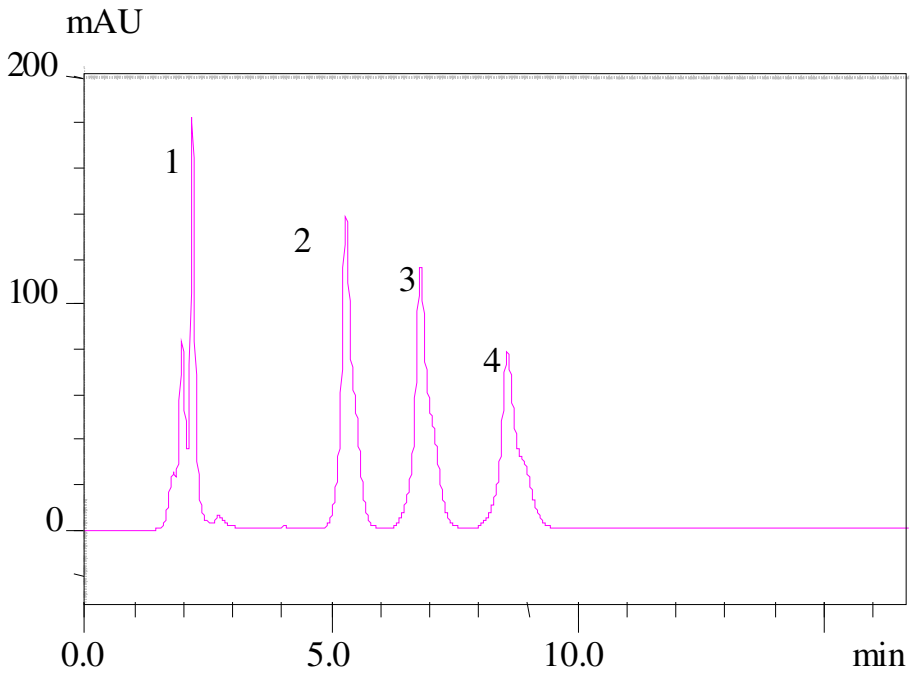

Figure 5. Representative chromatogram in the plasma (Peak 1 blank plasma peak) for 50 µg/ml SDZ (Peak 2), 100 µg/ml AcSMZ (Peak 3) and 100 µg/ml SMZ (Peak 4).

status was determined by finding the molar ratio of AcSMZ/SMZ. The frequency histogram for molar ratio of AcSMZ/SMZ was shown in the Figures 6 and 7 for male and female respectively. The maximum number of male and female subjects was fall in the AcSMZ/SMZ ratio of 0.01 to 0.6 while minimum number subjects were fall in the ratio of 1.5 to 2.00 for both male and female subjects. The relationship between plasma AcSMZ/SMZ ratio and the plasma SMZ and AcSMZ concentrations are shown in Figures 8 and 9, respectively. There was a positive correlation between plasma AcSMZ/SMZ acetylation ratio and plasma SMZ ($r = 0.3841$ $p < 0.01$) and AcSMZ ($r = 0.3895$ $p < 0.01$) concentrations. The acetylator phenotype pattern of distribution was found to be bimodal in both male and female healthy volunteers and it is similar to the findings reported elsewhere (Nhachi, 1988; Abzalov et al., 2000). Our data shows that the frequencies of slow and fast acetylator phenotypes were 38 and 62%, respectively, among male subjects while in female subjects, 40% were found to be slow and 60% fast acetylators. The percentage distribution of two acetylator phenotype among total of 100 subjects as well as in male (50) and female (50) was shown in Table 4. The distribution of acetylator phenotypes among populations of different geographic origins have described by several studies and found that the distribution of NAT2 phenotype differs widely among different populations (Lin et al., 1993) and epidemiological studies showed that Asian populations are fast acetylators by NAT2 enzyme (Gupta et al., 1984), 13.1% among Japanese pulmonary tubercular and non tubercular chest-disease subjects TB (Kohno et al., 1996) and 45% in the healthy subjects of with one degree of freedom and $\alpha=0.05$, there is no significant difference in phenotype distribution between the sexes as $\chi^2<3.832$ and similar results showed in another study girls showed non significantly lower proportion of fast acetylators than boys (Hadasova et al., 1990) however on second thought a significant effect of sexes was found upon reanalysis of Evans' original research data (Iselius and Evans, 1983). Results show that our population was slightly fast acetylator of NAT2 enzyme similar to other Asian populations where the fast acetylation phenotype is more common than slow acetylation phenotype. The distribution of slow acetylators was 14.6% among Indian pulmonary patients the Iranian population (Khalili et al., 2009) In Asians slow acetylator phenotype is much less frequent (10). In Nigerian population 41% slow acetylators were observed (Eze and Obidoa, 1978), in native Chinese population 19.8% slow acetylators were found after a single oral dose of 1 g Sulphamethazine (Xu and Jiang, 1990), similarly an apparent bimodal distribution of acetylator phenotype in 96 subjects (27% slow and 73% fast acetylators) was found from measuring the percentage of acetylation of sulphamethazine in 6 h plasma sample (Huang et al., 1992) which are similar to our findings as NAT 2 acetylation phenotype distribution in our study population was bimodal as 61 fast and 39% slow irrespective of gender. There was no significant $\chi^2<3.832$ difference of NAT 2 acetylation distribution in

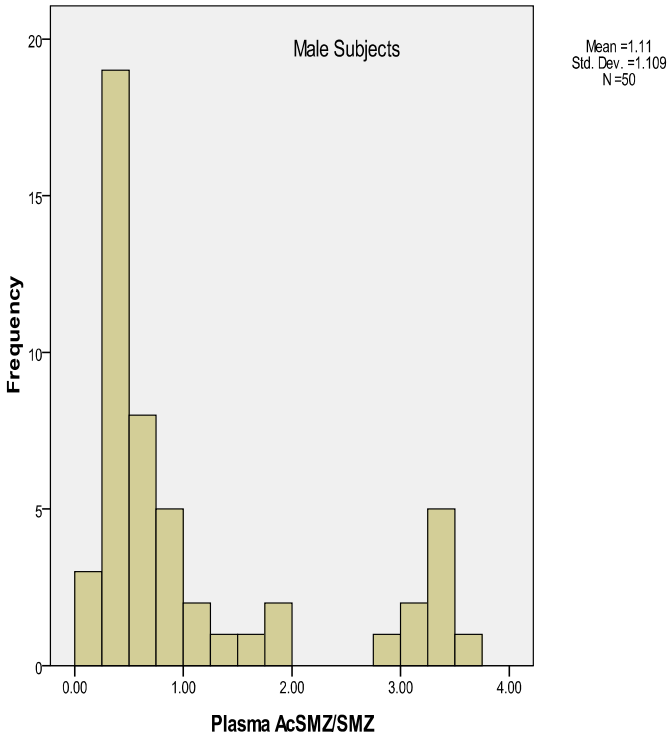

Figure 6. The frequency histogram for molar ratio of AcSMZ/SMZ for male.

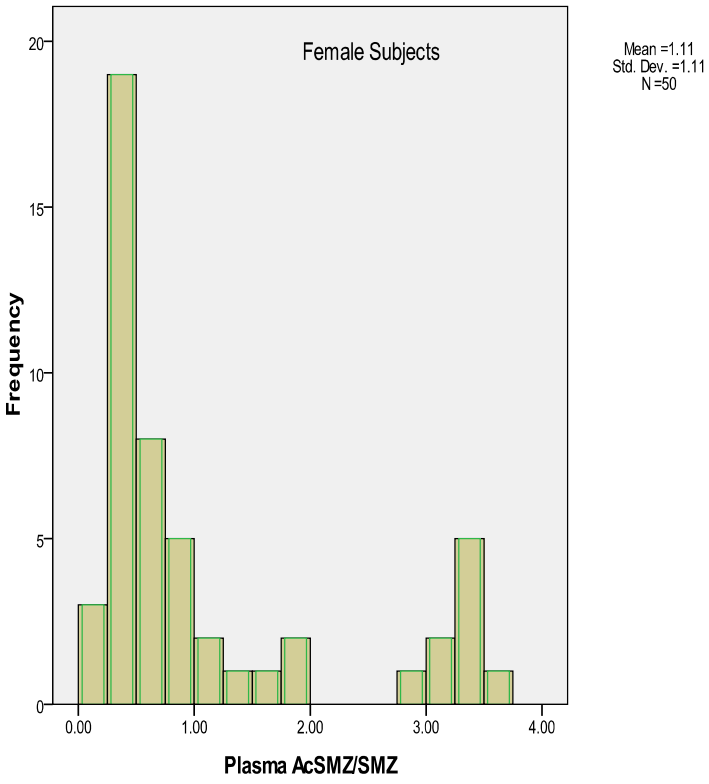

Figure 7. The frequency histogram for molar ratio of AcSMZ/SMZ for female.

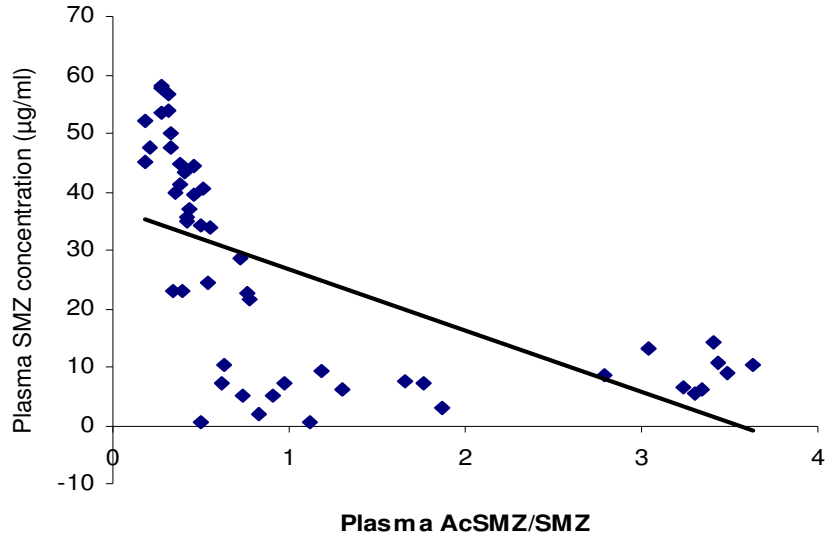

Figure 8. The relationship between plasma AcSMZ/SMZ ratio and the plasma SMZ.

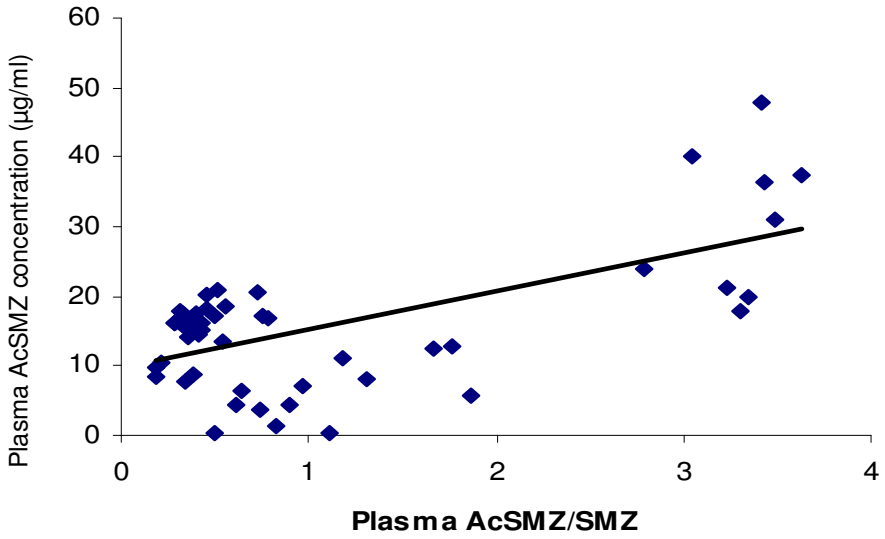

Figure 9. The relationship between plasma AcSMZ/SMZ ratio and the plasma AcSMZ.

male (62 % fast, 38 % slow) and female (60% fast, 40% slow) volunteers, similar results were obtained by caffeine test (Muscat et al., 2008), by sulphamethazine assay (Hadasova et al., 1990) and by dapsone test (Philip et al., 1984) all of them found that NAT2 acetylation was unaffected by gender distribution. The acetylator phenotype is a lifelong, stable characteristic of the individual and can be determined by using any of many probe drug e.g., sulphamethazine (Hein, 2002). Nevertheless, further investigations are required to establish the acetylation status affected by sex, age, weight, height, and food interaction etc of this enzyme in the Pakistani population. No such research was conducted in any other study published to date in Pakistani population.

Conclusion

In conclusion our local population was found to be slightly fast acetylators of NAT2 enzyme by sulphamethazine which is comparable to other Asian population and there was non significant higher proportion of fast acetylators among male than female, this data will provided the

Table 4. Distribution of the acetylator phenotype among 100 subjects (50 male and 50 female).

Acetylator distribution (%)	Fast	Slow
Total acetylators	61	39
Female acetylators	60	40
Male acetylators	62	38

basics for the future therapeutic implementations that considering the sex during dose regimen is not significant.

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