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Phenotyping of N-acetyltransferase 2 in Male Volunteers by HPLC

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ABSTRACT

The N-Acetyltransferase 2 (NAT2) 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 NAT2 acetylator phenotypes among populations of different geographic origins. The aim of this study was to investigate the acetylator phenotype of the healthy male subjects of Pakistani population. The polymorphic acetylation of sulphamethazine has been investigated in male volunteers (n=50) of Pakistani population. 500 mg Sulphamethazine was administered orally in capsule form to each volunteer. Sulphamethazine and acetylsulphamethazine were determined in the six hour plasma samples by reversed phase HPLC assay. Acetylator phenotype was determined from the metabolic ratio of acetylsulphamethazine to sulphamethazine in the plasma samples. The acetylation of sulphamethazine by NAT2 showed bimodal population frequency distribution. About 62% of the male volunteers were fast and 38% were slow acetylators. Data revealed that studied population is the fast acetylator by NAT2 enzyme. © 2011 Friends Science Publishers

Key Words: NAT2 phenotype; Sulphamethazine; Male volunteers; HPLC assay

INTRODUCTION

Arylamine *N*-acetyltransferases (NATs), EC 2.3.1.5 are found in almost all species from bacteria to humans (Butcher *et al.,* 2002) and exist as two polymorphic forms as *N*-acetyltransferase 1 (NAT1) and *N*-Acetyltransferase 2 (NAT2), both involved in drug and/or chemicals metabolism. A large number of clinically important drugs are metabolized by NAT2 (Probst *et al.,* 1992). The arylamine *N*-acetyltransferases expressed predominantly in liver and gut (Sim *et al.,* 2008), is polymorphic in humans (Deguchi *et al.,* 1990). These polymorphisms 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).

NAT2 enzyme polymorphism is an early example of drug metabolizing enzymes, which metabolizes many drugs as isoniazid and sulphamethazine, as well as other chemicals and carcinogens (Gross *et al.,* 1999; Butcher *et al.,* 2002). In 1950 during the metabolic investigation of the antituberculousis drug (isoniazid) the human acetylation polymorphism was discovered (Weber, 1990) and both activation and deactivation pathways of arylamine metabolism are affected by it (Hein, 1988).

NAT2 polymorphism is responsible for the inherited interindividual variation for the acetylation of the drugs such as isoniazid and sulphamethazine (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 & Houlston, 2000). The frequency of NAT2 phenotype varies markedly across the populations (Hein, 2002). The N-acetylation capacity has been investigated in different populations and classified the individuals either in bimodal (rapid or slow) or 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 & Zanger, 1997; Brans *et al.,* 2004).

The NAT2 phenotype can be determined by dosing the subjects with specific probe drug such as sulphamethazine and then measuring the acetylmetabolites in urine or plasma to categorized the subject as ''slow'' or

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''fast'' acetylator (Ognjanovic *et al.,* 2006). The NAT2 acetylation polymorphism is very central in clinical pharmacology and toxicology as of its primary role in the activation and/or deactivation of a large number of aromatic amine and hydrazine drugs used in clinical medicine, so the project has been designed to investigate the NAT2 phenotype acetylation in the male volunteers of Pakistani population by using HPLC assay, because high pressure liquid chromatographic methods are favored due to its accuracy and easiness.

MATERIALS AND METHODS

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

Subjects: A total of fifty healthy male subjects of local population of Pakistan of mean age, 22 years; range 19–28 years and mean body weight, 61 kg; range 45–90 kg took part in this study. The study was approved by the Ethics 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 and plasma biochemistry. 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. All were non-alcoholics and non smokers and were not allowed to take any caffeinated drinks during the sampling.

Reagents and chemicals: The reference standards of probe drug 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, distillary & 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). Sulphamethazine was the gift from Star laboratories, (PVT) LTD, Lahore 53800, Pakistan.

Sampling of the probe drug: Ten mL of venous blank blood sample was collected from each volunteer for blank plasma, complete blood picture and biochemical parameters determination after an overnight fasting. All volunteers were provided an oral dose of 500 mg sulphamethazine capsule with a glass of water and single blood sample (3 mL) was collected in heparinized centrifuge tubes at 6 h after dosing. Blood was centrifuged and immediately separated plasma to be stored at -20°C until analysis.

Equipment and 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 mm×15 mm), pore size 5 µm. The chromatographic data was collected and analyzed by using CSW 32 software.

HPLC assay for SMZ and AcSMZ: Plasma samples were quickly thawed, processed and determined according to Whelpton, *et al.* (1981) by using the mobile phase of Irshaid *et al.* (1991) with slight modification. 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 and then centrifuged at 3000 rpm for 10 min. The clear supernatant was taken and filter through syringe filter (0.45 µm), injected 20 µL directly into the column. The samples were eluted with 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. Isocratic mode was used for the analysis of sulphamethazine and acetyl sulphamethazine with flow rate of 1 mL/min and retention time was 7.9 and 6.6 min for SMZ and AcSMZ, respectively at the wave length of 254 nm.

Standard curve: Stock solutions for SMZ and AcSMZ (1.0 mg/mL) were prepared in distilled water and that of sulfadiazine (SDZ), the internal standard was prepared in aqueous acetonitrile (200 mL of acetonitrile per liter of water). Standard concentrations for SMZ and AcSMZ were prepared in the drug free plasma in the concentration range of 5, 10, 20, 50 and 100 µg/mL and concentration of quality controls (QCs) were 8, 40 and 80 µg/mL. Calibration curves for SMZ and AcSMZ were generated using regression analysis and obtained over the respective standard concentration range.

Calibration and linearity: Assay performance was done according to the ICH Guidance (ICH Guidance, 2005). Standard curves were constructed using 5 standard concentrations in plasma and run in duplicate daily for three consecutive days drug concentrations were reported as the ratios of peak height for SMZ and AcSMZ to the internal standard calibration curves were generated using regression analysis and obtained over the respective standard concentration range for sulphamethazine and acetylsulphamethazine.

Statistical analysis: Means±SD value was used for body weight, age and biochemical parameters. The CV % was calculated to determine precision.

RESULTS AND DISCUSSION

In this study total of 50 healthy male subjects were NAT2 phenotyped by SMZ. NAT2 acetylation was determined after an oral administration of 500 mg sulphamethazine in healthy male volunteers of mean age

Table I: Demographic data of healthy male volunteers (n=50)

Volunteer no.	Age (years)	Weight (kg)	Height (cm)	Body temperature (˚F)	Blood pressure (mm Hg)	
					Systolic	Diastolic
Mean	22.78	55.3	5.255	98.148	114.6	76.36
± SD	1.93	7.28	0.23	0.89	8.51	8.05
Maximum	28	90	6.1	100	130	100
Minimum	18	45	5.1	96	90	90

Table II: Intra-and inter-day precision and accuracy for sulphamethazine (SMZ) and acetylsulphamethazine (AcSMZ) in plasma

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

a Six quality control samples per concentration

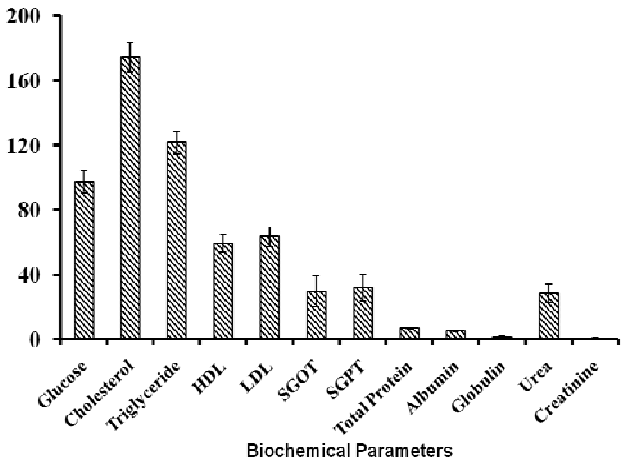
b Eighteen quality control samples or two standards per day per concentration for 3 days

22.26±2.29 years. The demographic data (Table I) and biochemical parameters (Fig. 1) were also determined for each volunteer. After drug administration blood samples were collected at 6 h and concentration of sulphamethazine and acetyl sulphamethazine were determined by High Performance Liquid Chromatography (HPLC) assay.

The representative chromatogram for the SDZ, SMZ and AcSMZ in plasma was with retention time of 5.0, 6.6 and 7.9 min for SDZ, AcSMZ and SMZ, respectively (Fig. 2). The plasma standard curve for SMZ and AcSMZ was found to be linear in the range of 5−100 µg/mL with a slope of 0.005 and 0.0066, a correlation coefficient of 0.999 for both and y-intercept of 0.0019 and 0.0043, respectively. For precision and accuracy, plasma samples of standards and replicate QCs at each concentration were analyzed on three consecutive days and then inter and intra-day means, standard deviation (SD), and coefficients of variation (CV %) were calculated (Table II) by standard methods (ICH Guidelines, 2005). 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%.

Phenotyping assay for NAT2 was done after HPLC determination of SMZ and AcSMZ in volunteer's plasma. The AcSMZ/SMZ molar ratio was used to check the acetylation status of healthy subjects. Subjects with acetylation ratio of acetylsulphamethazine to sulphamethazine more than 0.45 were denoted as fast, while less than 0.45 were rated as slow acetylators (Ladero *et al*., 1987). As regards frequency histogram for molar ratio of acetylsulphamethazine and sulphamethazine, a bimodal

Fig. 1: Mean ± SD biochemical parameters for healthy male volunteers (n=50)



acetylator phenotype pattern of distribution was found in healthy male subjects (Fig. 3). The percentage distribution of slow and fast acetylator phenotype was 38% and 62%, respectively in our study data and is similar to the findings reported elsewhere (Nhachi, 1988; Abzalov *et al.,* 2000).

Among the populations of different geographic origins the distribution of acetylator phenotypes have been described by several studies and found that the distribution of NAT2 phenotype differs widely among different population (Lin *et al.,* 1993). The epidemiological studies showed that Asian populations are fast acetylators by NAT2

Fig. 2: Representative chromatogram in plasma (peak 1 of blank plasma), 50 µg/mL SDZ (peak 2), 100 µg/mL AcSMZ (peak 3) and 100 µg/mL SMZ (peak 4)

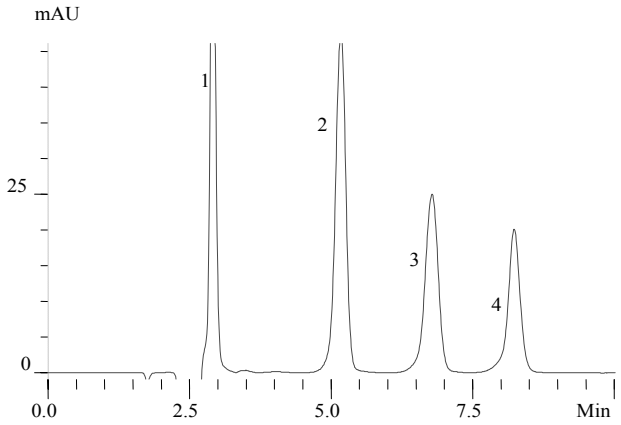
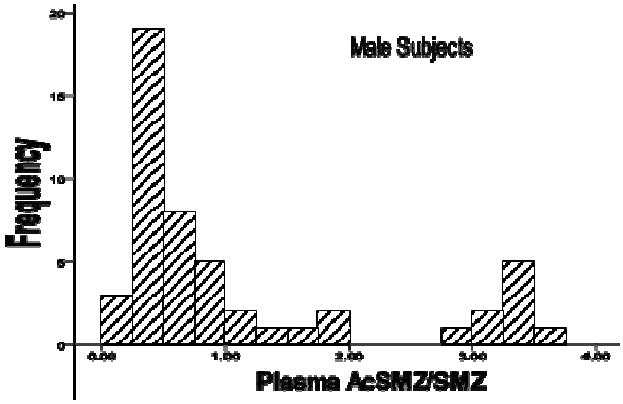


Fig. 3: The frequency histogram for molar ratio of AcSMZ/SMZ for male subjects (n=50)



enzyme as the frequency of the slow acetylation phenotype of Asian populations, such as Japanese, Chinese, Korean, and Thai, ranged from 10 to 30% (Meyer *et al.,* 1997). In an another study the frequency of slow acetylation was also less common in Asian population (15%) as compared to European (55%) and African (35%) populations indicating that Asians are fast acetylators by NAT2 enzyme (Yu *et al.,* 1994).

In Asians, slow acetylator phenotype is much less frequent (Hein, 2002) than the Caucasians (50 to 60%) and Northern Africans (90%) (Marchand *et al.,* 1996). Our studied population subjects were found to be the fast acetylator of NAT2 enzyme, which is similar to other Asians like distribution of slow acetylator phenotype was 14.6% among Indian pulmonary tubercular and non-tubercular chest-disease subjects (Gupta *et al.,* 1984) 13.1% among Japanese pulmonary TB patients (Kohno *et al.,* 1996) and 45% in the healthy subjects of the Iranian population (Khalili *et al.,* 2009). Similarly, in Nigerian population 41% (Eze & Obidoa, 1978) and in native Chinese population 19.8% (Xu & Jiang, 1990) slow acetylators were found after a single oral dose of 1 g sulphamethazine. An apparent bimodal distribution of acetylator phenotype in 96 subjects (27% slow & 73% fast acetylators) was observed from measuring the percentage of acetylation of sulphamethazine in 6 h plasma sample (Huang *et al.,* 1992). Our findings corroborate these data as NAT2 acetylation phenotype distribution in our study was bimodal (62% fast & 38% slow). NAT2 acetylation was also unaffected by gender distribution as revealed from results obtained by caffeine test (Muscat *et al.,* 2008), sulphamethazine assay (Hadasova *et al.,* 1990) and dapsone test (Philip *et al.,* 1984).

A large number of human studies for N-acetylation used the metabolic phenotyping methods (Bell *et al.,* 1993), because most often acetylation phenotype was used as a predictor for toxicity to various drugs and/or of dose requirement (Drayer & Reidenberg, 1977) as well as the genotype was comparable to extent of sulphamethazine acetylation (Hickman & Sim, 1991). The acetylator phenotype is a lifelong and stable characteristic of the individual and can be determined by using any of many probe drugs e.g., sulphamethazine (Weber, 1990; Hein, 2002).

CONCLUSION

The HPLC assay is a fast technique to determine the acetylation of NAT2 enzyme, since our population was found to be fast acetylators of NAT2 enzyme by sulphamethazine like other Asian population. Although present data provides the basics for the therapeutic implementations, further investigations are required to establish the more precise acetylation status of this enzyme in the Pakistani population.

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