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A Phenotypic Study of N-Acetyltransferase-1 (Nat1) In Local Human Population for Determination of N-Acetyl Metabolite of 5-Aminosalicylic Acid

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ABSTRACT

N-Acetyltransferase-1 (NAT1) is the phase II metabolizing enzyme, renders for the detoxification of many drugs and arylamines present in foods. NAT1 is polymorphic in nature, varies in different ethnic groups. The present study was designed to evaluate the acetylation capacity of NAT1 using 5-aminosalicylic acid in the healthy Pakistani population. The blood samples were collected at 3 hour from each volunteer for acetyl metabolites. The concentrations of acetyl metabolite and parent drug were determined in plasma samples by HPLC. The concentrations of 5-aminosalicylic acid (5-ASA), acetyl 5-aminosalicylic acid (Ac5-ASA), ratio of Ac5-ASA/5-ASA were determined to assign the population into slow or fast acetylators by using the statistical model probit plot. NAT1 acetylation activity by 5-ASA showed bimodal frequency distribution in the population. Almost 71% of the population was fast acetylator and 29% was slow acetylator.

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INTRODUCTION

Drug metabolism is usually described as comprising of phase 1 oxidation reactions and phases 2 conjugations reactions such as acetylation, methylation, glucuronidation and sulphation in the liver. In history, the drug metabolising polymorphism was firstly described over 50 years ago for acetylation. N-acetyltransferase (NAT) activity is controlled by two genes (NAT1 and NAT2) of which NAT1 is responsible for clinically significant metabolic polymorphisms (Shenfield, 2005; Patin et al., 2006). The N-acetyltransferases (NATs) are found in almost all species from microorganisms to humans. They catalyze the acetyl transfer from acetyl coenzyme A to hydrazine compound, aromatic amine, heterocyclic amines (Suzuki et al., 2007). N-acetyltransferase (NAT) enzymes are accountable for metabolizing heterocyclic and aromatic amines via N-acetylation, which may lead to detoxification of these aromatic amines, or via O-acetylation, which may lead to activation and formation of DNA adducts associated to the induction of cancer (Hein, 2002). NAT1 and NAT2 appear to establish some substrate specificity and variable levels of

expression in different organs and tissues (Chiu et al., 2005). The substrates of NAT1 enzyme are 5-aminosalicylic acid (5-ASA) (Suzuki et al., 2007), para-aminosalicylic acid (PAS), para-aminobenzoic acid (PABA) and sulfamethoxazole. 5-Aminosalicylic acid (5-ASA) is most commonly used in the treatment of inflammatory bowel diseases (IBDs), as ulcerative colitis and Crohn's diseases (Frieri et al., 2000; Sandborn and Hanauer, 2003; Bergman and Parkes, 2006). Oral administration of 5-ASA causes rapid absorption, N-acetyltransferase I enzyme metabolizes the 5-ASA in the gut wall and in the liver, mainly to its N-acetyl-5-ASA (N-Ac-5-ASA) metabolite. This major metabolite is present in blood (Wilding et al., 2003) and is therapeutically inert. In plasma, 5-ASA and N-Ac-5-ASA are found 40–50% and 80%, respectively bound to the plasma proteins (Nobilis et al., 2006). The pharmacokinetic studies and metabolism of 5-ASA from mesalazine-containing drugs is compulsory when new drug formulations are developed for the treatment of IBDs (Pastorini et al., 2008). In the metabolism of a wide range of hydrazine drugs, arylamine drugs and carcinogens N-Acetylation plays a significant role. Humans have genetically determined differences in

their N-acetyltransferase activities and are phenotypically categorized as rapid or slow acetylators (Martell et al., 1992). The data regarding the acetylation studies of NAT1 in Pakistani population was missing. Therefore present project was designed to study the acetylation of NAT1 using 5-ASA.

MATERIALS AND METHODS

NAT1 acetylation was determined in healthy male and female volunteers by using probe drug 5-aminosalicylic acid (5-ASA). The whole research was conducted in Pharmaceutical Research lab, Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan. The physical examination and different biochemical parameters of all volunteers were also checked.

Subjects

The project was designed and conducted according to the good clinical practice (GCP) and according to all ethical principles declared by world health organization (WHO 2008). The study was approved by the ethics Committee of the University. A total of one hundred and twelve subjects (male=52, female=60) local students and people took part in this study.

Collection of blood samples

The blank blood sample was collected from each volunteer and was used for biochemical parameters. All volunteers were given an oral dose of 800 mg (2 tablets). 5-aminosalicylic acid (5-ASA) with a glass of water after an overnight fasting. All volunteers were given similar breakfast (free of juices) and single blood sample (3 mL) was collected in heparinized centrifuge tubes at 3 hour after an oral administration of 5-ASA. The plasma was separated and stored at -20°C till analysis.

Chemicals and Reagents

5-aminosalicylic acid and its acetyl metabolite was purchased from MP Biomedical, para-aminosalicylic acid was purchased from (BDH) AnalaR. EDTA, ethidium bromide were purchased from Sigma, Deionized distilled water was obtained (Advanced GS-590, Distillery and CPW-200, Japan) from Central High Tech Lab, University of Agriculture, Faisalabad, Pakistan. Acetonitrile was from Merck, methanol from Lab scan. All chemicals and solvents were of high purity and HPLC grade (Sigma/Lab Scan/Bio Basic Int.). Drug free plasma was obtained from Cheniot Dialysis Centre. 5-amino salicylic acid was purchased from Care Pharmacy Faisalabad.

Equipment and Instrumentation

The high performance liquid chromatographic (HPLC) system was consisted of a Shimadzu communication bus module (CBM-20A/ 20 A system controller), SPD-M20A UV-Visible photodiode array detector, LC-10AT pump with FCU-10AL VP flow controller valve,

(DGU-20A3/DGU-20A5) online degasser, and LC-20AT solvent delivery Module. The separation of 5-aminosalicylic acid and its acetyl metabolite was done on LiChrospher® 100 RP-18e column (250 mm × 4.6 mm, 5 µm, Merck, Germany). The chromatographic data was analyzed and collected by using CSW 32 software. The mobile phase was a mixture of 1 % acetic acid, acetonitrile and methanol (6: 3: 1) mixed by magnetic stirrer (Gallen Kamp England), filtered under vacuum by filtration assembly using nylon filters (Sartorius, AG. 37070 Geottingen, Germany) of pore size 0.45 µm and 47 mm diameter. Other equipment includes; Sonicator (Elma, E 60 H), Thermostat Water Bath (HH-4), pH meter (WTW series 720 Inolab), Micropipette (Gilson and Eppendroff), Glass Distillery (GS-590, distillery and CWP-200), Microcentrifuge (Hettich Germany), Electronic balance (Shimadzu Japan), Vortexer (VELP Scientific), Centrifuge Machine (YJ03-043-4000 China), clinical chemistry analyzer (Roche).

Preparation of Stock Solutions and Standards

The stock solutions (1000 µg/mL) of 5-aminosalicylic acid (5-ASA), acetyl 5-aminosalicylic acid (Ac 5-ASA) and internal standard para-aminosalicylic acid (PAS) were prepared in methanol. While the solubility of 5-ASA in methanol was increased by adding 1 M HCl. The solutions of these stocks were stored at 4°C and were used to prepare plasma standards and quality controls (QCs) of known quantity. The plasma standards and quality controls (QCs) were prepared in drug free plasma.

Analysis of 5-Aminosalicylic acid (5-ASA) & Acetyl 5-aminosalicylic acid (Ac 5-ASA)

The standards and plasma samples of 5-aminosalicylic acid and acetyl 5-aminosalicylic acid were determined on the HPLC at 288 nm by using the method of Cantilena et al. (2004). This method was optimized for the determination of 5-ASA and Ac 5-ASA in plasma samples. The processing of standards or plasma samples involves 500 µL of standard or plasma sample, 1000 µg/mL para aminosalicylic acid was added as an internal standard and 100 µL of the 30 % perchloric acid was added to deproteinize the plasma protein. The standards and plasma samples were vortexed, centrifuged at 10,000 xg for 10 min. The supernatant was taken, filtered through syringe filters of pore size 22 µm and 20 µL of the sample was injected directly to the HPLC column. For sample elution through column the mobile phase (pH 3.5) used. For analysis of samples isocratic mode was used and the solvent flow rate was 0.8 mL/min. The retention time for para-aminosalicylic acid (I.S), 5-aminosalicylic acid and acetyl 5-aminosalicylic acid was 4.9, 3.88 and 5.4 minutes respectively. Representative chromatograms of blank plasma, concentration of 5-ASA, AcASA and I.S

of volunteer plasma sample shown in Figures 1 and 2 respectively.

RESULTS AND DISCUSSION

In the present project the concentration of the 5-ASA and Ac-5ASA in the plasma samples at 3 hour was determined through HPLC method. The method was established for the convenient sample processing and quick result determination of NAT1 phenotype (Vasbinder et al., 2004; Pastorini et al., 2008). NAT1 acetylation phenotype was evaluated by measuring the Ac-5ASA and 5-ASA metabolite ratios in the 3 hour plasma samples after single dose. The measurement of metabolite ratio to drug fractions in plasma is more precise way to determine the acetylation phenotype than calculating the urinary metabolite ratios (Hein, 2002).

The concentrations of 5-ASA, Ac5-ASA, ratio of Ac5-ASA/5-ASA and distribution of slow or fast acetylators of healthy male and female subjects were determined. The slow and fast acetylator phenotype status was determined from the molar ratio of Ac5-ASA/5-ASA by using probit plot (Akhtar et al., 2011). The cut off point for 5-ASA was 1.2, the subjects with molar ratio less than 1.2 were considered slow acetylators while those having molar ratio more than 1.2 were considered to be fast acetylators. The frequency histogram was plotted against molar ratio of Ac5-ASA/5-ASA for the male and female subjects (Figure 3) showed bimodal distribution in the subject for fast and slow acetylation phenotypes. The Ac5-ASA/5-ASA ratio for male subjects were ranged from 0.39 to 8.97, maximum number of subjects have molar ratio ranging from 0.92 to 1.3. While Ac5-ASA/5-ASA ratio for female subjects was ranged from 0.61 to 9.81, maximum number of subjects has molar ratio ranging from 1.14 to 1.5. The distribution of acetylator phenotype showed that 65.4% (34/52) males were fast acetylator and 34.6% (18/52) males were slow acetylators (Table 1). While female distribution of acetylator phenotype showed that 76.7% (46/60) were fast acetylator and 23.3% (14/60) were slow acetylators (Table 1).

There was difference in the distribution of acetylation phenotype for 5-ASA drug in the male and female subjects. The distribution of slow acetylators in male subjects is almost (33%) while in females it is (23%). The frequency of fast and slow acetylators in the male and female was significantly different ($P < 0.05$) for 5-ASA. These results were concordant with the results reported by Weber and Vatsis (1993) demonstrated that males have slightly high acetylation activity as compared to females using PABA as a specific substrate. This difference could not be explained, the

Table 1: Distribution of acetylator phenotype for 5-aminosalicylic acid in healthy male and female volunteers (n=112)

Volunteers	Fast	Slow
Male %	65.4% (34/52)	34.6% (18/52)
Female %	76.7% (46/60)	23.3% (14/60)
Total	71.4% (80/112)	28.6% (32/112)

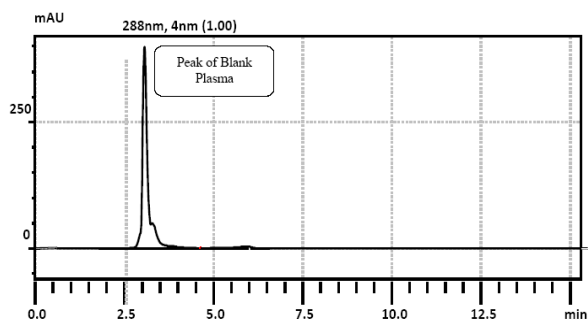


Fig. 1: Representative chromatogram of blank plasma of human volunteer.

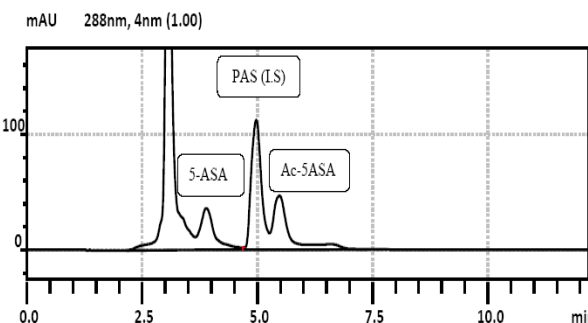


Fig. 2: Representative chromatogram of plasma sample of human volunteer showing peaks of 5-ASA, PAS (I.S) and Ac-5ASA.

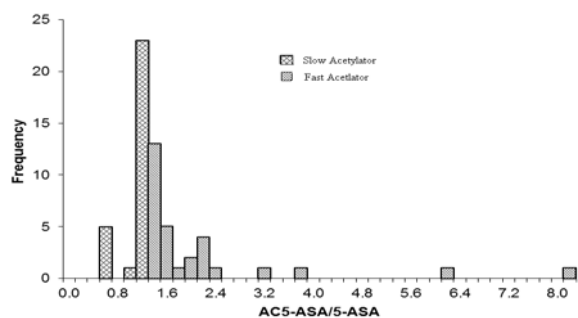


Fig. 3: Frequency histograms of molar ratio of AcASA/5-ASA showing cut off point of 1.2 for all the male and female volunteers (n=112) & showing the bars before 1.2 are slow acetylators and after 1.2 are fast acetylators.

sex dissimilarity is perhaps not significant. The effect of sex on the acetylator phenotype frequency was not properly established and appeared to give contradictory results in different populations.

The studies conducted in Japanese subjects (n=126) the 5-ASA was used as probe for phenotyping and genotyping studies, the Ac-5-ASA/5-ASA molar ratio was used for correlation of genotype (Yokogawa et al., 2001). Hughes et al. (1998) categorize phenotypically the individuals and estimated the acetylation rates by using the PAS through the ratio of PAS to N-acetylated PAS (AcPAS) in the urine and plasma. The AcPAS/PAS ratio in the urine did not give the reliable results while AcPAS/PAS ratio in the plasma was give more reliable results. Depending on the genetic phenotype, the elimination half-life and apparent oral clearance of 5-aminosalicylic acid are approximately 14 hours and 40 ml/min (slow acetylators) or 6 hours and 150 ml/min (fast acetylators), respectively (Klotz, 1985).

The studies conducted to check the genetic polymorphism of the NAT1, para-aminosalicylate (PAS) was used as probe drug to categorizes the individuals (n=24) into slow and fast acetylators reported by Hein et al. (2000). A study conducted on 5-aminosalicylic acid acetylation by NAT1, showed less 50% phenotypic variations *in vivo* was associated to the variation in the NAT1 activity. The lesser number of slow acetylators in the population made it difficult to find the associations (Ricart et al., 2002). The deviation from the present study may be due to different sample sizes, studies conducted in different populations and due to biological variations.

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