Genetic polymorphism of UDP-glucuronosyltransferase (UGT2B15) and glucuronidation of paracetamol in healthy population

Huma Mehboob[1]*, Tahira Iqbal[1], Amer Jamil[1] and Tanweer Khaliq[2]
[1]Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan
[2]Department of Physiology and Pharmacology, University of Agriculture Faisalabad, Pakistan

Abstract: Inter individual variability in polymorphic UDP-glucuronosyltransferase (UGT2B15) has been associated with varied glucuronidation level. The present project was designed to determine the genetic polymorphism of UDP-glucuronosyltransferase (UGT2B15) and glucuronidation of paracetamol in healthy (male=59 and female=50) population. The association between genotype (UGT2B15) and phenotype (paracetamol glucuronidation) has been evaluated. According to trimodal model, genotypes and phenotypes were categorized as fast, intermediate and slow glucuronidators. Presence of wild type allele illustrated a UGT2B15 genotype as fast glucuronidator. The glucuronidation status was investigated by HPLC analysis of paracetamol. Ratio of paracetamol glucuronide to paracetamol was determined with two antimodes at glucuronidation ratio of 0.3 and 1.8. In our study, 7% and 12% of population was distributed as slow glucuronidators by phenotype and genotype, respectively and association between phenotype and genotype was good for analysis of glucuronidation status as displayed by kappa value (0.792).

Keywords: Paracetamol, paracetamol glucuronide, UDP-glucuronosyltransferase (UGT2B15), slow, intermediate, fast glucuronidators

INTRODUCTION

Glucuronidation, a major metabolic pathway catalyzed by the UDP-glucuronosyltransferase (UGT) super family is accountable for removal of harmful exobiotic and endobiotic compounds in humans (Desai *et al*., 2003). The super family UGT is divided into four subfamilies. This classification is kept on sequence similarity at the level of amino acid. UDP-glucuronosyltransferase (UGT1A and UGT2B) genes are highly polymorphic. The isoforms of UGT2B subfamily emerge to be encoded by a rigid cluster of separate genes located on chromosome four in humans (Turgeon *et al*., 2001). UGT2B15 gene is one of sixteen human UGTs that have been known to date. The genetic alterations in UGT2B15 gene transform the glucuronidation activity of the human volunteer carrying that polymorphism. The genetic polymorphism in UGT2B15 gene results in an amino acid change from aspartic acid to tyrosine at position 85. Although this enzyme was originally identified for glucuronidation of steroid hormones (Chen *et al*., 1993), following studies with recombinant UGTs (UGT2B15) signify that this enzyme is likely to have an important function in the metabolism of xenobiotics and other drugs (Green *et al*., 1994). Many drugs that serve as substrates for UGT2B15 include paracetamol, oxazepam, E-4-hydroxytamoxifen, 5-hydroxyrofecoxib, eugenol, 8-hydroxyquinoline, phenolphthalein, 4-hydroxyphenytoin, and nandrolone (Navarro *et al*., 2013; Green *et al*., 1994; Court *et al*., 2002; Nishiyama *et al*., 2002; Kuuranne *et al*., 2003; Zhang *et al*., 2003). Paracetamol (PAR), N-4-hydroxyphenyl acetamide analgesic drug, is of significant attention as it is primarily excreted by glucuronidation and is widely used in humans (Court *et al*., 2013). UGT makes PAR molecule more water-soluble by shifting the glucuronosyl moiety from UDP-glucuronic acid. Moreover, the glucuronidation of paracetamol by human volunteers is recognized to be polymorphic. Individuals having mutated enzymes may differ from normal individuals in their vulnerability to certain diseases. Some specific phenotypes for these polymorphic enzymes are related to increased vulnerability to prostate cancer (Vidal *et al*., 2013).

A prior study of paracetamol biotransformation in human volunteers by Pabba *et al*., (2002) recognized paracetamol glucuronide as the major metabolite of paracetamol in human plasma and urine. The various level of glucuronidation activity classify the human volunteers as fast and slow glucuronidators (bimodal model) (Jakobsson *et al*., 2006) and slow, intermediate, and fast glucuronidators (trimodal model) (Bock *et al*., 1994; Holthe *et al*., 2003).

The objectives of this study were to find out the glucuronidation status of paracetamol and the polymorphisms of UGT2B15 gene and establish association between UGT2B15 genotype and glucuronidation phenotype in healthy female and male volunteers.

MATERIALS AND METHODS

The present project was designed to determine the genetic polymorphism of UDP-glucuronosyltransferase

**Corresponding author:* e-mail: moona_farooqi21@yahoo.com

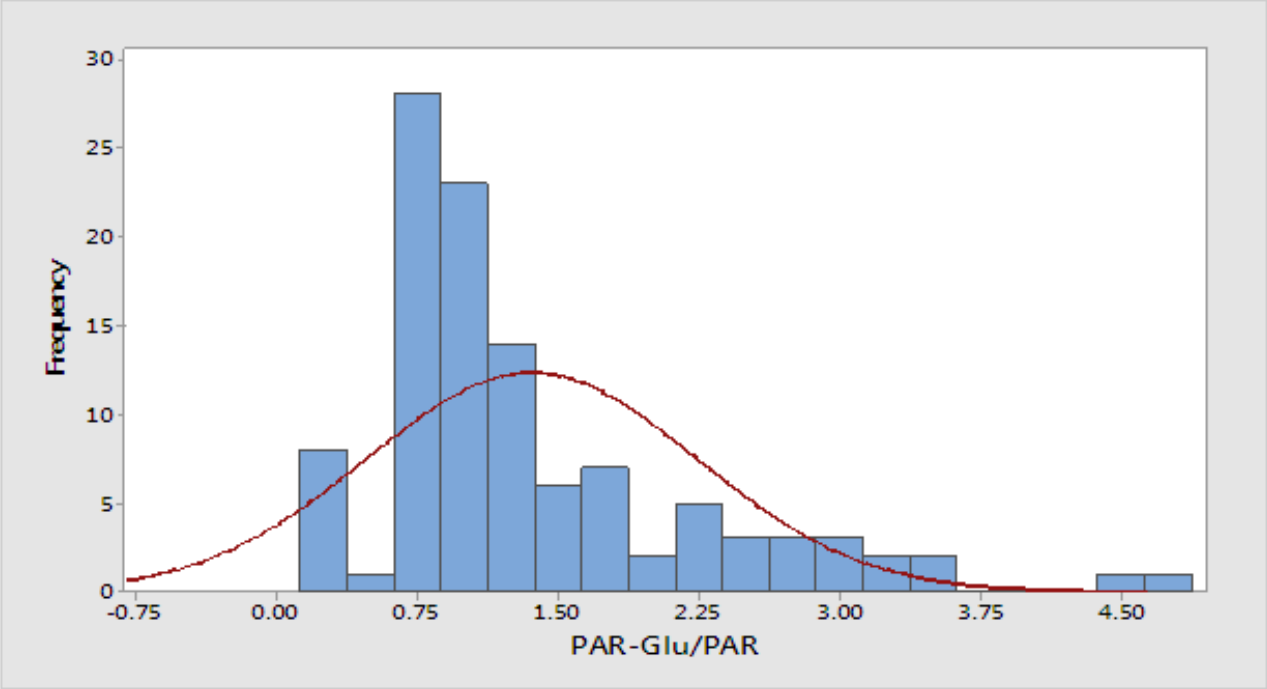

Fig. 1: Frequency histogram of ratio of PAR-Glu/PAR showing trimodal distribution (slow, intermediate and fast) of healthy male and female volunteers (n=109).

(UGT2B15) and glucuronidation of paracetamol in healthy population was determined by administering two tablets of paracetamol (500 mg).

Reagents

The paracetamol and paracetamol β-D-glucuronide reference standards were purchased from Sigma-Aldrich and Fluka. Paracetamol (Panadol) manufactured by Pharmatec Pvt. Ltd, Karachi, Pakistan was purchased from the market. All the solvents used were of high purity and of HPLC grade. De-ionized distilled water was obtained (Advanced GS-590, Distillery and CPW-200 Japan) from Central High Tech Lab, University of Agriculture, Faisalabad. Blood DNA isolation kit (Bio basic Inc.) and TaqMan probes (Applied Bio Systems, USA).

Volunteers

Healthy individuals (male and female) enrolled for this study were students of University of Agriculture, Faisalabad. The study was officially approved by the ethics committee of the University and written informed consent was taken from all volunteers. The demographic data included age, body weight, height, body temperature and blood pressure was recorded from each subject. The mean ages of male and female subjects were 22.19 years and 22.26 years, respectively. The physical examination and laboratory tests were performed to check the health status of volunteers. All the volunteers were on normal diet and no one was on any medication. All were non alcoholics and not permitted to take any caffeinated drinks and juices during the sampling.

Sample collection

Blank blood samples were collected from all volunteers before drug administration. Then two tablets of paracetamol (500mg) were administered orally to all volunteers and 3mL of blood sample was withdrawn at 1.5hour time point in EDTA vials. The samples were centrifuged at 4000 rpm for 10 minutes and plasma was separated and stored at -20°C till analysis.

Paracetamol Glucuronidation assay

Analysis of paracetamol and paracetamol glucuronide in plasma samples was carried out *via* HPLC method as demonstrated in detail previously with minor changes (Vertzoni *et al*., 2003). Briefly, 200µL of the 15% perchloric acid was added to 100µL of plasma to deproteinize the plasma proteins. The plasma samples were vortexed for 1minute and then centrifuged at 13,000 rpm for 10 minutes. The supernatant was taken, filtered through syringe filters of the pore size 22µm and 15 µL of the sample was injected directly on to Hypersil BDS-C_{18} column (250mm x 4.6mm, internal diameter, 5µm, Thermo Electron Corporation, USA). For elusion, the mobile phase used was composed of aqueous buffer solution of 0.05M KH_2PO_4 (475mL) in 5% acetic acid (pH 6.5) and methanol (25mL). For analysis, isocratic mode was used and the solvent flow rate was 1mL/min. The analysis detects paracetamol and paracetamol glucuronide, respectively. Paracetamol glucuronide/ paracetamol ratio was calculated for all volunteers to find the distribution of slow, intermediate and fast glucuronidators.

Genotyping of UGT2B15 gene

Genotyping of UGT2B15 gene was done by using standard protocols of TaqMan nuclease assay.

Isolation of DNA

For genomic DNA isolation, 2mL of blank blood sample was drawn from both male and female volunteers in BD Vacutainer® EDTA tubes and stored at -80°C. Blood samples were thawed before isolation and DNA was isolated by Bio Basic (EZ-10 Spin Column Genomic DNA Minipreps) isolation kit. Isolated DNA was quantified on Gen5™. High quality DNA samples obtained were stored at -40°C for further analysis.

RT-PCR of UGT2B15 gene

Genotyping of the UGT2B15 gene polymorphism (rs1902023; 253 A>C) in exon 1 region was completed by using sequence specific TaqMan probes in real time PCR. TaqMan probes containing FAM and VIC dyes were used for UGT2B15 gene polymorphisms. The results were analyzed with ViiA *7*™ Software (Applied Biosystem, USA). First of all, the 40ng DNA template was aliquoted in 384-well plate for drying. The optimized reaction mixture consisted of 2.50µL of TaqMan genotyping master mix (2X), 0.25µL of TaqMan genotyping assay mix (20X) and 2.25µL of DNase-free, RNase-free water. The thermal cycling conditions of qPCR were consisted of a pre-read stage of 30 seconds at 60°C, hold stage of 10 minutes at 95°C, PCR stage containing denaturation and annealing at 95°C for 15 seconds and 62°C for 1 minute, respectively.

STATISTICAL ANALYSIS

Allele and genotype frequencies are presented in percentages. The Hardy-Weinberg equilibrium was used for calculating genotype and allele frequencies. The probit plot was used for the distribution of volunteers into fast, intermediate and slow glucuronidators on the basis of cutoff points. K-statistics is used to measure level of agreement between phenotype and genotype. SPSS statistics version 17 was used to describe the association between phenotype and genotype, (Kappa test). The comparison of parameters between male and female volunteers was evaluated by Chi square test.

RESULTS

The glucuronidation of paracetamol by UDP-glucuronosyltransferase (UGT2B15) was determined in healthy (male=59 and female=50) subjects after oral administration of 1g paracetamol (two tablets of panadol). The concentration of paracetamol and paracetamol glucuronide was measured in blood at 1.5-hour time point. The data was analyzed as molar ratio of PAR-Glu/PAR. The PAR-Glu/PAR ratio for the male volunteers was ranged from 0.23 to 4.72. The distribution of paracetamol glucuronidation illustrated that 7% (4) were slow, 37% (22) were intermediate and 56% (33) male volunteers were fast glucuronidators while in female volunteers, the PAR-Glu/PAR ratio was ranged from 0.26 to 2.95. The frequency distribution of slow, intermediate and fast glucuronidators among female volunteers is 8% (4), 32% (16) and 60% (30), respectively. The frequency histogram of PAR-Glu/PAR ratio of healthy (male=59 and female=50) volunteers is shown in fig. 1. The frequency histogram shows apparent trimodal distribution of slow, intermediate and fast glucuronidators at glucuronidation ratio of 0.3 and 1.8. The volunteers with molar ratio of less than 0.3 were thought to be slow glucuronidators whereas those with molar ratio of greater than 0.3 were intermediate and those with molar ratio of greater or equal to 1.8 were considered fast glucuronidators.

The 109 volunteers (male=59, female=50) were genotyped in this study. A single nucleotide polymorphism (rs1902023) was studied in exon 1 region of UGT2B15 gene. The frequency distribution of homozygous wild type, heterozygote and homozygous variant alleles and the genotype frequency for the SNP (rs1902023) is summarized in table 1. The minor allele frequencies for SNP (rs1902023) in male and female are 27.9% and 37.8%, respectively. They are in Hardy Weinberg equilibrium. The genotype frequency of UGT2B15 (rs1902023) is significantly different in female and male volunteers. For SNP rs1902023, the percentage of homozygous wild type, heterozygote and homozygous variant genotype is 54.2%, 35.6% and 10.2%, respectively in male volunteers whereas in female volunteers, this percentage is 40%, 46% and 14%. On the basis of genotype (rs1902023), 32 (54.2%) male volunteers are fast, 21 (35.6%) are intermediate and 6 (10.2%) are slow glucuronidators whereas for female fast, intermediate and slow glucuronidators are 20 (40%), 23 (46%) and 7 (14%), respectively.

Correlation or association was computed as measure of agreement between UDP-glucuronosyltransferase (UGT2B15) phenotype and genotype. The k-statistics showed good agreement between phenotype and genotype with kappa value of 0.793 in both male and female for SNP rs1902023 as shown in Table 2. A total of 51 (46.79%), 37 (33.94%) and 8 (7.34%) female and male volunteers were categorized as fast, intermediate and slow glucuronidators, respectively and 12 volunteers demonstrated deviation from this distribution. Out of these 12 volunteers, 7 volunteers were fast glucuronidators by (rs1902023) genotype but classified as intermediate glucuronidators and 5 volunteers were slow glucuronidators by PAR phenotype but categorized as fast and intermediate glucuronidators genotypically. The kappa value 0.793 (79.3%) illustrates a good concordance overall in both male and female volunteers.

Table 1: Allele and genotype frequency distribution of UGT2B15 (rs1902023) in healthy volunteers

UGT1A1 polymorphisms	Allele frequency		Genotype frequency	
Rs1902023	Male (n=59)	Female (n=50)	Male (n=59)	Female (n=50)
A/A	72.1% (0.721)	62.2 % (0.622)	54.2% (0.542)	40% (0.40)
A/C			35.6% (0.355)	46% (0.46)
C/C	27.9% (0.279)	37.8 % (0.377)	10.2% (0.102)	14% (0.14)

Table 2: The comparison of genotype (rs1902023) and paracetamol phenotype in male and female volunteers

		Genotype			Total
		Fast	Intermediate	Slow	
Paracetamol Phenotype	Fast	51	1	0	52
	Intermediate	7	37	0	44
	Slow	4	1	8	13
Total		62	39	8	109

DISCUSSION

We first time demonstrate the genetic polymorphism of UDP-glucuronosyltransferase (UGT2B15) in healthy population of Pakistan. The volunteers are categorized as slow, intermediate and fast glucuronidators on the basis of genotype and glucuronidation of paracetamol. Inter ethnic and inter individual variability is observed in drug metabolism most often. This can affect both the therapeutic and toxic drug response. In drug metabolizing capacity, glucuronidation is the set example of genetic polymorphism. In present study, inter individual variability in glucuronidation was measured by computing paracetamol glucuronide/paracetamol ratio as was determined by Court *et al*., (2004) who calculated S/R oxazepam ratio for glucuronidation activity in liver microsomes. In our study, the volunteers having variant alleles show reduced glucuronidation of paracetamol and excretion pattern was similar in both male and female volunteers (7% and 8%). Navarro *et al*. (2011) reported that the paracetmol half-life was longer in individuals with UGT2B15*2/*2 genotypes which supports the present study. In present study, Isocratic, reverse phase high performance liquid chromatography was used for determining glucuronidation of paracetamol after administration of a single oral dose at 1.5 hour time point as was reported by Flouvat *et al*., (2004). The prevalence of major and minor alleles of UGT2B15 gene in our healthy male and female volunteers is 72.1%, 62.2%, 27.9%, 37.8%, respectively. The distribution of UGT2B15 alleles is differed by ethnicity. Lamp *et al*., (2000) reported that 32.2% of Caucasians were homozygous for substituted or variant alleles. The data proposed that the distribution of UGT2B15 polymorphisms is comparable to Caucasians. In our study, the major genotypes for SNP (rs1902023) are A/A and A/C in both male and female volunteers. At the end association was analyzed between UGT2B15 genotype and glucuronidation of paracetamol. Our results showed good association as was obtained from the kappa value (0.793) 79.3% between genotypic and phenotypic methods for evaluating glucuronidation status in healthy volunteers.

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