A Practical Non-Extraction Direct Liquid Chromatography Method for Determination of Thiopurine S-Methyltransferase Activity in Inflammatory Bowel Disease

Fariborz Bahrehmand[1,2], Amir Kiani[3], Asad Vaisi-Raygani[1,2], Homayoun Bashiri[4], Mehdi Zobeiri[4], Ali Moini[4], and Tayebeh Pourmotabbed[5]

[1] Fertility and Infertility Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran

[2] Department of Clinical Biochemistry, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran

[3] Regenerative Medicine Research Center, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran

[4] Department of Internal Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran

[5] Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, USA

Received: 28 Jan. 2016; Accepted: 28 May 2016

Abstract- Thiopurine drugs remain pivotal therapies for the wide varieties of diseases such as inflammatory bowel disease (IBD). Here, thiopurine S-methyltransferase (TPMT) phenotype, the main metabolizing enzyme of thiopurine-drugs, was studied. This is for the first time that TPMT activity is measured in Iranian IBD patients. We used an improved direct liquid chromatography assay without need for solvent extraction and minimize excess labor handling making it ideal for use in routine referral medical centers. TPMT activity in whole blood was determined by a non-extraction HPLC method. We evaluated 427 individuals including 215 IBD patients and 212 unrelated healthy individuals as control group from Iran's western population. TPMT phenotyping of this study demonstrated no frequency for deficient, 2.8 % for low and 97.2% for normal activity, which is different with results of other studies. There was a significant negative correlation between TPMT activities as calculated based on nmol/grHb/h and the Hb-levels in IBD and control groups (r= -0.54, P<0.001 and r= -0.27, P<0.001), respectively. Interestingly a significant positive correlation between Hb levels and TPMT-activities were seen when the activity calculated in mU/L in IBD patients and control subjects (r=0.14, P=0.05 and r=0.43, P<0.001), respectively. We strongly suggest the use of international unit (mU/L) is more appropriate than nmol6MTG/grHb/h for expressing TPMT-activity in IBD patients. In addition, in comparison with other providers of TPMT test activity and centers around the world the risk of toxicity is much lower after utilizing thiopurine drugs for IBD patients in this region.

Acta Med Iran 2017;55(6):360-367.

Keywords: HPLC; IBD; Phenotypes; Thiopurine drugs; TPMT

Introduction

Thiopurines, azathioprine (AZA) and 6-mercaptopurine (6-MP) are the most important immunosuppressive drugs which are used in the treatment of inflammatory bowel disease (IBD), other inflammatory diseases such as rheumatoid arthritis, dermatitis and in the management of acute lymphoblastic leukemia (ALL) and to prevent transplant organ rejection. They have a pivotal role in inducing and maintaining remission of patients with Crohn's disease (CD) and ulcerative colitis (UC), principal types of IBD. The use of 6-thioguanine (6-TG) has been proposed as an alternative for classical thiopurines (1). Azathioprine is a pro-drug converted by the sulphydryl compounds, glutathione and cysteine, in red blood cells to 6-MP after absorption, and 6-MP is subsequently undergoing activation, via a multi-step enzymatic pathway, into the 6-thioguanine nucleotides (6-TGN), as well as 6-methylmercaptopurine (6-MMP) which is thought to be inactive. The therapeutic mechanism of action of 6-TGN is unclear, it may achieve therapeutic efficacy primarily by virtue of their cytotoxic action (2). In patients with ALL there is a positive correlation between RBC 6-TGN

Corresponding Author: A. Kiani
Regenerative Medicine Research Center, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran
Tel: +98 918 8316929, Fax: 98 834274623, E-mail address: amir1kiani@yahoo.com

concentration and risk of leucopenia (3). This association has been confirmed in some but not all studies of patients with UC and CD (4). 6-TGN may incorporated as false bases into newly synthesized DNA resulting in breaks in DNA strands (2), preventing proliferation of the target bone marrow and white blood cells, and also interfere with other biochemical pathways. The primary toxicity associated with AZA and 6-MP is leukopenia, possibly due to accumulation of 6-TGN in neutrophils (5). One major catabolic enzyme of AZA and 6-MP is thiopurine methyltransferase (EC 2.1.1.67) (2). However, patients show diversity in metabolism of these drugs due to genetic differences of TPMT enzyme. The activity of TPMT is absent in approximately 1 in 300 individuals (≈0.3%), is at low level in 11% of the population (6) and has variation in ethnic groups (7,8). Severe risk of side-effects (myelosuppression) involves patients with low or no detectable TPMT activity, and we would enable to adjust the dose of thiopurine drugs in these patients by the measurement of TPMT activity (5). Traditionally, clinical monitoring of bone marrow toxicity measuring white cell count only identifies susceptible patients after toxicity has occurred, but nowadays, pharmacogenetics strategy therapy has become more effective and better tolerated and positive enormous financial implications (9). Although genotyping is possible, TPMT activity analysis (phenotyping) in red blood cell lysates establishes the enzyme activity prior to commencing any treatment (10). Care must be taken to ensure that the assays measure the activity, not the concentration, since the common genetic polymorphisms affects enzymatic activity not necessarily enzymatic concentration levels (11). TPMT activity is typically measured in erythrocytes accessible and demonstrates the level of the enzyme activity and immunoreactive protein which are representatives of those which are found in tissues, such as the liver, where the majority of thiopurine metabolism takes place (12). Since there are major ethnic differences in the prevalence of particular TPMT variants and their activity, it is important for each country to study their own prevalence to estimate the role of TPMT variants-related thiopurines toxicity in the population. Indeed, in the USA, the Federal Drug Administration has discussed about adding advice to thiopurine drug packaging recommending that TPMT status should be checked (13). Regarding the above information, we found that such a necessary evaluation has not been performed in Iran. As a consequence to measure the enzyme activity, we used a direct HPLC TPMT assay. To determine TPMT activity influencing 6-thioguanine as a substrate, the product 6-methyl thioguanine (6-MTG) was measured after its extraction from the incubate using HPLC with fluorescence detection (14,15). In this non-extraction method unlike previous traditional extraction methods, we overcame the solvent extraction steps, which reduce inherent imprecision using heating. More explanation about heating effect, the possibility of laboratory errors and the potential for further automation may make it appropriate for the routine enzyme assay.

Materials and Methods

Study participants

The subjects for this case-control study consisted of 215 unrelated inflammatory bowel disease (IBD) patients consisting of 85 males and 130 females (mean age 35.75±13.2 years) who underwent an evaluation for IBD by a gastroenterologist at Mahdieh clinic of the Kermanshah University of Medical Sciences and 212 unrelated healthy individuals (96 males and 116 females; mean age 33.1±14.2 years) as control group. The Patient and the control groups were matched according to their gender, age and race.

Chemicals

S-adenosyl-L-methionine (SAM, A.7007), 2-amino-6-methyl mercaptopurine (6-MTG, A.9546) and 6-thioguanine (6-TG A. 4882) were obtained from Sigma-Aldrich.

Substrate incubation and mixture preparation

A substrate incubation mixture containing 600 µM 6 TG and 80 µM SAM in 0.1 M potassium phosphate buffer, pH 7.4 was aliquoted and stored at -70˚ C for up to 1 month prior to use.

Stock standard preparation

A stock standard 6-MTG was made in 0.1 M NaOH and serial dilutions prepared in H2O. 5 mg of 6-Methylthioguanine was dissolved in 2 mL of 0.1 M NaOH and made up to a final volume of 100 mL with deionized distilled water. Thus 50,000 ng/mL stock standards were made.

Quantitative measurement method

For this reason two series of standard solutions were prepared (a) aqueous standard solution and (b) whole blood standard solution.

Aqueous standard solutions preparation

Concentrations of 31.25, 62.5, 125, 250 ng/mL 6-MTG standards were prepared from stock standard. After injection to HPLC chromatogram of them earned and height (light unit) of peaks in concentration was drawn and standard curve was obtained (Figure 1a).

Standard solutions in whole blood to assess recovery

First aqueous standards 312.5, 625, 1250, 2500, 5000 ng/mL of 6-MTG were prepared from stock standard. Then 20 µL of each above concentrations were added to 180 µL of whole blood so the final working concentrations 31.25, 62.5, 125, 250, 500 ng/mL of 6-MTG in whole blood were made, which after injection to HPLC recovery standard curve was drawn (Figure 1b).

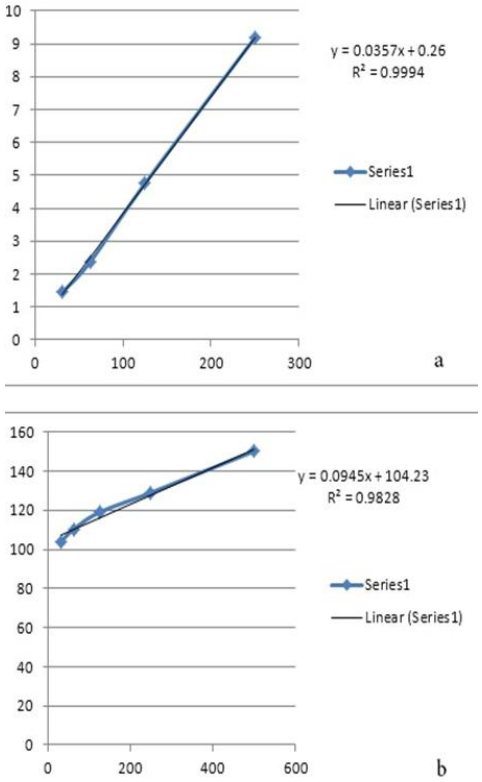

Figure1. (a). 6-MTG standard solutions 31.25, 62.5, 125, 250 ng/mL compared with the corresponding peak height (LU). (b). Recovery of 6-MTG standard solutions 31.25, 62.5, 125, 250, 500 ng/mL from whole blood

Apparatus and chromatographic conditions

The chromatographic apparatus consisted of an Agilent technologies model 1200 series quaternary pump (Germany). The stationary phase was a MZ analytical 5 µ reverse-phase HPLC column (C18, 150 X 4.6 mm Germany) at ambient temperature protected with a 4 mm x 3 mm security guard column (Phenomenex, UK). The mobile phase (flow-rate, 2 ml/min) was an isocratic solution consisted of water and methanol (80:20 v/v) and 100 mM of triethylamine (TEA) which final pH was adjusted to 6.2 with concentrated ortho phosphoric acid. The fluorescence detector was set at excitation 315 nm and emission 390 nm.

Incubation tubes were 2 ml screw top plastic vials. Reaction tubes were incubated in a Memmert (Germany) water bath and finally in a dry plate incubator. The hemoglobin content of whole blood was determined by Zist Shimy hemoglobin kit (Iran).

Ethical considerations

The human subject study protocol was approved by the Ethics Committee of the Kermanshah University of Medical Sciences (KUMS), Iran and was in accordance with the principles of the Declaration of Helsinki II and all the subjects provided written informed consent.

Sample collection, storage, hemolysate preparation and enzyme activity assay

The Blood samples were collected into EDTA tubes and stored for a maximum of 2 days at 4˚ C prior to analysis. The whole blood samples (200 µl aliquots)

were frozen for 15 minutes at -70˚ C to disrupt the cells without the need of lysate solution. The cells were thawed by the addition of 600 µl of whole blood suspension buffer (0.1 M KH2PO4 titrated with 0.1 M K2HPO4 to pH 7.4), and vortexed. The blood lysates (200 µl) were then added to 6 TG/SAM substrate solutions in phosphate buffer and the mixtures were incubated at 37˚ C for 1 h. The enzyme reaction was stopped by incubating the mixtures at 90˚ C for 10 min. the samples were cooled, centrifuged at 1400 g for 5 min and 20 µl of supernatants were injected to HPLC loop. A reaction blank was prepared by adding 200 µl potassium phosphate buffer (0.1 mol/l. pH 7.4) to the substrate incubate instead of 200 µl blood lysates and sample blank was prepared by adding 200 µl of lysate and then stopping the reaction at time zero. The 6-MTG component was eluted from the column as a sharp narrow peak that was completely isolated from the rest of the components. The retention time of 6-MTG (5.75 min) on the column was specific to the compound and the peak height was exactly proportional to its concentration. The chromatogram of the 6-MTG standard, reaction blank, low, normal and high TPMT activities are presented in Figure 2.

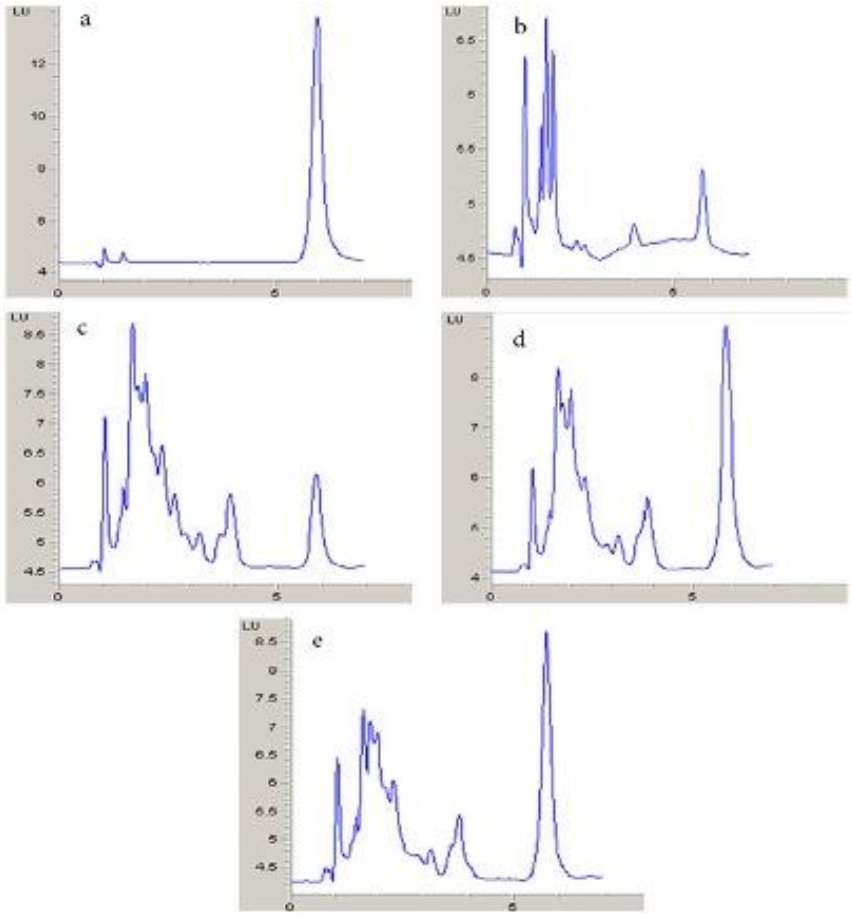

Figure 2. High performance liquid chromatography of 6-methyl thioguanine. (a) standard 1.38 nmol/ml 6-MTG, (b) reaction blank, (c) low activity patient (44 mu/l), (d) normal activity individual (133 mu/l), (e) high activity individual (156 mu/l)

Standardization

A standard curve was shown to be linear over the range 0-2.76 nmol 6-MTG with the equation of the curve being y=0.0357x+0.26, (R^2=0.9994).

Method imprecision

The imprecision of the method was determined using EDTA whole blood collected from a volunteer. The within and between-batch imprecision are shown in Table 1. The coefficient of variation between duplicate results was (5.2%).

Accuracy

The accuracy of the method assessed by means of recovery studies as mentioned before at 5 levels. Known concentrations of the product 6-MTG were added to a lysate preparation in order to make 5 different levels, and analysis performed Table 2.

Limit of detection

The limit of detection was given as 0.4 nmol/mL (6.6 mU/L) this being twice the value that the blank gave when calculated as a test, though in routine practice lower values can be expressed by subtracting the blank value from a test.

Statistical analyses

Data were analyzed first for normality of distribution using the Kolmogorov-Smirnov test. Results were expressed as mean±SD for normally distributed data, median and interquartile range (IQR) for non-normally distributed data, and percentages for categorical data. Comparison of groups was carried out with Student's t test, Mann-Whitney U test and one-way ANOVA as appropriate. The correlation values of TPMT activities in mU/L and in nmol/grHb/h, Hb levels, BMI and age of IBD and control groups were calculated using Pearson correlation. TPMT activities in mU/L and in nmol/grHb/h, Hb concentration, BMI and age in IBD according to gender and between genders were compared to control groups by independent-Samples t-Test and nonparametric independent-sample Mann-Whitney analyses, respectively. Statistical significance was assumed at the $P<0.05$. The SPSS statistical software package version 19 was used for the statistical analysis.

Table 1. Within-batch and between-batch imprecision of the TPMT

Mean TPMT activity mU/L	CV%	n
Within-batch imprecision	3.1	10
Between-batch imprecision	7.6	10

Table 2. Recovery of 6-methyl thioguanine

Spiked value(ng/mL)	31.25	62.5	125	250	500
Mean recovery	30	61	130	256	496
CV%	2.3	1.7	0.84	0.89	0.57
n	5	5	5	5	5

Results

The clinical, laboratory and demographic characteristics of the participants are summarized in Table 3. There was no significant difference between the mean of TPMT activity in mU/L, age, BMI, Hb and sex of the two groups. However, when TPMT was calculated in nmol6MTG/grHb/h, the concentration of TPMT in IBD patients was false-fully significantly higher than control group (46.5(40.9 -53.8) vs 45(41.2 -50.6), $P=0.026$, respectively).

Table 3. The demographic characteristics and distribution of the thiopurine methyltransferase (TPMT) activities and other risk factors in inflammatory bowel disease (IBD) patients and control groups in a population from Kermanshah province

	IBD patient (n=215)	Control subjects (n=212)	*P*
Age (years)	35.9 ±13.2	34 ±14.2	0.58
Sex (M/F)	86/129	96/116	0.27
TPMT activity (nmol6MTG/grHb/h)	*46.5(40.9 -53.8)	45(41.2 -50.6)	0.026
TPMT activity (mU/L)	108.9±20.1	111.7±19.8	0.15
Hb (g/dL)	13.8±1.85	14.6±1.73	<0.001
BMI (Kg/m^2)	24±3.97	24.1±4.51	0.87

6MTG =6methylthioguanine, *Median and interquartile range (IQR) for non-normally distributed data, and percentages for categorical data

Correlations between TPMT activities in mU/L and in nmol6MTG/grHb/h with Hb concentration, BMI and age in IBD patients and control group are shown in Table 4. There was a significant negative correlation between TPMT-activities as calculated based on nmol6MTG/grHb/h and the Hb levels in the IBD and control groups (r= -0.54, $P<0.001$ and r= -0.27, $P<0.001$), respectively. Interestingly a significant

positive correlation between Hb levels and TPMT activities were seen when the activity calculated in mU/L in IBD patients and control subjects (r=0.14, *P*=0.05 and r=0.43, *P*<0.001), respectively.

Table 4. Correlation of TPMT activities in mU/l and nmol6mtg/grHb/h with Hb concentration, BMI and age in IBD patients and control group.

	IBD patients					Control group				
	TPMT activity (mU/L)	**TPMT activity (nmol6MTG/grHb/h)**	**gr/Hb**	**BMI**	**Age**	**TPMT activity (mU/L)**	**TPMT activity (nmol6MTG/grHb/h)**	**gr/Hb**	**BMI**	**Age**
TPMT activity (mU/L)	r=1	r=0.72, *P*<0.001	r=0.14 *P*=0.05	r=0.03 *P*=0.7	r=0.05 *P*=0.5	r=1	r=0.73, *P*<0.001	r=0.43 *P*<0.001	r= -0.07 *P*=0.9	r= -0.15 *P*=0.029
TPMT activity (nmol6MTG/grHb/h)	r=0.72, *P*<0.001	r=1	r= -0.54 *P*<0.001	r=-0.08 *P*=0.3	r= -0.07 *P*=0.34	r=0.72, *P*<0.001	r=1,	r= -0.27 *P*<0.001	r= -0.02 *P*=0.77	r= -0.08 *P*=0.26
Hb gr/dL	r=0.14 *P*=0.05	r= -0.54, *P*<0.001	r=1	r= 0.12 *P*=0.1	r= -0.04 *P*=0.54	r= 0.43 *P*<0.001	r= -0.27, *P*<0.001	r=1	r= 0.03 *P*=0.7	r= -0.07 *P*=0. 3
BMI (Kg/m^2)	r=0.03 *P*=0.68	r=-0.08 *P*=0.3	r= 0.12 *P*=0.1	r=1	r= 0. 3 *P*<0.001	r= -0.01 *P*=0.9	r= -0.02, *P*=0.77	r= 0.03 *P*=0. 7	r=1	r= 0.4 *P*<001
Age (year)	r=0.05 *P*=0.5	r=0.07 *P*=0.34	r= -0.04 *P*=0.6	r= 0. 3 *P*<0.001	r=1	r= -0.15 *P*=0.029	r= -0.08 *P*=0.26	r= -0.07 *P*=0. 3	r= 0.4 *P*<001	r=1

The IBD patients and control group were divided into two subgroups based on Hb concentrations less than 12 and ≥12 gr/dL. Comparison of clinical, laboratory features and risk factors in subgroups of IBD patients and control groups are demonstrated in Table 5. As expected, in both groups of IBD patients and control group with Hb levels <12 gr/dL had false-fully significantly higher TPMT activities in nmol6MTG/grHb/h compared in both groups of IBD patients and control group with Hb levels≥12 gr/dL (57.2(54.7-68) vs 45.4(40.5-50.1). *P*<0.001, and 55.6(48.8-60) vs 44.6 (40-49.3); *P*<0.001, respectively). It can be predicted, in both groups of IBD patients and control group with Hb levels less than 12 gr/dL compared with both groups with Hb concentrations≥12 gr/dL had lower TPMT activity in mU/L (101.9 vs 110.; *P*=0.041, and 101 vs 112.3.; *P*=0.036, respectively).

Table 5. Comparison of TPMT activities in mU/l and nmol6mtg/grHb/h, Hb concentration and BMI between subjects with Hb concentration less than 12 and 12≥gr/dl in IBD patients and control groups separately

	Control group		IBD patients	
	Hb<12 gr/dl	**Hb≥12 gr/dl**	**Hb<12 gr/dl**	**Hb≥12 gr/dl**
	N=13	**N=199**	**N=30**	**N=179**
TPMT activity (mU/L)	101 ±12.4	112.3±19.9	101.9 ±20.2	110±19.6
	P=0.036		*P*=0.041	
TPMT activity (nmol6MTG/grHb/h)	55.6(48.8-60)	*44.6 (40-49.3)	*57.2(54.7-68)	*45.4(40.5 – 50.1)
	P<0.001		*P*<0.001	
Hb gr/dl	12.4(11.6-12.75)	15(14.2-16)	12.1(11.2-12.5)	14.4(13.7-15.6)
	P<0.001		*P*<0.001	
BMI (Kg/m^2)	23.7±3.4	24.1±4.6	23.1±3.8	24.3±4
	P=0.78		*P*=0.15	

*Median and interquartile range (IQR) for non-normally distributed data, and percentages for categorical data

As shown in Table 6, we compared TPMT activities in mU/L and nmol6MTG/grHb/h, Hb concentration and BMI between IBD and control groups according to gender. We found that TPMT activity in nmol6MTG/grHb/h in female IBD patients was significantly higher than that in control group (47.3(41.7-54.3) vs 44.9(40.4-50.4), *P*=0.016); however TPMT activity in mU/L in male IBD was lower than that in control subjects (114.9±24 vs 121.2±18.47, *P*=0.024). The Hb concentration in IBD male and female was significantly lower than in control groups.

Table 6. Comparison of TPMT activities in mU/l and nmol6mtg/grHb/h, Hb concentration and BMI between IBD and control groups in males and females separately

	Males		Females	
	IBD patients N=82	**Control group N=96**	**IBD patients N=127**	**Control group N=116**
TPMT activity (mU/L)	114.9±24	121.2±18.47	105.8±16.4	103.8±17.2
	P=0.024		*P*=0.39	
TPMT activity (nmol6MTG/grHb/h)	46.3±10.7	45.7±7.7	*47.3(41.7-54.3)	44.9(40.4-50.4)
	P=0.65		*P*=0.016	
Hb gr/dL	14.7±1.9	15.7±1.5	13.2±1.5	13.7±1.3
	P<0.001		*P*=0.004	
BMI (Kg/m^2)	24±3.7	24.3±4.9	24.2±4.2	23.8±4.1
	P=0.56		*P*=0.43	

*Median and interquartile range (IQR) for non-normally distributed data, and percentages for categorical data

Furthermore, we compared of TPMT activities in mU/L and nmol6MTG/grHb/h, Hb concentration, BMI and age between males and females in IBD and control groups separately (Table 7). TPMT activity in mU/L and Hb concentration were significantly higher in males compared with females in IBD patients and control group (114±24 vs ±16.4, *P*=0.004 and 121.2±18.5 vs 103.8±17.2, *P*<0.001, respectively).

Table 7. Comparison of TPMT activities in (mU/l) and (nmol6mtg/grHb/h), Hb concentration, BMI and age between males and females in IBD and control groups separately

	IBD patients		Control group	
	Males n=82	**Females n=127**	**Males n=96**	**Females n=116**
TPMT activity (mU/L)	114±24	105.8±16.4	121.2±18.5	103.8±17.2
	P=0.004		*P*<0.001	
TPMT activity (nmol6MTG/grHb/h)	46.7±10.7	48.5±10.4	45.7±7.75	45±7.5
	P=0.18		*P*=0.54	
Hb gr/dL	14.8±1.9	13.2±1.5	15.7±1.5	13.7±1.3
	P<0.001		*P*<0.001	
BMI (Kg/m^2)	24±3.7	24.2±4.2	24.3±5	23.8±4.1
	P=0.6		*P*=0.41	
Age (year)	36±14.3	35.9±12.5	35.8±15.9	32.5±12.6
	P=0.96		*P*=0.09	

Discussion

This is for the first time that the activity of TPMT enzyme was evaluated in Iran's IBD patients. We have used non-extraction isocratic HPLC method to determine concentration of 6-MTG produced to assess TPMT enzyme activity in erythrocytes. We think that this modified robust assay might be suitable for use in the routine clinical laboratory to predict the risk of thiopurine toxicity in (IBD) patients prior to receiving thiopurine drugs.

Although majority of centers around the world are using the TPMT activity in relation to hemoglobin or RBC content of the blood cell lysate to determine the toxicity of thiopurine drugs, we found a significantly negative correlation between TPMT activity in nmol6MTG/grHb/h and the Hb levels in both the study groups. Graham shown that the expressing patient whole blood activity as mU/L gives a result which classifies patients equally well against their underlying genotype (5).

In addition, we found that correlation of TPMT activity in IBD patients is significantly affected by hemoglobin level. TPMT activity is significantly greater in IBD than control group when it is calculated in nmol6MTG/grHb/h, whereas, when TPMT activity is calculated in mU/L, no statistically significant difference was seen between the two study groups. The observed difference might be due to the decrease in hemoglobin level in the IBD patients compared to the control group. In support of this theory, we divided the IBD patients and the control group into two subgroups based on Hb concentrations, less than 12 and ≥12 gr/dL. Also we noticed that in some patients with low hemoglobin level,

misleading high results can be obtained and this was not seen when results are expressed as mU/L TPMT. For encouraging other providers to move to mU/L reporting format and expression into routine use, we need further presentations.

As noted above, since this is the first report on IBD patient's TPMT level in Iranian population and our findings showed that there is no frequency for deficient and 2.8 % for low activity, Therefore thiopurine drugs can be more confidently prescribed to treat IBD patients in Iran. In addition, based on our data, we strongly suggest the use of international unit format mU/L is more appropriate than nmol6MTG/grHb/h for expressing TPMT-activity for routine reporting.

Acknowledgments

This work was performed in partial fulfillment of requirements for a Ph.D by Research degree in Clinical Biochemistry, Kermanshah University of Medical Sciences, Kermanshah, Iran (Fariborz Bahrehmand). All authors contributed equally to this study.

References

1. Al Hadithy A, De Boer N, Derijks L, Escher J, Mulder C, Brouwers J. Thiopurines in inflammatory bowel disease: pharmacogenetics, therapeutic drug monitoring and clinical recommendations. Digestive and liver disease. 2005;37:282-97.
2. Lennard L. The clinical pharmacology of 6-mercaptopurine. Eur J Clin Pharmacol 1992;43:329-39.
3. Lowry P, Franklin C, Weaver A, Pike MG, Mays D, Tremaine W, et al. Measurement of thiopurine methyltransferase activity and azathioprine metabolites in patients with inflammatory bowel disease. Gut 2001;49:665-70.
4. Sandborn WJ, Tremaine WJ, Wolf DC, Targan SR, Sninsky CA, Sutherland LR, et al. Lack of effect of intravenous administration on time to respond to azathioprine for steroid-treated Crohn's disease. Gastroenterology 1999;117:527-35.
5. Graham V. Thiopurine methyltransferase phenotyping and genotyping in clinical practice preferred access arrangement: University of Birmingham; 2010.
6. Darakhshan F, Khojeini EV, Balaii H, Naderi N, Firouzi F, Farnood A, et al. Epidemiology of Inflammatory Bowel Disease in Iran: A review of 803 cases. Gastroenterol Hepatol Bed Bench 2009;1:19-24.
7. McLeod HL, Siva C. The thiopurine S-methyltransferase gene locus-implications for clinical pharmacogenomics. Pharmacogenomics 2002;3:89-98.
8. Holme S, Duley J, Sanderson J, Routledge PA, Anstey AV. Erythrocyte thiopurine methyl transferase assessment prior to azathioprine use in the UK. QJM 2002;95:439-44.
9. Evans WE, Horner M, Chu YQ, Kalwinsky D, Roberts WM. Altered mercaptopurine metabolism, toxic effects, and dosage requirement in a thiopurine methyltransferase-deficient child with acute lymphocytic leukemia. J Pediatr 1991;119:985-9.
10. O'Kane DJ, Weinshilboum RM, Moyer TP. Pharmacogenomics and reducing the frequency of adverse drug events. Pharmacogenomics 2003;4:1-4.
11. Ossaily S, Zgheib NK. The pharmacogenetics of drug metabolizing enzymes in the Lebanese population. Drug Metab Drug Interact 2014;29:81-90.
12. Szumlanski CL, Honchel R, Scott MC, Weinshilboum RM. Human liver thiopurine methyltransferase pharmacogenetics: biochemical properties, liver-erythrocyte correlation and presence of isozymes. Pharmacogenet Genomics 1992;2:148-59.
13. Ford LT, Berg JD. Determination of thiopurine S-methyltransferase activity in erythrocytes using 6-thioguanine as substrate and a non-extraction liquid chromatographic technique. J Chromatogr B 2003;798:111-5.
14. Kröplin T, Weyer N, Gutsche S, Iven H. Thiopurine S-methyltransferase activity in human erythrocytes: a new HPLC method using 6-thioguanine as substrate. Eur J Clin Pharmacol 1998;54:265-71.
15. Kröplin T, Fischer C, Iven H. Inhibition of thiopurine S-methyltransferase activity by impurities in commercially available substrates: a factor for differing results of TPMT measurements. Eur J Clin Pharmacol 1999;55:285-91.