

Thiopurine S-Methyltransferase Assay by HPLC in Acute Lymphoblastic Leukemia Patients and a Healthy Iranian Population

Sima Mozafari¹, Nahid Einollahi², Elham Shahgholi³, Safoura Vardasbi¹, Abolfazl Golestani¹

¹ Department of Clinical Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

² Department of Medical Laboratory Sciences, School of Allied Medical Sciences, Tehran University of Medical Sciences, Tehran, Iran

³ Department of Pediatrics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

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Abstract- Thiopurine s-methyltransferase (TPMT) plays a key role in the metabolism of the drug; 6-MP in children with acute lymphoblastic leukemia (ALL). The wild-type TPMT*1 allele and mutant alleles are associated with normal and intermediate enzyme activity, respectively. In patients with intermediate or deficient TPMT activity, normal dose of the drug may cause serious side effects such as bone marrow toxicity. The aim of this study was to assay the enzyme activity by HPLC for right ordering of chemotherapy drug doses in the patients. TPMT activity was measured in RBC of healthy adults (n=73) and children (n=10). Also, TPMT (*2, *3A, *3B and *3C) genotype of the samples were assessed by ARMS-PCR and RFLP-PCR. No indication of gender and age differences in the TPMT activity was found. This study showed that the HPLC method was sensitive, accurate and precise and can be used in routine clinical laboratory tests.

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Introduction

Leukemia is the most common cancer in childhood which is characterized by an immature lymphoid cell proliferation in bone marrow and peripheral blood altering hematopoiesis (1). The main treatment for childhood leukemia is chemotherapy which destroys cancer cells, usually by stopping the cancer cell ability to grow and divide. Usually, chemotherapy is employed, and a combination of anti-cancer drugs is used. One of the most effective medications for the treatment of acute lymphoblastic leukemia in the maintenance phase is 6-MP (2-4).

6-MP is an antimetabolite which has been used for 40 years in the treatment of leukemia. There are three different pathways for 6-MP metabolism. In the first, 6-MP enzymatically converts to 6-MTIMP and 6-TGN and the latter triggers cell-cycle arrest and induces apoptosis in malignant cells. In the second pathways, 6-MP converts to 6-MMP catalyzed by TPMT; and in the third, a minor amount of 6-MP converts to thiourea acid by xanthine oxidase. Therefore, a major amount of 6-MP will be inactivated by converting to 6-MMP and the rest of it will be converted to 6-TGN and 6-MTIMP (5-

6).

Incorporation of 6-TGN into DNA may trigger cell apoptosis of cancerous and bone marrow stem cells which may lead to myelotoxicity. Therefore, 6-TGN is responsible for both therapeutic efficacy and myotoxicity (7). Recently, several studies have demonstrated that the level of 6-TGN is related to TPMT activity. Thiopurine S-methyltransferase is a cytosolic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds (8). In the normal conditional, the level of 6-TGN produced is suitable for therapeutic purposes, but in high TPMT activity the 6-TGN level is not sufficient, and in low TPMT activity, the level of 6-TGN is high, leading to therapeutic insufficiency and myelotoxicity respectively. So, right therapeutic dose ordering of the drug depends on the activity of TPMT. Currently, there are two methods to detect TPMT deficiency. The first method is enzymatic assay (phenotyping test) which measures metabolites such as 6-MMP and 6-TGN by HPLC. The second is genotyping that detects the patient's individual genetic makeup for producing the TPMT enzyme (9).

Genetic mutations in the thiopurine S-methyl transferase (TPMT) gene, which results in decreased

Corresponding Author: A. Golestani

Department of Clinical Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 64053387, Fax: +98 21 64053385, E-mail address: golsetan@tums.ac.ir

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enzyme activity, have been linked to increased risk of myelotoxicity, due to 6-TGN overproduction. The mutation in TPMT can be explained by 40 sequence variants in the TPMT gene, that most of them show reduced enzyme activity (10). A low dose of 6-MP is

required for patients with homogeneous and heterogeneous mutations (11) (Table 1) including TPMT*2, TPMT*3A, TPMT*3b and TPMT*3c variants which are the most common mutant alleles (12).

Table 1. TPMT gene classification and its implications for therapy

Genotype	TPMT activity level	Frequency in the population (%)*	Dose adjustments**
Wild type	Normal and High	89	Normal dose may need to increase
Heterogeneous	Intermediate	11	30-70% normal dose
Homogenous	Deficiency	0.33	<10% of normal dose

*Based on the data presented in Ref. 13

**Based on the data presented in Refs. 11, 14-16

Several studies have revealed the relationship between polymorphism and TPMT activity, but there is no consistency in this regard. On the other hand, polymorphism studies have demonstrated several mutations with low enzyme activities in the populations under study. Based on the results obtained, it is highly recommended to measure the enzyme activity before beginning the therapy with the drug.

In the present study, we set up a HPLC method for measurement the enzyme activity in children with acute lymphoblastic leukemia (ALL) for right dose ordering of chemotherapy drug and determined the TPMT activity distribution among the studied subjects.

Materials and Methods

Reagents and chemicals

6-MP, 6-MMP, and SAM were obtained from MP Biomedical (France), Aldrich (South Korea) and Sigma-Aldrich (USA), respectively. DMSO, Methanol grade HPLC and TCA were obtained from Merck (Germany). The restriction enzymes and other molecular biology reagents were purchased from fermentas (Lithuania).

Study population

Blood samples were collected from 73 healthy volunteers (36 male and 37 female) and 10 sick children (3-15 years old). The study protocol was approved by the ethical committee of Tehran University of Medical Sciences and written informed consent was obtained from the adult participants and the parents of the children.

Preparation of erythrocyte

Three ml of whole blood was collected in lithium heparin tubes. For the preparation of hemolysate, plasma and leukocytes were removed by centrifugation at 3500,

4 °C for 5 minutes. Erythrocytes were washed with PBS and then mixed with 4 volumes of deionized water. The prepared samples were kept at -80 °C until use.

Enzymatic reaction assay

The assay was based on the TPMT catalysis of 6MP to 6-MMP using S-adenosyl-methionine as a methyl donor.

To prepare the reaction solution, 50 µl of phosphate buffer (0.1M, pH 7.4), was mixed with 20 µl of 24 mM 6-MP and 10 µl of 0.4 mM SAM. The reaction was initiated by addition of 200 µl of hemolysate. The mixture was incubated at 37 °C for 1 hour, and after that, the reaction stopped by adding 50 µl of TCA (100% v/w).

HPLC analysis

The HPLC systems, consisting of two pumps, degasser and PDA-UV detector used in this study was from Knauer (Germany). The analytical column was an OD s-3C18 (250 mm×4.6 mm I.D), 5µm particle size obtained from MZ (Germany) and was protected by a guard cartridge.

The mobile phase was 0.02 M phosphate buffer-methanol (70.30 v/v), pH=6.7 and the product was detected at 290 nm.

Validation of HPLC method

All validation factors *i.e.* specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ) and robustness were confirmed. The specificity of the method was assessed by 6MP, SAM, and blank hemolysate. Intraday and interday precision study of 6-MMP was carried out by calculating the corresponding responses for 3 times on the same day and on 3 different days for 50, 150 and 200 ng/ml of 6-MMP. The accuracy of the method was

determined by calculating percentage recovery of 6-MMP for 5 concentrations ranged from 50 to 300 ng/ml. The limit of detection and limit of quantitation were calculated as follows; LOD is 3.3 (SD)/S and LOQ are 10 (SD)/S, where the SD is the standard deviation of response (top area) and S=average of the slope of the calibration curve. For robustness assessment, parameters such as column length, flow rate, the percentage of methanol in and pH of the mobile phase were changed.

DNA extraction and PCR method

Genotyping of four major polymorphism of the gene encoding TPMT including TPMT*2 (G238C), TPMT*3

(TPMT*3B (G460A) and TPMT*3C (A719G)) were analyzed by ARMS-PCR and RFLP respectively. The genomic DNA of the whole blood samples (2ml) was obtained by enzyme-salting out extraction method with a minor modification (Sambrook and Russell, 2012). The DNA prepared, was dissolved in TE buffer (pH 8.0) for further use. The quality and quantity of the isolated DNA were determined by agarose gel electrophoresis (1%) and Nanodrop apparatus (Thermo, USA), respectively. To detect the type of TPMT polymorphism primer sequences were selected according to the previous studies (Table 2).

Table 2. Primer sequence for PCR study

Polymorphism		Primer sequence	Annealing (TM)	PCR product (BP)
TPMT*2	F _w	GTATGATTTTATGCAGGTTTC 3'	58	254
	F _m	GTATGATTTTATGCAGGTTTG 3'		
	R	TAAATAGGAACCATCGGACAC 3'		
TPMT*3B	F	ATAACAGAGTGGGGAGGCTGC 3'	60	365
	R	CTAGAACCCAGAAAAAGTATAG 3'		
TPMT*3C	F	TGTTGGGATTACAGGTGTGAGCCAC 3'	64	372
	R	TGTCATTTACTTTTCTGTAAGTAGTT 3'		

PCR reaction was performed in a total volume of 25 µl, containing 200 ng of template DNA, 1x PCR buffer, 3 µM of dNTP, 3-4 mM of MgCl₂, 3 µM of each primer, and 2 U of Taq DNA polymerase. PCR cycling was performed at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at a specific temperature for 30 s, extension at 72 °C for 1 minute and final extension at 72 °C for 5 minutes. Following amplification, 10 µl of the PCR reaction was electrophoresed on a 2% agarose gel with a 50-bp ladder. Restriction enzymes; MWOI and ACCI were used to detect G460A and A719G mutation, respectively.

Data analysis

The software SPSS 17.0 was used for statistical analysis. The data were examined for normality of distribution by using the Shapiro-Wilk test of normality. In addition, Comparisons between two groups were analyzed by t-test

Results

Validation of HPLC method

The HPLC method was validated by studying linearity, selectivity, accuracy, precision, LOD and

LOQ, quality control and robustness.

The linearity was assessed from 15.625 to 1000 ng/ml, and the corresponding linear regression equation was $y=1125x+18087$ with the regression coefficients (R^2) 0.994. For studying of selectivity, 3 compounds (6-MP, SAM, and 6-MMP) were injected and showed 4.5, 2.8 and 13.5 minutes as retention time, respectively, which confirmed no interfered peaks with 6-MMP. In addition, the experiment showed that neither mobile phase or other analytes in hemolysate interfere with 6-MMP peaks.

The relative standard deviation for intra-day and inter-day (n=3 days) analysis was in the range of 1.19-9.87 % and 2.06-6.46 % respectively. These values demonstrate an acceptable precision of the method. The relative standard deviation for the accuracy of analysis was in the range of 0.3-4.18% which was less than 10% for assessment of 6-MMP.

The LOD and LOQ for 6-MMP measurement by HPLC were 0.02 and 0.06 ng/ml respectively, which demonstrates the high sensitivity of the method. The chromatogram shows a good resolution and asymmetry factor (Figure 1). The results from the robustness study showed that the chromatography parameters for measurement of 6-MMP were acceptable.

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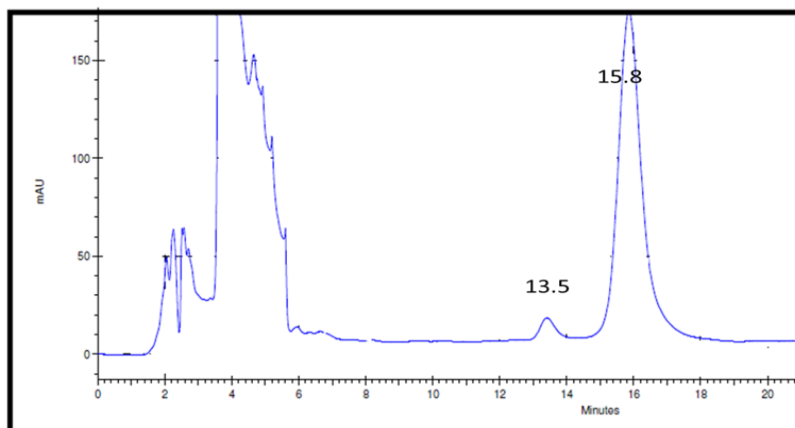


Figure 1. Chromatogram of an erythrocyte lysate sample from the reference population

TPMT Activity

The TPMT activity was investigated in 73 healthy volunteer subjects (36 men and 37 women) and 10

children, which their demographic and clinical features are reported in Table 3.

Table 3. Sample size and features

Group	Number of samples	Age (year)	Clinical feature
1	73 (36 Male and 37 Female)	22-32	Health
2	10 (6 Boy and 4 Girl)	3-15	Sick

The range of TPMT activity was between 10.10, and 31.51 nmol 6-MMP/ml_{RBC}/h and the frequency

histogram showed a bimodal distribution (figure 2).

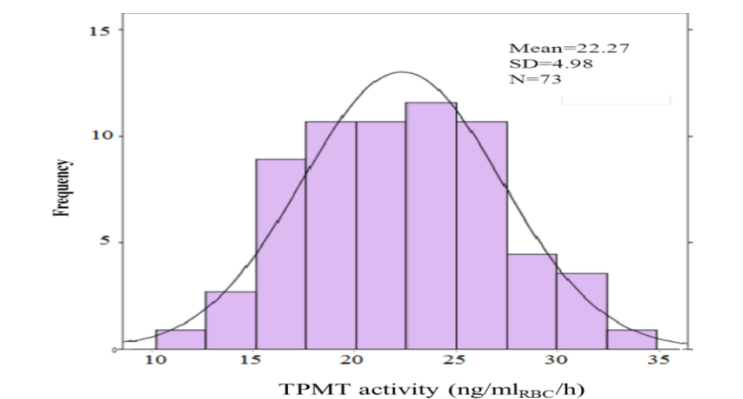


Figure 2. Frequency distribution histogram of TPMT activity in adult subjects

The subjects were classified according to their TPMT activities into deficient, intermediate and normal

activity groups (Table 4).

Table 4. Range of TPMT activity in adult subjects

TPMT activity level	Range (ng/ml _{RBC} /h)	Frequency in adult subjects (%)
Deficient	<12.3	0.01
Intermediate	12.31-17.29	0.17
Normal and high	>17.30	0.82

TPMT genotyping

In the population studied (n=73), about 95.89% of the cases had wild type, and 4.11% showed heterozygote TPMT genotype. No homozygous mutant was determined. The distribution of the TPMT

polymorphism is shown in Table 5 and as is shown the most common variant was *3B. The other variants (2,3C and 3A) were not found in the population studied. In addition in children group, 100% of the polymorphism were detected as wild-type (TPMT*1/1).

Table 5. Distribution of the TPMT polymorphism in both adults and children

Group	TPMT*1/1	TPMT*1/2	TPMT*1/3b	TPMT*1/3c	TPMT*1/3a
Adults (n=73)	95.89%	0%	4.11%	0%	0%
Children (n=10)	100%	0%	0%	0%	0%

Discussion

Nowadays there are many different methods for measuring TPMT activity. In this study, two of these methods *i.e.* phenotyping (assessment of enzyme activity) and genotyping (polymorphism) were performed. We set up the HPLC method for measurement of the enzyme activity and report for the first time, a reference range of TPMT activity in Iran.

Based on the previous studies, the method used is rapid, specific, accurate, and precise for routine analysis of TPMT activity

In the study, the intra-assay coefficient of variation (CV) ranged from 1.8% to 3.4% and the interassay CV was from 5.6% to 6.1%, and accuracy ranged from 0.3% to 4.18%. The coefficients of variation of precision and accuracy were less than 10%. Therefore they were considered as acceptable. In addition, the CV for precision and accuracy parameters were less than the amounts obtained by Indjova *et al.*, (2003) (16), Mauric *et al.*, (2005) (17) and Hawwa *et al.*, (2009) (18) which showed that the present study is reliable.

The method for determining the 6-MMP concentration and TPMT activity is similar to the other studies. The TPMT activities obtained in RBC lysate samples ranged from 10.10 to 31.51 nmol 6-MMP/ml RBC/h and had a bimodal distribution in the population sample. A normal TPMT activity was detected in 82.19 % of the subjects.

No significant difference was detected in TPMT activity in males and females. This result is consistent with the results of Garristen *et al.*, (19) and Serpe *et al.*, (20), and inconsistent with the study of Schaeffeler *et al.*, (9) which showed that TPMT activity was higher in males than females, significantly.

The measurement of TPMT activity in RBC can be sometimes misleading, for example in patients who have received blood in the last 3 months or are taking certain

medications. For example, Chrazanowski *et al.*, (21) showed that measurement of TPMT activity in children with ALL receiving 6-MP was 20% higher than that of those at the time of ALL diagnosis. In these cases, the genotyping test can be helpful. So, in this study, the TPMT genotypes were determined for all subjects using PCR method to detect the wild type (TPMT*1) and the most prevalent mutant alleles (TPMT*2, TPMT*3A, TPMT*3B, TPMT*3C). The frequency of wild type TPMT alleles was 97.95% in adult population, consistent with results of other studies in Iran (22-24).

The common mutant alleles are different in various races and ethnicities. The most common variant in our study determined to be *3b which is consistent with the study of Cheung *et al.*, (25) and inconsistent with studies of Rossi *et al.*, (2001, Italy) (26), Ganiere *et al.*, (2004, France) (27) and Zeglam *et al.*, (2015, Lebanon) (28).

In this study the relationship between TPMT genotypes and phenotypes was 84.93% which is similar to the study of Indjova *et al.*, (2001) (16), higher than the value reported by L. Serpe *et al.*, (2009) (20) and lower than Schaeffeler *et al.*, (2004) (9). Studies have shown that the relationship between TPMT genotypes and phenotypes does not reach 100% and the genotype variations account for two-thirds of the total variance in the level of RBC enzyme activity. This means that genetic polymorphism is a very important factor resulting in variation of TPMT activity. The enzyme activity depends on some other factors such as gene expression, interacting and environmental factors including chemicals, drugs or diet components.

This study shows that the screening of TPMT activity by HPLC method appears to be more reliable than that of the enzyme genotyping for treatment purposes and prevention of myelosuppression and other side effects of the drug used.

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