의학석사 학위 논문

Measurement of thiopurine nucleotides in erythrocytes and clinical application to pediatric acute lymphoblastic leukemia

적혈구 내 티오퓨린 측정과 소아 급성림프구성백혈병에의 적용

2017년 7월

서울대학교 대학원

임상의과학과

문 수 영

A thesis of the Degree of Master of Science

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Measurement of thiopurine nucleotides in erythrocytes and clinical application to pediatric acute lymphoblastic leukemia

by

Soo Young Moon, M.D.

A thesis submitted to the Department of Clinical Medical Sciences in partial fulfillment of the requirements for the Degree of Master of Science in Medicine at Seoul National University College of Medicine

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Approved by Thesis Committee:

Professor _____ Chairman

Professor _____

Professor _____

ABSTRACT

Introduction: Mercaptopurine (6MP) is a purine analog. Its oral form is administered daily in maintenance therapy for pediatric acute lymphoblastic leukemia. This prodrug is absorbed and metabolized in the blood cells to 6-thioguanine (6TG) nucleotide and 6-methylmercaptopurine (6MMP) nucleotide that inhibit DNA replication and purine synthesis. In this study, those metabolites in RBC were hydrolyzed into 6TG and 6MMP using a simple preparation method, and their concentrations were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS). This method was validated for clinical use and applied to blood samples from acute lymphoblastic leukemia (ALL) patients to investigate its effect on conventionally monitored indices to determine efficacy and compliance for therapy.

Methods: Centrifuged RBC were hemolyzed and deproteinized using perchloric acid, followed by hydrolysis for 1 hour at 100℃. For liquid chromatography, C18 column was used for the stationary phase, and distilled water and acetonitrile were used for the mobile phase. In mass spectrometry, the mass transitions were 168>150.9 for 6TG and 167>125.1 for 6MMP in the positive ion mode, and isotope-substituted 6TG and

6MMP were used as internal standards. To evaluate precision of the method, two different concentrations of each drug were added to erythrocytes from three healthy persons, then measured for seven days. In order to determine the quantitative measurement limits, the experiment was repeated thrice at four low concentrations for four days. We measured the concentrations of 6TG and 6MMP in erythrocytes of children with ALL undergoing maintenance therapy for more than one month. We also analyzed whether leukocyte counts and liver enzyme levels, which were the factors determining the drug dose, could be predicted by 6MP metabolites.

Results: The coefficients of variation of 6TG and 6MMP were about 5.7–8.1%, and the bias was within 5% of the target concentration. Limits of quantification with acceptable precision of 20% were set at 54 ng/mL for 6TG and 1,036 ng/mL for 6MMP. In total, 74 blood samples were collected from 37 patients for 6MP metabolite analysis after 1–26 months of maintenance therapy. Concentration of 6TG was measured in the range of 16.1 to 880 pmol/8×10^8 RBC and that of 6MMP was measured in the range of 55 to 20,937 pmol/8×10^8 RBC. There was positive correlation between 6TG and 6MMP concentrations and 6MP doses. In addition, concentration of 6TG significantly increased with patient's age ($r=0.448$, $p<0.001$). Concentrations of 6MP metabolites were not correlated with neutrophil and leukocyte counts. Liver enzymes

were positively correlated with both 6TG and 6MMP concentrations, showing higher ALT activity (difference 61.4 IU/L, $p<0.001$) in the group of 6MMP above 5,700 pmol/8×10^8 RBC. However, these correlations were not significant in multivariate analysis when adjusted with drug dose and duration of treatment.

Conclusion: In this study, an analytical method for 6TG and 6MMP in RBC was established and applied to clinical specimens. The correlation with drug dose was good, but that with clinical indices for determining drug dose remains to be elucidated.

Keywords: Mercaptopurine, 6-thioguanine,

6-methylmercaptopurine, acute lymphoblastic leukemia

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LIST OF ABBREVIATIONS

6MMP	6-methylmercaptopurine
6MP	mercaptopurine
6TG	6-thioguanine
6TGN	6-thioguanosine nucleotide
ACN	acetonitrile
ALL	acute lymphoblastic leukemia
ALT	alanine aminotransferase
ANC	absolute neutrophil count
AST	aspartate aminotransferase
BSA	body surface area
CCG	Children's Cancer Group
CI	confidence interval
CV	coefficient of variation
DTT	dithiothreitol
DW	distilled water
GVHD	graft-versus-host disease

HPRT	hypoxanthine guanine phosphoribosyl transferase
IBD	inflammatory bowel disease
IS	internal standard
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOQ	limit of quantification
MeTIMP	6-methylthioinosine monophosphate
MTX	methotrexate
OR	odds ratio
PBSCT	peripheral blood stem cell transplantation
RER	rapid early responder
SE	standard error
SER	slow early responder
TdGTP	thiodeoxyguanosine triphosphate
TIMP	thioinosine monophosphate
TPMT	thiopurine methyltransferase
XO	xanthine oxidase

INTRODUCTION

Mercaptopurine (6MP) is a prodrug with similar structure as that of purines. Its metabolic active form is involved in DNA replication, DNA repair, and purine biosynthesis (1, 2). On entering the cell, 6MP enters the purine salvage pathway, which starts from reaction with hypoxanthine guanine phosphoribosyltransferase (HPRT) to generate thioinosine monophosphate (TIMP), followed by base modification and phosphorylation to yield 6-thioguanosine nucleotide (6TGN) (Figure 1). The deoxy form of 6TGN, thiodeoxyguanosine triphosphate (TdGTP) is structurally similar to dGTP, which is an essential substrate in DNA replication. This analogue nucleotide incorporates into the double stranded DNA, activating the mismatch repair system and causing DNA strand breaks, although the exact mechanism is not clear (3).

Two other major pathways competing with purine salvage pathway are oxidation and thiol-methylation. Oxidation is a major catabolic route of 6MP when absorbed through intestine mucosa and liver, which is mediated by xanthine oxidase to form the inactive metabolite thiouric acid. The second pathway is thiol-methylation, mediated by intracellular thiopurine methyltransferase (TPMT), which is responsible for methylation of several nucleotides in the purine salvage

pathway. TPMT converts 6MP and TIMP to 6-methylmercaptopurine (6MMP) and 6-methylthioinosine monophosphate (MeTIMP), thereby reducing 6TGN production. Moreover, a recent study showed that MeTIMP can inhibit the purine *de novo* pathway, resulting in dependence of the target cells on the purine salvage pathway, thereby hindering DNA replication (4) (Figure 1). TPMT activity is inherited in a co-dominant pattern: persons with homozygote of the variant genotype have reduced activity of the enzyme, although allele frequency of the variant is slightly low in far-east Asians (5). Standard dose of 6MP in patients with homozygote variant of TPMT would produce extensive 6TGN, which may cause life threatening myelosuppression (6, 7).

6MP is used as an immune suppressant in the treatment of inflammatory bowel disease (IBD), and as an antineoplastic agent in maintenance therapy for acute lymphoblastic leukemia (ALL). Most treatment protocols of ALL require 2-3 years of daily oral 6MP and weekly oral methotrexate (MTX) ingestion to suppress relapse after induction of remission (8). During this long period, maintaining a proper level of myelosuppression is important for favorable prognosis (9). In contrast, overdose of 6MP is related to severe neutropenia, resulting in cessation of maintenance therapy and increased risk of secondary malignancies (10). Most physicians evaluate myelosuppression with while blood cell (WBC) count and absolute neutrophil count

(ANC), and adjust the dose of 6MP or MTX to acquire proper myelosuppression. However, identical number of WBC and ANC does not reflect identical level of myelosuppression between different patients (11, 12). Some patients have difficulty reaching their target ANC due to side effects such as hepatotoxicity, and ANC may be also affected by intake of other drugs during maintenance therapy. In addition, to determine compliance of the patients to the prescribed doses, precise observational indicators are needed.

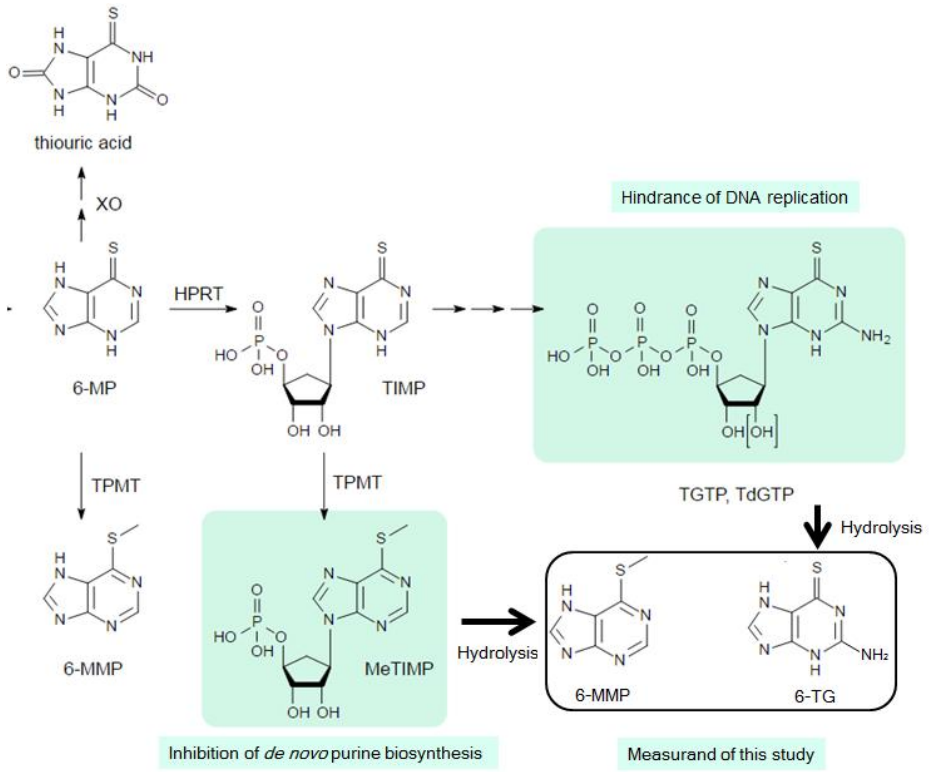
Therefore, proper drug delivery to the target cell is determined by measuring 6MP and its metabolites. The half-life of plasma concentration of the 6MP parent drugs is very short (1-2 hour), and its intra-individual bioavailability varied widely even in one patient repeatedly receiving the same dose of 6MP (13). As a substitute, 6TGN, the final metabolite that accumulates in RBC and incorporates into DNA, have prolonged half-life. 6TGN in RBC was measured to determine correlations with the abovementioned indicators and prognosis of patients. After a few days of oral administration of 6MP, concentration of 6TGN in RBC reaches a steady state (14). This concentration was reported to be associated with myelotoxicity, duration of remission, and risk of disease relapse (15, 16). Naturally, 6TGN was found to be lower in patients with low compliance to therapy (17), increased in patients with variant TPMT genotype/low activity of TPMT (18), and correlated with the

concentration of thioguanine incorporated in leukocyte DNA (19). Moreover, MeTIMP, the methylated nucleotide accumulated in RBC increased when 6MP dose increased, and was co-related with increased levels of liver enzymes (20).

Procedures for measurement of 6TGN and MeTIMP levels in RBC have been developed since decades. To quantify concentrations of intracellular thionucleotide metabolites, RBC should be hemolyzed and deproteinized, and the nucleotides should be hydrolyzed into bases and phosphoribosyl groups with heat and acid treatment. Then, concentrations of the cleaved bases, 6TG and 6MMP, were measured using liquid chromatography with UV detection, which was first introduced 30 years ago (21), or by recently adopted liquid chromatography-tandem mass spectrometry (LC-MS/MS) (18). Besides, concentration variation of about 2.7 times may occur depending on the hydrolysis and extraction process (22).

In this study, we developed a method for quantification of 6MP metabolites in RBC using LC-MS/MS. After validation of the analytical performance, 6TG and 6MMP in RBC were measured in samples from pediatric ALL patients undergoing maintenance therapy. Correlation with clinical characteristics, including drug dosage and duration of treatment, was analyzed.

Figure 1. Metabolic pathway of 6MP *in vivo*.



Abbreviations: 6-MP, mercaptopurine; XO, xanthine oxidase; HPRT, hypoxanthine guanine phosphoribosyl transferase; TIMP, thioinosine monophosphate; TGTP, thioguanine triphosphate; TdGTP, thiodeoxyguanosine triphosphate; TPMT, thiopurine methyltransferase; 6-TG, 6-thioguanine; 6-MMP, 6-methylmercaptopurine; MeTIMP, 6-methylthioinosine monophosphate.

MATERIALS AND METHODS

1. Chemicals and materials

6TG and 6MMP were prepared at a purity of 98% or more (Sigma Aldrich, St. Louis, MO, USA), and chemically identical molecules with replaced isotopes (6TG−$^{13}C2^{15}N$, 6MMP−D3) were used as internal standards (IS) (Toronto Research Chemicals, North York, ON, Canada). The stock solution of each material was prepared as follows: 6TG 2 mg/mL in 1M sodium hydroxide, 6MMP 2 mg/mL in 1M sodium hydroxide, 6TG IS 1 mg/mL in DMSO, and 6MMP IS 2.5 mg/mL in methanol. Perchloric acid (70%) and dithiothreitol (DTT) used in the hydrolysis process were purchased from Sigma Aldrich. Acetonitrile (ACN) used in LC was purchased from J.T. Baker (Center Valley, PA, USA), and formic acid was purchased from Samchun pure chemicals (Pyeongtaek, South Korea).

Working solutions used for validation and analysis of clinical samples were prepared by diluting the stock solutions with distilled water (DW). For using the working solutions as calibrators, 7 concentrations were prepared as follows: 125, 250, 500, 1,000, 2,000, 4,000, and 8,000 ng/mL for 6TG; 2500, 5,000, 10,000, 20,000, 40,000, 80,000, and 160,000 ng/mL for 6MMP. For the working solution to be used as QC materials,

two concentrations were prepared as follows: 1,250 and 2,500 ng/mL for 6TG; 25,000 and 50,000 ng/mL for 6MMP. Each working solution of IS was prepared at a concentration of 1 μg/mL for 6TG IS and 2.5 μg/mL for 6MMP IS.

2. Sample preparation

The samples were prepared using the method described by Dervieux and Boulieu et al. (23) and modified by Shipkova et al. (22). One milliliter of EDTA-anticoagulated whole blood was centrifuged at 1,000 *g* for 10 minutes at room temperature. After removing plasma, buffy coat, and upper layer of packed RBC, the remaining RBC portions were washed with saline solution and centrifuged twice at 1,000 *g* for 10 minutes at room temperature. Packed RBC were diluted 10-fold with saline solution, and 250 μL of the solution was dispensed into a polypropylene tube and stored at −80℃ until further use. Cryopreserved RBC from healthy persons were used as matrices for calibration, QC, and method validation. RBC samples from patients included in this study were separated and diluted on the day they were received.

The hydrolysis and extraction process were performed as follows: diluted RBC solution (250 μL) was mixed with 20 μL of

IS, 20 μL of 1.1 mol/L DTT, and 50 μL of DW, vortexed for 30 seconds, and spun down. To prepare the samples for calibration, QC, and method evaluation, 20 μL of calibrator, QC working solution or method evaluation material were added to the mixture, instead of 20 μL of DW. Then, 34 μL of 70% perchloric acid was added, vortexed for 30 seconds, and centrifuged at 3,000 *g* for 15 minutes at room temperature. The supernatant (270 μL) was transferred to another polypropylene tube, and hydrolyzed at 100℃ for 1 hour. After cooling at room temperature, 50 μL solution was used for analysis.

3. LC-MS/MS analysis

The LC instrument was a 1200 series infinity system (Agilent Technologies, Santa Clara, CA, USA), equipped with HiP sampler, binary pump, and column compressor. Chromatographic separation was performed using an Eclipse plus C18 column (3.5 μm particle size, 4.6 cm×100 mm, Agilent Technologies). Mobile phase A was 0.1% formic acid in DW and mobile phase B was 0.1% formic acid in ACN. The flow rate was 1 mL/min. The gradient condition was A 90% for the first minute, A 50% from 2 minutes to 2.6 minutes, followed by A 100% from 2.7 minutes till the end. The injection volume was 5

μL, and the total run-time per specimen was 5 minutes. The retention time of 6TG and 6TG IS was approximately 2.39 minutes, and that of 6MMP and 6MMP IS was approximately 2.85 minutes (Figure 2).

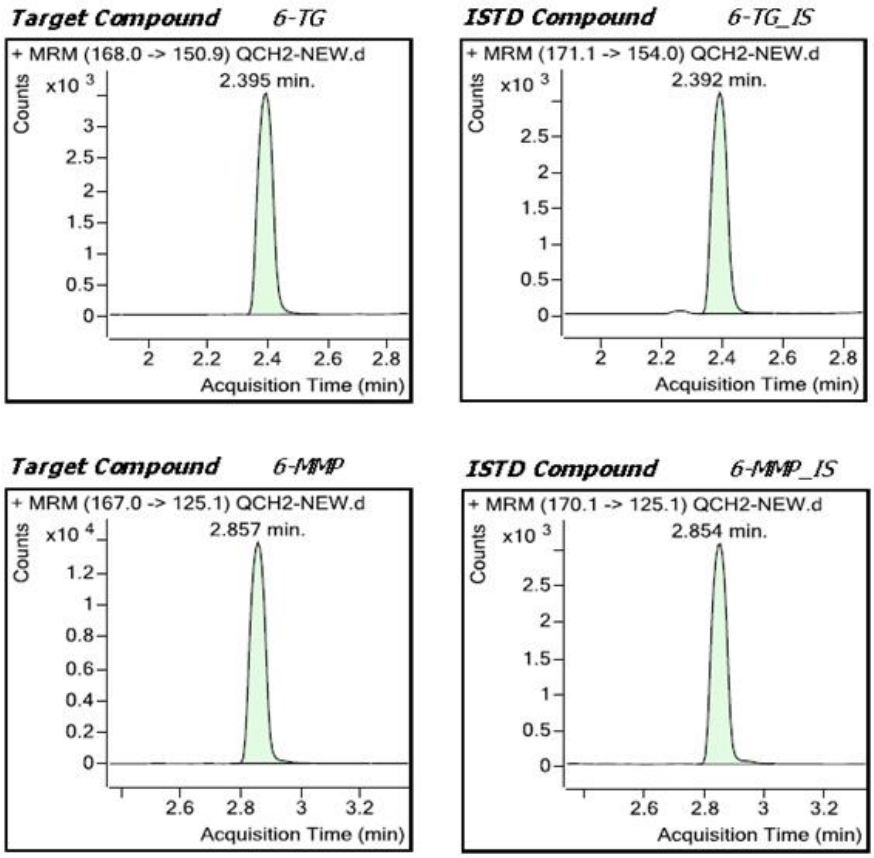
The LC instrument was connected to a 6490 triple quadrupole MS (Agilent Technologies). Sample analysis was performed using electrospray ionization in the positive-ion mode. In the multiple reaction monitoring mode, the *m/z* transition values were set at 168>150.9 for 6TG, 171.1>154 for 6TG IS, 167>125.1 for 6MMP, and 170.1>125.1 for 6MMP IS. Nitrogen gas was used for nebulization and collision. The collision energy used for 6TG, 6TG IS was 26 V, and for 6MMP, 6MMP IS was 30 V. Detailed specifications of MS/MS are as follows: capillary voltage 3.5 kV, dwell time 50 ms, sheath gas flow 11 L/min at 200℃, desolvation gas flow 16 L/min at 200℃. The ratio of the peak area of 6TG or 6MMP to the peak area of each IS was used for calculating the concentration with MassHunter Workstation software (Agilent Technologies) by regression with 1/x-weighted concentrations. Concentrations of 6TG and 6MMP were obtained in the ng/mL units by LC-MS/MS. To compensate for difference from RBC numbers of each sample, RBC number per microliter and hematocrit were measured before sample preparation were used to transform to pmol/8×10^8 RBC units, according to the equations described

below. Used RBC volume was 25 μL, and molecular weights of the respective analytes were 167 and 166. Dividing the value in ng/mL by six gives the approximate value of pmol/8×10^{8} RBC.

$$RBC-6TG(pmol/8\times10^{8}RBC) = \frac{\frac{RBC-6TG(ng/mL)}{100}\times8\times10^{8}\times1000}{\frac{25\mu L}{hematocrit}\times100\times RBC\ number\times167}$$

$$RBC-6MMP(pmol/8\times10^{8}RBC) = \frac{\frac{RBC-6MMP(ng/mL)}{100}\times8\times10^{8}\times1000}{\frac{25\mu L}{hematocrit}\times100\times RBC\ number\times166}$$

Figure 2. Representative chromatograms of 6TG, 6MMP, and respective internal standards.



4. Validation procedure

The analytical performance of the developed method was measured in terms of precision, limit of quantification (LOQ), and recovery. In order to evaluate precision of the method, two concentrations of QC working solutions were spiked into the diluted RBC from healthy donors, as described above. After five days of protocol familiarization period, the two samples were analyzed in triplicate for seven consecutive days. Using this approach, within-run precision, which reflects imprecision in sample preparation and analysis, between-day precision, and total precision could be obtained. As there was no reference method for analysis of 6TG and 6MMP, accuracy was evaluated by calculating recovery rate of spiked concentrations in the same procedure of precision analysis. Linearity was evaluated by linear regression analysis of results from seven concentrations of calibrators during validation period. For determining the limit of quantification, four concentrations of 6TG and 6MMP were prepared by two-fold serial dilution of the second lowest calibrator (250 ng/mL for 6TG and 5,000 ng/mL for 6MMP). After pretreatment process, each diluted sample was measured in triplicate for four consecutive days.

5. Clinical application

Seventy-seven venous blood samples were collected from 38 patients with ALL who underwent maintenance therapy at the Seoul National University Children's Hospital. All patients were under the age of 18 at the time of blood sampling, and were on the first remission status. They were prescribed a daily oral 6MP and weekly oral MTX for more than four weeks as maintenance therapy, except three exclusion cases. Two samples were excluded because administration of oral 6MP and MTX was stopped due to neutropenic fever for 7 days and 16 days. In these patients, 6TG concentrations were the lowest, while 6MMP concentrations were not. One sample was also excluded due to suspicious non-compliance. The patient started 6MP dosage with the highest dose (80 mg/m^2 per day), but the patient did not take 6MP because of fever after the start of therapy. The concentrations of 6TG and 6MMP measured after two weeks of prescription were under the LOQ.

Treatment protocol was largely classified into five types: CCG1882, CCG1952, 0601RER, 0601SER, and post PBSCT. Among the different treatment protocols, the method of adjusting 6MP dose was as follows: initial dose of 6MP at the start of maintenance therapy was administered, and the dose was adjusted according to WBC and ANC. In addition to analysis of 6MP metabolites in RBC, CBC and liver enzyme tests were also performed. The protocol was approved by the institutional review board of Seoul National University Hospital, and a

written informed consent was obtained from the patients.

6. Statistical analysis

EP evaluator 11 (Data Innovations, Burlington, VT, USA) was used for processing and interpreting data from method validation studies, and IBM SPSS statistics 23 (IBM Corporation, Armonk, NY, USA) and MedCalc 12 (MedCalc Software, Ostend, Belgium) was used for processing clinical data. In EP evaluator 11, complex precision module compatible for EP5-A2 was used for calculation of imprecision, and LOQ module compatible for EP17-A2 was used for estimation of LOQ. The correlation values between 6MP metabolites in RBC and various clinical parameters were expressed as Spearman's rho, when comparing two consecutive variables. To explore difference of a variable in two groups divided by a certain cutoff, Mann-Whitney test was performed. Multiple regression analysis with enter and stepwise methods was performed to predict myelosuppression and hepatotoxicity by adjusting the effects of other factors related to drug administration. Appropriate myelosuppression and hepatotoxicity were classified under a certain threshold, and the risk was analyzed by logistic regression analysis using enter and forward methods.

RESULTS

1. Validation of method

Results of the experiments evaluating the imprecision and recovery are shown in Table 1. These experiments were performed by adding 6TG and 6MMP to RBC from healthy people, and pretreating them, similar to the RBC from patients, as described in the method section. As the specimens were analyzed in triplicate for seven days, a total of 21 data points were obtained per concentration. However, on one day, one of the triplicate measurements was less than half of the expected concentration; hence, the data from this day were regarded as outlier and excluded from data analysis, finally resulting in 18 data points per concentration. The within-run CV were 4.0-4.9% for 6TG and 5.7-7.0% for 6MMP. The total CV were 7.3-8.1% for 6TG and 5.7-7.0% for 6MMP. The recovery rates in the same experiment as precision analysis ranged from 96.9% to 102.3%. The R^2 values from linear regression during validation period were above 0.99 for both 6TG and 6MMP (Figure 3).

To determine the LOQ, triplicate measurements at four different concentrations on four days were performed, and the imprecision of each concentration was calculated (Figure 4). The mean values of measured concentrations and CV were as

follows: 165 (10.0%), 92 (15.0%), 51 (18.9%), and 34 ng/mL (21.0%) for 6TG, and 3,655 (11.7%), 1,907 (14.2%), 911 (19.2%), and 557 ng/mL (20.1%) for 6MMP. As we aimed for imprecision level of 20%, the LOQ values were set as 54 ng/mL for 6TG and 1,036 ng/mL for 6MMP by EP evaluator. When the ng/mL unit was converted to the pmol/8×10^8 RBC unit using average hematocrit and RBC number, the LOQ values for 6TG and 6MMP were estimated to be 9.2 pmol/8×10^8 RBC and 179 pmol/8×10^8 RBC, respectively. Analysis of clinical samples showed that 3 out of 74 samples showed concentrations below LOQ for 6MMP, and one sample showed concentrations below LOQ for 6TG.

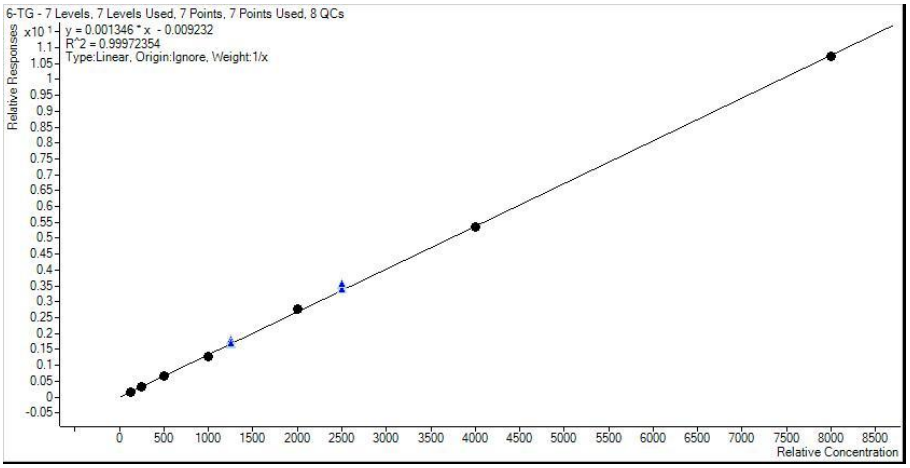
Table 1. Imprecision and recovery rate of 6TG and 6MMP in RBC.

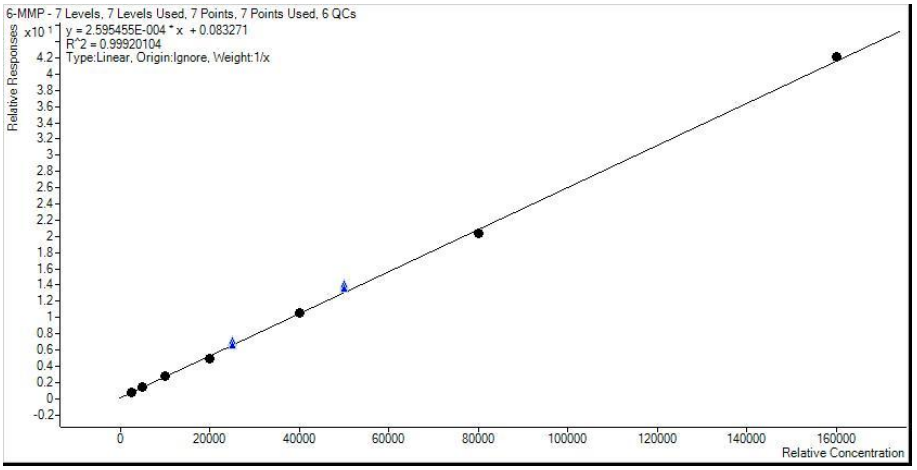
	6TG		6MMP	
Target value (ng/mL)	1,250	2,500	25,000	50,000
Within-run CV% (n=3)	4.9	4.0	5.7	7.0
Between-day CV% (n=6)	5.4	7.1	0	0
Total CV% (n=18)	7.3	8.1	5.7	7.0
Mean values (ng/mL)	1,227	2,423	25,523	51,152
Recovery (n=18)	98.2%	96.9%	102.1%	102.3%

Abbreviations: 6TG, thioguanine; 6MMP, 6-methyl mercaptopurine; CV, coefficient of variation.

The specimens were analyzed in triplicate for six days, and a total of 18 data points were obtained per concentration.

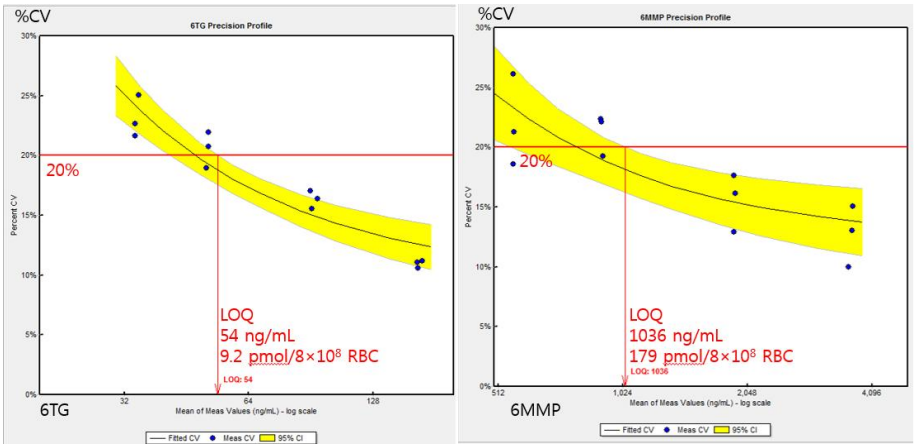
Figure 3. Linearity in response of 6TG and 6MMP in RBC at different concentrations.





Seven calibrators, ranging from 125 ng/mL to 8,000 ng/mL of 6TG and from 2,500 ng/mL to 160,000 ng/mL of 6MMP were used for each analysis run. Linear regression analysis was performed in 1/x weighted manner with ignored origin, and the resulting R^2 value was over 0.99.

Figure 4. Limit of quantification in measurement of 6TG and 6MMP in RBC.



Triplicate measurements were performed at four concentrations on four days by two-fold dilution of the second lowest calibrator. In each analyte, acceptable imprecision level for determining the limit of quantification was 20% of CV.

2. Clinical application

In total, 74 blood samples from 37 patients were used for clinical application. There were 17, 7, and 2 patients whose blood samples were collected 2, 3, and 4 times in the study period, respectively. Most of them were under treatment following Children's Cancer Group protocol, and two patients underwent PBSCT. All samples were collected after four weeks of maintenance therapy. Therefore, the drug concentrations were thought to have reached a steady state when the patients were enrolled in this study (Table 2).

The drug dose, concentration of 6MP metabolites, other clinical indices, and their correlations analyzed in 74 cases are summarized in Table 3. The drug dose was calculated by dividing the amount of drug taken per day or week by the body surface area (BSA) at that time. Concentrations of both 6MP metabolites in RBC were positively correlated with dose of drugs per BSA. The 6MMP concentration was more associated with 6MP than with 6TG concentration, and 6MP dose was more associated with 6MP metabolites than MTX dose. The concentrations of both metabolites were positive correlated each other.

Table 2. Clinical characteristics of enrolled patients.

Participant (n=37)	
Age, median (range), years	6 (2–16)
Male sex, n (%)	29 (78%)
Maintenance treatment protocol, n (%)	
CCG1882	9 (24%)
CCG1952	11 (30%)
0601RER	11 (30%)
0601SER	4 (11%)
Post PBSCT	2 (5%)
Number of measurements of 6MP metabolites in one patient, n (%)	
1 time	11 (30%)
2 times	17 (46%)
3 times	7 (19%)
4 times	2 (5%)
Duration of maintenance therapy, median (range), months	7 (1–26)

Abbreviations: CCG, children's cancer group; RER, rapid early responder; SER, slow early responder; PBSCT, peripheral blood stem cell transplantation; 6MP, 6-mercaptopurine.

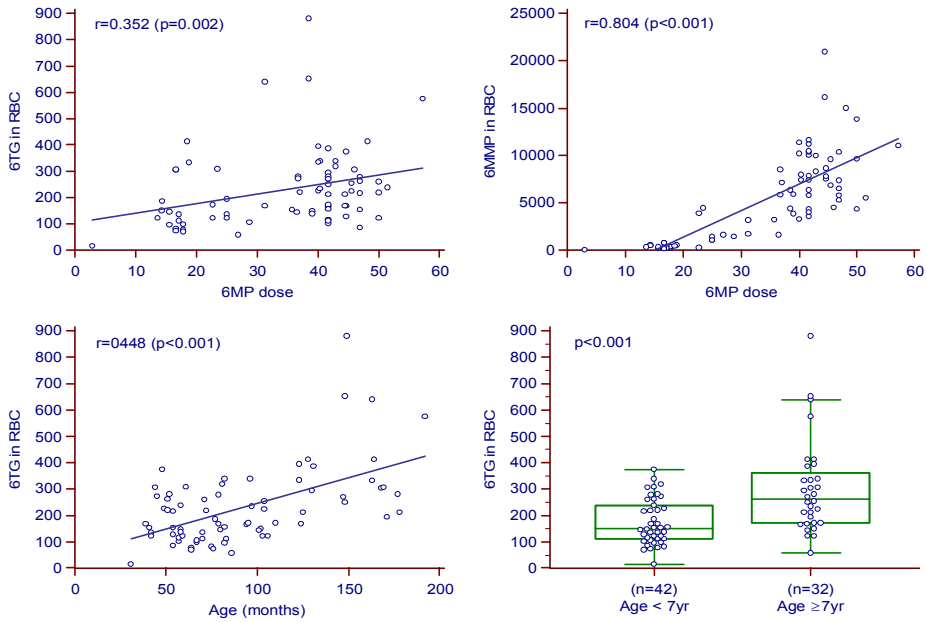
Table 3. 6MP metabolites concentrations in RBC and correlations with clinical indices.

Measurement (n=74)	Median(range)	Correlation with 6TG, Spearman's r (p value)	Correlation with 6MMP, Spearman's r (p value)
Age, months	80 (31–192)	0.448 (<0.001)*	0.128 (0.3)
Daily 6MP per BSA, mg/m²	40.0 (3.0–57.1)	0.352 (0.002)*	0.804 (<0.001)*
Weekly MTX per BSA, mg/m²	14.6 (2.1–21.4)	0.195 (0.097)	0.577 (<0.001)*
6TG, pmol/8×10^8 RBC	203.7 (16.1–880.0)	–	0.495 (<0.001)*
6MMP, pmol/8×10^8 RBC	4,904 (55–20,937)	0.495 (<0.001)*	–
ANC, number/㎕	1,653 (310–5,177)	0.122 (0.3)	0.102 (0.4)
WBC, number/㎕	3,050 (1,300–6,880)	−0.039 (0.7)	−0.045 (0.7)
ALT, IU/L	52 (8–733)	0.381 (0.001)*	0.521 (<0.001)*
AST, IU/L	29 (17–147)	0.263 (0.024)*	0.495 (<0.001)*

Abbreviations: 6MP, mercaptopurine; MTX, methotrexate; BSA, body surface area; 6TG, 6-thioguanine; 6MMP, 6-methyl mercaptopurine; ANC, absolute neutrophil count; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

The drug dose was calculated by dividing the amount of drug taken per day or week by the body surface area at that time. Correlation coefficient r and p values were calculated by Spearman's rank correlation test. *p value <0.05.

Figure 5. Factors related to concentrations of 6MP metabolites



in RBC (n=74).

Units: 6TG and 6MMP in pmol/8×10^{8} RBC unit; dose of daily oral 6MP per BSA and weekly oral MTX per BSA in mg/m^{2}.

The lines in each graph indicate the regression line, and the r and p values were calculated by Spearman's correlation test or the Mann-Whitney test.

In addition, we examined the effects of other clinical factors on concentrations of 6TG and 6MMP. We found that the 6TG concentrations increased significantly with age of the patients. Six cases showing 6TG above 400 pmol/8×10^8 RBC, which is known to be associated with cytopenia, were all from patients older than 10 years (Figure 5, lower). However, 6MP dose and 6MMP concentration were not correlated with age ($p=0.5$ and 0.3, respectively). This age-related increase could be an independent characteristic of 6TG in RBC. Other factors like sex, protocol, and the duration of maintenance therapy did not affect concentrations of 6MP metabolites.

There was no statistically significant correlation between 6MP metabolites and ANC or WBC values (Table 3). In addition, there was no significant correlation between the 6MP metabolites and ANC or WBC values obtained after 14 days and 28 days of 6MP metabolites measurements. Similar results were observed on comparing mean values of 6TG and 6MMP with those of ANC and WBC in patients whose blood was collected multiple times. Instead, duration of maintenance therapy was more correlated with WBC ($r=0.352$, $p=0.002$) and ANC ($r=0.448$, $p<0.001$).

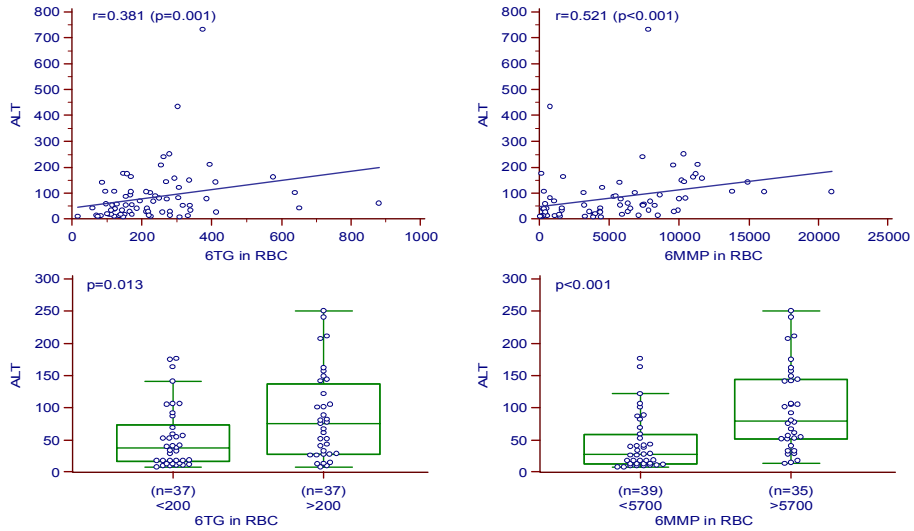
In multiple regression analysis, age, dose of 6MP or MTX, or 6MP metabolites did not have a significant effect on ANC and WBC (Table 4). Previous treatment period was the only

significant variable correlating with WBC (β=49.8, p=0.01) and ANC (β=56.2, p<0.001). When low counts of WBC and ANC were defined as under 2,500/μL and 1,000/μL, 18 cases and 15 cases were classified under low WBC group and low ANC group, respectively. Logistic regression analysis adjusted to other factors revealed that MTX dose and duration of maintenance showed significant odds ratio (OR) of 1.176 (95% confidence interval, 1.022–1.354) and 0.893 (0.812–0.982) for low WBC, and OR of 1.226 (1.032–1.457) and 0.826 (0.708–0.964) for low ANC. Thus, longer duration of maintenance before therapy seemed to be the only factor affecting low neutrophils and leukocytes.

The concentration of 6MMP was positively correlated with ALT (r=0.521, p<0.001), and that of 6TG was also positively correlated with ALT (r=0.381, p=0.001). Additionally, AST also showed relatively weak correlation with two 6MP metabolites (Table 3, Figure 5 upper). When patients were divided into two groups based on threshold value of 5,700 pmol/8×10^8 RBC, patients with 6MMP concentrations above the threshold values showed significantly higher ALT than patients below the threshold (mean difference of ALT 61.4, p<0.001). Similarly, in case of 6TG, patients showing values above threshold had higher ALT values than those below threshold (difference 60.1, p=0.013). These threshold values were based

on the previous studies on correlation between concentration and likelihood of hepatotoxicity (24, 25). ALT and AST were also significantly correlated with 6MP and MTX doses and duration of maintenance. ALT showed a negative correlation with duration of maintenance therapy ($r=-0.269$, $p=0.02$), and ALT and AST showed highest correlation coefficients with doses of MTX ($r=0.628$, $p<0.001$ and $r=0.509$, $p<0.001$).

Figure 6. Correlation between concentrations of 6MP metabolites and liver enzyme activity (n=74).



Units: 6TG, 6MMP in pmol/8×10^8RBC; ALT in IU/L.

The lines in each graph indicate the regression fit, and r and p values were calculated using the Spearman's correlation test or the Mann-Whitney test. Two cases with ALT values over 300 are not shown on this graph for visual distinction between the groups, but were included in the statistical analyses (ALT 434 and 733 IU/L).

In the results of multivariate analysis considering interaction with various variables, correlation of 6MP metabolites with liver enzymes was not clearly observed (Table 5). In the multiple regression analysis using the stepwise method, the MTX dose was the only significant variable for both ALT (β=6.53, p=0.007) and AST (β=1.5, p=0.004). Using threshold values of 120 IU/L for ALT and 40 IU/L for AST, 16 and 20 cases were grouped into increased ALT and AST categories, respectively. Logistic regression analysis using forward method showed that MTX dose (OR 1.363, 95% CI 1.114-1.668) and duration of maintenance therapy (OR 0.91, 0.829-1.0) remained significant risk factors for increase in ALT. Duration of maintenance therapy was a significant risk factor for elevation of AST (OR 0.913, 0.84-0.992), but 6MMP showed marginally increased OR 1.0002 (1.0001-1.0004).

		Multiple regression						Logistic regression (low WBC<2500/μL, low ANC<1000/μL)					
		Enter method			Stepwise method			Enter method			Forward method		
		β	SE	p	β	SE	p	OR	95% CI	p	OR	95% CI	p
WBC	Age	−5.741	4.526	0.209				1.012	0.992–1.032	0.255			
	6MP dose	−14.189	20.558	0.492				1.01	0.92–1.108	0.837			
	MTX dose	−26.92	37.782	0.479				1.209	1.018–1.435	0.03*	1.176	1.022–1.354	0.024*
	6TG	−0.227	1.32	0.864				0.999	0.994–1.004	0.617			
	6MMP	0.049	0.054	0.374				0.9999	0.9997–1.0002	0.622			
	Duration of maintenance	52.178	19.539	0.009*	49.812	18.759	0.01*	0.881	0.794–0.978	0.018*	0.893	0.812–0.982	0.02*
	Overall significance	Adjusted R^2=0.064		0.107	Adjusted R^2=0.077		0.01	R^2=0.284		0.016	R^2=0.258		0.001
ANC	Age	0.623	3.599	0.863				1.003	0.978–1.029	0.818			
	6MP dose	12.142	16.346	0.46				0.987	0.877–1.112	0.833			
	MTX dose	−21.215	30.042	0.483				1.282	1.046–1.572	0.017*	1.226	1.032–1.457	0.021*
	6TG	−0.169	1.05	0.873				0.999	0.993–1.005	0.633			
	6MMP	0.027	0.043	0.53				0.9999	0.9996–1.0003	0.751			
	Duration of maintenance	53.881	15.536	0.001*	56.209	14.804	<0.001*	0.821	0.698–0.967	0.018*	0.826	0.708–0.964	0.015*
	Overall significance	Adjusted R^2=0.130		0.016	Adjusted R^2=0.155		<0.001	R^2=0.386		0.002	R^2=0.368		<0.001

Table 4. Multivariate analysis of factors related to white blood cells counts.

Abbreviations: SE, standard error; OR, odds ratio; CI, confidence interval. *p value <0.05.

Table 5. Multivariate analysis of factors related to liver enzymes.

		Multiple regression						Logistic regression (high ALT>120 IU/L, high AST>40 IU/L)					
		Enter method			Stepwise method			Enter method			Forward method		
		β	SE	p	β	SE	p	OR	95% CI	p	OR	95% CI	p
ALT	Age	−0.034	0.357	0.924				1.01	0.985−1.034	0.444			
	6MP dose	−1.121	1.62	0.491				1.003	0.903−1.113	0.958			
	MTX dose	4.747	2.977	0.116	6.53	2.361	0.007*	1.31	1.037−1.656	0.024*	1.363	1.114−1.668	0.003*
	6TG	0.147	0.104	0.164				1.002	0.996−1.007	0.589			
	6MMP	0.005	0.004	0.236				1.0001	0.9998−1.0003	0.575			
	Duration of maintenance	−2.733	1.54	0.08				0.88	0.788−0.983	0.024*	0.91	0.829−1.000	0.05*
	Overall significance	Adjusted R^2=0.109		0.031	Adjusted R^2=0.083		0.007	R^2=0.425		0.001	R^2=0.362		<0.001
AST	Age	0.065	0.077	0.401				1.006	0.986−1.027	0.543			
	6MP dose	−0.483	0.351	0.173				0.958	0.871−1.053	0.371			
	MTX dose	1.665	0.645	0.012*	1.501	0.503	0.004*	1.111	0.949−1.299	0.19			
	6TG	0.002	0.023	0.935				0.999	0.994−1.004	0.665			
	6MMP	0.001	0.001	0.281				1.0003	1.0001−1.0005	0.028*	1.0002	1.0001−1.0004	0.001*
	Duration of maintenance	−0.894	0.333	0.009*	−0.751	0.322	0.022*	0.903	0.822−0.992	0.033*	0.913	0.84−0.992	0.032*
	Overall significance	Adjusted R^2=0.141		0.012	Adjusted R^2=0.077		0.01	R^2=0.351		0.002	R^2=0.301		<0.001

Abbreviations: SE, standard error; OR, odds ratio; CI, confidence interval. *p value <0.05.

DISCUSSION

In this study, a reliable method for measuring 6TG and 6MMP in RBC was developed and validated using LC-MS/MS, and applied to patient samples. The imprecision of the method ranged from 4.0 to 8.1%, which was satisfactory for clinical use and comparable to previous reports (18, 22, 26). The recovery rate and linearity values showed bias of less than 5% and R^2 values of more than 0.99. In the familiarization and validation period, some QC materials showed reduced concentrations, which was attributed to errors in preparing the samples. The hydrolysis and extraction methods used in this experiment were based on the method described by Dervieux and Boulieu et al. (23) and modified from Shipkova et al. (22). This one-step method using perchloric acid for deproteinization and heating for hydrolysis showed a better recovery rate with less labor-intensive procedure, as compared to the double extraction method described by Lennard and Singleton et al. (27) and modified by Cuffari et al. (18, 28). However, careful pipetting was essential for reducing variation between samples, as the spun down RBC layer may precipitate even after centrifugation, thus resulting in variation in the number of RBC when pipetting.

For LOQ, concentrations of 54 ng/mL and 1,036 ng/mL were chosen for 6TG and 6MMP, respectively, with acceptable imprecision of 20%. Assessment of detection capacity in previous studies mostly used certain signal-to-noise ratios of chromatogram, rather than the evaluation of imprecision as recommended by CLSI EP 17-A2 (29). The LOQ for 6MMP, 179 pmol/8×10^8 RBC, was higher than those reported in previous studies: 110 (18), 150 (30), and 120 pmol/8×10^8 RBC (26). This difference may be due to between-day imprecision and instability of 6MMP in acidic conditions, as reported previously (31, 32). According to the CLSI EP17-A2, LOQ experiments should be designed to reflect variation between different days, the LOQ value from this study may be more realistic than those from previous studies. Decrease in 6MMP concentration was not prominent on using high concentration of 6MMP. Therefore, further investigation of instability of low concentration 6MMP in acidic condition is required. In the clinical samples, concentration below the LOQ was observed once for 6TG, which was considered non-compliant for drugs. Three samples showed below LOQ values for 6MMP. As there may be delay in analysis of clinical settings, effect of several days of storage should be verified.

We collected 77 samples from 38 pediatric ALL patients and measured 6MP metabolites. Among them, 74 samples from 37 patients were investigated to search for the factors related to 6MP metabolites. The concentrations of 6MP metabolites in this study were similar to or slightly lower than those observed in previous studies with pediatric ALL patients (15, 24, 33). One definite outlier was excluded from the data analysis due to non-compliance. When measured using a microelectronic device that checked opening of the pill bottle, the observed mean compliance rate was approximately 90%, but the values were lower in certain groups, including elderly and single mother caregivers (33, 34). Lower compliance rate was closely associated with higher relapse rate (9, 34). Therefore, 6MP metabolites measurements might help to identify non-compliance, and encourage proper medication intake in these patients.

6TG and 6MMP concentrations were positively correlated with the dose of daily 6MP per BSA. The relationship of drug dose and concentrations of metabolites is controversial. In some studies, 6TG concentration and 6MP doses were not related to the drug dose (13, 35, 36), but there were significant correlations in other studies (37, 38). However, the dose of prescribed 6MP could be changed to obtain adequate myelosuppression with tolerable hepatotoxicity, and 6TG accumulated in RBC may reflect 6MP exposure over several

weeks (2, 14). Thus, the relationship between dose and metabolites requires further validation.

Interestingly, the concentration of 6TG was increased in the older patients. This phenomenon seems to be a distinct characteristic of 6TG concentration, as 6MP dose and 6MMP concentration were not increased. Older studies reported no association of survival with age (16, 35, 36), but a recent study regarding pharmacokinetics discovered age-related increase in 6TG, but not in 6MMP concentration, which is consistent with our finding (24). The cause of this relation is not well understood. TPMT activity did not change significantly with age, except in RBC from newborns and cord blood, which expressed 1.6 times higher TPMT activity than those from adults and children (39, 40). This age-related increase in 6TG might be derived from age-related change in 6MP metabolism. Therefore, younger patients may need larger doses of 6MP to achieve the same concentrations of 6TG as older patients. Additional research is required to establish the scientific basis of this phenomenon and to reveal its clinical significance.

In theory, the methylation pathway and the purine salvage pathway are competitive, suggesting that concentration of 6TG should exhibit inverse correlation with 6MMP concentration (20).

However, this study showed a positive correlation between 6TG and 6MMP. Similar results were obtained from a study based on 327 ALL patients, in which both concentrations were low in a significant number of patients, and none of the cases showed high concentration of both 6TG and 6MMP (33). Maybe because of unclearly identified non-compliance, some patients did not take the medicine properly or were not prescribed the proper amount.

We could not find any statistically significant correlation between concentrations of 6MP metabolites and ANC or WBC levels. One possible explanation could be the inter-individual variability of baseline CBC, which was mentioned in the introduction section. Previous studies reported inverse correlation of 6TG with WBC and ANC in childhood ALL patients (26), showing neutropenia two weeks later in childhood ALL patients (38, 41) and mean WBC levels in ALL patients (36). However, most of these small studies were conducted about 30 years ago. They repeatedly measured 6MP metabolites in one patient, and the mean value was used for establishing clinical correlation. More recently, a multicenter study was published suggesting that variation in 6TG was associated with compliance, continuity of prescription, and dose intensity of 6MP, and this was related to the risk of relapse (9). In this study, 6MP metabolites and CBC were measured once or twice in most patients,

so it was difficult to estimate the average value or the degree of variation.

To find other factors affecting myelosuppression, the high numbers of ANC and WBC were associated with longer duration of maintenance therapy. In the multivariate analysis, other drug-related factors did not significantly affect WBC or ANC, whereas duration of maintenance therapy turned out to be a significant factor. This duration-related decreased myelosuppression might be caused by several reasons. One possible explanation was that the compliance rate for the drug decreased, and the patient had not taken the medication over time, which was well described in the study used electro-monitoring system of pill bottle (34). Another possibility was that myelosuppression could be achieved efficiently because of the resistance to antimetabolite drugs in the body. TPMT activity increased in patients receiving chemotherapy and decreased during off-therapy (5). Perhaps metabolism of 6MP, including TPMT activity, increased further with long period of drug use, suggesting that the degree of myelosuppression gradually decreased even with the same dose of drugs. In IBD patients, who were able to determine response to therapy and regulate dose by the response, 6TG did not increase, in spite of an increase in 6MP dose, whereas methylation pathway was activated to produce more 6MMP (42).

Liver enzymes were associated with the 6MP metabolites in the correlation analysis, but the association was not significant in the multivariate analysis that adjusted the effects of age, 6MP, MTX dose, and duration of treatment. Similar to previous reports (25, 42), 6MMP concentrations of more than 5,700 pmol/8×10^8 RBC were associated with ALT elevation, but ALT elevation was also significantly affected by the 6MP dose, MTX dose, and treatment duration. Multivariate analysis showed that MTX dose and duration of treatment were predictive factors for ALT and AST elevation. This duration-related decreased hepatotoxicity might be explained with similar concepts of duration-related decreased myelosuppression, such as non-compliance or resistance to the drugs. MTX doses were found to have a significant effect on ALT and AST as well as on ANC and WBC levels. The time included in the study after maintenance therapy varied from patient to patient, and the interval between measurement times was not constant between patients, which limiting the interpretation of the results.

In conclusion, we developed and validated the method for quantification of 6MP metabolites in RBC using LC-MS/MS, and this method was applied to pediatric ALL patients. This method showed reliable performance in the range of concentrations detected in

patients, except for low concentrations of 6MMP, indicating uncertainty. Concentrations of 6MP metabolites were well correlated with the doses of 6MP and MTX. The concentration of 6TG increased with increasing age, while the doses of 6MP and 6MMP were not associated with age. Elevated activity of ALT and AST were associated with increased 6MP metabolite concentration, but this association was not evident in the multivariate analysis. ANC and WBC were higher and ALT and AST were lower, respectively, as the duration of previous maintenance treatment was longer, which may be due to non-compliance or resistance to drugs over time.

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국문 초록

서론: Mercaptopurine(6MP)은 소아 급성림프구성 백혈병의 유지 요법 시 2-3년간 매일 경구 복용하는 퓨린 유사체이다. 체내에 흡수되어 혈액 세포 내 여러 대사과정을 거친 후, 6-thioguanine (6TG) nucleotide와 6-methylmercaptopurine (6MMP) nucleotide와 같은 활성 물질로 변하여 DNA 복제를 방해하거나 purine 합성을 저해한다. 본 연구에서는 적혈구 내에서 두 대사체를 간단한 전처리 과정을 통해 6TG와 6MMP로 분해시킨 후 액체크로마토그래피-탠덤질량분석기로 그 농도를 측정하는 방법을 개발하였고, 임상 검체에 적용 가능한지 검증하였다. 또 소아 급성림프구성 백혈병의 유지 요법 중인 환자의 혈액에서 위 방법으로 6TG와 6MMP를 측정하여 어떤 임상적 유용성이 있는지 분석하였다.

방법: 원심분리한 적혈구를 과염소산으로 용혈 및 단백질 제거를 거친 후 섭씨 100도에 한 시간 동안 가수분해 과정을 거쳤다. 액체크로마토그래피에서는 C18 컬럼을 정지상으로, 정제수와 acetonitrile을 이동상으로 사용하였고 탠덤질량분석기에서는 6TG와 6MMP를 각각 분자량 대 전하 값 168>150.9와 167>125.1에서 농도를 측정하였다. 각각의 내부기준물질은 방사성 동위원소로 치환한 물질들을 사용하였다. 정밀도를 평가하기 위해 3개의 건강인의 적혈구에 각각

2가지 농도의 약물을 첨가하여 전처리 후 측정하는 실험을 7일간 진행하였고, 정량적 측정 한도를 설정하기 위해 4가지 농도에서 3번 반복 측정하는 실험을 4일간 진행하였다. 6MP를 1달 이상 복용한 소아 급성림프구성 백혈병 환자에서 적혈구 내 6TG와 6MMP를 측정하였고 측정 당시 나이, 성별, 약물용량, 치료기간, 혈구 검사, 간효소 검사와의 상관관계를 살펴보았다. 유지 치료 시 약물 용량을 결정하는 인자인 백혈구 수, 호중구 수, 간효소 검사 수치를 예측할 수 있는지 여부도 분석하였다.

결과: 정밀도 평가 결과 6TG, 6MMP의 변동계수가 5.7−8.1% 정도로 양호한 수준이었고 측정값은 목표한 농도의 5% 내 편차를 보였으며 측정 농도의 선형성 또한 R^2>0.99 를 만족하였다. 정밀도 20%를 만족하는 정량적 측정 한도는 6TG는 54 ng/mL, 6MMP는 1,036 ng/mL로 설정되었다. 37명의 환자에서 유지 요법 시작 1−26개월 후 채혈되었으며 한 환자에서 최대 4번까지 반복 측정하여 총 74검체에서 6MP 대사체를 분석할 수 있었다. 6TG는 16.1−880 pmol/8×10^8RBC 범위에서 측정되었고 6MMP는 55−20,937 pmol/8×10^8RBC 범위에서 측정되었다. 6TG, 6MMP 농도는 체표면적당 투약한 6MP 용량에 따라 증가하였고, 6TG는 나이가 증가하면 농도가 함께 증가하였는데 (r=0.448, p<0.001) 투약 용량과 6MMP는 나이와 관계가 없었다. 6MP 대사체 농도는 과립구와 백혈구 수와 상관관계가 없었으며 다변량 분석에서도 마찬가지였다. 간세포 효소는 6TG, 6MMP 두 가지 모두와 양의 상관관계가 있었는데 그 중 6MMP가 5700 이상인 그룹이 이하인

그룹보다 알라닌전이효소가 61.4 더 높았으나 ($p<0.001$), 약물 용량, 치료기간 등을 고려한 다변량 분석 시 이러한 상관관계는 유의하지 않았다.

결론: 본 연구를 통해 적혈구 내 6TG와 6MMP를 측정하는 방법을 수립하고 임상 검체에 적용하였으며 약물 용량과 좋은 상관관계를 보였으나 현재 쓰이는 임상 지표와의 연관성에 대해서는 좀 더 밝혀져야 할 것으로 보인다.

주요단어: Mercaptopurine, 6-thioguanine, 6-methylmercaptopurine, **급성림프구성 백혈병**

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