Development and Method Validation of 6-Thioguanine and 6-Methylmercaptopurine in Erythrocytes Using LC-MS/MS

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ABSTRACT

Aim: Previous studies had shown that the safety and efficacy of 6-mercaptopurine in acute lymphoblastic leukemia (ALL) patients was mainly determined by the concentrations of its metabolites in erythrocytes, which are 6-thioguanine (6-TGN) and 6-methylmercaptopurine (6-MeMP). The aim of this research is to develop and validate a method to quantify concentrations of 6-thioguanine and 6-methylmercaptopurine in erythrocytes and apply the validated method in ALL patients undergoing 6-mercaptopurine treatment.
Methods: We develop a method to determine 6-TGN and 6-MeMP on LC-MS/MS system, using a 1.8 µm column (2.1 x 50 mm). Mobile phase used for analysis were formic acid in acetonitrile and formic acid in H_2O (60:40). Extraction from erythrocytes was done using NH_4OH in acetonitrile. Bisoprolol was used as internal standard. The method validation was performed according to the European Medicines Agency (EMEA) guideline.
Results: Detection for 6-TGN, 6 MeMP and bisoprolol were done using ion transitions, m/z 168.08>151.02, 167.1>152.06 and 326.12>116.03, respectively The calibration curves were linear over the concentration range of 9.9 – 1979 ng/mL for 6-TGN and 10 – 2000 ng/mL for 6-MeMP. The other validation criteria, which consists of selectivity, accuracy, precision, lower limit of quantification, carry over, dilution integrity, matrix effect and stability, were achieved. Concentrations of 6-TGN and 6-MeMP in ALL patients underwent 6-MP treatment were found in the range of 21.05 – 109.56 pmol/8x10^8 erythrocytes and 13.06 – 80.27 pmol/8x10^8 erythrocytes, respectively.
Conclusion: The present LC-MS/MS method is valid for the quantification of 6-thioguanine and 6-methylmercaptopurine in erythrocytes and can be applied in ALL children treated with 6-MP.

Keywords: 6-mercaptopurine, 6-thioguanine, 6-methylmercaptopurine, LC-MS/MS, Acute Lymphoblastic Leukemia

INTRODUCTION

Mercaptopurine (6-MP) is the main drug used for the treatment of acute lymphoblastic leukemia (ALL) in the maintenance phase [1-3]. Mercaptopurine is a prodrug that needs to be activated to form its active metabolite, 6-thioguanine nucleotide (6-TGN). Following oral administration, mercaptopurine undergoes first pass metabolism by xanthine oxidase (XO)

enzyme and converted 6-MP to inactive 6-thiouric acid, that occurs right after absorption, followed by formation of 6-thioguanine nucleotide by (6-TGN) using hypoxanthine guanine phosphoribosyl transferase (HGPRT). 6-TGN is an active metabolite that bound into DNA in nucleated cells and then lead to DNA strand breaks and apoptosis. Afterwards, fractions of 6-MP will be catalyzed by thiopurine methyl transferase (TPMT) enzyme to form inactive metabolite 6-methyl mercaptopurine (6-MeMP), thus reducing the formation of 6-TGN [4-7].

Previous study had shown that a 6-TGN levels in erythrocytes corresponds to the efficacy and hematologic toxicity, while concentrations of 6-MeMP correlates with hepatotoxicity of mercaptopurine [8-10]. In acute lymphoblastic leukemia patients who received standard dose of 6-MP, wide inter-individual variability was observed, mainly in the erythrocyte concentrations of 6-TGN and 6-MeMP [1,11,12]. Thus, measurement of 6-MP metabolites in erythrocytes is important in order to monitor the safety and efficacy of mercaptopurine. In relation to drug monitoring in patients, a sensitive and valid method to quantify 6-thioguanine and 6-methylmercaptopurine in acute lymphoblastic leukemia patients undergoing mercaptopurine treatment is needed. The aim of the present study was to develop and validate a method to quantify 6-TGN and 6-MeMP in erythrocytes using LC-MS/MS.

MATERIALS AND METHODS

Reagents and Chemicals

HPLC grade acetonitrile, formic acid and NH_4OH (Merck), and ultrapure water from a Milli-Q instrument (Millipore, Watford, UK). 6-Thioguanine (6-TGN) and 6-methylmercaptopurine (6-meMP) were purchased from Sigma Aldrich (St. Louis, MO, USA), while bisoprolol was obtained from Rusan Pharma.

Instrument and Chromatographic Conditions

We used Liquid Chromatography-Mass Spectrometry/Mass Spectometry (LC-MS/MS)-Xevo Waters, Acquity. Mass spectrometry detection was performed on Waters, TQD Xevo MS/MS. The chromatography performed on Waters Acquity HSS T3 1.8 µm column (2.1 x 50 mm). Mass Lynx V4.1 software was used for data analysis. The flow rate was 0.3 ml/min using an isocratic elution, with mobile phase: 0.1% formic acid in acetonitrile and 0.1% formic acid in H_2O (60:40). Column temperature was 40^0C and auto-sampler temperature was set at 20^0C.

Preparation of standard solution

Five milligrams of 6-thioguanine and 17.95 mg of 6-methylmercaptopurine riboside (equivalent to 10 mg of 6-methylmercaptopurine) were weighed, put in separate volumetric flask, then were dissolved with 0.5% NH_4OH in aquadest into volume and mix homogeneously. The concentration of each standard stock solution was 1000 µg/mL and stored at -20^0C until required. For calibration curves, concentration of each solution were ranged from 10-1000 ng/mL. Bisoprolol was used as internal standard. A measure of 5.89 mg bisoprolol fumarate (~5.00 mg bisoprolol) was put into 10 mL volumetric flask, then was dissolved with acetonitrile to reach concentration 500 µg/mL and stored at -20^0C. All working solutions were freshly prepared by diluting stocks solutions in aquadest.

Sample preparation

Whole blood in EDTA-tube was centrifuged at 3000 x g for 10 minutes at 4^0C to separate plasma from erythrocytes. The plasma and buffy coat was discarded. The remaining erythrocytes were washed once with 2 ml NaCl 0.9% and packed by centrifugation for 10 minutes at 3000 x g. The isolated erythrocyte were stored at -20^0C until required for further processing. Erythrocyte count was determined before storage, and used to normalize metabolite concentrations to pmol/$8x10^8$ erythrocytes.

A 0.4 mL volume of erythrocytes containing 6-TGN and 6-MeMP were mixed with 40 µL of bisoprolol 800 ng/mL (internal standard), 600µL of 0.2% NH_4OH in acetonitrile, were mixed for 30 seconds using vortex mixer,then centrifuged at 14000 rpm for 5 minutes. The organic layer then was transferred into another micro-tube, and was evaporated under stream of nitrogen gas in water-bath at 55^0C. Then, the dried sample was reconstituted with 300 µL 0.3% NH_4OH in acetonitrile 50% by vortex mixing for 30 seconds. The solution was transferred into another micro-tube, then centrifuged at 14000 rpm for 5 minutes. Afterwards, a volume of 2 µL sample aliquot was injected into the LC-MS/MS system.

Method Validation

The method validation was performed according to the European Medicines Agency (EMEA) guideline on bioanalytical method validation. Parameters of validation include selectivity, carry-over, lower limit of quantification, calibration curve, accuracy, precision, dilution integrity, matrix effect, and stability [13].

Application to Children with Acute Lymphoblastic Leukemia

This study was part of a clinical study that was approved by the Ethics Committee of Medical Faculty, Universitas Indonesia number: 269/UN2.F1/ETIK/2017. Ten acute lymphoblastic leukemia children undergoing 6-mercaptopurine therapy in maintenance phase were included in this study.

RESULTS AND DISCUSSION

Detection of 6-thioguanine and 6-methylmercaptopurine

Mass spectrometry detection for 6-TGN, 6-MeMP and bisoprolol were done using ion transitions, m/z 168.08>151.02, 167.1>152.06 and 326.12>116.03, respectively (Figure 1).

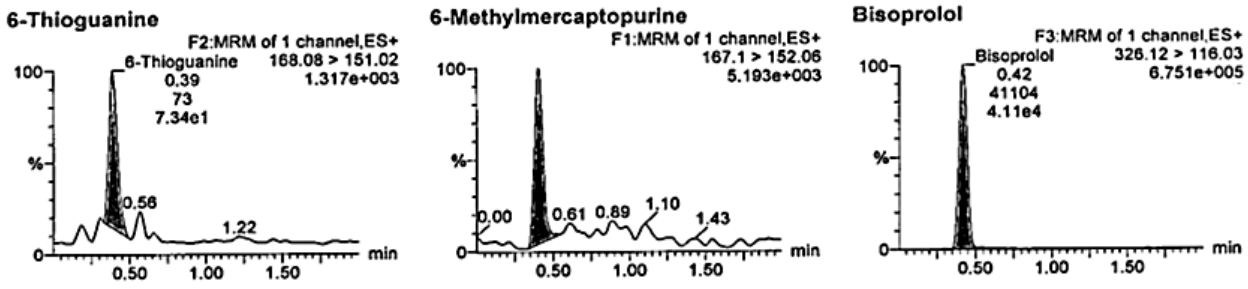

Figure 1: Chromatogram of 6-thioguanine, 6-methylmercaptopurine, and bisoprolol (internal standard)

Lower Limit of Quantification (LLOQ)

The lower limit of quantification was 9.9 ng/mL for 6-TGN and 10.0 ng/mL for 6-MeMP with a coefficient of variation less than 20%. The limits corresponds to the quantification limits of 12 pmol/8 x 10^8 erythrocytes for 6-TGN and 7 pmol/8 x 10^8 erythrocytes for 6-MeMP (assuming 4 x 10^6 erythrocytes per µl of packed erythrocytes)

Selectivity

Selectivity was evaluated using six different individual red blood cells compared by LLOQ sample for each different individual red blood cells. The peak appeared at the same retention time with the analyte was less than 20% of LLOQ area and with the internal standard was less than 15%. Interference of 6-TGN, 6-MeMP, and bisoprolol in blank red blood cells were ranged from 1.67 – 14.29%, 0.29 – 14.19%, and 0.01 – 0.73%, respectively.

Carry Over

Carry-over test was conducted by injecting blank sample after the highest concentration of calibration curve. Carry over value was 4.26% of LLOQ for 6-TGN and 0.02% for internal standard. No carry over was observed for 6-MeMP. The carry over result met the acceptance criteria for analyte <20% and for the internal standard <5%.

Calibration Curve

A blank sample, a zero sample, and samples with 7 concentration levels were prepared. A calibration curve was done for 5 consecutive days. The calibration curves were linear over the concentration range of 9.9 – 1979 ng/mL for 6-TGN and 10 – 2000 ng/mL for 6-MeMP, with coefficient correlation >0.99. From five replicate calibration curves on different days, the coefficient correlation each metabolite was >0.99 (Fig. 2 and Fig. 3).

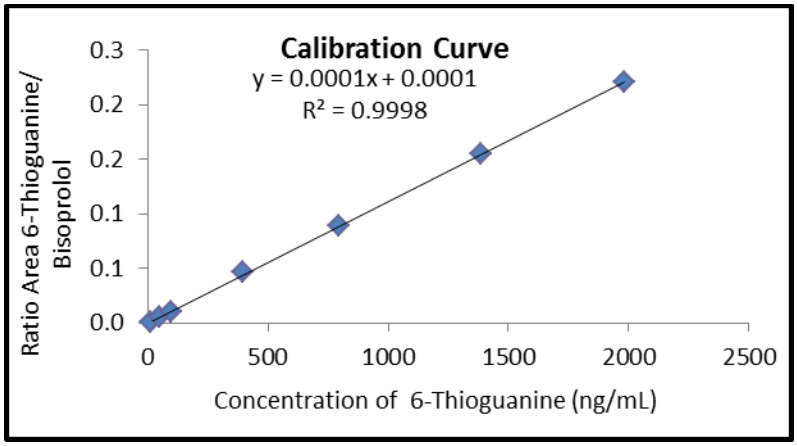

Figure 2: Calibration curve of 6-thioguanine

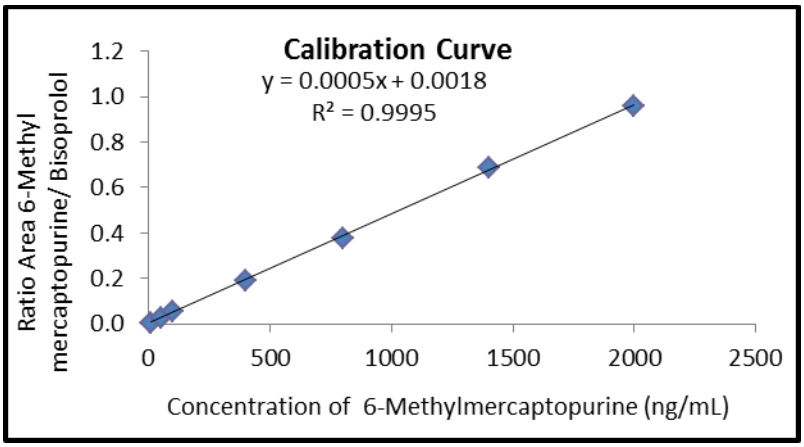

Figure 3: Calibration curve of 6-methylmercaptopurine

Accuracy and Precision

Within-run accuracy and precision were conducted for 4 level concentrations (LLOQ, low QC, medium QC, and high QC) for 5 replicates in a single run. Between-run accuracy and precision were conducted for 4 level concentrations for 5 replicates in 2 runs, on 3 different days. Within-run accuracy and precision for 6-TGN were -7.72 – 9.55% difference and 3.05 – 14.79% coefficient of variation (CV), respectively; For 6MeMP were -8.40 – 6.29% difference and 3.82 – 7.45% CV. Between-run accuracy and precision for 6-TGN were -10.25 – -3.28% diff and 3.39 – 9.82% CV, respectively; For 6-MeMP were -11.15 – 5.59% diff and 2.41 – 5.70% CV. The CV of within-run and between-run accuracy and precision of both 6-TGN and 6-MeMP were less than 20%, indicated the adequate reliability and reproducibility of the method within the analytical range (Table 1).

Table 1: Accuracy and Precision of 6-TGN and 6-MeMP

Analyte	6-TGN				6-MeMP			
	Actual conc	Mean conc	% Diff	% CV	Actual conc	Mean conc	% Diff	% CV
LLOQ	9.91	10.07	1.57	14.79	10.00	10.63	6.29	5.77
QCL	29.74	32.57	9.55	5.87	30.01	28.93	-3.60	7.45
QCM	991.19	945.10	-4.65	7.12	1000.22	973.44	-2.68	3.82
QCH	1486.78	1372.02	-7.72	3.05	1500.33	1374.38	-8.40	3.91

LLOQ = lower limit of quantification; QCL = quality control low concentration; QCM = quality control medium concentration; QCH = quality control high concentration

Dilution Integrity

Dilution integrity was conducted for concentration above of upper limit of quantification (ULOQ) concentration at 5 replicates by diluted concentration above ULOQ ½ and ¼ times from initial concentration with red blood cells. Measurement of dilution integrity for 6-TGN showed deviations of calculated concentration to nominal value were -7.62% difference (2.13% CV) and -9.68% difference (0.73% CV) at quarter and a half ULOQ, respectively. Dilution integrity for 6-MeMP, deviations of calculated concentration to nominal value were 2.19% difference (6.65% CV) and 0.14% difference (8.93% CV) at quarter and a half ULOQ, respectively. Mean

concentration difference was within 15% of the nominal value and CV value did not exceed 15% for the test samples.

Matrix Effects

Six lots of blank matrix from individual red blood cells were used to investigate matrix effect. The coefficient variation (CV) of internal standard (IS) normalized matrix factor (MF) for 6-TGN was 11.25% – 13.44% and for 6-MeMP was 3.79% – 7.74%. The matrix effect of both metabolites were within acceptable limit.

Stability

Working solution stability testing was performed by storing 6-TGN, 6-MeMP, and bisoprolol working solution at room temperature for 6 hours and at refrigerator (2-8^{0}C) for 0, 1, 2, 6, and 7 days. All working solution were stable at room temperature for 6 hours and at storage condition for 7 days (Table 2). Seven days of stability measurement was considered acceptable as for the application of therapeutic drug monitoring in children with ALL children treated with 6-MP, the results need to be completed immediately.

Table 2: Stability of Working Solution of 6-TGN, 6-MeMP, and Internal Standard

Analyte	Time	Temperature	Actual concentration	% Difference
6-TGN	6 h	Room	QCL	-6.5
			QCH	6.6
	1-7 days	Refrigerator	QCL	-12.0 – 0.34
			QCH	-7.34 – 3.63
6-MeMP	6 h	Room	QCL	-5.34
			QCH	2.73
	1-7 days	Refrigerator	QCL	-12.77 – 12.94
			QCH	-9.85 – 2.07
Bisoprolol	6 h	Room	795.36 ng/mL	4.69
	0-7 days	Refrigerator	795.36 ng/mL	-13 – 2.5

QCL = quality control low concentration (29.74 ng/ml for 6-TGN and 30.01 ng/ml for 6-MeMP); QCH = quality control high concentration (1486.78 ng/ml for 6-TGN and 1500.33 ng/ml for 6-MeMP); h = hour

Short term stability testing was conducted by storing QC low and high concentration in red blood cells at room temperature for 0, 6, 13, 18 hours. 6-TGN and 6-MeMP in red blood cells were stable at room temperature for 13 hours. After 18 hours at room temperature, deviation of mean concentration of both metabolites were greater than 15% (Table 3).

Table 3: Stability of 6-TGN and 6-MeMP in Red Blood Cells

Stability	Time	Temperature	Actual conc	% Diff	
				6-TGN	6-MeMP
Short term	0,6,13 h	Room	QCL	-0.4 – 8.24	-8.17 – 11.97
			QCH	-8.97 – 7.30	-8.85 – -4.81
	18 h	Room	QCL	0.4	-20.74
			QCH	-15.66	-26.41

Long-term	112 days	-20^{0}C	QCL	-4.71	1.6
			QCH	-0.72	2.83

QCL = quality control low concentration (29.74 ng/ml for 6-TGN and 30.01 ng/ml for 6-MeMP); QCH = quality control high concentration (1486.78 ng/ml for 6-TGN and 1500.33 ng/ml for 6-MeMP); h = hour

Long term stability was performed by storing QC low and high concentration of 6-TGN and 6-MeMP in red blood cells at -20^{0}C for 112 days and then thaw to 28^{0}C. The result showed that both metabolites were stable for 112 days (Table 3).

Application to Patient Samples

The method developed has been applicated for determination of 6-TGN and 6-MeMP in 10 children (2 – <18 years old) with Acute lymphoblastic leukemia who underwent 6-mercaptopurine treatment in maintenance phase therapy. All patients received 50 mg/m^2 body surface once a day according to the Indonesian ALL treatment protocol [14], for at least 1 month. Concentrations of 6-TGN and 6-MeMP measured in ALL children were presented in Table 4.

Table 4: Concentration of 6-TGN and 6-MeMP in ALL Children

Patient	6-TGN concentration (pmol/8x10^8 erythrocytes)	6-MeMP concentration (pmol/8x10^8 erythrocytes)
1.	42.24	41.22
2.	109.56	50.16
3.	89.36	20.66
4.	41.12	16.31
5.	21.05	28.58
6.	30.00	47.36
7.	69.54	80.27
8.	24.15	13.84
9.	44.77	13.06
10.	34.17	41.73

We chose LC-MS/MS method to improve the detection limit to quantify 6-TGN and 6-MeMP in erythrocytes. We investigated several combinations of mobile phase and flow rate, and found that combination mobile phase: 0.1% formic acid in acetonitrile and 0.1% formic acid in H_2O (60:40) with flow rate 0.3 ml/min using an isocratic elution, resulting separately analytes with good peak and short retention time (Figure 1). The selectivity, carry-over, calibration curve, accuracy, precision, dilution integrity, and matrix effect meet the acceptance criteria according to EMA guidelines. Both 6-MP metabolites, 6-TGN and 6-MeMP, in red blood cells were stable at room temperature for 13 hours and stable at -20^{0}C for 112 days.

The advantages of our method are a shorter run time (2 minutes) compared with study of Dervieux et al [9], and a lower LLOQ of 10.0 ng/mL for 6-MeMP compared with Kim et al [15]. Our LLOQ was sufficient enough to detect the sub therapeutic concentrations of 6-TGN and possible toxic concentrations of 6-TGN and 6-MeMP [9].

The efficacy and side effects of 6-MP is known influenced by inter-individual variability of erythrocytes concentration of 6-TGN and 6-MeMP. Several alternative treatment strategies have been done in improving the 6TGN/MeMP ratio [6]. In this study, ten samples of ALL children who underwent 6-MP 50 mg/m^2 body surface area daily in maintenance phase therapy were included in our analysis. We successfully detected 6-TGN and 6-MeMP in erythrocytes of patients undergoing mercaptopurine therapy. Mardini et al, 2005, founded that RBC 6-TGN level of > 450 pmol/8 x 10^8RBC was associated with myelotoxicity [16]. Beaumais et al, 2011, founded that a 6-MeMP threshold of 5000 pmol/8 x 10^8 RBC was associated with an increased risk of hepatotoxicity [8]. In all ten patients, no toxic concentrations of 6-TGN and 6-MeMP were found.

CONCLUSION

The present LC-MS/MS method is valid for measuring erythrocytes 6-Thioguanine and 6-methylmercaptopurine simultaneously according to the EMA guideline and can be applied in acute lymphoblastic leukemia children undergoing mercaptopurine treatment.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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