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Original Article

Simultaneous Analytical Method Development of 6-Mercaptopurine and 6-Methylmercaptopurine in Plasma by High Performance Liquid Chromatography-Photodiode Array

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ABSTRACT

Objective: 6-Mercaptopurin is antineoplastic drug that is included in antimetabolite group and is used in acute lymphoblastic leukemia medication. 6-Mercaptopurin is inactive pro-drug that will be metabolized into metabolites. One of its metabolites is 6-methylmercaptopurine. This study is aimed to optimize the analytical conditions and perform validation for the analysis of 6-mercaptopurine and 6-methylmercaptopurine in plasma. **Method:** Separation was performed using Waters 2996 HPLC, C18 Sunfire™ column (5µm, 250 x 4.6 mm) with the mobile phase containing water-methanol-acetonitrile with gradient elution, and detected at 303 nm. 5-Fluorouracil was used as internal standard. Plasma fraction was done by liquid-liquid extraction using dichloromethane. **Result:** The method was linear at concentration range of 2.0 – 200.0 ng/mL with r > 0.9991 for 6-mercaptopurine and 20 – 2000 ng/mL with r > 0.9993 for 6-methylmercaptopurine. Accuracy and precision within-run and between-run fulfill the acceptance criteria with % RE and relative standard deviation (% RSD) ≤ 20 % (LLOQ) and ≤ 15% (QC samples). 6-Mercaptopurine and 6-methylmercaptopurine was stable in plasma at least for 21 days when stored at -20°C. **Conclusion:** The bio-analytical method was sensitive, selective and all the parameters fulfilled the acceptance criteria of the EMA Bio-analytical Method Validation Guideline, 2011.

Keywords: 5-fluorouracil, 6-mercaptopurine, 6-methylmercaptopurine, HPLC, validation

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INTRODUCTION

6-Mercaptopurine is an antineoplastic drugs which belongs to antimetabolites drug and is widely used in childhood acute lymphoblastic leukemia medication.[1] It is inactive prodrug that will go through three metabolic pathways. First, 6-mercaptopurine will be transformed by hypoxanthine phosphoribosyl transferase (HPRT) into thioinosine monophosphate (TIMP) and subsequently into 6-thioxanthosine-5-monophosphate (TXMP) and 6-thioguanosine nucleotides (TGN mono-, di- and triphosphate). TGN is active metabolite that will incorporate with DNA and induce the breaking of DNA chains. TIMP inhibit ribosyl-5-phosphate formation, and conversion of inosine-5-monophosphate (IMP) into adenine and guanine nucleotides. Second, 6-mercaptopurine will be hydroxylated by xanthine oxidase (XO) into inactive metabolite, 6-thiouric acid. Third, 6-mercaptopurine will be methylated by thiopurine methyltransferase (TPMT) into 6-methylmercaptopurine (6-MMP). TPMT can also methylate TIMP into methylated thioinosine monophosphate (MTIMP). TIMP and MTIMP will inhibit glutamine-5-phosphoribosyl pyrophosphate amidotransferase, the first enzyme at de novo pathway to synthesize purine ribonucleotides.[2]

6-Mercaptopurine dosing is based on body surface area or patient weight so individual therapeutic windows should be noticed. Moreover, there are wide inter-individual differences in 6-mercaptopurine metabolism among patients receiving identical doses of 6-mercaptopurine.[3] Due to narrow therapeutic index of 6-mercaptopurine, small amount of dosage raise can cause toxicity.[4] Based on these reasons, monitoring drug concentration in plasma is necessary to assess the safety and efficacy. In the present study, the determination of 6-MP and its metabolites in biological fluids by reverse phase HPLC have been developed in order to characterize 6-MP pharmacokinetics and pharmacogenetics. Some methods of analysis of 6-mercaptopurine and 6-methylmercaptopurine in plasma have been performed using high performance liquid chromatography with a diode array detector (HPLC-DAD), which produces a detection limit of 6-mercaptopurine and 6-methylmercaptopurine of 20 nM.[5] Analysis of 6-mercaptopurine and its metabolites in erythrocytes and plasma using HPLC with ultraviolet detector (UV) has been developed by Hawwa *et al*. in 2008. In the study LLOQ 6-mercaptopurine was obtained at 3 pmol / 8×10^8 in erythrocytes and 2 ng / mL in plasma. While LLOQ 6-methylmercaptopurine is 3 pmol / 8×10^8 in erythrocytes and 20 ng / mL in plasma. However, these methods have difficult and complex extraction with derivatization procedure. Most of these methods are still using protein precipitation method for the sample preparation that causes less purity of extract. Such methods will be adapted to modify the method for analysis of 6-mercaptopurine and 6-methylmercaptopurine in plasma using HPLC-PDA. This research developed the simple extraction, quick, and accurate analytical methods of 6-mercaptopurine and 6-methylmercaptopurine with 5-fluorouracil as internal standard simultaneously using HPLC-PDA. The sample preparation used liquid-liquid extraction. In this current research, *in vitro* validation will be performed referring to the European Medicine Agency (EMEA) 2011 in term of lower limit of quantification (LLOQ), the linearity of the calibration curve, selectivity, carry-over, accuracy, precision, recovery, dilution integrity, and stability.

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MATERIAL AND METHOD

Chemicals and Reagents

6-Mercaptopurine (6-MP), 6-mercaptopurine (6-MMP), 5-fluorouracil (5-FU) were obtained from Sigma Aldrich (St. Louis, MO, USA), HPLC grade acetonitrile from Merck, HPLC grade methanol from Merck, ammonium acetate, NH_4OH, dichloromethane. All water was HPLC grade and prepared using a Millipore Direct-QTM 5Water System (Millipore, Watford, UK) and filtrated using Sartorius membrane filters [0.45_m] obtained from Sartorius (Epsom, UK). Plasma was obtained from Indonesian Red Cross and stored at -20°C until required.

Instruments

High performance liquid chromatography consists of separation module (Alliance 2996; Waters), column heater (Alliance series; Waters), and photodiode array detector (Waters 2996). The data were acquired and analyzed using Empower ™ software.

HPLC Conditions

Separation of 6-MP and 6-MMP achieved using reverse phase analytical column (C-18 Sun fire™, 5 µm; 250 x 4.6 mm). There are two mobile phase. Mobile phase A consists of water-methanol (90:10) and mobile phase B consists of water-acetonitrile (75:25). Samples separation was performed with gradient elution. The samples were eluted with mobile phase A at the first 6 min and changed to mobile phase B in 7 – 13.50 min. Elution was continued with mobile phase A to the end of the run at 16 min. The gradient elution condition profile is shown in Table 1.

Flow rate was maintained at 1 mL/min with column temperature of 30°C. The photodiode array detector was set at wavelength of 303 nm.

Preparation of Stock Solutions, Calibrators, and Quality Control Solutions

The stock solutions of 6-MP and 6-MMP were prepared by dissolving appropriate amount of each compound in 0.1 N NH_4OH and then diluting it with water-methanol (50:50). Stock solution of 5-FU was prepared by dissolving appropriate amount of 5-FU in water. Stock solutions were stored at −20°C until required. Calibrators and quality control solutions were prepared by spiking separate stock solutions to human plasma. The ranges of the calibration curves were 2 ng/mL, 5 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, 150 ng/mL, 200 ng/mL for 6-MP and 20 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL, 1 µg/mL, 2µg/mL for 6-MMP.

Plasma preparation

A 500 µL plasma that contained 6-MP 10 µg/mL and 6-MMP 30 µg/mL were mixed with 50 µl 5-FU 30 µg/mL by vortex mixing for 1 min in a centrifuge tube. The solutions were added with 5 mL dichloromethane and then were vortexed for 2 mins. The tube was then centrifuged for 20 min at 3000 rpm. The organic phase as much as 3 mL was evaporated with N_2 for 20 min at 60°C. The residue was then reconstituted with 200 µL mobile phase (air-methanol-acetonitrile, 90:7:3, v/v, and 30 µL aliquot was injected onto the HPLC system.

Method Validation

Lower Limit of Quantification (LLOQ)

LLOQ was validated using five replicates of plasma which were spiked with 6-MP and 6-MMP at LLOQ concentration (5% Cmax). % RE and % RSD should be ≤ ± 20%.

Calibration curves

A blank sample, a zero sample and sample at 8 calibration concentration levels were prepared. The slope, intercept and correlation coefficient of each calibration curve were determined. % RE should be ≤ ± 15% for each calibration concentration and ≤ ± 20% for LLOQ.

Selectivity

Selectivity is the ability of analytical method to differentiate the compounds of interest with the endogen components in the matrix or other compounds in the sample using at least 6 individual sources of matrix. The analysis was performed using plasma spiked with the compounds of interest at lower concentration (LLOQ). % RE and % RSD should be ≤ ±20% for LLOQ and 5% for internal standard.

Carry Over

Carry-over should be minimalized during method development. Carry over was performed by injecting blank sample after upper limit of quantification (ULOQ) concentration sample. A carry over ≤ ±20% for LLOQ and ≤ ±5% for internal standard are acceptable.

Accuracy, Precision and Recovery

Intra and inter-day accuracy and precision for the developed method were determined by analyzing plasma replicates (n= 5) at four different concentration (LLOQ, low-, medium-, and high-quality control). Inter-day analysis was performed on three different days and at five replicates of each concentration level whereas intra-day analysis was done on the same day. The LLOQ, low-, medium-, and high-quality control concentrations of 6-MP were 2 ng/mL, 6 ng/mL, 100 ng/mL and 150 ng/mL, while for 6-MMP were 20 ng/mL, 60 ng/mL, 1000 ng/mL and 1500 ng/mL.

Accuracy was calculated as the percent error of the measured concentrations with the expected concentrations or expressed as % RE. Precision was expressed as % RSD (relative standard deviation). % RE and % RSD should be ≤ ± 15% for the QC samples and should be ≤ 20% for LLOQ. There are two kinds of recovery; absolute and relative. Absolute recovery was determined by comparing peak areas of extracted plasma samples with peak areas of aqueous solutions containing the same concentration of interest compounds which were injected directly into the column without extraction. Relative recovery was determined by comparing measured concentration with the actual concentration.

Stability

Evaluation of stability was determined by analyzing plasma samples containing 6-MP and 6-MMP at low-, and high-quality control concentrations. The stability evaluation consisted of the stock solutions, working solutions of the analyte and internal standard stability, freeze and thaw stability, short term stability, long term stability and auto sampler stability.

RESULT AND DISCUSSION

Determination of the Lower Limit of Quantification (LLOQ)

Based on the research, LLOQ of 6-mercaptopurine was 2 ng/mL and had a value of %diff (-4.62%)- (-3.87%) and % RSD of 3.89%. While LLOQ of 6-methylmercaptopurine was 20 ng/mL and % diff was 4.24% to 14.11% and % RSD of 3.67%. The value of % diff and % RSD still met the requirements so this concentration is elected as LLOQ. The chromatogram is shown in Figure 1.

Calibration Curve

A calibration curve was made from eight-level concentrations of 6-mercaptopurine and 6- methylmercaptopurine in the plasma. The concentrations of 6-merkaptopurin made were 2, 5, 10, 25, 50, 100, 150, and 200 ng/mL. While concentrations of 6-methylmercaptopurine made were 20, 50, 100, 250, 500, 1000, and 1500 ng/mL. Calibration curve of 6-mercaptopurine had the correlation coefficient of 0.9991 with the line equation y = 0.0004 + 0,0025x. While 6-mercaptopurine had a coefficient correlation of 0.9993 with the line equation y = -0.0014 + 0,0006x. Correlation value and % diff obtained from the calibration curve met the requirement, that no more than ± 15% for concentration besides LLOQ and no more than ± 20% for LLOQ concentration.

Selectivity

This selectivity test was performed at a concentration of 2 ng/mL for 6-mercaptopurine and 20 ng/mL for 6-methylmercaptopurine using six different human plasma blank to see the effect of plasma impurities from different sources. In this research % RE values were obtained between 9.27% to 14.93%, relative standard deviation (%RSD) obtained was 1.89%. Values of %RE and % RSD met the requirements i.e. no more than ± 20%, and there was no interference at the retention time of analyte, metabolite, and internal standard. Based on the result the method can be considered to be selective.

Carry Over

Carry-over of 6-mercaptopurine after injection of ULOQ to the LLOQ concentration was 10.49%, 6-methylmercaptopurine was 5.87%, and internal standard was 0.7%.

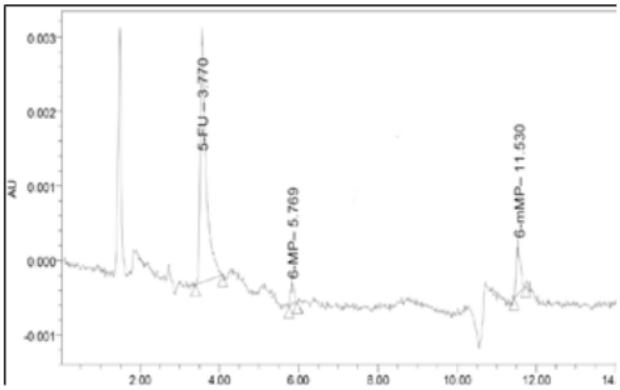

Figure 1: 6-Mercaptopurine and 6-methylmercaptopurine in plasma at LLOQ concentration with 5-fluorouracil as internal standard

Table 1: Gradient elution condition profile

Time (minutes)	Mobile phase
0	A
1	A
7	B
13.50	B
14	A
15	A

Table 2: Accuracy and Precision of 6-Mercaptopurine Resulted from Method Analysis

Conc.	Day 1			Day 2			Day 3		
	Measured Conc. (ng/mL)	% RSD	% RE	Measured Conc. (ng/mL)	% RSD	% RE	Measured Conc. (ng/mL)	% RSD	% RE
2.00	1.93	2.53	-3.71	1.83	2.28	-8.51	2.22	2.37	11.05
	1.95		-2.48	1.90		-5.00	2.19		9.41
	1.83		-8.66	1.94		-2.78	2.17		8.65
	1.93		-3.40	1.91		-4.39	2.20		9.95
	1.92		-4.18	1.87		-6.32	2.09		4.34
6.00	5.67	1.69	-5.43	6.02	1.47	0.27	5.97	1.81	-0.49
	5.91		-1.51	5.86		-2.40	5.92		-1.25
	5.86		-2.33	6.03		0.53	5.73		-4.48
	5.72		-4.73	5.88		-2.06	6.01		0.14
	5.77		-3.81	5.86		-2.26	5.90		-1.74
100.00	99.92	2.28	-0.08	101.67	2.75	1.67	110.46	4.82	10.46
	102.28		2.28	101.19		1.19	109.54		9.54
	100.21		0.21	100.17		0.17	101.61		1.61
	105.06		5.06	97.43		-2.57	100.88		0.88
	104.32		4.32	95.26		-4.74	100.12		0.12
150.00	160.52	0.63	7.01	150.46	0.61	0.30	150.45	0.86	0.30
	160.09		6.73	148.68		-0.88	149.35		-0.43
	158.26		5.51	150.93		0.62	149.15		-0.57
	160.60		7.07	150.87		0.58	149.17		-0.55
	159.13		6.09	150.27		0.18	146.94		-2.04

Table 3: Accuracy and Precision of 6-Methylmercaptopurine Resulted from Method Analysis

Conct. (ng/mL)	Day 1			Day 2			Day 3		
	Measured Conct. (ng/mL)	% RSD	% RE	Measured Conct. (ng/mL)	% RSD	% RE	Measured Conct. (ng/mL)	% RSD	% RE
20.00	21.30	2.57	6.50	20.47	1.55	2.36	21.63	0.88	8.15
	21.02		5.08	19.81		-0.96	22.02		10.08
	20.27		1.35	20.38		1.88	21.76		8.80
	20.51		2.55	20.35		1.75	22.01		10.07
	20.02		0.08	20.65		3.23	21.64		8.22
60.00	59.45	0.88	-0.92	54.05	1.62	-9.92	60.86	1.31	1.43
	60.14		0.24	52.08		-13.19	59.44		-0.93
	59.30		-1.16	52.10		-13.16	60.08		0.13
	60.22		0.36	53.41		-10.98	60.86		1.44
	59.04		-1.60	53.24		-11.26	59.16		-1.40
1000	999.80	0.83	-0.02	1042.67	1.05	4.27	1025.57	1.40	2.56
	982.04		-1.80	1055.49		5.55	1007.23		0.72
	998.73		-0.13	1054.51		5.45	993.96		-0.60
	995.20		-0.48	1040.29		4.03	993.22		-0.68
	984.73		-1.53	1028.93		2.89	993.93		-0.61
1500	1608.81	0.12	7.25	1556.30	0.82	3.75	1506.54	0.82	0.44
	1607.10		7.14	1556.11		3.74	1510.17		0.68
	1610.19		7.35	1529.30		1.95	1508.66		0.58
	1609.86		7.32	1553.39		3.56	1503.58		0.24
	1605.50		7.03	1561.52		4.10	1480.42		-1.31

Accuracy and precision

Accuracy and precision test were performed intra-day and inter-day. On the test of intra-day of 6-mercaptopurine, the values of % RE were between -5.43% and 7.07% for QCL, QCM, and QCH concentrations. While % RE for LLOQ concentrations were -8.66% to -2.48%. In intra-day accuracy test, % RE of 6-methylmercaptopurine were obtained between -1.80% and 7.35% for QCL, QCM, and QCH concentrations and between 0.08% to 6.50% for LLOQ concentration. In the inter-day accuracy test of 6-mercaptopurine, the % RE were between -5.43% to 10.46% for QCL, QCM, and QCH concentrations. While % RE for LLOQ concentrations were -8.66% to 11.05%. The inter-day accuracy test of 6-methylmercaptopurine obtained % RE between -13.19% to 7.35% for QCL, QCM, and QCH concentrations and % RE between -0.96% to 10.08% for LLOQ concentration. The precision could be seen from the relative standard deviation (% RSD). The intra-day precision of 6-mercaptopurine at LLOQ concentration, low, medium, and high concentration were respectively 2.53%; 1.69%; 2.28%; and 0.63%. The intra-day precision of 6-methylmercaptopurine at LLOQ concentration, low, medium, and high concentration were respectively 2.57%, 0.88%, 0.83%, and 0.12%. The inter-day precision of 6-mercaptopurine at LLOQ, low, medium, and high concentration were respectively 7.04%; 1.89%; 3.94%; and 3.30%. Inter-day precision of 6-methylmercaptopurine at LLOQ concentration, low, medium, and high concentration were respectively 3.57%, 5.96%, 2.53%, and 2.96% (Table 2 & 3).

Recovery

The results of relative recovery of 6-mercaptopurin at low, medium, and high concentrations were 94.57% - 100.53%; 95.26% - 110.46%; and 97.96% - 107.07%, respectively. The relative standard deviation (% RSD) at low, medium, and high concentration were 1.89%, 3.94%, and 3.30%, respectively. While the value of relative recoveries of 6-methylmercaptopurine at low, medium, and high concentration were 86.81% - 104%; 98.20% - 105.55%; and 98.69% - 107.35%, respectively. The RSD at low, medium, and high concentration were 5.96%; 2.53% and 2.96%, respectively. The absolute recovery of 6-mercaptopurine at low, medium, and high concentrations were 92.93% - 109.13%; 95.11% - 102.53%; and 95.66% - 102.05%, respectively. The relative standard deviation (% RSD) at low, medium, and high concentration were 8.56%, 3.87%, and 2.53%, respectively. The absolute recovery of 6-methylmercaptopurine at low, medium, and high concentrations were 77.16% - 86.02%; 89.46% - 91.97%; and 51.39% - 58.23%, respectively. The relative standard deviation (% RSD) at low, medium, high concentration were 5.43%; 1.52%; and 6.23%, respectively. It can be concluded that the recovery had good extraction efficiency and repeatability.

Dilution integrity

Intra-day accuracy study of 6-mercaptopurine by the dilution factor of 1/2 times obtained % RE between -0.56% to 1.02%, while for the dilution factor of 1/4 times acquired % RE between -10.83% to -6.45%. In the inter-day accuracy test 6-merkapatopurin by a factor dilution of 1/2 times obtained % RE between -5.02% to 1.02% while for the factor dilution 1/4 times acquired % RE between -10.83% to 0.71%. The intra-day precision of 6-mercaptopurine by the dilution factor of 1/2 times and 1/4 times consecutively obtained % RSD of 0.63% and 1.89%. As for inter-day precision of 6-mercaptopurine with a dilution factor of 1/2 times and 1/4 times consecutively acquired % RSD 1.72% and 4.32%. The

intra-day accuracy of 6-methylmercaptopurine by the dilution factor of 1/2 times obtained % RE between -1.31% to 2.15%, while for the dilution factor of 1/4 times acquired % RE between -4.85% to 0.75%. In the inter-day accuracy test of 6-methylmercaptopurine by the dilution factor of 1/2 times obtained % RE between -1.31% to 2.15%, while for the dilution factor of 1/4 times acquired % RE between -6.8% to 1.24%. The intra-day precision of 6-methylmercaptopurine by the dilution factor of 1/2 times and 1/4 times obtained % RSD of 1.44% and 2.27%. Inter-day precision of 6-methylmercaptopurine by the dilution factor of 1/2 times and 1/4 times obtained % RSD of 1.03% and 2.24%.

Stability Test

Stock Solutions

Stock solution stability test aimed to see how long stock solution remains stable and can be used, and will affect the efficiency of solution preparation time. With stability data obtained, the researchers did not need to set up a new stock solution every analysis. Researchers made only one stock solution that can be used along the research as long as it still meets the limit of stability requirements. The stock solution was stable if % RE was not more than ± 2%. The stability test of 6-mercaptopurine stock solution for one day at room temperature gave the % diff of -0.51% to 0.77%. While the stability test of 6-methylmercaptopurine stock solution for one day at room temperature gave % RE of -1.76% to 1.18%. Long-term storage at 4°C of 6-mercaptopurine and 6-methylmercaptopurine stock solution remained stable at least for 21 days with % RE of -1.99% up to -0.66% for 6-mercaptopurine and -1.97% to -1.27% for 6-methylmercaptopurine.

Freeze-Thaw Stability

The freeze-thaw stability test was performed using solutions at QCL and QCH concentrations and then stored for three cycles of freeze and thaw. The solutions were stable if % RE values were not more than ± 15%. From the research, % RE at low and high concentration of 6-mercaptopurine were -14.52% to -0.19% and -13.05% to 1.22%, respectively. While the % RE of 6-methylmercaptopurine at low and high concentration were -12.55% to 0.97% and -7.08% to 1.93% respectively. Therefore, the solutions were stable after three cycles of freeze and thaw.

Short-term Stability

Short-term stability is done by store the QCL and QCH solutions for 24 hours and performed the injection to be analyzed on the hour 0, hour-6, and the 24th hour. Solution declared stable after storage for 24 hours at room temperature, if the value of % RE not more than ± 15%. From the research value of % RE QCL and QCH concentrations of 6-mercaptopurine were -12.44% to -2.81% and -2.75% to 3.03%, respectively, while for 6-mercaptopurine were -10.22% to 2.72% and -2.05% to 6.40%, respectively. Thus, it can be stated that the solution stable at room temperature for 24 hours.

Long-term Stability

Long-term stability was performed at low and high concentrations. The samples were stored on days 0, 7, 14, and 21. The solution was stated stable for storage after preparation in the plasma if % RE stability is not over ± 15%. Based on the result, QCL and QCH of 6-mercaptopurine and 6-methylmercaptopurine were still stable until day 21st with % RE value for 6-mercaptopurine were -14.79% to 4.38% and -5.68% to 4.50%, respectively, while for 6-methylmercaptopurine were -10.96% to 1.59% and 3.56% to 7.46%, respectively.

Auto sampler Stability

Auto sampler stability test conducted on hour-0 and 24 after the solution was extracted and stored in the auto sampler. This test was performed at QCL and QCH concentrations. The solution was declared stable after storage for 24 hours in auto sampler if the % RE is not more than ± 15%. Based on the results, QCL and QCH of 6-mercaptopurine and 6-methylmercaptopurine were stable during storage in the auto sampler for 24 hours, with %RE for 6-mercaptopurine were 12.11% to 12.64% and -0.21% to 1.19%, while for 6-methylmercaptopurine were 13.01% to 14.03% and 3.99% to 4.98%. Thus, it can be stated that solutions were stable during storage in the auto sampler for 24 hours.

DISCUSSION

This research developed analytical methods of 6-mercaptopurine and 6-methylmercaptopurine in plasma with 5-fluorouracil as the internal standard simultaneously using photodiode array detector (PDA). The sample preparation used liquid-liquid extraction with dichloromethane as the solvent and the separation was conducted using gradient elution on C_{18} column. The extraction process used 500 µL plasma contain 6-mercaptopurine, 6-methylmercaptopurine and 5-fluorouracil then extracted with 5 mL dichlormethane (1:10 v/v).

The method had been validated refers to the European Medicine Agency (EMA) 2011 in term of lower limit of quantification (LLOQ), the linearity of the calibration curve, selectivity, carry-over, accuracy and precision, recovery, dilution integrity, and stability. LLOQ value associated with the sensitivity of a method for measuring the levels of analytes in biological matrices. The smaller the LLOQ, then these methods will be more sensitive to measure the low levels of analytes in biological matrices, moreover there was the metabolites in the sample. LLOQ value is one per twenty maximum concentration of the analyte in the plasma (Cmax).

All validation parameters fulfill the EMA 2011 criteria with LLOQ of 6-mercaptopurine was 2 ng / mL, while the LLOQ of 6-methylmercaptopurine was 20 ng/mL which lower than 5% of Cmax. The method of 6-mercaptopurine was linear in range concentration of 2 ng/mL - 200 ng/mL, while 6-methylmercaptopurine was linear in the range of 20 ng/mL - 1500 ng/mL. Typical HPLC chromatograms of blank plasma and blank plasma spiked with 6-mercaptopurine and 6-methylmercaptopurine LLOQ and IS were shown in Figure 1. Retention times of 6-mercaptopurine and 6-methylmercaptopurine and IS were 1.04 and 0.95 min, respectively. No significant interfering peak was observed around the 6-mercaptopurine and 6-methylmercaptopurine and IS.

Based on the data, the method was accurate and precise and was stable for 3 freeze-thaw cycles, 24 hours in room temperature, 24 hours on auto sampler, and 21 days at -20°C. Furthermore, LLE provided clean extracts and the recovery had good extraction efficiency and repeatability with no interferences presented on the chromatograms.

The method can be applied for supporting the therapeutic drug monitoring on Childhood Leukemia Lymphocytic Acute patients.

CONCLUSION

The analytical method was valid and linear at concentration range of 2.0 – 200.0 ng/mL for 6-mercaptopurine and 20 – 2000 ng/mL for 6-methylmercaptopurine. All the parameters fulfilled the acceptance criteria of the EMA Bioanalytical Method Validation Guideline, 2011.

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CONFLICT OF INTEREST

No conflict of interest are declared.

ABBREVIATIONS USED

HPLC-PDA: High Performance Liquid Chromatography-Photodiode Array; **6-MP:** 6-Mercaptopurine; **6-MMP:** 6-Methylmercaptopurine; **HPRT:** Hypoxanthine Phosphoribosyl transferase; **TIMP:** Thioinosine monophosphate; **TXMP:** Thioxanthosine Monophosphate; **TGN:** Thioguanosine Nucleotides; **DNA:** Deoxyribonucleic Acid; **IMP:** Inosine Monophosphate; **XO:** Xanthine Oxidase; **TPMT:** Thiopurine Methyltransferase; **MTIMP:** Methylated Thioinosine Monophosphate; **EMEA:** European Medicine Agency; **LLOQ:** Lower Limit of Quantification; **ULOQ:** Upper Limit of Quantification; **5-FU:** 5-fluorouracil; **RE:** Relative Error; **RSD:** Relative Standar Deviation; **QC:** Quality Control; **QCL:** Quality Control Low; **QCM:** Quality Control Medium; **QCH:** Quality Control High.

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