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Research Article

ANALYSIS OF 6-MERCAPTOPURINE AND 6-METHYLMERCAPTOPURINE IN DRIED BLOOD SPOTS USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY AND ITS APPLICATION IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS

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ABSTRACT

Objective: To analyze and validate 6-mercaptopurine (6-MP) and 6-methylmercaptopurine (6-MMP) in dried blood spots (DBS) using liquid chromatography-tandem mass spectrometry (LC/MS-MS).

Methods: Bio-sampling dried blood spot with DBS-CAMAG® paper diameter of 8 mm and extracted with acetonitrile-methanol (1:3) containing internal standard 5-fluorouracil (5-FU). Separation was performed with C_{18} column Acquity® 1.7 µm (2.1 mm × 100 mm), with a mobile phase mixture of 0.1% formic acid in water 0.1% formic acid in acetonitrile with gradient elution and flow rate 0.2 ml/min. Mass detection was Waters Xevo TQD with positive electrospray ionization (ESI) for 6-MP, 6-MMP and negative ESI for 5-FU in multiple reaction monitoring modes. The ions of 6-MP was detected at m/z 153.09->119.09, 6-MMP at m/z 167.17->126.03, and 5-FU at m/z 129.15->42.05.

Results: This method fulfill the requirements of selectivity, linearity, lower limit of quantification, accuracy, precision, carry-over, matrix effects, and stability which refers to the european medicines agency (EMEA) guidelines. The linearity of 0.99 was 26-1000 ng/mL for 6-MP and 6-MMP, respectively. The validated method was applied to two childhood ALL maintenance phase. Retrieved 6-MP levels of 10.37 pmol/8×10^8 erythrocytes, respectively. The levels of 6-MMP gained 16.59 pmol/8×10^8 erythrocytes, respectively.

Conclusion: The developed LC/MS-MS method is valid to analysis 6-MP and 6-MMP in DBS simultaneous *in vitro* according to EMEA guidelines. The method was successfully applied to authentic capillary blood samples from two childhood patients with ALL in the maintenance phase.

Keywords: 6-mercaptopurine, 6-methylmercaptopurine, Dried blood spots, Acute lymphoblastic leukemia.

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INTRODUCTION

6-mercaptopurine (6-MP) is an antineoplastic drug which belongs to antimetabolites drug and widely used in childhood acute lymphoblastic leukemia (ALL) medication in single or combination dose [1]. It is a prodrug with individual dose based on patient's body surface are because of the reason, the administration of the drug should be monitored. The usual bio-sampling method for therapeutic drug monitoring is performed through a vein (venipuncture) [2]. This method has many disadvantages such as requires a large volume, inefficient in storage and distribution, need experts to obtain the blood, and painful to the patients [3,4]. Dried blood spot (DBS) sampling has many potential advantages such as minimum invasive because using a sterile lancet needle in the fingers, toes or heel, small sample volumes ranging from 10 to 80 µl, efficient storage and distribution, prolonged sample stability, lower risk of infections, and comfortable for the subject [5,6]. Because the sample is taken from a peripheral vein in small volume, causing the analyte and the hematocrit interfering with the quantitative analysis of the drug molecule. Therefore, it needs rapid, sensitive, and selective method for quantification with liquid chromatography-tandem mass spectrometry (LC-MS/MS) [7-9].

Analysis of 6-MP and its metabolites in erythrocytes and plasma using high performance liquid chromatography (HPLC) and LC-MS/MS has been developed [1,10]. There is no report described the development of an analytical method to determine 6-MP in DBS. The novelty of the research is describes a simple, rapid, sensitive, and selective LC-MS/MS method for determining 6-MP and 6-methylmercaptopurine (6-MMP) in DBS and its application of this method in childhood ALL patients. This study performed assay validations, according to the european medicines agency (EMEA) guidelines [11].

METHODS

Chemicals and materials

6-MP ($C_5H_4N_4S$), 6-MMP ($C_6H_6N_4S$), and 5-fluorouracil (5-FU) ($C_4H_3N_2O_2F$) were purchased from Sigma-Aldrich (St. Louis, MO, USA), acetonitrile and methanol of HPLC grade were purchased from Merck (USA), purified water was prepared by a Millipore Direct-QTM 5 water system (Millipore, Watford, UK), sampling paper DBS w

as purchased from CAMAG, and whole blood from a blood bank (Indonesian Red Cross, Indonesia).

Instrument and chromatographic condition

The chromatography was performed on Waters Acquity® UPLC bridged ethylene hybrid C_{18} column 1.7 µm (2.1 mm × 100 mm). The flow rate was 0.2 ml/min using a gradient elution starting with 95% water with 0.1% formic acid and 5% acetonitrile with 0.1% formic acid. The mass selective detector operated in electrospray ionization positive mode for 6-MP and 6-MMP, a negative mode for internal standard (IS) 5-FU as internal standard (IS). Mass spectrometric detection was performed on Waters Xevo TQD Triple Quadrupole (Waters, Milford, USA); multiple reaction monitoring was employed with mass resolutions of wide for MS1 and widest for MS2. High purity nitrogen was used as source and collision gas. For data analysis, Waters Masslynx

software was used.

Standard solutions

All stock solutions were prepared using ammonium hydroxide at a concentration of 1.0 mg/ml and stored at −20°C, for calibration curve standard the stock solutions were diluted to yield 6-MP concentrations of 26-1000 ng/ml and 6-MMP concentrations of 13-500 ng/ml.

DBS sample preparation and extraction

An aliquot of 40 µl spiked blood was spotted on the sampling paper with a graded capillary tube and dried for a minimum of 3 h. Cut the spot with 8 mm diameter. After that transferred it into a 5 ml polypropylene tube, add 100 µl IS, then extract it with 3 ml methanol and 1 ml acetonitrile, vortex for about 30 s and sonicated for 25 min at 60°C temperature, then centrifuged (room temperature, 3100 ×*g*, 15 min). Supernatant transferred in the sample tube and evaporated under a gentle stream of nitrogen at 40°C temperature. After reconstituting the extract with 100 µl mobile phase, vortex for about 30 s and centrifuge (room temperature, 3100 ×*g*, 5 min), 10 µl of each sample was analyzed by LC-MS/MS.

Method validation

Assay validation was performed according to the EMEA guidelines for validation of bioanalytical assays [11]. In addition, experiments were conducted to determine the effects of volume of blood used to prepare the DBS, the effects of different hematocrit values, and the effects of DBS on the selectivity, carry-over, lower limit of quantitation, calibration curve, accuracy, precision, dilution integrity, matrix effect, and stability of the method.

Application of the method to authentic samples

Capillary blood from two patients spotted on a sampling paper and dried for at least 3 h. After drying, then the spots were packed in a sealed plastic bag with desiccant and stored at room temperature until analysis as described above.

The study protocol was approved by the Committee of the Medical Research Ethics of the "Dharmais" Cancer Hospital, with a KEPK/019/IV/2016 reference number for notice of approval.

RESULTS

Blood sampling procedure

For a sampling of an actual blood volume, we screened from several volumes; then, the spiked blood was spotted on papers DBS is 20 µl, 40 µl, and 80 µl. Accurate blood volume spotted on DBS card 40 µl.

Optimizing sample preparation

Extraction process from DBS sample is optimized by following parameters composition and volume of extraction, solution temperature and time of sonication. The preparation method was chosen based on area IS an analyte. The first experiment was performed with acetonitrile 1 ml, 1.5 ml, methanol 1 ml, 1.5 ml of methanol and a mixture of both as much as 1 ml. The recovery obtained from all the method above was unqualified. Therefore, for further optimization, we add variation in composition and volume of extraction solution, and the best recovery obtained in the mix methanol-acetonitrile (3:1) in increments of 4 ml (Fig. 1).

The recovery of 6-MP and 6-MMP was low on the organic solvent, due to different polarity between analyte and solvent. To overcome the shortcomings regarding solubility, we optimized the sonication temperature. Increasing the temperature of sonication is expected to increase the solubility of analyte in methanol and acetonitrile. Temperatures then optimized by 50°C, 60°C, and 65°C, respectively, for 30 min. The optimum temperature is 60°C (Table 1), increasing the temperature of sonication resulting a significant increase in area. It is believed that temperature is increasing the solubility of the analyte, so the amount that also extracted increases. After the temperature is optimized, we optimized the time of sonication in 15, 25, and 30 min, the optimum is 25 min. The increased time of sonication will lower 6-MP area, but increasing 6-MMP area.

Validation assay

Selectivity and hematocrit

The selectivity of the method was determined with blank blood spots and spiked blood spots for lower limit of quantification (LLOQ) concentration from six different blood lots. The proportion of cells in a blood sample is termed hematocrit; there are no definitive levels for normal human hematocrit value, as the levels change with age, sex, and general health. The optimized methods were selective for 6-MP and 6-MMP in different hematocrit value (25%, 35%, 40%, 45%, and 50%); no interfering signals were observed (Figs. 2 and 3). Selectivity was 9.93% coefficient of variation (CV) and −16.6-11.28% diff for 6-MP, 12.85% CV and −17.71-15.37% diff for 6-MMP.

Carry-over

Carry-over should be assessed by injecting blank samples after a high concentration sample or calibration standard at the upper limit of quantification. Spiked quality control samples containing the analytes in concentrations representing the top range of the calibration standard were extracted and analyzed (n=5). Immediately after injection of the high concentration quality control sample, a blank matrix sample was injected. The analyte measured in the blank matrix samples was required to be ≤20% of the LLOQ, for the IS ≤5%. No carry-over was observed for any of the analytes including the IS (Table 2).

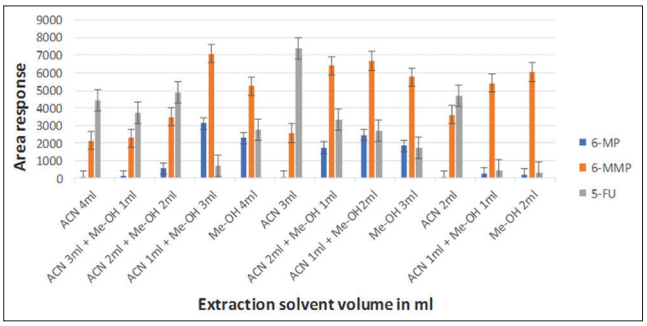

Fig. 1: Influence of the extraction acetonitrile (ACN) and methanol (Me-oh) on the analyte response peaks

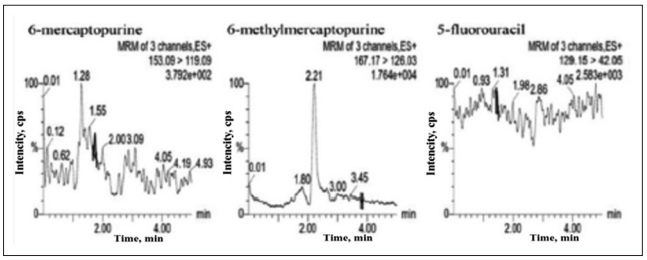

Fig. 2: Chromatograms showing the result of the extraction of blank

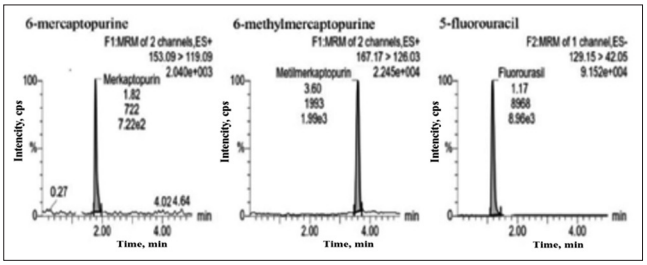

Fig. 3: Chromatograms showing the result of the extraction of lower limit of quantification

Table 1: Optimized temperature of sonication

Sonication temperature	Area			Time retention (min)		
	6-MP	6-MMP	5-FU	6-MP	6-MMP	5-FU
50°C	27,984±75.78	59,098±85.15	10,670±46.07	1.76±0.12	3.78±1.01	1.57±1.21
60°C	22,739±63.67	63,991±94.04	11,945±53.89	1.73±1.10	3.76±0.98	1.58±0.01
65°C	20,873±59.10	79,578±98.99	4853±47.78	1.73±1.11	3.76±1.21	1.57±1.11

Each value in the table was represented as mean±SD (n=3). SD: Standard deviation, 6-MP: 6-mercaptopurine, 6-MMP: 6-methylmercaptopurine, 5-FU: 5-fluorouracil

Table 2: Parameters of carry-over

Parameters	Mean measured blank area	Mean measured LLOQ area	Mean measured carry-over (%)
6-MP	100.80±50.61	713.51±46.74	14.05±6.69
6-MMP	70.20±27.44	1973.92±120.32	3.57±1.43
5-FU	78.8±68.01	7236.88±182.46	1.08±0.94

Each value in the table was represented as mean±SD (n=5). SD: Standard deviation, 6-MP: 6-mercaptopurine, 6-MMP: 6-methylmercaptopurine, 5-FU: 5-fluorouracil, LLOQ: Lower limit of quantification

Calibration curve, linearity, and LLOQ

A calibration curve was prepared for 5 consecutive days. The relationship between the quotient peak area of the analyte/peak area of the IS and the concentration was linear for 6-MP, and 6-MMP within the range of the calibration curve. Linearity was evaluated by comparing the slopes of the calibration curves. The coefficients of correlation (6-MP ≥0.99 for 26-1000 ng/ml, 6-MMP ≥0.99 for 13-500 ng/ml) were determined. For determination of the LLOQ quality control samples with low concentrations were analyzed (n=5). The LLOQ, defined as the lowest concentrations that could be determined with a precision displaying a % CV and % diff of ≤20%. The LLOQ is 26 ng/ml for 6-MP and 13 ng/ml for 6-MMP. The purpose of the development of this method is the monitoring of drug therapy, where the levels of drug C_{max} was taken at the time so that the LLOQ on this dry blood sample can be used for monitoring therapeutic drug 6MP for achievements LLOQ meet in the range of C_{max}.

Accuracy and precision

Within-run and between-run precision and accuracy were calculated from data obtained during a 3 d validation period. Each the 3 d sample spiked with the chosen concentrations (each concentration n=5) were analyzed. Four selected concentrations covered a high, medium and low range and the lower limit of quantification of the calibration curve. Accuracy was evaluated by calculating the difference between measured values and nominal values (% diff); precision was calculated based on the % CV. Within-run accuracy and precision, respectively, were −12.76-11.90% diff and 3.79-6.95% CV for 6-MP. For 6-MMP −19.92-9.41% diff and 13.15-6.13% CV. The between-run accuracy and precision ranged between −15.40-17.96% diff and 3.09-9.22% CV for 6-MP, −15.52-19.62% diff and 0.91-4.28% CV for 6-MMP. Therefore, all analytes were detected with sufficient accuracy and precision (±20% for LLOQ, ±15% for quality control low concentration [QCL], quality control medium concentration, and quality control high concentration [QCH]) (accuracy and precision data in Tables 3 and 4).

Matrix effects

Matrix effects were investigated for 6-MP and 6-MMP using 6 lots of individual human blood. The matrix factors (MF) were calculated for 6-MP and 6-MMP to obtain the IS normalized MF (MEIS). The response of post-spiked samples with known concentrations (QCL, and QCH) was compared with the response of non extracted solutions of the analyte at corresponding concentrations. The IS normalized MFs (MEIS) were found to be 8.40-10.06% CV for 6-MP and 7.66-8.07% CV for 6-MMP, and therefore the variability was within acceptable limits (Table 5).

Dilution integration

This parameter aims to see that the dilution of samples in the analysis affect the accuracy and precision or not. Integrity dilution demonstrated by spiking the matrix with the analyte concentration above upper limit of quantification (ULOQ) and diluting this mixture with blank matrix (at least five determinations per dilution factor). Dilution integrity at half ULOQ was 11.37% CV and −6.68-13.55% diff for 6-MP, 6.58% CV and −13.36-4.30% diff for 6-MMP. At a concentration quarter, ULOQ was 3.14% CV and 5.73-13.88% diff for 6-MP, 9.49% CV and −10.14-4.30% diff for 6-MMP. Therefore, all analytes were detected with sufficient accuracy (±15% relative error of the nominal value) and precision (within 15% relative standard deviation).

Stability

The stock solution stability was determined for one concentrations (1 mg/ml; each concentration n=5) under different conditions. Store the solution in room temperature for 24 h and at −20°C for 16 d. All solutions were stable under these conditions. The accuracy and precision was 0.21-0.60% CV and −0.34-0.43% diff for 6-MP, 0.04-0.93% CV and −0.03-0.66% diff for 6-MMP, 0.09-2.54% CV and −0.06-1.80% diff for 5-FU (Table 6).

To the determine the short-term stability, store the DBS in sealed plastic bags containing silica gel desiccants for 24 h, then determined by analyzing blood spots containing the analytes at two concentrations (QCL and QCH; each concentration n=3). The calculated concentrations, accuracy, and precision between samples immediately after drying for 3 hrs and the different time were determined and compared. The accuracy and precision was 2.64-8.20% CV and −8.61-12.24% diff for 6-MP, 4.25-6.42% CV and −14.11-13.72% diff for 6-MMP (Table 7).

The stability of the extracted samples in the autosampler for 24 h was determined by analyzing blood spots containing the analytes at two concentrations (QCL and QCH; each concentration n=3). The calculated concentrations, accuracy, and precision between samples measure immediately after extraction and samples remaining in the autosampler for 24 h until measurement were calculated and compared. The accuracy and precision was 2.79-5.47% CV and −5.33-13.23% diff for 6-MP, 1.60-4.90% CV, and −14.59-13.79% diff for 6-MMP. The result indicates that the compounds in the DBS and extracted samples were stable under the tested conditions (Table 8).

Analysis of authentic capillary blood samples from DBS

Subjects, were included, in this study are patients with ALL in children who are undergoing treatment in the maintenance phase. Retrieved two patients indicated its willingness to be a subject. Samples then carried out to the preparation and analyzed with methods that have been validated. The concentration of 6-MP found in erythrocytes were 6.69 and 10.37 pmol/8 x 10^8 erythrocytes, for 6-MMP were 6.02 and 16.59 pmol/8 x 10^8 erythrocytes (with 3.91 x 10^8 and 4.33 x 10^8 erythrocytes per 100 µl of packed erythrocytes in patient 1 and 2).

DISCUSSION

Accurate blood volume spotted on DBS cards 40 µl and it is spread evenly and absorbed rapidly. As for extraction method, we choose protein precipitation using methanol-acetonitrile (3:1) good recovery the analytes (Fig. 1). As shown in Table 1, its indicated sonication and evaporation step following the precipitation were added to concentrate its analyte they increase the response of analyte. Recovery of 6-MP and 6-MMP more than 90%, it concludes that the extraction method was successful producing high extraction yield. Validation assay was

Table 3: Accuracy and precision of 6-MP

Analyte	Actual concentration (ng/ml)	Mean measured concentration (ng/ml)±SD (n=5)	Precision (% CV)	Bias (% difference)
Within-run	LLOQ (26)	25.56±1.74	6.82	(−10.17-8.36)
	QCL (104)	103.59±3.82	3.69	(−4.36-4.28)
	QCM (520)	539.73±21.07	3.90	(−0.11-8.99)
	QCH (820)	877.70±44.07	5.03	(−0.67-14.07)
Between-run	LLOQ (26)	25.36±2.86	11.29	(−14.04-17.06)
	QCL (104)	98.26±3.72	3.78	(−12.08-3.21)
	QCM (520)	508.79±22.80	4.48	(−14.08-8.39)
	QCH (820)	806.81±57.56	7.13	(−13.59-13.34)

LLOQ: Lower limit of quantification, QCL: Quality control low concentration, QCM: Quality control medium concentration, QCH: Quality control high concentration, 6-MP: 6-mercaptopurine, SD: Standard deviation, CV: Coefficient of variation

Table 4: Accuracy and precision of 6-MMP

Analyte	Actual concentration (ng/ml)	Mean measured concentration (ng/ml)±SD (n=5)	Precision (% CV)	Bias (% difference)
Within-run	LLOQ (13)	10.96±0.71	6.44	(−19.84-7.04)
	QCL (52)	51.42±5.69	11.07	(−7.21-8.58)
	QCM (260)	261.44±19.18	7.34	(−10.59-8.19)
	QCH (416)	401.95±24.84	6.18	(−9.71-5.91)
Between-run	LLOQ (13)	12.86±0.61	4.70	(−14.54-19.62)
	QCL (52)	54.32±2.09	3.86	(−11.96-13.79)
	QCM (260)	261.08±6.76	2.59	(−10.08-8.54)
	QCH (416)	403.03±4.47	1.11	(−12.42-7.69)

LLOQ: Lower limit of quantification, QCL: Quality control low concentration, QCM: Quality control medium concentration, QCH: Quality control high concentration, SD: Standard deviation, CV: Coefficient of variation, 6-MMP: 6-methylmercaptopurine

Table 5: Matrix effects for 6-MP and 6-MMP

Blood lot	Concentration (ng/ml)	MF 6-MP (%)	MF IS (%)	MEIS (%)	MF 6-MMP (%)	MF IS (%)	MEIS (%)
1	QCL (104 for 6-MP, 52 for 6-MMP)	69.57	82.00	0.84	83.91	82.00	1.02
2		83.43	80.65	1.03	79.19	80.65	0.98
3		83.46	83.15	1.00	83.83	83.15	1.01
4		78.61	82.64	0.95	97.67	82.64	1.18
5		80.67	80.67	1.00	95.58	80.67	1.18
6		90.09	82.72	1.08	83.14	82.72	1.01
1	QCH (820 for 6-MP, 416 for 6-MMP)	76.66	82.00	0.93	91.31	82.00	1.11
2		70.64	80.65	0.87	80.39	80.65	0.99
3		66.94	83.15	0.80	88.51	83.15	1.06
4		63.58	82.64	0.76	80.58	82.64	0.97
5		67.13	80.67	0.83	73.46	80.67	0.91
6		85.24	82.72	1.03	85.18	82.72	1.03

MF: Matrix factors, MEIS: Internal standard normalized matrix factors, QCL: Quality control low concentration, QCH: Quality control high concentration, 6-MMP: 6-methylmercaptopurine, 6-MP: 6-mercaptopurine

Table 6: Stock solution stability of 6-MP, 6-MMP, and 5-FU

Analyte	Temperature	Time	Mean measured concentration (ng/ml)±SD (n=3)	Precision (% CV)	Bias (% diff)
6-MP	Room	0 hr	834.77 ± 58.60	0.60	(0.43-3.87)
		6 hrs	857.81 ± 88.81	0.27	(0.19-5.00)
		24 hrs	987.54 ± 50.18	0.31	(0.22-3.76)
	−20°C	8 days	984.77 ± 62.70	0.21	(0.15-4.77)
		16 days	998.81 ± 78.81	0.47	(−0.34-10.34)
6-MMP	Room	0 hr	878.54 ± 91.50	0.84	(0.59-9.23)
		6 hrs	875.64 ± 100.45	0.15	(−0.11-6.43)
		24 hrs	885.70 ± 99.18	0.26	(−0.18-10.01)
	−20°C	8 days	932.87 ± 101.72	0.04	(−0.03-5.99)
		16 days	977.41 ± 120.11	0.93	(0.66-6.43)
5-FU	Room	0 hr	911.24 ± 95.53	2.49	(1.76-2.98)
		6 hrs	953.62 ± 92.35	1.70	(−1.21-1.78)
		24 hrs	979.17 ± 72.98	0.29	(0.21-5.21)
	−20°C	8 days	1084.37 ± 102.72	0.09	(−0.06-3.22)
		16 days	1003.31 ± 120.16	2.54	(1.80-1.98)

Stock solution concentrations for 6-MP, 6-MMP and 5-FU 1000 ng/ml. 6-MMP: 6-methylmercaptopurine, 6-MP: 6-mercaptopurine, 5-FU: 5-fluorouracil, CV: Coefficient of variation

Table 7: Short stability of 6-MP, 6-MMP as DBS at room temperature storage in sealed plastic bags containing silica gel desiccants

Analyte	Concentration (ng/ml)	Time	Mean measured concentration (ng/ml)±SD (n=3)	Precision (% CV)	Recovery (%)
6-MP	QCL (104)	0 hr	99.81±7.29	6.01	94.98
		12 hrs	106.60±5.03	3.89	101.48
		24 hrs	103.45±7.29	5.79	98.48
	QCH (820)	0 hr	878.95±40.53	2.64	108.14
		12 hrs	843.60±43.23	4.19	102.34
		24 hrs	866.71±86.95	8.20	105.15
6-MMP	QCL (52)	0 hr	51.83±4.24	6.31	104.98
		12 hrs	5.98±3.05	4.44	107.17
		24 hrs	50.56±2.80	4.25	102.53
	QCH (416)	0 hr	389.35±21.64	4.50	93.59
		12 hrs	379.37±19.62	4.60	97.06
		24 hrs	391.37±31.04	6.42	94.08

6-MMP: 6-methylmercaptopurine, 6-MP: 6-mercaptopurine, CV: Coefficient of variation, QCL: Quality control low concentration, QCH: Quality control high concentration, DBS: Dried blood spots

Table 8: Autosampler stability of 6-MP, 6-MMP

Analyte	Concentration (ng/ml)	Time	Mean measured concentration (ng/ml)±SD (n=3)	Precision (% CV)	Recovery (%)
6-MP	QCL (104)	0 hrs	104.93±4.74	3.69	99.88
		24 hrs	110.44±7.35	5.47	105.16
	QCH (820)	0 hrs	885.64±29.91	2.79	106.21
		24 hrs	823.81±39.57	3.50	108.01
6-MMP	QCL (52)	0 hrs	55.30±1.15	1.60	109.16
		24 hrs	48.76±3.12	4.90	98.17
	QCH (416)	0 hrs	388.08±10.99	2.29	93.29
		24 hrs	373.01±16.46	3.57	90.70

QCL: Quality control low concentration, QCH: Quality control high concentration, 6-MMP: 6-methylmercaptopurine, 6-MP: 6-mercaptopurine, CV: Coefficient of variation

Table 9: Result of 6-MP and 6-MMP in patients DBS sample

Donor	Concentration (pmol/8 x 10^8 erythrocytes)	
	6-MP	6-MMP
1	6.69	6.02
2	10.37	16.59

6-MMP: 6-methylmercaptopurine, 6-MP: 6-mercaptopurine, DBS: Dried blood spots

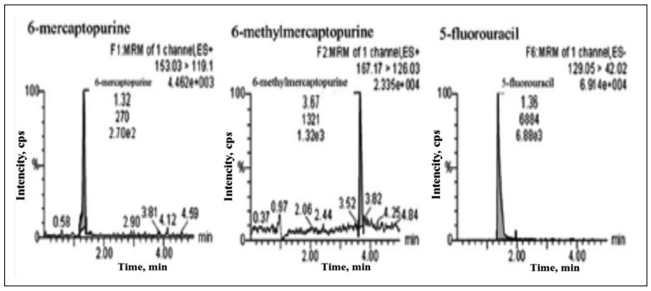

Fig. 4: Chromatograms showing the result of patient

performed and as reported above, in selectivity parameter found no interfering signals from different hematocrit value from the blood (Fig. 2). The result of carry-over was given in Table 2; no carry-over was observed for any of the analytes including the IS. Values of accuracy and precision within-run and between-run fulfill the range of ≤ ±15% for QC samples and ≤20% for LLOQ (Tables 3 and 4). As shown in Table 5, the MF value than less 100%, it concludes that indicated matrix compound causes ion suppression that can interfere with analyte ionization process, but % CV values were within limits requested by EMEA guidelines [2,11-14]. Analytes in stock solution stable at least for 16 days storage of −20°C. Analyte in DBS was stable for 6 days at room temperature and 24 hrs in the autosampler (stability data in Tables 6-8). The method has several advantages as compared to the literature [1,10] such as minimum volume sample, simple sample preparation procedures, short analysis time (5 min per sample), and high sensitivity which rendered for the purpose of its application. The result of application toward childhood ALL patients in maintenance phase showed the concentration of 6-MP and 6-MMP; the results indicated that the methods used are not disturbed by the metabolic processes *in vivo* (Fig. 4). Monitoring the levels of 6-MP and 6-MMP were necessary to get the right information related to the availability of the metabolite 6-MP both the active metabolite and inactive metabolites.

CONCLUSION

The developed LC/MS-MS method is valid to analysis 6-MP and 6-MMP in DBS simultaneous *in vitro* according to EMEA guidelines. The method was successfully applied to authentic capillary blood samples from two patients with ALL maintenance phase.

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