



Simultaneous Analytical Method Of 6-Mercaptopurine and 6-Methylmercaptopurine In-vitro Study With Bio-Sampling Venipuncture and Dried Blood Spot

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Abstract

Objective: 6-Mercaptopurine (6-MP) is a cancer chemotherapeutic agent. Metabolic pathway by thiopurine S-methyltransferase (TPMT) to become 6-methylmercaptopurine (6-MMP). Bio-sampling is required to obtain biological sample, using two technique; invasive (venipuncture) and minimum invasive (dried blood spot). This study aims to obtain an optimization and validation analysis 6-MP and 6-MMP in vitro study with bio-sampling venipuncture and dried blood spot (DBS). Method: Plasma from venipuncture method extraction was done using dichloromethane. Separation was performed using Waters HPLC, C18 Sunfire™ column (5µm, 250 x 4.6 mm), with gradient elution, flow rate 1 mL/min and detected at UV-PDA wavelength of 303 nm. Bio-sampling dried blood spot with DBS CAMAG® paper diameter of 8 mm and extracted with acetonitrile-methanol (1:3). Separation was performed with Waters LC-MS/MS UPLC C18 column (1.7 µm, 2.1 x 100 mm) with gradient elution, flow rate 0.2 mL/min. 5-fluorouracil (5-FU) was used as internal standard. Result: The method venipuncture was linear at concentration range of 2-200 ng/mL for 6-MP and 20-2000 ng/mL for 6-MMP. The method dried blood spot using Waters Xevo TQD for mass detection with positive electrospray ionization (ESI) for 6-MP, 6-MMP and negative ESI for 5-FU in Multiple Reaction Monitoring mode. Linear with the range 26-1000 ng/mL for 6-MP and 13-500 ng/mL for 6-MMP. Conclusion: The developed method is valid for 6-MP and 6-MMP simultaneously *in vitro* from venipuncture using HPLC and from dried blood spot using LC-MS/MS and showed good selectivity, linearity, accuracy and precision, matrix effect and stability.

Keywords: 6-mercaptopurine, 6-methylmercaptopurine, Venipuncture, Dried blood spot.

Introduction

One of method that can be used to therapeutic drug monitoring is bioanalysis. Bioanalysis is a drug and metabolite analysis method in biological sample, such as; blood, plasma, and saliva [1].

Biological sample can be obtained with bio-sampling venipuncture method, taking blood from vein with syringe [2]. The other bio-sampling method is dried blood spot, collecting blood with minimum invasive through fingers, toes or heel, then blood samples blotted and dried on special paper, then can be stored or directly analyzed.[3] 6-Mercaptopurine (6-MP) is a cancer chemotherapeutic agent. Metabolic pathway by thiopurine S-methyltransferase (TPMT) to

become 6-methylmercaptopurine (6-MMP), 6-MP also displays a range of possible adverse drug reactions and a narrow therapeutic index. Hence, the therapeutic index for each individuals need to be monitored [4]. 6-Mercaptopurine is cytostatic drug that has narrow therapeutic index, so it requires therapeutic drug monitoring.

Therefore, therapeutic drug monitoring of 6-MMP is required to estimate that 6-MMP concentration is at a safe range. Common 6-mercaptopurine dose given to a child suffers from ALL is 50-75 mg/m², variety of doses implies to patients depend on body surface area[5]. This study aims to obtain an optimization and validation analysis in vitro

study 6-MP and 6-MMP with bio-sampling venipuncture and dried blood spot. Analysis of 6-mercaptopurine and its metabolites in erythrocytes and plasma with venipuncture using HPLC has been developed, these methods have difficult and complex extraction with derivatization procedure[2,6]. This research developed a simple extraction, quick, and accurate analytical methods of 6-mercaptopurine and 6-methylmercaptopurine with 5-fluorouracil as internal standard simultaneously using HPLC. The sample preparation used liquid-liquid extraction.

There is no report described the development of an analytical method to determine 6-MP in DBS. This method has the advantage of minimum pain for patients, for the blood is taken using sterile needle lancet on the fingers, toes, and heels. Another advantage is the small amount of blood taken (10-80 μ L), dried blood sample also has convenience in storage and distribution, analyses in a sample of dried blood is relatively stable, As well as reduce the risk of infection and convenient to the subject [7]. Analytical method of 6-MP and 6-MMP simultaneously in dried blood spot sample with UPLC-MS/MS using 5-fluorouracil as internal standard. The sample preparation was using protein precipitation.

Materials and Methods

Chemicals and Reagent

6-Mercaptopurine (6-MP), 6-methylmercaptopurine (6-MMP), 5-fluorouracil (5-FU) were obtained from Sigma Aldrich (St. Louis, MO, USA), acetonitrile HPLC grade, methanol HPLC grade, formic acid, ammonium acetate, NH_4OH , dichloromethane were purchased from Merck(USA), purified water was prepared by a Millipore Direct-QTM 5 water system (Millipore, Watford, UK), sampling paper DBS was purchased from CAMAG, plasma and whole blood from a blood bank (Indonesian Red Cross, Indonesia).

Instruments and Chromatographic Conditions

High performance liquid chromatography (HPLC) 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 TM

software. Separation of 6-MP and 6-MMP was achieved using reverse phase analytical column (C-18 Sun fire™, 5 μ m; 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 7-13.5 min. Elution was continued with mobile phase A to the end of the run at 16 min. Flow rate were maintained at 1 mL/min with column temperature of 30°C.

The photodiode array detector was set at wavelength of 303 nm. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed on Waters Acquity® UPLC BEH (*Bridged Ethylene Hybrid*) C_{18} column 1, 7 μ m (2,1 mm x 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 (ESI) positive mode for 6-MP and 6-MMP, a negative mode for internal standard (5-FU). Mass spectrometric detection was performed on Waters Xevo TQD Triple Quadrupole (Waters, Milford, USA), multiple reaction monitoring (MRM) 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. The run was performed with gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Flow rate was 0.2 mL/min and injection volume was 10 μ L.

Sample Preparation

Plasma (Venipuncture)

A 500 μ L plasma that contained 6-MP 10 μ g/mL and 6-MMP 30 μ g/mL were mixed with 50 μ L 5-FU 30 μ g/mol 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 μ L mobile phase (air-methanol-acetonitrile, 90:7:3, v/v, and 30 μ L aliquot were injected onto the HPLC system.

Whole Blood (Dried Blood Spot)

An aliquot of 40 μ L 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 μ L IS, then extract it with methanol-acetonitrile (3:1), vortex for about 30 s and sonicated for 25 min at 60 °C temperature, then centrifuged (room temperature, 3100 \times 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 μ L mobile phase, vortex for about 30 s and centrifuge (room temperature, 3100 \times g, 5 min), 10 μ L of each sample was analyzed by LC-MS/MS.

Method Validation

Validation of the developed method was carried out as per EMEA guidelines for selectivity, carry-over, LLOQ, accuracy, precision, linearity, dilution integrity, matrix effect, and stability [1, 8].

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). % diff and % CV should be $\leq \pm 20\%$ for LLOQ and 5% for internal standard.

Carry-Over

Carry-over was performed by injecting blank sample after upper limit of quantification (ULOQ) concentration sample. A carry over $\leq \pm 20\%$ for LLOQ and $\leq \pm 5\%$ for internal standard are acceptable.

Lower Limit of Quantification (LLOQ)

LLOQ was validated using five replicates of matrix which were spiked with 6-MP and 6-MMP at LLOQ concentration. % diff and % CV should be $\leq \pm 20\%$.

Calibration Curves

A blank sample, a zero sample and sample at 7 calibration concentration levels were prepared. The slope, intercept and correlation coefficient of each calibration curve were determined. % diff should be $\leq \pm 15\%$ for each

calibration concentration and $\leq \pm 20\%$ for LLOQ.

Accuracy and Precision

Accuracy and precision of the method were performed within-run and between-run. Accuracy and precision were determined at four concentrations stated as LLOQ, LQC, MQC, HQC. Accuracy was calculated as the mean percent deviation from the actual concentration expressed as % diff while precision was expressed by % CV calculated. Both parameters should be $\leq \pm 15\%$ for the QC samples and should be $\leq 20\%$ for LLOQ.

Dilution Integrity

Dilution of samples should not affect the accuracy and precision. If applicable, dilution integrity should be demonstrated by spiking the matrix with an analyze concentration above the ULOQ and diluting this sample with blank matrix (at least five determinations per dilution factor). Accuracy and precision should be within the set criteria, within $\pm 15\%$.

Matrix Effect

Matrix effects should be investigated when using mass spectrometric methods. Matrix effect was assessed using at least 6 lots of blank matrix from individual donors. This was done by comparing the peak areas in the presence of matrix with the peak areas in pure solution of analyze. Matrix effect should be done at LQC and HQC.

Stability

Evaluation of stability was determined by analyzing samples containing 6-MP and 6-MMP at LQC and HQC. The stability evaluation consisted of the stock and working solutions of the analyze and internal standard stability, freeze and thaw stability (no for DBS), and long term stability.

Result and Discussion

Bio-sampling Venipuncture Analyzed Using HPLC

Result of lower limit of quantification (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 - 14.11% and % RSD of 3.67%. The value of % diff and % RSD still meet the requirements so this concentration is selected as LLOQ.

Selectivity test was performed at a concentration of 2 ng/mL for 6-MP and 20 ng/mL for 6-MMP using six different human

plasma blank to see the effect of plasma impurities from different sources no more than $\pm 20\%$ values of % diff and % CV.

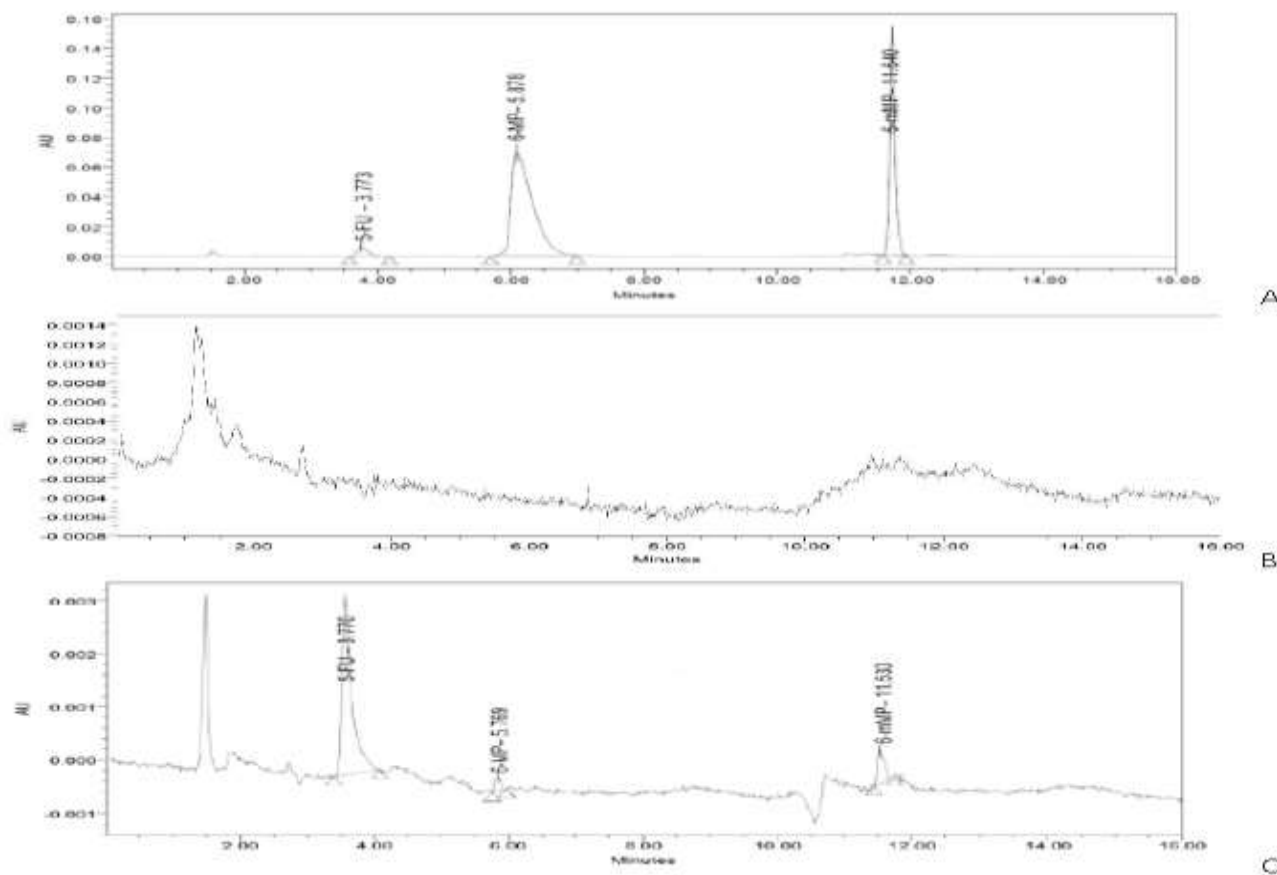


Figure 1: Chromatograms HPLC of standards solution (A), blank plasma (B), and LLOQ (C)

Therefore, there was no interference at the retention time of analyze, metabolite, and internal standard (shown in figure 1). A calibration curve of 6-MP made were 2-200 ng/mL, the correlation coefficient of 0.9991 with the line equation $y = 0.0004 + 0,0025x$ and 6-MMP made were 20-1500 ng/mL, had a coefficient correlation of 0.9993 with the line equation $y = -0.0014 + 0,0006x$. That no more than $\pm 15\%$ for concentration besides LLOQ and no more than $\pm 20\%$ for LLOQ concentration. Accuracy and precision test

were performed within-run and between-run. 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 coefficient of variation (% CV). Therefore, all analyses were detected with sufficient accuracy and precision ($\pm 20\%$ for LLOQ, $\pm 15\%$ for LQC, MQC, HQC) (accuracy and precision data in Table 1 and 2).

Table 1: Accuracy and precision of 6-mercaptopurine

| Analyze | Actual concentration (ng/ml) | Mean measured concentration (ng/ml) \pm SD (n=5) | Precision (% CV) | Bias (% diff) |
|-------------|------------------------------|--|------------------|---------------|
| Within-run | LLOQ (2) | 1.91 \pm 0.05 | 2.53 | (-2.48-8.66) |
| | LQC (6) | 5.78 \pm 0.09 | 1.69 | (-1.51-5.43) |
| | MQC (100) | 102.34 \pm 2.33 | 2.28 | (-0.08-5.06) |
| | HQC (150) | 159.72 \pm 1.00 | 0.63 | (5.51-7.01) |
| Between-run | LLOQ (2) | 2.17 \pm 0.05 | 2.37 | (4.34-11.05) |
| | LQC (6) | 5.91 \pm 0.11 | 1.81 | (-4.48-0.14) |
| | MQC (100) | 104.52 \pm 5.04 | 4.84 | (0.12-10.46) |
| | HQC (150) | 149.01 \pm 1.28 | 0.86 | (-2.04-0.30) |

Table 2: Accuracy and precision of 6-methylmercaptapurine

| Analyte | Actual concentration (ng/ml) | Mean measured concentration (ng/ml) ± SD (n=5) | Precision (% CV) | Bias (% diff) |
|-------------|------------------------------|--|------------------|---------------|
| Within-run | LLOQ (20) | 20.62 ± 0.56 | 2.57 | (0.08-6.50) |
| | LQC (60) | 59.63 ± 0.52 | 0.88 | (-1.60-0.36) |
| | MQC (1000) | 992.41 ± 8.19 | 0.83 | (-0.02-1.53) |
| | HQC (1500) | 1608.29 ± 1.97 | 0.12 | (7.03-7.35) |
| Between-run | LLOQ (20) | 21.81 ± 0.19 | 0.88 | (8.15-10.08) |
| | LQC (60) | 60.08 ± 0.78 | 1.31 | (-1.40-1.44) |
| | MQC (1000) | 1002.78 ± 4.02 | 1.40 | (-0.68-2.56) |
| | HQC (1500) | 1501.89 ± 12.24 | 0.82 | (-1.31-0.68) |

Note: LLOQ = lower limit of quantification, LQC = low quality control, MQC = medium quality control, QCH = high quality control

Dilution integrity at half ULOQ was 0.63 % CV and -0.56-1.02 % diff for 6-MP, 1.03 % CV and -1.31-2.15 % diff for 6-MMP. At a concentration quarter ULOQ was 1.89 % CV and -10.83-0.71 % diff for 6-MP, 2.24 % CV and -4.85%-0.75% % diff for 6-MMP. Therefore, all analyses were detected with sufficient accuracy ($\pm 15\%$ relative error of the nominal value) and precision (within 15% relative standard deviation).

Stock solution stability test remained stable at least for 21 days for storage at 4°C. 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 freeze-thaw stability test was performed using LQC and HQC and then stored for three cycles of freeze and thaw. The freeze-thaw stability 6-MP was -14.52--0.19 % diff for LQC and -13.05-1.22 % diff for HQC. While the % diff of 6-MMP at LQC and HQC were -12.55-0.97% and -7.08-1.93%, respectively. Therefore, the solutions were

stable after three cycles of freeze and thaw. Long-term stability was performed at LQC and HQC. The samples were stored on days 0, 7, 14, and 21. Based on the result, LQC and HQC of 6-MP and 6-MMP were still stable until day 21st with % diff value for 6-MP were -14.79-4.38% and -5.68-4.50%, respectively, while for 6-MMP were -10.96-1.59% and 3.56-7.46%, respectively.

Bio-sampling Dried Blood Spot Analyzed Using LC-MS/MS

Mass detection was performed on Waters Xevo TQD with electrospray ionization (ESI) positive for 6-MMP and 6-MMP and ESI negative for 5-FU. The following operational parameters of the MS detector were optimized; MS ion mode, Precursor and product ions cone and collision energies are presented in table 3. 6-Mercaptopurine was detected at m/z 153.03>119.10, 6-methylmercaptapurine was detected at m/z 167.17>126.03 (shown in figure 2) and 5-fluorouracil was used as internal standard at m/z 129.055>42, 02.

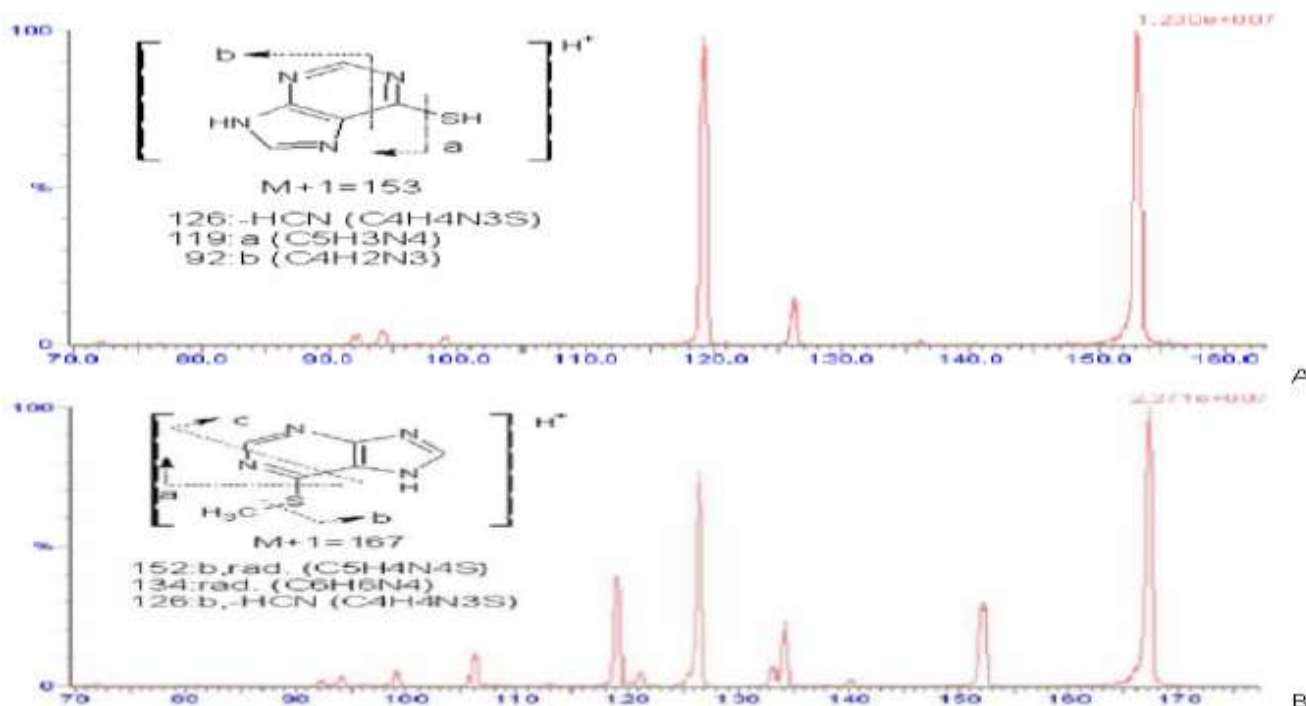


Figure 2: Fragmentation of ion mass spectra of 6-mercaptopurine (A) and 6-methylmercaptapurine (B)

Validation Assay

The calibration curves were linear over the concentration range of 26-1000ng/mL for 6-MP and 13-520 ng/mL for 6-MMP with a correlation coefficient of 0.99. The correlation coefficient from five replicate calibration curves on different days was more than 0.99.

The lower limit of quantification (LLOQ) was 26 ng/mL (6-MP) and 13ng/mL (6-MMP) with a coefficient of variation of less than 20%. The selectivity result showed that there was no interference endogenous compound from the blank whole blood of the six different sources, whereas the % diff both of 6-MP and 6-MMP were less than 20% (shown in figure 3).

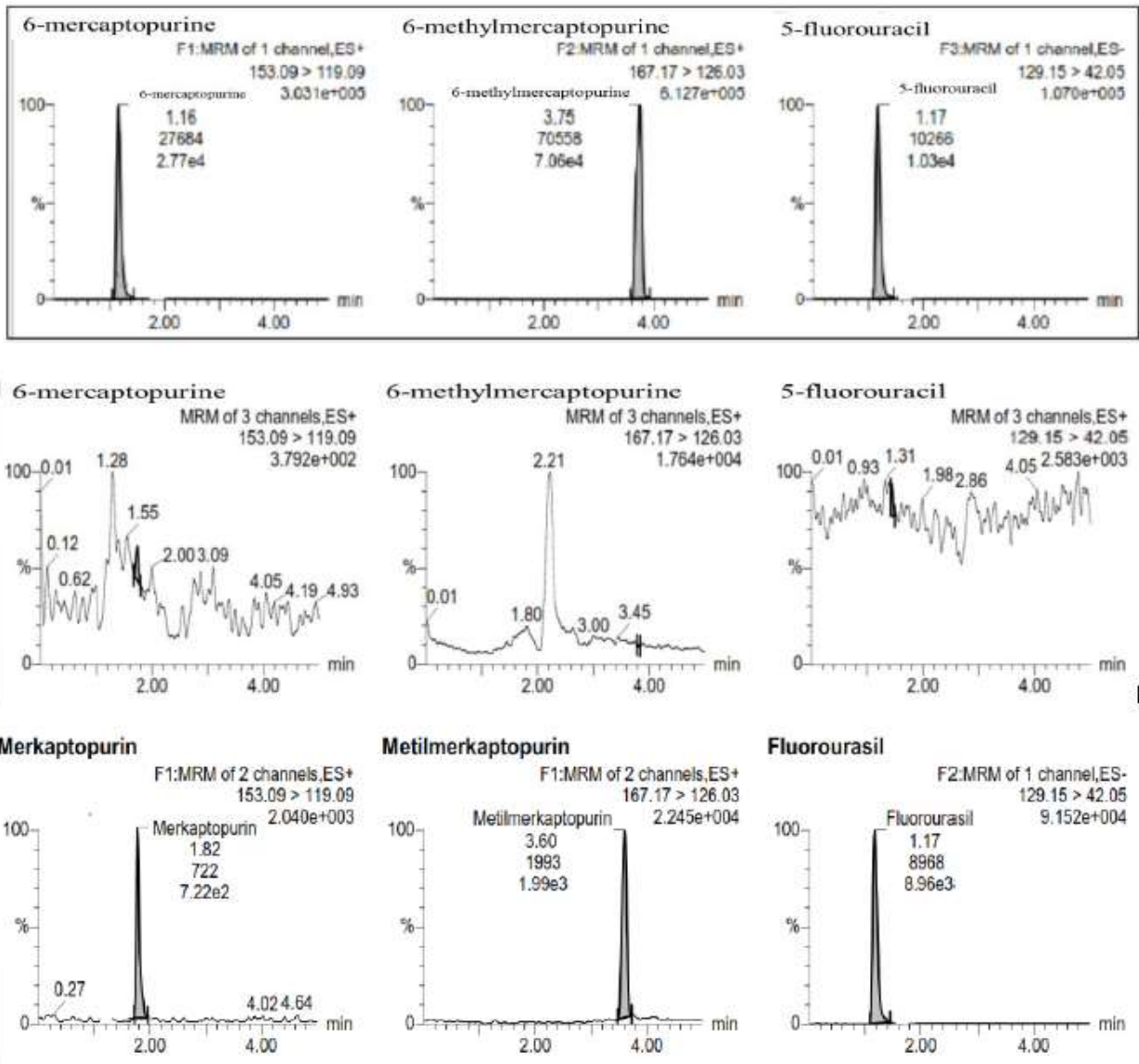


Figure 3: Chromatograms LC-MS/MS of standards solution (A), blank matrix (B), and LLOQ (C)

Carry-over value after high concentration injection was 14.05% for 6-MP and 3.57% for 6-MMP from LLOQ response while the carry-over of internal standard was 1.08%.

Precision and accuracy were calculated by within-run and between-run variation of QC sample in five replicates at four concentrations. Accuracy was evaluated by calculating the difference between measured values and nominal values (% diff), precision was calculated based on the coefficient of

variation (% 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 was -19.92-9.41 % diff and 13.15-6.13 % CV. The between run accuracy and precision ranged -15.40-17.96 % diff and 3.09-9.22% CV for 6-MP, for 6-MMP was -15.52-19.62% diff and 0.91-4.28% CV. Therefore, all analyses were detected with sufficient accuracy and precision ($\pm 20\%$ for LLOQ, $\pm 15\%$ for LQC, MQC, and HQC). The within-run and between-run precision and accuracy

values indicate the adequate reliability and reproducibility of the method within the analytical range.

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 analyses were detected with sufficient accuracy ($\pm 15\%$ relative error of the nominal value) and precision (within 15% relative standard deviation) Matrix effects were investigated for 6-MP and 6-MMP using 6 lots of individual human blood. Ion increasing effects due to matrix constituents

were observed which the value of matrix factors (MF).

The internal standard normalized matrix factors (MEIS) were found to be 8.40-10.06 % CV for 6-MP, 7.66-8.07 % CV for 6-MMP. The MF value than less 100%, it concludes that indicated matrix compound causes ion suppression that can interfere with analyze ionization process, but % CV values were within limits requested by EMEA guidelines [1,9] (matrix effects data in Table 3). The stability test of 6-MP, 6-MMP and 5-FU were evaluated under different temperature and storage condition and were performed at LQC and HQC in three replicates.

Table 3: Matrix effects for 6-mercaptopurine and 6-methylmercaptopurine

| Blood lot | Concentration (ng/ml) | MF 6-MP (%) | MF IS (%) | MEIS (%) | MF 6-MMP (%) | MF IS (%) | MEIS (%) |
|-----------|-----------------------------------|-------------|-----------|----------|--------------|-----------|----------|
| 1 | LQC (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 | HQC (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 |

The result of stability test showed that analyses in stock solution stable at least for 16 d storage of -20 °C. Analyte in DBS was stable for 6 d at room temperature and 24 h in the autosampler.

Conclusion

The developed method is valid for 6-MP and 6-MMP simultaneously *in vitro* from venipuncture using HPLC and from dried blood spot using LC-MS/MS and showed good selectivity, linearity [10], accuracy and precision, matrix effect and stability.

Abbreviations

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