INTRODUCTION
Leukemia is a disease that affects the blood and blood-forming tissues in the bone marrow of the spinal cord [1]. In patients with leukemia, the spinal cord produces abnormal white blood cells, which are called leukemia that will continuously produce cells to disrupt normal blood cells [2]. In 2010, a total of 43,000 men and women in the United States were diagnosed with leukemia, and 21,840 of them died. Leukemia accounts for 33% of the cancer incidence in children and kills 1340 children per year. Leukemia is divided into acute lymphocytic leukemia (ALL), acute non-lymphocytic leukemia, acute myelocytic leukemia (AML), and chronic myelocytic leukemia; among these, the acute types account for the highest prevalence of 97% (82% ALL and 18% AML) [3]. One of the curative treatments for ALL is chemotherapy [4].

6-mercaptopurine (6-MP) is an antimetabolite drug that is often used for cancer chemotherapy, especially in ALL, in both children and adults [5]. 6-MP undergoes three metabolic pathways. In the first pathway, mercaptopurine is transformed into thioguanine monophosphate by the enzyme hypoxanthine phosphoribosyl transferase and is subsequently converted to its active metabolite 6-thioguanine (TG) nucleotide, which is easily hydrolyzed into 6-TG. In the second pathway, 6-MP is bio-hydroxylated by xanthine oxidase to an inactive metabolite 6-thiouric acid. The final 6-MP pathway involves methylation to 6-methylmercaptopurine (6-MMP) by thiopurine methyltransferase (TPMT) [6]. High concentrations of 6-TG have been found to correlate with therapeutic efficacy, and high 6-MMP concentrations were associated with liver toxicity [7]. Since the metabolism formation of 6-MP is affected by TPMT enzymes that undergo polymorphism, the effects of 6-MP treatment vary widely among individuals. Therefore, monitoring the efficacy of a therapeutic drug regimen becomes important in ALL.

One method for monitoring drug therapy is analyzing the drug and its metabolites in biological samples, such as blood, plasma, urine, and saliva [8]. One of the biosampling methods for monitoring drug therapy is dried blood spot (DBS) or dry blood sample on paper. The DBS method entails collection of blood from the human body, usually from the fingers or heels, followed by spotting of the sample on a special paper until the blood becomes dry for immediate analysis or storage. This method has several advantages. First, the solid form of DBS makes the analyze less reactive than that in the liquid form. This will increase the stability even if it is not stored in a cold environment, unlike other matrices. This method also has the advantage of being minimally invasive to the patient because the collection of blood uses only sterile lancet needles. Another advantage is the small volume of required blood sample, which is usually 10–80 μL [9].

Several analysis methods of 6-MP and 6-TG in whole blood or plasma have been done before; ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) had been used to analyze both 6-MP and 6-TG in plasma samples [7] and 6-MP metabolites in whole blood samples [10]. On the other hand, a method for analysis of dry blood is still not available.

Although the DBS method has many advantages, it also has some limitations, such as the small volume of blood used, which would require a selective and sensitive method of analysis. For such purpose, the UPLC-MS/MS can be used, with 6-MP 6-TG, and 5-fluorouracil (5-FU) as the internal standards. The advantage of using UPLC-MS/MS is rapid analysis, as well as the selectivity and high sensitivity for detecting the test substance [8].

To date, a validated method of analyzing 6-MP and 6-TG in DBS using UPLC-MS/MS remains unavailable. Therefore, this study aimed to obtain and validate a method of bioanalysis of DBS using UPLC-MS/MS with 6-MP 6-TG, and 5-FU as internal standards. The optimum method will be validated to meet the requirements listed in the European Medicine Agency 2011. This method is expected to be applied for monitoring 6-MP drug therapy in pediatric patients with ALL.

MATERIALS AND METHODS

Materials
The reagents included 6-MP (Sigma-Aldrich), 6-TG (Sigma-Aldrich), 5-FU (Sigma-Aldrich), HPLC-grade acetonitrile (Merck), HPLC-grade formic acid in water, 0.1% formic acid in acetonitrile, and methanol with a gradient elution. Detection was performed using Waters Xevo TQD.
methanol (Merck), and formic acid (Merck). The other materials used were ultrapure water (Sartorius Water Filter), CAMAG DBS paper, and blood samples (Palang Merah Indonesia).

**Instruments**

UPLC-MS/MS (Waters Xevo TQD Triple Quadrupole) comprised quaternary solvent manager (Acquity UPLC H-Class); sample manager (Acquity UPLC); nitrogen generator compressor (PEAK Scientific); an amide acuity UPLC BEH Column (100 mm x 2.1 mm; 1.7-μm); mass analyzer in the form of triple quadruple (Xevo TQD) with ionization source (ZsprayTM); data processing software (Masslynx Software) and computer (Lenovo); analytical scales (AND); ultrasonic stirrer (Elmasonic); 4°C refrigerators; gas bleach (Elmasonic 540H); vortex (Maxi Mix II); centrifugator (Digiysystem DSC-300SD); freezer temperature at -20°C (Biomedical Labtech Deep Freezer); evaporator (TurboVap LV); sample tubes (Falcon); Eppendorf micropipes (Socorex); blue tip; yellow tip; and glassware.

**Stock solution**

The standard compounds 6-MP, 6-TG, and 5-FU, which were carefully weighed to a maximum of 5.0 mg each, were placed into a 5.0-mL measuring flask. Each substance was dissolved in 1N NH4OH to make a 2-mL solution; this was followed by addition of methanol to fill in the entire measuring flask. The standard compound solution obtained had a concentration of 1.0 μg/mL and was diluted accordingly to obtain a certain concentration. The solution was stored at 4°C until analysis.

**Preparation of DBS samples**

The DBS samples were placed on paper containing 6-MP and 6-TG at certain concentrations. The paper was then cut into 8-mm pieces, which were placed into 15-mL tubes with methanol to extract as much as 4 mL of solution. Thereafter, the solution was mixed with a vortex for 2 min, followed by sonication at 40°C for 20 min and centrifugation for 10 min at 3100 rpm. The supernatant was collected and placed into a test tube before making it evaporate to dry for 30 min at 40°C with the use of N2 gas. The obtained residue was then reconstituted with 100-μL 50% acetonitrile-water; the solution was mixed with a vortex for 2 min and underwent sonication for 2 min before it was transferred into the test tube. After centrifugation of the solution at 3100 rpm for 5 min, as much as 5 μL of the final solution was injected into the UPLC-MS/MS system.

**System suitability test**

As much as 5 μL of a mixture containing 1000 ng/mL of 6-MP and 6-TG with 100 μg/mL of 5-FU was injected into UPLC-MS/MS at a selected mobile phase, flow rate, and solvent. The injection was repeated 5 times. The coefficient of variation, ratio of peak area, and retention time of the analyte and internal standard were determined. System suitability test was defined to have qualified if the coefficient of variation was lower than 5%.

**Chromatography condition**

Chromatography was performed using an amide column (100 × 2.1 mm; 1.7 μm) aquity UPLC BEH. The analysis was performed in a mobile phase with elution gradient conditions as shown in Table 1.

The flow rate used was 0.2 mL/min at a temperature of 25°C. Mass detection was performed using Waters Xevo TQD with positive electrospray ionization (ESI) for 6-MP and 6-TG and negative ESI for 5-FU in multiple reaction monitoring mode. Detection of 6-MP, 6-TG, and 5-FU was 153.09+119.09, 168.09+107.06, and 129.15+42.05, respectively.

**Validation of analysis methods**

**Lower quantitation limit determination**

Five replicas were made using 25 ng/mL of 6-MP and 6-TG concentrations spotted on a DBS paper. The DBS sample preparation was performed at selected optimum conditions. A total of 5 μL of the final solution was injected into the UPLC-MS/MS system under selected chromatographic conditions. The values of % diff and % CV, with requirements not exceeding ±20%, were calculated.

**Linearity of calibration curve**

The following were prepared: (1) A blank sample (whole blood on a DBS paper without an internal standard); (2) one zero sample (blood on a DBS paper with an internal standard); and (3) non-zero samples of analytes, including lower limit of quantitation (LLOQ), with 6-MP at concentrations of 25, 50, 100, 200, 500, 800, and 1000 ng/mL and 6-TG at concentrations of 25, 50, 100, 200, 500, 800, and 1000 ng/mL. The concentration of each calibration curve was spotted on the DBS paper, which was cut into pieces and placed in a container with 100 μL of the internal standard 5-FU at a concentration of 100 μg/mL. Thereafter, as much as 5 μL of each solution was extracted and injected into the UPLC-MS/MS. The correlation coefficient of the linear regression equation was calculated to determine the linearity of the curve. In addition, the % diff was calculated with a limit value not exceeding ±15% for all concentration levels, except for LLOQ, which had a limit value of not exceeding ±20%.

**Selectivity**

A 40-μL whole blood blank was prepared and sample preparation was performed. Under selected conditions, as much as 5 μL of the final solution was injected into the UPLC-MS/MS system. Tests on this blank sample were done in duplicate using six blood samples collected from different sources. Next, blood containing a standard solution of 6-MP and 6-TG was prepared at LLOQ concentration; as much as 5 μL of the final solution was injected into the UPLC-MS/MS system. Testing of the LLOQ concentration was done in duplicate using six blood samples collected from different sources. The analyte and the standard were simultaneously observed for retention time and any interference from the blood extract; the response obtained was not >20% in the LLOQ analytes and not >5% in the internal standard.

**Carryover**

Carryover was performed by preparing the blank of DBS, and samples were prepared according to sample preparation conditions. The final solution was injected into the UPLC-MS/MS system after injecting the standard solution which contained 6-MP and 6-TG at the concentration of the upper limit of quantitation. Peak area of 6-MP, 6-TG, and the internal standard that appeared from the blank was observed. This test was replicated 5 times.

**Accuracy and precision**

Blank blood samples were mixed with a working solution at LLOQ concentration and a control solution at low-quality concentration (QCL), medium concentration, and high concentration (QCH) solution. Each mixture was added the internal standard with a concentration of 100-μg/mL and extracted according to the sample preparation procedure. Then, 5 μL of the final solution was injected into the UPLC-MS/MS system. This test was repeated for 5 times within the day (within-run) and between days (between-run). The % diff was calculated as the accuracy parameter and % CV was calculated as the precision parameter. The values of % diff and CV did not exceed ±15% for each concentration, except at LLOQ concentrations (i.e., not exceeding ±20%).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile phase A (%)</th>
<th>Mobile phase B (%)</th>
<th>Mobile phase C (%)</th>
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<tbody>
<tr>
<td>0.0</td>
<td>85</td>
<td>5</td>
<td>10</td>
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<tr>
<td>2.0</td>
<td>25</td>
<td>65</td>
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<td>3.0</td>
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<tr>
<td>4.0</td>
<td>85</td>
<td>5</td>
<td>10</td>
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</tbody>
</table>

*Mobile phase A= (Formic acid 0.2% in water solution), *mobile phase B= (formic acid 0.1% in acetonitrile solution), *methanol
Dilution integrity
A blood sample solution containing 6-MP and 6-TG at 2000 ng/mL, which was twice as the concentration of QCH, was prepared. Then, it was diluted with blank blood to get half and quarter concentrations. The diluted samples were prepared accordingly, and as much as 5 μL of the final solution was injected into the UPLC-MS/MS system. The test was repeated 5 times, and the corresponding parameters of accuracy and precision were determined.

Matrix effect
A total of 40 μL of blood blank was prepared and sample preparation process was done as described above. The supernatant was then mixed with 6-MP and 6-TG at QCL and QCH, as well as internal standard with a concentration of 100 μg/mL. A total of 5 μL of the final solution was injected into the UPLC-MS/MS system. The matrix effect was determined by calculating the matrix factor (MF), which was the ratio of area 6-MP, 6-TG, and internal standard after extraction compare to area of 6-MP, 6-TG, and internal standard without extraction or in solution. The normalized internal standard MF was calculated by dividing the analytic MF by the internal standard MF. Calculation of the %CV obtained did not exceed ±15% for each concentration.

Stability
Stability of the stock solution
The stock solution of 6-MP, 6-TG, and internal standard was prepared in a concentration of 1000 μg/mL. As much as 5 μL of each solution was injected into the UPLC-MS/MS system and then was stored at room temperature for 0, 6, and 24 h before analysis.

Short-term stability
DBS samples of 6-MP and 6-TG at QCL and QCH were prepared and stored at room temperature for 0, 6, and 24 h. The internal standard solution was added to the extracted solution, followed by sample preparation. A total of 5 μL of the solution was injected into the UPLC-MS/MS system. The instability of the substance was observed by calculating the % diff and shape of each chromatogram. The test was performed thrice for each concentration.

Long-term stability
DBS with 6-MP and 6-TG of QCL and QCH were prepared and stored at room temperature for 30 days. The internal standard was added to the extracted solution, followed by sample preparation. A total of 5 μL of the solution was injected into the UPLC-MS/MS system. The instability of the substance was observed by calculating the % diff and shape of each chromatogram. The test was performed thrice for each concentration.

Autosampler stability
DBS samples of 6-MP and 6-TG at QCL and QCH were prepared, injected, and left on the autosampler vials for 24 h. Analyses were done on the 0-h and 24-h time points. A total of 10 μL of the solution was injected into the UPLC-MS/MS system. The instability of the substance was observed by calculating the % diff and shape of each chromatogram. The test was performed thrice for each concentration.

RESULTS AND DISCUSSION
Test system sustainability
The CV values obtained were <2.0% in the area and in the retention time of each analyte area. The analysis time for each injection was 5 min.

The LLOQ
The LLOQ of 6-MP was determined as 25 ng/mL with a % diff value of -12.85% and %CV of 11.32%; these values met the requirements. When the LLOQ was decreased to half at 12.5 ng/mL, the obtained % diff of -11.84% and %CV of -13.25% did not meet the requirement of % diff of <20% to obtain an LLOQ concentration for 6-MP of 25 ng/mL. Meanwhile, the 25-ng/mL LLOQ value for 6-TG rendered a % diff value of -14.85–9.30% and % CV of 11.93%; these values met the requirements. When the LLOQ was decreased to half at 12.5 ng/mL, the obtained % diff of -14.85–9.30% and %CV of 11.93% did not meet the requirement of % diff of <20% to obtain the desired LLOQ concentration for 6-TG.

The calibration curves
The calibration curve of 6-MP yielded linear regression equation of
\[ y = 0.5789 + 0.0193x \]
with \( r^2 = 0.9981 \)

Where \( x \) represented the 6-MP concentration (ng/mL) and \( y \) represented the peak area ratio between 6-MP and the internal standard 5-FU.

The 6-TG calibration curve yielded a linear regression equation
\[ y = 0.4307 + 0.0222x \]
with \( r^2 = 0.9964 \)

The value of CV ranged from 0.67% to 12.84% (average \( r^2 = 0.9977 \) for 6-MP and from 0.66% to 9.94% (average \( r^2 = 0.9966 \) for 6-TG.

Accuracy and precision
Within-run measurement of the accuracy of 6-MP obtained a % diff value that ranged from -12.58% to +14.00% for LLOQ concentration and -10.66% to +12.63% for QC concentrations. Between-run measurement of the precision of 6-MP obtained CV values of 10.82% for LLOQ concentration and +9.70% for QC. Within-run measurement of the accuracy of 6-TG obtained a % diff value that ranged from -17.79% to +9.76% for LLOQ concentration and -6.22% s.d. +11.84% for QC concentrations. Within-run measurement of the precision of 6-TG obtained CV values of 13.25% for LLOQ concentration and -6.99% for QC. From the results obtained, the within-run tests for accuracy and precision of both analytes qualified for the % CV and % diff values of below 20% for LLOQ and below 15% for QC.

Between-run measurement of the accuracy of 6-MP obtained a % diff value that ranged from -15.48% to +17.81% for LLOQ concentration and -12.87% to +12.63% for QC concentrations. Between-run measurement of the precision of 6-MP obtained %CV values of 10.93% for LLOQ concentration and -8.32% for QC. The % diff and % CV values in both the between-run measurements met the accuracy and precision requirements. Between-run measurement of the accuracy of 6-TG obtained a % diff value that ranged from -18.61% to +1.52% for LLOQ concentration and -13.20% to +11.84% for QC concentrations. Between-run measurement of the precision of 6-MP obtained CV values of 13.25% for LLOQ concentration and -6.99% for QC. The % diff and % CV values in both between-run measurements met the accuracy and precision requirements.

Accuracy, which was represented by the value of % diff, was a parameter that measured the proximity between the measured concentrations in the analysis and the actual concentration; on the other hand, precision was a parameter that measured the repeatability of the analyte measurement. The accuracy and precision tests performed on both analytes for either the within- or between-run measures met the requirements set forth in the EMEA.

Selectivity
The results showed that the interference at LLOQ concentration was 7.81–14.22% for 6-MP; 1.49–6.91% for 6-TG; and 1.89–4.66% for the internal standard.

Carryover
The carryover yield was 7.46–15.02% for 6-MP; 0.48% s.d. 8.66% for 6-TG; and 1.89–4.66% for the internal standard.

Dilution integrity
The results of the dilution integrity test for 6-MP met the requirements, with % diff and % CV values of -6.73–3.85% and 4.45%, respectively.
for half-dilution, and −7.98–4.38% and 5.28%, respectively, for quarter dilution. Likewise, the results of the dilution integrity test for 6-TG met the requirements, with % diff and % CV values of −1.19–5.19% and 2.56%, respectively, for half-dilution, and −12.31–6.32% and 7.72%, respectively, for quarter dilution.

Test of matrix effects
In this study, the average MF and % CV for 6-MP were 0.76% and 8.89%, respectively, at a QCL concentration and 0.81% and 7.01%, respectively, at a QCH concentration. The average MF and % CV for 6-TG were 0.79% and 7.71%, respectively, at QCL concentration and 0.77% and 3.58%, respectively, at QCH concentration. The results obtained showed matrix values of <1, which indicated that there was suppression of the ion. The mechanism of ionizing or ion enhancement in UPLC-MS/MS depends on the sample matrix, sample preparation, and ionization type. ESI was more susceptible to the matrix effects than to the APCI. The most influential factors in the suppression or enhancement of ions in bioanalysis are the biological matrix and the extraction method [11-14]. The % CV values of both analytes met the EMEA-specific requirements (i.e., the % CV obtained from six different blood sources does not exceed 15%).

Stability test
Stability of the stock solution
The % diff and % CV values of the stock solutions at room temperature for 24 h were −2.00–1.96% and 0.03%, respectively, for 6-MP, −1.03–0.80% and 0.17%, respectively, for 6-TG, and −0.2% and 0.39%, respectively, for the internal standard. The data above fulfilled the requirements of % diff and % CV not exceeding ± 5%. Therefore, it can be concluded that all the stock solutions of both analytes and the internal standard were stable at room temperature for 24 h. In addition to testing at room temperature, stability test of the stock solution was performed at −20°C for 20 days. The long-term stability test results showed % diff and % CV values at −0.74–0.40% and 0.24%, respectively, for 6-MP, and −0.50%–0.12% and 0.27%, respectively, for 6-TG, and a % diff of −1.98–1.56% for the internal standard. From these data on long-term stability of the stock solution, we concluded that the stock solution remained stable for 20 days at −20°C.

Short-term stability
The results of the short-term stability test for 6-MP obtained % diff values of −6.58–8.94% at QCL and −12.50–3.73% at QCH. The results of the short-term stability test for 6-TG obtained % diff values of −6.74–9.63% at QCL and −12.94–9.94% at QCH. All the % diff values for both 6-MP and 6-TG were below 15%; therefore, it can be concluded that the analyte will be stable on DBS paper that has been dried for 24 h.

Long-term stability
From the results of the research, the values of % diff for both 6-MP at QCL (6.93–9.08%) and QCH (10.49–12.58%) and 6-TG at QCL (−1.69–3.71%) and QCH (1.49–8.03%) remained stable until the 16th day. It can be concluded that 6-MP and 6-TG can remain stable in DBS for up to a minimum of 16 days.

Post-preparation stability (autosampler)
The results of the stability tests of the samples in autosampler obtained % diff values of −5.66–10.95% at QCL and −4.29–3.92% at QCH for 6-MP and −7.24–6.07% at QCL and −8.60–1.83% at QCH for 6-TG. All the % diff values from both 6-MP and 6-TG were below 15%. Therefore, both can be deemed as stable solutions for storage in an autosampler for 24 h.

CONCLUSION
The developed UPLC-MS/MS method was useful and optimal for the determination of 6-MP and 6-TG in DBS using 5-FU as internal standard. These methods fulfilled the validation requirements of the EMEA bioanalytical guideline of 2011; met the linearity criteria of the calibration curve, LLOQ, accuracy and precision, dilution integrity, selectivity, carry over; stability and matrix effects; and can be applied to in vivo studies.

CONFLICTS OF INTEREST
All authors have none to declare.

REFERENCES