



Evaluation of Stability of Thiopurine Metabolites Using a Validated LC-MS/MS Method

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Measurement of thiopurine metabolites is helpful to monitor adverse effects and assess compliance in patients on thiopurine treatment. The purpose of this study was to develop and validate an analytical method for measurement of thiopurine metabolites, thioguanine nucleotides (6-TGN) and 6-methylmercaptopurine nucleotide (6-MMPN), in RBCs. We developed and validated a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the quantification of 6-TGN and 6-MMPN and evaluated the stability of the thiopurine metabolites in RBC and whole blood states without any preprocessing at various storage conditions. The linear range was 0.1–10 $\mu\text{mol/L}$ and 0.5–100 $\mu\text{mol/L}$ for 6-TGN and 6-MMPN, respectively. The mean extraction recovery at the two concentrations was 71.0% and 75.0% for 6-TGN, and 102.2% and 96.4% for 6-MMPN. Thiopurine metabolites in preprocessed RBC samples were stable at 25°C and 4°C after storage for 4 hours and at –70°C for up to 6 months. However, 6-TGN decreased by 30% compared with the initial concentration when stored at –20°C for 180 days. In whole blood states, 6-TGN decreased by about 20% at four days after storage at 4°C. We validated a reliable LC-MS/MS method and recommend that the patient's whole blood sample be preprocessed as soon as possible.

Key Words: Thiopurine metabolites, 6-Thioguanine nucleotide, 6-Methylmercaptopurine nucleotide, Mass spectrometry, Stability

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The thiopurines, 6-mercaptopurine (6-MP), azathioprine (AZA), and 6-thioguanine (6-TG), are immunosuppressive drugs used in the treatment of acute leukemia, inflammatory bowel disease (IBD), and other autoimmune diseases. Thiopurines are prodrugs that require activation by several drug-metabolizing enzymes, including thiopurine S-methyltransferase (TPMT) [1, 2]. 6-Thioguanine nucleotides (6-TGN) are formed from 6-MP by several enzymes, and 6-MP is inactivated by the action of TPMT, producing 6-methylmercaptopurine nucleotides (6-MMPN) [3]. Genetic polymorphisms and activities of enzymes involved in thiopurine metabolic pathways might determine the relative concentrations of 6-TGN and 6-MMPN related to thiopurine efficacy and various adverse effects, respectively [4]. Additionally, the

monitoring of thiopurine metabolites can be useful for adjusting thiopurine dosage to improve treatment outcome and avoid adverse effects [3]. Two thiopurine metabolites are currently measured in washed red blood cells (RBCs) as a surrogate to determine the intracellular levels of these metabolites [5, 6]. The therapeutic thresholds associated with high treatment efficacy (6-TGNs > 235 pmol/8 × 10⁸ RBCs), increased risk for leukopenia (6-TGNs > 450 pmol/8 × 10⁸ RBCs), and increased risk for hepatotoxicity (6-MMPNs > 5,700 pmol/8 × 10⁸ RBCs) can be used to determine an optimum dosage of thiopurines [7-10].

HPLC and liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been used to measure the concentrations of 6-TGN and 6-MMPN in RBCs [5, 11, 12]. As the metabolites

are unstable and can be altered during sample preparation and storage, control of every pre-analytical step is important to ensure reliable assay results. However, to our knowledge, stability of thiopurine metabolites has rarely been studied [13, 14]. For the first time in Korea, we developed and validated an LC-MS/MS method for the quantification of 6-TGN and 6-MMPN in RBCs. Additionally, we investigated the stability of thiopurine metabolites under various storage conditions to identify appropriate sample processing and storage conditions.

We prepared stock solutions of 3.0 mM 6-TGN and 30 mM 6-MMPN using standard materials (Sigma-Aldrich, St. Louis, MO, USA) and a stock solution of internal standards (IS) containing 1 mg/mL of each of 6-TG- $^{13}\text{C}_2^{15}\text{N}$ and 6-MMP- d_3 (Toronto Research Chemicals, Toronto, Canada) in methanol. These solutions were stored at -70°C . We diluted the stock solutions in 50 mM NaOH to prepare calibrators. We prepared the calibrators and control samples using whole blood (matrix-matched) spiked with the prepared drug standard solutions. Then, we validated the accuracy of our method for both metabolites. Six concentrations of calibrators (0.5, 2, 4, 6, 8, 10 $\mu\text{mol/L}$ for 6-TGN and 5, 20, 40, 60, 80, 100 $\mu\text{mol/L}$ for 6-MMPN) and quality-control materials at two concentrations for each analyte (2.5 $\mu\text{mol/L}$ and 8.0 $\mu\text{mol/L}$ for 6-TGN, and 25.0 $\mu\text{mol/L}$ and 80.0 $\mu\text{mol/L}$ for 6-MMPN) were measured during each analysis.

Residual EDTA-treated whole blood samples from patients under thiopurine treatment ($N=4$) were centrifuged (4,000 rpm, 10 minutes), and the plasma and buffy coat were discarded. This study was approved by the Institutional Review Board of Samsung Medical center, Seoul, Korea (IRB No. SMC-04-106-014). Two washing steps were performed, and the RBC count was determined using an automatic hematological cell counting device, XE-2100 (Sysmex Corp. Kobe, Japan), after diluting 100 μL of RBC three times in 200 μL of saline. The isolated RBCs were stored at -70°C until further analysis. After thawing the stored samples, 50 μL of each sample was transferred into a tube containing 200 μL of 0.2 M D,L-dithiothreitol (DTT) and 30 μL of working internal standards (0.5 mg/L for 6-MMPN and 0.125 mg/L for 6-TGN). After vortexing twice, the sample was rapidly deproteinized by the addition of 40 μL of 70% perchloric acid (GFS Chemicals, Columbus, Ohio, USA). After vortexing at the highest frequency, the deproteinized samples were centrifuged at 15,000 rpm for 10 minutes. The supernatant (100 μL) was transferred to a new tube that was sealed well and heated for one hour at 100°C to hydrolyze thiopurine nucleotides into their individual bases. After cooling, a 5- μL sample aliquot in 200 μL of mobile phase A of the chromatograph (water contain-

ing 2 mM ammonium acetate and 0.1% formic acid) was injected into the column as described below.

Chemical analyses were performed on an API 4000 tandem mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with an Agilent 1200 HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) and an electrospray ionization (ESI) source operated in positive-ion detection mode. Chromatographic separation of 6-TGN and 6-MMPN was conducted on an X-bridge Shield RP18 (Imtakt, Portland, OR, USA; 1.7 μm , 2.1×150 mm). The flow rate was 0.5 mL/min. Quantitative analysis was performed in multiple reaction-monitoring mode (m/z 168.0 \rightarrow 150.9 for 6-TGN, 158.0 \rightarrow 110.0 for 6-MMP, 171.0 \rightarrow 154.0 for 6-TG- $^{13}\text{C}_2^{15}\text{N}$, 161.1 \rightarrow 110.1 for 6-MMP- d_3), with a total running time of 3 minutes for each sample. Quantification was performed using the ratio of the integrated peak areas of 6-TGN and 6-MMPN to that of the IS, and calculations were performed using Analyst MD software (AB Sciex, Foster City, CA, USA) and a calibration curve with a weighting factor of $1/x$.

The chromatogram showed no interfering peaks for 6-TGN and 6-MMPN. For the IS, retention times were 0.94 minutes for 6-TG- $^{13}\text{C}_2^{15}\text{N}$ and 1.19 minutes for 6-MMP- d_3 (Fig. 1). The within-run ($N=5$) and between-run ($N=10$) precision and accuracy were determined at different concentrations of 6-TGN and 6-MMPN (2.5 $\mu\text{mol/L}$ and 8.0 $\mu\text{mol/L}$ for 6-TGN and 25.0 $\mu\text{mol/L}$ and 80.0 $\mu\text{mol/L}$ for 6-MMPN). Within- and between-run imprecisions evaluated by using quality control materials demonstrated coefficient of variation (CV) values less than 10% (see Supplemental Data Table S1). The linear assay range was 0.1–10 $\mu\text{mol/L}$ and 0.5–100 $\mu\text{mol/L}$ for 6-TGN and 6-MMPN, respectively ($R^2>0.999$ for both 6-TGN and 6-MMPN). The lower limit of quantitation (LLOQ) was defined as the lowest concentration with a signal to noise ratio of $>10\%$ and $<20\%$ of the CV. The LLOQ was 0.1 $\mu\text{mol/L}$ for 6-TGN and 0.5 $\mu\text{mol/L}$ for 6-MMPN. Matrix effects were evaluated by comparing the average peak areas of post-extraction blank samples spiked with 6-TGN and 6-MMPN to those of pure standard solutions of the corresponding concentrations. Samples were analyzed in quadruplicate at each concentration. The matrix effect ranged from 67.7% to 70.0% for 6-TGN and from 111.0% to 114.1% for 6-MMPN. The extraction recovery of 6-TGN and 6-MMPN was determined. The mean values of extraction recovery at two concentrations were 71.0% and 75.0% for 6-TGN, and 102.2% and 96.4% for 6-MMPN. Low extraction recovery of 6-TGN ($\sim 60\%$) was reported in a previous study [15].

Freeze-and-thaw stability was tested at two concentrations of 6-TGN and 6-MMPN (2.5 $\mu\text{mol/L}$ and 8.0 $\mu\text{mol/L}$ for 6-TGN

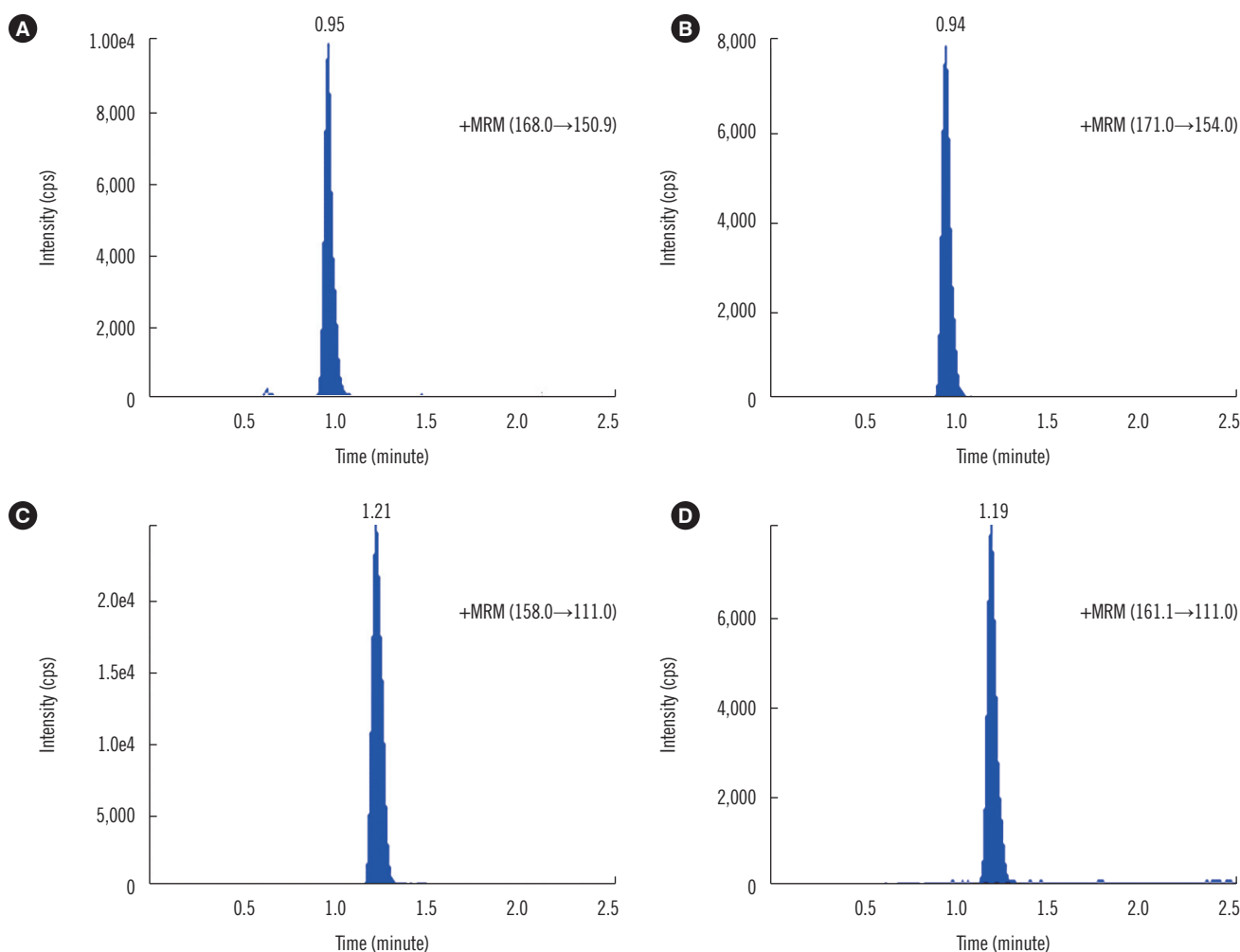


Fig. 1. Representative chromatograms of (A) 6-TGN, (B) 6-TG- $^{13}\text{C}_2^{15}\text{N}$ (IS for 6-TG), (C) 6-MMPN, and (D) 6-MMP- d_3 (IS for 6-MMP). Abbreviations: IS, internal standard; MRM, multiple-reaction monitoring; cps, counts per second.

and 25.0 $\mu\text{mol/L}$ and 80.0 $\mu\text{mol/L}$ for 6-MMPN) four times repeatedly. The materials were subjected to three freeze-and-thaw cycles (-70°C) with none of the materials showing significant changes in concentration; the decrease in concentration materials was less than 5% for both materials.

Short-term stability was investigated by analyzing two concentrations of each of 6-TGN and 6-MMPN (2.5 $\mu\text{mol/L}$ and 8.0 $\mu\text{mol/L}$ for 6-TGN and 25.0 $\mu\text{mol/L}$ and 80.0 $\mu\text{mol/L}$ for 6-MMPN) stored at 25°C and 4°C for 0.5, 1, 2, and 4 hours. There was no significant concentration change in either 6-TGN or 6-MMPN at either temperature after storage for 4 hours (Fig. 2). Long-term stability was investigated by analyzing samples stored at -70°C and -20°C until assayed (7, 14, 21, 28, 90, and 180 days). When stored at -70°C for 180 days, 6-TGN decreased to approximately 5% of the initial concentrations (2.5 $\mu\text{mol/L}$ and 8.0 $\mu\text{mol/L}$), but when stored at -20°C for 180 days, 6-TGN decreased by

30% compared with the initial concentrations (2.5 $\mu\text{mol/L}$ and 8.0 $\mu\text{mol/L}$). 6-MMPN decreased by approximately 10% of the initial concentration after 180 days at both temperatures and was rather stable (Fig. 2). Previously, Pike *et al* [14] reported that 6-TGN concentration decreased by about 12% after six months of storage at -80°C and that 6-MMPN showed almost no change in concentration after six months. Therefore, samples for long-term storage should be kept at a temperature lower than -70°C to prevent loss of 6-TGN.

Because whole blood samples cannot be pretreated immediately in a clinical laboratory, it is necessary to evaluate the stability of the thiopurine metabolites in the whole blood. To this end, we stored pooled patient blood samples ($N=4$) at room temperature (25°C) in the whole blood state and measured 6-TGN and 6-MMPN concentrations for up to seven days. For each concentration, four repeated measurements were conducted. Com-

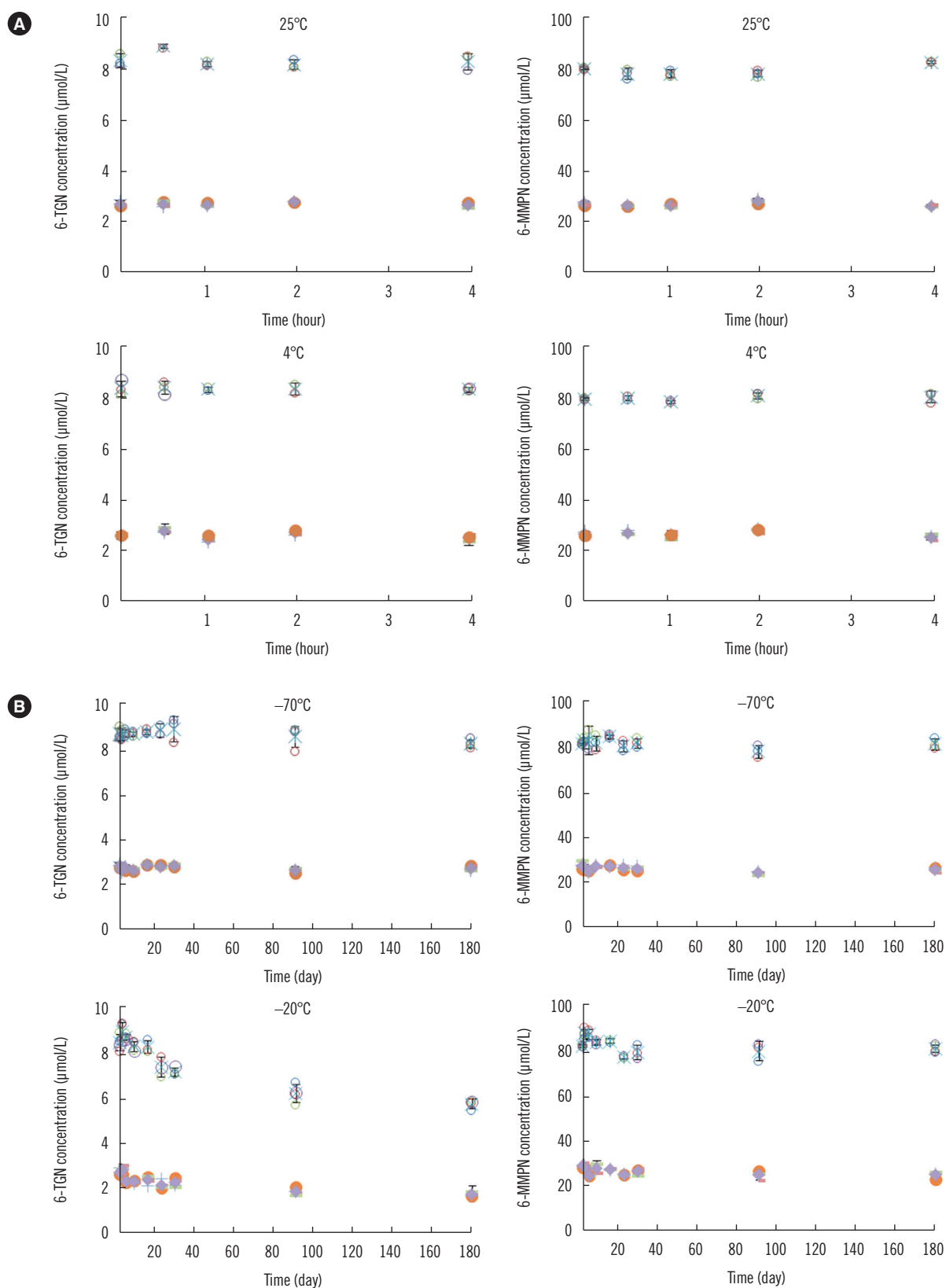


Fig. 2. Short-term (A) and long-term (B) stability of 6-TGN and 6-MMPN at different temperatures. Each dot represents a measurement (N=4). Abbreviations: 6-MMPN, 6-methylmercaptapurine nucleotide; 6-TGN, 6-thioguanine nucleotide.

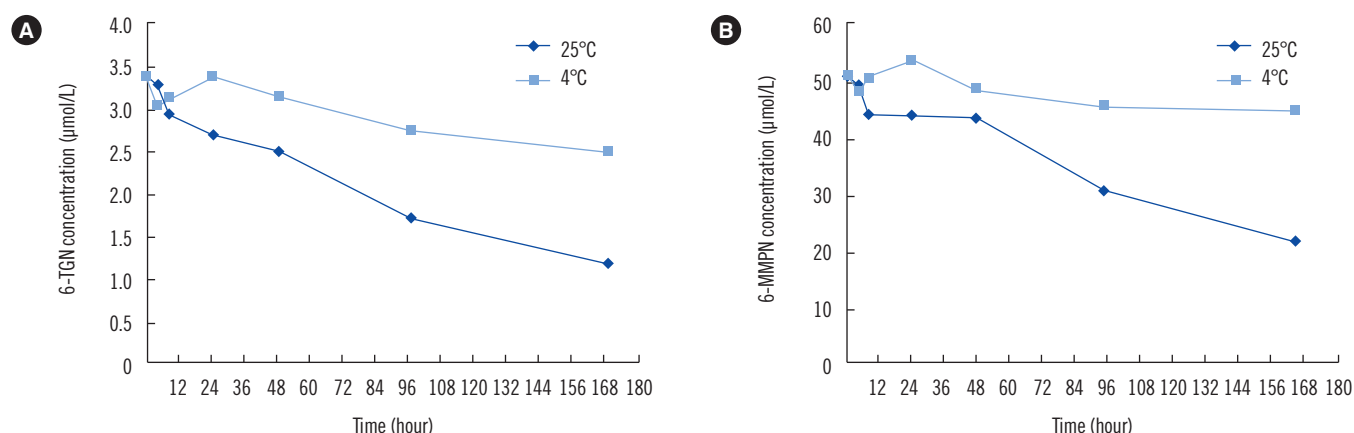


Fig. 3. Whole blood stability tests for (A) 6-TGN and (B) 6-MMPN. Abbreviations: see Fig. 2.

pared with the initial concentrations, 6-TGN decreased by more than 20% on day 2, and 6-MMPN decreased more than 40% on day 4 (Fig. 3). Although the degree of degradation is somewhat different, Pike *et al* [14] also found that the 6-TGN concentration decreased by 2–4% per day at room temperature in the whole blood state. Additionally, they found that the loss of 6-TGN was 1% after one week and 12% after 24 weeks at 80°C, indicating that 6-TGN is reasonably stable in whole blood, and 6-MMPN did not show a significant change when stored in the whole blood state for 24 weeks [14]. 6-TGN and 6-MMPN were relatively stable at 4°C without pretreatment. However, the 6-TGN concentration decreased by about 20% after four days of storage at 4°C. Therefore, pretreatment should be carried out within four days when samples are stored at 4°C.

In conclusion, we developed and validated a reliable LC-MS/MS method for quantification of RBC 6-TGN and 6-MMPN and comprehensively analyzed their stability. Based on the stability test results, we recommend –70°C for long-term storage of pretreatment samples. The whole blood sample should be preprocessed as soon as possible.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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Supplemental Data Table S1. Precision and accuracy of the LC-MS/MS method for the measurement of 6-TGN and 6-MMPN

Metabolite	Target ($\mu\text{mol/L}$)	Within-run (N=5)				Between-run (N=10)			
		Mean ($\mu\text{mol/L}$)	SD	CV (%)	Bias (%)	Mean ($\mu\text{mol/L}$)	SD	CV (%)	Bias (%)
6-TGN	2.5	2.7	0.1	4.5	8.8	2.8	0.2	7.8	10.3
	8.0	8.8	0.3	3.9	10.1	8.9	0.4	4.3	11.4
6-MMPN	25.0	26.9	1.0	3.9	7.5	27.7	1.4	5.1	10.8
	80.0	85.0	3.2	3.8	6.2	86.9	4.6	5.3	8.6

Abbreviations: CV, coefficient of variation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; 6-MMPN, 6-methylmercaptopurine nucleotide; SD, standard deviation; 6-TGN, 6-thioguanine nucleotide.