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# Therapeutic Monitoring of Thiopurine Metabolites: Validation of an HPLC Method and Preliminary Findings from a Small Cohort of Malaysian Patients with Inflammatory Bowel Disease

(Pemantauan Terapeutik Metabolit Tiopurina: Pengesahan Kaedah HPLC dan Penemuan Awal daripada Kohort Kecil Pesakit Malaysia dengan Penyakit Radang Usus)

SHENG ZHANG LIM[1], RAJA AFFENDI RAJA ALI[2], SUZANA MAKPOL[3] & ENG WEE CHUA[1,*]

*[1]Centre for Herbal and Drug Development, Faculty of Pharmacy, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Federal Territory, Malaysia*
*[2]Department of Medicine, Faculty of Medicine, Universiti Kebangsaan Malaysia, 56000 Cheras, Kuala Lumpur, Federal Territory, Malaysia*
*[3]Department of Biochemistry, Faculty of Medicine, Universiti Kebangsaan Malaysia, 56000 Cheras, Kuala Lumpur, Federal Territory, Malaysia*

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ABSTRACT

Thiopurine therapy of inflammatory bowel disease (IBD) is guided by the relative blood concentrations 6-thioguanine nucleotides (6-TGN) and 6-methylmercaptopurine (6-MMP). However, their action is altered by *in vivo* phosphorylation, and this is not normally measured in clinical studies. Hence, we trialled a novel method for profiling phosphorylated thiopurine metabolites and revisited the association between thiopurine metabolites and IBD treatment outcomes. We first optimised and validated a published high-performance liquid chromatography (HPLC) method for measuring the blood levels of thioguanosine monophosphate (TGMP), thioguanosine diphosphate (TGDP), thioguanosine triphosphate (TGTP), and methylthioinosine monophosphate (MeTIMP). Then, we assembled a small cohort of IBD patients ($n$ = 20), who had been treated with azathioprine for at least three months, and obtained blood samples for analysis of the metabolites. The patients received treatments at the Universiti Kebangsaan Malaysia Specialist Centre between March 2018 and April 2019. They were classified as responders ($n$ = 12) or non-responders ($n$ = 6) to azathioprine based on their disease activity scores (CDAI or Mayo score). The HPLC method was precise with intraday and interday variation < 15% for all the tested metabolites, and the relative accuracy ranged from 40.2 to 114.0%. We noted that the responders had higher median 6-TGN but lower median TGTP levels than the non-responders. However, the differences were not statistically significant (Wilcoxon rank-sum tests; 6-TGN, $p$ = 0.925; TGTP, $p$ = 0.189). The higher median 6-TGN level detected in the responders is in keeping with the findings of prior studies, suggesting that HPLC analysis of phosphorylated thiopurine metabolites is both technically feasible and clinically useful.

Keywords: High-performance liquid chromatography; inflammatory bowel disease; therapeutic drug monitoring; thiopurine metabolites

ABSTRAK

Rawatan tiopurina untuk penyakit radang usus (IBD) adalah berpandukan kepekatan nukleotida 6-tioguanina (6-TGN) dan 6-metilmerkaptopurina (6-MMP) dalam darah. Akan tetapi, tindakan metabolit tiopurina berubah-ubah mengikut tahap fosforilasi *in vivo* dan fenomena ini sukar dikesan secara tepat dalam kajian klinikal. Oleh itu, kami menguji satu kaedah baharu bagi pengesanan metabolit tiopurina terfosforilasi dan mengkaji semula hubungan antara metabolit tiopurina dan hasil rawatan IBD. Kami mengoptimumkan dan menilai prestasi satu kaedah kromatografi cecair prestasi tinggi (HPLC) yang dilaporkan dalam kajian lepas untuk penentuan paras darah tioguanosina monofosfat (TGMP), tioguanosina difosfat (TGDP), tioguanosina trifosfat (TGTP) dan metiltioinosina monofosfat (MeTIMP). Kemudian, kami merekrut sekumpulan kecil pesakit IBD ($n$ = 20) yang telah dirawat dengan azatioprina untuk sekurang-kurangnya tiga bulan dan memperoleh sampel darah untuk analisis paras metabolit. Pesakit-pesakit tersebut menerima rawatan di Pusat Perubatan

Universiti Kebangsaan Malaysia antara Mac 2018 dan April 2019. Mereka dikelaskan kepada kumpulan pesakit yang menunjukkan respons baik terhadap azatioprina, atau responden ($n$ = 12) dan kumpulan yang gagal rawatan, atau bukan responden ($n$ = 6), berdasarkan aktiviti penyakit (CDAI atau skor Mayo). Kaedah HPLC tersebut didapati tepat dengan variasi dalam tempoh sehari dan antara hari < 15% bagi semua metabolit dan ketepatan relatif antara 40.2% dan 114.0%. Kami mendapati bahawa kumpulan pesakit responden mempunyai paras median 6-TGN yang lebih tinggi tetapi paras median TGTP yang lebih rendah daripada pesakit bukan responden. Walau bagaimanapun, perbezaan tersebut tidak signifikan secara statistik (ujian Wilcoxon; 6-TGN, $p$ = 0.925; TGTP, $p$ = 0.189). Selaras dengan kajian terdahulu, kajian ini mendapati bahawa paras median 6-TGN adalah lebih tinggi dalam kalangan pesakit responden. Ini mencadangkan bahawa analisis HPLC metabolit tiopurina terfosforilasi boleh dilaksanakan dan adalah berguna untuk amalan klinikal.

Kata kunci: Kromatografi cecair berprestasi tinggi (HPLC); metabolit tiopurina; pemantauan ubat terapeutik; penyakit radang usus

## Introduction

Mercaptopurine, azathioprine, and thioguanine, also known collectively as 'thiopurines', structurally resemble endogenous purines. They are commonly used in the treatment of acute lymphoblastic leukaemia and inflammatory bowel disease (IBD). Inside cells, thiopurines are converted by successive metabolic steps into 6-thioguanine nucleotides (6-TGNs), which contribute to the immunosuppressive effects of the drugs (Figure 1). Two other pathways that compete with the formation of 6-TGNs, namely methylation and oxidation of thiopurines, are mediated by thiopurine methyltransferase

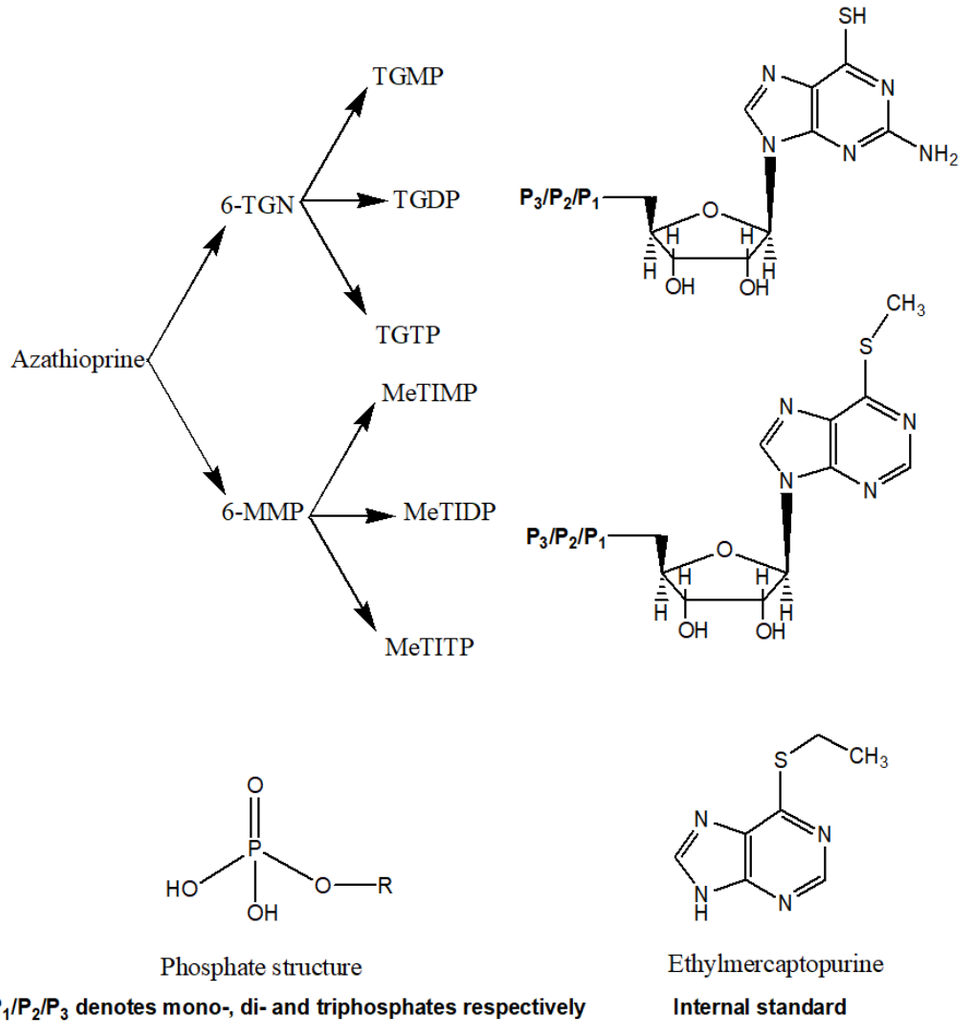


FIGURE 1. The metabolites of azathioprine

(TPMT) and xanthine dehydrogenase, respectively. The incorporation of deoxy-TGNs into DNA and suppression of GTPase Rac1 by thioguanosine triphosphate (TGTP) are the primary mechanisms suggested to cause cell apoptosis and inhibit the proliferation of T-lymphocytes (Zaza et al. 2010). Methylthioinosine monophosphate (MeTIMP), a putative toxic metabolite, may also be important to thiopurine effects and toxicity, as it was found to be a potent inhibitor of *de novo* purine synthesis and cell growth (Karim et al. 2013).

Proactive monitoring of thiopurine metabolites is recommended in patients who experience toxicity or exhibit subtherapeutic responses (Feuerstein et al. 2017; Vande Casteele et al. 2017). However, thiopurine metabolites are usually measured using a method that cannot distinguish the mono-, di-, and triphosphates of TGNs and methylmercaptopurines (Lennard & Singleton 1992). TGTP is the predominant phosphorylated form of thiopurines in red blood cells (RBCs) and is responsible for the bioactivity of the drugs. Measuring the levels of TGTP and the other minor phosphorylated metabolites may provide additional insights into the association between metabolite levels and treatment efficacy (Vikingsson et al. 2009). A threshold serum TGTP level of 100 pmol/8 × $10^8$ RBC was found to predict positive responses to treatments, and an elevated fraction of thioguanosine diphosphate (TGDP) attenuated therapeutic response (Neurath et al. 2005).

In this study, we revisited the relationship of thiopurine metabolites with therapeutic response in IBD patients, using an HPLC method that can distinguish the phosphorylated thiopurine metabolites (Vikingsson et al. 2013). We first validated the HPLC method in-house to ensure it was precise and accurate. Then, we recruited a small cohort of IBD patients treated with azathioprine and measured their serum metabolite levels using the validated HPLC method. Through a series of statistical analyses, we assessed the association between metabolite levels and the outcomes of IBD treatment.

## Materials and Methods

### Chemicals and Reagents

Thioguanosine monophosphate (TGMP), TGDP, TGTP, and MeTIMP were purchased from Jena Bioscience GmbH (Germany). Ethylmercaptopurine and tetrabutylammonium bisulphate were purchased from Merck Sdn. Bhd. (Malaysia). The purity of all the analytes was ≥ 95%. Potassium permanganate, 30% hydrogen peroxide, sodium bicarbonate, sodium chloride, sodium hydroxide, phosphoric acid, dichloromethane, acetonitrile, and methanol were purchased from Fisher Scientific (Malaysia). All the reagents were of the HPLC grade.

The internal standard (150 µM ethylmercaptopurine in 50 mM phosphoric acid, pH adjusted to 7.4 with sodium hydroxide), precipitation solution (dichloromethane-methanol 10:35 v/v), derivatisation solution (0.5 M sodium bicarbonate with its pH adjusted to 10.5 with sodium hydroxide), potassium permanganate (1% w/w), and hydrogen peroxide (10% w/w) were freshly prepared before the experiments. Drug-free blood used for preparing the standards, controls, and validation samples was obtained from healthy volunteers.

### Chromatography

The HPLC system consisted of a Waters 600 Controller module, a 2707 Autosampler, a 2998 Photodiode Array Detector, and a 2475 Multi-Wavelength Fluorescence Detector (Waters Analytical Instrument Sdn. Bhd., Malaysia). The analytes were separated on a Phenomenex Gemini C18 column (150 × 4.6 mm, 5 µM) with a Phenomenex Gemini C18 guard column (4 × 3 mm) – both purchased from LT Resources Sdn Bhd (Malaysia).

Table 1 details the HPLC gradient. Mobile phase A consisted of phosphoric acid (40 mM), tetrabutylammonium sulphate (5 mM), and acetonitrile (1% v/v; pH adjusted to 5 with sodium hydroxide). Mobile phase B was a mix of phosphoric acid (20 mM), tetrabutylammonium sulphate (5 mM), and acetonitrile (26% v/v; pH adjusted to 5 with sodium hydroxide). TGMP, TGDP, and TGTP were detected by fluorescence (excitation 329 nm, emission 403 nm, gain 10), while ethylmercaptopurine and MeTIMP were detected by UV absorbance at 289 nm.

### Standards and Controls

Six standard solutions of varied concentrations were prepared in MilliQ water and stored at -20 °C. To prepare the working standards, 10 µL of the standard solution was mixed with 10 µL of the internal standard (300 µM ethylmercaptopurine) and 80 µL of a blank matrix. The concentrations of the standard solutions are given in Table 2.

TABLE 1. Gradient profile

| Time (min) | Mobile Phase A (%) | Mobile Phase B (%) |
|---|---|---|
| 0 | 100 | 0 |
| 0.1 | 85 | 15 |
| 12.5 | 78 | 22 |
| 24.0 | 20 | 80 |
| 31.0 | 20 | 80 |
| 31.2 | 100 | 0 |
| 35.0 | 100 | 0 |

TABLE 2. The concentrations of the standard solutions

| Metabolites | Concentration (pmol/100 µL lysate) | | | | | |
|---|---|---|---|---|---|---|
| | Level 1 | Level 2 | Level 3 | Level 4 | Level 5 | Level 6 |
| TGMP | 0.30 | 10 | 21.25 | 85 | 170 | 340 |
| TGDP | 1.80 | 10 | 50 | 250 | 1000 | 2000 |
| TGTP | 1.80 | 10 | 50 | 250 | 1000 | 2000 |
| MeTIMP | 30 | 50 | 100 | 1125 | 2250 | 4500 |

TGMP: thioguanosine monophosphate; TGDP: thioguanosine diphosphate; TGTP: thioguanosine triphosphate; MeTIMP: methylthioinosine monophosphate

Three levels of quality control solutions were prepared in MilliQ water and stored at -20 °C. To prepare the control samples, 10 µL of the control solution was mixed with 10 µL of the internal standard (300 µM ethylmercaptopurine) and 80 µL of a blank lysate. Considering the rapid interconversion between TGTP and TGDP (Vikingsson et al. 2013), we prepared the control solutions of only TGMP and TGTP. Table 3 shows the concentrations of quality control samples used in the method validation.

TABLE 3. The concentrations of the quality control samples

| Metabolites | Concentration (pmol/100 µL lysate) | | |
|---|---|---|---|
| | Low | Medium | High |
| TGMP | 1 | 15 | 125 |
| TGTP | 20 | 300 | 240 |
| MeTIMP | 50 | 200 | 1000 |

TGMP: thioguanosine monophosphate; TGTP: thioguanosine triphosphate; MeTIMP: methylthioinosine monophosphate

## EXTRACTION PROCEDURE

The HPLC method was adapted from a published protocol (Vikingsson et al. 2013). To maintain the stability of the analytes, the samples were kept on ice throughout the extraction procedure. Blood samples were drawn using 5 mL EDTA tubes. Erythrocytes were extracted from whole blood, washed, and resuspended with sodium chloride (0.9% w/v). The resultant

erythrocyte suspension, termed blank lysate when it was obtained from a healthy volunteer and devoid of the target analytes, was stored at -80 °C prior to use in subsequent analysis.

In preparation for HPLC, the blood lysate was thawed on ice, and its haemoglobin (Hb) content was measured with the HemoCue Hb 201+ system. Then, 80 µL of the lysate was mixed with 20 µL of a cold internal standard solution and 175 µL of a cold precipitation solution. The resultant suspension was vortexed for 3 s and centrifuged at 17,530 × g and 4 °C for 5 min; 100 µL of the supernatant was transferred to a fresh tube and stored at -20 °C. This was the blank matrix used to prepare the working standards.

Then, the analytes in the lysate were converted into fluorescent derivatives by the addition of the derivatisation solution. After 3 min of incubation, 20 µL of hydrogen peroxide (10% w/w) was added to induce the formation of a precipitate, which was later removed by centrifugation at 17,530 × g and 4 °C for 5 min. Finally, 15 µL of the supernatant was injected into the chromatographic system. An overview of the sample preparation procedure is shown in Figure 2.

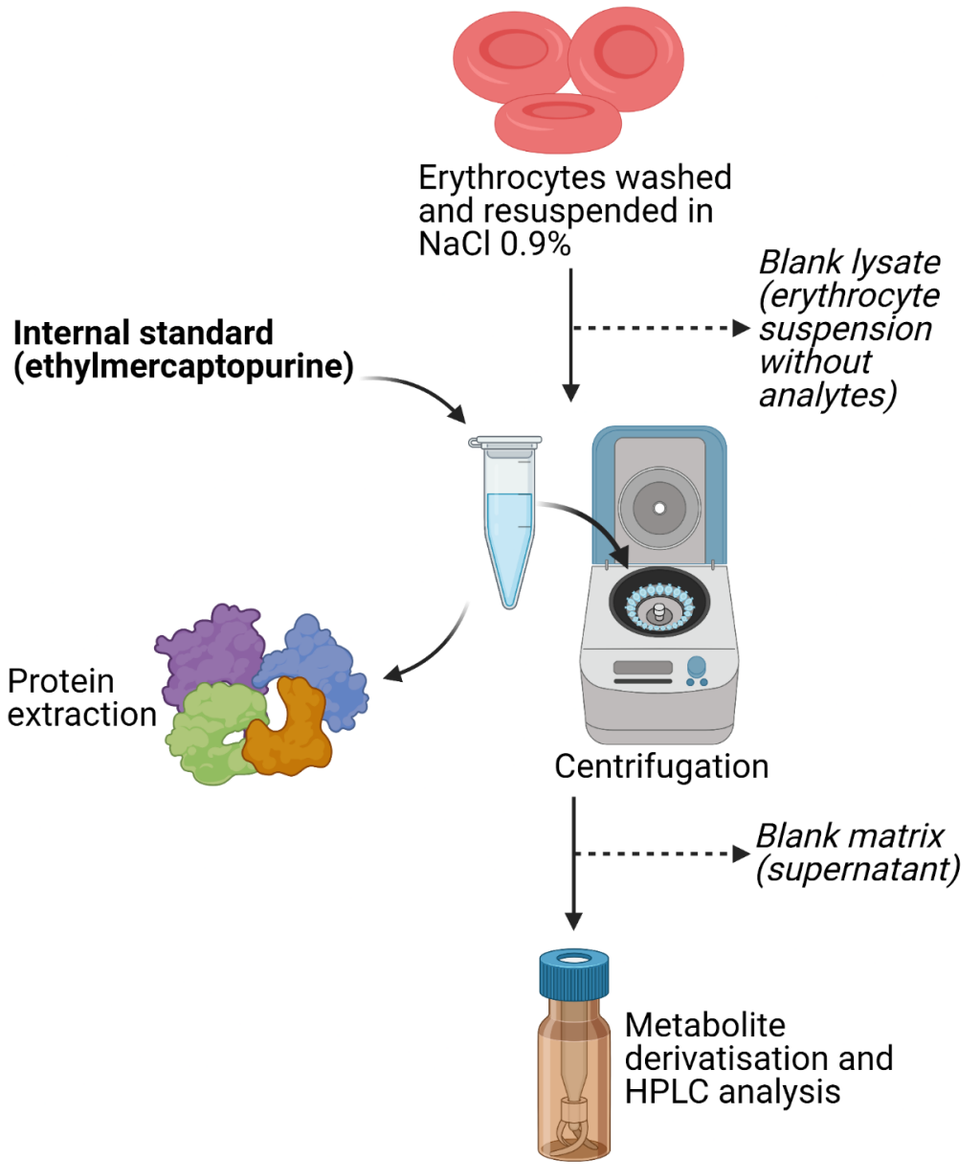


FIGURE 2. Sample preparation prior to HPLC analysis
(Created with BioRender.com)

### METHOD VALIDATION

The method validation was based on the U.S. FDA's Bioanalytical Method Validation Guidance for Industry (FDA 2018). We did not perform a full validation, as the method (Vikingsson et al. 2013) was intended for use in only research and not in establishing a clinical diagnosis of thiopurine resistance. Instead, we performed a partial validation of the method to ensure that it was precise and accurate.

### PRECISION AND ACCURACY

Three technical replicates of each of the controls were analysed in the same run to estimate within-run precision. Also, the controls were analysed on three different days to estimate between-day precision and accuracy.

### METABOLITE MONITORING STUDY

This is a prospective, observational study involving 20 IBD patients treated with azathioprine and a preliminary analysis of the relationship between the blood concentrations of phosphorylated thiopurine metabolites and therapeutic response. The patients were recruited from the Universiti Kebangsaan Malaysia Specialist Centre by convenience sampling between March 2018 and April 2019. Eligible participants were identified from patients receiving treatments or returning for follow-up visits at the centre for a variety of gastrointestinal or liver diseases. Before clinical consultations, two of the investigators (one of them a gastroenterologist) screened the patients, assessed their eligibility, and obtained their informed consent for participating in the study.

All the patients were ≥ 18 years old and had been treated with azathioprine for at least three months so that the blood levels of thiopurine metabolites had reached the steady state (Derijks et al. 2004; Pozler et al. 2010; Relling et al. 2011). Patients who were also on corticosteroids, biologics, or allopurinol were excluded. Therapeutic response was evaluated based on the patients' disease activity scores. Patients with Crohn's Disease Activity Index (CDAI) < 150 (Best et al. 1976) or total Mayo score ≤ 2 without any of the criteria scored > 1 (D'Haens et al. 2007; Rutgeerts et al. 2005) were classified as having achieved remission i.e., *responders*. Patients who had active disease despite receiving standard doses of azathioprine were classified as *non-responders*. The study was approved by the Research Ethics Committee (Human) of National University Malaysia (Reference: UKM PPI/111/8/JEP-2016-693).

### STATISTICAL ANALYSIS

The correlation between metabolite levels and drug responsiveness was assessed by the Spearman's rank-order correlation test. The Wilcoxon rank-sum test was used to analyse the differences in metabolite levels between the responders and non-responders. All the statistical analyses were performed using SAS University Edition v9.4M6 (SAS Institute Inc., Cary, NC). A $p$-value of $< 0.05$ was considered statistically significant.

## RESULTS

### CHROMATOGRAM

Figures 1 and 2 show the chromatograms of the analytes detected by fluorescence and UV absorbance, respectively. All the analytes were well separated with well-defined peaks, indicating satisfactory selectivity and sensitivity. Although some baseline noise was observed throughout the UV chromatogram, it did not affect the detection and quantification of the internal standard and MeTIMP. The retention times of TGMP, TGDP, and TGTP were 5.06, 17.2, and 29.06 min, respectively. TGDP and TGTP were retained longer in the column than TGMP because of the steric hindrance caused by the higher number of phosphate groups. The retention time of ethylmercaptopurine (internal standard) was 25.72 min and that of MeTIMP was 26.69 min. The internal standard and MeTIMP had similar retention times owing to their chemical properties. Both compounds are non-polar because of the alkyl functional groups in their structures.

### PRECISION AND ACCURACY

The HPLC method was found to be precise. As shown in Table 4, none of the coefficients of variation for gauging the within-run and between-day precision exceeded 15%, which was the acceptable cut-off recommended by the FDA (FDA 2018). Overall, the coefficients of variation were slightly higher than those reported by Vikingsson et al. (2013), most probably owing to variation related to the detectors and the analytical column.

The accuracy of the method ranged from 17.6 to 56.4%. We noted a significant improvement in the accuracy after correction for analyte loss, which resulted in relative accuracy ranging from 40.2 to 114.0% (Table 5).

TABLE 4. Within-run precision (a) and between-day precision (b) gauged using quality control samples of low, medium, and high concentrations

| | TGMP | TGDP | TGTP | MeTIMP |
|---|---|---|---|---|
| (a) Within-run precision [a], CV (mean, range), pmol/100 µL lysate | | | | |
| Low | 8.9 (1.16, 1.05-1.24) | 12.2 (4.18, 3.72-4.72) | 6.7 (8.49, 7.92-9.06) | 8.7 (95.23, 92.96-97.49) |
| Medium | 10.6 (11.91, 10.81-13.29) | 9.8 (41.11, 38.28-45.71) | 12.4 (121.47, 105.31-135.22) | 4.4 (266.31, 255.43-278.82) |
| High | 12.7 (63.33, 56.58-72.27) | 5.0 (549.79, 518.94-571.74) | 5.7 (455.79, 462.20-471.11) | 2.6 (1359.34, 1322.32-1393.95) |
| (b) Between-day precision [b], CV (mean, range), pmol/200 µL lysate | | | | |
| Low | 3.2 (1.18, 1.21-1.16) | 8.4 (4.09, 3.84-4.34) | 9.1 (7.92, 7.43-8.43) | 4.1 (92.96, 90.29-95.63) |
| Medium | 3.4 (11.62, 11.23-11.99) | 3.3 (39.33, 38.71-39.94) | 1.2 (135.22, 133.06-137.41) | 0.2 (278.82, 277.2-280.45) |
| High | 1.2 (61.16, 60.32-62.0) | 0.1 (558.68, 561.40-555.98) | 0.6 (426.20, 426.85-425.56) | 4.9 (1322.22, 1284.17-1360.04) |

[a] Number of replicates, $n = 3$; [b] The experiments were repeated on three different days, each with three replicates; CV: coefficient of variation; TGMP: thioguanosine monophosphate; TGDP: thioguanosine diphosphate; TGTP: thioguanosine triphosphate; MeTIMP: methylthioinosine monophosphate

TABLE 5. Between-day accuracy (a) and relative accuracy calculated using data obtained from the internal standard (b)

| | TGMP | TGDP +TGTP | MeTIMP |
|---|---|---|---|
| (a) Accuracy | | | |
| Low | 56.4% | 31.0% | 33.2% |
| Medium | 41.0% | 30.4% | 36.0% |
| High | 22.0% | 17.6% | 31.1% |
| (b) Relative accuracy | | | |
| Low | 114% | 63.3% | 75.1% |
| Medium | 77.1% | 54.1% | 71.9% |
| High | 50.6% | 40.2% | 62.2% |

TGMP: thioguanosine monophosphate; TGDP: thioguanosine diphosphate; TGTP: thioguanosine triphosphate; MeTIMP: methylthioinosine monophosphate

### PATIENT CHARACTERISTICS

A total of 20 patients were recruited. Two of the patients were excluded because they had 6-TGN levels < 100 pmol/8 × $10^8$ RBC and were probably not compliant with treatments (Selinger et al. 2019). The remaining 18 patients were divided into two groups based on the outcomes of azathioprine treatment i.e. having achieved remission (responders; $n$ = 12) or with active disease (non-responders; $n$ = 6; Table 6). The mean age of the patients was 42; 13 of the patients were diagnosed with Crohn's disease (CD), and five had ulcerative colitis (UC). None of the patients reported any adverse drug reactions. We found that azathioprine was not effective for most of the UC patients, with 4 out of 5 patients still having active disease after six months of azathioprine treatment.

TABLE 6. Patient characteristics

| Characteristics | Total ($n$ = 18) | Remission [a] ($n$ = 12) | Active disease [a] ($n$ = 6) |
|---|---|---|---|
| Age (years), mean (SD) | 42.77 (17.99) | 44.58 (19.62) | 39.16 (15.19) |
| Gender, $n$ (%) | | | |
| Male | 10 (55.6) | 6 (50) | 4 (66.7) |
| Female | 8 (44.4) | 6 (50) | 2 (33.3) |
| Ethnicity, $n$ (%) | | | |
| Malay | 11 (61.1) | 8 (66.6) | 3 (50) |
| Chinese | 3 (16.7) | 2 (16.7) | 1 (16.7) |
| Indian | 4 (22.2) | 2 (16.7) | 2 (33.3) |
| Body weight (kg) | 60.11 ± 9.88 | 59.89 ± 8.96 | 60.55 ± 12.44 |
| Azathioprine dose (mg) | 90.28 ± 38.48 | 77.08 ± 37.62 | 116.67 ± 25.82 |
| Disease subtype, $n$ (%) | | | |
| CD | 13 (72.2) | 11 (91.7) | 2 (33.3) |
| UC | 5 (27.8) | 1 (8.3) | 4 (66.7) |
| Concurrent 5-aminosalicylate, $n$ (%) | 6 (33.3) | 2 (16.7) | 4 (66.7) |

[a] Disease status after azathioprine treatment of at least six months; SD: standard deviations; CD: Crohn's disease; UC: ulcerative colitis

### CORRELATION BETWEEN METABOLITE CONCENTRATIONS AND DISEASE ACTIVITY INDICES

Because of the small sample size, the statistical analysis was conducted using non-parametric tests. The Spearman's rank-order correlation test was used to examine the relationship between metabolite concentrations and disease activity indices. Table 7 shows the correlation between the mean concentrations of the metabolites and the disease activity scores. We found that TGMP had a significant inverse correlation with CDAI (Table 7; $r$ = -0.58; $p$ = 0.047). The other metabolites were not found to be significantly correlated with any of the disease activity indices.

TABLE 7. The Spearman's rank-order correlation analysis of the relation between metabolite concentrations (pmol/30 mg Hb) and disease activity scores. The metabolite concentrations are presented as means alongside standard deviations in parentheses

| Metabolites [a] | CDAI ($n$ = 13) | Correlation coefficient, r ($p$-value) | Mayo score ($n$ = 5) | Correlation coefficient, r ($p$-value) |
|---|---|---|---|---|
| 6-TGN | 186.78 (98.64) | -0.09 (0.747) | 118.01 (77.1) | 0.41 (0.492) |
| TGMP | 31.43 (41.62) | -0.58 (0.047) | 14.09 (17.45) | -0.66 (0.218) |
| TGDP | 69.6 (35.95) | 0.02 (0.929) | 33.63 (18.47) | 0.41 (0.492) |
| TGTP | 88.17 (74.10) | 0.19 (0.517) | 70.29 (44.28) | 0.41 (0.492) |
| MeTIMP | 609.12 (637.36) | −0.01 (0.957) | 1012.11 (1586.39) | 0.31 (0.683) |

[a] TGMP, TGDP, and TGTP are phosphorylated forms of 6-TGN; CDAI: Crohn's disease activity index; 6-TGN: 6-thioguanine nucleotides; TGMP: thioguanosine monophosphate; TGDP: thioguanosine diphosphate; TGTP: thioguanosine triphosphate; MeTIMP: methylthioinosine monophosphate

### COMPARISON OF METABOLITES CONCENTRATIONS BETWEEN PATIENTS

The Wilcoxon rank-sum test was used to compare the metabolite concentrations between the responders and the non-responders. The patients who had achieved remission were found to have a higher median 6-TGN level but a lower TGTP concentration than those who had failed the azathioprine treatment. However, the difference was not statistically significant (Table 8; 6-TGN, $p$ = 0.925; TGTP, $p$ = 0.189).

TABLE 8. Comparison of the metabolite concentrations (pmol/30mg Hb) between azathioprine responders and non-responders by the Wilcoxon rank-sum test. The metabolite concentrations are presented as medians alongside values for the interquartile range in parentheses

| Metabolite [a] | Responders [b] ($n$ = 12) | Non-responders [c] ($n$ = 6) | Two-sided $p$-value (Pr> \|Z\|) |
|---|---|---|---|
| 6-TGN | 182.29 (80.36-211.55) | 121.35 (107.031-216.378) | 0.925 |
| TGMP | 20.70 (5.99-26.19) | 7.20 (3.99-15.932) | 0.159 |
| TGDP | 60.43 (33.05-94.54) | 41.49 (30.38-61.39) | 0.707 |
| TGTP | 48.39 (34.37-74.92) | 75.04 (57.94-125.45) | 0.189 |
| MeTIMP | 229.73 (139.65-1085.18) | 313.61 (224.17-1022.36) | 0.527 |

[a] TGMP, TGDP and TGTP are phosphorylated forms of 6-TGN; [b] the responders were patients who achieved remission after six months of treatment; [c] the non-responders were patients whose disease status remained active after six months of treatment; 6-TGN: 6-thioguanine nucleotides. TGMP: thioguanosine monophosphate; TGDP: thioguanosine diphosphate; TGTP: thioguanosine triphosphate; MeTIMP: methylthioinosine monophosphate

## DISCUSSION

Proactive monitoring of metabolite concentrations to guide thiopurine dosage is a standard clinical practice for patients with active IBD or those experiencing toxicity while receiving thiopurine treatments (Feuerstein et al. 2017; Vande Casteele et al. 2017). However, patients who achieve therapeutic 6-TGN levels do not always respond favourably to thiopurines, and this can be due to ethnicity- and age-related variation in thiopurine sensitivity. The problem is complicated further by the existence of phosphorylated metabolites that produce (sometimes) contrasting pharmacological effects, resulting in complex variation in treatment outcomes. Therefore, we used an HPLC method (Vikingsson et al. 2013) that can distinguish the phosphorylated forms of 6-TGN and MeTIMP to revisit the relationship between metabolite concentrations and the effectiveness of thiopurine treatments in IBD patients. We validated the method in-house before trialling it in a group of IBD patients treated with azathioprine.

Given their polarity, all the nucleotides were expected to be poorly retained in reversed phase chromatography (Vikingsson et al. 2010). However, in our study, all the analytes were well separated, as shown in Figures 1 and 2. This was achieved by optimising the elution gradient, pH, and ionic strength of the mobile phases. The addition of tetrabutylammonium ions in the mobile phase is crucial for retaining the analytes through ion-pairing, thereby ensuring selectivity (Neurath et al. 2005; Vikingsson et al. 2013, 2009). Without tetrabutylammonium ions in the mobile phase, the C18 column would not be able to retain the analytes, as they would be eluted within the first minute of the HPLC run (data not shown). In addition, the elution gradient of the two mobile phases with different degrees of polarity was optimised such that the more polar metabolites would be eluted first, and the less polar compounds would be retained longer in the column. This would ensure that all the nucleotides could be distinguished in the chromatogram with a good spread of retention times across the 35 min run time.

The method devised by Vikingsson et al. (2010) yields blood concentrations that reflect the true physiological levels of thiopurine metabolites. This is different from the conventional method, which hydrolyses thiopurine nucleotides back to purine bases, causing them to lose their phosphate groups. Thus, the old method is unable to distinguish the mono-, di-, and triphosphate forms of thiopurine metabolites (Lennard & Singleton 1992). Analysis of the phosphorylated metabolites is challenging because of rapid interconversion between di- and triphosphates inside cells (Vikingsson et al. 2013). Addition of EDTA may inhibit the interconversion (Rabel et al. 1995); however, this strategy has not been thoroughly investigated (Vikingsson et al. 2013). Preanalytical handling of samples is foreseeably difficult if the goal is to effectively curb the interconversion between the metabolites.

The accuracy of the method was suboptimal, ranging from 17.6 to 56.4%. The values did not meet the FDA requirements i.e., the accuracy should be within 15% of the nominal values for all quality control levels (FDA 2018) (Table 5). The method suffered from limited extraction efficiency (Vikingsson et al. 2013), which the addition of ethylmercaptopurine, an internal standard, failed to overcome. The previously reported limits of quantification were 0.3, 3, 2 and 30 pmol/8 × $10^8$ RBC for TGMP, TGDP, TGTP, and MeTIMP, respectively (Vikingsson et al. 2013). Using the data obtained from the internal standard to correct for analyte loss caused by sample preparation yielded relative accuracy that was better than the unadjusted accuracy. However, the improvement was not sufficient to render the method accurate according to the FDA requirements (FDA 2018). This is possibly because ethylmercaptopurine is not a nucleotide, so it may differ from the thiopurine metabolites in chemical behaviour. Also, the Vikingsson method requires dual modes of analyte detection – fluorescence and UV absorbance – as the methylated and the non-methylated metabolites have distinct chemical properties. This limits the ability of ethylmercaptopurine, which is UV-reactive but not fluorescent, to compensate for potential fluctuations in chemical reactions, analyte stability, and detector responses.

Vikingsson et al. (2013) speculated that the subpar accuracy may be caused by the different matrices used in the preparation of the controls and the standards. The controls were prepared using blank lysates (extracted RBCs) while the standards were prepared using blank matrices (the supernatants that were left after protein extraction). However, the inconsistency is inevitable and also difficult to rectify. Spiking the analytes into blank lysates to prepare the standards will trigger the interconversion of di- and triphosphates, rendering the calibration curve inaccurate. Adding EDTA into blank lysates before they are mixed with the analytes may allow the use of standards prepared in blank lysates, eliminating the bias caused by the limited extraction recovery (Vikingsson et al. 2013).

In the second phase of this study, we found that most of the UC patients failed to achieve remission, but patients with CD responded relatively well to azathioprine. This could be due to the different criteria used in calculating the CDAI and Mayo scores. CDAI is based on comprehensive evaluation of clinical presentations and laboratory findings. In contrast, Mayo scores are determined by only four criteria that include endoscopic evaluations. Moreover, Mayo scores are ascertained using a more stringent scheme than CDAI, with each of the criteria being scored on a narrow scale of 0 to 3. Hence, for a UC patient to be classified as having achieved 'remission', they would have to achieve the 'normal' status (score 0) in at least two categories and 'mild' (score 1) for the remaining criteria. These differences may explain why the reported correlation between 6-TGN levels and treatment responses has been inconsistent.

Of all the detected metabolites, only TGMP showed a significant inverse correlation with CDAI. However, this may not be clinically meaningful because TGMP makes up only a small fraction (~ 2%) of 6-TGNs (Vikingsson et al. 2013). In a previous study, high blood levels of TGDP and TGTP were detected in IBD patients taking azathioprine, but TGMP was often undetected owing to its extremely low blood levels (Neurath et al. 2005). Similar observations were reported by other studies using different HPLC methods (Karner et al. 2010; Vikingsson et al. 2009). Hence, TGMP is often regarded as a quality control metabolite, and high TGMP levels suggest improper handling and storage of blood samples (Karner et al. 2010).

Through the Wilcoxon rank-sum tests, the responders were found to have a higher median 6-TGN concentration than the non-responders (Table 7). Although the difference is not statistically significant, this observation is consistent with the findings of other studies (Fangbin et al. 2016; Feng et al. 2018; Lee et al. 2017; Liu et al. 2016). We have also observed that the median concentration of TGTP was lower in the responders than the non-responders. This contradicts a prior hypothesis that patients with higher TGDP levels are prone to fail thiopurine treatments (Neurath et al. 2005). Nevertheless, having higher TGTP concentrations does not guarantee positive responses to thiopurine treatments. This is probably because GTPase Rac1 retains some activity after it is bound to TGTP. The TGTP-Rac1 adduct remains biologically active until it is converted into TGDP-Rac1 by RhoGAP (Shin et al. 2016). This may explain the lower TGTP levels in the responders and the discrepancy between our findings and those of other studies. In addition, the interconversion between TGDP and TGTP may have skewed the results, giving rise to inconsistent observations across studies.

The non-responders were also found to have higher MeTIMP levels than the responders. Although MeTIMP may mediate (partly) the immunosuppressive effect of thiopurines by inhibiting *de novo* purine synthesis (Karim et al. 2013), there is a lack of definitive evidence that corroborates the relation between MeTIMP concentrations and thiopurine responses. The synthesis of MeTIMP is driven by TPMT; so high TPMT activity would increase MeTIMP production (Coulthard et al. 2002) and augment the immunosuppressive effects. However, 6-TGN is thought to play a more important role than MeTIMP in causing the immunosuppressive effects of thiopurines. The hypothesis is rooted in two oft-reported observations. First, patients with deleterious TPMT polymorphisms are susceptible to adverse reactions attributed to excessive 6-TGN-induced immunosuppression, such as leukopenia. Second, increased MeTIMP production impairs 6-TGN synthesis, resulting in low serum 6-TGN concentrations and treatment failures (Haglund et al. 2011). This is in line with our observation, whereby patients with active disease had higher MeTIMP levels but lower 6-TGN levels.

The unit of measurement for the HPLC method used in our study is pmol/30 mg Hb, instead of the more widely used pmol/$8 \times 10^8$ RBC. Normalising the metabolite concentrations against Hb content rather than RBC count eliminates the need for cell counting and allows blood samples to be frozen and transported over long distances (without inducing substantial degradation of the thiopurine metabolites). RBC counting is not possible with frozen blood samples (Vikingsson et al. 2013). The disadvantage of this approach is that it is not equivalent to the conventional method, though a variety of formulas have been suggested for converting the measurements into corresponding values. Vikingsson et al. (2013) suggested that $8 \times 10^8$ RBCs is equivalent to 30 mg Hb; but this approximation may not be entirely accurate, as there is a lack of mathematical calculation to support it.

Other researchers used formulas that yielded considerably varied results. For instance, D'Alessandro et al. (2017) estimated that one RBC should contain ~ $2.7 \times 10^8$ Hb molecules; therefore, $8 \times 10^8$ RBCs should be equivalent to 23 mg Hb. Lavi and Holcenberg (1985) estimated that 25 mg Hb is equivalent to $8 \times 10^8$ RBCs.

There are two major limitations of this study. First, the limited extraction efficiency and the low accuracy warrant further optimisation. Second, the sample size for the metabolite monitoring study was small; and this limited the power of the study to detect meaningful

associations between metabolite levels and treatment outcomes. We adopted a passive approach to subject recruitment with only one recruitment site i.e., the Universiti Kebangsaan Malaysia Specialist Centre. Moreover, patients who had achieved remission did not require frequent treatment changes or follow-ups at the clinic, further limiting the number of patients that were available for our study. Patients who did not respond well to azathioprine were quickly switched to other medications such as biologics, rendering them no longer eligible for this study. Hence, the results from our study should be considered as preliminary and interpreted with caution.

## Conclusion

We carried out an in-house validation of a novel method for measuring the phosphorylated metabolites of thiopurines in blood samples and identified several challenges arising from the chemical behaviour of the metabolites. The method was limited by its suboptimal accuracy and low extraction efficiency. A follow-up metabolite monitoring study involving a small cohort of 18 IBD patients showed that of all the tested metabolites, only TGMP showed a significant inverse correlation with CDAI. We also showed that the patients who achieved remission had higher 6-TGN concentrations than those who did not respond well to azathioprine. This observation is consistent with the findings reported by other studies. In contrast, the patients who failed azathioprine treatment had higher TGTP and MeTIMP levels.

As we gain a better understanding of the different roles of phosphorylated thiopurine metabolites in influencing the outcomes of IBD treatments, methods that can distinguish the metabolites may become increasingly important and eventually replace existing methods of therapeutic monitoring of thiopurines in the clinical setting. Our findings offer new insights into the clinical utility of such methods in monitoring the blood levels of thiopurine metabolites, alongside their advantages and drawbacks. Future studies should focus on optimising sample preparation to curb the interconversion between TGDP and TGTP, which we speculate is (partly) the cause of the suboptimal accuracy of the methods and the inconsistent relation observed in prior studies between thiopurine metabolites and the efficacy of IBD treatments. In addition, a more sensitive mode of detection such as mass spectrometry may be considered to further improve the sensitivity, selectivity, and accuracy of the methods.

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*Corresponding author; email: cew85911@ukm.edu.my