Bulletin of Pharmaceutical Research 2025;15(1):196
An Official Publication of Association of Pharmacy Professionals
ISSN: 2249-6041 (Print); ISSN: 2249-9245 (Online)
DOI: 10.21276/bpr.2025.15.1.4

RESEARCH PAPER

A VALIDATED CHROMATOGRAPHIC METHOD FOR DETERMINATION OF THIOPURINE DRUGS METABOLITES CONCENTRATIONS IN PATIENTS WITH INFLAMMATORY BOWEL DISEASE

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Received: Dec 02, 2024 / Revised: Apr 30, 2025 / Accepted: Apr 30, 2025

Thiopurine drugs are characterized by a small therapeutic window, which make therapeutic drug monitoring of metabolites [6-thioguanine nucleotides (6-TGN) and 6-methylmercaptopurine (6-MMP)], mandatory for maintaining adequate exposure. The aim of this study was to develop and validate a simple and reliable high-performance liquid chromatography (HPLC) method for the quantification of 6-TGN and 6-MMP concentrations in patients with Inflammatory Bowel Diseases (IBD). HPLC method was performed and the chromatographic separation was achieved on a C18 column at 30°C. The retention times were 4.67 and 3.5 min for 6-TGN and 6-MMP respectively. The present method is specific and linear over the range of 0.26–5.2 µg/ml with regression coefficient of 0.998 and 1.3–21.05 µg/ml with regression coefficient of 1 for 6-TGN and 6-MMP, respectively. In this study, the method employed could be suitable for routine determination and dosing adjustment of 6-TGN and 6-MMP in patients to support therapeutic drug monitoring.

Key words: Thiopurine, 6-Thioguanine nucleotides, 6-Methylmercaptopurine, HPLC, IBD.

INTRODUCTION

Azathioprine and 6-mercaptopurine (6-MP) are immunomodulating thiopurine drugs commonly used for the treatment of various diseases including acute lymphoblastic leukaemia, inflammatory bowel disease (IBD), and other autoimmune diseases [1-3]. Thiopurine metabolism is complex and involves multiple enzymes [4]. Major metabolites of interest are 6-thioguanine nucleotides (6-TGN) and 6-methylmercaptopurine (6-MMP) [5]. 6-TGN is considered the most active metabolite of the thiopurines and it is responsible for therapeutic efficacy and correlate with myelotoxicity [6]. In contrast, 6 MMP levels have not been associated with clinical efficacy but correlate with hepatotoxicity [3]. Thus measuring metabolites concentration and adjusting the azathioprine and 6-MP dose is essential to obtain optimal therapeutic effects.

Wide inter-individual variability in thiopurine drugs metabolism have been observed among patients who have undergone the same treatment and this has been associated with genetic polymorphisms in the enzymes involved in the metabolism, in particular the gene encoding for thiopurine methyltransferase (TPMT) [7, 8]. Furthermore, thiopurine drugs

are characterized by a small therapeutic window, which make therapeutic drug monitoring of thiopurine metabolites mandatory for maintaining adequate exposure [9, 10].

Several liquid chromatographic methods have been developed for the simultaneous determination of 6-TGN and 6-MMP from purified red blood cells (RBC) as surrogate marker of drug concentration in the target cells [10-13]. The results of these determinations have been often reported as pmol/8x 108 RBC and discriminant levels for determining prediction of response or adverse events have been proposed [14, 15]. Moreover, the use of purified RBC can be time consuming and requires large sample volumes.

Other studies have developed analytic method for dosing thiopurine drugs in plasma of patients [16, 17]. However, thiopurine drugs are inactive drugs that have short half-lives in plasma [18].

The aim of this study was to develop and validate a simple and reliable high-performance liquid chromatography (HPLC) method for the quantification of 6-TGN and 6-MMP concentrations in whole blood of patients with IBD treated by thiopurine drugs.

EXPERIMENTAL

Reagents and chemicals

6-TGN and 6-MMP pure powder were provided by SIGMA-ALDRICH, India. Potassium phosphate (KH_2PO_3) was purchased from SUVCHEM, India which is used for preparation of mobile phase solution. Methanol HPLC grade was purchased from SIGMA-ALDRICH, India. Dithiothreitol (DTT) pure powder, used as internal standard (IS) was provided by SIGMA-ALDRICH, India. Acetonitrile HPLC grade and perchloric acid ($HClO_4$) used for the extraction of 6-TGN and 6-MMP from human whole blood were purchased from SUVCHEM, India and SIGMA-ALDRICH, India respectively. Sodium hydroxide (0.1 M NaOH) was obtained from SIGMA-ALDRICH. Water used for the preparation of the mobile phase was filtered through a 0.18 µm filter using an ultra-pure water system

Instrumentation

Chromatographic separation was developed basing on the stationary and mobile phase compositions, flow rate, injected volume, detection wavelength, temperature and internal standard. The chromatographic separation was performed using a chromatograph Ultimate 3000 (Thermoscientific, Germany) equipped with a data processing unit, diode array detector, heated column compartment, auto-sampler and auto-injector.

Stock solutions and working standards

6-TGN and 6-MMP stock solutions were prepared in NaOH (0.1 M) at a concentration of 1g/l and stored at -20°C. 6-TGN and 6-MMP standards were prepared by dilution in distilled water at a concentration of 0.05 mg/ml and 0.1 mg/ml respectively. DDT solution was prepared in distilled water at a concentration of 200 mg/ml.

Processing samples

Samples of venous blood were collected into a tube containing EDTA (ethylenediamine tetraacetic acid) at 2 hours after medication intake and immediately sent to the Pharmacology Laboratory of the Faculty of Medicine of Monastir and stored at 4°C. Patients should have been under continuous administration of thiopurine drugs for a minimum period of 3 weeks and should not have received packed red cell transfusions in the 6 weeks preceding blood sample collection.

Sample preparation and extraction procedure

Extraction procedures

In conical-bottom glass centrifuge tubes put 900 µl of whole blood and 100 µl DDT and vortex for 10 sec then add 200 µl acetonitrile and vortex for 10 sec followed by addition of 100 µl perchloric acid and vortex for 1 min. Subsequently, the mixture was centrifuged at 4000 rpm for 20 min at 4°C. Take the supernatant to another conical tube and vortex for 30 sec. Heat the extracts for 45 min at 150°C in the tube heater with vortexing from time to time during heating to reach a final volume of 1 ml which centrifuged at 4000 rpm for 20 min at 4°C. Take the supernatant (200 µl) and fill in micro-inserts to be injected into the column.

Calibration curves

A blank and five fortified whole blood samples were used for 6-TGN calibration curve: 0.26, 0.52, 1.05, 2.6 and 5.2 µg/ml. Calibration curves were performed by plotting the height ratio of 6-TGN/IS versus the respective points of 6-TGN concentration range (0-5.2 µg/ml). In case of 6-MMP, calibration curves were created by plotting the high ratios relative to the IS against the various metabolite's concentrations (0, 1.3, 2.6, 5.2, 10.5, 21.05 µg/ml).

Chromatographic conditions
Chromatographic separation was achieved using a C18 column (5µm, 150 × 4.6 µm). The mobile phase consisted of Potassium phosphate (KH_2PO_3) 0.02 M filtered using 0.45-µm filter paper and dissolved gases were degassed from the solution. Methanol pumped using isocratic elution which achieved by using the Potassium phosphate solution and methanol. Several assays were carried out to find the optimal mobile phase composition. Three flow rates (0.8, 1 and 1.2), three temperature ranges (20°C, 25°C and 30°C) and three volumes of injection (20 µl, 50 µl and 80 µl) were tested to determine the most suitable conditions of separation, detection and quantification. Signals were monitored at wavelength (λ) of 304 nm, 320 nm and 341 nm.

Methods validation
Metabolites dosing method's was validated according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines and the reviewer guidance of the center for drug evaluation and research (CDER) [5, 19, 20]. The parameters that have been assessed for the purpose of method validation were as follows: selectivity, linearity, limit of detection (LOD) and lower limit of quantification (LLOQ), accuracy and precision.

Selectivity
Selectivity assessed by comparing a chromatogram of the blank blood sample, a chromatogram of a fortified blood sample and a chromatogram of one patient taking the medication.

Limits of detection and quantification
They were determined using the Signal-to-Noise method according to ICH [21, 22]. Usually LOD is the concentration at which the signal equals 3 times the baseline signal, and LLOQ is the concentration at which the signal equals 10 times the baseline signal.

In this study, the LOD and LLOQ were calculated using the following equations [23, 24]:

$LOD = 3.3\sigma / S$
$LLOQ = 10\sigma / S$

Where σ is the standard deviation of the response, and S is the slope of the calibration curve.

Linearity
The linearity of the developed method was assessed by creating calibration curves for each 6-TGN and 6-MMP individually over three consecutive days. Each series consisted of five concentration samples as we mentioned in the preparation of the calibration curves. Linear regression analysis was used to find out the intercept, slope and correlation coefficient of the calibration curves. The relation between height ratio (drug/IS) (y) and drug concentration (x) was expressed using the following equation:

$y = ax + b$

Where (a) The slope of the line, (b) the intercept

Accuracy
The degree of proximity of the determined value to the true value of the analyte is known as the accuracy of the analytical method. Using the same procedures of injection in the linearity study, the accuracy was evaluated by calculation of differences between the recovered concentrations of the metabolites and the concentration in the pure solutions. The mean recovery and its confidence interval were estimated.
Intra- and inter-assay precision
Inter- and intra-day accuracy and precision of the method were calculated using the data collected from the within day and between days concentrations. Intra-day (in single day) precision were calculated using three replicates at each concentration from a single day run, while inter-day precision were calculated using one replicate at each concentration over three consecutive days. They were assessed using three series of 6-TGN and 6-MMP solutions over three days, each series contains six samples of the concentration 2.6 µg/ml and 5.2 µg/ml respectively. An accepted precision according to the U.S. Food and Drug Administration (FDA) guidelines should be within 15% of the actual value for the quality control samples. However, for the lower limit of quantification, an accepted deviation should be equal or less than 20% [5, 24, 25].

Statistical methods and data analysis
All HPLC data were analysed on Chromeleon 7. software (Thermo Scientific). Linearity was calculated using linear regression analysis. Means, standard deviations and CV% were calculated using Microsoft® Excel.

The linearity of the method was assessed using the correlation coefficient (r) of the linear equation of height ratio *vs* 6-TGN and 6-MMP concentrations; Student test for the comparison of the intercept with the 0; Test of homogeneity of variance using Cochran test; and Fisher test for regression line validity.

The accuracy of the method was evaluated using homogeneity of variance by Cochran test and Test of the validity of the mean using Fisher test. The relative standard deviation (RSD) of repeatability and RSD of intermediate precision were calculated to evaluate the method's precision. The accepted upper limit of RSD is 15%, as required by the Washington Conference for bioanalytical methods [26]. Besides, we performed the test for homogeneity of variance using Cochran test.

RESULTS

Sample processing and chromatographic conditions

We have chosen to use of whole blood for the determination of metabolites concentration. The extraction protocol is relatively simple and common for these two metabolites. 6-TGN and 6-MMP metabolites were extracted simultaneously from patient's blood to be injected from the same extracted sample into the auto-sampler HPLC instrument. Several assays were carried out to find the optimal mobile phase composition. Various ratios of potassium phosphate solution and methanol composition were tried to achieve good resolution, symmetric peak shapes as well as short runtime. The chosen mobile phase was composed of the potassium phosphate solution and methanol (95:5) in case of 6-TGN or (80:20) for 6-MMP analysis. The optimal chromatographic conditions were as follows: a flow rate of 1.2 ml/min as it allows the minimization of the retention time and the maintaining of a good resolution (**Figure 1**). The optimal absorption wavelengths for detection of 6-TGN and 6-MMP were 341 nm and 304 nm, respectively. We chose a temperature of the column of 30°C as the peak symmetry was optimal. The injection volume of 50 µl was chosen to achieve the suitable peak height of the metabolites and the IS. These technical features make our method suitable for routine analysis in our clinical pharmacology laboratory.

Validation method

Quantification method for 6-TGN and 6-MMP was validated as described in the materials and methods section.

Selectivity

To test the selectivity for each metabolite, three chromatogram profiles of blank whole blood from untreated healthy volunteer, spiked whole blood with 6-TGN, 6-MMP and DDT; and patient's whole blood were compared, respectively. Each chromatogram of the spiked whole blood and the chromatogram of the patient revealed two peaks of the tested metabolite and IS. We note that each metabolite and IS were well separated, with retention times of 4.67 and 10.93 min for 6-TGN and IS respectively (**Figure 1**). While in the chromatogram of 6-MMP, the retention times were 3.52 and 4.77 min for 6-MMP and IS respectively. The chromatogram of the blank whole blood does not represent any peak that could interfere with the peak of the metabolites or IS (**Figure 2**).

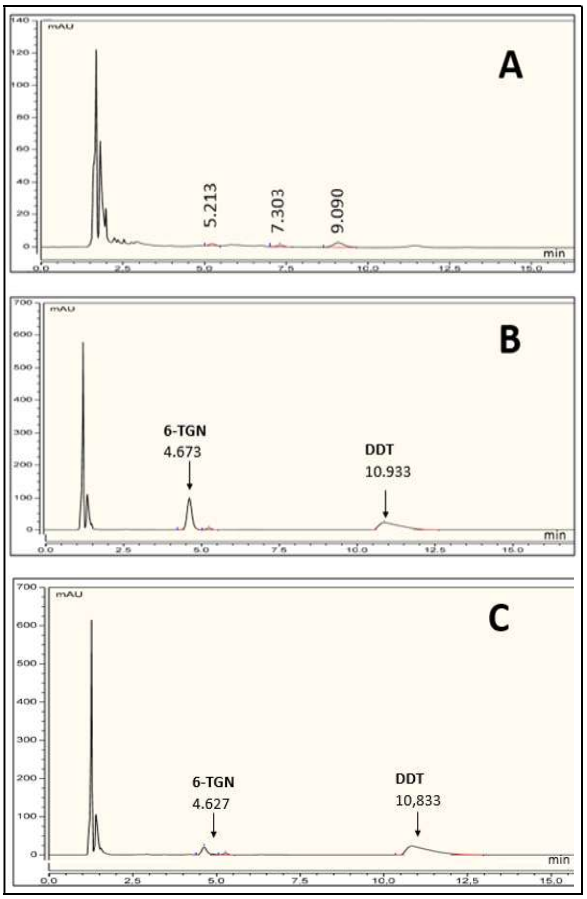

Fig. 1. Chromatograms of (A) whole blood from untreated healthy volunteer, (B) Spiked whole blood with 6-TGN and DDT (IS) and (C) patient's whole blood after administration of drugs.

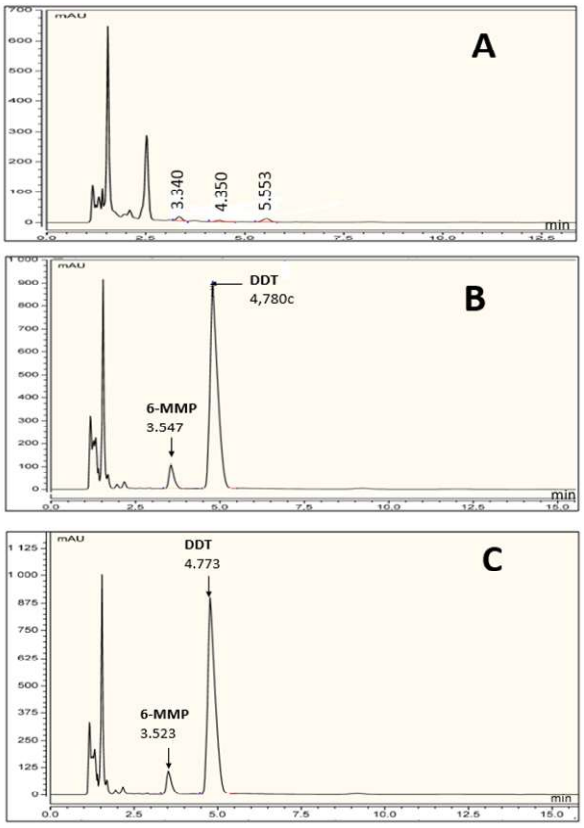

Fig. 2. Chromatograms of (A) whole blood from untreated healthy volunteer, (B) Spiked whole blood with 6-MMP and DDT (IS) and (C) patient's whole blood after administration of thiopurine drugs.

Detection limit

The detection limits of 6-TGN and 6-MMP were 0.01 µg/ml and 0.014 µg/ml, respectively with a signal-to-noise ratio of 3:1, considered as acceptable for detection according to ICH [5, 25].

Quantification limit

The quantification limit of 6-TGN and 6-MMP were 0.034 µg/ml and 0.048 µg/ml, respectively with a signal-to-noise ratio of 10:1. This concentration level, determined with accuracy and precision, is suitable for these metabolites quantification in patients receiving treatment.

Linearity

Calibration curves of the five points of means (n=3) of 6-TGN and 6-MMP were linear over the concentration ranges of 0.26-5.2 µg/ml and 1.3-21.05 µg/ml, respectively as shown in **Figure 3**.

According to Student test, the values of "t calculated" were less than the upper acceptance limit; *i.e.* "t (0.05;13)". Thus, we can conclude that the intercept is not significantly different from "0" at the probability limit of p = 95% (**Table 1**) for the metabolites. Test for homogeneity of variance, *i.e.* Cochran test, has shown that variances of the different levels for 6-TGN and 6-MMP are homogeneous, as "C calculated" were less than the upper limit of acceptance, *i.e.* "C (0.05;5;2)" (**Table 2**). Assessment of regression line validity test using Fisher test has shown that adjustment error is not significantly different of experimental error (6-TGN F calc = 0.098; 6-MMP F calc = 0.069 and F (0.5;3;13) = 3.71) (**Table 3**). Thus, results of different statistical tests confirmed linearity of the present method within calibration ranges.

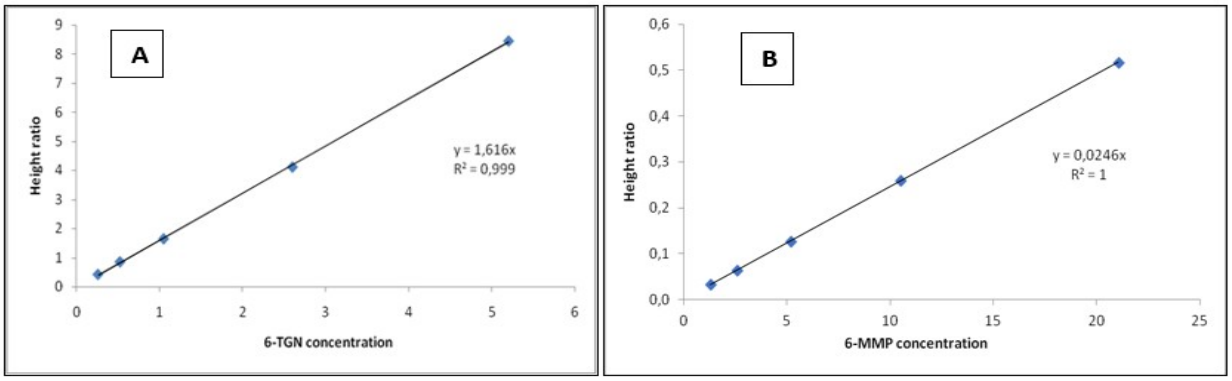

Fig. 3. (A) Calibration curve over the concentration range of 0.26–5.2 µg/ml of 6-TGN, (B) Calibration curve over the concentration range of 1.3–21.05 µg/ml of 6-MMP

Table 1. Comparison test of the intercept with the Student test

	t calculated	t (0.05;13)
6-TGN	0.306	2.16
6-MMP	0.559	2.16

Table 2. Test for homogeneity of variance: Cochran test

	C calculated	C (0.05;5;2)
6-TGN	0.645	0.68
6-MMP	0.634	0.68

Table 3. Regression line validity test: Fisher test

	F calculated	F (0.5;3;13)
6-TGN	0.098	3.71
6-MMP	0.069	3.71

Accuracy

Homogeneity of variances was confirmed using Cochran test (**Table 4**), so the variances considered homogenous at the 5% probability limit. Fisher test for the validity of means, has shown that "F calculated" is less than" F (0.05;4;10)" for each metabolite at the confidence level of 95 % (**Table 5**), so there are no significant differences between the inter-days means concentrations (6-TGN F calculated = 0.244; -6-MMP F calculated=0.566 and F (0,5;3;13) = 3.48). The calculated mean recovery was 101.66% for 6-TGN with confidence interval (IC 95%) was [94.98%; 108.33%]. While the mean recovery for 6-MMP was 102.93% with required accepted IC 95% of [90.81%; 109.73%].

Table 4. Test for homogeneity of variance: Cochran test

	C calculated	C (0.05;3;5)
6-TGN	0.409	0.68
6-MMP	0.273	0.68

Table 5. Test of the validity of means : Fisher test

	F calculated	F (0.05;4;10)
6-TGN	0.244	3.48
6-MMP	0.566	3.48

Intra-assay and inter-assay precision

The assessment of both intra-assay and intermediate precision using Cochran test has shown that the variances of different concentrations were homogeneous at 5% risk (**Table 6**). As shown in **Table 7**, the calculated Relative Standard Deviation (RSD) of intra-assay and inter-assay precision for both metabolites are lower than the accepted upper limit of Relative Standard Deviation (15%) as required by the Washington Conference for bioanalytical methods.

Table 6. Test for homogeneity of variance: Cochran test

	C calculated	C (0.05;3;6)
6-TGN	0.563	0.677
6-MMP	0.551	0.677

Table 7. The calculated RSD of intra- and inter-assay precision for 6-TGN and 6-MMP

	RSD of intra-assay precision	RSD of inter-assay precision
6-TGN	1.23%	1.00%
6-MMP	0.97%	1.30%

Application of the developed method

The developed method was applied to the analysis of 6-TGN and 6-MMP metabolites in whole blood samples collected from patients with IBD treated by thiopurine drugs. All patients' samples were analyzed within one week of blood collection. The therapeutic range of concentrations was between 230 and 450 pmol/8×10^8 RBC for 6-TGN and >7000 pmol / 8×10^8 RBC for 6-MMP [6]. Each analytical run included QC samples, calibration standards and a batch of processed patient samples. Calibration curves were generated for 6-TGN and 6-MMP in each analytical run.

DISCUSSION

In this study, we presented an improved and validated method for the simultaneous measurement of 6-TGN and 6-MMP concentrations in the whole blood of IBD patients treated with thiopurine drugs. The extraction protocol is straight forward and applicable to both metabolites. 6-TGN and 6-MMP were simultaneously extracted from patient blood samples and analyzed using an HPLC auto-sampler. Previous liquid chromatographic methods have been developed to determine 6-TGN and 6-MMP in purified red blood cells (RBCs) as surrogate markers of drug concentration [10-13]. For instance, a method was developed to quantify thiopurine metabolites in RBCs by separating the plasma through centrifugation and washing the RBCs with a diluted saline solution [27]. However, using RBCs involves laborious purification and hydrolysis steps with potentially toxic and expensive chemicals. Additionally, it is time-consuming and requires large sample volumes.

Therefore, some other studies proposed the use of whole blood as an alternative. Indeed, it is demonstrated that there was no significant difference between the concentrations of metabolites measured from whole blood and those obtained from purified RBCs in a sample of IBD patients treated with thiopurine drugs. The use of whole blood in our method allowed to extract all of the metabolites and to reduce the time required for the extraction by avoiding the RBC purification step.

It has been demonstrated by several studies that the concentration of 6-TGNs is well correlated with the efficacy of thiopurine drugs on the one hand, and with the occurrence of hematotoxicity on the other hand [28, 29]. Thus, the TDM of this metabolite has been proposed as a preferential marker for estimating the efficacy of treatment with azathioprine or 6-mercaptopurine. Different efficacy thresholds for IBD patients have been suggested in the literature, ranging from 200 to 260 pmol/8×10^8 RBC. A 2006 meta-analysis confirmed a threshold value for 6-TGN between 230 and 260 pmol/8×10^8 RBC. These findings were supported by a meta-analysis which indicated that concentrations of 235 and 250 pmol/8×10^8 RBC are strongly correlated with thiopurine efficacy (OR=4.71, p<0.001, CI [2.31-9.62] and OR=2.66, p<0.001, CI [1.94-3.66], respectively). Thus, a minimum threshold of 235 pmol/8×10^8 RBC is recommended to ensure the efficacy of thiopurine drugs.

Regarding toxicity, it is identified that a 6-TGN level above 450 pmol/8×10^8 RBC is significantly associated with hematotoxicity. For this purpose, the majority of studies have recommended a target therapeutic range of 6-TGN between 235 to 450 pmol/8.108 RBC [28, 29]. The 6-MMP assay is primarily used to prevent liver toxicity. It is estimated that the risk of occurrence of hepatotoxicity in patients with IBD is multiplied by three when the level of 6-MMP is greater than 5700 pmol/8×10^8 RBC. Several methods for assaying thiopurine metabolites have been reported in the literature, such as spectrophotometry, but HPLC remains the technique of choice. Despite the short plasma half-life of thiopurine drugs (3 to 5 hours), metabolite measurement cannot be performed at treatment initiation; it takes several weeks to reach therapeutic equilibrium or “steady state”. For 6-TGN, with a half-life of 4 to 5 days, dosage can only be accurately performed at least 20 days after starting or modifying the thiopurine dosage. Additionally, for patients who have received a blood transfusion, a delay of 6 weeks is necessary because metabolite concentrations are normalized to RBC levels. These timelines were adhered to in our study to ensure the specificity of our assay method. We chose a liquid/liquid extraction protocol for its simplicity, speed, and reliability. Consistent with other studies, we used liquid phase extraction to determine thiopurine metabolites. Perchloric acid was used in the extraction protocol to deproteinize samples and hydrolyze RBCs, allowing metabolite extraction. Some studies have used dichloromethane during this step which is more toxic than perchloric acid [30]. At the end of the extraction step, we heated the tubes for 45 min at 150°C. This step allowed to hydrolyze the purine bases (nucleotides) bound to the metabolites and convert 6-MMP into 4-amino-5-methylthiocarbonyl imidazole (AMTCI). Indeed 6-MMP is undetectable with UV in order to be able to quantify this metabolite with a DAD detector it must be converted into AMTCI [31]. To improve assay accuracy and precision, DTT was added as an internal standard. DTT was chosen because it has similar physical and chemical properties to thiopurine metabolites and does not react with them. Additionally, DTT protects the thiol function of 6-TGNs against oxidation during deproteinization, preventing degradation and improving recovery rates. For 6-MMP, the thiol function is already protected by a methyl group. We used a C18 reverse phase

column (5 µm, 250 × 4.6 mm), which is suitable for analyzing alkaline compounds. For the elution of the thiopurine metabolites we used a mobile phase composed of phosphate buffer (0.02 mol/l) (way A) and methanol (way B). Indeed, the phosphate buffer is used to neutralize the pH of the mobile phase in order to avoid the degradation of thiopurines. We carried out an elution in isocratic mode by modifying the percentages of two channels in order to obtain the best metabolite peaks. According to the chromatograms obtained, the best percentages of mobile phase were the following: 95% TP and 5% methanol for the elution of 6-TGN, and 80% TP and 20% methanol for 6-MMP. The flow rate of the mobile phase which allowed separating the chromatographic peaks of 6-TGN, 6-MMP and DTT was 1.2 ml/min with an oven temperature of 30°C. The total analysis time recommended in this method was 15 min with a retention time of 4.6 min for 6-TGN and 3.5 min for 6-MMP. In some studies, the analysis time exceeded 25 min, making the technique more restrictive.

The second stage of the study was to validate our method of analysis in accordance with the guidelines dictated by the CIH, in order to be able to apply it subsequently in IBD patients treated with thiopurine drugs. Our method of analysis was specific and selective, since the peaks of 6-TGN and 6-MMP were present in the chromatograms of a patient treated with azathioprine and absent in those of a blank sample (whole blood unloaded). In order to test the linearity of our method, we used a calibration range composed of five increasing concentrations of each metabolite. The calibration curves obtained were perfectly linear over the whole range for the two metabolites with a linearity coefficient r^2 of 0.999 for the 6-TGNs and 1 for the 6-MMP. The linearity parameters relating to the two regression equations were validated by Cohran's and Fisher's tests. Fidelity and accuracy are major validation criteria for an analytical assay method. Indeed, a method is exact if the recovery rates, which represent the ratio between the concentration estimated by the mathematical model and the actual theoretical value, are less than 15%. These rates were 0.38% for 6-TGNs and 0.52% for 6-MMP with respective average recovery of 100.25±2.6% and 100.3±1.4%, which confirms that our method is correct. The judgment of the fidelity of our assay method was based on the calculation of the coefficients of variation of repeatability and intermediate fidelity. The values obtained confirm the fidelity of our analytical method when the coefficients of variation of repeatability and reproducibility were all less than 15%. These results were validated by the Cohran test.

CONCLUSION

In this study, a validated HPLC method was developed for the simultaneous measurement of 6-TGN and 6-MMP in patients undergoing thiopurine therapy. The proposed method is linear, sufficiently sensitive, accurate and precise, as shown by appropriate statistical tests recommended by ICH. The use of the whole blood allows avoiding the need for RBC separation, which facilitate the analytic procedure and save time. Thus, the method employed here could be suitable for routine determination and dosing adjustment of these two metabolites in patients treated with azathioprine to support therapeutic drug monitoring.

REFERENCES

1. Gearry RB, Barclay ML. Azathioprine and 6-mercaptopurine pharmacogenetics and metabolite monitoring in inflammatory bowel disease. *J Gastroenterol Hepatol*. 2005;20(8):1149-57. doi:10.1111/j.1440-1746.2005.03832.x
2. McLeod HL, Krynetski EY, Relling M V, Evans WE. Genetic polymorphism of thiopurine methyltransferase and its clinical relevance for childhood acute lymphoblastic leukemia. *Leukemia*. 2000;14(4):567-72. doi:10.1038/sj.leu.2401723
3. Deshpande AR, Abreu MT. Optimizing therapy with 6-mercaptopurine and azathioprine: To measure or not to measure? *Therap Adv Gastroenterol*. 2010;3(5):275-9. doi:10.1177/1756283X10376121
4. Qasim A, Buckley M, O'Morain CA. Thiopurine metabolites and the role of thiopurine methyl transferase activity in inflammatory bowel disease. *Gut*. 2002;51(1):141-2.
5. Cangemi G, Barabino A, Barco S, Parodi A, Arrigo S, Melioli G. A validated HPLC method for the monitoring of thiopurine metabolites in whole blood in paediatric patients with inflammatory bowel disease. *Int J Immunopathol Pharmacol*. 2012;25(2):435-44. doi:10.1177/039463201202500213
6. Bradford K, Shih DQ. Optimizing 6-mercaptopurine and azathioprine therapy in the management of inflammatory bowel disease. *World J Gastroenterol*. 2011;17(37):4166-73. doi:10.3748/wjg.v17.i37.4166
7. Abaji R, Krajinovic M. Thiopurine S-methyltransferase polymorphisms in acute lymphoblastic leukemia, inflammatory bowel disease and autoimmune disorders: Influence on treatment response.

Pharmgenomics Pers Med. 2017;10:143-56. doi:10.2147/PGPM.S108123
8. Coelho T, Andreoletti G, Ashton JJ, Batra A, Afzal NA, Gao Y, et al. Genes implicated in thiopurine-induced toxicity: Comparing TPMT enzyme activity with clinical phenotype and exome data in a paediatric IBD cohort. *Sci Rep*. 2016;6:34658. doi:10.1038/srep34658
9. Lennard L. Implementation of TPMT testing. *Br J Clin Pharmacol*. 2014;77(4):704-14. doi:10.1111/bcp.12226
10. Sahasranaman S, Howard D, Roy S. Clinical pharmacology and pharmacogenetics of thiopurines. *Eur J Clin Pharmacol*. 2008;64(8):753-67. doi:10.1007/s00228-008-0478-6
11. Supandi S, Harahap Y, Harmita H, Andalusia R. Quantification of 6-mercaptopurine and its metabolites in patients with acute lympoblastic leukemia using dried blood spots and UPLC-MS/MS. *Sci Pharm*. 2018;86(2):18. doi:10.3390/scipharm86020018
12. Lennard L, Singleton HJ. High-performance liquid chromatographic assay of the methyl and nucleotide metabolites of 6-mercaptopurine: Quantitation of red blood cell 6-thioguanine nucleotide, 6-thioinosinic acid and 6-methylmercaptopurine metabolites in a single sample. *J Chromatogr B Biomed Sci Appl*. 1992;583(1):83-90. doi:10.1016/0378-4347(92)80347-S
13. Karran P, Attard N. Thiopurines in current medical practice: Molecular mechanisms and contributions to therapy-related cancer. *Nat Rev Cancer*. 2008;8(1):24-36. doi:10.1038/nrc2292
14. Lennard L, Gibson BES, Nicole T, Lilleyman JS. Congenital thiopurine methyltransferase deficiency and 6-mercaptopurine toxicity during treatment for acute lymphoblastic leukaemia. *Arch Dis Child*. 1993;69(5):577-9. doi:10.1136/adc.69.5.577
15. Cuffari C, Théorêt Y, Latour S, Seidman G. 6-Mercaptopurine metabolism in Crohn's disease: Correlation with efficacy and toxicity. *Gut*. 1996;39(3):401-6. doi:10.1136/gut.39.3.401
16. Keuzenkamp-Jansen CW, Van Baal JM, De Abreu RA, De Jong JGN, Zuiderent R, Trijbels JMF. Detection and identification of 6-methylmercapto-8-hydroxypurine, a major metabolite of 6-mercaptopurine, in plasma during intravenous administration. *Clin Chem*. 1996;42(3):380-6. doi:10.1093/clinchem/42.3.380
17. Chan GLC, Erdmann GR, Gruber SA, Matas AJ, Canafax DM. Azathioprine metabolism: Pharmacokinetics of 6-mercaptopurine, 6-thiouric acid and 6-thioguanine nucleotides in renal transplant patients. *J Clin Pharmacol*. 1990;30(4):358-63. doi:10.1002/j.1552-4604.1990.tb03606.x
18. Stefan C, Walsh W, Banka T, Adeli K, Verjee Z. Improved HPLC methodology for monitoring thiopurine metabolites in patients on thiopurine therapy. *Clin Biochem*. 2004;37(9):764-71. doi:10.1016/j.clinbiochem.2004.05.025
19. International Conference on Harmonization (ICH), Guidance for Industry Q (R1): V of APM. International Conference on Harmonization (ICH) Guidance for Industry, Q2 (R1): Validation of Analytical Procedures: Methodology. Available from US Food and drugs administration,1995.
20. Center for Drug Evaluation and Research (CDER), Reviewer Guidance: Validation of chromatographic methods. US Food and drugs administration, 1994.
21. Taleuzzaman M. Limit of blank (LOB), limit of detection (LOD), and limit of quantification (LOQ). *Org Med Chem Int J*. 2018;7(5):127-31. doi:10.19080/OMCIJ.2018.07.555722
22. Gupta PC. Method validation of analytical procedures. *PharmaTutor*. 2015;3(1):32-9.
23. Cangemi G, Barabino A, Barco S, Parodi A, Arrigo S, Melioli G. A validated HPLC method for the monitoring of thiopurine metabolites in whole blood in paediatric patients with inflammatory bowel disease. *Int J Immunopathol Pharmacol*. 2012;25(2):435-44. doi:10.1177/039463201202500213
24. Hawwa AF, Millership JS, Collier PS, McElnay JC. Development and validation of an HPLC method for the rapid and simultaneous determination of 6-mercaptopurine and four of its metabolites in plasma and red blood cells. *J Pharm Biomed Anal*. 2009;49(2):401-9. doi:10.1016/j.jpba.2008.10.045
25. Vashist SK, Luong JHT. Chapter 4. Bioanalytical requirements and regulatory guidelines for immunoassays. Handbook of Immunoassay Technologies: Approaches, performances, and applications, Elsevier Inc.; 2018: 81-95. doi:10.1016/B978-0-12-811762-0.00004-9
26. Hartmann C, Massart DL, McDowall RD. An analysis of the Washington Conference Report on bioanalytical method validation. *J Pharm Biomed Anal*. 1994;12(11):1337-43. doi:10.1016/0731-7085(94)00083-2
27. Erdmann GR, Steury JC, Carleton BC, Stafford RJ, Bostrom BC, Canafax DM. Reversed-phase high-performance liquid chromatographic approach to determine total lymphocyte concentrations of 6-thioguanine, methylmercaptopurine and methylthio guanine in humans. *J Chromatogr B Biomed Sci Appl*. 1991;571(1-2):149-56. doi:10.1016/0378-4347(91)80441-E
28. Estevinho MM, Afonso J, Rosa I, Lago P, Trindade E, Correia L, et al. A systematic review and meta-analysis of 6-thioguanine nucleotide levels and clinical remission in inflammatory bowel disease. *J Crohns Colitis*. 2017;11(11):1381-92. doi:10.1093/ecco-jcc/jjx089
29. Dubinsky MC, Lamothe S, Yang HY, Targan SR, Sinnett D, Théorêt Y, et al. Pharmacogenomics and metabolite measurement for 6-mercaptopurine therapy in inflammatory bowel disease. *Gastroenterology*. 2000;118(4):705-13. doi:10.1016/S0016-5085(00)701 40-5
30. Vikingsson S, Almer S, Peterson C, Carlsson B, Josefsson M. Monitoring of thiopurine metabolites - A high-performance liquid chromatography method for clinical use. *J Pharm Biomed Anal*. 2013;75:145-52. doi:10.1016/j.jpba.2012.11.027
31. Oliveira BM, Romanha AJ, Alves TMA, Viana MB, Zani CL. An improved HPLC method for the quantitation of 6-mercaptopurine and its metabolites in red blood cells. *Braz J Med Biol Res*. 2004;37(5):649-58. doi:10.1590/s0100-879x2004000500004

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