Clinica Chimica Acta 499 (2019) 24–33

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Determination of 6-thioguanine and 6-methylmercaptopurine in dried blood spots using liquid chromatography-tandem mass spectrometry: Method development, validation and clinical application

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ARTICLE INFO

Keywords:
Azathioprine
6-thioguanine
6-methylmercaptopurine
Dried blood spot samples
Therapeutic drug monitoring
Inflammatory bowel disease

ABSTRACT

Background: Therapeutic drug monitoring of azathioprine metabolites is required for pharmacotherapy individualisation in patients with inflammatory bowel disease. Currently mainly hemolysates are used, requiring long sample preparation and showing limited analytes stability. Therefore, a quantitative LC-MS/MS method for determination of 6-thioguanine (6-TG) and 6-methylmercaptopurine (6-MMP) in dried blood spot samples (DBS) was developed.

Methods: Analysis involves liquid extraction from 30 µL blood spot, hydrolysis and quantification with LC-MS/MS.

Results: Method met the validation criteria in terms of selectivity, linearity, accuracy, and precision in a range from 50 to 5300 pmol/8 × 10^8 Ery for 6-TG and from 260 to 5300 pmol/8 × 10^8 Ery for 6-MMP. Range can be increased to 8000 pmol/8 × 10^8 Ery. No matrix effect was observed and the recovery was > 80%. DBS specific validation parameters were confirmed: spot homogeneity, no influence of blood spot volume (> 30 µL) on 6 mm DBS disk, and absence of haematocrit effect. DBS samples were stable for at least one month at temperatures from −20 to 40 °C. Clinical validation confirmed that DBS method and routine clinical method with hemolysate samples give comparable results and enable similar clinical decisions.

Conclusions: The newly developed DBS method is simple and presents an alternative to conventional methods for therapeutic drug monitoring of azathioprine metabolites.

1. Introduction

Inflammatory bowel disease (IBD) is characterized by recurrent, destructive inflammation of the gastrointestinal tract caused by dysregulation of immune system especially in individuals with genetic predisposition. It consists of two major phenotypes, Crohn's disease and ulcerative colitis, that differ in affected part of gastrointestinal tract and their macroscopic and histological features [1]. Current treatment endpoint is remission where absence of signs and symptoms of IBD, normalization of inflammatory parameters and mucosal healing is observed [2]. The therapy of IBD includes stepwise use of anti-nflammatory drugs, immunosuppressives, and biologic drugs, which were lastly introduced into the clinical practice [3].

Immunosuppressive drugs belonging to the thiopurine group *e.g.* azathioprine and 6-mercaptopurine (6-MP) are traditionally used for treatment of IBD either as monotherapy or combination therapy, where combination with infliximab is currently well recognised in the clinical practice [4]. The metabolism of azathioprine and 6-MP is complex and many studies aimed to investigate the association of drug metabolism and their therapeutic or toxic activity [5]. Neither azathioprine nor 6-MP has intrinsic activity; they have to undergo extensive metabolic transformations to exert their clinical efficacy. Azathioprine is converted to 6-MP nonezymatically or based to some studies by glutathione-S-transferase [3,5]. 6-MP undergoes several metabolic transformations that lead to production of two different active nucleotides, namely 6-thioguanine nucleotides (6-TGN) and 6-methyl-mercaptopurine ribonucleotides (6-MMPr) and inactive metabolite 6-thiouric acid [6]. The three main enzymes responsible for these metabolic

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https://doi.org/10.1016/j.cca.2019.08.024
Received 12 March 2019; Received in revised form 22 August 2019; Accepted 22 August 2019
Available online 23 August 2019
0009-8981/

transformations are hypoxanthine guanine phosphoribosyltransferase, thiopurine *S*-methyltransferase (TPMT) and xanthine oxidase. 6-TGN and 6-MMPr exert different effects on the target cells. 6-TGN are presumably responsible for the anti-inflammatory response; however, elevated levels may also lead to myelosuppression. On the other hand, elevated levels of methylated metabolites *i.e.* 6-methyl-mercaptopurine (6-MMP) and its riboside, are related to hepatotoxic effect [6]. The key enzyme in the formation of methylated metabolites is TPMT which has highly variable inter-individual activity partly caused by single nucleotide polymorphisms in the *TPMT* gene. To overcome potential toxicity of thiopurine drugs and optimize their therapeutic effects, different strategies leading to personalised medicine are used in the therapy of IBD. The current biomarkers largely accepted in the clinical practice are TPMT genotype or phenotype and concentrations of 6-TGN and 6-MMP. Several studies attempted to define therapeutic threshold of 6-TGN, and majority of studies proposed the range from 230 to 280 pmol/8×10^8 erythrocytes (Ery) [7–12]. Moreover, hepatotoxic threshold for 6-MMP was studied and the currently accepted concentration is 5700 pmol/8×10^8 Ery [7].

A number of analytical approaches using HPLC [13–15], UPLC-MS/MS [16] and LC-MS/MS [17–19] have been reported for assay of thiopurine metabolites. The described methods differ in terms of biological matrix, sample preparation techniques, and the chemical forms of analytes (nucleosides or pure bases).

The collection of whole blood, erythrocytes or peripheral blood mononuclear cells is the current standard for active nucleosides 6-TGN and 6-MMPr determination, while drops of whole blood collected on filter paper represent a patient-friendlier alternative of sample collection. The collection of the blood using dried blood spot (DBS) approach on special cards can be non-volumetric where blood volume is unknown *e.g.* fingerprick DBS samples, or volumetric by depositing known volume of the blood. More common non-volumetric approaches use DBS sub-punches for analysis whereas volumetric one use whole spot area. DBS sampling offers a number of advantages over conventional whole blood, plasma, or serum sample collection. It is a less-invasive sampling method in comparison with venipuncture and requires a smaller blood volume (10–50 μL). Additionally, DBS sampling offers a simple storage, easier sample transfer, and potential increase in sample stability compared to liquid blood, plasma, or serum samples [20]. DBS approach is also very attractive sampling technique since it enables home self-sampling without the need of specially trained personal and therefore presents a valuable advantage for patients whose therapy is individualized by therapeutic drug monitoring (TDM) [21]. As this technique is not limited to blood sampling but also renders it possible to sample other biological materials, its application in TDM fields is additionally supported [22–25]. Recent advances in the field of sampling techniques enabled development of new volumetric microsampling approaches that enable collection of a defined volume, making microsampling attractive to quantitative analysis in general [26–28]. Besides clinical advantages, the DBS approach also has several advantages from a bioanalytical perspective since it can cause adsorption of several matrix compounds on paper while enabling extraction of analytes, it substantially reduces the volume of extraction solvent, and reduces time of sample preparation [29].

Despite many potential benefits of DBS sampling there are several challenges that have to be addressed properly during the development of the method. To overcome complex matrix sample and low amount of the analyte in the sample, optimisation of extraction and use of sensitive detection methods are required. Moreover, to overcome the lack of specific regulatory guidance for bioanalytical method validation from European Medicines Agency (EMA) [30] and Food and Drug Administration (FDA) [31], DBS specific validation parameters *e.g.,* haematocrit effect, spot volume, homogeneity, spot to spot carry-over, internal standard addition, extraction recovery, matrix effect, and stability issues regarding the storage and transport have to be properly addressed. Moreover, to ensure consistency of results obtained from the DBS analysis with traditional analysis, for example in plasma or erythrocytes, clinical validation performed through the comparison of DBS and plasma or erythrocytes concentration originating from the same patients has to be performed along with appropriate statistical analysis [20,32,33].

We have developed a quantitative LC-MS/MS method for determination of 6-thioguanine (6-TG) and 6-MMP in whole blood samples using the volumetric DBS approach and known value of erythrocyte number in the blood. The method has been rigorously validated in accordance with Guideline on bioanalytical method validation published by EMA. Moreover, DBS specific validation parameters and clinical evaluation have been determined and proved that the method is suitable for routine determination of 6-TG and 6-MMP in patients with IBD on azathioprine therapy.

2. Experimental

2.1. Chemicals and materials

2-Amino-6-mercaptopurine riboside hydrate (98%, *w/w*), 6-Methylmercaptopurine riboside (≥99%, *w/w*) and DL-Dithiothreitol (≥99.0%, *w/w*) were obtained from Sigma-Aldrich (Steinhein, Germany). Sodium hydroxide (> 98%, *w/w*), methanol for liquid chromatography LiChrosolv® (≥99.9%, *w/w*), and formic acid (98–100%) were obtained from Merck (Darmstadt, Germany); acetonitrile LC-MS grade ≥ 99.9% (Chromasolv®) and perchloric acid (69.0–72.0%, *w/w*) from ACS Reagent Honeywell Fluka (Seelze, Germany). The stable isotopes labelled 2-amino-6-mercaptopurine-$^{13}C_2$,^{15}N (6-TG-IS) and 6-methylmercaptopurine-D_3 (6-MMP-IS) were obtained from Santa Cruz Biotechnology (California, USA) and used as internal standards (IS). Ultrapure water (18.2 MΩ cm) was obtained using the Milli-Q Advantage A10 Ultrapure Water Purification System (Bedford, USA). For the collection of DBS samples, Whatman™, 903 Protein Saver Card from GE Healthcare (Dassel, Germany) were used. In the experiments with punched DBS samples, commercially available stainless steel hole puncher from Rayher Hobby&Art (Laupheim, Germany) was used.

2.2. Preparation of solutions and standards

Stock solutions of 6-TG riboside and 6-MMPr at 0.2 mg/mL were prepared by dissolving appropriate amounts of each analyte in water. The primary solution was prepared by mixing both stock solutions and further diluting it in water to obtain a concentration 0.1 mg/mL for each analyte. Working solutions in range from 1.00 mg/L to 70.0 mg/L were prepared by diluting appropriate volumes of the primary solution with water. Primary solution and working solutions were used to prepare DBS calibrators and quality control (QC) samples.

The internal standards, 6-TG-IS and 6-MMP-IS, were dissolved in 50 mM sodium hydroxide to obtain 1 or 2.5 mg/mL, respectively. Stock solutions were further diluted with water, to obtain 180 mg/L in case of 6-TG-IS and 450 mg/L in case of 6-MMP-IS, aliquoted and frozen. The extraction solvent was a mixture of methanol and water (80:20; *v/v*), containing 2.0 and 1.5 μg/L of 6-TG-IS and 6-MMP-IS, respectively. 0.5 M DL-dithiothreitol (DTT) and 4 M sodium hydroxide solution were prepared by dissolving standards in water.

2.3. Preparation of DBS calibrators and quality control samples

Drug-free venous blood was collected into the 6 mL EDTA blood collection tubes from healthy volunteers, who signed an informed consent. Differential blood analyses were measured using Microsemi CRP hematology analyzer from Horiba (Kyoto, Japan) and volunteers haematocrit (Hct) values ranged from 0.41 to 0.48, while number of erythrocytes ranged from 4.3 to 5.4 10^{12}/L. DBS calibrators and QC samples were prepared by diluting the primary solution and working

solutions with the obtained blood in the proportion 20:230 (*v/v*), respectively. The spiked calibrators ($n = 8$) in concentration range from 80.0 to 8000 μg/L were obtained. Concentration of 6-TG QC at low (QC_L), medium (QC_M), and high level (QC_H) was 240, 2400, and 6000 μg/L, respectively. Concentration of 6-MMP QC samples was 1200, 2400, and 6000 μg/L for QC_L, QC_H and, QC_M, respectively. All mass concentrations of calibrators and quality control samples refer to riboside standards, which were used for the preparation of the stock solutions.

30 μL of the spiked blood or patient blood were pipetted onto the Whatman™, 903 Protein Saver Card and kept at room temperature for at least 3 h. Cards were inserted in sachets of low gas permeability with moisture absorbent packets and stored at room temperature until extraction.

2.4. DBS sample preparation

30 μL spot of DBS calibrator, QC sample or patient sample was cut out with scissors and transferred to a 2 mL micro tube with a cap. A 600 μL of the extraction solvent containing both IS, 60 μL of DTT solution, and 60 μL of perchloric acid were added, followed by 20 min sonication in ultrasonic bath and 45 min heating at 100 °C. Afterwards, a 600 μL aliquot of the obtained solution was transferred to a 2 mL micro tube and neutralized with 94 μL of 4 M NaOH. After centrifugation for 10 min at 16000 × *g* the supernatant was transferred to autosampler vials with inserts and 2.5 μL was injected into the LC-MS/MS system.

2.5. Chromatographic and mass spectrometry conditions

The extracted DBS samples were analysed by an Agilent 1290 Infinity liquid chromatograph coupled with 6460 Triple Quadrupole detector (Agilent Technologies, Santa Clara, USA) equipped with a JetStream™ electrospray. After sample injection, the chromatographic separation was performed on a Luna Omega Polar C18 column 100 × 2.1 mm, with 1.6 μm particles (Phenomenex, Torrance, USA) maintained at 30 °C and using a linear gradient elution with a flow rate of 0.3 mL/min of mobile phase consisting of 0.05% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) with the following gradient steps (time [min], %B): (0.0, 0), (4.0, 1), (6.2, 20), (7.5, 40), (8.0–10, 50), (10.50–20, 0). The total run time including re-equilibration was 20 min. The mass spectrometer was operated in positive ionization mode using the following ion source parameters: drying gas flow (and temperature): 5 L/min (275 °C), nebulizer pressure: 45 psi, sheath gas flow (and temperature): 11 L/min (350 °C) and the capillary voltage was 3500 V. Both quadrupoles were set for all analytes at wide mass resolution (1.2 amu) except for 6-TG where it was set at the widest (2.5 amu). The multiple-reaction monitoring (MRM) details for all compounds are listed in Supplementary material (Table 1S). The dwell time for each *m/z* transition was 50 ms. For sensitivity purposes, the MRM recording was divided in two time-segments: from 3.75 to 5.4 min, and from 5.4 to 8.25 for 6-TG and 6-MMP quantitation, respectively.

2.6. Validation studies

To demonstrate that the developed bioanalytical method was fit for purpose, validation was conducted based upon Guideline on bioanalytical method validation of EMA [30]. Absolute and relative matrix effect was estimated according to Matuszewski et al. [34].

The method was validated in terms of selectivity, linearity, lowest limit of quantification, precision, accuracy, matrix effects, recovery, carry-over, and dilution integrity. Moreover, DBS specific validation parameters as are effect of haematocrit, blood spot size, blood spot homogeneity, and stability of DBS were determined. Finally, clinical evaluation of the method was determined on patients with IBD on azathioprine therapy.

2.6.1. Selectivity

Blank DBS samples from 6 different volunteers were processed as described in the section 2.4. DBS sample preparation was performed with and without ISs to verify the presence of chromatographic peaks that might interfere with the detection of 6-TG and 6-MMP. Blank sample chromatograms were compared with chromatograms of the lower limit of quantification (LLOQ). The average response of LLOQ samples for 6-TG and 6-MMP was required to be at least five times greater than the average blank sample response.

2.6.2. Linearity and lower limit of quantification

DBS calibrators were prepared in duplicate ($n = 2$) with exception of the lowest points of the curves, which were analysed in six replicates ($n = 6$). A calibration curve for each analyte was obtained by plotting the ratio of analyte/internal standard peak area against nominal (theoretical) concentration. An 1/x weighted linear regression was applied. The back calculated concentration of the calibration standards should be within ± 15% of the nominal value, except for the LLOQ for which it should be within ± 20%. At least 75% of the calibration standards, with a minimum of six calibration standard levels, must fulfil this criterion. The LLOQ was defined as the lowest concentration on the calibration curve where accuracy and precision did not exceed 20%. The 6-TG and 6-MMP peak areas had to be at least 5-times higher than corresponding peak areas of the blank sample, and the signal-to-noise ratio (peak-to-peak, area based) had to be above 10:1.

2.6.3. Precision and accuracy

For both analytes the intra- and inter-day accuracy and precision were determined by analysing QC DBS samples at three concentration levels: QC_L, QC_M, and QC_H. The QCs were analysed in six replicates on three separate days. Concentrations were calculated from a peak area ratio of analyte *versus* IS using the equation of calibration curve and the obtained concentrations were compared with the nominal values and presented in percent values to determine intra- and inter-day accuracy. Moreover, intra- and inter-day precision was calculated as the relative standard deviation of replicates. The method was considered accurate and precise if the percentage values were between 85 and 115% and relative standard deviation did not exceed 15%.

2.6.4. Recovery, matrix effects

Absolute matrix effect was determined according to Matuszewski et al. [34]. Three differently prepared QC samples in two replicates were used to evaluate recovery and absolute matrix effects: DBS QC samples extracts (pre-extracted) prepared from extracts of spiked venous blood; blank DBS extracts obtained from drug-free venous blood and spiked with analytes corresponding to 100% recovered levels after extraction (post-extracted) and neat analytes solution samples (unextracted). Recovery and overall process efficiency was calculated as the ratio of the mean response A/IS of the analytes in extracted QCs (pre-extracted) to the mean response A/IS of the analytes in post-extracted QCs or neat analytes solution, respectively. Absolute matrix effect was calculated as the ratio of the mean response A/IS of the analytes in post-extracted QCs to mean response A/IS of the analytes in unextracted solvent QCs.

The absence of relative matrix effect is an even more important parameter than the evaluation of the absolute matrix effect. It assures that the method accuracy is not compromised by matrices originating from different individuals. According to Matuszewski [34] we evaluated the relative matrix effect by constructing six standard curves prepared as described in the section 2.4. DBS sample preparation using drug-free venous EDTA anticoagulated blood from six different sources. The calculated coefficient of variation of the calibration line slopes in each matrix should not exceed 4% in order for the method to be considered reliable and free from significant relative matrix effect.

2.6.5. Carry-over

Carry-over was assessed for both analytes by the injection of a blank sample immediately following the highest concentration standard (two-fold QCH), which was prepared for the dilution integrity test.

2.6.6. Dilution integrity

A sample above the upper limit of quantification (> ULOQ), *i.e.*, two-fold QC_H, was prepared in duplicate as described previously. The final extract after the sample preparation was subsequently diluted 2-fold with the final extract of a processed blank DBS. The response ratio of analyte *versus* IS of the sample > ULOQ was compared with the response ratio of the QC_H. The criterion for confirmation of the dilution integrity was deviation < 15%.

2.6.7. Haematocrit effect

Drug-free EDTA anticoagulated venous blood was adjusted to a range of Hct values as are expected in clinical practice. The blood samples with Hct values 0.20, 0.40, and 0.60 were prepared by adding or removing the appropriate volume of blood plasma from the blood with defined volume and known Hct value [35]. For each Hct level, QC_L and QC_H samples were spiked and analysed in triplicate. The obtained response ratio of the analyte *versus* IS of the QC samples at selected Hct value was compared with response ratio of the QC sample at Hct value 0.4. The absence of the haematocrit-based overall bias was confirmed if deviation was < 15%.

2.6.8. Blood spot size

This investigation was conducted to demonstrate that after selection of a disc size for analyses, the obtained quantitative results were not affected by the volume of blood deposited presuming there is uniformity in the spread of the spot on DBS card. To investigate the blood volume effect DBS calibrators with 30 μL blood spots and DBS QC samples at low and high levels in triplicates with 20, 30, 40 and 50 μL blood spots were prepared. 6 mm discs were punched from the spots center and prepared according to the section 2.4. DBS sample preparation. Concentrations of QC samples calculated from the calibration curve obtained with punched calibrators were compared to their nominal concentrations. A deviation of < 15% confirmed the absence of the blood spot size effect.

2.6.9. Blood spot homogeneity

Blood spot homogeneity was investigated together with the blood spot size, however, it was estimated only for 40 and 50 μL blood spots. 6 mm discs were punched from the periphery of the spot. The concentration of the sample was determined equally as in the experiment for evaluation of blood spot size effect using equal criteria.

2.6.10. Stability of dried blood spots

We investigated the effect of storage time and storage temperature on the stability of spiked DBS samples. Three replicates of DBS QC samples at two concentrations (QC_L and QC_H) were prepared and dried at room conditions for three hours. The samples were then stored in sealed plastic bags with desiccant packages at four temperatures, *i.e.* -20 °C, 2–8 °C, room temperature (20 to 25 °C), and 40 °C. Stability was tested from day 0 till 2 months. After the storage period, samples were analysed and the concentrations were calculated from a peak area ratio of analyte *versus* IS using the equation of freshly prepared calibration curve. The samples were considered stable if their calculated concentration did not deviate > 15% from the nominal concentration.

2.6.11. Application of the method

The method was applied to patients on therapy with azathioprine or 6-MP sent to routine TDM. The study protocol was approved by the Slovenian National Medical Ethics Committee (0120–013/2016–2). The venous blood samples were collected into 3 mL Vacutainer tubes containing potassium EDTA. DBS samples in duplicates were prepared as described above in the section 2.4. DBS sample preparation. The remaining of the blood was used for routine TDM of 6-TG and 6-MMP in hemolysates using HPLC method with UV detector according to Dervieux *et al* with some modifications [15]. In brief, hemolysate samples were prepared by three times consecutive centrifugation and washing of erythrocytes with phosphate buffer saline. The final pellet was reconstituted with cold water to initial volume of the blood, haemolysed, aliquoted in 200 μL of hemolysate and stored at −80 °C till further analysis. 25 μL of 0.5 M DTT and 20 μL of 70% perchloric acid was added to unfrozen hemolysates. After centrifugation supernatants were heated at 100 °C for 45 min. Cold samples were centrifuged again and supernatants were injected into the Agilent 1260 Infinity Series HPLC system from Agilent (Waldbronn, Germany). Sample separation was obtained on Agilent Zorbax Eclipse plus C18 (150 mm × 4.6 mm, 5 μm) and isocratic elution with 20 mM phosphate buffer pH 2.0 and acetonitrile (98:2, *v:v*). The 6-TG and 6-MMP were detected at 342 and 303 nm, respectively. The method was linear from 25 to 1100 and 200–10,000 pmol/8 × 10^8 Ery for 6-TG and 6-MMP, respectively. To compare the determined patient concentrations in DBS and hemolysate samples, DBS analyte concentrations expressed in μg/L were calculated to pmol/8 × 10^8 Ery considering the analyses' molecular weight and individual patient number of erythrocytes in the blood. As number of erythrocytes per blood volume is equal to ratio of haematocrit and mean corpuscular volume, molar concentrations per number of erythrocyte can be calculated also from individual haematocrit and mean corpuscular volume (Supplementary material). The obtained concentrations were analysed using Deming regression and Bland-Altman approach.

3. Results

3.1. General method validation

3.1.1. Selectivity

Chromatograms of six blank DBS samples were superimposed with 6-TG and 6-MMP LLOQ sample chromatograms (Supplementary material, Fig. 1S). No significant interferences at the retention times of the analytes were detected.

3.1.2. Linearity and lower limit of quantification

The developed method is linear from 80.0 to 8000 μg/L for 6-TG and from 400 to 8000 μg/L for 6-MMP as determined from back-calculated calibrators. All calibrators deviated within 0.01 and 11.5% except LLOQ which deviated < 16.1%. The mean slope and intercept values for the calibration curves are given in Table 1. The lower limit of quantification was 80.0 μg/L for 6-TG and 400 μg/L for 6-MMP.

3.1.3. Precision and accuracy

The results of precision and accuracy studies are presented in Table 2. The obtained values were within the recommendations of EMA [30]. The intra and inter-day precision of 6-TG and 6-MMP was below 7.8 and 11.2%, respectively, while intra and inter-day accuracy was within 98.1 to 110.3 and 97.1 to 102.9%, respectively.

3.1.4. Recovery and matrix effects

Table 3 shows that based on the quantitative assessment, the estimated absolute matrix effect on 6-TG and 6-MMP was found to be within 92.2% and 104.5%, which indicates that the method is free from any major ion suppression or enhancement for both analytes. Mean recovery at the two quality control sample levels were within 79.7% and 103.1%. The method displayed good overall process efficiency (above 80%).

Relative matrix effect was estimated by comparing slopes of the calibration curves, constructed from DBS calibrators prepared from six different sources. Coefficient of variability between slopes was 0.60% for 6-TG and 1.96% for 6-MMP. No significant difference in slopes

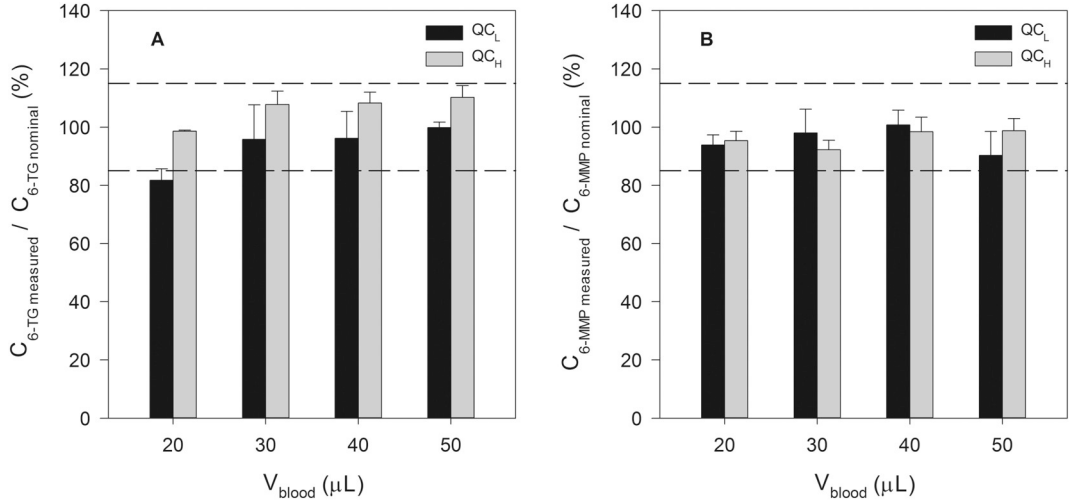

Fig. 1. Influence of blood spot volume on concentration accuracy of 6-TG (A) and 6-MMP (B). DBS were prepared from blood volumes ranging from 20 to 50 µL and 6 mm punches were used for analysis. Determined concentrations are presented *versus* nominal for QC samples at low and high concentrations (mean ± standard deviation; $n = 3$). The dotted lines on the figure present ± 15% deviation.

Table 1
Linear response range, mean slope, intercept values, and coefficient of determination (r^2) for calibration curves of 6-TG and 6-MMP.

	Linear response range (µg/L)	Intercept	Slope	r^2
6-TG				
1. day	80.0–8000	0.0143	0.00213	0.997
2. day		−0.0282	0.00250	0.999
3. day		0.00278	0.00249	0.998
6-MMP				
1. day	400–8000	−0.00476	0.000214	0.999
2. day		−0.00331	0.000228	0.999
3. day		−0.00400	0.000215	0.997

indicates a lack of relative matrix effects for both analytes.

3.1.5. Carry-over

No carry-over was observed for 6-TG and 6-MMP at the injection of a blank sample following immediately the highest concentration standard (two-fold QC_H), which was prepared for the dilution integrity test.

3.1.6. Dilution integrity

The > ULOQ sample of 12,000 µg/L was prepared and diluted to the concentration of 6000 µg/L equivalent to the QC_H. The comparison of response ratio of analyte *versus* IS for > ULOQ and QC_H showed that the precision of the diluted > ULOQ samples were for 6-TG and 6-MMP 2.7 and 12.9%, respectively. Therefore, it was concluded that samples exceeding the ULOQ could be diluted by the studied procedure.

3.2. DBS specific validation

To further demonstrate the suitability of the method to be applied on DBS samples, the specific DBS validation parameters were studied.

3.2.1. Haematocrit effect

The study was designed to cover Hct levels in typical patients on azathioprine therapy. QC samples at low and high concentrations of 6-TG and 6-MMP in blood with Hct values 0.2, 0.4, and 0.6 were prepared. Whole spot area was used for sample extraction, samples were further analysed and ratios of response (analyte / IS) of the QC sample at specific concentration *versus* response of the QC sample at 0.4 Hct value were calculated. The ratios of 6-TG ranged from 92.4 to 100.4% with RSD < 6.6% at both concentration levels and all three Hct values. Moreover, the ratios for 6-MMP ranged from 97.5 to 100.0% with RSD < 4.7% at all studied samples. The results show the absence of haematocrit-based overall bias.

3.2.2. Blood spot volume

To investigate the effect of different volumes of blood used for preparation of the blood spots, blood volumes in the range from 20 to 50 µL were spotted on DBS cards. The results on the Fig. 1 show that determined concentrations accuracy is within 15% deviation. The only exception was the result obtained with 20 µL spot at QC_L level for 6-TG due to reduced saturation of the DBS card or not entirely filled 6 mm punch in comparison with 30 µL spots which were used for calculation of the calibration curve in this experiment. The results confirm that blood spot volume does not affect the accuracy of the concentrations for both analytes.

3.2.3. Blood spot homogeneity

The results obtained with the 6 mm disk punched from periphery of 40 and 50 µL blood spots were analysed. The accuracy of the 6-TG QC samples at low level was within the range from 87.0 to 97.8% and 107.9 to 108.1% for high level, irrespectively of the blood volume. Moreover, accuracy of the 6-MMP QC samples was within 99.7 to 103.8% regardless of the concentration level and volume of blood spot. The results confirm homogeneity of the blood spot irrespective of the studied blood volume.

3.2.4. Stability of dried blood spots

The stability of 6-TG and 6-MMP in DBS samples was evaluated by exposing DBS samples to different conditions (time and temperature) at two QC concentration levels for 6-TG and 6-MMP in three replicates. The results were compared with those obtained for the freshly prepared DBS samples. All DBS samples are stable for two months if stored at room temperature (RT), 2–8 °C or − 20 °C. However, storage at 40 °C caused a significant degradation of 6-TG after two months: the concentration fell to 62–69% if compared with a freshly prepared sample. No significant degradation of 6-MMP at that condition was observed. We can conclude that 6-TG DBS samples are stable for one month if stored at 40 °C. Collected data are shown in Fig. 2.

3.3. Clinical evaluation

The clinical evaluation of the method was performed on 34 samples of patients on therapy with azathioprine who were submitted to routine control of the 6-TG and 6-MMP levels. The concentrations of the

Table 2
Intra and inter-day precision and accuracy of 6-TG and 6-MMP determination.

6-TG									
Intra-day precision					Inter-day precision				
(n = 6)	Level	Nominal (μg/L)	Mean (μg/L)	R.S.D. (%)	(n = 3)	Level	Nominal (μg/L)	Mean (μg/L)	R.S.D. (%)
	LLOQ	80.0	85	4.02		LLOQ	80.0	88.2	6.49
	QC_L	240	236	2.82		QC_L	240	246	7.78
	QC_M	2400	2417	3.34		QC_M	2400	2416	2.78
	QC_H	6000	6147	2.38		QC_H	6000	6149	2.50
Intra-day accuracy					Inter-day accuracy				
(n = 6)	Level	(%)			(n = 3)	Level	(%)		
	LLOQ	106.7				LLOQ	110.3		
	QC_L	98.1				QC_L	102.3		
	QC_M	100.7				QC_M	100.7		
	QC_H	102.5				QC_H	102.5		
6-MMP									
Intra-day precison					Inter-day precision				
(n = 6)	Level	Nominal (μg/L)	Mean (μg/L)	R.S.D. (%)	(n = 3)	Level	Nominal (μg/L)	Mean (μg/L)	R.S.D. (%)
	LLOQ	400	397	9.67		LLOQ	400	396	11.19
	QC_L	1200	1193	6.98		QC_L	1200	1165	8.13
	QC_M	2400	2469	8.53		QC_M	2400	2447	7.16
	QC_H	6000	5842	7.83		QC_H	6000	6079	6.45
Intra-day accuracy					Inter-day accuracy				
(n = 6)	Level	(%)			(n = 3)	Level	(%)		
	LLOQ	99.3				LLOQ	98.9		
	QC_L	99.4				QC_L	97.1		
	QC_M	102.9				QC_M	102.0		
	QC_H	97.4				QC_H	101.3		

QC_L - quality control samples at low concentration; QC_M - quality control samples at medium concentration; QC_H - quality control samples at high concentration; R.S.D. - relative standard deviation.

Table 3
Absolute matrix effect, recovery and overall process efficiency for DBS analysis of 6-TG and 6-MMP in blood samples.

	6-TG		6-MMP	
Nominal conc. (μg/L)	240 (QC_L)	6000 (QC_H)	1200 (QC_L)	6000 (QC_H)
Absolute matrix effect (%)	104.5	102.7	102.1	92.2
Recovery (%)	89.0	79.7	103.1	87.5
Process efficiency (%)	93.1	81.9	105.3	80.7

The results are presented as mean from two replicates.

analytes were measured with routine HPLC-UV method in hemolysates and with the novel method employing DBS samples and LC-MS/MS. A typical chromatogram obtained with LC-MS/MS by analysing a DBS sample from a patient on therapy with azathioprine is shown in the Fig. 2S (Supplementary material). The concentrations of 6-TG and 6-MMP were in five and four samples below the LLOQ, respectively. The concentrations of 6-TG and 6-MMP are clinically presented in pmol/8×10^8 Ery which is approximately equivalent to 200 μL of blood [19]. 6-TG concentrations ranged from 79 to 802 pmol/8×10^8 Ery and from 96 to 813 pmol/8×10^8 Ery, for hemolysates and DBS samples, respectively. 6-MMP concentrations were within 320 to 6246 pmol/

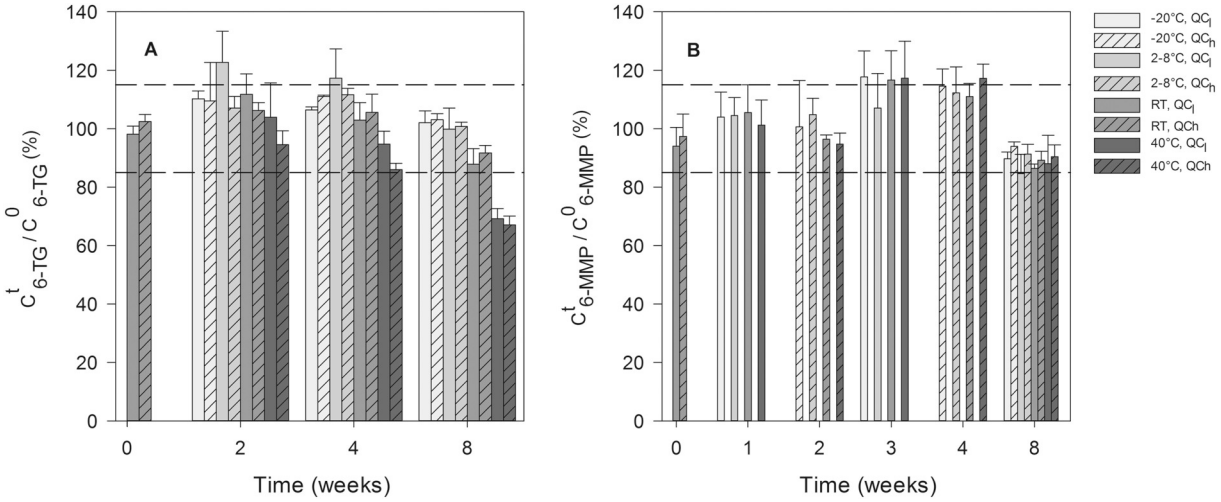

Fig. 2. Long-term stability of 6-TG (A) and 6-MMP (B) on DBS card at four different temperatures (−20 °C, 2–8 °C, room temperature, 40 °C) and two concentrations (QC_l and QC_h). The results are presented as mean ± standard deviation of three replicates.

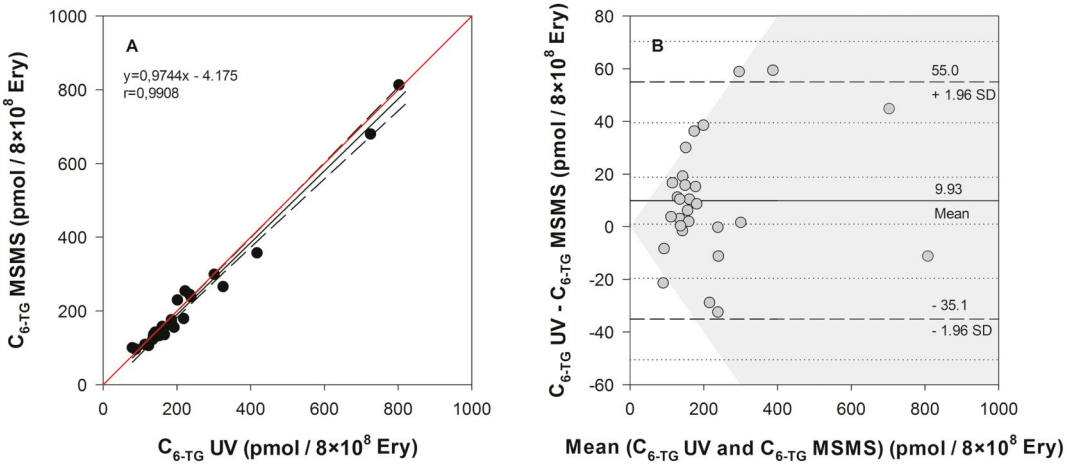

Fig. 3. Comparison of 6-TG concentrations in patients (n = 29). Weighted Deming regression of 6-TG concentrations measured by HPLC-UV and plotted against concentrations measured by LC-MS/MS (A). Solid black line is Deming fit (y = 0.9744× - 4.175; r = 0.9908), dashed lines are 95% CI of fit. Red line is identity line. Bland-Altman plot for 6-TG determined by using HPLC-UV and LC-MS/MS method (B). The mean line represents the bias between the measured concentrations by both methods and 1.96 SD dashed lines represent 95% limits of agreement. Dotted lines represent 95% CI. Shaded area represents the 20% difference between both methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

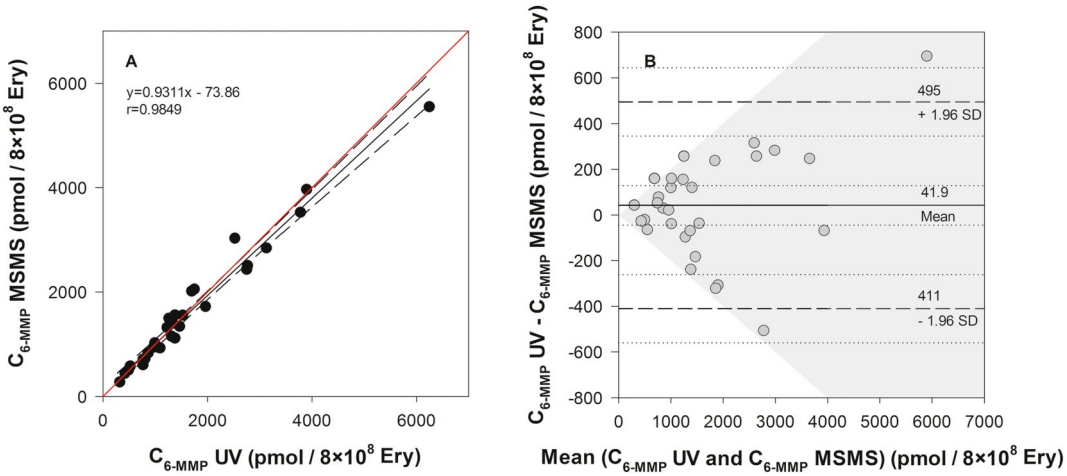

Fig. 4. Comparison of 6-MMP concentrations in patients (n = 30). Weighted Deming regression of 6-TG concentrations measured by HPLC-UV and plotted against concentrations measured by LC-MS/MS (A). Solid black line is Deming fit (y = 0.9311× – 73.86; r = 0.9849), dashed lines are 95% confidence interval of fit. Red line is identity line. Bland-Altman plot for 6-MMP determined by using HPLC-UV and LC-MS/MS method (B). The mean line represents the bias between the measured concentrations by both methods and 1.96 SD dashed lines represent 95% limits of agreement. Dotted lines represent 95% CI. Shaded area represents the 20% difference between both methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

8×10^8 Ery and 277 to 5551 pmol/8×10^8 Ery for hemolysates and DBS samples, respectively. The results of the clinical evaluation are presented using Deming regression and Bland-Altman analysis (Figs. 3 and 4). The slope and intercept of Deming regression analysis for 6-TG were 0.974 (95% CI, 0.92 to 1.03) and − 4.175 (95% CI, −19.1 to 10.8), respectively (Fig. 3A). On the other hand, Deming regression slope and intercept for 6-MMP was 0.931 (95% CI, 0.87 to 1.00) and − 73.9 (95% CI, −58.7 to 206), respectively (Fig. 4A). Moreover, the Pearson correlation coefficient for 6-TG and 6-MMP was 0.991 and 0.985, respectively. The observed values of slope are close to unity and their 95% CI contains 1 for both analytes. It is also evident that 95% CI of intercepts contains 0, and that Pearson coefficients are > 0.985 for both analytes which indicates a good agreement between both methods. Bland Altman analysis presented on Fig. 3B and 4B shows that there is a small mean difference between concentration obtained with HPLC-UV and LC-MS/MS for 6-TG (9.9 pmol/8×10^8 Ery) and for 6-MMP (42 pmol/8×10^8 Ery). The 95% confidence intervals of the mean difference are quite narrow and include or are close to zero suggesting absence of bias. Additionally, the width of the span between the lower and upper limit of agreement is relatively low. Finally, based on 20% acceptance criterion [36] only one sample for both analytes lies outside the range with deviation < 24%, thus confirming good agreement between methods.

4. Discussion

Therapeutic drug monitoring of azathioprine metabolites is one of the strategies to optimize pharmacotherapy in patients with IBD. TDM is recommended to optimize therapeutic effect after 4 weeks of initiation of the therapy with azathioprine or 6-MP and in the follow-up period of the therapy to prevent toxicity and identify the cause for lack of response to thiopurine therapy [5,6,19]. The target cells of the azathioprine therapy are the lymphocytes, however, Ery are used as surrogate markers, due to their abundance in the blood and comparable concentrations of 6-TG and 6-MMP in lymphocytes [37]. As the metabolites are accumulated in the cells 6-TG and 6-MMP are not detected in the plasma [13]. Therefore, majority of clinical laboratories use either Ery or whole blood samples as both matrices can be interchangeably

used for TDM of thiopurine drug metabolites determination [13]. The isolation of Ery is time consuming as it involves steps of blood washing with saline buffer. Moreover, isolated Ery and whole blood samples have to be frozen to prevent analyte degradation and this can additionally increase the costs of analysis. To overcome these problems, we aimed to develop an analytical method for determination of 6-TG and 6-MMP using DBS samples.

An analytical method for determination of 6-MP and its metabolites, 6-MMP and 6-thioguanosine-5′-monophosphate, using DBS sampling was recently published [38]. The authors presented the method development, general method validation and final application to the patients diagnosed with lymphoblastic leukemia and treated with 6-MP. Regardless of the published method, we think that our work is novel, since it is focused to the patients with IBD. Moreover, we believe that our method has several advantages in the analytical part and more elaborated clinical evaluation which are presented in the following text.

The optimisation of extraction procedure began with the selection of appropriate DBS cards. We selected Whatman 903 card, which is also one of the most commonly used [32]. 6-TGN and 6-MMPr are very polar compounds, therefore, polar extraction solvent should facilitate their extraction. However, large percentage of water in the extraction solvent enables extraction of haemoglobin and other polar haematic compounds, which can produce severe matrix effect [39]. Methanol, acetonitrile and their mixtures with water in ratios 80/20 and 60/40 *v/v* were first explored as extraction solvent using samples from DBS cards where standard solution without the blood was deposited on cards. Samples were centrifuged and supernatant (without the DBS disk) was exposed to hydrolysis. Acetonitrile and both mixtures of acetonitrile/water were found to be unsuitable due to low recoveries of both analytes. Mixture of methanol/water in 80/20 *v/v* ratio showed highest recoveries, which were however low when DBS samples were used. The recoveries from DBS samples were further improved by including sonication before centrifugation step. Finally, the centrifugation step was omitted, and extraction mixture including the DBS disk was exposed to acid hydrolysis at high temperature. Recoveries > 80% were obtained for both analytes. The HPLC UV method used the perchloric acid for nucleotides hydrolysation in hemolysates. We decided to substitute perchloric acid with more volatile trifluoracetic acid, which is more suitable for mass spectrometry applications. The effect of trifluoroacetic acid concentration and time of hydrolysis at 100 °C were studied. None of the conditions enabled determination of comparable concentrations of 6-TG and 6-MMP as when hydrolysis was performed with perchloric acid. Therefore, method development optimization continued by using perchloric acid. The appropriateness of the selected conditions for extraction and hydrolysis are supported by high linearity, recoveries, process efficiencies, and absence of relative and absolute matrix effect for 6-TG and 6-MMP (Tables 1 and 3).

The general method validation parameters revealed that the method meets the required criteria. The method range is from 50 to 5300 pmol/8×10^8 Ery and from 260 to 5300 pmol/8×10^8 Ery for 6-TG and 6-MMP, respectively (average number of Ery in volunteers blood, 4.1×10^{12} Ery/L, was used for calculation). Moreover, dilution integrity showed that the upper limit of quantification can be increased up to 8000 pmol/8×10^8 Ery for both analytes, without compromising the accuracy or precision. Therefore, we can appropriately cover the predisposed therapeutic range for 6-TGN [7–12] and toxicity threshold of 6-MMP [7]. Moreover, the method range would also enable the TDM of patients on combinational therapy of azathioprine and infliximab, where the proposed threshold level of 6-TGN for detection of patients with unfavourable infliximab pharmacokinetic is 105 pmol/8×10^8 Ery [40]. The concentration ranges of comparable methods for determination of thiopurine metabolites in whole blood samples were determined by Kirchherr *et al* [19] and ranged from 30 to 10,000 pmol/8×10^8 Ery for 6-TG and 6-MMP, while Cangemi *et al* [13] determined the range for 6-TG from 19 to 608 pmol/8×10^8 Ery and 143 to 4560 pmol/8×10^8 Ery for 6-MMP. The method for determination of 6-MMP in DBS samples ranged from 25.5 to 1020 μg/L [38]. Our method has comparable ranges with methods using whole blood samples, and a higher ULOQ in comparison with the recently published DBS method [38]. This offers an important advantage for identification of patients at risk for development of myelosuppression and hepatotoxicity. Moreover, the matrix effect in DBS samples reported by Supandi *et al* [38] was higher (> 10%) than ours, since we obtain < 8% for absolute and only 2% for relative matrix effect. One of the differences between methods is the inclusion of isotope labelled analogues as IS for both analytes in our method. Chosen internal standards result in more efficient control of matrix effect, which was additionally confirmed by comparison with results where matrix effect was calculated without considering IS.

Method validation process was also intensively focused on DBS specific validation parameters. Different study approaches can be used to determine the Hct effect [26,41]. We studied the Hct-based overall assay bias by using the whole DBS area. The parameter was studied on the wide range of Hct values from 0.2 to 0.6, which is also supported by the Hct values of patients included in the study which had Hct values in a range from 0.260 to 0.478. The results showed that the analytes response was not influenced by haematocrit values confirming the absence of Hct-based overall bias [26,41]. The sample preparation method which was performed with whole spot area was also upgraded by demonstrating that punched disks of 6 mm can be used as well. The study of blood spot volume confirmed that blood volumes higher than 30 μL do not affect the method accuracy and precision and thus enable to use the method on DBS samples where blood volume used for preparation of the spot is unknown. Simultaneously, we studied the blood spot homogeneity. We proved that central or peripheral punched disk do not affect the method accuracy and therefore both areas can be used for preparation of the samples. DBS sampling offers also possible increase in analytes stability. We demonstrated that both analytes are stable for at least two months if stored at 40 °C, room temperature (RT), 2–8 °C or − 20 °C, except 6-TG which was stable only for one month at 40 °C. This is also expected since the free thiol group on 6-TG is more prone to oxidation process than the methylated one on 6-MMP. Nevertheless, DBS samples show superior stability in comparison with samples of Ery or whole blood, where stability was demonstrated only for a few days [42] or even hours [43] at RT or under refrigeration. Additionally, the frozen Ery or whole blood samples at − 20 or − 80 °C showed comparable stability as our DBS samples [42,43]. High stability of analytes in DBS samples renders it possible to use regular mail at room temperature for the shipment.

Clinical validation of 6-TG and 6-MMP in DBS samples was studied for the first time. We demonstrated that by recalculation of the concentrations in DBS samples from μg/L to pmol/8×10^8 Ery and by using individual number of erythrocytes in the patients' blood, comparable concentrations as in hemolysates are obtained. The mean difference between the methods was 9.93 and 41.9 pmol/8×10^8 Ery for 6-TG and 6-MMP, respectively, which means that DBS method determines slightly lower concentrations than the method with hemolysates. However, the 95% confidence intervals of the mean differences were narrow indicating no significant bias between both methods. In addition, the differences between both methods are clinically not relevant since the therapeutic range for 6-TG and the toxic threshold for 6-MMP are approximately one to two orders above the obtained difference. To further demonstrate the clinical usefulness of the DBS samples, concentrations determined in patients were classified according to therapeutic range for 6-TG and toxicity threshold for 6-MMP. The patients were classified as low, within the range or in risk for toxicity if 6-TG concentrations were lower than 250, within 250 to 450, and higher than 450 pmol/8×10^8 Ery, respectively. Moreover, based on 6-MMP concentrations, they were classified as no risk or at risk for toxicity if their concentrations were lower or higher than 5700 pmol/8×10^8 Ery, respectively. The 6-TG concentrations in hemolysates showed that 24 patients were classified as low, 3 within the range, and

2 at risk for toxicity. Similar results were obtained also with DBS samples except that one patient, which had the hemolysate concentration 222 and DBS concentration 254 pmol/8 × 10^8 Ery, was classified to the within the range group based on DBS concentration. Based on the 6-MMP concentrations, 29 patients were classified to no risk group and one to risk for toxicity group, irrespective of the determination method. These results, in addition to previous ones, confirm that methods using hemolysates or DBS samples are equally acceptable from analytical and clinical point of view.

The presented method enables determination of 6-TG and 6-MMP in DBS samples by using individual number of erythrocyte per blood volume or haematocrit and mean corpuscular volume. Quantitative DBS strategies are focusing on methodologies that enable analyte determination based on information that can be obtained only from DBS sample. Different methodologies are described in the literature that enable determination of haematocrit from the DBS sample using non-destructive methodologies as UV-VIS [44] and near infrared spectrometry [45] or image analysis [46] and a destructive one as well *e.g.* measurements of haemoglobin [47], potassium ions [48] or lipids [49] from DBS extracts. We demonstrated that by using individual haematocrit values and a fixed population typical value of mean corpuscular volume, comparable concentrations as those from hemolysates can be obtained (Supplementary materials, Fig. 3S). Therefore, we can speculate that our approach could be used on samples of unknown haematocrit values *e.g.* fingerprick DBS samples, as well. However, as this approach was not confirmed, further investigations of methodologies that combine volumetric application on the DBS paper and haematocrit estimation from DBS samples are warranted.

5. Conclusions

We developed an analytical method for determination of 6-TG and 6-MMP in DBS samples intended for TDM of patients with IBD and therapy with azathioprine. The method fulfilled the validation criteria in range from 50 to 8000 and 260 to 8000 pmol/8 × 10^8 Ery for 6-TG and 6-MMP, respectively, which is clinically feasible. The clinical validation confirmed that DBS method and routine clinical method which uses hemolysate samples give comparable results and enable similar clinical decisions. The major advantages of DBS sampling such as the simple sample preparation and the long term stability at room temperature can facilitate the method application to the clinical practice.

Acknowledgements

This work was supported by the Slovenian Research Agency (Grants P1-0189 and P1-0153).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.08.024.

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