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Limited stability of thiopurine metabolites in blood samples: Relevant in research and clinical practise

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ABSTRACT

Background: Monitoring of thiopurine metabolites 6-thioguanine nucleotides (6-TGN) and 6-methylmercaptopurine (6-MMP) is used to assess compliance and explain adverse reactions in IBD-patients. Correlations between dosage, metabolite concentrations and therapeutic efficacy or toxicity are contradictive. Research is complicated by analytical problems as matrices analyzed and analytical procedures vary widely. Moreover, stability of thiopurine metabolites is not well documented, yet pivotal for interpretation of analytical outcomes. Therefore, we prospectively investigated metabolite stability in blood samples under standard storage conditions.
Methods: Stability at room temperature and refrigeration (22 °C, 4 °C) was investigated during 1 week and frozen samples (−20 °C, −80 °C) were analyzed during 6 months storage. Ten patient samples were analyzed for each study period.
Results: Median 6-TGN concentrations on day 7 decreased significantly to 53% and 90% during storage at ambient temperature or refrigeration. Median 6-MMP concentrations on day 7 decreased significantly to 55% and 86%, respectively. Samples stored at −20 °C also showed significant decreases in both 6-TGN and 6-MMP in comparison with baseline values. At −80 °C, only 6-MMP showed a significant decrease in values compared to baseline.
Conclusion: The stability of thiopurine metabolites is clearly a limiting factor in studies investigating utilisation of TDM and correlations with therapeutic outcome in IBD-patients. This has to be accounted for in clinical practice and (multi-center) trials investigating thiopurine drugs.

1. Introduction

The thiopurines azathioprine (AZA) and 6-mercaptopurine (6-MP) are widely used immunosuppressive drugs in the treatment of inflammatory bowel disease (IBD) and efficacy has been demonstrated in inducing and maintaining remission of disease [1–4].

The clinical pharmacology of thiopurines is complex and subject to large inter- and intra-individual variations (see Fig. 1). In short, AZA is converted into 6-MP after oral administration. Subsequent metabolism along three competing routes is mediated by xanthine oxidase (XO), thiopurine S-methyl transferase (TPMT) and hypoxanthine phosphoribosyl transferase (HPRT). Former two enzymes lead to formation of 6-thiouric acid (6-TUA) and 6-methyl mercaptopurine (6-MMP). The latter enzyme system results in formation of 6-TIMP, which is converted to the active 6-thioguanine nucleotides (6-TGN) by inosine monophospate dehydrogenase (IMPDH) and guanosine monophosphate synthetase (GMPS). The 6-TGN metabolites consist of 6-thioguanosine 5′-monophosphate (6-TGMP), 6-thioguanosine 5′-diphosphate (6-TGDP) and 6-thioguanosine 5′-triphosphate (6-TGTP).

Thiopurines were originally thought to exert their action as antimetabolites [5–8]. However, new research has revealed that 6-TGN contributes significantly to the overall molecular immunosuppressive effect of thiopurines in IBD-patients by inhibition of the small GTPase Rac1 [9–11].

Prediction of therapeutic response is cumbersome and correlations between dosage, thiopurine metabolite concentrations and therapeutic efficacy or toxicity still have to be established [12–14]. 6-TGN levels greater than 230–260 pmol/8 × 10^8 red blood cells (RBC) are associated with an increased likelihood of remission or optimal therapeutic response [15,16]. Additionally, 6-MMP levels above 5700 pmol/8 × 10^8 RBC have been associated with

Abbreviations: IBD, inflammatory bowel disease; AZA, azathioprine; 6-MP, 6-mercaptopurine; TDM, therapeutic drug monitoring; 6-TG, 6-thioguanine; 6-TGN, 6-thioguanine nucleotide; 6-MMP, 6-methylmercaptopurine; RBC, red blood cell; PBS, phosphate buffered saline; CI, confidence interval.

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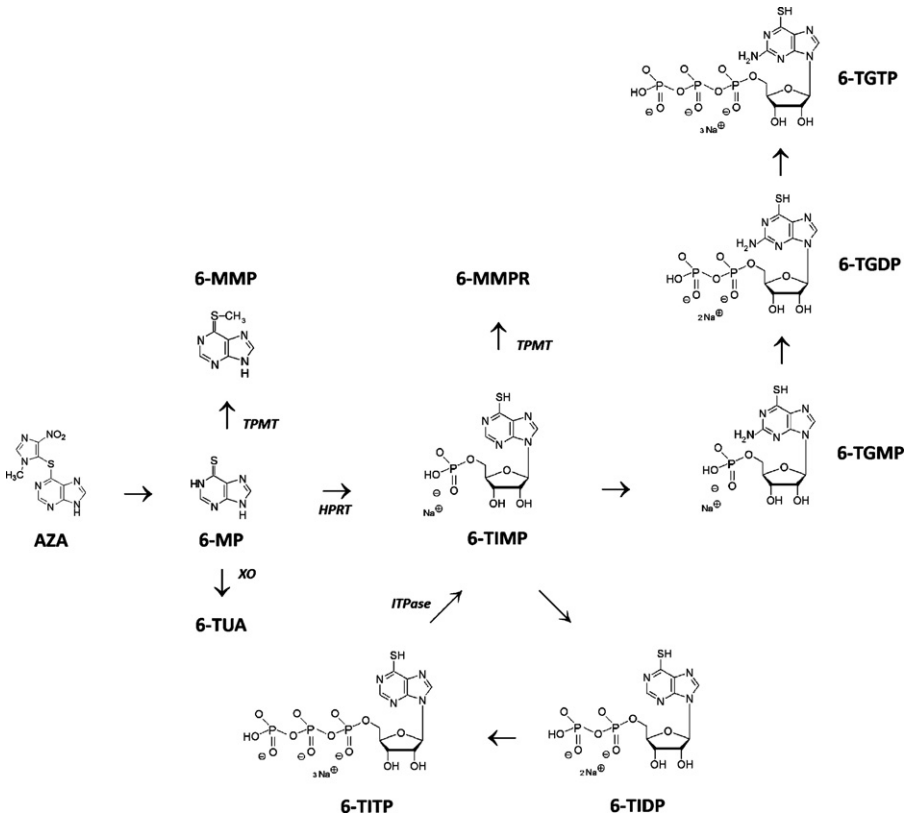

Fig. 1. The metabolic pathway of thiopurine metabolism in humans.

hepatotoxicity [16]. Several other studies, however, did not find such a correlation [17–19]. In daily IBD-practice, therapeutic drug monitoring (TDM) of thiopurine metabolites is used for monitoring therapeutic compliance and to predict chance of efficacy or occurrence of adverse reactions.

As well as utilisation of various matrices used for analysis of thiopurine metabolites, analytical procedures also differ in sample preparation procedures, extraction steps, analytical targets, elution procedures and final detection. To complicate matters even more, various units have been used to report the results of 6-TGN measurements. The analytical procedures by Lennard or Dervieux are most widely used in clinical practice and the former has served as the basis for establishing the current therapeutic ranges for 6-TGN and 6-MMP [20,21].

Apart from these critical analytical problems, stability of thiopurine metabolites themselves is an issue that is often overlooked. We therefore prospectively investigated the stability of 6-TGN and 6-MMP metabolites in patient blood samples under various storage conditions after validating the assay of these metabolites in the laboratory of our clinical pharmacy department.

2. Study design

Being part of our validation procedure, stability of thiopurine metabolites was investigated in patient samples stored at four standard storage conditions: room temperature (22 °C), refrigerated (4 °C) and frozen at −20 °C and −80 °C, respectively. Stability was investigated at room temperature and refrigeration during 1 week (study A) and in frozen samples during 6 months storage (study B); ten patients were sampled for each study period.

In study A, each sample was divided into equal portions at the laboratory of the clinical pharmacy after homogenization. RBC-isolation and metabolite analysis in each sample was performed on days 0, 1, 4, 5, 6 and 7 after sampling. In study B, red blood cells were isolated and counted *before* sample storage. Analysis of the frozen samples was performed at day 0 and in weeks 1, 2, 4, 6, 8, 12, 16, 20 and 26 after sampling. The design for study B was chosen, as freezing of blood samples results in erythrocyte lysation and cell counting is technically impossible on the day of analysis for reporting in standard units (pmol/8.0 × 10^8 RBCs). We have published the results from study A earlier in a short letter [24].

3. Materials and methods

3.1. Acquisition of patients and blood sampling procedure

Blood was sampled in 10 mL lithium-heparin tubes from IBD-patients visiting the Outpatient Department of the section Gastroenterology and Hepatology in the VU University Medical Centre as part of their routine visit. All samples were immediately homogenized and transported to the laboratory of the clinical pharmacy.

3.2. Drugs and reagents

All reagents were of the highest available chemical purity. 6-Thioguanine (6-TG), 6-MMP, dithiothreithol (DTT) and 5-bromouracil (5-BU) were purchased from Sigma (St. Louis, USA). Phosphoric acid 85% (PA), and perchloric acid 70% (PCA) were obtained from Merck (Darmstadt, Germany) and acetonitrile (ACN; HPLC Supra-gradient) from Biosolve (Valkenswaard, The Netherlands). Stock solutions of 6-TG (600 pmol/μl) and 5-BU (5 pmol/μl) were prepared in 0.1 M NaOH; the stock solution of 6-MMP (600 pmol/μl) was prepared in HCl 0.1 M (in purified water; Millipore Synergy UV purification system). All stock and DTT solutions were prepared fresh before each run.

3.3. Red blood cell isolation and counting

All samples were treated according to the study design. Tubes were centrifuged at 160 × *g* for 10 min in a Hettich Rotixa/AP centrifuge at ambient temperature. The plasma layer and buffycoat were discarded and replaced by an equal amount of phosphate buffered saline (PBS). After careful mixing the sample was centrifuged at 160 × *g* for 10 min one more time, the upper layer was discarded and replaced by PBS. This procedure was repeated once again at 640 × *g* for 10 min after careful mixing. The final cell isolates were diluted with equal amounts of PBS and the exact red blood cell counts per sample volume were determined using an automatic hematologic cell counting device ('CELL-DYN Sapphire™', Abbott, IL, USA).

3.4. Sample preparation

Our routine assay as well as the handling of patient samples was based on the method described by Shipkova et al. [22]. In brief, 20 μl internal standard (300 pmol/μl 5-BU), 20 μl DTT 1.1 M and 30 μl water was added to 250 μl of isolated erythrocytes in a glass test-tube and mixed carefully. Subsequently, 45 μl perchloric acid 70% was added for deproteinization and the tube was vortex-mixed for 90 s. After centrifuging for 15 min at 4000 rpm, the supernatant was transferred to a glass tubes (100 mm × 16 mm) with screw caps and incubated for 1 h at 100 °C in a block heater UBD4 (Grant; Shepreth, UK) for hydrolysis of the 6-TGN to free 6-TG. After cooling to room temperature, a 50 μl aliquot was injected into the column.

3.5. Chromatographic procedure and performance characteristics

A reversed phase gradient HPLC system was used for separation of 6-TG, 6-MMP, 5-BU and DTT, using a Symmetry C18 column as the stationary phase (150 mm × 3.9 mm; particle size 5 μm; Waters Corp.), a Waters (Milford, MA, USA) 717 Plus autosampler, a P680 LPG pump and a UVD340U Diode Array detector, both from Dionex (Sunnyvale, CA, USA).

Gradient elution at a flow of 1.2 mL/min was performed using two different mobile phases. Mobile phase A consisted of 3% v/v ACN in potassium dihydrogenphosphate 20 mM at pH 3.5; mobile phase B consisted of 100% ACN. The following gradient was performed: T = 0–10 min: 100% A to 80% A; T = 10–10.5 min: back to 100% A; T = 10.5–15 min: 100% A. Elution was performed at ambient temperature. Concentrations of 6-TG, 6-MMP and 5-BU were detected by UV-absorption at 343 nm, 303 nm and 280 nm respectively by switching wavelengths between the peaks. Chromeleon software (v6.6; Dionex) was used for data-acquisition and calculating peak surfaces.

Each run was calibrated using a 4-point calibration curve. Concentration standards of 6-TG (at 300 pmol, 600 pmol, 900 pmol and 1500 pmol) and 6-MMP (at 1500 pmol, 3000 pmol, 5000 pmol, 8000 pmol) were used, made by adding known concentrations of 6-TG and 6-MMP to standard RBC volumes. Calculations of the 6-TGN and 6-MMP concentrations were made by interpolation of peak height ratios on the acquired calibration curve.

Quality-control samples consisted of drug-free erythrocytes to which fixed concentrations of 6-TG and 6-MMP were added (807 pmol 6-TG and 4131 pmol 6-MMP respectively) and analyzed in each run. Both standards and QA-samples were stored at −80 °C and prepared fresh each 3 months. The allowed deviation from target values was <15% (according to international guidelines [23]) and 6-TGN and 6-MMP concentrations were reported in pmol/8 × 10^8 RBC, units in line with the international literature.

3.6. Assessment of performance characteristics

The detection limit and lower limit of quantification for 6-TGN and 6-MMP were calculated by a signal-to-noise ratio of 3 and 10, respectively. For this purpose, the baseline noise signal was obtained from a segment of the chromatograms that preceded the 6-TG and 6-MMP peak.

Linearity, within- and between-run precision and analytical recovery were established using drug-free RBC standards to which known concentrations of 6-TG and 6-MMP were added, resulting in fixed concentrations of 6-TG and 6-MMP in 8.0 × 10^8 RBC. The linearity of the method was established by constructing calibration curves (n = 3) at 300 pmol, 600 pmol, 900 pmol, 1200 pmol, 1800 pmol and 3600 pmol 6-TG and 1200 pmol, 2400 pmol, 3600 pmol, 4800 pmol, 7400 pmol and 15,000 pmol 6-MMP respectively. Within- and between-run imprecision and extraction efficiency were studied with 6-TG and 6-MMP standards of 400 pmol or 600 pmol (6-TG) and 1100 pmol or 4600 pmol (6-MMP). The analytical recovery for each method was determined using 6-TG and 6-MMP standards of 400 pmol and 600 pmol and 1100 pmol and 4600 pmol respectively. The recovery was calculated by comparing the measured concentrations with the expected concentrations.

3.7. Statistical methods

Reductions in median content with respect to the original values determined at T = 0/baseline and comparisons between different storage conditions were calculated by Wilcoxon rank-sum tests. The statistical software package 'R' v 2.1 was used for data analysis.

4. Results

4.1. Performance characteristics

Performance characteristics of the procedure met the requirements of our internal validation procedures and were comparable to results published by Dervieux and Shipkova ([21,22]; see Table 1). Standard chromatograms of a blank sample spiked with 6-TG/6-MMP and a patient sample obtained by our assay can be seen in Figs. 2 and 3. Acceptable precision values were obtained by our assay for 6-TGN and 6-MMP, respectively, and no known interferences were observed on the chromatographs. Determination of linearity yielded a slope of 2.04 × 10^{-4} and a Y-intercept of 0.04 for 6-TGN and a slope of 2.20 × 10^{-4} and a Y-intercept of 0.16 for 6-MMP. The Sheward-plot of quality-control samples showed no deviations according to international guidelines (data not shown).

Table 1
Performance characteristics of the analytical procedure as established in the laboratory of our clinical pharmacy.

Characteristics	6-TGN		6-MMP	
LLQ[a]	20		20	
Limit of detection[a]	5		5	
Linearity (n = 3)	0–3600 (r = 0.999)		0–14,500 (r = 0.999)	
Selectivity	No known interferences		No known interferences	
Conc QC[a]	400	1160	1080	4600
Precision (CV, %)				
Within-run (n = 6)	5.7	4.4	4.9	1.6
Between-run (n = 6)	6.9	7.0	7.2	3.6
Accuracy (%)				
Within-run (n = 6)	107	85	112	110
Between-run (n = 6)	107	99	101	100

[a] As pmol/8 × 10^8 erythrocytes.

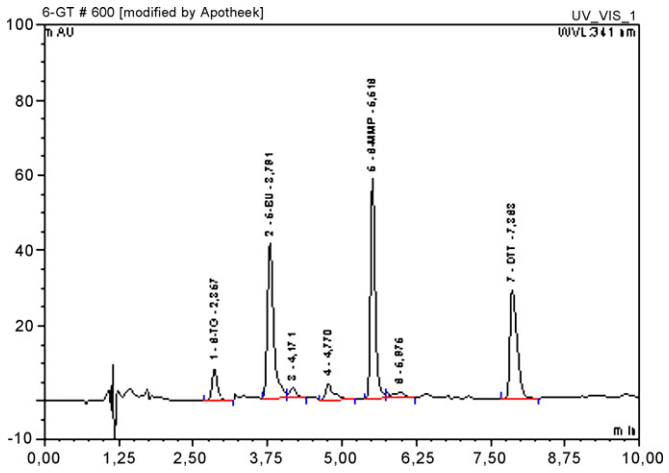

Fig. 2. Standard chromatograms by our assay of a blank sample spiked with 6-TG and 6-MMP.

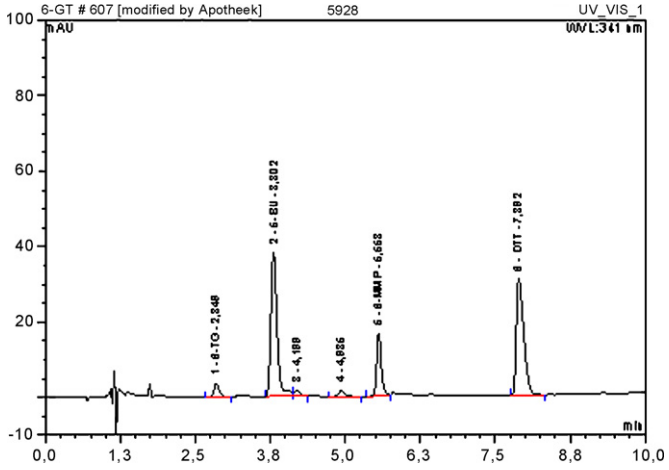

Fig. 3. Standard chromatograms by our assay of a patient sample with 6-TG and 6-MMP.

4.2. Stability of thiopurine metabolites in patient samples

The median 6-TGN concentration at day 7 decreased significantly to 53% during storage at 22 °C ($V = 0$, $p = 0.002$, 95% CI 48–70%) and to 90% under refrigeration (not significant: $V = 10$, $p = 0.155$, 95% CI 82–105%; see Fig. 4). Inter-day variation explains the non-significance in the latter results, as a clear trend to reduction in metabolite levels can be seen after days 5 and 6 under refrigeration. Concerning 6-MMP, median concentrations at day 7 decreased significantly to 55% at ambient temperature ($V = 0$, $p = 0.014$, 95% CI 40–69%) and to 86% under refrigeration ($V = 2$, $p = 0.018$, 95% CI 76–96%; see Fig. 5). Decreases in median concentrations in both metabolites were significantly less pronounced from day 4 to day 7 during refrigeration.

Study B showed significant decreases at $t = 26$ in both 6-TGN and 6-MMP at −20 °C in comparison with baseline values at $t = 0$ ($V = 3.5$, $p = 0.028$, 95% CI 82–96 and $V = 3$, $p = 0.010$ 95% CI 71–91

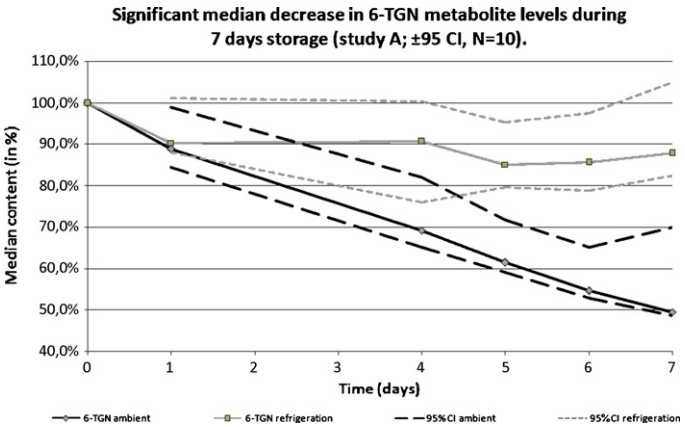

Fig. 4. Significant median decrease in 6-TGN metabolite levels during 7 days storage (study A; ±95 CI, $N = 10$).

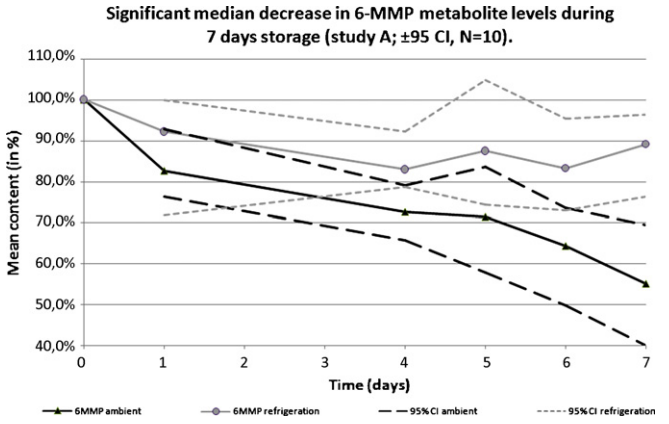

Fig. 5. Significant median decrease in 6-MMP metabolite levels during 7 days storage (study A; ±95 CI, $N = 10$).

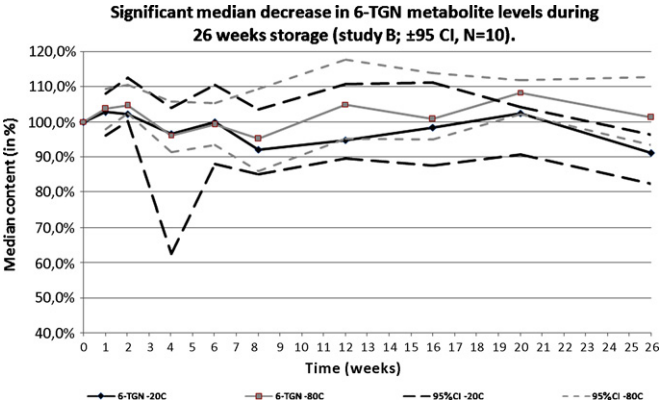

Fig. 6. Significant median decrease in 6-TGN metabolite levels during 26 weeks storage (study B; ±95 CI, $N = 10$).

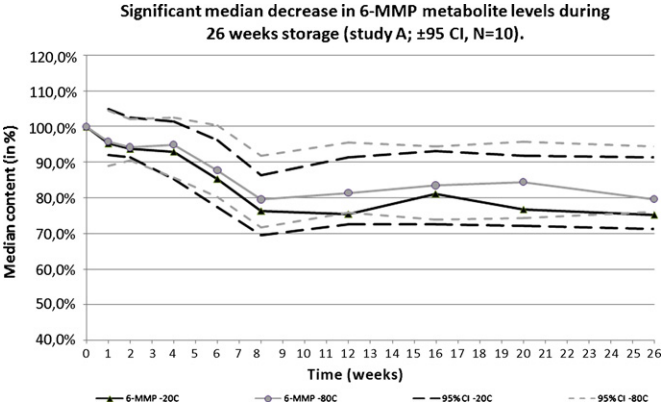

Fig. 7. Significant median decreases in 6-MMP metabolite levels during 26 weeks storage (study B; ±95 CI, $N = 10$).

respectively; see Figs. 6 and 7). At −80 °C, the decrease was less pronounced as only 6-MMP showed a significant decrease in values ($V = 6$, $p = 0.027$ 95% CI 76–94 and $V = 34$, $p = 0.557$, 95% CI 93–112 for 6-TGN; Fig. 7).

5. Discussion

In this prospective study, we demonstrated clinically relevant decreases in 6-TGN and 6-MMP concentrations compared to their baseline values in blood samples of IBD-patients during various controlled storage conditions and periods.

Reports on 6-TGN and 6-MMP stability are sparse, but a significant decrease in 6-TGN concentrations after sampling is described [25,26]. Similar reductions in 6-TGN of 2–4% per day at ambient temperature and up to 75% on day 4 and <35% on day 7 in samples stored at 20 °C and 4 °C have been reported by Sauviat and Bolon [26]. Less dramatic decreases in 6-TGN concentrations reported Pike: 14–28% decrease on day 7 during ambient temperature storage [25], although this reduction is still relevant in clinical and research context.

The differences with our study may be largely attributable to study design and analytical methods used. Exact storage temperatures were not mentioned in the referred studies, making comparison of results difficult. Variations in nucleotide hydrolysis techniques may lead to incomplete hydrolysis of nucleotides (for example in the method of Lennard et al.) and, subsequently, lower 6-TGN concentrations. However, we considered this of minor importance, as our analytical procedure was validated and comparable to the method described by Shipkova (in which complete hydrolysis of nucleotides occurs). Besides, instability of thiopurine metabolites is independent of the analytical method used.

Correlations between metabolite levels and efficacy/toxicity have been demonstrated [12,27–30], but are also contradictive in several other studies [17,31,32,18]. Analytical differences can explain these conflicting outcomes [22,31], but instability of thiopurine metabolites is also a potential explanation. Inadequate or prolonged storage of samples can result in decrease of thiopurine metabolite concentrations, as we show that higher storage temperatures will result in a more substantial decrease of 6-TGN and 6-MMP concentrations during time. This is an important detail in clinical practice and (multi-centre) studies investigating correlations between therapeutic drug monitoring and clinical outcome in IBD-patients. Published studies are inconsistent in describing the exact storage conditions and/or sampling procedures. For example, Teml regarded TDM to remain controversial in patients treated with thiopurines for IBD [33]. However, only 4 of 23 TDM-studies provided details concerning sample storage prior to and during investigation and a central laboratory was utilised in 11 studies for determination of thiopurine metabolites. A recent publication by Reinshagen also showed no positive attribution of TDM to clinical response, but sample handling or storage conditions were not specified [34].

In conclusion, we found thiopurine metabolites to be unstable under various storage conditions. This has to be accounted for in (multi-center) clinical trials and evaluation of pharmacotherapy in daily practice. A significant delay between patient sampling and analysis can be expected when central analysis of the thiopurine metabolites is performed. Current publications concerning TDM in IBD-patients should be re-evaluated in the absence of detailed information regarding sample handling.

Conflicts of interests

None declared.

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