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Monitoring of thiopurine metabolites – A high-performance liquid chromatography method for clinical use

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A high-performance liquid chromatography method capable of measuring thiopurine mono-, di-, and triphosphates separately in red blood cells (RBCs) was developed. RBCs were isolated from whole blood using centrifugation. Proteins were precipitated using dichloromethane and methanol. The thioguanine nucleotides (TGNs) were derivatised using potassium permanganate before analysis. Analytes were separated by ion-pairing liquid chromatography using tetrabutylammonium ions and detected using UV absorption and fluorescence. The method was designed for use in clinical trials in thiopurine therapy and proven valid by analysis of authentic patient samples.

The method measured thioguanosine mono- (TGMP), di- (TGDP), and triphosphate (TGTP), as well as methylthioinosine mono- (meTIMP), di- (meTIDP) and triphosphate (meTITP) in RBCs collected from patients treated with thiopurine drugs (azathioprine, 6-mercaptopurine, and 6-thioguanine).

LOQ was 0.3, 3, 2, 30, 30 and 40 pmol/8x10⁸ RBC, for TGMP, TGDP, TGTP, meTIMP, meTIDP and meTITP, respectively. Between-day precision were below 14% for all analytes at all concentrations and samples were stable at 5 °C for 8 hours after sampling.

Thiopurines are immunosuppressive and cytotoxic drugs used in the treatment of childhood acute lymphoblastic leukaemia (ALL) and inflammatory bowel disease (IBD). At present, three different drugs are used, mercaptopurine, azathioprine, and thioguanine. All are prodrugs and their therapeutic effects, although not completely understood, are probably mediated through the formation of thioguanine nucleotides. The main mechanism has been suggested to be apoptosis mediated by the mismatch repair system, caused by incorporation of thioguanine nucleotides (TGNs), into DNA [1]. However, recently the methylthioinosine nucleotides (meTINs), also known as methyl mercaptopurine nucleotides (MMP), or methylthioinosine monophosphate (meTIMP), and especially their ability to inhibit purine de novo synthesis, has been emphasised as being important for thiopurine effects and toxicity [1, 2].

A majority of patients respond well to thiopurine drugs, but up to a third have to modify or discontinue their treatment due to adverse events or lack of treatment effect [3]. Large interindividual differences in the formation of the thiopurine metabolites contribute to the variable response. Some of the differences, but far from all, can be explained by genetic factors, such as mutations in the enzyme thiopurine methyltransferase (TPMT) [4].

Many laboratories around the world monitor thiopurine metabolites to individualize the treatment, in combination with TPMT geno- and/or phenotyping.

Analysis of thiopurine nucleotides is challenging due to their physical and chemical properties. Nucleotides consist of a purine base, a sugar moiety (ribose or deoxyribose) and one, two or three

phosphate groups as seen in figure 1. Due to their polarity, all nucleotides are poorly retained in reversed phase chromatography.

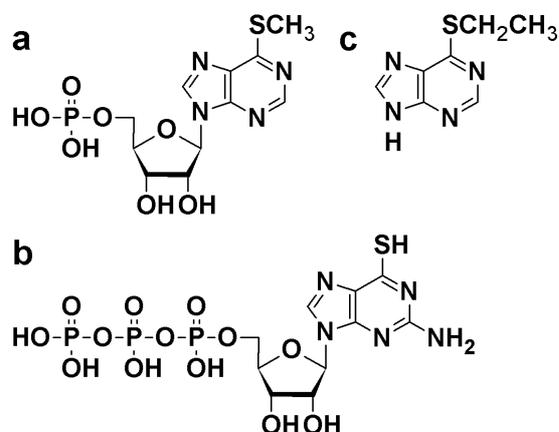


Figure 1 - The structures of methylthioinosine monophosphate (a), thioguanosine triphosphate (b), and internal standard ethylmercaptopurine (c).

Moreover, nucleotides are used as sources of energy in many biological processes in the human body through cleavage of phosphate bonds. Although adenosine triphosphate (ATP) is the most abundant nucleotide, most nucleotides can be used as an energy source in this way. This makes sampling and preanalytical handling difficult, as the interconversion between di-, and triphosphates is very rapid and needs to be stopped immediately at sampling to ensure a true quantitative measurement. Previously published methods for thiopurine monitoring avoid these difficulties by hydrolysing the thiopurine nucleotides back to purine bases, thereby losing the ability to distinguish between mono-, di-, and triphosphates [5].

Thiopurine metabolites, TGN and in many cases m^eTIN, are currently measured in red blood cells (RBCs) [6, 7] or whole blood [8]. However, the choice of matrix rests on practical concerns rather than biological. Most effects of the thiopurines, such as incorporation into DNA and immunosuppression, occur in white blood cells rather than in RBCs, but the latter are present in abundance and easy to separate, making it the logical choice as a surrogate matrix.

In a meta study, Osterman et al [9] showed that about a third of patients above the suggested threshold concentration of TGN, lacked treatment effect while about a third of patients below the threshold had a good effect. A number of ways to improve the predictive power have been suggested [10]. Measuring the metabolites in white blood cells, is one, and measuring the triphosphates separately, is another. Containing more energy, the triphosphates are responsible for most thiopurine effects. Even though the idea has been suggested for a long time, clinical studies are lacking, possibly due to a lack of good methodology to measure the nucleotides. The study of Neurath et al (47 patients) found a correlation between a high thioguanosine diphosphate (TGDP) to thioguanosine triphosphate (TGTP) ratio and worse clinical outcome compared to those with a lower ratio [11]. However, they did not measure m^eTINs.

There are very few published methods for measuring thiopurine nucleotides in RBC. Most of them use similar methodology [11-15]. One recent method by Hofmann et al [16] used LC-MS/MS detection instead of fluorescence and/or UV absorption used by the others. The methodology consists of an extraction step and, when applicable, derivatisation to make the TGNs fluorescent.

The extraction protocol used was first published by Rabel et al [15]. A combination of methanol and dichloromethane was used for protein precipitation. Lavi and Holcenberg [17] used an alternative extraction procedure based on absorption to a mecurial cellulose resin. However, the use of mercury is highly controversial and hardly motivated when developing a novel method as other methodology exists.

The derivatisation procedure was first described by Finkel [18]. By using potassium permanganate the sulphur moiety on thioguanine (or its nucleotides) was oxidized to a sulphonate. Excess potassium permanganate was reduced by hydrogen peroxide. Me^eTINs were not affected,

possibly due to inactivation of the sulphur moiety by methylation.

To retain and separate the highly polar thiopurine nucleotides on an HPLC column, most recent methods used chromatography based on ion-pairing with tetrabutylammonium ions [11-13], while others including Hofmann et al used ion-exchange chromatography [14, 16, 17]. Some methods to separate endogenous nucleotides, using HPLC columns specially designed for polar compounds, such as porous graphitic carbon (PGC) [19] and HILIC columns [20], have been published. It has proven difficult to transfer these techniques to thiopurine nucleotides, possibly due to the increased polarity and/or reactivity caused by the free sulphur moiety, figure 1. Rabel et al [15] used capillary electrophoresis instead of chromatography.

In the light of recent findings regarding thiopurine mechanisms, TGNs are not the only metabolites of interest when monitoring the thiopurines, particularly the meTINs have been suggested to be important for thiopurine effects [1], and therefore they were included in the assay. Previously only two methods did include the meTINs [14, 16] while another two include meTIMP but not the di-, and triphosphates [12, 17].

To be used in clinical studies, especially considering the poor stability of thiopurine nucleotides, methods need to be designed for sampling at multiple centres. It is apparent that a great deal of standardisation and training is needed for every pre-analytical step to ensure good data quality in multicentre studies [21]. Limiting the number of such steps would reduce the need for both standardisation and training and the possibility of sampling and pre-analysis errors.

Also, the analytical method needs to produce reliable data. To do this the method must be well characterized and robust. The latter is facilitated by the use of an internal standard, especially as some reagents are both viscous and volatile, making them difficult to handle. Two internal standardisation strategies for the methanol-dichloromethane extractions were found in the literature. Etylmercaptapurine was previously used by us [12], and deuterated analogs were used by Hofmann et al [16]. However, the latter can only be used in combination with mass spectroscopy.

None of the available methods meet the demands of planned clinical studies. The studies were designed to assess the benefits of measuring thiopurine nucleotides separately when predicting treatment outcome in IBD and childhood ALL. Some of the methods lack important metabolites [11-13, 15, 17], some lack the precision needed, or documentation thereof [11, 14, 15, 17], and none have pre-analytical protocols suitable for multicentre studies.

The aim of this work was therefore to develop a method that measured TGN and meTIN in RBCs. It was to be sensitive, simple, inexpensive, robust and well characterized enough to be used for clinical studies.

Materials and methods

The analytical procedure consisted of four steps. Isolation of RBC's from whole blood by centrifugation (1), precipitation of protein using dichloromethane and methanol (2), derivatisation using potassium permanganate (3), and chromatographic separation and detection using UV-absorption and fluorescence (4). A flow chart of the preparation of samples, standards, and controls is shown in figure 2.

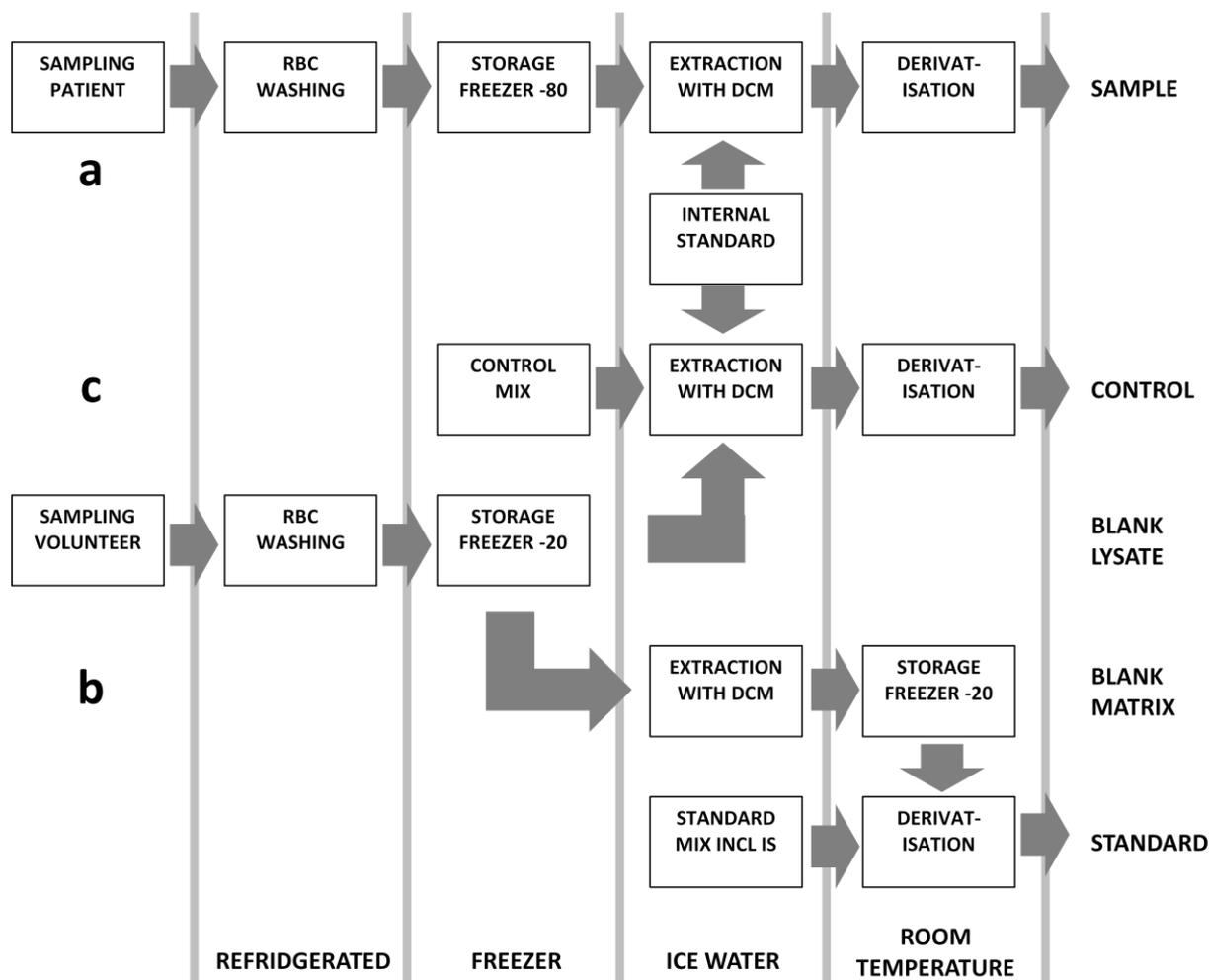


Figure 2 - Flow chart of the preparation of samples (a), standards (b) and controls (c). DCM dichloromethane IS internal standard.

Both qualitative and quantitative aspects were considered in the design of this method. Qualitatively, we wanted to depict a metabolite profile that can be used to identify if the treatment will be successful or not. Quantitatively, the profile was created by quantifying six nucleotides central to the thiopurine metabolism.

Materials

Thioguanosine monophosphate (TGMP), TGDP, TGTP, meTIMP, methylthioinosine diphosphate (meTIDP) and methylthioinosine triphosphate (meTTIP) were purchased from Jena Bioscience GmbH (Jena, Germany) and ethylmercaptapurine was purchased from Sigma-Aldrich Sweden AB (Stockholm, Sweden). The purity of all analytes was equal or better than 95%.

Potassium permanganate, 30% hydrogen peroxide, sodium bicarbonate and tetrabutylammonium bisulfate were purchased from Sigma-Aldrich Sweden AB. Sodium chloride, sodium hydroxide, phosphoric acid and dichloromethane were purchased from Merck KGaA (Darmstadt, Germany). Acetonitrile and methanol were purchased from Labscan Limited (Dublin, Ireland). Water was obtained from a Milli-Q gradient water purification system (Millipore, Bedford MA).

Drug free blood used for standards, controls and validation samples were obtained from volunteers at the laboratory. Patient samples were obtained from patients visiting the gastroenterological clinic at Linköping university hospital treated with thiopurine drugs for Crohn's disease or ulcerative colitis and informed consent was collected from all

subjects. The study was approved by the local ethics committee (Dnr 01-016).

Sample preparation

Samples were drawn using 5-ml tubes containing ethylenediaminetetraacetic acid (EDTA) and immediately placed at +4°C until isolation of RBC's. (figure 2a) Within eight hours of sampling, the samples were centrifuged (1200 g, 5 min, 4°C) and the plasma and buffy coat were removed. The RBC:s were washed twice and resuspended in sodium chloride (0.9%, w/v) at a concentration of approximately 4×10^9 RBC/ml. The suspension was frozen at -80 °C in 500 µl aliquots.

The lysates were thawed 45 min on ice and 80 µl were placed in microcentrifuge tubes on ice. 20 µl of cold internal standard solution, phosphoric acid (50 mM) - ethylmercaptapurine (150 µM, internal standard)(pH 7.4 with sodium hydroxide), was added. After brief vortex mixing, 175 µl of cold precipitation solution, dichloromethane-methanol (10:35, v/v), was added. The samples were mixed by vortex for 3 s and centrifuged (17 530 g, 5 min, 4 °C). 100 µl of the supernatants were transferred to fresh tubes.

14 µl of derivatisation solution, sodium bicarbonate (0.5 M; pH 10.5 with sodium hydroxide) - potassium permanganate (1%, w/w), were added and the samples were mixed by vortex. Following a 3 min incubation, 20 µl hydrogen peroxide (10%, w/w) were added and the samples were mixed by vortex. The resulting precipitate was removed by centrifugation (17530 g, 5 min, 4 °C) and 8 µl of the supernatant were injected onto the HPLC column.

Hemoglobin (Hb) concentration was measured in the thawed lysates within 4 hours of thawing using the HemoCue® B-Hemoglobin system (HemoCue AB, Ängelholm, Sweden) and used to normalize nucleotide concentrations.

Chromatography

The HPLC system consisted of an Alliance 2695 Separation module, a 2487 Dual Absorbance Detector and a 2475 Multi-Wavelength Fluorescence Detector, all from Waters Sverige AB (Sollentuna, Sweden). The separation was carried out on an Xbridge C18 column (150x3 mm, 3.5 µm, Waters) with a Gemini C18 guard column (4x2 mm) from Phenomenex (Torrance, CA).

Table 1 - Gradient profile.

Time (min)	%A	%B
0	100	0
0.1	85	15
12.5	78	22
24.0	20	80
28.0	20	80
28.2	100	0
32.0	100	0

A 32 min gradient detailed in table 1 going from 100% mobile phase A, phosphoric acid (40 mM) - tetrabutylammonium sulphate (5 mM) - acetonitrile (1%, v/v)(pH* 5 with sodium hydroxide), to 80% mobile phase B, phosphoric acid (20 mM) - tetrabutylammonium sulphate (5 mM) - acetonitrile (26%, v/v)(pH* 5 with sodium hydroxide). TGMP, TGDP, and TGTP were detected using fluorescence (excitation 329 nm, emission 403 nm, gain 50), and ethylmercaptapurine, meTIMP, meTIDP and meTTTP using UV-absorption at 289 nm.

Standards and Controls

Standards were prepared and stored in MilliQ water. 20 µl of standard were mixed with 80 µl of blank matrix, blank lysate precipitated without internal standard, and were derivatised as described above (figure 2b). Six standards were used for TGMP (0.300-340 pmol/100 µl lysate), TGDP (1.80-2000 pmol/100 µl lysate), TGTP (1.80-2000 pmol/100 µl lysate), meTIMP (30.0-4500 pmol/100 µl lysate), meTIDP (30.0-4500 pmol/100 µl lysate) and meTTTP (38.0-5600 pmol/100 µl lysate).

Table 2 - Conversion of analytes during preparation of standards (matrix) and controls (lysate). Mono-, di-, and triphosphates were added individually to study conversion during sample preparation. meTIDP methylthioinosine diphosphate; meTIMP methylthioinosine monophosphate; meTIN methylthioinosine nucleotides; meTITP methylthioinosine triphosphate; TGDP thioguanosine diphosphate; TGMP thioguanosine monophosphate; TGN thioguanine nucleotides; TGTP thioguanosine triphosphate.

TGN - Controls

		Found (in%)		
		TGMP	TGDP	TGTP
Added	TGMP	100%	0%	0%
	TGDP	2%	17%	81%
	TGTP	0%	16%	84%

TGN - Standards

		Found (in%)		
		TGMP	TGDP	TGTP
Added	TGMP	100%	0%	0%
	TGDP	1%	98%	1%
	TGTP	0%	3%	97%

meTIN - Controls

		Found (in%)		
		meTIMP	meTIDP	meTITP
Added	meTIMP	96%	0%	4%
	meTIDP	0%	13%	87%
	meTITP	0%	13%	87%

meTIN - Standards

		Found (in%)		
		meTIMP	meTIDP	meTITP
Added	meTIMP	96%	0%	4%
	meTIDP	0%	100%	0%
	meTITP	1%	3%	96%

Three levels of quality control samples were prepared and stored in MilliQ water. 20 µl of control were mixed with 80 µl of blank lysate and the controls were prepared together with the samples (figure 2c). Due to interconversion (see discussion and table 2) the controls were prepared using only mono- and triphosphates. The concentrations were 2.00, 30.0, and 250 pmol/200 µl lysate for TGMP; 40.0, 600, and 5000 pmol/200 µl lysate for TGTP; 100, 400, and 2000 pmol/200 µl lysate for meTIMP; and 500, 2000, and 10000 pmol/200 µl lysate for meTITP.

Method evaluation and validation

Experiments designed to characterise observed equilibriums between thiopurine mono-, di- and triphosphates as well as method validation were carried out. The method validation was based on an FDA guideline for bioanalytical method validation [22]. However, considering the method at hand, some aspects were added, modified or omitted. The method was validated with regards to specificity, sensitivity, precision, accuracy, and stability.

Equilibrium studies

The six different analytes as well as the internal standard were spiked into blank

lysate and analysed separately without internal standard. Also, mixtures containing either mono-, di- or triphosphates were used to spike either blank lysate or blank matrix.

Recovery, Sensitivity and Specificity

The recovery of the extraction was estimated by comparing the peak height of the medium and high controls with a calculated peak height at 100% recovery based on standards 5 and 6. Due to the interconversion between di- and triphosphates, recovery was given as a sum of the total.

Sensitivity of the method was estimated by analysing 4 replicates of serial dilutions of the low control. LOQ was defined as the lowest level at which precision was equal or below 15% of the mean and accuracy were within 85-115% of expected value. LOD was defined as the lowest added concentration producing a peak with a signal to noise ratio of at least 3.

To determine specificity, blank samples from five sources were analysed (3 male, 2 female, aged 27-36). The same samples were also spiked at a concentration corresponding to a high control diluted six times (40, 830, 330 and 1700 pmol/200 µl lysate, for TGMP, TGTP, meTIMP, and meTITP, respectively) to analyse matrix effects.

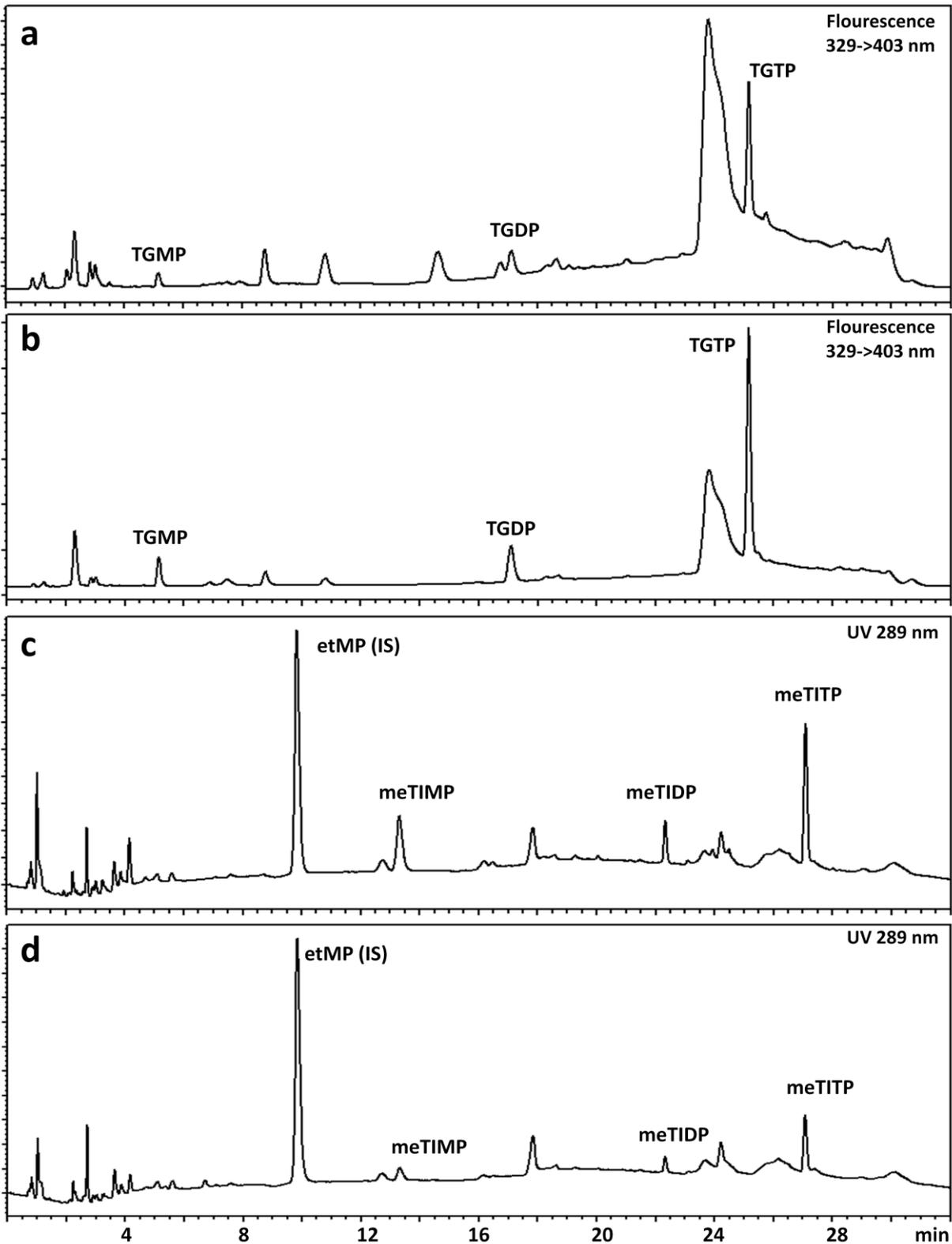


Figure 3 - Chromatograms of patient sample (6-mercaptopurine 75 mg/day, 0.6 mg/kg body weight/day) and medium control. Fluorescence ex 329 nm, em 403 nm for patient (a), 4.7, 18, and 59 pmol/200 μ l lysate for TGMP, TGDP, and TGTP respectively, and control (b) 22, 59, and 240 pmol/200 μ l lysate for TGMP, TGDP, and TGTP, respectively. UV absorption at 289 nm for patient (c), 1400, 920, and 3600 pmol/200 μ l lysate for meTIMP, meTIDP, and meTITP, respectively, and control (d), 380, 340, and 1600 pmol/200 μ l lysate for meTIMP, meTIDP, and meTITP, respectively. etMP ethylmercaptopurine; meTIDP methylthioinosine diphosphate; meTIMP methylthioinosine monophosphate; meTITP methylthioinosine triphosphate; TGDP thioguanosine diphosphate; TGMP thioguanosine monophosphate; TGTP thioguanosine triphosphate.

Precision and Accuracy

To estimate the precision of the method six replicates of each control were analysed in the same run to estimate within-run precision. Also, controls were analysed on six different days to yield between-day precision and accuracy data. Given the interconversion, accuracy data for the di- and triphosphates were given as a sum of the total.

Stability

Stability of the samples was investigated by comparing metabolite concentrations in RBCs isolated from whole blood stored 8 or 24 h at room temperature or +4°C, with concentrations in RBCs isolated immediately after sampling.

The stability of thawed lysates was estimated by thawing the lysates on ice 45 min, 1.5 h, and 3 h. Freeze/thaw stability of lysates was investigated by thawing (1h on ice) and refreezing (>1 h at -80°C) samples from five different patients three times. Stability of the calibration curves was assessed by comparing 4 different curves run on different days.

Patient samples

Ten patient samples from IBD patients treated with thiopurines were analysed on three different days to estimate the ranges found in patient samples and between-run precision in patient samples.

Results

The presented method combined gradient ion-pair chromatography and dual detection principles (fluorescence and UV absorption) to analyse derivatised (TGN) and non-derivatised (meTIN) analytes. Although interconversion was observed during sample preparation, results showed low between-day variability combined with high selectivity and sensitivity.

Even though the principle of the method was complex, it was efficient and robust. Mobile phases, a calibration curve, controls, and ten samples in duplicate were all prepared within 4 hours and were analysed within 24 hours.

All analytes were well retained on the column. The first peak, TGMP, eluted at 5.2 min (>7 void volumes). The last peak, meTTP, eluted at 27 min.

Equilibrium studies

Analysis of each standard individually showed that none of the analytes, nor their impurities, interfered with the quantification of other analytes. Interconversion between di-, and triphosphates was seen when spiked into blank lysate, equivalent to preparation of controls, but not when spiked into blank matrix, equivalent to preparation of standards, as can be seen in table 2. No conversion into monophosphates, nor between meTINs and TGNs, was observed. The interconversion was very rapid, reaching equilibrium within seconds of addition (data not shown).

Table 3 - Concentrations of 5 different thiopurine free matrices spiked with 40, 830, 330, 1700 pmol/200 µl lysate, for TGMP, TGTP, meTIMP, and meTITP, respectively (pmol/200 µl lysate). meTIDP methylthioinosine diphosphate; meTIMP methylthioinosine monophosphate; meTITP methylthioinosine triphosphate; TGDP thioguanosine diphosphate; TGMP thioguanosine monophosphate; TGTP thioguanosine triphosphate.

Source	meTIMP	meTIDP	meTITP	TGMP	TGDP	TGTP
A	272	216	1371	27,7	63,6	303
B	325	270	1668	34,0	84,0	398
C	267	259	1471	30,9	81,3	345
D	266	267	1416	29,8	81,0	334
E	216	238	1131	24,2	68,9	244
CV	14%	9%	14%	12%	12%	17%

Recovery, Sensitivity and Specificity

The recoveries of TGMP, TGDP+TGTP, meTIMP, meTIDP+meTITP, and internal standard were estimated to 50, 31, 65, 61, and 68%, respectively.

LOQ was estimated to 0.3, 3, 2, 30, 30, and 40 pmol/200 µl lysate, for TGMP, TGDP, TGTP, meTIMP, meTIDP, and meTITP, respectively. LOD estimated to 0.2, 0.6, 0.8, 20, 10, and 40 pmol/200 µl lysate, for TGMP, TGDP, TGTP, meTIMP, meTIDP, and meTITP, respectively.

Although baseline fluctuations throughout the chromatograms and a number of interference peaks were seen in both the UV and fluorescence traces (figure 3), none of them affected the quantification, nor were

any severe matrix effects observed as shown in table 3. The artefact seen in the fluorescence trace around 24 min might be caused by ion-pairing agent being retained on the column at the beginning of the gradient.

Precision and accuracy

Within-run imprecision was shown to be below 8% for all control levels and analytes. Between-day imprecision was below 14% for all control levels and analytes, with the largest variability observed at the low control level (at medium and high level, maximum imprecision was 9%, table 4.

Accuracy ranged between 47 and 96%. Relative accuracy ranged from 98 – 102% except for TGDP+TGTP in the low control (106%), table 5.

Table 4 - Within-day precision (a) and between-day precision (b) of low, medium and high controls. CV (mean, range in pmol/200 µl lysate) n=6. meTIDP methylthioinosine diphosphate; meTIMP methylthioinosine monophosphate; meTITP methylthioinosine triphosphate; TGDP thioguanosine diphosphate; TGMP thioguanosine monophosphate; TGTP thioguanosine triphosphate.

a. Within-run precision, CV (mean, range), pmol/200 µl lysate

	TGMP	TGDP	TGTP
Low	2.3% (1.56, 1.51-1.60)	3.6% (4.27, 4.12-4.53)	5.8% (17.1, 15.7-18.6)
Medium	3.7% (22.9, 22.1-24.2)	4.9% (60.7, 57.9-65.7)	5.4% (244, 229-264)
High	3.5% (194, 186-204)	5.7% (473, 433-504)	5.4% (2030, 1860-2170)
	meTIMP	meTIDP	meTITP
Low	7.8% (90.5, 82.5-101.7)	7.8% (66.9, 61.9-70.2)	4.1% (408, 382-445)
Medium	3.0% (373, 363-388)	3.0% (320, 303-336)	3.8% (1580, 1500-1670)
High	3.2% (1890, 1790-1950)	3.2% (1600, 1500-1670)	3.7% (7800, 7290-8320)

b. Between-day precision, CV (mean, range), pmol/200 µl lysate

	TGMP	TGDP	TGTP
Low	6.6% (1.47, 1.29-1.56)	13.1% (4.06, 3.35-4.55)	9.9% (16.4, 14.8-19.0)
Medium	5.0% (21.5, 19.6-22.7)	7.1% (55.4, 49.6-59.9)	3.4% (229, 219-239)
High	3.6% (183, 174-191)	7.4% (432, 390-482)	5.0% (1900, 1780-2020)
	meTIMP	meTIDP	meTITP
Low	7.9% (94.7, 87.1-106.6)	10.8% (78.8, 67.3-88.0) ^a	7.3% (385, 344-423)
Medium	4.7% (374, 351-390)	9.4% (306, 263-338)	6.1% (1500, 1350-1590)
High	3.3% (1860, 1760-1930)	8.1% (1530, 1330-1650)	4.9% (7450, 6790-7790)

^a n=5, one replicate removed due to chromatographic problem.

Table 5 - Between-day accuracy and relative recovery. *meTIDP* methylthioinosine diphosphate; *meTIMP* methylthioinosine monophosphate; *meTITP* methylthioinosine triphosphate; *TGDP* thioguanosine diphosphate; *TGMP* thioguanosine monophosphate; *TGTP* thioguanosine triphosphate.

Accuracy				
	TGMP	TGDP+TGTP	meTIMP	meTIDP+meTITP
Low	76%	51%	96%	92%
Medium	74%	48%	94%	90%
High	76%	47%	94%	89%
Mean	76%	49%	95%	91%

Recovery relative to mean				
	TGMP	TGDP+TGTP	meTIMP	meTIDP+meTITP
Low	101%	106%	101%	102%
Medium	98%	98%	100%	99%
High	101%	96%	99%	99%

Stability

The samples were stable for 8 h at +5 °C (Relative concentrations between 93 and 107%), but degradation of triphosphates into mono- and diphosphates were seen after 24 h. While only modest reductions of triphosphate levels were observed, marked increases of mono- and diphosphates present at lower concentrations were seen. (TGMP 147%, TGDP 128%, meTIDP 125%). At room temperature the samples were degraded after 8 hours.

The lysates were not stable on ice after thawing, nor during three freeze/thaw cycles. Similar degradation patterns, as those seen during sample storage were observed.

Extracts were stable on the autosampler at 4°C for at least 48 hours. Comparison of the calibration curves from four different days showed that the method must be calibrated each day, mostly due to high variability at low concentrations.

Patient samples

Chromatograms from a patient sample and a low control are shown in figure 3. The maximum difference from the mean in all patients for all analytes was 24% or lower (mean 8%).

The following concentration ranges were observed, (pmol/200 µl lysate (mean)); TGMP 5-18(10), TGDP 5-45(27), TGTP 58-

160(103), meTIMP 63-1500(500), meTIDP <60-770, meTITP 140-4000(1700). 3 samples were below LOQ for meTIDP.

Discussion

A method for determination of TGMP, TGDP, TGTP, meTIMP, meTIDP and meTITP separately in RBCs was presented. Compared to earlier studies [9, 11], more information about the metabolism can be obtained by measuring the nucleotides individually and by including the meTINs in a single run. The added information might increase the ability to predict thiopurine response and side effects in a clinical setting.

The validation showed that in terms of specificity, accuracy, and precision, the presented method satisfies the demands of a bioanalytical method as defined in FDA guidelines [22] as well as the demands of the ongoing clinical studies. The latter include the possibility of sampling at multiple centers.

The major challenge in development of the sample preparation was how to handle conversion between di- and triphosphates. It was very rapid and the equilibrium was reached within a few seconds regardless of the combination of di- and triphosphates added, indicating a dynamic equilibrium (table 2). Due to inter-conversion, a true physiological measurement was not possible. Thus, a semi-quantitative approach was

adopted aiming at ex vivo measurements correlating with in vivo concentrations.

Rabel et al [15] stated that 50 mM EDTA at high pH was inhibiting the conversion. Even though the role of EDTA addition was poorly investigated, it was included in all later methods [11-16]. However, the use of EDTA is of limited interest for patient samples, as the equilibrium between di- and triphosphates is reached within seconds of sampling, and no net-conversion occurs after that. However, if EDTA is added to blank lysate before mixing with analytes it could facilitate the use of standards prepared in blank lysate, possibly eliminating the bias seen in the presented method due to limited extraction recovery. This approach was used by Hofmann et al [16] while other aspects were prioritised during the development of the presented method, as discussed below.

It was shown that the equilibrium shifted from triphosphates towards diphosphates after thawing. During sample preparation it was important to keep the samples on ice until precipitation to avoid such a shift of the equilibrium. However, after the precipitation as well as on the autosampler the analytes were shown to be stable.

To minimise the risk of shifting the di- and triphosphate equilibrium, the method has conditions during sample preparation as similar to those in vivo as possible. The high pH EDTA buffer used by other methods [11-16] was replaced by a low concentration phosphate buffer at pH 7.4. Raising the temperature to 37 °C did not affect the equilibrium, and was abandoned due to severe degradation of the nucleotides (data not shown).

A number of modifications were made in the sample preparation described by Rabel et al [12, 15] making the sample preparation easier, quicker and more robust. The number of additions was minimized by preparing mixtures of reagents (i.e. dichloromethane with methanol, and potassium permanganate with sodium carbonate buffer), and a more efficient precipitation was achieved by

increasing the proportion of methanol in the precipitation reagent. Methanol was crucial to precipitation efficacy, possibly by mediating phase transfer (data not shown).

The precision of the presented method was improved by the addition of an internal standard. Ethylmercaptapurine was selected since it was commercially available and suited the chromatography. This internal standard is a stable analog to the thiopurine nucleotides lacking the reactive phosphate groups, the ribose and the free sulphur, figure 1c. However, it is not a nucleotide, nor is it detectable by fluorescence, limiting the ability to compensate for differences in reaction chemistry, stability and detector response. In the method presented Hofmann et al [16], LC-MS/MS detection enabled the use of deuterated analogs as internal standards. However, in their study degradation of triphosphates into mono- and diphosphates was observed (Up to 20% of the total triphosphate concentration.) [16]. It is possible that such conversion also affected the deuterated analogs, limiting their suitability as internal standards. Also, the deuterated analogs used were not commercially available.

Analyte measurements were normalised by measuring Hb-content of the lysate rather than the RBC count used by most methods. This correction was first used by Lavi and Holcenberg [17], and enabled the use of frozen and thawed samples. The amount and complexity of preanalytical procedures were drastically reduced and the need for cell counting equipment at each center eliminated, facilitating the use of the method in multi-center studies. To compare results with other methods it was assumed that 8×10^8 RBC contained 30 mg of Hb and diluted 1+1 with saline corresponded to 200 μ l.

The presented method is probably the most cost effective method measuring TGNs and mTINs separately, mainly due to the use of fluorescence and UV detection instead of LC-MS/MS, as well as the simple, quick, robust and inexpensive sample preparation.

High selectivity and sensitivity was achieved by separating all analytes from the large number of artefacts present in the extract, figure 3. This was achieved by using an HPLC column with high efficiency (Xbridge), as well as optimization of a shallow gradient elution. Optimization included modification of pH, ion strength, tetrabutylammonium concentration, and gradient slope to enhance selectivity. The final gradient included several steps, table 1, as well as different ion strengths in mobile phases A and B, respectively.

The standards used in the method were prepared using previously precipitated and pooled blank RBCs (figure 2). If standard mixtures were to be added to the lysates before precipitation the di- and triphosphated would have converted, as the lysates lacked EDTA. Precision, table 4, accuracy, table 5, and matrix effect data, table 3, showed that the recovery was stable and not affected by analyte concentration, nor by matrix components, despite being limited. Our ability to compare different samples to each other, and to predict clinical outcome, was not limited by any bias originating from limited extraction recovery.

The presented method was well suited, in terms of range and sensitivity, for the patient samples of our ongoing clinical studies. Most difficult was the quantification of TGMP, representing only about 2% of total TGN, and the meTINs, which were almost nonexistent in some patients. In fact, it was

one of the most sensitive methods, only rivalled by the LC-MS/MS method by Hofmann et al [16]. Their method was about three times more sensitive for meTIN, while the method presented here was about six times more sensitive for TGMP. The similar sensitivity of the methods might be attributed to high ion strength of the eluate used by Hofmann et al [16] having a negative impact on LC-MS/MS sensitivity.

The method will be used in our ongoing clinical study for the analysis of 100 well characterized patient samples from IBD patients with active disease or in remission to evaluate the ability to predict remission using this method. However, the problem of poor sample stability needs to be addressed, in order for this type of methodology to be used in a clinical routine,

Conclusion

The method presented in this study to measure meTIN and TGN in RBCs was sensitive, simple, cheap, robust and well characterized. It was well suited for our ongoing clinical studies, but not for routine monitoring.

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