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High-performance liquid chromatographic assay of methylthioguanine nucleotide

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Abstract

This paper describes a specific and sensitive reversed-phase HPLC assay for the measurement of 6-methylthioguanine (methyl-TG) and methyl-TG nucleotides (methyl-TGNs) in red blood cells (RBCs), which is suitable for routine clinical use. Briefly, an ethyl acetate extract of RBCs is evaporated and reconstituted in O.1 *M* HCl. The methyl-TG is separated from other thiopurines by reversed-phase HPLC and quantitated using UV detection. For the measurement of methyl-TGNs the free base (methyl-TG) is obtained by acid hydrolysis of the nucleotide back to the parent thiopurine. The intra-assay C.V. over the concentration range of 0.055–1.10 nmol methyl-TG per 4×10^8 (100 μl) RBCs ranged from 2.8 to 8.5%, and the mean recovery of methyl-TG over the calibration range was 61.6% (coefficient of variation, C.V., 3.8%). The lower limit of reproducibility was 0.055 nmol extracted from 100 μl RBCs. Analysis of blood samples from children with leukaemia receiving 6TG chemotherapy, revealed RBC methyl-TGNs at concentrations ranging from 323 to 1365 pmol per 8×10^8 RBCs. No methyl-TG was detected in any of the patient samples. © 1998 Elsevier Science B.V.

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1. Introduction

The purine analogue drugs 6-thioguanine (6TG; 2-amino 6-mercaptopurine) and 6-mercaptopurine (6MP) have been used in the treatment of acute leukaemias since the mid 1950's. The prolonged oral use of these two drugs is well established, traditionally 6TG is used in myeloid leukaemias whilst 6MP is used in the continuing chemotherapy of lymphoblastic leukaemias. The anti-leukaemic effect of 6MP in childhood acute lymphoblastic leukaemia (ALL) can be related to drug derived 6-thioguanine nucleotides (TGNs), active metabolites which are easily measured using the red blood cell (RBC) as a surrogate tissue. 6TG also undergoes intracellular transformation to 6TGNs. However, 6TG has not been widely used in the treatment of childhood ALL mainly because of custom and practice [1].

In children taking identical doses of 6MP there are wide interindividual variations in RBC TGNs [2].

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One major factor influencing the formation of TGNs is the inherited activity of thiopurine methyltransferase (TPMT), a genetically regulated and variable intracellular enzyme [3,4] which forms methylated metabolites at the expense of TGNs. Those children who inherit very high levels of TPMT activity form low concentrations of 6TGNs and do not experience cytotoxicity at standard 6MP dosages. They may be at an increased risk of disease relapse [5]. This sub-group of children could perhaps form therapeutic TGN concentrations more reliably and predictably from 6TG rather than 6MP [6], and this viewpoint is supported by the superior cytotoxic potency of 6TG when compared to 6MP in leukaemic cells isolated from patients with ALL [7]. A direct comparison of oral 6TG versus 6MP in the treatment of ALL is the subject of current clinical trials in Germany, the United Kingdom and in the USA.

Both 6MP and its initial nucleotide metabolite, 6-thioinosine 5′-monophosphate acid, can be *S*-methylated by TPMT [8]. 6TG can be *S*-methylated by TPMT, but TGNs are very poor substrates, for example 6-thioguanosine 5′-monophosphate has a high K_M (1040 μ*M*) and low V_{MAX} [4]. RBC 6TGN concentrations from oral 6TG can be ten-fold higher than those produced by the same patient on 6MP [7], raising the prospect of methyl-TGN formation as TGN concentrations approach the TPMT K_M. Alternatively, methyl-TGNs could be formed by the sequential action of a nucleoside phosphorylase and a nucleoside kinase on methyl-TG. To investigate RBC methyl-TGN concentrations we have developed a sensitive assay for the determination of this drug metabolite. The aim was to develop an assay for methyl-TG and methyl-TGNs using modifications of HPLC techniques used for routine clinical assays of thiopurines [9]. Briefly, an ethyl acetate extract of RBCs is evaporated and reconstituted in 0.1 *M* HCl. The methyl-metabolite is separated from other thiopurines by reversed-phase HPLC and quantitated using UV detection. As in other assays published from this laboratory for the measurement of intracellular thionucleotides [9], the free base of the methyl-TG is obtained by acid hydrolysis of the nucleotide back to the parent thiopurine. The assay was then used to determine methyl-TGN formation in the RBCs of leukaemic children receiving 6TG and leukaemic children receiving 6MP continuing chemotherapy.

2. Experimental

2.1. Materials

6TG (2-amino-6-mercaptopurine; MW anhydrous=167), methyl-TG (2-amino-6-methylmercaptopurine; MW anhydrous=181), 6-methylmercaptopurine (methyl-MP; MW anhydrous=166), methyl-MP riboside (6-methylmercaptopurine riboside; MW anhydrous=298), 6MP (monohydrate; MW=170), 6-thioinosine 5′monophosphate (TIMP; MW anhydrous barium salt=499.6) and DL-dithiothreitol (DTT) were obtained from Sigma (London, UK). Stock solutions of 6TG, methyl-MP and methyl-MP riboside were made at 100 μg/ml as described previously [9]. A stock solution of methyl-TG (100 μg/ml) was prepared in an alkaline solution by dissolving 10 mg in 5 ml of 0.1 *M* NaOH in a light-protected container before making to a final volume of 100 ml with water. The alkaline stock solution was stable for 3 mth when stored in aliquots (1.5-ml micro-tubes) at −15°C. For direct injection onto the HPLC the drug stock was diluted in 0.1 *M* HCl containing 1 m*M* DTT. For spiking RBCs the drug stock was diluted in water, and the final residue (see 2.3.) was reconstituted in 0.1 *M* HCl.

Methanol (HPLC grade) and ethyl acetate were obtained from Rathburn (Walkerburn, UK). All other reagents were of analytical grade and obtained from Merck (Poole, UK). The water for all experimental procedures was distilled and further purified via a Milli-Q Plus system (Millipore, Watford, UK).

2.2. Sample preparation

Washed RBCs were prepared from blood samples collected in lithium heparin tubes as previously described [9]. To enable easier pipetting of the washed RBCs the packed cells were resuspended in one volume of Hanks balanced salt solution (Life Technologies, Paisley, Scotland) to a concentration of 4×10^8 cells per 100 μl prior to storage at −20°C.

2.3. Extraction and HPLC procedure

RBCs (4×10^8 cells in 100 μl) were added to 800 μl of 3.75 m*M* in a DTT 10-ml screw neck test tube. To this was added 500 μl of 1.5 *M* H_2SO_4 and the tubes were heated at 100°C for 15 min in a Dri-

Block (Techne, Cambridge, UK). In this step thionucleotides are hydrolysed to the parent thiopurine. After cooling, and to each tube in turn, 500 μl of 3.4 *M* NaOH were added immediately followed by 6 ml of ethyl acetate. The tubes were shaken gently for 15 min and centrifuged for 10 min at 900 *g* (4°C). Then, 4.5 ml of ethyl acetate was transferred to a conical test-tube and evaporated to dryness under vacuum for 20 min at 30°C using a Buchler vortex-evaporator (Baird and Tatlock, Romford, UK). The residue was reconstituted in 200 μl 0.1 *M* HCl.

Samples (50 μl in 0.1 *M* HCl) were injected through a Waters 717 autosampler (Waters Chromatography, Watford, UK) onto a Waters RCM 8×10 radial compression system containing 0.8 cm×10 cm Resolve C_{18} cartridge, 5μm particle size (Waters). The analytical column was protected by a Guard-Pak (Waters) guard column containing a Resolve C_{18} insert (0.5 cm×0.4 cm), 5 μm particle size. The methyl-thiopurine was detected using a Model 994 programmable photodiode array detector and the peak at 314 nm was traced onto a 5200 printer plotter (Waters). During the initial stages of assay development the methyl-metabolite of 6MP was also quantitated. Thus, the photodiode array detector was set to measure the peaks at 290 nm (methyl-MP) as well as those at 314 nm (methyl-TG).

A Model 510 chromatography pump (Waters), flow-rate 1 ml/min, was used for solvent delivery. The mobile phase of methanol-water (40:60, v/v) contained 100 m*M* triethylamine and was adjusted to pH 2.7 with orthophosphoric acid. DTT, 0.5 m*M*, was added to the mobile phase immediately prior to helium purging. Prior to use, the column was equilibrated with 60 ml of mobile phase and after use it was flushed with at least 150 ml of water. It was stored in methanol.

2.4. Calibration

Calibration graphs were constructed by spiking RBCs with methyl-TG standards in the range 0.055–1.10 nmol (10–200 ng) per 4×10^8 RBCs assayed. Since the methyl-TGN was not available the standard curves for methyl-TGN were constructed using methyl-TG spiked RBCs. Spiked control RBCs containing methyl-TG were prepared and treated in parallel with all patient RBC samples.

2.5. Assay optimisation

The extraction efficiency of methyl-TG was studied under varying conditions in quadruplicate experiments. With the exception of the variable under study the conditions of the assay were as stated above. The duration of the acid hydrolysis step was varied from 0 to 1 h using 100 μl RBCs spiked with 0.30 nmol (90 ng) of methyl-MP riboside and, at a fixed time of 15 min, with the amount of methyl-MP riboside used varied from 0.30–3.0 nmol (90–900 ng) per 100 μl RBCs. Methyl-MP riboside was used for the acid hydrolysis experiments as the corresponding metabolite of 6TG was not readily available. Spiked RBCs containing 0.30 nmol (50 ng) methyl-MP were prepared and treated in parallel. In addition, the acid hydrolysis step was investigated from 0 to 1 h using 100 μl RBCs spiked with 1.18 nmol (200 ng) 6MP treated in parallel with cells spiked with 1.18 nmol (588 ng) TIMP.

The pH of the extraction was varied from 2.1 to 9.0 by the addition of varying volumes of 3.4 *M* NaOH (400–550 μl) to the acid hydrolysate sample prior to the addition of ethyl acetate. At each pH 0.28 nmol (50 ng) of methyl-TG was extracted from spiked RBCs.

2.6. Patients

Blood samples were obtained 6 h post-dose from eleven children with acute lymphoblastic leukaemia receiving daily 6TG (31 to 65 mg/m^2, median 44). At 6 h post-dose plasma 6TG concentrations fall towards the lower limit of detection [6]. The protocol standard dose of 6TG is 40 mg/m^2, but this dosage is tailored to the individual child's response to treatment. The 6TG dose is adjusted downwards in response to neutropenia and/or thrombocytopenia and upwards in a protocol directed sequence if the blood cell counts remain within the normal range. Blood cells are counted at weekly or two weekly intervals.

For comparison with 6MP chemotherapy, RBC methyl-TGN concentrations were measured in eleven children taking daily 6MP (33 to 108 mg/m^2, median 76). The blood samples were obtained approximately 6 h post-dose [5]. The protocol standard 6MP dose is 75 mg/m^2, and like 6TG the dosage is adjusted in response to blood cell counts.

2.7. Statistics

Metabolite ranges between patients were compared by the Mann-Whitney test and within patients (paired data) by the Wilcoxon signed ranks test.

3. Results

3.1. Chromatography

Fig. 1A illustrates the chromatographic separation of the methyl-metabolites of 6TG and 6MP aqueous standards each at a concentration of 70 pmol per 50-μl injection. The thiopurines were eluted with retention times of 4.1 and 6.7 min for methyl-TG and methyl-MP, respectively. The diode array enabled each compound to be measured at its λ_{max} (314 nm and 290 nm for methyl-TG and methyl-MP, respectively). Fig. 1B shows the separation of an RBC extract spiked with 0.28 nmol of methyl-TG and methyl-MP, each metabolite at a concentration of 70 pmol per 50-μl injection. The peaks eluting between 4.5 and 6 min represent endogenous compounds.

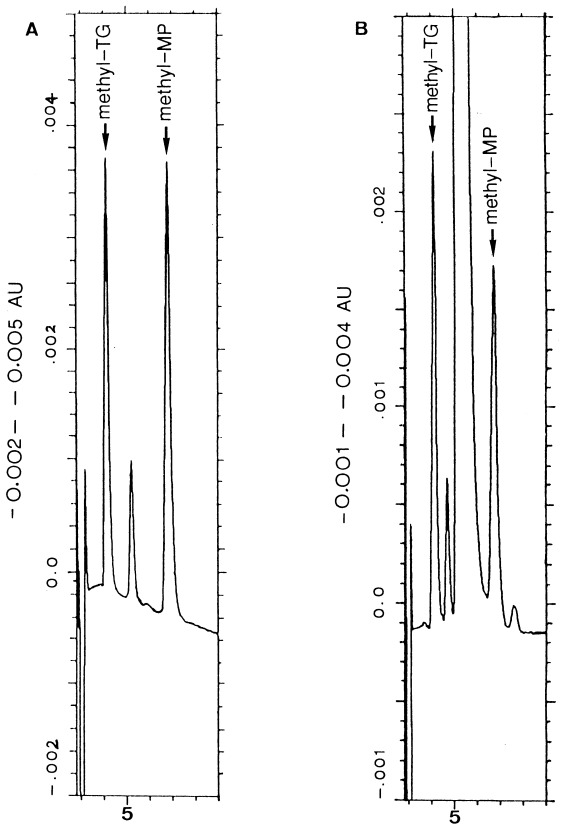

Fig. 1. (A) Chromatographic separation of an aqueous standard solution containing 70 pmol of methyl-TG and methyl-MP, 5.0 min peak-DTT: (B) Chromatogram showing an extract of RBCs spiked with 0.28 nmol of methyl-TG and methyl-MP, peaks between 5.0 and 6.2 mins – endogenous compounds.

For routine clinical assays the diode array detector was fixed at 314 nm. Although the chromatogram was recorded from 2.8 to 10 min the integrator output was set to register from 3 to 5 min. This was to maximise the methyl-TG peak height (retention 4.0 min) whilst ignoring the endogenous peak (retention 4. 9 to 6. 2 min). Fig. 2A shows the substrate blank. Fig. 2B illustrates the chromatographic separation of an RBC extract spiked with 70 pmol methyl-TG per 50-μl injection. Fig. 2C illustrates the separation of thiopurines extracted from the RBCs of a child under going 6TG chemotherapy. The 4 min peak represents the methyl-TG liberated from the methyl-TGNs. 6TG coelutes with the solvent front (retention 2.3 to 2.9 min).

3.2. Optimization of assay

3.2.1. Acid hydrolysis

The duration of the acid hydrolysis step was varied from 0 to 1 h: at each time point RBCs spiked with 0.30 nmol (90 ng) of methyl-MP riboside or 0.30 nmol (50 ng) of methyl-MP were heated at 100°C (Fig. 3a). The acid hydrolysis step was required to convert thionucleotides back to the parent thiopurine. No metabolite was extracted from the unheated methyl-MP riboside sample. Fig. 3b illustrates the decrease in recorded absorption units of extracted methyl-TG as the duration of the acid hydrolysis is increased. With the acid hydrolysis time fixed at 15 min the formation of methyl-MP liberated from the corresponding riboside was linear over the concentration range 0.30–3.0 nmol per 100 μl RBCs; $y=-0.17+0.021x$, $r^2=0.99$. The acid hydrolysis of 6MP nucleotide, TIMP, to 6MP is illustrated in Fig. 4. After 15 min 87%±5 of the nucleotide is converted to the parent purine. The difference in the final absorbance of the two curves

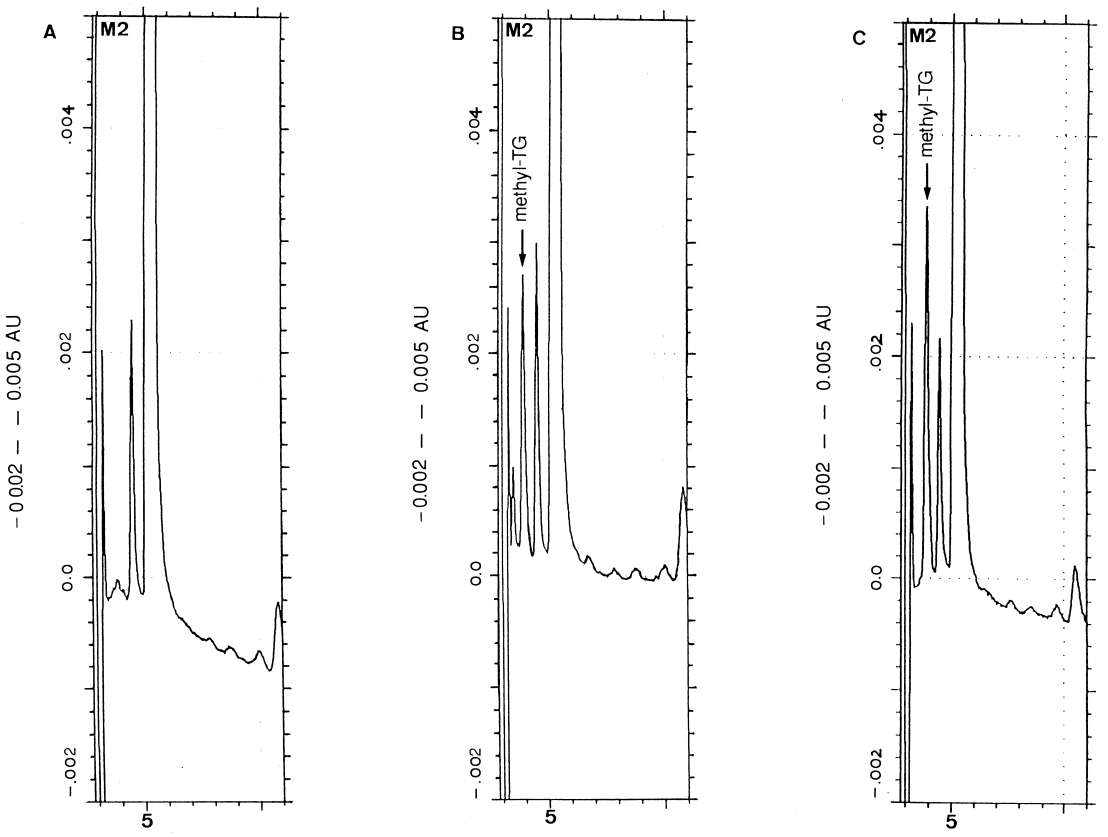

Fig. 2. Chromatograms showing the separation of methyl-TG (4.0 min) from endogenous compounds (4.9 to 6.2 mins) in extracted RBC samples. (A) Chromatogram showing the substrate blank; (B) chromatogram showing an RBC extract spiked with 70 pmol. methyl-TG; (C) chromatogram showing the presence of methyl-TG, liberated from methyl-TGNs, and extracted from the RBCs of a child undergoing 6TG therapy (dose=31 mg/m^2).

is presumably due to the degree of hydration of the 6MP and TIMP used for spiking the RBCs.

3.2.2. Effect of pH.

Fig. 5 illustrates the change in extraction efficiency as a function of the pH. Optimum extraction of methyl-TG occurred at pH 7.9 corresponding to the addition of 500 μl of 3.4 *M* NaOH.

3.3. Calibration curves

The methyl-TG calibration curves were linear with correlation coefficients >0.99. A typical calibration graph gave a regression of $y=-0.027+O.051x$, where y is the peak height and x is the RBC methyl-TG concentration.

3.4. Extraction efficiency, precision and accuracy

The mean recovery of methyl TG over the calibration range was 61.6% (coefficient of variation, C.V., 3.8%). Assay precision was evaluated from eight measurements at each point of the calibration curve (intra-.assay variation) and accuracy from six measurements of two quality control samples consisting of RBCs spiked with 0.28 and 1.10 nmol of methyl-TG taken over a two-month period (inter-assay variation). The intra-assay C.V. over the con-

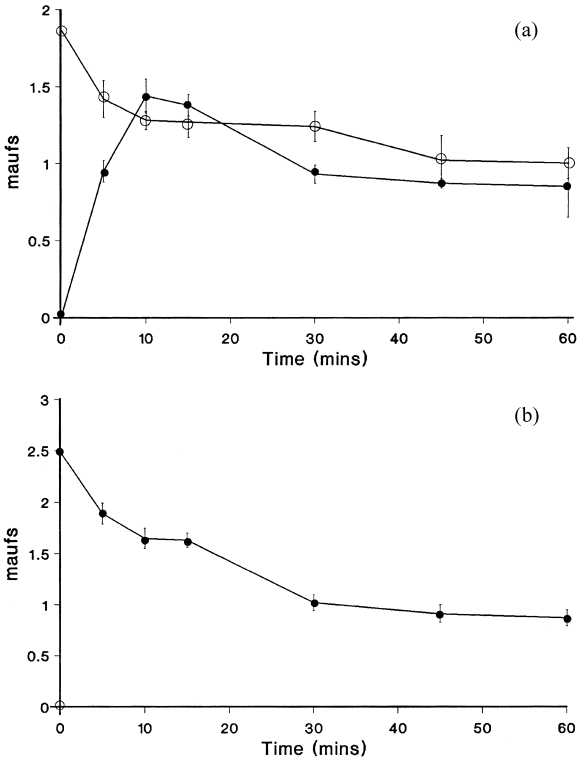

Fig. 3. Duration of the acid hydrolysis step and (a) the production of methyl-MP liberated from methyl-MP riboside (closed circle), with a methyl-MP heated control (open circle) (b) extraction of methyl-TG. Error bars=±1 S.D.

centration range of 0.055–1.10 nmol per 4×10^8 (100 μl) RBCs ranged from 2.8 to 8.5% (Table 1) and the quality control concentrations for methyl-TG at the

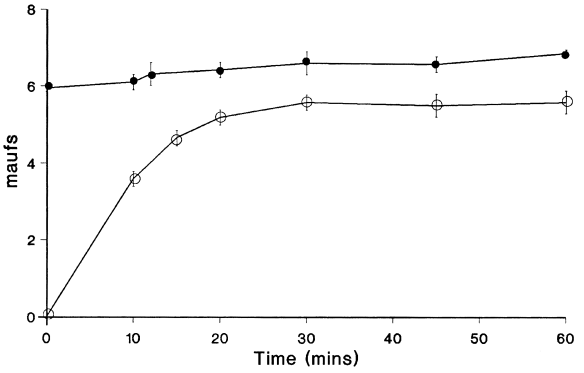

Fig. 4. Duration of the acid hydrolysis step and the extraction of heated MP (closed circle), and the extraction of MP liberated from heated TIMP (open circle).

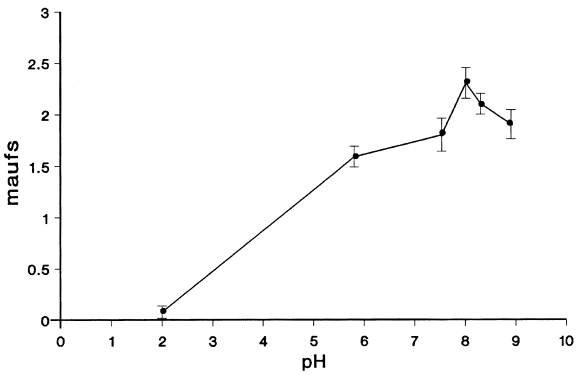

Fig. 5. Influence of the pH on the extraction efficiency of methyl-TG. Error bars=±1 S.D.

low and high concentrations were 0.281±0.021 nmol (C.V.=7.6%) and 1.12±0.025 nmol (C.V.=2.2%), respectively. The lower limit of reproducibility was 0.055 nmol extracted from 100 μl RBCs.

3.5. Assay performance

During the developmental stages of this work, methyl-MP riboside was used to study the acid hydrolysis step. Repeated analysis of RBCs spiked with both methyl-MP and methyl-TG showed that the inclusion of wavelengths <300 nm (λ_{min} of methyl-MP=290 nm) resulted in the detection of late retained peaks. Over a 14-h period, keeping the injection time at 10 min, the chromatography deteriorated. This, coupled with the fact that this assay could potentially be used on blood samples derived from children undergoing 6MP chemotherapy, made it impractical to use methyl-MP as an internal standard for the methyl-TGN assay

Table 1
Intra-assay reproducibility of methyl-thioguanine in red blood cells

Concentration (nmol/4×10^8 RBCs)	Absorbance (m.a.u.f.s) (mean±S.D., n=8)	C.V.(%)
0.055	0.40±0.034	8.5
0.110	0.77±0.062	8.0
0.275	2.06±0.136	6.6
0.550	4.12±0.350	8.5
1.10	8.42±246	2.8

3.6. Patient samples

During 6TG therapy RBC methyl-TGN concentrations ranged from 323 to 1365 pmol per 8×10^8 cells, median 1111 (Table 2). No child had any 6TG or methyl-TG in their RBCs. The spectra of the methyl-TG generated from methyl-TGN in a patient sample was superimposable on the authentic methyl-TG extracted standard. During 6TG therapy, RBC TGN concentrations ranged from 623 to 7090 pmol per 8×10^8 cells, median 2323, significantly higher than the methyl-TGN concentrations (median difference 1447 pmol, 95% C.I. 755 to 3139, $P<0.001$). RBC TGNs were quantitated using a previously published assay [9]. Methyl-TGNs represent 10 to 49% (median 30) of total TGNs.

Methyl-TGNs are tabulated alongside the TGN and methyl-MP concentrations [9] measured in eleven children. receiving 6MP therapy (Table 2). The methyl-MPs within patients RBCs are the nucleotide metabolites of methylmercaptopurine [9]. With 6MP chemotherapy lower TGN concentrations are produced (56 to 1164 pmol per 8×10^8 RBCs, median 285; median difference 2045 pmol, 95% C.I. 1532 to 2913, $P<0.0001$), and only two children produced methyl-TGNs (Table 2).

4. Discussion and conclusions

This paper describes a specific and sensitive reversed-phase HPLC assay for the measurement of methyl-TG and methyl-TGNs in RBCs which is suitable for routine clinical use. Analysis of blood samples from leukaemic children receiving 6TG chemotherapy, confirms the presence of RBC methyl-TGN, but in amounts significantly lower than TGN concentrations. No methyl-TG was detected in

Table 2
Red blood cell methyl-thioguanine nucleotide concentrations (a) From 6TG

Subject	Dose of 6TG (mg/m^2)	Methyl-TGNs (pmol/8×10^8 RBCs)	6-TGNs (pmol/8×10^8 RBCs)
1	65	1329	1401
2	44	1365	1988
3	51	1281	2323
4	44	323	623
5	42	1111	7090
6	31	551	1988
7	49	1006	2323
8	44	1150	3018
9	40	413	3617
10	37	922	3365
11	42	1089	2527

(b) From 6MP

Subject	Dose of 6MP (mg/m^2)	Methyl-TGNs (pmol/8×10^8 RBCs)	6-TGNs (pmol/8×10^8 RBCs)	Methyl-MPs (pmol/8×10^8 RBCs)
1	76	287	1164	174
2	71	–	168	48935
3	85	96	545	1218
4	91	–	266	13846
5	89	–	285	920
6	74	–	372	12959
7	108	–	56	37132
8	85	–	278	5497
9	76	–	384	13342
10	72	–	105	13471
11	33	–	456	290

any of the patient samples. In contrast, of the eleven children taking 6MP, only two had detectable amounts of RBC methyl-TGNs – the two patients with the highest RBC 6TGN concentrations. The preliminary observations presented in this paper indicate that in children taking 6MP chemotherapy the formation of methyl-TGNs is not a major metabolic route, whilst in children taking 6TG 10 to 49% of total TGNs are methylated.

The metabolism of 6TG is not as complex as 6MP. On oral dosing 6TG can be *S*-methylated by TPMT, phosphoribosylated by hypoxanthine guanine phosphoribosyltransferase (HGPRT) or deaminated by guanase. The latter catabolic route produces the xanthine oxidase substrate 6-thioxanthine, the final product is 6-thiouric acid. HGPRT catalysed phosphoribosylation is a very rapid reaction [10] compared to 6TG *S*-methylation [8], thus the major metabolic 6TG flux will be towards direct TGN formation. Methyl-TGNs could arise from two possible routes, the *S*-methylation of the high TGN concentrations formed during 6TG therapy and indirectly by the phosphorylation of methyl-TG derived methyl-TG ribosides. During 6MP therapy the initial nucleotide metabolite is 6-thioinosine monophosphate which undergoes metabolic conversions to eventually form TGNs. The formation of methyl-MP nucleotides from 6-thioinosine monophosphate is a major metabolic route [11]. These antimetabolites mimic the actions of inosine 5′-monophophate and inhibit de novo purine synthesis, but the methyl-TGNs are at least 10 times less effective than the methyl-MP nucleotides in inhibiting this metabolic step [12]. How these inhibitory properties of the methylated thionucleotides influence the action of thiopurine drugs in vivo are uncertain. The development of this assay will enable methyl-TGN formation to be investigated and its role elucidated.

The quantitation of methyl-TG in various biological samples has been previously reported using an ethyl acetate extract analysed by reversed-phase C_{18} HPLC followed by fluorimetric detection [13], or UV detection [14], and by anion-exchange HPLC and UV detection of the supernatent from a perchloric acid (PCA) precipitate [15]. These methods were developed for the measurement of methyl-TG in urine, lymphocytes and peripheral mononuclear cells, respectively, and quote lower limits of 500 ng/ml^{-1} urine, 5 ng per 5×10^6 lymphocytes and 150 n*M* (i.e. 15 pmol per 100 μl PCA extract injected). There is one reported method for the specific determination of methyl-TG and methyl-TGNs in RBCs [16]. The methyl-TG is detected by reversed-phase HPLC using a cyanopropylsilane column, a methanol/phosphate buffer mobile phase and UV detection. The methyl-TGNs are analysed by acid hydrolysis back to the parent purine and the lower limit of reproducibility is 25 ng methyl-TG per 8×10^8 RBCs [16]. However, during the acid hydrolysis step the methyl-TG is heated at 100°C for 45 min in the presence of 0.5 *M* sulphuric acid. This would produce a 100% conversion of nucleotide to purine, but we estimate that this could be accompanied by a decrease in the recorded absorbance units of methyl-TG by 68% (Fig. 3b). Heating at 100°C modifies the methyl group of methyl substituted 6-thiopurines [9]. In the assay reported in this paper we have optimised methyl-TG extraction whilst ensuring maximum conversion of the thionucleotides to the thiopurines by simply reducing the time of the 100°C heating step to 15 min. The HPLC assay reported here enables both the free base and thionucleotide of methyl-TG to be quantitated in 4×10^8 RBCs, approximately 50 μl of packed cells, with a lower limit of 55 pmol (10 ng). The time taken for chromatographic separation is less than 10 min, a suitable time interval for routine HPLC analysis.

Acknowledgements

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