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Short communication

Liquid chromatography–tandem mass spectrometry quantification of 6-thioguanine in DNA using endogenous guanine as internal standard

Jack H. Jacobsen [a], Kjeld Schmiegelow [a,b], Jacob Nersting [a,*]

[a] Department of Pediatrics, Rigshospitalet, Copenhagen, Denmark
[b] Institute of Gynecology, Obstetrics and Pediatrics, The Faculty of Medicine, University of Copenhagen, Denmark

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ABSTRACT

Thiopurines are S-substituted antimetabolites that are widely used in the treatment of hematological malignancies and as immunosuppressants. Because of extensive inter-individual variation in drug disposition and the significant toxicity associated with thiopurine therapy, there is a need for improved individualized treatment. We here present a fast and sensitive method for quantifying the pharmacological end-point of thiopurines, 6-thioguanine (TG) in chromosomal DNA. Purine nucleobases are released from DNA, etheno-derivatized with chloroacetaldehyde, separated by HILIC and quantified by tandem mass spectrometry using endogenous chromosomal guanine as internal standard. The method is linear up to at least 10 pmol TG/μg DNA and the limit of detection and quantification are 4.2 and 14.1 fmol TG/μg DNA, respectively. The matrix (DNA) had no effect upon quantification of TG. SPE recovery was estimated at 63% (RSD 26%), which is corrected for by the internal standard resulting in stable quantification. The TG levels found were above the LOQ in 18 out of 18 childhood leukemia patients on 6-mercaptopurine/methotrexate maintenance therapy (median 377, range 45–1190 fmol/μg DNA) with intra- and inter-day RSDs of less than 11%. The method uses 2 μg DNA/sample, which can easily be obtained from these patients.

1. Introduction

Thiopurines, such as 6-mercaptopurine (6MP) are S-substituted antimetabolites that are functional analogs to natural nucleobase precursors. They have been used for over 50 years in the treatment of leukemia, autoimmunity and inflammatory disorders, and to prevent graft rejection [1–3]. The cytotoxicity of thiopurines depends on their conversion into 6-thioguanine (TG) nucleotides (TGN), which are incorporated into DNA, causing cell death by post-replicative DNA mismatch repair [2,4,5]. Since the therapeutic index of thiopurines is low and patients' responses vary substantially, sensitive patients may experience life-threatening toxicity whereas low-responders are under-treated with standard dosing. High- and low-activity variants of the enzyme thiopurine methyltransferase (TPMT), which methylates and inactivates thiopurines and some of their metabolites [6] are major determinants, but variants in additional metabolizing enzymes have been identified [2]. Response to thiopurines can to some extent be predicted by pharmacogenomics [7,8], and current guidelines prescribe geno- and phenotyping of TPMT before commencing thiopurine therapy, as deficiency in this enzyme can lead to fatal immunosuppression from a single dose of 6MP [9]. Nonetheless, prediction of the thiopurine effects by genetics cannot be made with sufficient accuracy for dose adjustments in individual patients. Alternative strategies, such as measuring TGN in erythrocytes (E-TGN, a surrogate marker for lymphocyte DNA-TG) have likewise been tested. Although E-TGN correlates with treatment response in most [10–14] but not all [15] studies, the strength of the correlation is insufficient for dose adjustment in individual patients. Dosing to toxicity (white blood cell counts $<3.5 \times 10^6$/ml), is thus the standard thiopurine dosing regimen in acute lymphoblastic leukemia (ALL) in children [16], but toxicities and treatment failures remain major problems. Because of the need for a better outcome predictor we developed a method for quantifying the pharmacological endpoint of thiopurines, TG incorporated in chromosomal DNA of white blood cells, and applied it to 18 children on ALL maintenance therapy.

Abbreviations: 6MP, 6-mercaptopurine; ALL, acute lymphoblastic leukemia; CAA, chloroacetaldehyde; εA, etheno-adenine; εG, etheno-guanine; εTG, etheno-thioguanine; E-TGN, erythrocyte TGN; G, guanine; SPE, solid phase extraction; TG, 6-thioguanine; TGN, thioguanine nucleotide.

* Corresponding author at: Department of Pediatrics, Blegdamsvej 9, Rigshospitalet, 5704, DK-2100 Copenhagen, Denmark. Tel.: +45 3545 7091; fax: +45 3545 4524.
E-mail address: jacob.nersting@rh.regionh.dk (J. Nersting).

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2. Materials and methods

2.1. Chemicals and materials

All chemicals if available were LC/MS grade and from Sigma–Aldrich, Denmark except water and acetonitrile from Fisher-Scientific, Denmark. TG stocks at 5 mM were prepared in 310 mM NH_4OH and stored at −80 °C. Working solutions were made daily by diluting TG stocks with water. Solid phase extraction (SPE) columns were Strata-X-C, 33 μm particle size, 30 mg/1 ml, polymeric strong cation exchangers (Phenomenex, Denmark).

2.2. DNA sources and extraction

DNA was purified from EDTA blood with a Quickgene 810 system (Fujifilm, Sweden) according to the manufacturer's instructions, except that DNA was eluted with water because the included buffer inhibited detection of the thioguanine etheno-adduct (εTG). No attempts were made to identify the interfering component. Alternatively, DNA was manually extracted by sodium dodecyl sulfate/proteinase K digestion followed by NaCl and 2-propanol/ethanol precipitation of protein and DNA, respectively, which is a modified version of Sambrook and Russell [17]. The precipitated DNA was dissolved in water. DNA was quantified at A_{260} using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Denmark) and stored at −80 °C. Control DNA, i.e. DNA without any detectable TG, was extracted from blood of unexposed, healthy donors or bone marrow aspirates from leukemia patients prior to 6MP exposure.

2.3. Incorporation of TG into Jurkat DNA

DNA with known content of incorporated TG is not commercially available. We therefore produced DNA-incorporated TG by growing Jurkat cells in medium supplemented with 46 μM 6MP for the final 24 h of growth. At harvest the medium was aspirated, the cells were washed three times with phosphate buffered saline, and DNA was extracted manually.

2.4. Sample preparation

Two micrograms of DNA in 75 μl water was incubated with 75 μl derivatization buffer (1 M chloroacetaldehyde (CAA) in 90 mM potassium phosphate, pH = 5.0) for 3 h at 99.9 °C. The resulting etheno (ε)-derivatized sample was mixed with 850 μl 0.2% formic acid (FA) and loaded on the SPE-column, which had been conditioned and equilibrated with 1 ml each of methanol and 0.1% FA, respectively. The adsorbed sample was washed with 1 ml 0.1% FA, 1 ml 0.1% FA in 50% methanol and eluted with 450 μl 780 mM NH_4OH in 50% methanol. Washing and elution steps were done by centrifuging the SPE columns 50–200 × *g* for 1–3 min. The final eluate was dried for 15 min at 40 °C under N_2 streaming and reconstituted in 200 μl 95% acetonitrile, 0.1% FA.

2.5. Chromatography and MS

Samples were run on an Aquity UPLC system equipped with an Aquity Triple Quadrupole Detector (TQD) and a 2.1 mm × 50 mm HILIC BEH 1.7 μm column protected by a 2.1 mm × 5 mm Vanguard HILIC BEH 1.7 μm pre-column (all from Waters, Denmark). Separation at 35 °C was isocratic with 160 mM FA, 10 mM ammonium formate in 95% acetonitrile at a flow rate of 0.3 ml/min for 2.3 min followed by 0.8 ml/min for 0.7 min. Injection volume was 6 μl and total run time was 4 min/sample. *MS settings*: Source temperature was 140 °C. Desolvation gas (N_2) flow was 780 l/h at 490 °C. Argon (0.22 ml/min) was used for collision gas. Capillary and cone voltages were 500 and 33 V, respectively. TQD analyzer resolutions were set to 13 corresponding to peak widths of approx. 0.7 amu. at half height. TQD parameters were set to achieve maximal ion signals. Small or parallel changes in εTG, εG1 ionization were observed in response to varying these settings.

2.6. Calibration curves and internal standard (IS)

Due to the lack of a standard of TG in DNA, daily calibration curves were generated from dilutions of Jurkat DNA in control DNA (total 2 μg DNA/sample) that were derivatized in parallel with unknown samples. In order to estimate the TG-content of the Jurkat DNA, calibration curves were also generated from TG spiked into blank DNA (final 0–1875 fmol/μg DNA). We assume that incorporated TG in DNA and TG spiked to a blank DNA sample are derivatized equally. When we compared the two linear calibrations of εTG response vs. %-Jurkat and TG-concentration we found, based on two independent experiments, that 1% Jurkat DNA contained 100.5 fmol TG/μg DNA, assuming identical signal from equimolar free and DNA-incorporated TG. This conversion factor is used to convert all dilutions of Jurkat DNA to fmol TG/μg DNA. Chromatographic εTG peaks were normalized using εG1 (see Section 3.1) as IS, by calculating TG responses as εTG area/εG1 area. The quality of the IS as a normalizing factor inherent in DNA was tested by analyzing samples containing 0.5–5 μg of Jurkat DNA (500 fmol TG/μg DNA).

2.7. Method validation

DNA from 18 randomly selected childhood ALL patients on 6MP/methotrexate maintenance therapy (Rigshospitalet, Copenhagen), control DNA and Jurkat DNA was used for method validation and testing of clinical applicability. DNA from five of the patients was selected for evaluation of inter-day variation.

2.7.1. SPE recovery

Etheno-TG was produced by derivatizing 500 μg TG in 1 ml of derivatization buffer for 1 h at 99.9 °C. SPE purification of εTG was then performed as described in Section 2.4, except that FA was not added before loading the sample on the SPE column and εTG was re-dissolved in 6 mM NaOH after drying under N_2. The recovery efficiency of the SPE method was estimated, by spiking 2 μg of control DNA with approx. 100 fmol εTG in either the derivatized sample, the eluate from the SPE column or in the sample vial before LC/MS quantification, in triplicates.

2.7.2. Matrix effect

The effect of DNA on quantification was investigated by analyzing 256–10,000 fmol TG with 2 μg control DNA or with water only, resulting in two linear calibration curves whose slopes were compared. As guanine (G) is not to be found in the water sample, the εTG/εG1 response cannot be calculated as it normally would be. Instead, the εTG peak area was normalized to the response of a QC sample (Jurkat DNA) that was quantified in every sixth injection, and used as a correction for drift in MS detection. Additionally, samples were run twice.

3. Results

3.1. Derivatization and IS

During incubation with CAA, adenine (A), TG and G nucleobases are derivatized to their respective εA, εTG [18] and εG [19] adducts. Derivatization of Jurkat DNA (500 fmol TG/μg) with 0–1.25 M CAA for 0–3.5 h at 60–99.9 °C was tested and 0.5 M CAA for 3 h at 99.9 °C was found to be optimal. Under these conditions εA peaked at

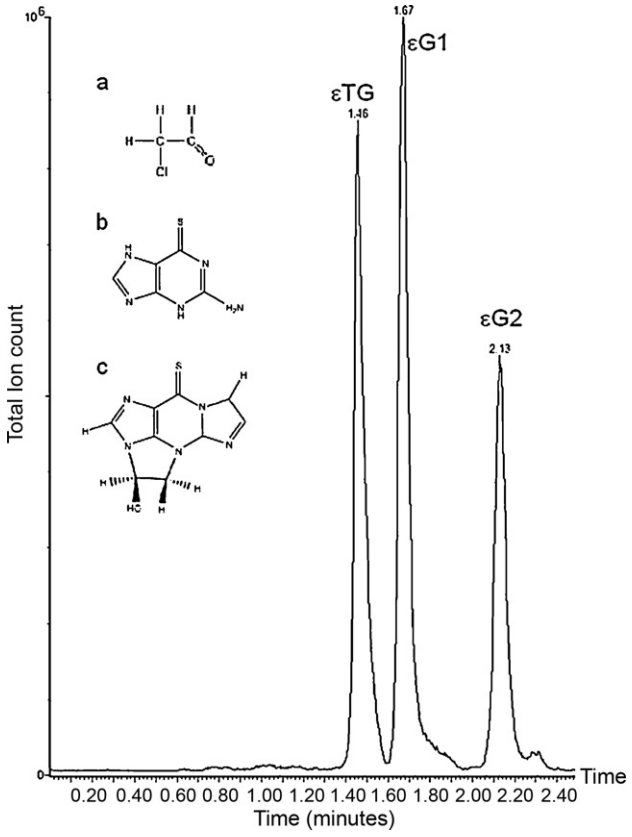

Fig. 1. Total ion count chromatogram from undiluted Jurkat DNA showing SRM activities of εTG and εG (233.95 → 190.95 and 175.93 → 94.00, respectively) in positive electrospray ionization (ESI+) mode. (a) CAA, (b) TG, and (c) εTG. The structure of C was determined by NMR spectrometry for a Ph.D. thesis (Karen Marie Olesen, unpublished data).

approximately 30 min followed by a rapid decline (not shown). In contrast, maximum levels of εTG and εG1 were reached within 50 min and remained stable up to at least 3.5 h, resulting in a stable εTG response (Fig. 2). Derivatization for 3 h was chosen since this stabilized variations in TG responses when varying CAA concentrations as compared to shorter incubations. Calculation of εTG responses was tested with both εG1 and εG2 as IS. Owing to the super-imposable derivatization kinetics, higher TQD signal, elution closer to εTG, and better stabilization of the TG response (lower RSD of repeated injections, not shown) εG1 was chosen for IS.

3.2. Chromatography

Whereas εTG eluted in 1.46 min, selected reaction monitoring (SRM)-activity of εG resolved in two peaks at 1.67 min (εG1) and 2.13 min (εG2) (Fig. 1). Varying the concentrations of water or FA in the mobile phase had minor and parallel effects on εG and εTG peak areas and hence did not influence the calculated TG responses. In contrast, larger εG and εTG peaks were observed at lower (<5 mM) ammonium concentrations, but these changes were not parallel and increased peak tailing was observed. Since method sensitivity was not an issue (see Section 4), 10 mM ammonium formate was used.

3.3. Method validation

3.3.1. Linear range, sensitivity and specificity

The TG response in serial dilutions of Jurkat DNA was linear up to at least 10,050 fmol TG/μg DNA (mean $r^2 > 0.999$; $n = 5$ experiments). LOD and LOQ, calculated as the response of control sample

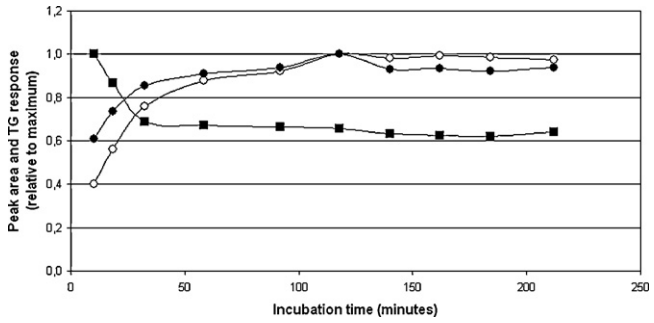

Fig. 2. Two micrograms of Jurkat DNA containing 500 fmol TG/μg DNA was derivatized with 1 M CAA at 99.9 °C for the indicated amount of time. The resulting εTG and εG1 peak areas as well as calculated TG response are shown relative to the maximum value observed. (○) εG1 peak area; (●) εTG peak area; (■) TG response (εTG/εG1). Varying CAA concentrations (0.56–1.25 M) or incubation times (1–3 h) had minimal effects on TG responses (not shown). Data points represent individual samples. Comparable results were found in 5 independent experiments.

plus 3 and 10 times the standard deviations of the response from six preparations of Jurkat DNA (25.1 fmol TG/μg DNA) were 4.3 and 14.1 fmol/μg DNA, respectively. Method specificity was confirmed with control DNA from 10 donors in which TG was not detected.

3.3.2. Method reproducibility

At TG levels above approximately 100 fmol TG/μg DNA, which corresponds to low TG-levels in patients, intra- and inter-day RSDs less than 11% were generally observed (Table 1), which is acceptable for clinical applications given the approx. 25-fold inter-patient variation (see Section 3.4).

3.3.3. SPE recovery, matrix effect and IS

The mean recovery of approx. 100 fmol εTG with 2 μg control DNA, relative to that spiked directly in vial, was 62.7% or 87.2% when spiked before loading on the SPE column or before drying, respectively (Table 1). There was no observable matrix effect as there was no difference in the calibration slopes of TG in water or in DNA. Deviations in TG response with varying amounts of DNA (0.5–5 μg) were approx. 10%/μg DNA (not shown), demonstrating that εG1 is an efficient normalization with regard to unexpected variations in amounts of DNA.

Table 1
Reproducibility, SPE recovery and matrix effects of the method are shown. Mean TG content with RSDs.

Reproducibility	fmol TG/μg DNA	RSD (%)	n
Intra-day			
Jurkat low	91	11.0	6
Jurkat medium	382	10.9	
Jurkat high	1053	3.7	
Inter-day			
Patient 7	145	11.4	5
Patient 12	261	9.4	
Patient 13	281	7.1	
Patient 18	528	7.9	
Patient 2	1166	4.6	

Recovery	%	RSD (%)	n
SPE + drying	62.7	26.3	3
Drying only	87.2	3.1	
No SPE or drying	100.0	2.3	

Matrix effect(normalized to QC)	1st run	2nd run
Cal. slope (DNA)	1.487	1.598
Cal. slope (water)	1.483	1.599

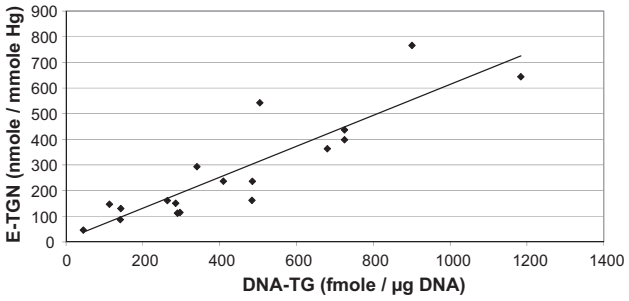

Fig. 3. Paired measurements of DNA-TG and E-TGN from eighteen patients with a fitted line (R^2 = 0.78). Hg: hemoglobin.

3.4. TG-metabolite levels in ALL patients

The median (range) TG level of the 18 leukemia patients was 377 (45–1190) fmol TG/μg DNA corresponding to 15–400 per 10^6 bases, which is well in line with previous findings [18,20]. Fig. 3 shows the relationship between the DNA-TG and E-TGN measurements [21]. The two parameters are correlated (R^2 = 0.78).

4. Discussion

The two peaks resulting from the reaction of G with CAA likely correspond to the known adducts, 1,N^2-εG and N^2, 3-εG [19]. The εG1 peak showed some tailing, which may be due to column overload because of the concentration of derivatized purines in the sample, which is in the μM range. Although SPE recovery was found to be somewhat low and to display some variation, the variation in recovery was restricted to the spiking before SPE, as the samples spiked after SPE and after drying had RSDs of 3.1% and 2.3%, respectively.

RSDs of reproducibility samples show that quantification is reproducible with the εG1 as IS. The εG adduct is used as IS, because G is present in all DNA in amounts that are species specific. Thus, the sample matrix will contain IS in fixed amounts relative to the amount of starting material. This is an advantage, as potential errors in quantification of DNA are corrected, resulting in a concentration-dependent response. One possible limitation is that the IS and analyte are not chemically identical, and thus could be quantified with different efficiency, but we have found them to behave very similarly with regard to ionization, derivatization and SPE recovery. Normalizing εTG to endogenous εG1 thus allows for a consistent quantification despite fluctuating MS detection efficiency and variable amounts of DNA in the sample.

As absolute accuracy is not determined because of the lack of a standard, it is reassuring to note the similarity of the clinical values to that of another, dissimilar method, that was based on enzymatic digestion of DNA and fluorescence detection of TG [20]. It suggests that quantification is, at least reasonably, accurate. In 18 patients, the lowest TG level found was more than three-fold the method LOQ, meaning that TG levels are quantifiable in essentially all leukemia patients, using small amounts of DNA. In contrast to our previous method where εTG and εA are measured in separate chromatographic runs [18], the present method allows for quantification in one run, which in combination with improved sample preparation effectively increases sample throughput. In summary, we have developed an easily applicable, fast and precise analysis to measure the TG content in genomic DNA, without the need for enzymes or multiple incubations. The potential of this method in thiopurine dose adjustment and outcome prediction is now being tested in Nordic and Baltic children with ALL on 6MP/methotrexate maintenance therapy.

Contributors

All authors participated in designing the study and writing the manuscript. K. Schmiegelow coordinated sample collection. J.H. Jacobsen and J. Nersting established the method and analyzed patient DNA.

Conflict of interest

The authors declare no conflicts of interest.

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