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Determination of thiopurine *S*-methyltransferase activity in erythrocytes using 6-thioguanine as substrate and a non-extraction liquid chromatographic technique

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Abstract

A non-extraction high-performance liquid chromatographic (HPLC) method has been developed for the determination of 6-methylthioguanine (6-MTG), as part of the determination of thiopurine *S*-methyltransferase activity (TPMT) in erythrocytes. Erythrocyte lysate is added to a glass vial containing substrates and incubation buffer, which is then sealed for the rest of the analysis. Enzyme incubation, sample preparation, and analysis are then undertaken without further sample-handling steps. The need for a solvent extraction step has been overcome by heating the incubate to 85 °C to stop the enzyme reaction. The heat inactivation step precipitates protein which upon centrifugation forms a thin film in the bottom of the glass vial enabling the supernatant to be injected directly onto the HPLC system. The assay shows excellent precision and recovery with a within-batch imprecision giving a co-efficient of variation of 2.9% (mean = 41.5 nmol 6-MTG/g Hb/h, $n = 10$) and 5.1% (mean = 12.6 nmol 6-MTG/g Hb/h, $n = 10$). The between-batch imprecision gives a co-efficient of variation of 8.2% (mean = 11.1 nmol 6-MTG/g Hb/h, $n = 11$) and 7.3% (mean = 41.0 nmol 6-MTG/g Hb/h, $n = 16$). Determination of the TPMT activity in 120 people shows a range of enzyme activity of 11.3–63.8 nmol 6-MTG/g Hb/h with a mean and median activity of 34.8 and 34.2 nmol 6-MTG/g Hb/h, respectively. TPMT is increasingly used in clinical practice to ensure optimisation of treatment with thioguanine drugs. This direct HPLC method minimises sample-handling, reduces inherent imprecision, the possibility of laboratory error and with the potential for further automation, makes it ideal for use in a regional referral laboratory.

Keywords: Enzymes; Thiopurine *S*-methyltransferase; 6-Thioguanine

1. Introduction

The thiopurine drugs azathioprine, mercaptopurine and 6-thioguanine (6-TG) are used to influence the immune response, in the treatment of inflammatory diseases such as ulcerative colitis, dermatitis and rheumatoid arthritis, and to stop rejection of tissues post-transplant [1]. However, individual patients vary greatly in susceptibility to the toxic effects of these drugs, as a result of genetic differences in drug metabolism and this limits their clinical use [2]. Thiopurine metabolism includes the enzyme thiopurine *S*-methyltransferase (EC 2.1.1.67; TPMT), the activity of which is absent in approximately 1 in 300 individuals, is at low activity in another 11% of the population [3] and is subject to ethnic variation [4,5]. Patients with low or no detectable TPMT activity are at severe risk of side-effects, and the study of TPMT activity enables the dose of thiopurine to be appropriately adjusted in such patients [5].

Historically, clinical treatment regimes have monitored patients on thiopurine drugs by looking for a drop in their white cell count to identify low metabolisers indirectly [6]. TPMT activity analysis in red blood cell lysates establishes a patient's enzyme activity prior to commencing treatment [7]. Current thinking suggests that phenotyping using assays for TPMT activity is a better predictor than a genotypic approach [8].

Monitoring bone marrow toxicity by measuring white cell count only identifies susceptible patients after toxicity has occurred. Genotyping is possible [9] but may not reflect clinically important patient difference. The preferred strategy is to assess TPMT phenotype by measuring enzyme activity [6,9]. Indeed, in the USA the Federal Drug Administration

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has discussed adding advice to thiopurine drug packaging recommending that TPMT status should be checked [10].

Early methods for determining TPMT activity used mercaptopurine as substrate and, after incubation, a radiochemical assay was used to determine the amount of the product methylmercaptopurine produced [11]. More recent assays have used HPLC to determine the methylmercaptopurine [12]. An alternative approach has been to use 6-thioguanine as a substrate with TPMT activity producing the product 6-methylthioguanine (6-MTG) which is measured after extraction from the incubate using HPLC with fluorescence detection [13,14].

Analysis of TPMT enzyme activity in erythrocytes involves several steps including lysate preparation, incubation and solvent extraction prior to the analysis of the 6-MTG produced. For the clinical hospital laboratory this procedure is relatively complex. Here we present a direct TPMT assay using 6-TG as substrate in a method which overcomes the need for solvent extraction, and minimises sample-handling making it ideal for use in a routine regional referral centre.

2. Experimental

2.1. Chemicals and samples

The substrates *S*-adenosyl-L-methionine (SAM, lot No. 052K7045) and 2-amino-6-methyl mercaptopurine (6-MTG, lot No. 81H0183) were obtained from Sigma–Aldrich (Poole, UK) and 6-thioguanine (6-TG, lot No. GR 234422X) was obtained from GlaxoSmithKline (Middlesex, UK). Solvents of HPLC grade were obtained from BDH (Poole, UK).

A stock standard solution of 6-MTG was made by dissolving 10 mg of 6-MTG in 4 ml of 0.1 M NaOH and was used to make a working standard of 400 ng/ml (2.2 nmol/ml).

The incubation mixture comprised final concentrations of 600 μmol/l 6-TG and 80 μmol/l SAM. This was made up by dissolving 6-TG (14 mg) in 4 ml of 0.1 mol/l sodium hydroxide. SAM (4.2 mg) was dissolved in 50 ml potassium phosphate buffer (0.1 mol/l, pH 7.4). The two substrates were mixed together and made up to a final volume of 100 ml with 0.1 mol/l potassium phosphate buffer (pH 7.4). The substrate mixture (0.5 ml) was then pipetted into incubation tubes, which were capped and stored at −70 °C for up to 1 month prior to use.

To establish the levels of TPMT activity expected in a population, an additional 4 ml EDTA sample was collected from patients attending phlebotomy for routine blood tests. Local ethics committee approval was given and informed consent was obtained from patients who entered the study. To help with long-term quality assurance studies, blood samples were taken from volunteers. The lysates produced were aliquoted, stored at −70 °C, and run on each assay as a quality assurance sample.

2.2. Apparatus and chromatographic conditions

The chromatographic apparatus consisted of a Gynkotech model 300 isocratic pump (Severn Analytical, UK), with a Hewlett Packard 1100 series fluorimeter connected to a Hewlett Packard 3394 series integrator (Agilent technologies, UK) and Chromosystems CLC 200 autosampler (Chromosystems, Diagnostics by HPLC, UK) fitted with a 50 μl loop.

The stationary phase was a Kingsorb 5 μ reverse-phase column (C18, 150 × 4.6 mm, 5 μm, Phenomenex, UK) at ambient temperature protected with a 4 mm × 3 mm Security-Guard guard column (Phenomenex, UK). The mobile phase (flow-rate, 2 ml/min) was 0.05 mol/l phosphate buffer; acetonitrile; tetrahydrofuran (91:5:4 v/v) final pH 6.2. The fluorescence detector was set at excitation 310 nm and emission 390 nm.

Incubation tubes were 2 ml screw top glass vials with caps with prefitted liner (Chromacol, UK, Cat No. PN 2-SVW & PN 9-SC(B)-ST1).

Reaction tubes were incubated in a Luckham WB 150 waterbath modified with a plastic insert that allowed 50 tubes to be incubated. A Griffin dry block was modified with locally manufactured aluminium blocks with 13 mm diameter and 180 mm deep holes to contain the glass vials.

The haemoglobin content of lysates was determined on an ADVIA 600 analyser.

2.3. Sample collection, storage and RBC lysate preparation

Blood samples were collected into EDTA tubes and stored at 4 °C before analysis. The blood samples (4 ml) were centrifuged at 1400 × *g* for 5 min. The serum and the buffy coat were discarded and 1.5 ml physiological saline added to wash the cells. Samples were mixed vigorously for 5 min, then the erythrocytes centrifuged at 1400 × *g* for 5 min and the saline wash removed. The washed erythrocytes (0.5 ml) were suspended in 2 ml phosphate buffer (0.02 mmol/l, pH 7.4) and mixed for 30 s to lyse the cells. For paediatric and other small samples, volumes were scaled down.

Quality control samples for within-batch and between-batch imprecision studies were obtained from healthy volunteers and stored as washed cells at −20 °C.

2.4. Incubation and HPLC analysis

The stored incubation tubes containing the buffer and substrates were pre-warmed in the water bath at 37 °C for 5 min and 200 μl of lysate added to tubes at 15 s intervals. Tubes were incubated for exactly 1 h, then the reaction was stopped by transferring tubes to the heating block and incubating for 10 min at 85 °C. Samples were cooled in cold water and centrifuged at 1400 × *g* for 5 min. The incubation tubes were then transferred to the autosampler for anal-

ysis with the 50 μl loop being filled. The frit in the guard column was changed when the back-pressure rose by more than 40 psi. A single analytical column has been used for more than 2000 injections.

2.5. Standardisation and blank

A standard curve was shown to be linear over the range 0–2.2 nmol 6-MTG with the equation of the curve being $y = 0.58X + 6.92$. In routine use a 0.44 nmol 6-MTG (80 ng) single-point calibrant was used to standardise the assay. A stock standard was prepared with 10 mg 6-MTG in 4 ml 0.1 mol/l sodium hydroxide, which was then diluted to give the working standard.

A standard blank was prepared by adding 200 μl NaCl (0.9 g/l) to the incubate. A sample blank was prepared by adding 200 μl of lysate and then stopping the reaction at time zero.

3. Results and discussion

3.1. Optimising the concentration of substrates 6-TG and SAM

The V_{max} and K_m values for 6-TG and SAM were calculated from Michaelis–Menten curves. For 6-TG the K_m was determined as 193 μmol/l and the V_{max} as 61 nmol 6-MTG/g Hb/h, and for SAM the K_m was 19 μmol/l and V_{max} 74 nmol 6-MTG/g Hb/h. We chose final incubate concentrations of 600 μmol/l for 6-TG and 80 μmol/l for SAM.

3.2. Non-extraction HPLC validation

3.2.1. Protein precipitation using heat inactivation

The use of heat both to stop the enzyme reaction and precipitate proteins was found to be superior to more conventional approaches such as adding solvents or perchloric acid. Not only does this produce a clear supernatant, but major changes in the solvent front due to additional acid or solvent in the sample is avoided. We have observed that 6-MTG is stable at 85 °C for 20 min, twice the duration of the heat inactivation step. The protein content of the supernatant after heating at 85 °C for 10 min and centrifugation fell to approximately 7% of the levels prior to heat treatment. For example, in a representative sample the incubate protein concentration was 1.98 g/l falling to 0.13 g/l after heating and centrifuging.

3.2.2. HPLC characteristics

Representative traces for a standard, blank and test incubate are shown in Fig. 1. There is a small amount of non-enzymatic 6-MTG produced in the incubate, and this is accounted for by use of a reagent blank, which is deducted from both the standard and test peaks.

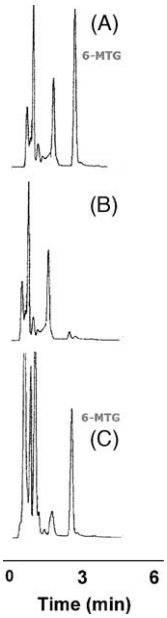

Fig. 1. High-performance liquid chromatography of 6-methyl thioguanine. (A) 2.2 nmol/ml 6-MTG standard (equivalent to 0.44 nmol 6-MTG produced). (B) Reaction blank. (C) An individual with an activity of 42 nmol 6-MTG/g Hb/h.

3.2.3. Response linearity and limit of detection

The detector response was linear to a series of 6-MTG standards in the range 0–1.5 nmol 6-MTG produced. The limit of detection was given as 5 nmol 6-MTG/g Hb/h this being twice the value that the blank gave when calculated as a test, though in practice lower values can be expressed by removing the blank value from a low test.

3.2.4. Accuracy and comparison with extraction method

The accuracy of the non-extraction 6-MTG measurement and comparison to the solvent extraction method according to Kroplin and co-workers [13,14] was assessed by means of recovery studies at three levels. Known concentrations of the product 6-MTG were added to a lysate preparation at three different levels, and analysis performed using both methods. The results, presented in Table 1, show that the direct method has a better precision and accuracy profile compared to solvent extraction.

3.2.5. Method imprecision

The imprecision of the method was determined at two levels by preparing incubates at normal, and low TPMT

Table 1
Recovery of 6-methyl thiopurine using conventional solvent extraction and the direct measuring technique

Method	Direct measurement			Solvent extraction		
Spiked value (nmol)	0.110	0.055	0.027	0.110	0.055	0.027
Mean recovery	0.109	0.056	0.031	0.109	0.045	0.031
CV (%)	1.0	0.7	2.5	3.5	20.3	29.0
n	5	5	5	5	5	5

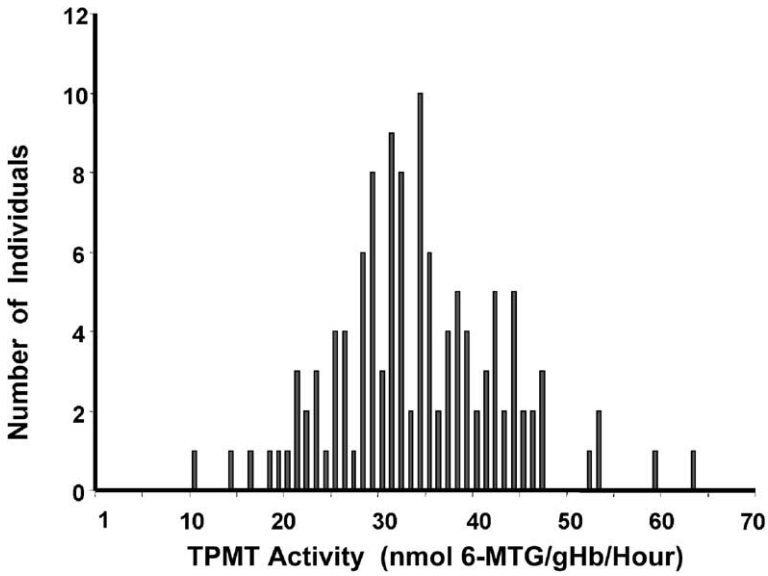

Fig. 2. Distribution of TPMT activity in 120 individuals.

Table 2
Within-batch and between-batch imprecision of the TPMT non-extraction assay

	Mean TPMT activity (nmol 6-MTG/g Hb/h)	CV (%)	*n*
Within-batch imprecision			
Level I	12.6	5.1	10
Level II	41.5	2.9	10
Between-batch imprecision			
Level I	11.1	8.2	11
Level II	41.0	7.3	16

activities, with the activities all calculated using the undiluted lysate hemoglobin concentration and the results are presented in Table 2.

3.2.6. Patient samples

The method was used to determine TPMT activity in erythrocytes in an initial population study. The distribution of enzyme activity in 120 individuals is presented in Fig. 2. Sample blanks showed no underlying peaks at the same retention time as 6-MTG in the 120 patients being studied, and also confirmed that heating leads to complete inactivation of enzyme activity in the lysate preparation.

4. Conclusion

A non-extraction isocratic HPLC method has been developed for the determination of 6-MTG as part of the assessment of TPMT activity in erythrocytes. Emphasis has been placed on developing an integrated assay, which is robust and suitable for use in the routine clinical laboratory. Sample-handling steps have been minimised. The addition of lysate to a reaction vial containing all other components of the incubate is the only liquid-handling step prior to the injection onto the chromatography system. The procedure is suited to further automation of the pipetting and incubation steps using suitable equipment.

The previously described method for TPMT analysis using 6-TG as substrate [13,14] requires an extraction step and, unusually, corrects for extraction losses only with external standardisation. The method presented here has removed the need for solvent extraction and all steps are undertaken in a sealed tube. The use of heat to stop the TPMT reaction and clean up the sample has also recently been described for the TPMT assay using mercaptopurine as substrate [15]. This gives excellent accuracy and imprecision characteristics and a method well suited to external standardistaion. Our initial population study of the distribution of TPMT activity is in broad agreement with that of Kroplin [13]. Further work is required to establish reference intervals in the population for this modification with 6-TG as substrate and a comprehensive prospective population study of phenotyping of TPMT activity with either 6-TG or the more conventional assay using mercaptopurine as substrate has yet to be reported [5]. In conclusion the present method is ideal for the routine analysis of TPMT activity in patients commencing on thiopurine drug therapy.

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