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Interference free and simplyfied liquid chromatography-based determination of thiopurine *S*-methyltransferase activity in erythrocytes

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Abstract

The determination of the thiopurine S-methyltransferase activity (TPMT; EC 2.1.1.67) has become an important issue during thiopurine therapy due to its known genetic polymorphism resulting in a wide range of TPMT activity. Therefore, the standard thiopurine drug regimen is associated with increased hematopoetic toxicity in patients with low or absent TPMT activity, whereas patients with high activity may be insufficiently treated. However, presently available methods are labour intensive and time consuming and tend towards too high or too low enzyme activity due to their methodological approach. The use of instable substrate solutions (6-MP or 6-TG), organic solvents like dimethyl sulfoxide and too high substrate and co-substrate saturation concentrations contribute to this phenomenon. We therefore, established an optimized and fast isocratic HPLC linked TPMT assay based on the enzymatic methylation of mercaptopurine or thioguanine in RBC lysates with *S*-adenosyl-L-methionine as methyl donor. Unspecific non-enzymatic methylation was not detectable. The recovery of 6-methyl-mercaptopurine was 97–102%, the intra- and interday variation between 1.0 and 5.0%, respectively. The assay dispenses with a time consuming extraction procedure with organic solvents, a heating step, and a gradient elution and is therefore, favourable for clinical routine application. The TPMT activity was measured in 62 untreated children with acute lymphoblastic leucemia at the time of diagnosis (activity = 34.0 ± 10.6 nmol/g Hb/h, range: 11.5–55.4 nmol/g Hb/h) and in 12 adult healthy volunteers (62.8 ± 7.7 nmol/g Hb/h, range: 48–82 nmol/g Hb/h) reflecting the wide measurable TPMT activity found in erythrocytes.

Keywords: Leukemia; Enzymes; Thiopourine *S*-methyltransferase; 6-Mercaptopurine; 6-Thioguanine

1. Introduction

The antimetabolites 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), analogues of guanine and hypoxanthine, have been used in the treatment of acute leukemia for almost 50 years. 6-MP became, for historical and not for scientific reasons, the drug of choice in the maintenance therapy of acute lymphoblastic leukemia (ALL), whereas 6-TG is preferred in acute myeloid leukemia (AML) [1]. On principle, the cytotoxic mechanism of both pro-drugs is based on incorporation in the DNA/RNA as thioguanine nucleotides [1–4]. The thiopurine *S*-methyltransferase (TPMT; EC 2.1.1.67) represents the key enzyme of the thiopurine degradation metabolism. An allelic polymorphism in the TPMT gene, however, causes wide individual differences in the activity of this enzyme: approximately 89 % Caucasians show high, 11% intermediate, and 1 of 300 is lacking TPMT activity [5–7]. Thus, the standard regimen of thiopurines results in a severe bone marrow depression with fatal outcome if low or absent TPMT activity is present, whereas patients with high activity may be insufficiently treated [8–12].

In recent years we have investigated the kinetics and metabolism of both, 6-MP and 6-TG, and provided extended evidence of a more direct catabolic mechanism of 6-TG resulting in a seven-fold higher thioguanine nucleotide (TGN)

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concentration leading to higher side-effects in comparison to 6-MP [14]. The higher rate of side-effects using 6-TG was confirmed in a large randomized trial by the *German co-operative ALL study group* (COALL-92) in which patients received either 6-TG or 6-MP during maintenance treatment. However, the event-free survival was equal in both groups after a median observation time of 6.6 years [15].

Enzyme activity assays are warranted to reduce adverse side effects and to optimise dosage regimens. There exist a number of non-radioactive labeled assays for quantitative analysis of the TPMT activity using a high-performance-liquid-chromatographic (HPLC) based assay, which was introduced first by Lennard and Jacqz-Aigrain in 1994, independently from each other [13,17–24]. However, despite the main advantage of dealing with non-radioactive agents handling and extraction still remains labour-intensive and time-consuming. Moreover, presently available methods tend towards too high or too low enzyme activities due to their methodological approach. Thus, the use of unstable substrate stock solutions (6-MP or 6-TG), organic solvents, i.e. dimethyl sulfoxide (DMSO), and too high substrate and/or co-substrate saturation concentrations contribute to this phenomenon. We therefore, established a novel high-sensitive HPLC based TPMT assay resulting in a higher accuracy in measuring enzyme activities. The described method renounce of time consuming extraction procedures with organic solvents, a heating step and gradient elution, making the assay easy and fast, and therefore, favourable for clinical routine application. For this propose, blood samples of 62 untreated children with ALL at the time of diagnosis were analyzed and compared with 12 adult healthy volunteers after establishment of the present TPMT assay where erythrocytes from banked donor blood were used. Moreover, we provide evidence of an interference free activity assay without a detectable non-enyzyme involved methylation (NEM), the determination and usage of an optimal substrate/co-substrate saturation concentration and pure compounds, respectively.

2. Experimental

2.1. Chemicals

All reagents used were of analytical grade except the chloride salt of S-adenosyl-L-methionine (SAM, 70%) and obtained from Merck (Merck, Darmstadt, GFR), if not otherwise indicated. SAM, 6-MP, 6-MMP (6-methyl-mercaptopurine), 6-TG and 6-MTG (6-methyl-thioguanine) were obtained from Sigma and Fluka in highest available purity (Sigma-Aldrich, Taufkirchen, GFR; Fluka, Taufkirchen, GFR). Purified water was produced with Purelab Plus (USF Seral, Ransbach, GFR). Phosphate buffer (pH 7.4) was prepared, according to Sörensen with 0.067 M Na_2HPO_4/KH_2PO_4 (81.8:18.2). Thiopurines are not soluble in water or buffer, the stock solutions of 6-MP and 6-TG (15 mM), 6-MMP (6.02 mM) and 6-MTG (5.52 mM) were prepared in 0.1 N NaOH. SAM (3.0 mM, corrected for purity) was dissolved in phosphate buffer. These stock solutions were aliquoted in 2.0 ml safe lock tubes (Eppendorf, Hamburg, GFR) and kept frozen (−21 °C) until use.

2.2. Sample source and preparation of the cell lysate

The present assay was established using erythrocytes from banked donor blood first. Thereafter, we analyzed 74 venous blood samples, 62 children with ALL and 12 healthy adult volunteers. Patients were recruited by the COALL group, a german multicenter study for the treatment of ALL. For morphological and immunological diagnosis and subtyping blood and bone marrow are sent to the central study lab in Hamburg. All enrolled children were untreated and none of the patients or healthy volunteers received banked donor blood prior to the blood sample. This investigation was performed in accordance with the ethical commitee of Hamburg and a written informed consent was obtained from the parents.

All samples were collected in EDTA tubes (Sarstedt, Nürnbrecht, GFR) and processed within 24 h. The red cell lysate for measuring TPMT activity was prepared in a standardized manner. In brief, erythrocytes were separated from EDTA blood by *Ficoll* separation or by centrifugation at 1000 g (Biofuge pico, Heraeus, Osterode, GFR) for 4 min at room temperature. Plasma and buffy coat were discarded. The red blood cells (RBC) were washed and centrifuged (1000 g) twice with isotonic saline (same volume as discarded). The washed and packed RBC were diluted with water (1:2.5), frozen for complete hemolysis and stored at −21 °C in 2 ml tubes until analysis. After thawing the RBC lysates were centrifuged for 4 min at 16,000 × *g* to remove cell debris. The supernatants were used to measure the TPMT activity and hemoglobin content (Celldyn 1600; Abbot, Wiesbaden, GFR).

2.3. TPMT activity assay

On principle the *S*-methylation of 6-MP and 6-TG is catalysed by TPMT, using SAM as methyl donor, resulting in the products 6-MMP and 6-MTG, respectively. In general quadruplicate measurements at a final volume of 500 μl were performed after an incubation period of 60 min at 37 °C using a dry-heating-block (Typ 50006101; Liebisch, Bielefeld, GFR). Twenty μl 0.1 M HCl were diluted with 315 μl 0.067 M phosphate buffer (pH 7.4) in a 1.5 ml safe lock tube and then 20 μl of the stock solution of 6-MP or 6-TG and of SAM were added (resulting in final concentrations of 600 μM (6-MP/6-TG) and 120 μM (SAM). The enzymatic reaction was started by adding 100 μl of the prepared RBC lysate. Following incubation the reaction was terminated by adding 25 μl 60% $HClO_4$. The remnant was centrifuged for 4 min at 13,000 × *g* after a protein precipitation of 3 min. Finally the clear supernatant was analysed by RP-HPLC without further pretreatment.

2.4. Chromatography

The RP-HPLC system (Thermo Electron, Egelsbach, GFR) consisted of a pump (P 200), an autosampler (AS 300) with Microvials (100 μl), a variable UV–vis detector (UV 2000) and an integrator (Chromjet), supplemented by a degasser system (DG 1310; Applica, Bremen, GFR).The separation was performed at room temperature on a reversed-phase column using Nucleosil 120, C18, particle size 3 μm (System Chromcard®, Macherey&Nagel, Düren, GFR). The analytical column (70 mm × 3 mm) was protected by a guard column (8 mm × 3 mm) containing the same phase. The flow rate was 0.5 ml/min, the injection volume 25 μl. The mobile phase consisted of 0.01 M sodium phosphate buffer adjusted to pH 2.7 with phosphoric acid (85%). Thereafter, 7.0% (determination of 6-MMP) or 3.5% (determination of 6-MTG) acetonitrile was added to the mobile phase. The thiopurines were detected at a wavelength of 290 nm for 6-MMP and 315 nm for 6-MTG. To determine substrates and co-substrate acetonitrile was omitted, the detection was performed at 322 (6-MP), 342 (6-TG) and 254 nm (SAM). The calibration standards (one-point-calibration) were diluted from the stock solutions with incubation medium supplemented with $HClO_4$, the final concentrations were 3.0 μM (6-MMP), 5.5 μM (6-MTG) and 0.5 M ($HClO_4$). This HPLC equipment including column is identical with this one we use for the determination of thiopurine metabolites [16], simply the percentage of acetonitrile must be changed.

2.5. Validation and optimization of the TPMT activity assay

We validated the described TPMT activity assay by vary different assay conditions. Some commercially available substrate reagents (Table 1) were investigated for chromatographic purity and enzymatic activity under optimal assay conditions. The linearity and proportionality of the enzymatic reaction regarding the incubation time was measured at 6 different incubation times (0–180 min). Five RBC lysate volumes of one sample (0, 25, 50, 100, and 200 μl) were taken resulting in different enzyme concentrations in order to describe the linearity and proportionality of the methylation reaction of 6-MP and 6-TG to 6-MMP and 6-MTG, respectively. Furthermore the incubation process was optimized by varying buffer concentration (0.017–0.067 M) and pH (7.2–7.8). The recovery of the reaction products was measured by adding 6-MMP in various concentrations (0.0, 0.221, 0.110, and 0.022 μM) to different erythrocyte lysate concentrations (Hb: 3.4, 6.7, and 13.5 g/dl). Finally, the intraday and interday assay stability was measured, processing frozen aliquots of three pooled erythrocyte lysates showing different activities ten times (intraday assay stability) and of one of these samples twelve times (interday assay stability) during a period of tree months, respectively.

2.6. Michaelis constants, non-specific methylation (NEM) kinetic and statistical analysis

Fourteen different concentrations were used to determine the saturation curves for both, substrate (6-MP: 0–2000 μM at a fixed SAM concentration of 600 μM) and co-substrate (SAM: 0–600 μM at a fixed 6-MP concentration of 2000 μM). For this purpose the usual incubation period of 60 min was doubled in order to receive detectable peaks above the baseline noise. Corresponding blank controls without erythrocytes were prepared to measure possible non-specific methylation, the saturation curves were the differences between whole and non-specific methylation. Enzyme kinetics analysis of K_M and V_{max} values were estimated by fitting double reciprocal regression (*Lineweaver-Burk Method*) using *Statview*, (Statview, version 5.0.1, SAS Institute Inc. Cary, NC, USA). All other figures used *Software for Scientific Graphing* (Origin, version 6.1, Origin Lab, Northampton, MA, USA). Quadruplicate measurements were carried out and results expressed as mean ± S.D., if not otherwise indicated; patient samples were analyzed in duplicate.

3. Results

3.1. Substrate-, co-substrate concentrations and non-specific methylation

The final saturation concentration of 6-MP and SAM was determined to be 600 μM for the substrate and 120 μM for the co-substrate, respectively (Fig. 1a–b). The calculation of K_M and V_{max} resulted in 72.71 μmol/l and 31.14 μmol/g Hb/h for 6-MP and 5.47 μmol/l and 28.54 μmol/g Hb/h

Table 1
Purity and activity of different lots of 6-MP and 6-TG[a]

No.	Substrate	Source	Lot	Interfering impurity	Activity [nmol/g Hb/h]
1	6-MP	Sigma M 7000	128 H 1145	None	49.7
2	6-MP	Sigma M 7000	45 F 044H	272 pmol/ml	57.0
3	6-MP	Fluka 377872/1	62100	None	48.5
4	6-TG	Sigma A 4882	119 F 4024	None	8.9
5	6-TG	Sigma A 4882	117 H 0599	113 pmol/ml	44.3
6	6-TG	Fluka 269521/1	133599	None	58.7

[a] The impurities were determined in solutions of different lots of both substrates (concentration and medium as used in the assay). All activities were measured in the same erythrocyte lysate.

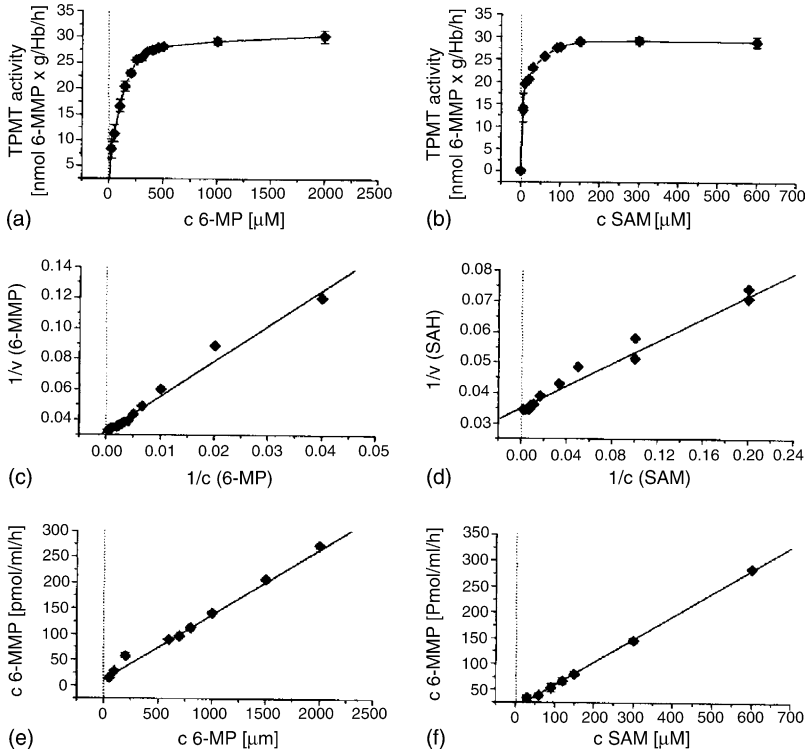

Fig. 1. TPMT assay kinetic. (a and b): Substrate (*a*) and co-substrate (*b*) saturation curves using 0–2000 μM 6-MP at a constant co-substrate concentration of 600 μM and 0–600 μM SAM at a constant substrate concentration of 2000 μM, respectively. The saturation concentration was reached at a substrate concentration of 600 μM and a co-substrate concentration of 120 μM; (c and d): double reciprocal plots of the substrate (*c*) and co-substrate (*d*) saturation curves for determination of K_M and V_{max}; (e and f): linear description of the substrate (*e*) and co-substrate (*f*) NEM blank curves corresponding to (a and b). The co-substrate concentration was fixed at 600 μM and the substrate concentration varied from 0–2000 μM (e), the substrate concentration was fixed at 2000 μM and the co-substrate concentration varied from 0–600 μM (f). The usually used incubation period was doubled to 120 min in order to receive detectable peaks above the baseline noise.

for SAM (Fig. 1c–d). No non-specific methylation was detectable at a substrate/co-substrate concentration of 600 μM 6-MP/120 μM SAM, respectively. A linear increase of non-specific methylation was observed when higher concentrations of substrate and co-substrate or a longer incubation time (>1 h) were used (Fig. 1e–f).

3.2. Enzymatic reaction

The enzyme reaction, using 6-MP as substrate, was linear up to 300 min and dependent on the used RBC lysate volume: (25 μl) $y = 0.77x^2 + 33.90x - 17.28$, $r^2 = 0.99$; (50 μl) $y = -0.33x^2 + 91.17x - 76.23$, $r^2 = 1.00$; (100 μl) $y = -1.66x^2 + 195.37x - 170.69$, $r^2 = 1.00$; (200 μl) $y = 0.64x^2 + 329.23x - 307.32$, $r^2 = 1.00$. The rate of 6-MMP formation remained constant within the incubation period of 300 min and an RBC lysate volume of 0–200 μl (Hb 4.0 g/dl). The pH optimum of the assay was pH 7.4. However, only minor variation of the TPMT activity were detected between pH 7.2 and pH 7.8 (33.7–35.1 nmol/g Hb/h). The optimal buffer concentration was 0.067 M, lower buffer concentrations resulted in a reduction of up to 9.0% detectable TPMT activity. After acid precipitation probes were found to be stable at room temperature for several hours (0, 18, and 36 h; maximum C.V. = 5.0%) and at −21 °C at least for 3 months (C.V. = 5.0%).

3.3. Chromatography

The total run-time for separation of 6-MMP and 6-MTG from stable supernatants to determine the TPMT activity was only 8.0 min (retention time 6.0 min) and 10.0 min (retention time 7.3 min), respectively. A high-quality separation of the compounds was obtained without underlying peaks at the same retention time as 6-MMP or 6-MTG, as demonstrated in Fig. 2a–c and Fig. 3a–c, respectively. Analysing samples of patients during treatment, the presence of interfering peaks in blanks (RBC incubated without substrate/co-substrate) has to be proved. The main thiopurine metabolites in erythrocytes, however, are methylated and unmethylated nucleotides, which do not coelute with the free bases, the concentration of 6-MMP and 6-MTG is negligible.The limit of detection (signal to noise ratio of 3) was 5 pmol in an injection volume of 25 μl for both, 6-MMP and 6-MTG. The concentration after incubation and extraction of a sample with an activity of 18 nmol 6-MMP/g Hb/h was approximately 3fold higher, the limit of quantitation therefore, is sufficient to identify patients even with very low TPMT activity.

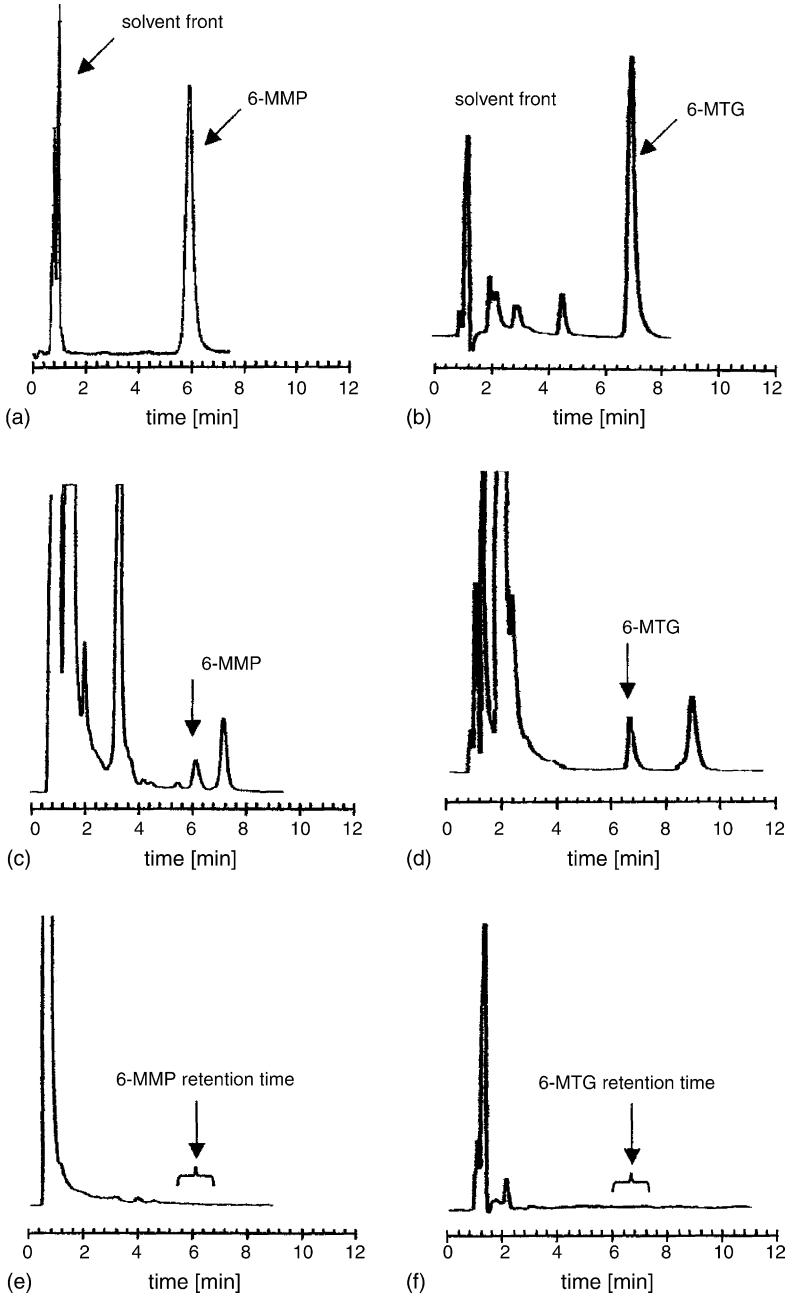

Fig. 2. Chromatographic separation of 6-MMP (a–c) and 6-MTG (d–f) in erythrocyte lysates. Chromatographic conditions: Nucleosil 120–3, C18, 70 mm × 3 mm with guard cartridge. The injection volume was 25 μl and the flow rate adjusted to 0.5 ml/min. The mobile phase consisted of phosphate buffer pH 2.7 with 7% (separation of 6-MMP) and 3.5% acetonitrile (separation of 6-MTG), respectively. 6-MMP was measured at 290 nm and 6-MTG at 315 nm; (a and d): calibration standards of 6-MMP (3.0 μM) and 6-MTG (5.5 μM) in assay buffer supplemented by $HClO_4$ (0.5 μM). The retention time was 6.0 min, the total run time 8.0 min for 6-MMP, and 7.3 and 11.0 min for 6-MTG, respectively. (b and e): Perchloric extract of an erythrocyte lysate, incubated with 6-MP (b) or 6-TG (d) and SAM for 1 h at 37 °C. Substrate and co-substrate are eluted within 2 min and cannot determined under these conditions; (c and f): Perchloric extract of a blank erythrocyte lysate (without substrate/co-substrate) after incubation for 1 h at 37 °C.

Substrates and SAM are eluted within the first 2 min and cannot be quantified under these conditions (c.f. 2.4).

3.4. Compound purity, activity and stability

All tested substrates are specified in Table 1. In our hands, samples No.1, 3 and 4 were the best choice under the described conditions, because they did not show any impurity coeluting with the corresponding methylated product and therefore, no falsification of the result towards higher levels. The concentration of the detected impurity in one sample of 6-MP (No 2) corresponded with 272 pmol/ml 6-MMP, and in one sample of 6-TG (No. 5) with 113 pmol/ml 6-MTG at the assay concentration of 600 μM. The measured enzyme

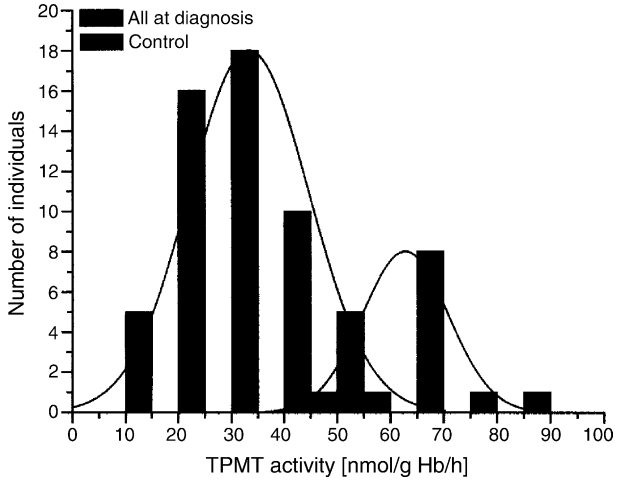

Fig. 3. TPMT activitiy in erythrocytes. Distribution of TPMT activity in 74 individuals, 66 children with ALL before onset of treatment and 12 adult healthy volunteers.

activities using different lots of 6-TG differed widely, the activity of lot No. 4 was up to 85% lower compared to the others. The stock solutions were stable and soluble for several months whereas less stability and precipitation occurred when the stock solution was neutralised with HCl (0.1 M) following the dissolving step.

3.5. Assay recovery and stability

The assay recovery for 6-MMP and 6-MTG was 97–102%. The assay was tested for stability by 10 measurements of three different samples within 1 day (intraday assay stability) and 12 analyses of the same a sample over a total period of 3 months (interday assay stability). Thereby the assay demonstrated an excellent stability: 113 nmol 6-MMP/g Hb/h (±4.1%); 49.6 (±1.4%) and 18 (±4.9%) for the intraday assays and 49.8 (±1%) for the interday assay.

3.6. Analysis of TPMT activity

The present assay was used to analyse the TPMT activity in isolated erythrocytes in a total of 74 individuals. The untreated patients with ALL ($n = 62$) were 7.2 ± 4.6 years old compared to 36.8 ± 15.6 years in the adult reference group with a mean activity of 33.3 ± 11.2 (range 11.4–55.4) and 62.8 ± 7.7 (48.0–82.0) nmol/g/Hb/h, respectively (Fig. 3).

4. Discussion

Quantitative measurement of TPMT activity is recommended to ensure an optimal treatment with thiopurines [5,8,10–29]. However, presently available methods have shown to be time consuming, labour intensive, probably do not ascertain the real TPMT activity, and therefore, are not suitable for clinical routine [13,17–24]. By deproteinization with perchloric acid, our optimized method renounces of both, the extraction of the methylated products with organic solvents and an heating step for stopping the enzymatic reaction. Extraction might be responsible for low recovery rates of the methylated products 6-MMP or 6-MTG, and the heating step may contribute to the non-enzymatic methylation reported in the literature [13,17–22]. Additionally, it is possible to inject the supernatant directly into the HPLC column without any further adjustment of the solution. It is stable at room temperature making this assay favourable for autosampling in clinical routine. The HPLC based assay uses an isocratic run with a simple mobile phase, containing phosphate buffer and small amounts of ACN, compared to the literature [13,19–22]. This elution process leads to a fast and high quality separation and therefore, to a significant decrease in the total run time of the probes. An oxidation process of the substrate was not observed under assay conditions (data not shown) and therefore, the addition of Dithiothreitol [17–20,22] was not necessary.

The substrate and co-substrate concentration used in the present assay are similar to those given by several groups [13,17,21,23], others are using much more higher substrate concentrations [18,20,22,24]. The neutralisation of substrates dissolved in NaOH directly with acid led to the precipitation of the substrates and therefore, to a lower concentration in solution and consequently a possible fall below of the needed saturation concentration. Kröplin et al. observed this phenomenon 2 h after preparation of the stock solution [13]. However, we detected a precipitation already within minutes, and therefore, left this way of neutralisation. The disadvantage of DMSO as a solvent is its methylating property resulting in an increase of measurable reaction products [17]. As we and Kröplin [13,21] have shown, non-enzymatic methylation furthermore can occur if the concentration of substrate and/or co-substrate is chosen too high. The calculated enzyme kinetics analysis of K_M and V_{max} for the present assay were found to be close to those in literature [13,17, 21,23].

In the recent years 6-MP or 6-TG have been used as substrates to determine the TPMT activity by different authors [13,17–23]. We have compared both substrates and decided to use 6-MP (proved for interfering peaks) instead of 6-TG for routine use based on a lower retention and total run time. Moreover, tremendous differences in measurable TPMT activities were found between different lots of 6-TG. This also was observed in an earlier investigation, the responsible impurities, however, are not yet identified [21].

A total of 74 probes were analysed after establishing the present assay reflecting once again the wide range of TPMT activity as previously described [5–7,10,24]. The TPMT activity was found to be higher in the adults compared to the untreated children with ALL. Our control group, however, was very small. Whether or not the TPMT activity indeed relates to age remains to be clarified [5,20,30,31]. Brouwer et al. recently demonstrated that children with other diseases show higher TPMT activities than leucemic children [32]. They

also observed lower activities before antileucemic treatment compared with those measured thereafter. This may be the result of the different age of the erythrocytes [33].

In conclusion, the present assay for quantitative measurements of TPMT activity is time saving in comparison to previously described methods, easy to apply due to the elimination of a number of assay steps and suitable for routine analysis. Moreover, the enzyme activity is measurable more precisely without accepting any summation and or subtraction effect caused by non-enzymatic methylation, substrate precipitation or the use of impure substrate compounds, respectively. We are investigating now TPMT activities in larger groups, it also remains to be proved, whether different antileucemic drugs can interfere with measurements or influence enzyme activities.

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