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Determination of thiopurine *S*-methyltransferase (TPMT) activity by comparing various normalization factors: Reference values for Estonian population using HPLC-UV assay

Kersti Oselin [a,*], Kaili Anier [a,c], Riin Tamm [b], Kristi Kallassalu [b], Uno Mäeorg [c]

[a] *Department of Pharmacology, Tartu University, 51014 Tartu, Estonia*
[b] *Institute of Cell and Molecular Biology, Tartu University, 51014 Tartu, Estonia*
[c] *Institute of Organic and Bioorganic Chemistry, Tartu University, 51014 Tartu, Estonia*

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Abstract

Thiopurine *S*-methyltransferase (TPMT; EC 2.1.1.67) is the key enzyme in the metabolism of thiopurine drugs. Determination of TPMT activity has been used for the individualization of thiopurine dose. We developed HPLC-UV assay for the determination of TPMT activity in human erythrocytes using 6-mercaptopurine as a substrate. Various extraction and chromatographic conditions were compared. In-house developed extraction with acetonitrile provided the lowest limit of quantification. TPMT activity was determined in 99 previously genotyped healthy Estonians. TPMT activity was expressed as the formation of 6-methylmercaptopurine ng/ml/h and normalized either to haemoglobin, haematocrit, erythrocyte count or protein content. The receiver-operating characteristic curve analysis revealed similar accuracy values for TPMT activity in predicting heterozygous and wild type individuals for each method of calculation. In healthy Estonians, TPMT activity varied from 21.5 to 129.6 ng/ml/h. For heterozygous individuals ($n = 18$), TPMT activity was 48.1 ± 11.7 ng/ml/h. Wild type individuals ($n = 81$) revealed significantly higher TPMT activity 79.3 ± 20.7 ng/ml/h ($P < 0.001$). This sensitive HPLC assay for quantitative determination of TPMT activity could easily be used in clinical settings. Under constant experimental conditions for haemolysate preparation no normalization is required.

Keywords: Thiopurine *S*-methyltransferase; 6-Mercaptopurine; Normalization

1. Introduction

Thiopurine *S*-methyltransferase (TPMT, EC 2.1.1.67) is a cytosolic enzyme involved in methylation of aromatic and heterocyclic sulphydryl compounds such as thiopurine drugs azathioprine, 6-mercaptopurine (6-MP) and 6-thioguanine [1]. Previous studies have shown that intracellular formation of thioguanine nucleotides has a major role in the efficacy and toxicity of thiopurines [2]. Alternatively, metabolic conversation of thiopurines via TPMT leads to the formation of inactive methylated metabolites. Several studies have shown negative correlation between erythrocyte thioguanine nucleotide level and TPMT activity. Individuals with decreased TPMT activity have higher risk for side effects when treated with standard doses of thiopurines. Determination of erythrocyte TPMT activity instead of parent compound or active metabolite serum concentration has been used for the individualization of thiopurine dose [3].

First method introduced for TPMT activity was based on the enzyme catalysed transfer of the radioactive ^{14}C-labelled methyl group of S-adenosyl-L-methionine (SAM) to the thiol group of 6-MP [4]. One unit (U) of TPMT activity was defined as a formation of 1 nmol of 6-methylmercaptopurine (6-MMP) per ml of packed red blood cells per 60 min incubation at 37 °C. Using the radioactive method, Weinshilboum et al. found trimodal distribution for TPMT activity among Caucasians [5]. 11.1% of individuals expressed intermediate enzyme activity of 1–9.5 U. 88.6% of individuals had high enzyme activity of >9.5 U, and 1 out of 300 had no active enzyme.

In 1994, Lennard et al. and Jacqz-Aigrain et al. introduced first non-radioactive HPLC methods for the determination of

* Corresponding author. Tel.: +372 737 4354; fax: +372 737 4352.
E-mail address: kersti.oselin@ut.ee (K. Oselin).

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in vitro formed 6-MMP by UV detection [6,7]. Several HPLC-based methods with different cut-off values for high, intermediate and low enzyme activity have been published so far [1,5–11]. Since generally accepted reference values do not exist for TPMT activity, the reference values for every laboratory should be established before TPMT activity measurements are introduced into routine clinical practice. Regardless of radioactive- or HPLC-based method used, formation of methylated metabolite in erythrocytes has been normalized. In addition to ml of packed red blood cells as supposed in original work by Weinshilboum et al. [4], normalization per gram of haemoglobin, per count of erythrocytes and per gram of protein have been used by others [1,7,8,11–17]. No studies have been performed to compare results for TPMT activity obtained using different normalization factors.

We have developed and validated a HPLC method with UV detection for the determination of TPMT activity in human erythrocytes using 6-MP as a substrate. In HPLC assays, the product of the enzymatic reaction is extracted by a liquid–liquid or liquid–solid extraction. We compared different procedures for sample preparation published previously for TPMT assays and found best results for our in-house developed extraction with acetonitrile. We analyzed TPMT activity in previously genotyped 99 healthy Estonian volunteers and performed phenotype–genotype correlations when different normalization factors were used. Genetic variability in the TPMT gene with wide ethnic differences is responsible for interindividual variations in TPMT activity [18]. Here, we present reference values of TPMT activity for Estonian population.

2. Experimental

2.1. Chemicals

6-Mercaptopurine (6-MP), 6-methylmercaptopurine (6-MMP), S-adenosyl-L-methionine (SAM) and dimethylsulfoxide (DMSO) were obtained from Sigma (MO, USA). Dithiothreitol (DTT) was from Boehringer Ingelheim (Heidelberg, Germany). Sodium phosphate monobasic dihydrate was from Janssen Chimica (Geel, Belgium). Seventy percent perchloric acid was from YA Kemira (Helsinki, Finland). Acetonitrile (ACN) and methanol were obtained from Rathburn Chemicals (Walkerburn, Scotland). Chloroform, 2-propanol and ethanol were from Fluka (Buchs, Switzerland). ACN and methanol were of HPLC grade, all other chemicals were of analytical grade. The Bio-Rad *DC* protein assay kit was from Bio-Rad Laboratories (CA, USA). PUREGENE® Genomic DNA Purification Kit was obtained from Gentra Systems (MN, USA).

6-MP solution was prepared immediately prior to use by dissolving 6-MP in 1 M NaOH and neutralized by the same volume of 1 M HCl. DTT and SAM were dissolved in distilled water. A stock solution of 6-MMP was prepared by dissolving 5.7 mg of 6-MMP in 1.14 ml 0.1 M NaOH and then diluting with 8.86 ml of distilled water to a final concentration of 0.5 mg/ml and stored at −80 °C.

2.2. Erythrocyte preparation and incubation conditions

The study was approved by the Ethics Review Committee on Human Research of the University of Tartu. Two hundred and fifty-three healthy blood donors from the Blood Centre of Tartu University Clinics were asked to participate in the study. After an informed consent, 7 ml of whole blood was collected in lithium heparin tubes. Blood was processed within 8 h of sampling. Plasma and leukocytes were removed after centrifugation at 3200 × *g* for 3 min and erythrocytes were washed twice with two volumes of PBS buffer. For the determination of haemoglobin, haematocrit and erythrocyte count 100 μl of red blood cells was resuspended in 100 μl of PBS buffer. One millilitre of erythrocyte suspension was transferred to another tube and lysed with 4 ml of distilled water. Samples were centrifuged at 13,000 × *g* for 10 min and supernatant was kept at −80 °C until analysis. TPMT activity has been shown to be stable in these storage conditions [1]. Haemoglobin, erythrocyte count and haemotocrit was determined by the United Laboratory of the Tartu University Clinics using assays applied for routine clinical analyses. Protein content in haemolysates was determined by the Bio-Rad *DC* protein kit according to the manufacturers protocol using bovine serum albumin as a standard.

Erythrocyte TPMT activity was determined in vitro using 6-MP as a substrate. Samples were incubated at 37 °C for 60 min in a total volume of 400 μl. Samples consisted of 125 μl of 0.1 M NaH_2PO_4 buffer (pH 7.4), 50 μl of 6-MP (final concentration 1 mM), 25 μl of SAM (40 μM) and DTT (1 mM) blend. The reaction was started by adding 200 μl of haemolysate.

2.3. Conditions for sample preparation

To find suitable method for sample preparation, we compared different extraction methods published previously in the literature with our in-house developed methods.

Extraction with chloroform–2-propanol was performed as described by Kröplin and Medard [10,19]. Briefly, the reaction was stopped by adding 1 ml of chloroform–2-propanol (90:10, v/v) or 5 ml of chloroform–2-propanol (80:20, v/v). After centrifugation, the organic phase was evaporated to dryness under the stream of air.

Extraction with 70% perchloric acid was performed according to Dervieux [17] and Khalil [8] with minor modifications. After incubation, reaction was stopped by adding 100 μl of 70% perchloric acid. After centrifugation at 13,000 × *g* for 10 min, supernatant was collected and 40 μl injected into HPLC system.

Extraction via sample heating has been performed by several authors [11,14]. We stopped the reaction by heating samples for 3 min at 120 °C. After cooling, samples were centrifuged at 2000 × *g* for 15 min and supernatant was analyzed.

Extraction with ethanol was performed by adding 200 μl of absolute ethanol to incubated samples [1]. After 10 min on ice, 355 μl solution of methanol–0.1 M HCl (20:80, v/v) was added, samples were centrifuged at 5000 × *g* for 5 min at 4 °C and 40 μl of supernatant was injected into HPLC system.

In-house developed extraction with methanol was performed by adding equal volume of methanol to a total volume of incu-

bated sample. After centrifugation at 4000 × *g* for 10 min at 4 °C, 40 μl of supernatant was injected into HPLC system.

In-house developed extraction with ACN was performed by adding 2 ml of ACN to incubated samples. Samples were vortexed and kept at −20 °C for at least 30 min. Separation of the two layers occurred at −20 °C. Upper organic layer (∼2 ml) was removed immediately and transferred to another tube. After thawing, aqueous layer was extracted again with 1 ml of ACN as described above. The collected organic layers were added and evaporated to dryness under the stream of air. The residue was reconstituted with 100 μl of mobile phase and 40 μl injected into HPLC system.

In several HPLC-based methods for TPMT activity determination, after sample preparation with extracting solvent organic phase has been evaporated to dryness under nitrogen [9,10,12,19]. To study 6-MMP recovery when exposed to oxygen, we compared evaporation under air stream with that obtained under mixture of gases (85% N_2: 10% H_2: 5% CO_2).

2.4. HPLC apparatus and chromatographic conditions

The HPLC system consisted of Waters™ 717 plus Autosampler (Waters Millipore, USA), Alltech 426 HPLC pump (Alltech Associates Inc., USA) and Waters 486 Tunable absorbance detector (Waters Millipore, USA). The analytical column was an Inertsil® ODS-3 C_{18} (150 mm × 4.6 mm I.D.), 5 μm particle size (GL Science Inc., Japan). It was protected by a Platinum C_{18} (7.5 mm × 4.6 mm I.D.), 5 μm particle size precolumn (Alltech GmbH, USA). Chromatography software Kromex, ver 32S (Akrom-EX, Estonia) was used for data acquiring. Samples were analyzed at a flow rate 1.3 ml/min in isocratical elution with a mobile phase of 0.04 M phosphate buffer–methanol (80:20, v/v), pH 7.9. 6-MMP adsorbance was detected at 290 nm.

2.5. Validation of the TPMT activity assay

TPMT activity was studied under various incubation, extraction and chromatographic conditions. The linearity of the enzyme reaction was studied at different time points from 0 to 120 min, and at fixed time the amount of lysate was varied from 0 to 300 μl. For the determination of Michaelis–Menten constants, incubations with various concentrations of 6-MP (0–1.2 mM, fixed SAM of 25 μM) and SAM (0–50 μM, fixed 6-MP of 0.6 mM) were performed. The validation of the analytical part was performed according to the FDA Guidance for Industry – Bioanalytical Method Validation [20]. Standard curves of 6-MMP (0–250 ng/ml, eight samples) were prepared by spiking haemolysate with appropriate dilutions of the stock solution. SAM, DTT and buffer were added as in incubated samples. These spiked samples were extracted in parallel with incubated samples.

2.6. DNA isolation and TPMT genotyping

Three hundred microlitres of whole blood was used for DNA isolation. DNA was isolated by the PUREGENE® Genomic DNA Purification Kit according to the manufacturers protocol. Genetic variants TPMT*2, *3A, *3B, *3C, *3D and *8 were detected with PCR-RFLP (paper in preparation).

2.7. Statistical analysis

TPMT activity was expressed as a formation of 6-MMP ng/ml after 60 min incubation at 37 °C. Formation of 6-MMP was normalized per gram of haemoglobin (Hb), per ml of packed red blood cells (PRBC), per 10^8 erythrocytes (10^8 RBC) and per gram of protein in haemolysates.

The apparent Michaelis–Menten constants K_m and V_{max} were estimated by fitting non-linear regression curves using the computer program GraphPadPrism, ver 4.0 (GraphPad Software, CA, USA).

Results are expressed as mean ± S.D. Comparisons of means were made by Mann–Whitney test, with a threshold of $P = 0.05$. A receiver-operating characteristic (ROC) curve analysis was used to estimate the cut-off point segregating between intermediate and high TPMT activity using the StatsDirect, ver 2.3.4 (StatsDirect, Cheshire, UK). Shapiro–Wilk *W* test was used to test for normality using the StatsDirect software.

3. Results

3.1. Extraction and chromatographic conditions

In-house developed extraction with ACN provided increased method sensitivity. Several parameters characterizing the quality of chromatographic separation were calculated for 6-MMP peak obtained after various extraction conditions (Table 1). 6-MMP peak was considerably higher, well separated and symmetric when different extraction methods were compared.

6-MMP recovery was determined when exposed to evaporation under the air stream and under the mixture of gases. No difference in 6-MMP recovery was determined, 83 ± 3 and

Table 1
Comparison of various extraction methods of sample preparation for TPMT activity assay

Parameter[a]	ACN	Chloroform–2-propanol (90:10, v/v)	Chloroform–2-propanol (80:20, v/v)	Perchloric acid (70 vol.%)	Heating	Ethanol	Methanol
Capacity factor (k')	6.92	9.54	9.22	10.25	7.40	8.10	9.12
Column efficiency (N)	4798	3042	3210	2804	2443	1274	2931
Asymmetry factor (A_s)	1.33	1.50	1.50	1.33	1.30	2.00	1.50
Resolution (R_s)	5.15	2.50	2.35	2.50	1.62	1.76	2.67
Selectivity (α)	1.45	1.23	1.24	1.22	1.17	1.23	1.24

[a] The parameters characterizing the quality of chromatographic separation were calculated as following [23].

82 ± 4% over the calibration curve, respectively. Since 6-MMP was not oxidized when exposed to air, in all further experiments organic phase was evaporated to dryness under the stream of air.

3.2. Assay validation

3.2.1. Calibration curve

The calibration plots of peak areas versus concentrations were linear with correlation coefficients >0.99 over the range of 6-MMP concentrations 3.91–250 ng/ml. Limit of quantification was the lowest concentration in the calibration curve (3.91 ng/ml) with acceptable accuracy 102% and precision 7% (Fig. 1A). Accuracy and precision were determined as a variation of back-calculated concentration at each concentration level.

3.2.2. 6-MMP stability

The 6-MMP stock solution (0.5 mg/ml) remained stable for 12 months at −80 °C (R.S.D. = 7.5%, $n = 12$). 6-MMP stability in mobile phase after sample preparation with ACN was studied by comparing samples analyzed immediately after preparation and after storage. 6-MMP was stable at room temperature for 24 h (8% change in peak areas, $n = 2$). In thawed samples analyzed after the storage at −80 °C for 24 h, no sample decomposition was observed (5% change, $n = 2$). Samples with poor chromatograms could be reanalyzed in next day or after storage at −80 °C.

3.2.3. Recovery

Recovery was determined by comparing 6-MMP peak areas over the calibration curve for aqueous solutions (100%) and spiked plasma samples after extraction. First, liquid–liquid extraction with 1 ml ACN was used. The assay recovery for 6-MMP varied from 50% at higher to 94% at lower concentrations. This indicated that at higher concentrations, 1 ml ACN was not sufficient for sample extraction. ACN volume was increased to 2 ml and after the organic layer was collected, the residue was extracted again with 1 ml ACN. 2 + 1 ml extraction with ACN gave the mean ± S.D. recovery for 6-MMP 83 ± 3% with R.S.D. 4% over the calibration curve in duplicates.

3.2.4. Kinetics of TPMT activity in red blood cell lysates

6-MMP formation was linear in respect to the lysate concentration 0–300 μl and incubation time 0–120 min. Further experiments were performed with 200 μl of haemolysate and 60 min incubation at 37 °C as indicated in previous studies.

The apparent Michaelis–Menten constants for 6-MP and SAM were estimated from three experiments using the Lineweaver–Burk plot. For 6-MP, the K_m was 0.4 ± 0.01 mM (R.S.D. = 3%) and the V_{max} was 178.3 ± 3.1 mM/h (R.S.D. = 2%). For SAM, the K_m was 11.4 ± 0.8 μM (R.S.D. = 7%) and V_{max} 162.3 ± 4.3 μM/h (R.S.D. = 3%). Further experiments were performed in a final incubate concentration of 1.0 mM 6-MP and 40 μM SAM.

6-MP in a concentration higher than 2.4 mM was not soluble in 1 M NaOH/HCl mixture. The organic solvent DMSO has been frequently used to produce high-concentration solutions of 6-MP. When 6-MP was dissolved in DMSO, samples incubated

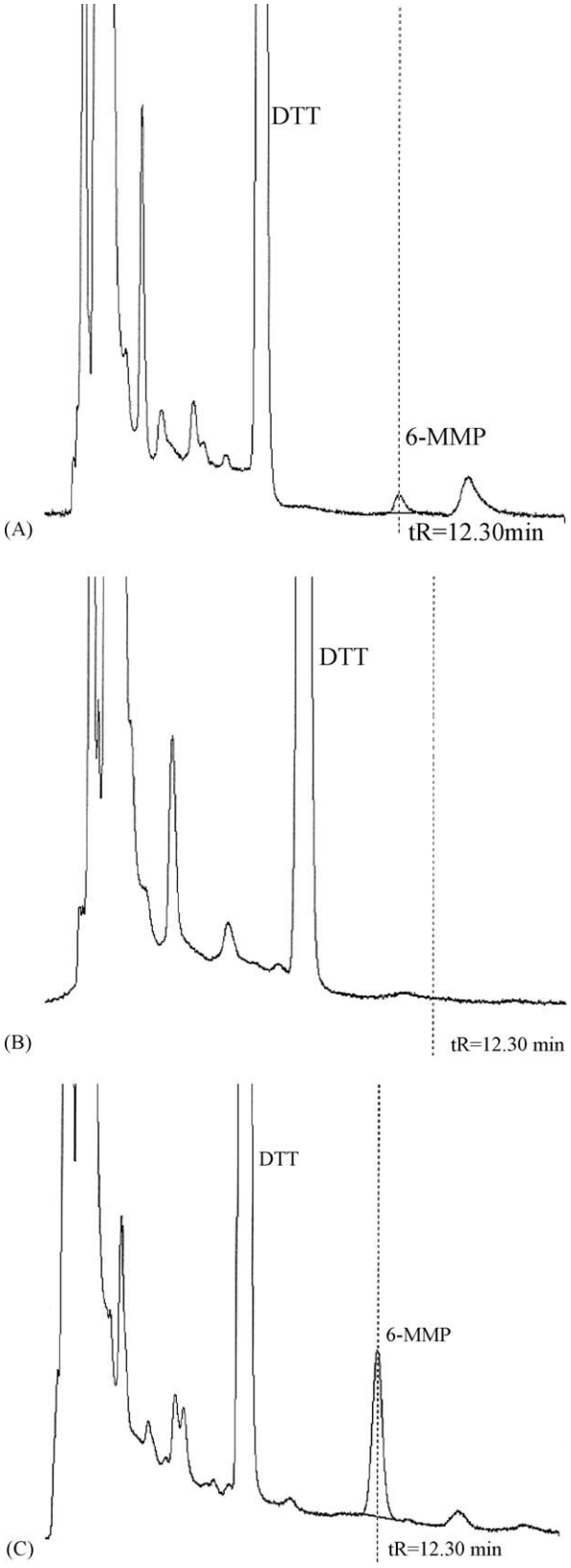

Fig. 1. Chromatographic separation of 6-methylmercaptopurine (6-MMP) in haemolysates. Panel A: the lower limit of quantification (the lowest concentration in the calibration curve 3.91 ng/ml). Panel B: calibration standard containing no 6-MMP. Panel C: 6-MMP formed by the enzymatic reaction in haemolysates during 60 min incubation at 37 °C. TPMT activity was calculated as 90.33 ng/ml/h.

without SAM (blank) showed 6-MMP formation 85% of that when samples were incubated with SAM. When 6-MP was dissolved in 1 M NaOH/HCl mixture, in sample incubated without SAM formation of 6-MMP was markedly lower than revealed in samples incubated with SAM (peak area 19.9 mV min versus 239.9 mV min).

3.3. TPMT activity expressed using different normalization factors

Formation of 6-MMP was determined in haemolysates of 99 previously genotyped healthy Estonian volunteers. Each run consisted of calibration curve samples (spiked into haemolysate, but not incubated), one quality control sample with known TPMT activity and unknown samples. For each sample, a blank incubated without SAM was included. Throughout the study, TPMT assay showed inter-run precision of 4%, based on the variation of quality control sample. Ten percent of the unknown samples were measured twice with accuracy 96–103%. Results of the first measurement were used for TPMT activity analysis.

Formation of 6-MMP was calculated by extracting and without extracting 6-MMP formed in blank sample (incubated without SAM). Good correlation between two approaches was observed (Spearman's $r^2 = 0.97$, $P < 0.001$) without any visual differences in distributions for heterozygous and wild type individuals in frequency distribution histograms. The Wilcoxon estimate of area under the ROC curve for TPMT activity segregating between heterozygous and wild type individuals was 0.91 and 0.93, extracting and without extracting blank, respectively. Since no significant differences were found for the Wilcoxon estimates, no blank is needed in further experiments allowing higher sample performance per day.

In further calculations, TPMT activity was expressed as 6-MMP formation per 60 min incubation at 37 °C, not extracting 6-MMP formed in blank sample. Formation of 6-MMP was normalized to Hb, PRBC, erythrocyte count and protein content in haemolysates. Spearman's rank correlation coefficients for all pairwise comparisons between different normalizations were ≥ 0.97 ($P < 0.001$) with similar distribution patterns in frequency distribution histograms by visual inspection. When formation of 6-MMP was not normalized, but expressed as a formation of 6-MMP ng/ml/h, good correlation with 6-MMP formation normalized to various factors was observed (Spearman's $r^2 \geq 0.97$, $P < 0.001$). All frequency distribution histograms revealed the same distribution patterns for heterozygous and wild type individuals regardless of the method used to express TPMT activity (Fig. 2). For each method of calculation, ROC curve analysis to define the cut-off level for TPMT activity segregating between heterozygous and wild type individuals was performed. The area under the ROC curves calculated by extended trapezoidal rule are presented in Fig. 3. All calculations used gave similar accuracy values. Its time- and cost-saving that no Hb, haematocrit, erythrocyte count or protein determination is needed in further experiments. Reference values of TPMT activity for Estonian population using the present method were expressed as 6-MMP formation ng/ml/h (without extracting blank nor using any normalization).

3.4. Reference values for TPMT activity in Estonian population

From 253 individuals genotyped, TPMT activity was determined in 18 heterozygous individuals found and in 81 randomly selected wild type individuals. TPMT activity among 99 healthy Estonian volunteers (52 male, 47 female, 32 ± 11 years, range 18–60 years) varied from 21.5 to 129.6 ng/ml/h. For heterozygous individuals ($n = 18$), TPMT activity was 48.1 ± 11.7 ng/ml/h. Wild type individuals ($n = 81$) revealed significantly higher TPMT activity 79.3 ± 20.7 ng/ml/h ($P < 0.001$). Cut-off value was arbitrarily set 58.8 ng/ml/h. There was some overlap between two phenotypes. No gender related differences in TPMT activity was found. Excluding heterozy-

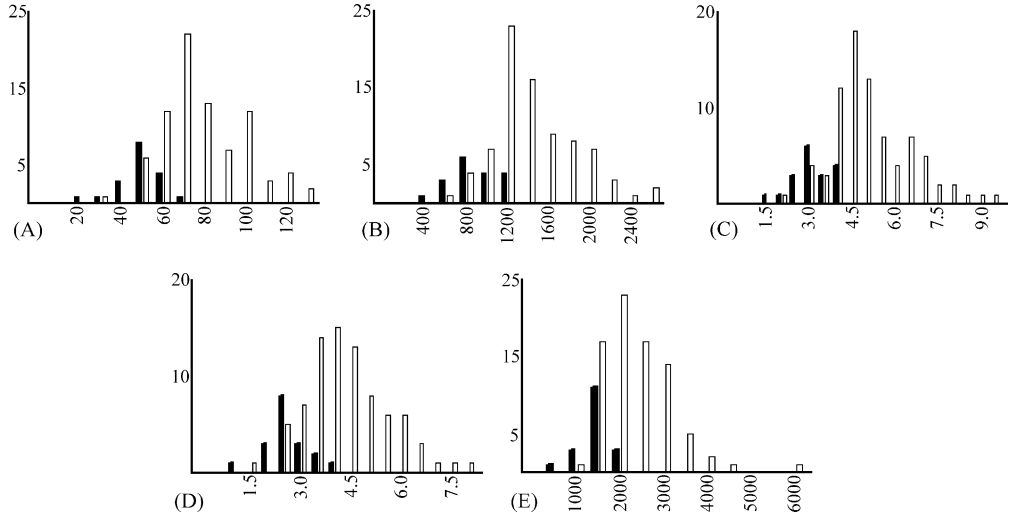

Fig. 2. Frequency distribution histograms of the TPMT activity for heterozygous (solid bars) and wild type (open bars) individuals. *Y*-axis indicates number of individuals. *X*-axis indicates TPMT activity. TPMT activity was expressed as: (A) 6-MMP formation ng/ml/h; and normalized to either; (B) Hb content ng/ml/h per g Hb; (C) Hkt ng/ml/h per ml PRBC; (D) erythrocyte count ng/ml/h per 10^8 RBC; or (E) protein content ng/ml/h per g protein.

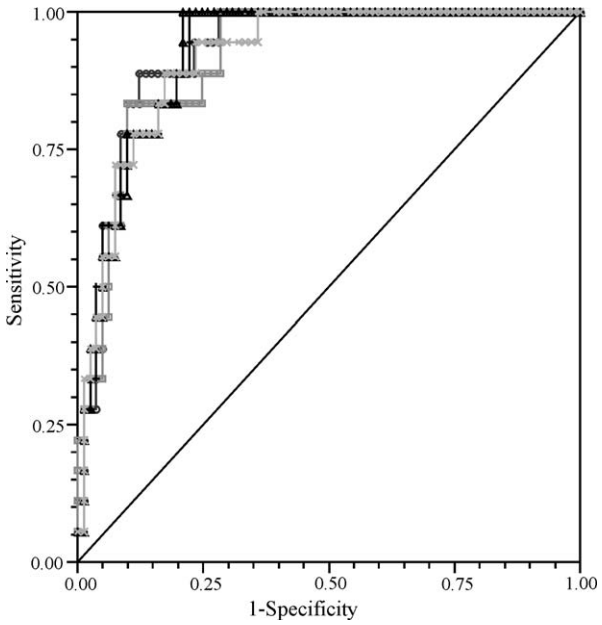

Fig. 3. The receiver-operating characteristic (ROC) plots. Wilcoxon estimate of area under the ROC curve and optimum cut-off point were obtained via the ROC analysis for each method of calculation used to express TPMT activity. Wilcoxon estimate of area under the ROC curve ranged from 0.92 to 0.93 indicating similar power of the assay to predict wild type and heterozygous individuals regardless of the method used to express TPMT activity.

gous individuals, a Shapiro–Wilk W test indicated a non-normal distribution of the wild type population ($W = 0.97$, $P < 0.05$). A small group of individuals with ultrahigh TPMT activity was detected.

4. Discussion

HPLC method for the determination of TPMT activity suitable for routine clinical use was developed. The method presented here requires no blank and Hb, haematocrit, erythrocyte count or protein determination, factors used to normalize 6-MMP formation in red blood cells. Therefore, the assay is cost- and time-saving allowing higher sample performance per day.

To express erythrocyte TPMT activity various normalization factors for 6-MMP formation in haemolysates have been used. We present for the first time that regardless of the factor used, the results for TPMT activity were highly comparable.

ROC plot is a graphical plot of the sensitivity versus 1-specificity for a binary classifier system and could be used to define the cut-off levels for diagnostic tests. In the present study, the ROC curve analysis revealed similar accuracy values for TPMT activity in predicting heterozygous and wild type individuals for each method of normalization. For the determination of an enzyme activity, formation of metabolite is standardized with protein content. However, an enzyme activity determined in erythrocytes is often calculated per Hb content. Under constant experimental conditions for the preparation of haemolysates no normalization with Hb content, haemotocrit, erythrocyte count or protein content was required. Normalization is generally used to compensate for differences in loading. Only the protein content in haemolysates would be appropriate for this purpose. However, when TPMT activity was normalized to protein content similar accuracy in ROC analysis was obtained compared with 6-MMP formation not normalized. Further studies should confirm if normalization of TPMT activity is needed when comparing different populations and clinical conditions, i.e. patients suffering from leukaemia and patients on thiopurine therapy.

Various extraction and chromatographic conditions were compared in preliminary experiments. While 6-MP has a low solubility in aqueous solution, organic solvent DMSO has been used in several studies [1,7,9,10,12]. When 6-MP was dissolved in DMSO, significant 6-MMP formation was observed in samples incubated without SAM due to non-enzymatic conversion or presence of endogenous SAM. This was shown already by Lennard et al. [6]. Khalil et al. [8] and Kröplin et al. [19,21] have shown non-enzymatic methylation if the concentration of the substrate and/or the co-substrate was chosen too high. In several HPLC methods for TPMT activity DMSO has been used to dissolve 6-MP and blank sample contained no substrate. However, non-specific conversion of 6-MP could not be determined when substrate was not added to incubate.

We tested different liquid–liquid extraction methods and found that in-house developed extraction with ACN gave good results with the lowest limit of quantification. In comparison with other solvents tested, ACN has higher polarity and hence extracts 6-MMP more effectively. The lower limit of quantification in our work was 3.91 ng/ml, perceptibly lower compared with others studies [1,6–9] and sufficient to identify patients even with very low TPMT activity.

Erythrocyte TPMT activity in healthy Estonians ranged from 21.5 to 129.6 ng/ml/h with a median for the total population of 70.45 ng/ml/h. TPMT activity was substantially lower for heterozygous individuals than for wild type individuals (48.1 ± 11.7 ng/ml/h versus 79.3 ± 20.7 ng/ml/h, $P < 0.001$) with no gender differences. Using a cut-off value of 58.8 ng/ml/h, the sensitivity and specificity of the TPMT assay for predicting wild type and heterozygous individuals was 89 and 88%, respectively. A Shapiro–Wilk W test indicated a non-normal distribution of the wild type population with a small group of individuals with ultrahigh TPMT activity. Several biotransformation enzymes express large interindividual differences in their activities. Previous studies have revealed ultrahigh enzyme activity of CYP2D6 due to gene duplication [22]. However, no genetic markers correlating with ultrahigh TPMT activity have been found. Knowledge of TPMT activity is of special importance when patients are treated with thiopurine drugs. Individuals with intermediate or absent TPMT activity have higher risk for side effects when treated with standard doses of these drugs [18]. Ultrahigh TPMT activity may provide a rational for increasing thiopurine dose.

In conclusion, here we present reference values of TPMT activity for Estonian population with similar distribution of intermediate and high activity as reported previously in white subjects. The HPLC assay for quantitative measurement of TPMT activity could easily be used in clinical settings since no additional determination of Hb content, haematocrit, erythrocyte count or protein content for normalization is needed.

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