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Thiopurine methyltransferase activity: new conditions for reversed-phase high-performance liquid chromatographic assay without extraction and genotypic–phenotypic correlation

Dany Anglicheau[a,b], Sylvia Sanquer[a], Marie-Anne Loriot[a], Philippe Beaune[a], Eric Thervet[a,b,*]

[a]*INSERM U490, Centre Universitaire des Saints Pères, 45 rue des Saints Pères, F-75270 Paris Cedex 06, France*
[b]*Service de Néphrologie et de Transplantation Rénale, Hôpital Saint Louis, 1 avenue Claude Vellefaux, F-75475 Paris Cedex 10, France*

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Abstract

Thiopurine methyltransferase (TPMT) is a cytosolic enzyme involved in the metabolism of thiopurine drugs. A genetic polymorphism is responsible for large inter-individual differences observed in TPMT activity. We report a new HPLC technique, which avoids an extraction step and the use of radioactive reagents, based on the conversion of 6-mercaptopurine (6-MP) to 6-methylmercaptopurine (6-MMP) using *S*-adenosyl-L-methionine (SAM) as methyl donor in red blood cell lysates (RBC). Intra- and inter-assay variation, within-day, within-run, between-day, and between-run variations showed high precision. The formation of 6-MMP was linear with respect to the lysate concentration and time. In a blinded assay of 61 samples, the results of HPLC method correlated with those of the radiochemical method ($r^2=0.82$, $P<0.0001$). Using a cut-off point of 8.5 nmol/h/ml packed RBC, positive predictive value of HPLC was 100% for heterozygous patients. Because of the absence of extraction step, this new HPLC technique of TPMT activity determination reduces analysis variation and is time-saving. This rapid, sensitive, and reproducible method is suitable for routine monitoring of TPMT activity and for fundamental studies.

Keywords: Genotypic–phenotypic correlation; Thiopurine methyltransferase; Enzymes

1. Introduction

Thiopurine methyltransferase (TPMT, EC 2.1.1.67) is a cytosolic enzyme involved in S-methylation of aromatic and heterocyclic sulphydryl compounds such as thiopurine drugs [1]. Azathioprine (AZA) and 6-mercaptopurine (6-MP) are thiopurine drugs which are used in organ transplant recipients, autoimmune diseases and as anti-cancer drugs [2,3]. AZA acts after conversion into 6-MP. In vivo 6-MP undergoes three competitive metabolic pathways [4] (Fig. 1). The anabolic pathway is catalyzed by the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT) which metabolize 6-MP into thio-inosine monophosphate and subsequently by a series of enzymes to 6-thioguanine nucleotides that are incorporated into DNA and RNA in nucleated cells. The first catabolic pathway for 6-MP is oxidation to thiouric acid by xanthine

*Corresponding author. Fax: +33-1-4249-4653.
E-mail address: eric.thervet@sls.ap-hop-paris.fr (E. Thervet).

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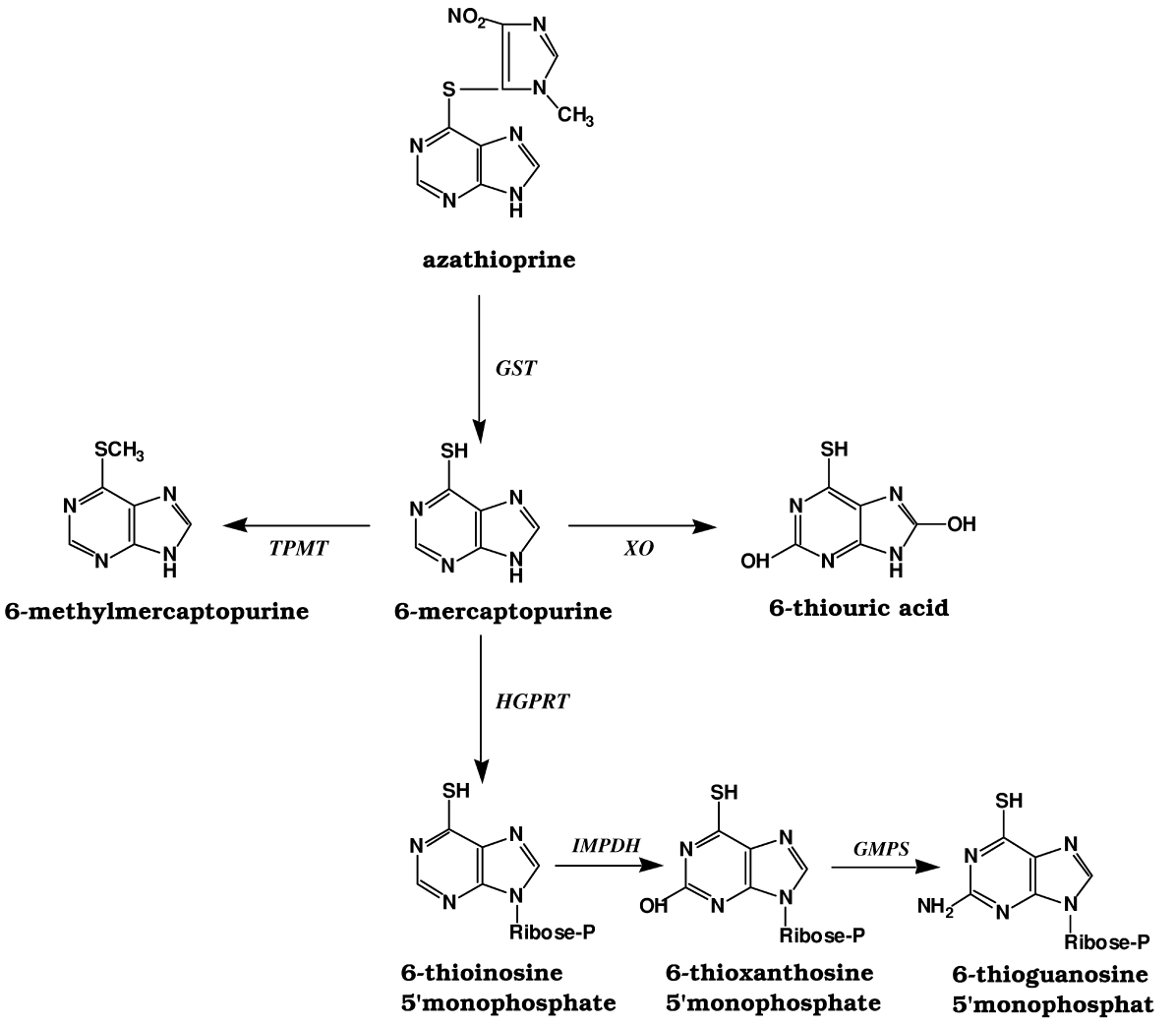

Fig. 1. Azathioprine metabolism. Azathioprine is mainly converted in the liver by glutathione-*S*-transferase (GST) into 6-mercaptopurine which undergoes three major pathways. Conversion of 6-mercaptopurine by the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT) leads to the formation of 6-thioguanine nucleotides which are responsible for the immunosuppressive activity of azathioprine. The thiopurine methyltransferase (TPMT) pathway leads to the methylation of 6-mercaptopurine forming methylmercaptopurine. Finally, conversion by the enzyme xanthine oxidase (XO) leads to the formation of thiouric acid. (IMPDH: inosine monophosphate dehydrogenase; GMPS: guanosine monophosphate synthase).

oxidase. Another catabolic pathway is methylation to 6-methylmercaptopurine (6-MMP). This reaction involves TPMT, the methyl group being provided by *S*-adenosyl-L-methionine (SAM). TPMT activity in vitro presents large inter-individual variations which has been associated with both efficacy [5–7], toxicity, variability [8,9], and drug interactions [10] in vivo. A genetic polymorphism is partly responsible for these wide interindividual differences observed in TPMT activity. The frequency distribution of TPMT activity in large population studies is trimodal: 1 in 300 subjects have undetectable activity, 11% have intermediate activity, and the remainder inherits high enzyme activity. The cloning of the gene has shown that these variations are related with different mutations [11–15]. However, it is still important to detect phenotypic activity variation in patients treated with AZA or 6-MP because of the clinical implications

and the lack of perfect correlation between genotypic and phenotypic assessment [16].

TPMT has been firstly measured by a radiochemical assay (RC) [17]. More recently, HPLC methods have been published [18–20]. All these methods are based on the in vitro conversion of 6-MP to 6-MMP or 6-thioguanine to 6-methylthioguanine, using SAM as the methyl donor. In these HPLC assays, the product of the enzymatic reaction is extracted by a liquid–liquid or liquid–solid extraction. We report here a new simple HPLC technique, which avoid extraction and therefore reduce the variation of the analysis.

2. Experimental

2.1. Reagents and chemicals

6-MP, 6-MMP, dithiothreitol, allopurinol, *S*-adenosyl-L-methionine (SAM) were supplied by Sigma–Aldrich (Saint-Quentin Fallavier, France). Dimethylsulfoxide (DMSO) was from Merck (Darmstadt, Germany). Chelex 100 was purchased from Bio-Rad (Richmond, CA, USA). Acetonitrile, absolute ethanol, methanol, chlorhydric acid were obtained from Prolabo (Paris, France). Acetonitrile was of HPLC grade, all other chemicals were of analytical grade.

2.2. Blood samples: preparation of lysates

TPMT activity assessment was clinically indicated for all patients because of auto-immune skin disease or inflammatory bowel diseases. TPMT activity was assessed using the same blood sample by radiometric (RC) and HPLC. Samples were prepared as previously described by Weinshilboum et al. [10]. Briefly, 7 ml of whole blood collected in lithium heparinized tube was centrifuged at 800 *g* for 10 min at +4 °C to isolate red cells. Plasma, leukocytes and the upper layer of the erythrocytes were removed. After washing the pellet twice with 4 ml of 0.154 *M* NaCl and centrifugation for 10 min at 800 *g*, 2 ml of red cells were resuspended in 2 ml of 0.154 *M* NaCl and the haematocrit was determined. Two ml of solution of red blood cells were lysed with 8 ml of cold distilled water and centrifuged at 13 000 *g* for 10 min at +4 °C. The supernatant was kept at −80 °C until analysis. It was shown that the activity did not decrease in these storage conditions.

2.3. Incubation conditions

Solutions of 6-MP in DMSO were prepared immediately prior to the incubation. Solutions of SAM, allopurinol and dithiothreitol in water were kept at −20 °C. After slow thawing, 900 μl of erythrocyte lysate was chelated by adding 100 μl of Chelex 100 resin, followed by gentle rotation for 1 h at +4 °C and centrifugation at 4000 *g* for 10 min at +4 °C. Aliquots of 100 μl of the supernatant were incubated with 25 μl potassium phosphate buffer (0.15 *M*, pH 7.5), 25 μl of a mixture containing SAM (final concentration 12 μ*M*), allopurinol inhibiting the xanthine oxidase pathway (final concentration 48 μ*M*), and dithiothreitol to protect the thiol group from oxidation (final concentration 1 m*M*), and 5 μl of 6-MP solution (final concentration 3.8 m*M*) in a final volume of 155 μl. The mixture was incubated for 1 h at 37 °C in a shaking water bath and stopped by adding 200 μl of absolute ethanol. After 10 min on ice, 355 μl solution of methanol–0.1 *M* HCl (v/v) was added, centrifuged at 5000 *g* for 5 min at +4 °C. A 100-μl aliquot of the supernatant was then injected into the HPLC system.

2.4. Chromatographic conditions

Stock solutions of 6-MMP in DMSO were kept at −20 °C. Standard curves of 6-MMP (25–500 n*M*) were prepared from appropriate dilutions of the stock solution.

The HPLC system consisted of a Spectra Series P200 pump (Thermo Separation Products, Fremont, CA, USA), an AS100 Autosampler and an UV100 UV detector. The analytical column was a C_{18}/5-μm particle size LiChrocart column (Merck) with a C_8/5-μm precolumn. The column size was 125 mm in length and 4 mm in diameter. The pre-column was used to protect the column from obstruction by impurities and early degradation and not for separation purpose. The mobile phase consisted of solution A (acetic acid 0.1%) and solution B (acetonitrile 100%) that formed the following linear gradient: 0 min (2% B), 5 min (6% B), 22 min (22% B), 23–24

min (50% B), 25–30 min (2% B). The flow-rate was 1 ml/min. The detection wavelength was 290 nm which is the maximal absorbance of 6-MMP. The total run time including the equilibration time for the next run was 30 min.

2.5. Radiochemical 6-MMP assay

The RC assay of TPMT activity was performed as previously described [17]. Briefly, 6-MP was converted in vitro into 6-MMP in the presence of SAM, the methyl donor, which methyl group was labeled with ^{14}C. Preparation of lysates and incubation conditions were identical to the HPLC method except for *S*-adenosyl-L-[^{14}C]methionine. The reaction was stopped by the addition of 0.5 ml of borate buffer 0.5 *M*, pH 10. Five ml of 20% isoamyl alcohol in toluene was added and the tubes were mixed for 10 s. Following centrifugation at 700 *g* for 10 min, 2 ml of the organic phase was placed in liquid scintillation counting vials that contained 2 ml of ACS II liquid and radioactivity was determined.

2.6. Detection of TPMT mutations by polymerase chain reaction

We used PCR-based methods to detect the most common functional mutations of TPMT gene as described [21]. Namely, we detected the wild-type allele *TPMT*1* and the variant alleles *TPMT*2* (G238C), *TPMT*3A* (G460A and A719G), *TPMT*3B* (G460A) and *TPMT*3C* (A719G). Briefly, for the detection of G238C transversion, an allele-specific PCR procedure was performed. Genomic DNA was amplified with primer P2W (5′-GTATGATTTTATGCAGGTTTG-3′) or P2M (5′-GTATGATTTTATGCAGGTTTC-3′) in association with primer P2C (5′-TAAATAGGAACCATCGGACAC-3′). Unpurified PCR products were analyzed after electrophoresis in 2.5% agarose gel stained with ethidium bromide. A DNA fragment was amplified with P2M and P2C primers when C238 (mutant) was present, whereas a DNA fragment was amplified with P2W and P2C primers when G238 (wild-type) was present. To detect the G460A mutation, a PCR assay using primers P460F (5′-ATAACAGAGTGGGGAGGCTGC-3′) and P460R (5′-CTAGAACCCAGAAAAAGTATAG-3′), 0.1 μl of each primer per reaction tube was done under similar conditions. The PCR product was digested with *Mwo*I (Ozyme, France) for 1 h at 60 °C. After analysis by gel electrophoresis, *Mwo*I digestion was performed. The 365-bp fragment was cut into 267 and 98 bp fragments in case of mutant C238 allele and was not cut in the case of wild-type G238 alleles. A PCR assay using primers P719R (5′-TGTTGGGATTACAGGTGTGAGCCAC-3′) and P719F (5′CAGGCTTTAGCATAATTTTCAATTCCTC-3′) was done under the described conditions to detect the A719G mutation. The PCR products were digested with *Acc*I for 2 h at 37 °C, and analyzed by gel electrophoresis. The A719G mutation created an *Acc*I restriction site in the amplified fragments and yielded fragments of 207 and 86 bp. Wild-type DNA yielded an uncleaved fragment of 293 base pairs.

2.7. Statistical analysis

TPMT activity is expressed in nanomoles of 6-MMP formed per milliliter of packed red blood cells per hour (nmol/h/ml PRBC). Results are expressed as means±standard deviation (mean±SD). Statistical analysis was performed with the Statview™ v5.0 program (Abacus Concepts, Berkeley, CA, USA). Percentage coefficient of variation (C.V.) is the quotient of the standard deviation and the mean value×100. Comparisons between groups were made by using Student's *t*-test. Probability values of less than 0.05 were considered significant. Linear regression analysis was analyzed between HPLC and RC TPMT values and the Pearson product moment correlation coefficient (r^2) was calculated. All data points for the in vitro assays represent the results of three determinations.

The apparent Michaelis–Menten constants [maximum velocity (V_{max}) and affinity constant (K_m)] were determined graphically using the program GraphPad Prism, Version 2.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. HPLC 6-MMP assay

Using the HPLC conditions described, we found that 6-MMP was well separated and easy to analyze. No interfering substances were present in both

cytosol of untreated normal subjects or patients (Fig. 2). The 6-MP and 6-MMP retention times were 4.3 and 15.5 min, respectively.

There was a linear relationship between the peak areas and 6-MMP concentration over the range of 0.2 and 1 nmol/ml of 6-MMP. The detection limit, defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy, was 0.05 nmol/ml.

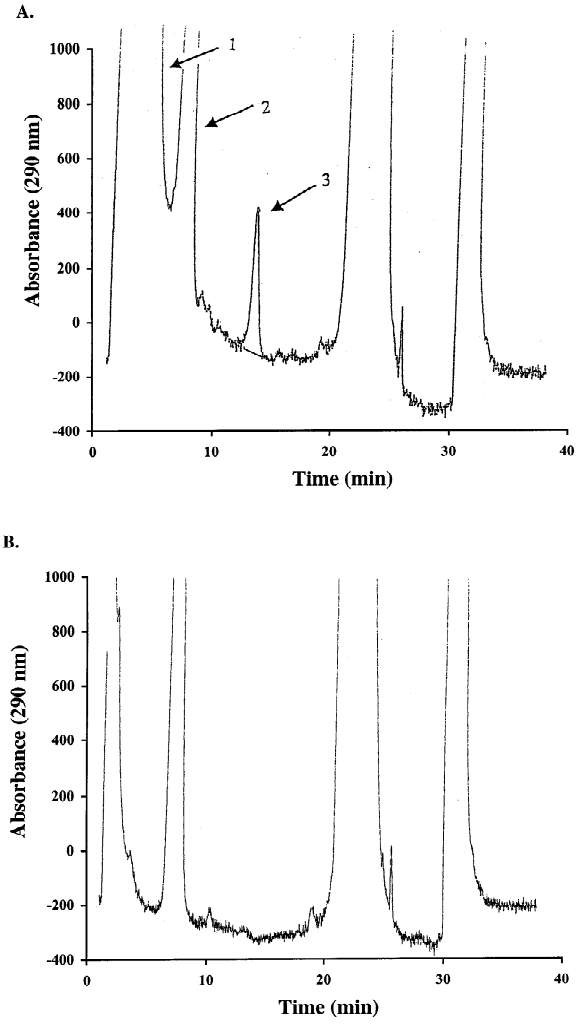

Fig. 2. Chromatographic separation of 6-methylmercaptopurine (6-MMP). The detection wavelength was 290 nm. The two runs represent the cytosol from the same patient. Panel A: cytosol was incubated with the substrate 6-mercaptopurine (6-MP). The peak with a retention time of 4.3 min represents the 6-MP in excess. The following peak with a retention time of 15.5 min represents the 6-MMP formed by enzymatic reaction during the incubation. Panel B: the lower chromatogram represents the result of incubation in the same conditions in absence of the substrate 6-MP (blank). As a consequence, no 6-MMP was formed in this case. Peaks: 1=6-MP, 2=dithiothreitol, 3=6-MMP. In this sample, 6-MMP formed was 0.6 nmol/ml and the corresponding thiopurine methyltransferase activity was 19 nmol/h/ml per RBC.

The inter-assay C.V. for assay of 0.2 and 1 nmol/ml ($n=5$) were 6.2 and 4.8%, respectively. The inter-assay C.V. for the slope of the calibration curve was 5.3% ($n=5$). The coefficients r^2 for the calibration curves were all >0.99.

The precision was studied by adding 6-MMP to erythrocyte lysate at three different concentrations (0.5, 1, and 5 nmol/ml). Lysates were analyzed in duplicate and each sample was injected twice in a single run. Samples were prepared ten times over a period of 15 days. Precision was good since the coefficient of variation between-day, between-run, within-day and within-run varied between 6.5 and 12%, 8.3 and 12%, 3.6 and 5.7%, and 3.4 and 7.9%, respectively (Table 1).

3.2. HPLC method for TPMT activity measurement

Intra-assay variation determined by measuring TPMT activity five times in a single run was 6.0%. Inter-assay variation, determined by measuring TPMT activity five times in different runs was 9.5%.

The stability of the samples was studied during 30 h at room temperature. After standard incubation, ten samples were pooled and stored at room temperature. Chromatograms were recorded for 30 h. The stability was demonstrated with a C.V. of peak area of 2.5%. The samples were also stored for 5 days at room temperature. The stability was assessed with a C.V. of peak area of 7.5% ($n=14$).

3.3. Kinetics of TPMT activity

The formation of 6-MMP was linear with respect to the lysate concentration (0–100 μl/assay) and time (0–60 min). The average equations observed were $y=127.17x+132.51$ ($r^2=0.9968$, $y=$6-MMP area, $x=$volume of packed RBC) and $y=241.07x+4253.2$ ($r^2=0.9881$, $y=$6-MMP area, $x=$incubation time), respectively. For the linearity assessment, each point was the mean of three determinations.

6-MMP formation was measured in the presence of various concentrations of 6-MP (0.15–8 mM) and SAM (1–12 μM). The apparent K_m was 426.5 μM

Table 1
Components of variance

	Sample					
	0.5 nmol/ml		2.5 nmol/ml		5 nmol/ml	
	SD (nmol/ml)	C.V. (%)	SD (nmol/ml)	C.V. (%)	SD (nmol/ml)	C.V. (%)
Between-day	0.039	6.5	0.296	11	0.673	12
Between-run	0.049	8.3	0.322	12	0.662	12
Within-day	0.034	5.7	0.155	3.7	0.136	3.6
Within-run	0.048	7.9	0.118	3.4	0.192	3.4

for 6-MP and 1.9 μ*M* for SAM. V_{max} was 11.5 n*M*/min for 6-MP and 5.0 n*M*/min for SAM (Fig. 3).

3.4. Correlation of TPMT activity using HPLC and radiometric assay

We analyzed 61 samples from patients using both RC and TPMT assay. TPMT activity was determined by RC and HPLC assays with standard red blood cell preparations. The range of TPMT activity was 4.3–28.7 nmol/h/ml PRBC by the HPLC method and 6.2–28 nmol/h/ml PRBC by the RC method. The mean TPMT activities obtained by the HPLC method and RC methods were 12.0±3.7 and 15.2±5.5, respectively.

Linear regression analysis of HPLC vs. RC TPMT activity yielded the following relationship: $TPMT_{RC} = 1.33x TPMT_{HPLC} + 0.71$ (Fig. 4). The Pearson product moment correlation coefficient (r^2) was 0.82 ($P<0.0001$). When the radiometric assay is used, a cut-off between high and intermediate activity at 10 nmol/h/ml PRBC is usually taken. Using the regression line obtained, we calculated the corresponding cut-off point for HPLC. The value we used was 8.5 nmol/h/ml PRBC (Fig. 4).

3.5. Phenotype–genotype correlation

After genotyping, individuals were classified according to the TPMT genotypes into group of high (HMs), intermediate (IMs) and deficient methylators (DMs). In the population studied, 58 patients carrying two functional alleles (genotype **1/*1*) were predicted as HMs (95%), three subjects heterozygous for one nonfunctional allele [**1/*3A* ($n=1$), **1/*3C*, ($n=2$)] were predicted as IMs (5%). We found no homozygous deficient subject in our population. Erythrocyte activity by both techniques was compared blindly. Subjects predicted as IMs exhibited a TPMT range from 4.3 to 7.7 nmol/h/ml PRBC for HPLC and between 7 and 10 nmol/h/ml PRBC for RC. Subjects predicted as HMs exhibited a TPMT activity range between 5.9 and 28.7 nmol/h/ml PRBC for HPLC and between 6.2 and 28 nmol/h/ml PRBC for RC. The median TPMT activity was significantly higher in predicted HMs than in predicted IMs. These values were, respectively, 11.7 nmol/h/ml PRBC versus 7 nmol/h/ml PRBC ($P<0.03$) using HPLC and 14.5 nmol/h/ml PRBC versus 8 nmol/h/ml PRBC ($P<0.03$) using RC. Phenotypes were in good agreement with genotypes for 90% of individuals using HPLC and 87% using RC (Fig. 5). More importantly for clinical practice, none IMs subjects exhibited TPMT activity above the cut-off value using either technique even though in six cases with HPLC and eight cases with RC, HMs subjects had a TPMT activity values below the cutoff value.

4. Discussion

We report here a new specific HPLC method to measure 6-MMP formation in red blood cell cytosols after incubation with 6-MP. This assay therefore measures the RBC TPMT activity, which has been shown to correlate with liver TPMT activity [22,23].

The metabolism of 6-MP involves three competitive pathways. There is little variation in the RBC HGPRT activity [24]. There is little interindividual variation in the activity of xanthine oxidase which catabolizes 6-MP into thiouric acid [25]. The other catabolic pathway is S-methylation to 6-MMP. This

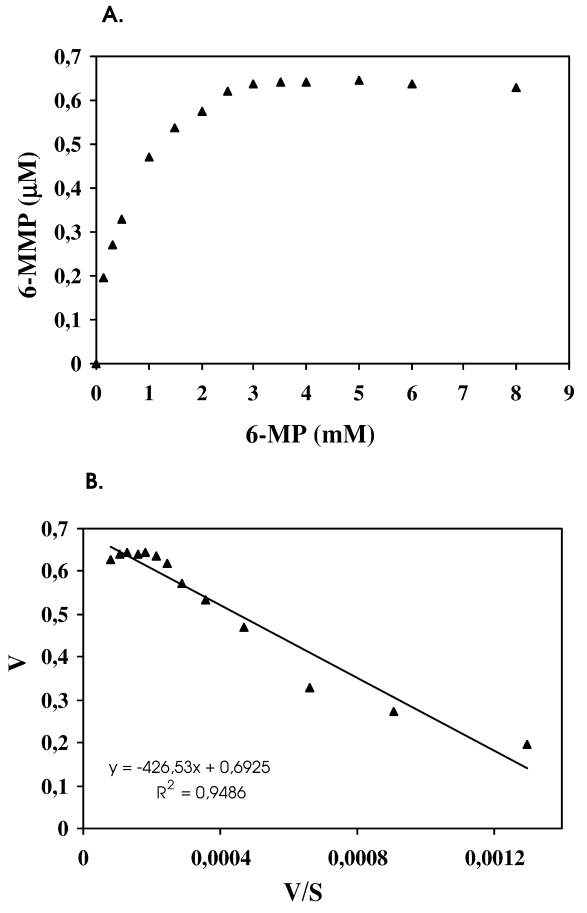

Fig. 3. Effects of substrate concentrations on TPMT activity (A: substrate curve; B: Eadie–Hofstee plot). A 100-μl aliquot of erythrocyte lysate were incubated with 25 μl of potassium phosphate buffer (0.15 *M*, pH 7.5), 25 μl of a mixture containing SAM (final concentration 12 μ*M*), allopurinol (final concentration 48 μ*M*), and dithiothreitol (final concentration 1 m*M*), and 5 μl of 6-MP solution at concentration ranging from 0.2 to 8 m*M*. After incubated for 1 h at 37 °C in a shaking water-bath the reaction was stopped by adding 200 μl of absolute ethanol. After 10 min on ice, 355 μl of a solution of methanol–0.1 *M* HCl (v/v) was added. After centrifugation at 5000 *g* for 5 min at +4 °C, 100 μl of the supernatant was then injected into the HPLC system. The detection wavelength was 290 nm. Each point is the mean of three determinations.

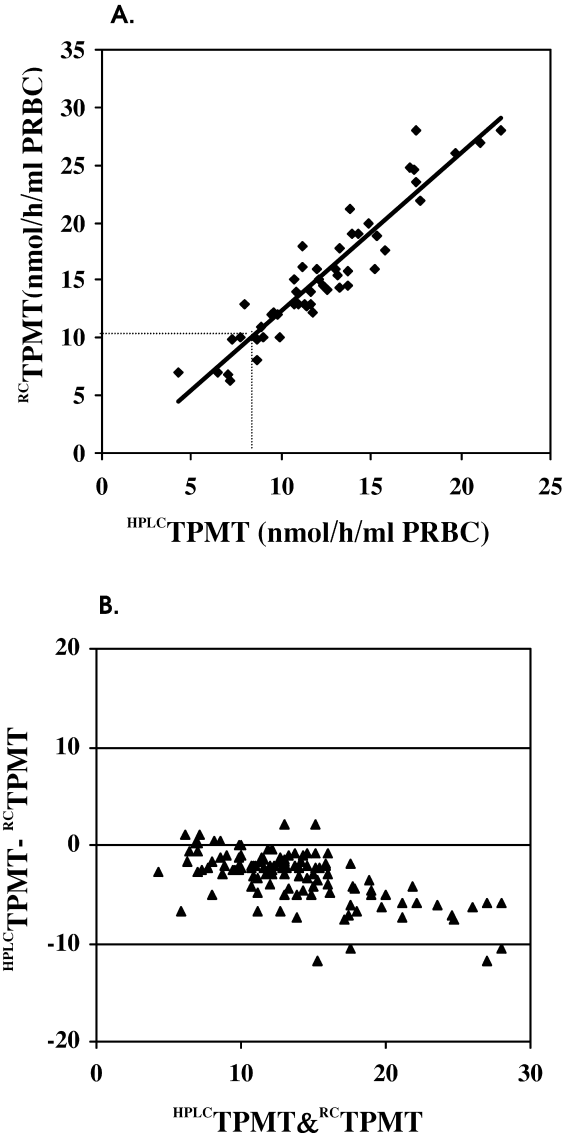

Fig. 4. (A) Linear regression of thiopurine methyltransferase (TPMT) values measured by the chromatographic ($TPMT_{HPLC}$) and radiometric ($TPMT_{RC}$) assays. TPMT activity is expressed as the amount of 6-methylmercaptopurine (nmol) formed per hour and per ml of red blood cells cytosol. The regression line is expressed as $y = 1.33x + 0.71$ ($r^2 = 0.82$, $P < 0.0001$). (B) Distribution of $TPMT_{HPLC}$–$TPMT_{RC}$ activity plotted according to the $TPMT_{HPLC}$ and $TPMT_{HPLC}$ absolute values.

reaction involves TPMT. Population studies have shown a trimodal distribution of TPMT activity which is inherited as an autosomal codominant trait [13,14]. Recent studies have demonstrated the role of unique or multiple point mutation to explain this loss of activity [21,26–28]. Null activity is associated with homozygote mutation, intermediate activity is associated with heterozygous. However, genotype cannot be the only method used since the best correlation to date between phenotypic and genotypic

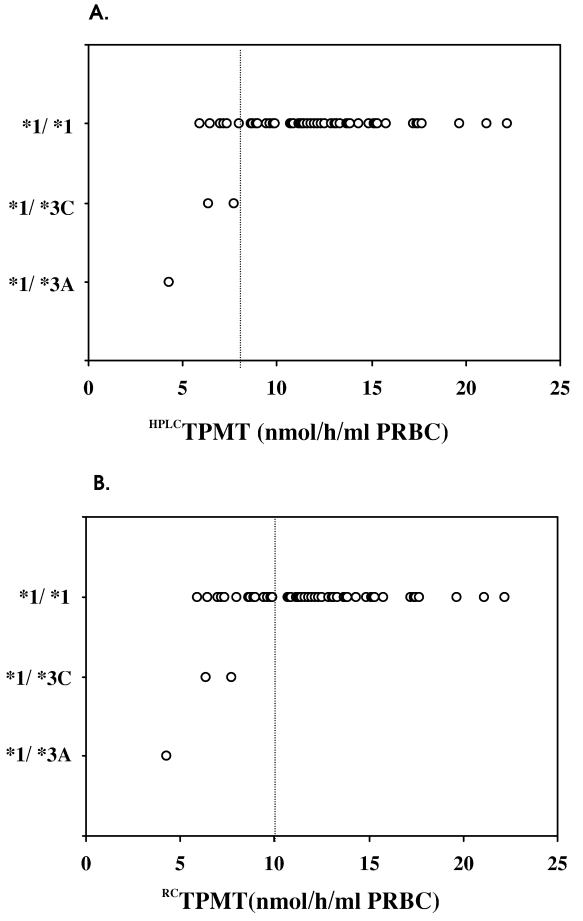

Fig. 5. Distribution of Thiopurine methyltransferase activity (TPMT) by (A) high-performance liquid chromatography ($TPMT_{HPLC}$) and (B) radiochemical ($TPMT_{RC}$) assay among 61 European individuals in relation with their TPMT genotypes. Individuals TPMT **1/*1* are homozygous for the functional allele and are predicted as high methylator phenotypes (HMs) whereas individuals genotyped for non-functional alleles (TPMT **1/*3A* and TPMT **1/*3C*) are predicted as intermediate methylator phenotypes (IMs).

assessment is 87% because of possible still unknown mutations [16]. We have also demonstrated that not only TPMT baseline activity but various pattern of TPMT activity induction after renal transplantation is associated with different clinical results [6,7]. Therefore, assessment of TPMT activity before and after treatment is needed.

In vitro, TPMT activity in red blood cells can be measured in phosphate buffer containing the cell lysate, 6-MP and SAM as the methyl donor. The RC TPMT assay is based on the conversion of 6-MP using radiolabeled SAM [^{3}H] or [^{14}C] whereas the HPLC assay uses unlabelled SAM. A hospital laboratory for clinical studies could easily use this HPLC assay, which does not involve the use of radioactive reagents. Using the present assay, we found a wide range of TPMT activity similar to reported values in a French population. In the present study, the apparent K_m was 426.5 μM and V_{max} was 11.5 nM/min. These results are in good agreement with those already published [18,22].

The previous method reported use of an extraction step. This extraction is associated with a recovery rate ranging from 70 to 89.9% which can be responsible for lack of precision in the lower activity. The extraction step is also onerous and is time consuming especially if large numbers of samples and/or serial evaluation of TPMT activity are asked for. Because of the avoidance of this extraction step and the relatively short run time, our method represents a significant improvement of the technique already described.

Reproducibility, repeatability and precision were satisfactory. The stability of the samples was good during 30 h at room temperature. This long stability is important because of the long chromatogram run time (two chromatograms per hour) and the necessity to measure the blank value for each patient.

The correlation between RC and HPLC techniques was good. The linear regression analysis yielded an intercept value of $+0.71$. Therefore, the cutoff value between normal and low activity is 8.5 nmol/h/ml PRBC for HPLC method.

The aim of baseline TPMT activity assessment before treatment is to detect the presence of a low or null activity in order to avoid potential life-threatening complications. We found that using our technique, the probability to detect a heterozygous subject was 100%. On the other hand, there was a relatively poor correlation between genotype and phenotype. A phenotypic deficient patient may present a mutation which is not detected during this routine genotyping assay. Our results are in accordance with the already published correlation between genotype and phenotype [16,21,26]. Our technique determines TPMT activity both quickly and accurately and allows therefore its routine use before

azathioprine or 6-MP treatment is started or for sequential analysis after treatment initiation.

In conclusion, we report a HPLC assay of TPMT activity which avoid both the use of radioactive reagents and an extraction step. This allows its use in a hospital laboratory and is time-saving when compared with previous assays. This may allow a larger use of phenotypic assessment coupled with genotypic assessment of TPMT activity in cancer patients or organ transplant recipients receiving either azathioprine or 6-MP.

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