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High-performance liquid chromatographic assay of human red blood cell thiopurine methyltransferase activity

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Abstract

An assay is described for the determination of red blood cell (RBC) thiopurine methyltransferase (TPMT) activity. TPMT S-methylates the antileukaemic drugs 6-mercaptopurine (6-MP) and 6-thioguanine and enzyme activity is inherited as a genetic trait. The assay is based on the TPMT-catalysed conversion of 6-MP to 6-methylmercaptopurine (methyl-MP) with non-radioactive S-adenosyl-L-methionine as the methyl donor. The methyl-MP metabolite is extracted into toluene as a phenyl–mercury adduct and back-extracted into 0.1 *M* HCl. Methyl-MP is quantitated by reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet detection using a C_{18} Resolve cartridge and a mobile phase of methanol–water (20:80, v/v) with 100 m*M* triethylamine adjusted to pH 3.2 with orthophosphoric acid. There was a strong correlation between the HPLC assay and the established radiochemical assay ($P < 0.0001$). TPMT activities were measured by both methods in a population study of 111 children. There was no significant difference between the two frequency distribution histograms ($P > 0.6$).

1. Introduction

6-MP is widely used in the chemotherapy protocols for the treatment of childhood acute lymphoblastic leukaemia, but 6-MP has no intrinsic anticancer activity; it is a prodrug. The initial activation of 6-MP, catalysed by the enzyme hypoxanthine phosphoribosyltransferase (EC 2.4.2.8), results in the formation of several active nucleotide metabolites; the major ones in the RBC are the 6-thioguanine nucleotides (6-TGNs), compounds which can be incorporated into DNA [1]. The antileukaemic effect of 6-MP can be related to the RBC concentration of 6-TGN active metabolites [2,3] and in children taking identical doses of 6-MP there are wide interindividual variations in RBC 6-TGNs which are related to subsequent bone-marrow suppression [2].

TPMT (EC 2.1.1.67) catalysed S-methylation of 6-MP is a separate metabolic pathway. TPMT activity is regulated by a common genetic polymorphism which is responsible for wide interindividual differences in enzyme activity [4]. Population studies have shown that enzyme activity is trimodally distributed; in a white Caucasian population 0.33% lack, 11% have intermediate and 89% have high TPMT activities [4]. The molecular mechanisms responsible for the TPMT genetic polymorphism remain unclear [5] but in addition to regulating RBC enzyme

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activity it also controls TPMT activity in all other cells and tissues [6–8].

Both 6-MP and its initial nucleotide metabolite can be S-methylated by TPMT and the rate of S-methylation can influence 6-TGN formation [9,10]. Very low TPMT activity is associated with grossly elevated RBC 6-TGN concentrations and profound myelosuppression after only a brief exposure to thiopurine drugs whilst those individuals who inherit very high levels of activity do not experience cytotoxicity on standard doses of 6-MP. Thus, TPMT activity can influence both the antileukaemic effect of 6-MP and the immunosuppressive effect of azathioprine, a slow-release formulation of 6-MP [9–11].

We have previously reported the measurement of the 6-MP metabolite 6-methylmercaptopurine–the product of the TPMT reaction–by reversed-phase HPLC with UV detection [12]. This paper reports a modification of that assay which reduced the lower limit of detection of the methylated metabolite 20-fold and enabled the development of a non-radioactive measure of TPMT activity.

2. Experimental

2.1. Materials

6-MP, methyl-MP, S-adenosyl-L-methionine. HCl and DL-dithiothreitol (DTT) were obtained from Sigma (London, UK). A stock solution of methyl-MP (100 μg/ml) was prepared in a neutral solution by dissolving 10 mg in 1.5 ml of 1 *M* NaOH in a light-protected container; 80 ml of water were then added prior to HCl (1.5 ml of 1 *M*) and a final dilution to 100 ml with water. A 250 μg/ml 6-MP solution in 0.01 *M* HCl containing 1 m*M* DTT was prepared immediately prior to use in the incubation; 6-MP (2.5 mg) was dissolved in 400 μl of 0.1 *M* NaOH in a light-protected container followed by 6 ml of water, 1.04 ml of 0.1 *M* HCL and 1 ml 10 m*M* of DTT before making to a final volume of 10 ml with water.

Methanol (HPLC grade) and toluene (glass-distilled grade) were obtained from Rathburn Chemicals (Walkerburn, UK). The preparation of the toluene solution containing 170 m*M* amyl alcohol (AnalaR grade, BDH, Poole, UK), and 1.3 m*M* phenyl mercury acetate (PMA; laboratory grade, BDH), has been described elsewhere [12]. All other reagents were of analytical grade and obtained from BDH. The water used for all experimental procedures was obtained from a Milli-Q Plus water purification system (Millipore, Watford, UK). The glassware used for the analysis of thiopurines was soaked overnight in 30% nitric acid and rinsed in distilled water.

2.2. Blood samples

Blood samples (5 ml) were collected in lithium heparin tubes. Routinely, blood was stored at 4°C and processed within 24 h of sampling. To investigate the effect of blood storage 20 ml of blood was stored at room temperature and at 4°C in 2-ml aliquots. The aliquots were shaken (Denley Spiramix blood mixer) three times daily. Red cell lysates were prepared immediately after sampling and then at 24 h intervals for each of the storage conditions.

Red cell lysates for the assay of TPMT were prepared as follows: The whole blood was centrifuged at 640 *g* for 10 min at 4°C, and the plasma and "buffy coat" were discarded. The RBCs were washed twice in two volumes of 0.9% NaCl, the packed RBCs were resuspended in 2 ml of 0.9% NaCl and a haematocrit obtained. The RBCs were lysed by adding 200-μl aliquots of the resuspended cells to 800 μl of ice-cold water in a microcentrifuge tube and vortex-mixing the sample. The diluted lysate was centrifuged (14 000 *g*, 10 min, 4°C), and the transparent supernatant carefully removed and stored at −80°C. Blood samples were taken under guidelines approved by the Sheffield Southern District Ethical Committee.

2.3. TPMT assay

Red blood cell TPMT activity was measured by a modification of the radiochemical method described by Weinshilboum and co-workers [8,13]. The assay is based on the TPMT-cata-

lysed conversion of 6-MP to 6-methyl-MP with non-radioactive S-adenosyl-L-methionine as the methyl donor. The methyl-MP is extracted and the concentration measured by a modification of the HPLC procedure described by Lennard and Singleton [12]. One unit of enzyme activity represented the formation of 1 nmol of methyl-MP per hour of incubation, per millilitre of packed red cells at 37°C.

2.4. Incubation

Red cell lysates were incubated in duplicate in a final volume of 237 μl. A 100-μl volume of lysate was placed in a 10-ml screw-neck glass test-tube with 90 μl of a 250 μg/ml solution of 6-MP in 0.01 *M* HCl and 15 μl of 250 m*M* sodium phosphate buffer pH 9.2. The tubes were preincubated for 10 min in a shaking water bath at 37°C prior to the addition of 32 μl of a 3:1 mixture of 250 μ*M* S-adenosyl-L-methionine (final concentration $2.5 \cdot 10^{-5}$ *M*) and 30 m*M* DTT (final concentration 10^{-3} *M*). The final pH was 7.5 and the 6-MP concentration 558 μ*M*. The tubes were incubated for 1 h and the TPMT reaction stopped, in each tube in turn, by the addition of 850 μl of ice-cold 3.5 m*M* DTT immediately followed by 500 μl of 1.5 *M* H_2SO_4 and placing the tube on ice. The tubes were removed from ice and equilibrated to room temperature prior to heating at 100°C for 2 h in a Dri-Block (Techne, Cambridge, UK). After cooling the tubes could be stored overnight at 4°C if required. With every incubation a quality control was included. This was a pooled lysate in which TPMT activity had been previously measured by the radiochemical method.

2.5. Extraction and HPLC procedure

To each tube in turn, 500 μl of 3.4 *M* NaOH were added immediately followed by 8 ml of the toluene–amyl alcohol–PMA mixture. The tubes were shaken gently for 10 min and centrifuged for 5 min at 900 *g* (10°C). Then, 6 ml toluene were transferred to a glass-stoppered conical test-tube and 0.2 ml of 0.1 *M* HCl added. After vortex-mixing for 4 × 20 s the tubes were centrifuged (5 min, 900 *g*, 10°C) and the toluene layer discarded [12].

Samples (50 μl in 0.1 *M* HCl) were injected through an ISS 101 autoinjector (Perkin-Elmer, Beaconsfield, UK) onto a Waters RCM 8 × 10 radial compression system containing a 10 × 0.8 cm I.D. Resolve C_{18} cartridge, 5 μm particle size (Waters Chromatography, Millipore, Watford, UK). The analytical column was protected by a Waters Guard-Pak guard column containing a 0.5 × 0.4 cm Resolve C_{18} insert, 5 μm particle size. The thiopurines were detected using a Waters 994 programmable photodiode-array detector and the peaks at 303 nm were traced onto a Waters 5200 printer plotter. A Waters 510 chromatography pump, flow-rate 1.5 ml/min, was used for solvent delivery. The mobile phase of methanol–water (20:80, v/v) contained 100 m*M* triethylamine and was adjusted to pH 3.2 with orthophosphoric acid. DTT, 0.5 m*M*, was added to the mobile phase immediately prior to use and the solution purged with helium. The column was equilibrated overnight with 60 ml of mobile phase prior to use.

2.6. Calibration

Calibration graphs were constructed by spiking substrate blank incubates (i.e. the incubation mix with 6-MP but without the cosubstrate S-adenosyl-L-methionine) with 6-methyl-MP standards in the range 12–600 pmol/100 μl lysate. These spiked incubates were treated in parallel with the TPMT assay incubation.

2.7. Assay optimisation

The TPMT reaction with 6-MP as a substrate was studied under varying conditions in quadruplicate experiments. With the exception of the variable under study the constituents of the assay mixture were as stated above. The rate of the TPMT reaction was studied in sodium phosphate, potassium phosphate and Tris-HCl buffers at pH 7.2, 7.5 and 7.8. The linearity of the reaction was studied at 9 time points from 0 to 120 min using 100 μl lysate and, at a fixed time of 60 min, with the amount of lysate used varied.

The maximum lysate volume feasible in the incubation mix was 100 μl. The stored lysate was a 5-fold dilution of RBC supernatant and the reaction rate was studied with the volume of this lysate varied, in 25-μl increments, from 0 to 100 μl. To study the effect of higher lysate volumes a 2.5-fold dilution of RBCs was prepared.

2.8. Michaelis constants

The K_M and v_{max} for 6-MP were calculated from quadruplicate experiments using 14 concentrations of 6-MP from 0 to 558 μ*M* at a constant 25 μ*M* of S-adenosyl-L-methionine. The volume of the lysate used was 100 μl. K_M and v_{max} values were estimated by fitting regression lines to the experimental data points using the Lineweaver–Burk method. To allow for the distortion of the error distribution during the Lineweaver–Burk transformation final values were calculated using non-linear regression.

2.9. Statistical analysis

Comparison of means was made by one-way-analysis of variance and comparison of medians by the Mann–Whitney U test. The correlation between normally distributed variables was assessed by linear regression and association between measurements of TPMT activity by the Spearman rank correlation coefficient (r_S).

3. Results

3.1. TPMT reaction

Substrate concentration

6-MP has a low solubility in aqueous solution. For this reason the organic solvent dimethyl sulphoxide (DMSO) is frequently used to produce high-concentration solutions. But, PMA extractable methyl-MP metabolite was produced in the absence of S-adenosyl-L-methionine when DMSO 6-MP solutions were used in the HPLC assay procedure. An 18 mg/ml solution of 6-MP in DMSO (5 μl in a 150-μl incubate, final concentration 3.53 m*M*), as used in the radiochemical assay [13], produced 18.8 μmol of methyl-MP per millilitre of packed RBCs per hour of incubation in the absence of S-adenosyl-L-methionine. The rate of methyl-MP formation exceeded the quoted range of RBC-TPMT activity by three orders of magnitude. This was attributed to non-enzymatic S-methylation of 6-MP by DMSO primarily during the acid hydrolysis step.

The maximum 6-MP concentration for an aqueous solution was found to be 250μg/ml; this solution was prepared immediately prior to use. To maximise the 6-MP concentration allopurinol was omitted from the incubate–the red cell lacks functional xanthine oxidase activity. The final 6-MP concentration was 558 μ*M*. An incubate blank containing 6-MP but without the essential co-substrate SAM was included in all the incubations.

Optimal incubation conditions

Analysis of variance showed no significant difference in the rate of TPMT activity at pH 7.5 in either sodium phosphate, potassium phosphate or Tris-HCl buffers ($P = 0.40$). Neither was any significant difference in enzyme activity observed over the pH range studied ($P = 0.23$; mean activity 13.4 U, C.V. 8.3%, $n = 36$).

The rate of the TPMT reaction was linear up to 120 min; $y = 1.09 + 0.205x$, $r^2 = 99.3$. With the incubation time fixed at 60 min the reaction rate was linear with respect to lysate volume up to 150 μl; $y = -0.04 + 0.013x$, $r^2 = 99.7$ (Fig. 1).

Metabolite extraction and chromatography

The duration of the acid hydrolysis step influences the amount of PMA-extractable methyl-MP metabolite formed. A previous assay [12] has shown that the amount of extractable methyl-MP increases steadily over a 3-h period but 89% is produced in the first 2 h. Thus, a 2-h hydrolysis was used in this assay. To optimise the chromatography of the methyl-MP metabolites the methanol concentration of the mobile phase was increased to 20% and the flow-rate adjusted to 1.5 ml/min. These modifications reduced the lower limit of detection of the methylated metab-

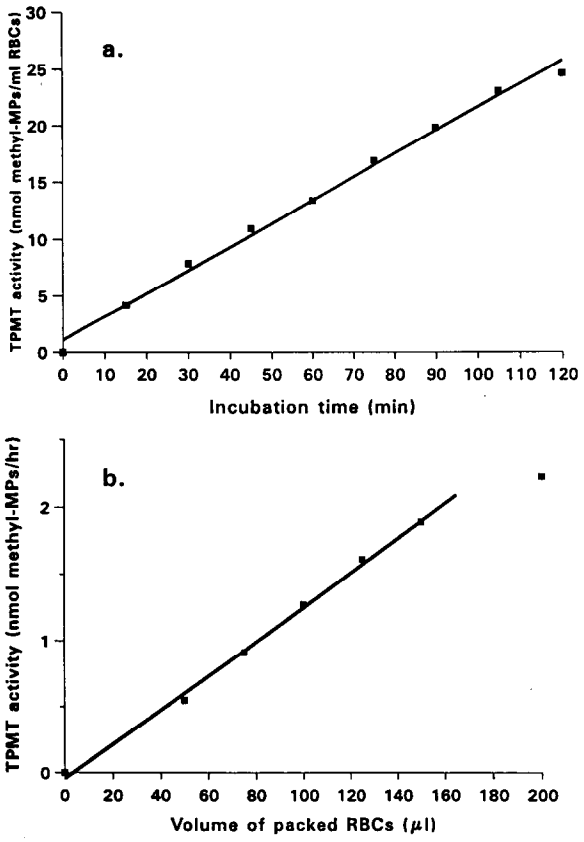


Fig. 1. TPMT activity measured (a) when the incubation time was varied from 0 to 120 min, and (b) when the equivalent of a 5-fold diluted lysate was adjusted from 0 to 200 μl.

olites 20-fold. Although the chromatogram was recorded from 0 to 10 min the integrator output was set to register from 4 to 6 min. This was to maximise the methyl-MP peak height (retention 4.7 min) whilst ignoring the 6-MP substrate (retention 3.2 to 4.5 min). Fig. 2 illustrates the chromatographic separation of methyl-MP from the substrate 6-MP.

3.2. Calibration curves and quality controls

The methyl-MP calibration curves were linear with correlation coefficients >0.99. A typical calibration graph gave a regression of $y = 0.024 + 0.042 \times$. The inter-assay C.V. over the concentration range of 12–600 pmol/100 μl spiked lysate, evaluated over 8 assays, ranged from 2.7 to 8.6%.

Throughout the TPMT incubations a quality control lysate was included. The activity measured by the radiochemical assay was 13.2 units TPMT. Enzyme activity measured by the HPLC assay (11 assays over an 8-month period) was 12.7 units TPMT, C.V. 10.1%.

3.3. Blood storage

Table 1 summarises the TPMT activities measured in the red cell lysates prepared from blood samples kept at room temperature (range 18–20°C) and 4°C for up to 4 days. Each lysate preparation was assayed in quadruplicate. No red cell lysis was observed in any of the blood samples during the storage procedures. There was no significant difference between the TPMT activity measured in the lysate prepared immediately and those stored for 1 day ($F = 0.21$, $P = 0.67$). There was no difference in TPMT activities measured in the blood samples kept at room temperature and those stored at 4°C ($F = 3.46$, $P = 0.073$). The mean TPMT activity (day 0 to day 4, all conditions) was 13.9 units (C.V. 10%).

3.4. Michaelis constants

The K_M and v_{max} for 6-MP were 83 μM and 15.6 μmol per millilitre packed RBCs/h respectively (Fig. 3).

3.5. Population studies

Red cell lysates were prepared from 130 control children. Of these samples 111 were available for analysis by both the radiochemical and HPLC methods. TPMT activities measured by the radiochemical assay ranged from 0.5 to 18.5 units (median 12.9). This data has been previously published [9]. TPMT activities measured by the HPLC method ranged from 0.65 to 18.8 units (median 12.9). The median difference between the two assays was 0.14 units, $P = 0.67$, N.S. (not significant). The frequency distribution histograms are illustrated in Fig. 4. The Spear-

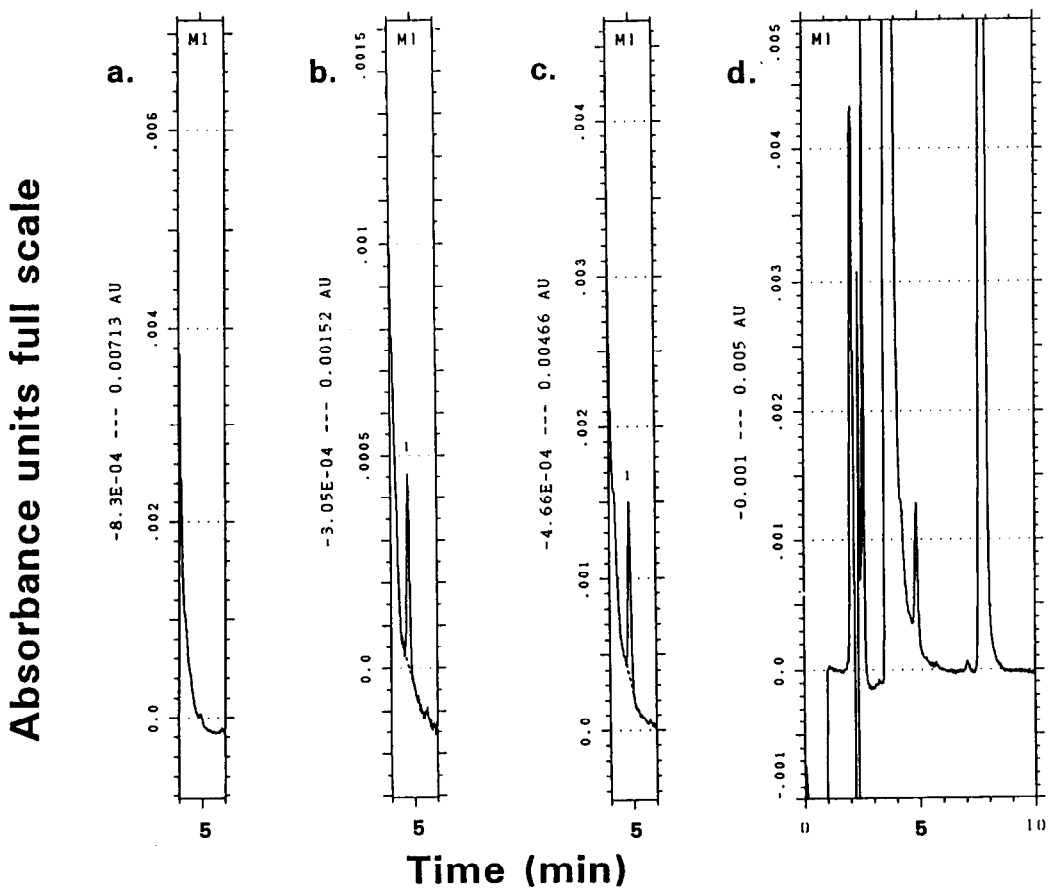


Fig. 2. Chromatographic separation of methyl-MP from the substrate 6-MP in extracted lysate samples. (a) Integrator output of the substrate blank; (b) integrator output of lysate spiked with 60 pmol of methyl-MP, methyl-MP elutes at 4.7 min; (c) integrator output of methyl-MP produced from the quality control subject lysate (13 nmol of methyl MP/ml packed RBCs); (d) full chromatogram showing the solvent front (2 to 2.5 min), 6-MP (3.2 to 4.5 min), methyl-MPs (4.7 min) and an endogenous peak (7.5 min).

man rank correlation coefficient was used to assess the association between TPMT activities measured by both methods, $r_S = 0.68$, $z = 7.13$, $P < 0.0001$ (Fig. 5).

Visual comparison of the two distributions in Fig. 4 shows the break point between the intermediate and high TPMT activities to be >9.5 and >8.5 with the radiochemical and HPLC assays respectively but no child was "misclassified" (Fig. 5). Comparison of the 100 children with high TPMT activities, assuming a normal distribution, gave mean values of 13.24 units (S.D. 1.57) and 13.07 units (S.D. 1.99) for the radiochemical and HPLC assays respectively ($F = 0.44$, $P = 0.51$, N.S.). Likewise, comparison of the boys versus the girls TPMT activity within the high group showed no significant difference ($P > 0.8$, both assays) and comparison of boys versus girls with respect to TPMT activity for the group as a whole showed no significant difference (Mann–Whitney, $P > 0.25$, both assays).

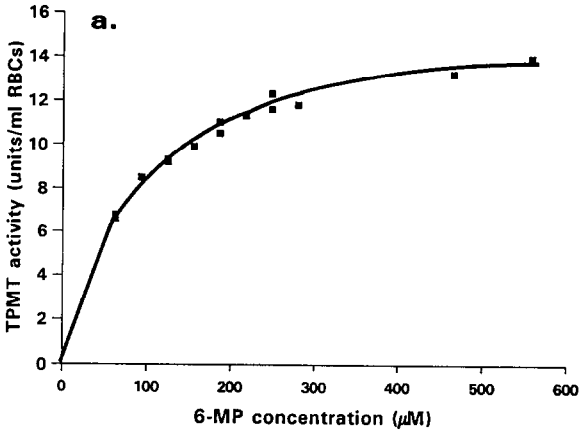
4. Discussion and conclusions

The HPLC assay of RBC-TPMT activity described in this paper has shown itself to be equivalent to the established radiochemical

Table 1
TPMT activities measured in red cell lysates

Day	TPMT activity (U/ml packed RBCs[a])	
	18–20°C	4°C
0	14.5 (6.1)	–
1	14.3 (3.0)	15.2 (3.8)
2	12.3 (6.4)	11.6 (6.7)
3	13.8 (3.1)	14.7 (9.5)
4	12.7 (7.3)	15.7 (3.7)

Prepared from blood samples kept at room temperature (range 18–20°C) and 4°C for up to 4 days. The day 0 sample was prepared immediately after venipuncture. Each lysate preparation was assayed in quadruplicate.
[a]Values in parentheses are coeffients of variation (%).



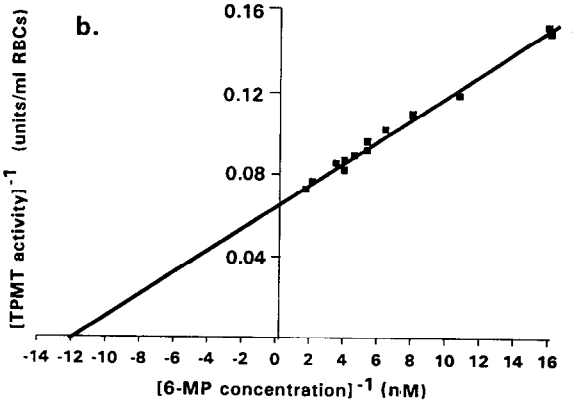


Fig. 3. TPMT reaction, kinetic data. The substrate curve (a) and double reciprocal plot (b).

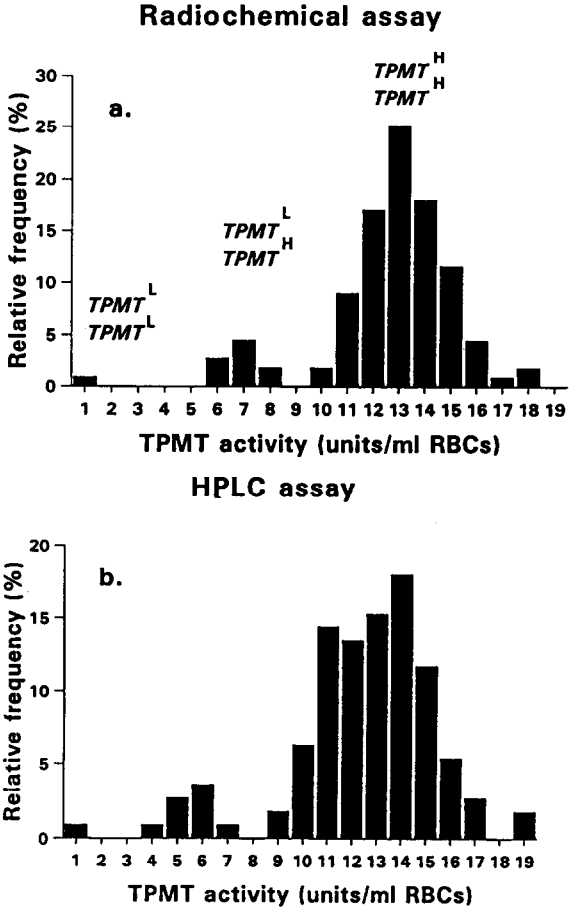


Fig. 4. TPMT frequency distribution histograms for RBC enzyme activities measured in 111 children by the radiochemical (a) and HPLC (b) methods. Proposed genotypes at the locus TPMT have been assigned on the basis of previous population, genetic and family studies [4].

method; there was a strong correlation between TPMT activities measured by both methods and in a population of control children there was no significant difference between the two frequency distribution histograms. The value of the apparent K_M for 6-MP measured by the HPLC method was 4-fold lower than that reported for RBC TPMT when measured by radiochemical techniques (83 μM compared to 320 μM, respectively). This may be a reflection of the lower 6-MP concentration used in the HPLC assay. The true K_M value for the RBC enzyme has been reported to be 290 μM [8].

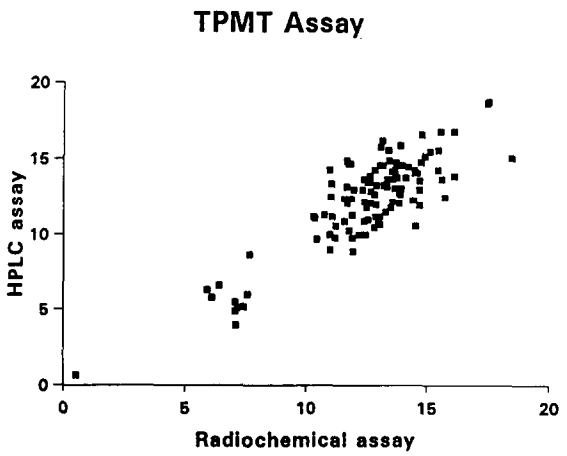


Fig. 5. Scatter diagram showing the correlation between the radiochemical and HPLC assays. The units on the x- and y-axis are TPMT activity (nmol methyl-MP per hour per millilitre packed RBCs).

It was not possible to use a DMSO 6-MP solution due to the non-enzymatic S-methylation of 6-MP by DMSO in the absence of S-adenosyl-L-methionine. S-Methylation of 6-MP, 6-MP ribonucleoside and 6-MP ribonucleotide can occur by a non-enzymatic process in vitro in the presence of S-adenosyl-L-methionine [14] and this has been observed in the buffer blank of the radiochemical assay [13]. But, in the radiochemical assay this non-enzymatic transfer produces counts of radioactivity 6.7-fold less than those recorded in the red cell lysate with the lowest TPMT activity. The high concentration of methyl-MPs produced by heating 6-MP and DMSO, in the absence of S-adenosyl-L-methionine, requires an additional explanation. The DMSO probably acts as a source of methyl groups for the non-enzymatic formation of methyl-MPs during the 100°C heating step. Acid hydrolysis is an essential part of the assay procedure, the methyl-MP is modified in such a way that it can be specifically extracted by phenyl–mercury adduct formation [12]. The use of aqueous 6-MP solutions results in lower drug incubate concentrations, approximately twice the K_M values, but this was sufficient for the quantitative determination of TPMT activities.

The HPLC assay of TPMT activity described in this paper uses analytical techniques similar to those employed in the routine measurement of 6-MP metabolites. The measurement of TPMT activity prior to drug therapy would enable those individuals who may be unduly sensitive or resistant to 6-MP to be quickly identified. A proposed study of TPMT activities in individuals outside our immediate area prompted us to investigate the stability of the enzyme in whole blood in which the enzyme lysate was not prepared immediately on receipt of the sample. When the red cells remain intact the enzyme activity appears to be relatively stable for up to 4 days.

TPMT-catalysed S-methylation of thiopurine drugs is a significant route of biotransformation. Individuals with congenital deficiency are at greatly increased risk for the occurrence of life-threatening myelosuppression when treated with these drugs [9–11] while patients with very high TPMT activity may be undertreated with standard doses [2,9]. A number of population studies describing the TPMT genetic polymorphism have now been published [4,15] and possible ethnic and gender differences in enzyme activity have been described [16–18], although the small absolute differences in TPMT activity reported indicate that this possible gender difference is of minor clinical importance [19]. Such a gender difference was not apparent in the population of children reported in this paper. It is clear that the response to thiopurine therapy is multifactorial but, one major factor is undoubtedly genetically-controlled TPMT activity and pre-therapy assay of this enzyme activity would help identify those patients with high-risk profiles.

Acknowledgements

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