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ASSAY OF 6-THIOINOSINIC ACID AND 6-THIOGUANINE NUCLEOTIDES, ACTIVE METABOLITES OF 6-MERCAPTOPURINE, IN HUMAN RED BLOOD CELLS

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SUMMARY

A highly sensitive reversed-phase high-performance liquid chromatographic assay, with ultraviolet detection, for 6-thioinosinic acid and the 6-thioguanine nucleotides (6TGNs) was developed. The 6TGNs are major red blood cell metabolites of the immunosuppressive agent azathioprine and the cytotoxic drugs 6-thioguanine and 6-mercaptopurine. The assay is based on the specific extraction, via phenyl mercury adduct formation, of the thiopurine released on acid hydrolysis of the thionucleotide metabolite. Red blood cell 6TGN concentrations in eighteen leukaemic children receiving chronic 6-mercaptopurine chemotherapy were measured and compared to a previously published spectrophotofluorometric assay. Linear regression analysis gave $r=0.991$; $P<0.001$; $y=40+0.94x$.

INTRODUCTION

The cytotoxic drug 6-mercaptopurine (6MP) has no intrinsic anticancer activity; it is a prodrug. After an oral dose 6MP undergoes extensive intestinal and hepatic metabolism along several possible pathways. The biological and biochemical effects of 6MP are attributed to the intracellular transformation of the thiopurine to its nucleotide [1]. This intracellular activation results in the formation of a variety of active nucleotide metabolites; the major ones in the human red blood cell (RBC) are the 6-thioguanine nucleotides (6TGNs) [2,3]. The 6TGNs are also the major RBC metabolites of the cytotoxic drug 6-thioguanine (6TG) and the immunosuppressive agent azathioprine [2]. The cytotoxicity of these purine analogue drugs is due, in part, to the incorporation of 6TGNs into DNA [4].

In previous studies we have shown that increased RBC 6TGN concentrations are related to neutropenia in leukaemic children receiving chronic 6MP chemotherapy [5–7] and that 6TGNs are a better index of a child's ability to form active

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cytotoxic metabolites than 6MP dose or plasma concentrations [7]. Elevated RBC 6TGN concentrations are directly related to bone marrow failure in adults receiving azathioprine immunosuppression [8,9]. In addition, the acute sensitivity to azathioprine and 6MP, shown by some individuals, has a pharmacogenetic basis [10].

There are a number of methodological problems associated with the development of non-radioisotopic techniques for the extraction and measurement of low concentrations of intracellular metabolites in small quantities of biological fluids. The major drawback in thiopurine drug analysis is the lack of commercially available standards for many of the active nucleotide metabolites. Excellent high-performance liquid chromatography (HPLC) separations of 6-thiopurines and their metabolites have been achieved enabling the measurement of in vitro [11,12] and in vivo [3] concentrations of these compounds. However, to separate fully the thionucleoside mono-, di- and triphosphates, these methods have used multi-step extraction procedures and linear gradients resulting in long retention times and, thus, are probably unsuitable for routine clinical use. Ion-pair HPLC for 6-thioinosinic acid and 6-thioguanosine monophosphate has been reported [13] and flow fluorometric HPLC analysis of oxidised 6-thioguanosine monophosphate, after a single-step extraction, is highly sensitive [14]. In the latter case the fluorophore formed is unstable, thus unsuitable for autoinjection, a necessity when handling large numbers of biological samples for HPLC analysis. In the light of the well documented nucleotide phosphate interconversions within the RBC [3,14,15] quantitation of thionucleotide monophosphates alone may not give an accurate estimation of the cytotoxic potential of intracellular active metabolites.

We have, for many years, been interested in the biochemical pharmacology of thiopurine drugs both as cytotoxic compounds and immunosuppressive agents. In previous studies we have used a spectrophotofluorometric assay [2] for the quantitation of intracellular 6TGN concentrations. This assay was based upon the partial purification of intracellular thionucleotide metabolites, hydrolysis to the parent thiopurine, specific extraction of the thiopurine adduct and fluorescence detection of released, oxidised thiopurine. The conditions of fluoresense were optimised to distinguish between 6TG produced from 6TGNs [2] and 6MP produced from thioinosinic acid [16]. Total 6TGN concentrations are measured to avoid errors associated with the time-dependent 6-thioguanosine mono-, di- and triphosphate interconversions [3,14,15].

We report here a specific and sensitive HPLC method for the quantification of RBC 6TGNs. The extracted thiopurines are stable and thus suitable for autoinjection. This assay is compared to our previously published direct fluorometric assay for the measurement of RBC 6TGN concentrations in leukaemic children receiving 6MP consolidation chemotherapy.

EXPERIMENTAL

Materials

6MP, 6-thioinosinic acid (6TIA; 6-mercaptopurine riboside-5′-phosphate), 6TG (2-amino-6-mercaptopurine), 6-thioxanthine (6TX; 2-hydroxy-6-mercap-

topurine) and DL-dithiothreitol (DTT) were obtained from Sigma (London, U.K.). 6-Thiouric acid (6TU; 2,8-hydroxy-6-mercaptopurine), 8-hydroxy-6-mercaptopurine (8-OH6MP), 8-hydroxy-6-thioguanine (8-OH6TG) and 6-thioguanosine 5′-monophosphate (6TGMP) were gifts from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.). Stock solutions (100 μg/ml) of the thiopurines were prepared by dissolving, e.g., 10 mg in 0.1 *M* sodium hydroxide (4 ml) and making up to volume with water (6TG, 6TX) or, for all other thiopurines, water plus 1 *M* hydrochloric acid to a final concentration of 0.1 *M* acid The thiopurine stocks were stable, at 4°C, for over one month, and all stock solutions were diluted prior to use in 0.1 *M* hydrochloric acid containing 1 m*M* DTT.

Methanol (HPLC grade) and toluene (glass-distilled) were obtained from Rathburn Chemicals (Walkerburn, U.K.). Amyl alcohol, AnalaR grade (BDH, Poole, U.K.), final concentration 170 m*M*, and phenyl mercury acetate (PMA; organic analytical standard; BDH), final concentration 1.3 m*M*, were added to the toluene before use. All stock solutions were protected from light during storage. Hanks-balanced salt solution (HBSS) was obtained from Flow Labs. (Rickmansworth, U.K.). All other reagents were of analytical grade and obtained from BDH. With the exception of the mobile phase, in which the water used was distilled, the water used for all solutions was distilled and deionised. The glassware used for the analysis of thiopurines was soaked overnight in 30% nitric acid and rinsed in distilled, deionised water.

Sample preparation

Whole blood was collected in lithium heparin tubes at least 6 h after an oral dose of 6MP. The whole blood was centrifuged at 160 g for 10 min at 4°C, and the platelet-rich plasma and "buffy coat" were discarded. The RBCs were washed twice in two volumes of HBSS and centrifuged firstly at 160 *g* (10 min, 4°C) and secondly at 640 *g* (10 min, 4°C). Finally, the RBCs were resuspended in one volume of HBSS and counted in a Model Dn cell counter (Coulter Electronics, Luton, U.K.). The diluted RBCs were stored, at a concentration of approximately $8 \cdot 10^8$ cells per 200 μl, at −20°C. Control RBCs obtained from the venous blood of normal healthy volunteers were similarly processed.

Extraction

RBCs (0.2 ml) were added to a 10-ml round-bottomed graduated test-tube containing 0.5 ml of water, 0.5 ml of 1.5 *M* sulphuric acid and 0.3 ml of 10 m*M* DTT. The tubes were gently mixed before heating at 100°C for 1 h in a Dri-Block (Techne, Cambridge, U.K.). To each tube, after cooling, was added 0.5 ml of 5 *M* sodium hydroxide followed by 8 ml of toluene containing 170 m*M* amyl alcohol and 1.3 m*M* PMA. The tubes were gently mixed for 10 min and then centrifuged at 900 *g* (5 min, 10°C). The toluene (6 ml) layer was transferred to graduated conical test-tubes and back-extracted with 0.2 ml of 0.1 *M* hydrochloric acid for 3×20 s on a Model 2601 multi-tube vortexer (Alpha Labs., Eastleigh, U.K.). The tubes were centrifuged, as before, and the toluene layer was discarded. An aliquot of the acid layer (50 μl) was injected.

HPLC procedure

Samples (50 μl) were injected through a WISP 710B autoinjector (Waters Assoc., Northwich, U.K.) onto a Z module radial compression system containing a 10×0.8 cm Nova-Pak cartridge, 5 μm particle size (Waters Assoc.). The analytical column was protected by a Guard-Pak (Waters Assoc.) guard column module containing a C_{18} insert. The thiopurines were detected with a Model 4020 variable-wavelength UV detector (Pye-Unicam, Cambridge, U.K.) set at 342 nm (6TG, 8-OH6TG, 6TX and 6TU) or 322 nm (6MP and 8-OH6MP) and a sensitivity of 0.01 a.u.f.s. Peaks were traced on a Model 6500 flat-bed potentiometer recorder (LKB, Croydon, U.K.) at 300 mm/h. A Model 6000A chromatography pump (Waters Assoc.) was used for solvent delivery. The mobile phase of methanol–water (10:90, v/v) contained 100 m*M* triethylamine, adjusted to pH 3.2 with orthophosphoric acid, and 0.5 m*M* DTT, the latter added immediately prior to use. The column was flushed daily and left standing in water.

Calibration

Calibration graphs were constructed by spiking control RBCs with 6TGMP and TIA aqueous standards in the range 30–3000 pmol per $8 \cdot 10^8$ RBCs. No suitable internal standard could be found for this assay; therefore, thiopurine RBC standards were prepared and treated in parallel with the patients' RBC samples.

RESULTS

Calibration curves

The assay was developed using 6TGMP and 6TIA. The concentration of 6TG and 6MP released from 6TGMP and 6TIA, respectively, after heating at 100°C for 1 h, was equivalent to that extracted from equimolar 6TG and 6MP standards added to tubes containing control RBCs prior to the heating step. The conversion of 6TGMP to 6TG, over two standard curves, was 98% (coefficient of variation, C.V. = 3.76%, $n = 12$). Due to a limited supply of 6TGMP (< 10 mg) the standard curves for 6TGN assay were constructed using 6TG added to RBCs prior to the addition of acid and the hydrolysis step. The calibration curve for 6TGNs was linear and passed through the origin (Table I). The conversion of 6TIA to 6MP was 100%. Calibration curves, constructed using 6TIA, were linear and passed through the origin (Table II). The retention times of 6TG and 6MP were 2.8 and 3.3 min, respectively, and the lower limit of detection for the assay, at a signal-to-noise ratio of 2.5:1, was 30 pmol 6TGNs and 33 pmol 6TIA per $8 \cdot 10^8$ RBCs for a 50 μl injection.

Extraction efficiency

The mean extraction efficiencies for 6TGNs and 6TIA at 600 pmol per $8 \cdot 10^8$ RBCs over a six-month period were 64.2% (C.V. 2.83%) and 65.5% (C.V. 2.45%), respectively. The extraction efficiency was dependent upon the PMA concentration in the organic phase. The PMA, at a final concentration of 1.3 m*M*, was added directly to toluene, containing 170 m*M* amyl alcohol, in an Oxford pipettor. Extracting the 6TG, produced from 6TGMP, from RBCs with 6, 8 or 10 ml of

TABLE I

ASSAY REPRODUCIBILITY IN RED BLOOD CELLS

Mean of twelve 6TGN curves over a three-month period. Due to a limited supply of 6-thioguanosine monophosphate standard curves for 6TGN assay were constructed using 6TG added to RBCs prior to the addition of acid and the hydrolysis step.

6TGN concentration (pmol per $8 \cdot 10^8$ RBCs)	Peak height (mean ± S.D.) (cm)	Coefficient of variation (%)
30	0.57 ± 0.04	7.8
60	1.20 ± 0.09	7.5
120	2.38 ± 0.10	4.3
300	5.91 ± 0.30	5.1
600	12.15 ± 0.54	4.5
1200	24.6 ± 1.6	6.5

organic phase produced extraction efficiencies of 55, 64 and 76%, respectively. The most convenient volume of organic phase, for use in 10-ml graduated test-tubes, was 8 ml. The PMA was dissolved in toluene in an attempt to overcome the stability problems associated with aqueous solutions of PMA used in previous assays [2,17]. The same batch of toluene–amyl alcohol–PMA was used over a two-month period with no decrease in extraction efficiency.

Stability of thiopurines

The extracted thiopurines, produced from the acid hydrolysis of thionucleotide, were stable in the 0.1 *M* hydrochloric acid back-extract for over seven days when kept at 4 °C and over 36 h when kept at room temperature. 6TGMP (300 pmol) and 6TIA (200 pmol) were added to, and the thiopurines produced on acid hydrolysis extracted from, RBCs. The 0.2-ml back-extracts from ten extractions were pooled and kept at 4 °C. Aliquots were removed and injected, via an autoinjector, onto the chromatograph three times daily, at 24 h intervals over seven

TABLE II

ASSAY REPRODUCIBILITY IN RED BLOOD CELLS

Mean of twelve 6TIA curves over a three-month period.

6TIA concentration (pmol per $8 \cdot 10^8$ RBCs)	Peak height (mean ± S.D.) (cm)	Coefficient of variation (%)
20	0.43 ± 0.04	9.5
40	0.90 ± 0.06	7.0
100	2.22 ± 0.10	4.7
200	4.43 ± 0.21	4.6
400	9.12 ± 0.43	4.7
1000	23.1 ± 1.49	6.4

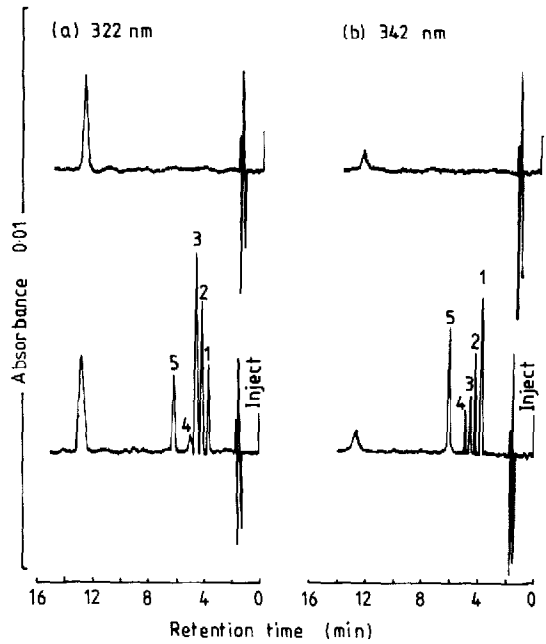

Fig. 1. Chromatographic separation of 6-thiopurines with the methanol concentration of the mobile phase reduced to 5%. A mixture containing 100 ng of each thiopurine was added to, and extracted from, $8 \cdot 10^8$ RBCs (lower traces). The upper traces represent the RBC blank. Peaks: 1 = 6-thioguanine; 2 = 8-hydroxy-6-mercaptopurine; 3 = 6-mercaptopurine; 4 = 8-hydroxy-6-thioguanine; 5 = 6-thioxanthine. The extraction efficiencies were 64, 55, 65, 19 and 62%, respectively. An endogenous red cell compound elutes at 12.5 min.

days. The mean ($\pm$S.D.) peak heights were 5.9 ± 0.9 cm and 4.4 ± 0.05 cm for 6TGMP and 6TIA, respectively. The mixture of 6TGMP and 6TIA gave the same peak heights as 6TGMP and 6TIA alone. There was no detectable loss of peak height when the same sample was kept for 36 h on the autoinjector.

Analysis of other thiopurines

The 6MP catabolites 6TU, 8-OH6TG, 8-OH6MP and 6TX were added to and extracted from RBCs at a concentration range of 500–5000 pmol catabolite per $8 \cdot 10^8$ RBCs. The addition of acid and the hydrolysis step of the thionucleotide assay was omitted. The catabolite plus RBCs, in 2 m*M* DTT, were adjusted to pH 14 with 50 μl sodium hydroxide prior to PMA extraction. The optimisation of PMA extraction conditions for 6MP and 6TG resulted in poor extraction of 2,8-substituted thiopurines from biological fluids. The extraction efficiencies of 6TU, 8-OH6TG, 8-OH6MP and 6TX, each at 100 ng per $8 \cdot 10^8$ RBCs, were 0, 19, 55 and 62%, respectively. Sharp symmetrical peaks were shown for 8-OH6MP and 6TX with retention times of 3.0 and 4.1 min, respectively. The retention times of 6TU and 8-OH6TG aqueous standards were 3.0 and 3.1 min, respectively.

The 8-hydroxythiopurines show poor recoveries from acid-precipitated RBCs. When the thionucleotide assay was followed, acid added, but the heating step omitted the recoveries of 6TU, 8-OH6TG, 8-OH6MP and 6TX, each at 100 ng

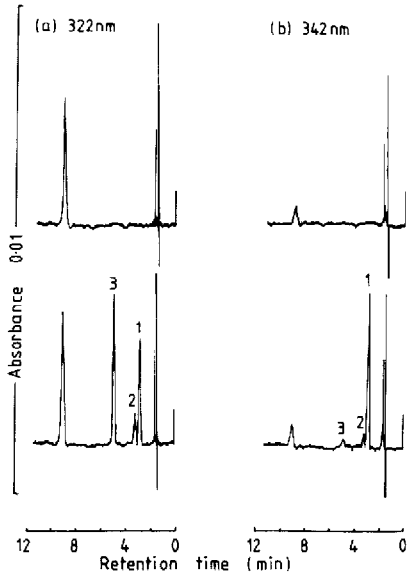

Fig. 2. Chromatographic separation of 6-thioguanine nucleotide (peak 1, 617 pmol per $8 \cdot 10^8$ RBCs) and 6-thioinosinic acid (peak 2, 86 mol per $8 \cdot 10^8$ RBCs) extracted from RBCs of a leukaemic child RB (lower trace). The nucleotides are detected as the parent purine, 6-thioguanine and 6-mercaptopurine, respectively. In addition to the endogenous RBC compound eluting at 9.0 min, peak 3 was detected in some of the children receiving chronic 6-mercaptopurine therapy. All the chromatographic traces from leukaemic children were identical to the control blank (upper traces) when either the heating step was omitted or PMA was omitted from the organic phase.

per $8 \cdot 10^8$ RBCs, were 0, 0, 11 and 62%, respectively. Including the heating step did not alter these recoveries. Reducing the methanol concentration of the mobile phase to 5% separated fully a mixture of 6TG, 8-OH6MP, 6MP, 8-OH6TG and 6TX added to, and extracted from, RBCs with retention times of 3.5, 4.1, 4.5 and 4.8 min, respectively (Fig. 1).

Analysis of patient samples

The assay reported here was used to quantify RBC 6TGN continuing in a group of leukaemic children receiving 6MP consolidation chemotherapy (Fig. 2). The drug is prescribed on a surface area basis and the daily dose of 6MP (75 mg/m^2) had been constant for at least seven days prior to assay. The 6TGN concentrations measured ranged from 168 to 1150 (mean 482) pmol per $8 \cdot 10^8$ RBCs. Five of the ten children studied also had 6TIA in their RBCs [range 39–86 (mean 63) pmol per $8 \cdot 10^8$ RBCs]. These five children also had an additional unidentifiable peak (max 300 nm) with a retention time of 4.8 min. RBC metabolite concentrations were measured in one child seven days after 6MP withdrawal. In addition to 6TGNs and 6TIA, 6TX nucleotide was detected. The thionucleotide concentrations were 138, 125 and 170 pmol per $8 \cdot 10^8$ RBCs, respectively. No child had any free 6MP, 6TG or 6TX in the RBCs. All the chromatograms from these eleven children were identical to the control blank when either the heating step

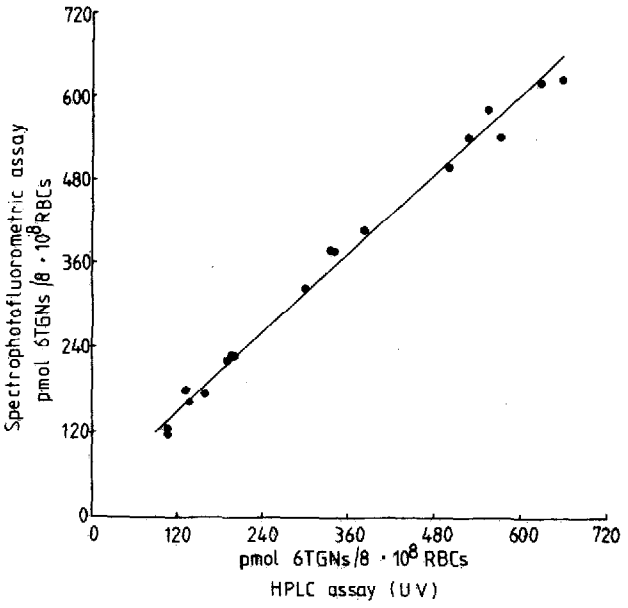

Fig. 3. Relationship between RBC 6TGN concentrations measured by the HPLC assay and the spectrophotofluorometric assay in eighteen leukaemic children receiving chronic 6-mercaptopurine chemotherapy ($r = -0.991$; $P < 0.001$; $n = 18$; $y = 40 + 0.94x$).

was omitted or PMA was omitted from the organic phase. Reassay of these samples using the mobile phase with the methanol concentration reduced to 5% confirmed nucleotide metabolites of 6MP, 6TG or 6TX, no 8-OH6MP or 8-OH6TG was detected.

Comparison of assays

Eighteen children had their RBC 6TGN concentrations quantified using both the HPLC assay detailed here and a previously published spectrophotofluorometric assay used in this laboratory [2]. Using the HPLC technique the children had a range of 108–659 (mean 335) pmol 6TGNs per $8 \cdot 10^8$ RBCs compared to a range of 120–653 (mean 352) pmol 6TGNs per $8 \cdot 10^8$ RBCs using the fluorometric assay. Linear regression analysis gave a regression coefficient of 0.991 (Fig. 3).

DISCUSSION

This paper describes a reversed-phase HPLC assay for the measurement of 6MP active metabolites, in the RBCs, which is suitable for routine clinical use. Analysis of patient samples, from leukaemic children receiving chronic 6MP chemotherapy, confirms that the 6TGNs are the major RBC thionucleotide metabolites with small quantities of 6TIA being detected in some children. No parent thiopurine was detected within the RBC during chronic 6MP dosing, previous studies using thin-layer chromatographic analysis of patient samples revealed no detectable 6-thioguanosine [2]. The RBC thionucleotide metabolites were easily measurable seven days after drug withdrawal when, in addition to 6TGNs and 6TIA, 6TX nucleotides were detected.

6TGNs were detected in all children receiving chronic 6MP therapy. The sensitivity of the assay was sufficient for routine clinical use even with the reduced extraction efficiencies caused by the low PMA concentrations during adduct formation. The concentration and amount of PMA used were kept as low as was consistent with adequate measurements of 6TGNs. To avoid any possible chronic effects associated with the use of this neurotoxin the handling of PMA and all manipulations of PMA-containing organic phase took place in a fume hood. The purity of the water used to prepare reagents can influence the formation of the phenyl mercury–thiopurine adduct [2]. Thiopurines are susceptable to oxidative degradation in aqueous solutions. The presence of contaminant ions and UV light promotes this process [18,19]. The addition of DTT both to the mobile phase and during drug extraction protects the thiol group from oxidation and, during extraction, it also prevents the binding of the thiopurine to the acid denatured proteins of the RBCs [20].

The extracted thiopurines were found to be very stable in the 0.1 *M* hydrochloric acid back-extract. No attempt was made to reconstitute the extracted drug directly into the mobile phase. Repeated injections of 0.1 *M* hydrochloric acid over a two-year period [17] had no adverse effects on the chromatogram. The control blank chromatogram traces contained no interfering peaks in the area of thiopurine drug elution. This assay compared well with the direct fluorometric assay used in this laboratory. Regression analysis showed that the direct fluorometric assay gave slightly higher readings on the extreme lower end of the standard curve than did the HPLC assay. This is perhaps due to the variable blank associated with the fluorometric measurement of oxidised thiopurines [2,16].

The 6MP active metabolites measured within the RBC may reflect those in other, less accessible, cells and tissues. This is true for 6MP catabolic enzyme thiopurine methyltransferase (TPMT). TPMT catalyses the S-methylation of thiopurine drugs such as 6MP and its activity within the human RBC is controlled by a common genetic polymorphism [21]. The genetic polymorphism that regulates RBC TPMT activity also regulates the enzyme activity in other cells and tissues [22,23]. We have previously shown that 6TGN concentrations are inversely related to TPMT activities in the RBCs of leukaemic children receiving 6MP [10].

RBCs are easily accessible and only small quantities (100 μl packed cells) are required for 6TGN assay. The 6TGN assay reported here is suitable for routine clinical use in children with acute lymphoblastic leukaemia. The monitoring of drug metabolism to active intracellular metabolites during 6MP continuing, or "remission maintenance", chemotherapy, when the peripheral blood is free of detectable neoplastic cells, may identify those children who may be resistant to the cytotoxic effects of 6MP [10] at an early stage in therapy. Improvements in the use of thiopurine drugs, as antileukaemic agents, will depend on a more precise therapy based upon a better knowledge of the biochemical pharmacology of these compounds.

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