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High-performance liquid chromatographic assay of the methyl and nucleotide metabolites of 6-mercaptopurine: quantitation of red blood cell 6-thioguanine nucleotide, 6-thioinosinic acid and 6-methylmercaptopurine metabolites in a single sample

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ABSTRACT

A reversed-phase high-performance liquid chromatographic assay was developed to quantify intracellular metabolites of the cytotoxic drug 6-mercaptopurine in the human red blood cell. The 6-thioguanine nucleotides, 6-thioinosinic acid and 6-methylmercaptopurine metabolites are measured in a single sample. A similar assay measures the parent thiopurine compounds. The limit of quantitation of the assay is 0.03, 0.03 and 0.12 nmol per $8 \cdot 10^8$ red blood cells for the 6-thioguanine nucleotides, 6-thioinosinic acid and the 6-methylmercaptopurine metabolites, respectively.

INTRODUCTION

6-Mercaptopurine (6-MP) is an important drug in the conventional therapy of childhood acute lymphoblastic leukaemia. The antileukaemic effect of 6-MP is not related to drug dose but to the red blood cell concentration of a set of active metabolites, the 6-thioguanine nucleotides (6-TGNs) [1]. One major factor influencing 6-TGN formation is 6-MP S-methylation, a catabolic pathway catalysed by the enzyme thiopurine methyltransferase (TPMT) [2]. TPMT activity is under the control of a common genetic polymorphism, and is thus constitutionally determined [3].

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The intracellular metabolism of 6-MP results in the formation of a variety of active nucleotide metabolites. We have previously reported the measurement of the 6-MP metabolites 6-thioinosinic acid (6-TIA) and 6-TGN in red cells obtained from leukaemic children undergoing 6-MP chemotherapy [4]. Assays based on this original paper are in routine clinical use as part of an extensive study into the inter-patient variability in the response to 6-MP therapy. During the course of this study HPLC technology has changed dramatically and column packings evolved such that the original assay has been extensively modified. Advances in spectroscopy have enabled a range of 6-MP metabolites to be recorded at their λ_{max}, in a single sample, and an additional metabolite of 6-MP to be detected in the chromatogram. The "unknown" compound mentioned in an earlier assay [4] has been identi-

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fied as a 6-methylmercaptopurine (methyl-MP) metabolite.

This assay is based on previously published assays in which thiopurines [*e.g.* 6-thioguanine (6-TG), 6-MP, 6-thioxanthine (6-TX)] are separated by reversed-phase HPLC and quantitated using UV detection [4,5]. For the measurement of intracellular thionucleotides the free base of each 6-MP metabolite is obtained by acid hydrolysis of the nucleotide back to the purine. The resulting purine, a 6-thiopurine, is specifically extracted from the biological matrix, by phenyl mercury adduct formation, into toluene [6]. Back-extraction of this organic phase with 0.1 *M* HCl splits the adduct and liberates the free thiopurine into the acid layer.

There are many reasons why children may not respond to standard doses of 6-MP. Children who inherit high activities of TPMT, the enzyme responsible for forming 6 methyl-MP, form low concentrations of 6-TGNs at standard 6-MP dosages. They require a higher dose of 6-MP than do the majority of children, to form cytotoxic concentrations of 6-TGNs. This assay modification was developed in order to quantitate both the cytotoxic 6-TGNs and the methyl metabolites of 6-MP in the same biological sample.

EXPERIMENTAL

Materials

6-MP, 6-TG (2-amino-6-mercaptopurine), 6-TX (2-hydroxy-6-mercaptopurine), 6-TIA (6-mercaptopurine riboside-5′-monophosphate), methyl-MP and DL-dithiothreitol (DTT) were obtained from Sigma (London, UK). Stock solutions of the thiopurines were made at 100 μg/ml [4].

Methanol (HPLC grade) and toluene (glass-distilled) were obtained from Rathburn (Walkerburn, UK). Toluene containing 170 m*M* amyl alcohol (AnalaR grade, BDH, Poole, UK) was added to phenyl mercury acetate (PMA; laboratory grade, BDH) to prepare a saturated solution of PMA (1.3 m*M*) in toluene. The PMA powder was dissolved in the organic phase by gentle mixing for 1 h; the solution was protected from light during storage. 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.

Sample preparation

Washed red blood cells (RBCs) were prepared from blood samples collected in lithium heparin tubes as previously described [4]. To enable easier pipetting of the washed RBCs the packed cell pellet was resuspended in one volume of Hanks balanced salt solution (INC Flow, High Wycombe, UK) to a concentration of $8 \cdot 10^8$ cells per 200 μl prior to storage at −20°C.

Extraction and HPLC procedure

RBCs ($8 \cdot 10^8$ cells in 200 μl) were added to 800 μl of 3.75 m*M* DTT in a 10-ml screw neck test tube (12.5 × 1.6 cm culture tube with PTFE-lined screw cap; BDH). To this were added 500 μl of 1.5 *M* H_2SO_4 and the tubes were heated at 100°C for 1 h in a Dri-Block (Techne, Cambridge, UK). In this step thionucleotides are hydrolysed back to the parent thiopurine and methyl-MP is modified in such a way that it can be extracted by phenyl mercury adduct formation. After cooling, and 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 subsequent steps in the assay have been fully described [4]. Briefly, the tubes are shaken gently for 10 min and centrifuged. Toluene (6 ml) is transferred to a glass-stoppered conical test-tube and 200 μl of 0.1 *M* HCl are added. After vortex-mixing for 3 × 20 s the tubes are centrifuged and the toluene layer is discarded.

Samples (50 μl in 0.1 *M* HCl) were injected through a ISS 101 autoinjector (Perkin-Elmer, Beaconsfield, UK) onto a Waters RCM 8 × 10 radial compression system containing a 0.8 cm × 10 cm Resolve C_{18} cartridge, 5 μm particle size (type/control No. 8C_{18}5μ/P1297A01, Waters Chromatography, Watford, UK). The analytical

column was protected by a Guard-Pak (Waters) guard column containing a Resolve C_{18} insert (0.5 cm × 0.4 cm), 5 μm particle size. The thiopurines were detected using a Model 994 programmable photodiode array detector and the peaks at 303, 322 and 342 nm were traced onto a 5200 printer plotter (Waters). A Model 510 chromatography pump (Waters) was used for solvent delivery. The mobile phase of methanol–water (7.5:92.5, 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 helium purging. Prior to use the column was equilibrated with 60 ml of mobile phase and after use it was flushed with at least 150 ml of water. It was stored in methanol.

Other assay modifications

This basic assay can be used to quantitate RBC 6-MP and 6-TG concentrations by the omission of the 100°C heating step [4]. Plasma (500 μl) can be substituted for RBCs in order to quantitate plasma thiopurine concentrations [4,5] but the heating step must be included to measure plasma methyl-MP.

Calibration

Calibration graphs were constructed by spiking RBCs with 6-TG and 6-TIA aqueous standards in the range 0.03–0.9 nmol (5–150 ng 6-TG; 15–450 ng 6-TIA) per $8 \cdot 10^8$ RBCs assayed, and methyl-MP in the range 0.3–30 nmol (0.05–5 μg) per $8 \cdot 10^8$ RBCs assayed. During the initial stages of assay development the thionucleotide standards 6-thioguanosine-5′-monophosphate (a 6-TGN) and 6-TIA were used. The conversion of the thionucleotides to their respective thiopurines during the hydrolysis step is 100% [4]. Due to a limited supply of 6-TGN the standard curves for 6-TGN were constructed using 6-TG-spiked RBCs. Spiked control RBCs containing 6-TG, 6-TIA and methyl-MP were prepared and treated in parallel with all patient RBC samples.

RESULTS

Chromatography

Fig. 1A illustrates the chromatographic separation of 6-TG, 6-MP and 6-TX aqueous standards each at a concentration of 75 pmol per 50-μl injection. The thiopurines were eluted with retention times of 4.1, 5.0 and 6.7 min for 6-TG, 6-MP and 6-TX, respectively. Fig. 1B illustrates the chromatographic separation of an RBC extract spiked with 0.6 nmol of 6-TG, 0.6 nmol of 6-TIA and 6 nmol of methyl-MP at concentrations of 0.15, 0.15 and 1.5 nmol per 50-μl injection for 6-TG, 6-TIA and methyl-MP, respectively. Approximately 600 50-μl injections of thiopurines in 0.1 *M* HCl have been separated on the Resolve column per month for over twelve months with no deterioration in column properties. The pre-column was changed monthly.

Fig. 2 shows the separation of the thiopurines extracted from the RBCs of two children undergoing 6-MP chemotherapy. The 4.2- and 5-min peaks represent the 6-TG and 6-MP liberated from their nucleotides 6-TGNs and 6-TIA, respectively. The 8.2-min peak has been previously reported eluting at 4.8 min on a Nova-Pak column with the methanol concentration of the mobile phase at 10%; the UV detector was fixed at 322 nm [4]. The diode array detector enables this compound to be measured at its λ_{max} (303 nm).

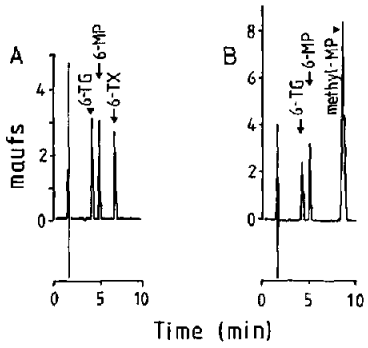

Fig. 1. HPLC separation of (A) a standard solution of thiopurines containing 75 pmol of 6-thioguanine (6-TG), 6-mercaptopurine (6-MP) and 6-thioxanthine (6-TX) and (B) an extract of RBCs spiked with 0.6 nmol of 6-TG, 0.6 nmol of 6-thioinosinic acid (peak = 6-mercaptopurine, 6-MP) and 6 nmol of 6-methylmercaptopurine (methyl-MP).

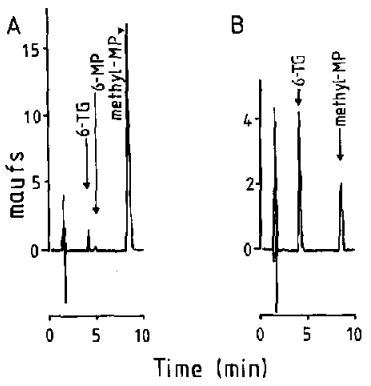

Fig. 2. HPLC separation of 6-thioguanine (6-TG), 6-mercaptopurine (6-MP) and 6-methylmercaptopurine (methyl-MP), liberated from the 6-thioguanine nucleotides, 6-thioinosinic acid and the methyl-MP metabolite, respectively, and extracted from the RBCs of two children (A and B) undergoing 6-MP therapy (dose = 75 mg/m^2).

Identity of 8.2-min peak

The observation that this metabolite was present in large amounts in children who formed only low concentrations of 6-TGN metabolites (Fig. 2A) whilst in those children producing high cytotoxic concentrations of 6-TGNs this metabolite was present in low amounts (Fig. 2B) suggested that we could be measuring a product of the TPMT reaction, *i.e.* methyl-MP.

The compound eluting at 8.2 min in the patient samples had the same retention time and λ_{max} as methyl-MP aqueous standard taken through the assay procedure. The spectra of the 8.2-min eluting compound gave a match of 995/1000, over a wavelength range of 250–400 nm, against the methyl-MP standard (Fig. 3).

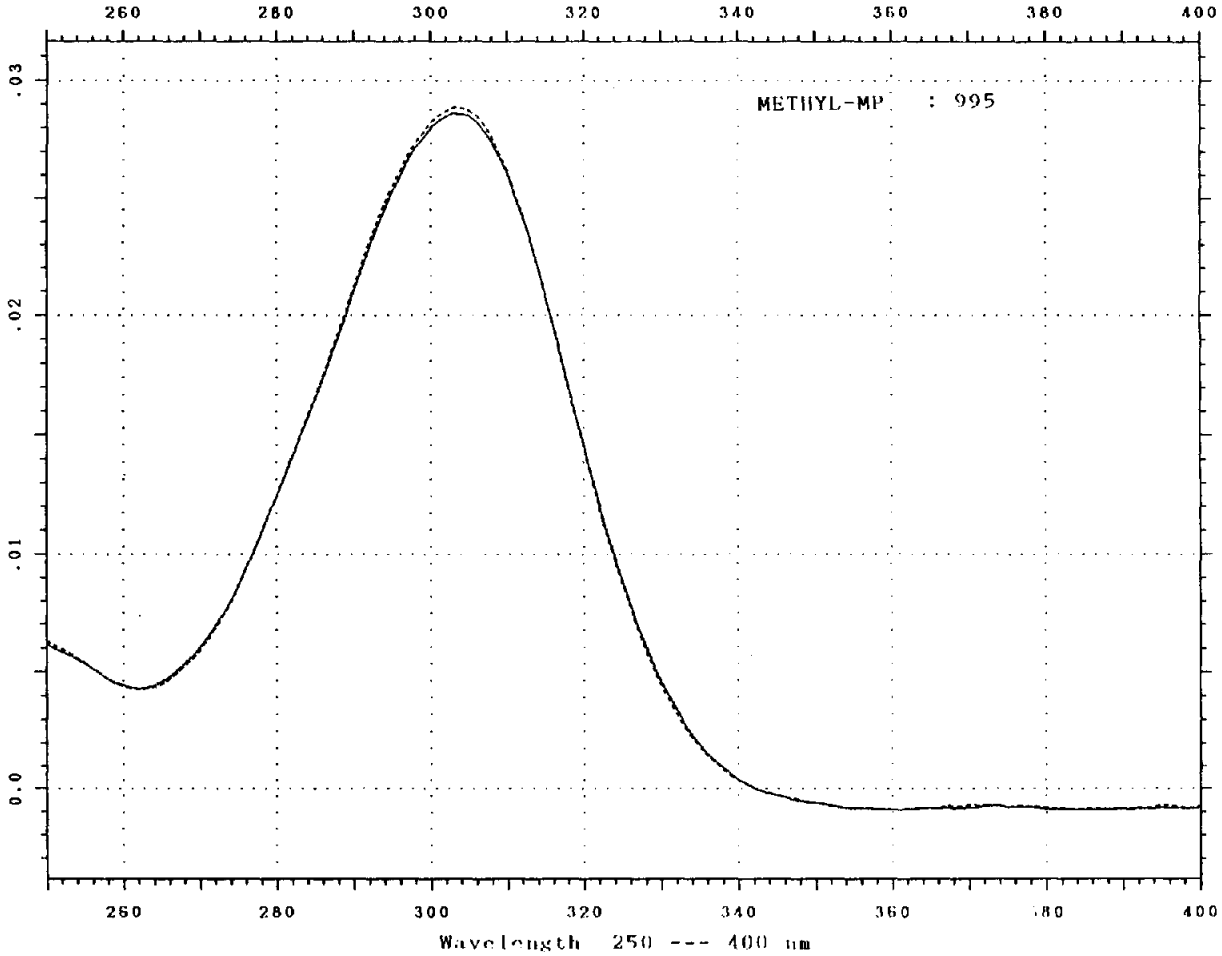

Fig. 3. Spectra of the metabolite eluting at 8.2 min in the patient samples superimposed on the 6-methylmercaptopurine (methyl-MP)-extracted standard.

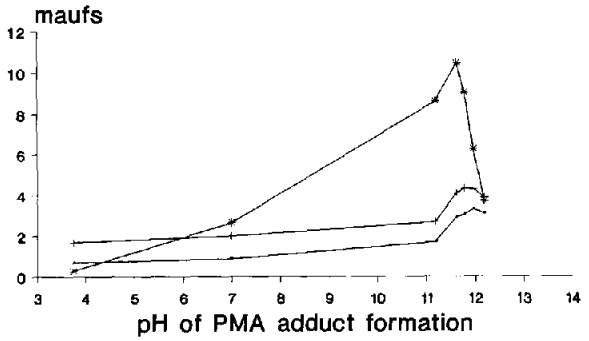

Fig. 4. Influence of the pH of phenyl mercury adduct formation on the extraction of 6-thioguanine (■), 6-mercaptopurine (+) and 6-methylmercaptopurine (*).

Optimization of assay

Adduct formation. The pH of phenyl mercury adduct formation was varied from 3.7 to 12.2 by the addition of varying volumes of 4 *M* NaOH (380–500 μl) to the acid hydrolysate sample prior to the addition of the toluene–amyl alcohol–PMA mixture. At each pH a mixture of 600 pmol (100 ng) of 6-TG, 660 pmol (100 ng) of 6-MP and 6 nmol (1 μg) of methyl-MP was extracted from spiked RBCs. Fig. 4 illustrates the change in extraction efficiency as a function of the pH of adduct formation. Optimum extraction of 6-TG, 6-MP and methyl-MP occurred at pH 11.6 corresponding to the addition of 425 μl of 4 *M* NaOH (for practical reasons 500 μl of 3.4 *M* NaOH was used in the routine assay).

Acid hydrolysis. The duration of the acid hydrolysis step was varied from 0 to 3 h; at each time point RBCs spiked with 6 nmol of methyl-MP were heated at 100°C (Fig. 5). The acid hydrolysis step was required to produce the methyl-MP metabolite extracted by phenyl mercury adduct formation. Increasing the time of the acid hydrolysis step produced more extractable methyl-MP.

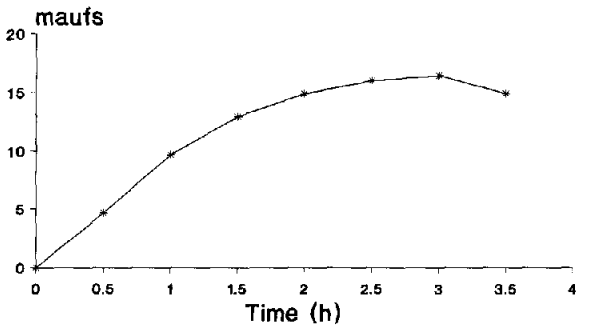

Fig. 5. Duration of the acid hydrolysis step and the production of the methyl-MP-extractable metabolite.

No metabolite was extracted from the unheated sample. In addition, if, after the acid hydrolysis step and pH adjustment to 11.6, PMA was omitted from the toluene–amyl alcohol mixture no extraction of methyl-MP metabolite occurred.

Calibration curves

Calibration curves were linear with correlation coefficients greater than 0.99. A typical calibration graph gave a regression of $y = -0.103 + 4.66x$ for 6-TG, $y = -0.072 + 5.47x$ for 6-TIA and $y = -0.072 + 1.48x$ for methyl-MP, where y is the peak height and x is the RBC thiopurine concentration. The peak height (a.u.f.s.) and the peak area (a.u.f.s. min) recorded on the chromatogram output were correlated with coefficients greater than 0.99.

Extraction efficiency

Phenyl mercury–6-MP adduct formation is the limiting factor in the extraction of thiopurines into toluene and thence into 0.1 *M* HCl [4]. The recoveries for 6-TG and 6-MP (derived from 6-TIA) over the calibration range were 68.6% (coefficient of variation, C.V., 3.6%) and 73% (C.V. 3.6%), respectively. Re-extracting the biological phase with an additional 6-ml toluene mixture (total volume 8 ml) and back-extracting this with the *same* 200 μl of 0.1 *M* HCl used in the first extraction will increase the extraction efficiency to 84% for 6-TG and 91% for 6-MP. The methyl-MP metabolite behaves in a similar fashion with a second toluene extraction increasing the recorded absorption units by 45%.

Reproducibility

The reproducibility of the assay was evaluated from five measurements at each point of the calibration curve (intra-assay variation) and from eight measurements of a quality control sample consisting of RBCs spiked with 0.3 nmol of 6-TG, 0.3 nmol of 6-TIA and 3 nmol of methyl-MP

TABLE I

INTRA-ASSAY REPRODUCIBILITY IN RED BLOOD CELLS

Concentration (nmol/8 · 10^8 red cells)	Absorbance (mean ± S.D.) (m.a.u.f.s.)	C.V. (%)
Methyl-MP		
0.3	0.37 ± 0.017	4.6
0.6	0.82 ± 0.076	9.3
1.2	1.62 ± 0.125	7.7
3.0	3.95 ± 0.223	5.6
6.0	9.05 ± 0.771	8.5
12.0	20.7 ± 1.02	4.9
30.0	55.9 ± 2.20	3.9
6-TG		
0.03	0.12 ± 0.007	5.8
0.12	0.47 ± 0.029	6.2
0.30	1.22 ± 0.026	2.1
0.60	2.56 ± 0.158	6.2
0.90	4.00 ± 0.160	4.0
6-TIA		
0.03	0.17 ± 0.008	4.7
0.12	0.65 ± 0.022	3.4
0.30	1.67 ± 0.051	3.0
0.60	3.41 ± 0.169	5.0
0.90	5.28 ± 0.199	3.8

taken over a two-month period (inter-assay variation). The intra-assay C.V. ranged from 2.1 to 9.3% (Table I) whilst the quality control concentrations for 6-TG, 6-TIA and methyl-MP were 0.297 ± 0.017 nmol (C.V. = 5.6%), 0.295 ± 0.012 nmol (C.V. = 4.0%) and 3.03 ± 0.082 nmol (C.V. = 2.7%), respectively. The lower limit of reproducibility was 0.03 nmol for 6-TG, 0.03 nmol for 6-TIA and 0.12 nmol for methyl-MP extracted from 8 · 10^8 RBCs.

Patient samples

Blood samples were obtained from six children with acute lymphoblastic leukaemia receiving daily 6-MP (75 mg/m^2) as part of their continuing chemotherapy. The children routinely took their 6-MP prior to breakfast; the blood samples were obtained at least 6 h post-dose. Thiopurines and the methyl-MP metabolite were measured in both RBCs and plasma, intracellular thionucleotides were measured in the RBCs. Neither 6-MP nor methyl-MP were detected in the 6-h post-dose plasma sample. The RBCs did not contain any thiopurines. RBC 6-TGN concentrations ranged from 156 to 880 pmol per 8 · 10^8 cells. Three children also had 6-TIA in their RBCs. RBC methyl-MP metabolites ranged from 1.66 to 20.9 nmol per 8 · 10^8 cells (Table II).

DISCUSSION

We have identified an unknown compound observed in the chromatogram of leukaemic children receiving 6-MP therapy [4] as a methyl-MP metabolite. The aim of the assay modification reported in this paper was to optimize the measurement of the methyl-MP metabolite extracted by phenyl mercury adduct formation without losing sensitivity in the 6-TGN and 6-TIA assays. This was achieved by decreasing the pH of thio-

TABLE II

RED BLOOD CELL 6-MERCAPTOPURINE METABOLITE CONCENTRATIONS

Subject	6-Thioguanine nucleotides (pmol/8 · 10^8 RBCs)	6-Thioinosinic acid (pmol/8 · 10^8 RBCs)	6-Methylmercaptopurine metabolite (nmol/8 · 10^8 RBCs)
1	156	0	8.4
2	329	0	2.7
3	401	60	8.3
4	335	98	10.7
5	880	0	1.7
6	174	130	20.9

purine–phenyl mercury adduct formation down to 11.6. This doubled the amount of methyl-MP extracted without decreasing sensitivity in the already established 6-TGN assay. The photodiode array detector enables each metabolite to be reported at its λ_{max}.

A number of important observations were made with respect to the assay of the methyl-MP metabolite. Firstly, the peak eluting at 8.2 min is not methyl-MP *per se*. Methyl-MP does not form an adduct with PMA — it has no free thiol group. Methyl-MP required heating to extract it into the toluene layer. If PMA was omitted from the toluene mixture it did not extract, thus, one can presume the formation of a PMA adduct. The heating step presumably modified the methyl-MP so that, after the addition of NaOH, a lone-pair on the sulphur centre could complex with the soft Lewis acid (Hg^{2+}) represented by PMA [7].

The possibility exists that the methyl-MP inside the RBC is not the free base but a nucleotide metabolite. However, the assay does not differentiate between these two 6-MP metabolites. The acid hydrolysis step modifies methyl-MP such that a free thiol group is formed but it would also break down any nucleotide back to the purine. No methyl-MP was detected in the plasma even when intracellular methyl-MP metabolite concentrations were in excess of 20 nmol per $8 \cdot 10^8$ RBCs. The form of methyl-MP metabolite inside the RBC is stable for at least 48 h at 20°C, it does not leak out of the RBC or equilibrate with the plasma. These observations are compatible with a nucleotide metabolite.

A similar assay to that reported here was described in 1987 using a Nova-Pak C_{18} cartridge (type/control No. 8NVC$_{18}$5μ/P4150D01, Waters Chromatography) and this cartridge is still in use [4]. But, since the publication of this first assay the manufacturing process for the Nova-Pak cartridge has changed [8] and the end-capping procedure modified. The Nova-Pak cartridge is now fully end-capped and will not separate mixtures of 6-TG and 6-MP, even with adjustments in the methanol concentration, or the other constituents, of the mobile phase. The Resolve column, which has no end-capping, retains the polar thiopurines and the separation of a mixture of 6-TG, 6-MP and 6-TX is very similar to that previously reported for the Nova-Pak [4].

The quantitation of a variety of 6-methylthiopurines, 6-MP and 6-TG metabolites, in cultured cells, has previously been reported using high-performance cation-exchange chromatography and UV detection. But, the complete separation of all the compounds in a single sample takes 140 min [9]. There are no other reported methods for the quantitation of both 6-thiopurines and 6-methylthiopurines on the same HPLC system but there are a number of separate HPLC methods which have been developed for the quantitation of thiopurines or methylthiopurines, either as the free base or the nucleotide metabolite, in clinically relevant concentrations. Assays have been developed which measure the free base released after the acid hydrolysis of 6-TIA and the 6-TGNs. The extracted thiopurines are separated on a reversed-phase column and detected by the UV absorption of 6-MP or 6-TG [4] or the fluorescence of the permanganate oxidised thiol group [10]. Thionucleotide metabolites have been quantitated by UV detection using either a strong anion-exchange column with gradient elution [11] or by ion-pair HPLC using a reversed-phase C_{18} column [12]. Methylthiopurines have been measured in biological fluids with reversed-phase C_{18} HPLC followed by fluorimetric detection [13] or by separation on a cyanopropylsilane column and UV (diode array) detection [9].

The HPLC assay reported here enables two important sets of 6-MP metabolites, the cytotoxic 6-TGNs and the methyl-MP metabolites, to be quantitated simultaneously in 100 μl of packed RBCs. The time taken for chromatographic separation is 10 min, a suitable time interval for routine HPLC analysis. The assay of both methyl and nucleotide 6-MP metabolites in the same sample enables those children who do not respond to 6-MP, for whatever reason (*e.g.* drug metabolism, absorption or compliance), to be quickly identified.

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