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BIOMEDICAL APPLICATIONS

## Determination of extracellular and intracellular thiopurines and methylthiopurines by high-performance liquid chromatography

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### Abstract

The thiopurine antimetabolites 6-thioguanine and 6-mercaptopurine are important chemotherapeutic drugs in the treatment of childhood acute lymphoblastic leukaemia. Measurement of metabolites of these thiopurines is important because correlations exist between levels of these metabolites and the prognosis in childhood acute lymphoblastic leukaemia. The reversed-phase method for the determination of extracellular thiopurine nucleosides and bases was previously developed and has been modified such that methylthiopurine nucleosides, bases, thioxanthine and thiouric acid can be measured also. The anion-exchange method enables the determination of intracellular mono-, di- and triphosphate (methyl)thiopurine nucleotides in one run. Extraction on ice with perchloric acid and dipotassium hydrogenphosphate results in good recoveries for (methyl)thiopurine nucleotides in lymphoblasts and peripheral mononuclear cells and for methylthioinosine nucleotides in red blood cells. Measurement of the low concentrations of mono-, di- and triphosphate thioguanine nucleotides in red blood cells (detection limit 20 pmol/10<sup>9</sup> cells) is possible after extraction with methanol and methylene chloride, followed by oxidation of thioguanine nucleotides with permanganate and fluorimetric detection.

### 1. Introduction

Purines and pyrimidines play an essential role in human cell metabolism. The thiopurine antimetabolites 6-thioguanine (6TG) and 6-mercaptopurine (6MP) are important chemotherapeutic drugs in the treatment of childhood acute lymphoblastic leukaemia (ALL). Since 1953 6MP and 6TG have been administered orally in cases of childhood ALL and other leukaemias. Recent-

ly the administration of high-dose 6MP infusions has started in clinical trials [1].

Several studies showed correlations between levels of extracellular and intracellular metabolites of low-dose oral 6MP and prognosis of childhood ALL [2,3]. Because 6MP and 6TG are prodrugs and exhibit their cytotoxic effects by various intracellular metabolic routes, it is important to develop methods by which extracellular as well as intracellular metabolites can be measured as nucleosides and nucleobases and as mono-, di- and triphosphate nucleotides.

The metabolism of 6TG and 6MP is indicated

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in Fig. 1. Thioguanine nucleotides (TGN), which cause a delayed cytotoxic reaction after incorporation into DNA and RNA, and methylthioinosine monophosphate (MetIMP), which is an inhibitor of the purine de novo synthesis, are responsible for the cytotoxic action of thiopurines in vitro [4–6]. Conflicting data exist about the relative importance of each cytotoxic pathway in vivo. Because thiopurine methyltransferase, an important enzyme involved in the metabolism of thiopurines and the formation of MetIMP, shows a genetic polymorphism [7], it is important to elucidate the intracellular cytotoxic

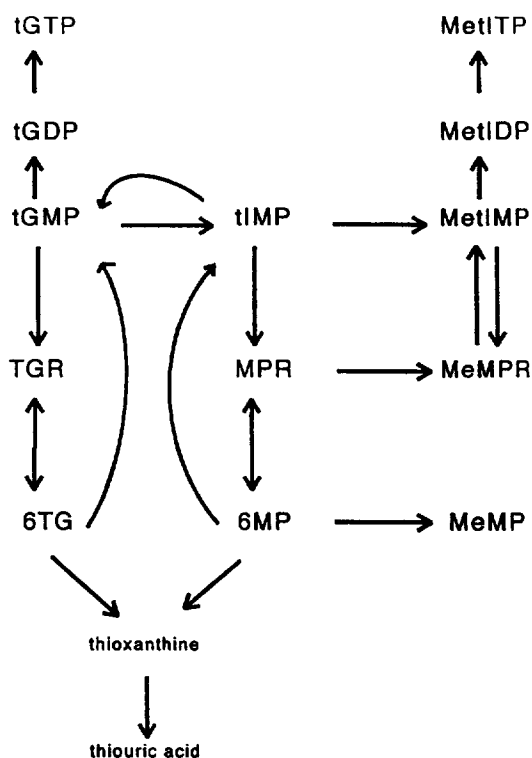


Fig. 1. Thiopurine metabolism. (Methyl)thiopurine nucleotides: tGM(D,T)P = 6-thioguanosine mono-(di-, tri-)phosphate, MetIM(D,T)P = 6-methylthioinosine mono-(di-, tri-)phosphate, tIMP = 6-thioinosine monophosphate. (Methyl)thiopurine nucleosides: TGR = 6-thioguanine riboside, MPR = 6-mercaptopurine riboside, MeMPR = 6-methylmercaptopurine riboside. (Methyl)thiopurine bases: 6TG = 6-thioguanine, 6MP = 6-mercaptopurine, MeMP = 6-methylmercaptopurine.

pathways in vivo in order to select patients at risk for high toxicity or for undertreatment.

Previously, we published HPLC methods for the detection of purine and pyrimidine nucleotides [8], nucleosides and bases [9] and thiopurine nucleosides and bases [10,11]. In the present article we describe two HPLC methods, one for measurement of methylthiopurine and thiopurine nucleosides and bases, and another for measurement of (methyl)thiopurine nucleotides. Both methods are used in the clinical randomized ALL-8 trial of the Dutch Childhood Leukaemia Study Group.

Several methods have been described for the measurement of (methyl)thiopurine nucleotides in red blood cells (RBC) [12–18] and in lymphoid cells [19–21]. Many of these methods [13–16,19] measure these nucleotides only indirectly after hydrolysis to their bases and thus require an extra measurement of (methyl)thiopurine nucleosides and bases before hydrolysis to establish which part of the base originates from the hydrolyzed nucleotides. Moreover, no distinction can be made between the concentrations of mono-, di- and triphosphate nucleotides. An ion-pairing HPLC assay enables the measurement of thioguanosine monophosphate (tGMP) and thioinosine monophosphate (tIMP) [20] and an anion-exchange fluorimetric method detects tGMP, tGDP and tGTP [21] in human neoplastic cells. Extraction of tGMP, tGDP and tGTP from RBC was performed with mercurial cellulose resin and mercaptoethanol, with extraction efficiencies of 75–90% [17]. A less laborious procedure for extraction of TGN from RBC has recently been developed by Rabel et al. [18]. RBC are deproteinized and TGN extracted with methanol and methylene chloride. After this TGN are oxidized with permanganate [18,21], separated by capillary electrophoresis and detected with laser-induced fluorescence [22]. This procedure results in extraction efficiencies of 86–95% [18] and in attomole detection limits [22].

The anion-exchange HPLC method we describe in this article enables the detection in one run of separate (methyl)thiopurine nucleotides in lymphoblasts and peripheral mononuclear cells.

Detection of methylthioinosine nucleotides (MeTIN) and TGN in RBC still has to be done by two separate runs in our system.

## 2. Experimental

### 2.1. Chemicals

(Methyl)thiopurine nucleosides and bases, thioinosine monophosphate (tIMP), dithiothreitol, 5-phosphorylribose-1-pyrophosphate, bovine serum albumin, xanthine oxidase and hypoxanthine guanine phosphoribosyltransferase were obtained from Sigma (St. Louis, MO, USA). Thioinosinetriphosphate (tITP) was from Pharmacia/LKB (Woerden, Netherlands). All other chemicals were obtained from E. Merck (Darmstadt, Germany). Water used for all buffers was purified in a Milli-Q System (Millipore, Bedford, MA, USA).

### 2.2. Enzymatic preparation of thiouric acid

Thiouric acid was produced by enzymatic oxidation of thioxanthine with xanthine oxidase, according to the procedure described by the manufacturer of the enzyme. The conversion into thiouric acid was 100% as determined by HPLC.

### 2.3. Preparation of TGN

tGMP was prepared by enzymatic conversion of 6TG with hypoxanthine guanine phosphoribosyltransferase in the presence of 5-phosphorylribose-1-pyrophosphate (PRPP). The reaction was performed at 37°C overnight with 500  $\mu$ l 0.4  $\mu$ M 6TG, 500  $\mu$ l 8 mM PRPP, 480  $\mu$ l Tris-MgCl<sub>2</sub> buffer (0.5 M Tris + 0.05 M Tris-MgCl<sub>2</sub>, pH 7.4), 20  $\mu$ l hypoxanthine guanine phosphoribosyltransferase (250 units in 500  $\mu$ l) in Tris-MgCl<sub>2</sub> buffer and 50  $\mu$ l Triton X-100 10%. The conversion into tGMP was 100% as determined by HPLC.

Thioguanosinediphosphate (tGDP) and thioguanosinetriphosphate (tGTP) were obtained by incubating Molt-F4 lymphoblasts with

6-thioguanine and separating the TGN by HPLC from the cell free extracts.

### 2.4. Preparation of MeTIN

MetIMP was synthesized by methylation of tIMP: 0.026 mmol of tIMP was dissolved in 500  $\mu$ l 2 M ammonia, 5  $\mu$ l 0.40 mM methyl iodide was added and the mixture was mixed immediately and incubated at room temperature for 2 h. The suspension was flushed with N<sub>2</sub> for 10 min in order to remove the excess of ammonia, frozen and lyophilized.

The same procedure was used to synthesize methylthioinosinetriphosphate (MetITP) and methylthioinosinediphosphate (MetIDP) from a mixture of tITP and thioinosinediphosphate (tIDP). With this procedure the methylation of thioinosine nucleotides was 100% as determined by HPLC.

### 2.5. Stock solutions

Stock solutions of the (methyl)thiopurine nucleosides and bases were prepared as described before [10]. The exact concentrations were determined spectrophotometrically. Molar absorption coefficients were used at pH 4.6 as described [23].

Stock solutions of tIMP and tITP were prepared by dilution of 10 mg nucleotide in 10 ml distilled water. Exact concentrations were calculated at pH 4.6 with:  $\epsilon_{\max} = 27.6 \text{ mM}^{-1} \text{ cm}^{-1}$  at  $\lambda_{\max} = 322 \text{ nm}$ . TGN and MeTIN were prepared as described above and the concentrations were measured spectrophotometrically at pH 4.6 with  $\epsilon_{\max} = 26.7 \text{ mM}^{-1} \text{ cm}^{-1}$  at  $\lambda_{\max} = 342 \text{ nm}$  for TGN and  $\epsilon_{\max} = 18.9 \text{ mM}^{-1} \text{ cm}^{-1}$  at  $\lambda_{\max} = 291 \text{ nm}$  for MeTIN [23].

### 2.6. Extraction of (methyl)thiopurine nucleosides and bases

Extraction of plasma was performed with 1/20 volume of ice-cold 8 M perchloric acid (PCA). The suspension was mixed and kept on ice for 10 min. After centrifugation at 10,000 g for 5 min

the supernatant was adjusted to pH 6–7 with 1/10 volume of ice-cold 4 M dipotassium hydrogenphosphate, and kept on ice for another 10 min. The precipitated potassium perchlorate was removed by centrifugation at 10,000 g for 5 min and the supernatant was stored at  $-20^{\circ}\text{C}$  until measurement.

### 2.7. Extraction of (methyl)thiopurine nucleotides

Peripheral mononuclear cells were separated from defibrinated blood by a ficoll isolation procedure. Contaminating RBC were removed by a  $\text{NH}_4\text{Cl}$ -shock [24]. Lymphoblasts of the Molt-F4 T-ALL cell line were cultured as described [5]. Cell pellets of peripheral mononuclear cells or Molt-F4 cells were resuspended in 100  $\mu\text{l}$  phosphate buffered saline with 1% (w/v) bovine serum albumin and 5% (w/v) dithiothreitol. Extraction was performed on ice with 50  $\mu\text{l}$  of 1.2 M PCA and the supernatant was adjusted to pH 6–7 with 25  $\mu\text{l}$  of 4 M dipotassium hydrogenphosphate.

RBC were isolated from heparinized blood and washed thrice with 0.9% saline. One volume of RBC was resuspended in approximately 2 volumes 0.9% saline with 1% (w/v) dithiothreitol. Extraction of MeTIN was performed with PCA as described above with 1/20 volume of 8 M PCA and 1/10 volume of 4 M dipotassium hydrogenphosphate. Extraction of TGN from 100  $\mu\text{l}$  RBC was performed with 150  $\mu\text{l}$  55 mM EDTA (pH 10.5), 100  $\mu\text{l}$  methanol and 500  $\mu\text{l}$  methylene chloride as described by Rabel et al. [18]. All cell extracts were stored at  $-80^{\circ}\text{C}$  until measurement.

### 2.8. Oxidation of TGN in RBC

Oxidation of TGN was performed with 100  $\mu\text{l}$  RBC extract, 10  $\mu\text{l}$  1 M sodium carbonate (pH 10.1), 100  $\mu\text{l}$  0.24% potassium permanganate and 10  $\mu\text{l}$  hydrogen peroxide as described by Rabel et al. (22).

### 2.9. HPLC apparatus

Measurements were performed with a Thermo Separation Products HPLC system, which con-

sists of a ternary HPLC pump (SP 8800), an automatic sampler (SP 8880) and a variable UV-Vis absorbance detector (SpectraFocus 2000 HR system), set at three wavelengths (290, 320 and 342 nm). Fluorimetric detection was performed with a Japan Spectroscopic Co fluorescence detector (Model 821-FP). For measurement of TGN  $\lambda_{\text{ex}} = 329$  nm and  $\lambda_{\text{em}} = 410$  nm [17]. Measurements were performed at room temperature and during elution all solutions were deaerated with helium. Injection volumes were 100  $\mu\text{l}$ .

### 2.10. Chromatography of (methyl)thiopurine nucleosides and bases

Separation of (methyl)thiopurine nucleosides and bases was performed in 1-h runs on a reversed-phase column (Supelcosil LC-18-DB,  $250 \times 4.6$  mm I.D., particle size 5  $\mu\text{m}$ , Supelco, Bellefonte, PA, USA). Chromatography was carried out as presented in Table 1.

### 2.11. Chromatography of (methyl)thiopurine nucleotides

Separation of (methyl)thiopurine nucleotides was performed on an anion-exchange column

Table 1  
Mobile-phase sequence used for separation of (methyl)thiopurine nucleosides and bases

| Time (min) | A (% v/v) | B (% v/v) |
|------------|-----------|-----------|
| 0          | 98        | 2         |
| 5          | 96        | 4         |
| 8          | 85        | 15        |
| 10         | 80        | 20        |
| 20         | 40        | 60        |
| 22         | 40        | 60        |
| 25         | 20        | 80        |
| 45         | 20        | 80        |
| 48         | 98        | 2         |
| 60         | 98        | 2         |

A = 0.025 M potassium dihydrogenphosphate; B = 0.05 M potassium dihydrogenphosphate, 25% (v/v) methanol. Flow-rate 1.25 ml/min.

Table 2  
Mobile-phase sequence used for separation of (methyl)thiopurine nucleotides

| Time (min) | A (% v/v) | B (% v/v) | C (% v/v) |
|------------|-----------|-----------|-----------|
| 0          | 10        | 90        | 0         |
| 6          | 20        | 80        | 0         |
| 8          | 44        | 44        | 12        |
| 14         | 40        | 40        | 20        |
| 30         | 10        | 10        | 80        |
| 68         | 10        | 10        | 80        |
| 69         | 10        | 90        | 0         |
| 90         | 10        | 90        | 0         |

A = 0.05 M potassium dihydrogenphosphate, 3% acetonitrile, adjusted to pH 3.25 with phosphoric acid; B = 3% acetonitrile; C = 0.5 M potassium dihydrogenphosphate, 1.5% acetonitrile, adjusted to pH 5.25 with potassium hydroxide. Flow-rate 1.30 ml/min.

(Partisil-10-SAX, 250 × 4.6 mm I.D., particle size 10 μm, Whatman, Clifton, NJ, USA). Chromatography was carried out with a minor modification of the method described earlier [8]. The gradient, indicated in Table 2, allows a reduced

runtime as compared to the method described before [8] without loss of resolution.

### 2.12. Recovery of (methyl)thiopurine nucleosides, bases and nucleotides

Dithiothreitol was added in a concentration of 60 mg/l [10] to plasma, RBC and suspensions of peripheral mononuclear cells or Molt-F4 lymphoblasts, an ALL cell line used for the study of thiopurine metabolism [5]. Standards of (methyl)thiopurines were added in various concentrations and extractions were performed immediately as described above.

## 3. Results

### 3.1. Chromatograms

Retention times of standards of the (methyl)thiopurines are indicated in Table 3. Chromatograms of these compounds, measured in plasma, urine and RBC of patients treated

Table 3  
Retention times of (methyl)thiopurines

|                               | Retention time (min)    | Optimal wavelength (290, 320 or 342 nm)        |
|-------------------------------|-------------------------|--|
| <i>Nucleosides and bases:</i> |                         |  |
| Thiouric acid                 | 5                       | 342  |
| Thioguanine                   | 10                      | 342  |
| 6-Mercaptopurine              | 11                      | 320  |
| Thioxanthine                  | 13                      | 342  |
| Thioguanine riboside          | 17                      | 342  |
| Mercaptopurine riboside       | 18                      | 320  |
| Methylthioguanine             | 28                      | 320  |
| Methylmercaptopurine          | 33                      | 290  |
| Methylmercaptopurine riboside | 42                      | 290  |
| <i>Nucleotides:</i>           |                         |  |
| MetIMP, MetIDP, MetITP        | 12, 23, 35 <sup>a</sup> | 290  |
| tIMP, tIDP, tITP              | 13, 26, 39              | 320  |
| tGMP, tGDP, tGTP              | 15, 30, 50              | 342  |
| Oxidized tGMP, tGDP, tGTP     | 12, 23, 34 <sup>a</sup> | $\lambda_{ex}$ 329 nm<br>$\lambda_{em}$ 410 nm |

<sup>a</sup> After the oxidation procedure for TGN MeTIN are not detectable with fluorimetric detection.

with a 6MP infusion ( $1300 \text{ mg/m}^2$  in 24 h) are shown in Figs. 2 and 3.

### 3.2. Calibration

Concentrations of thiouric acid were calculated from the amount of thioxanthine from which it was prepared. Since MetIDP and MetITP were

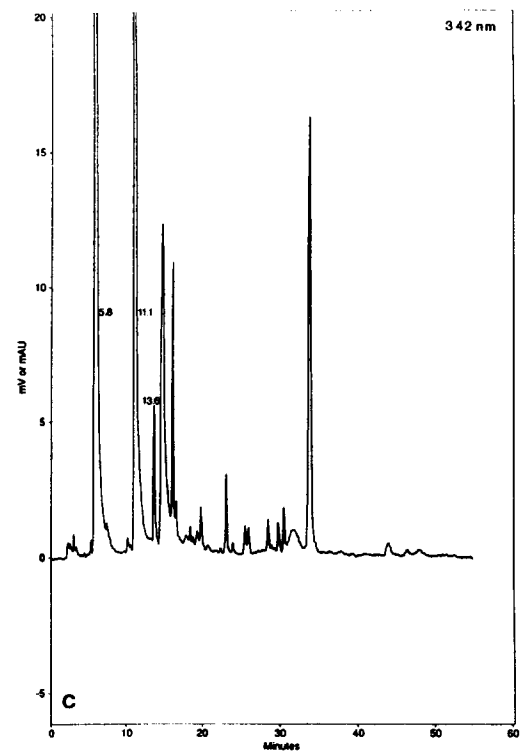
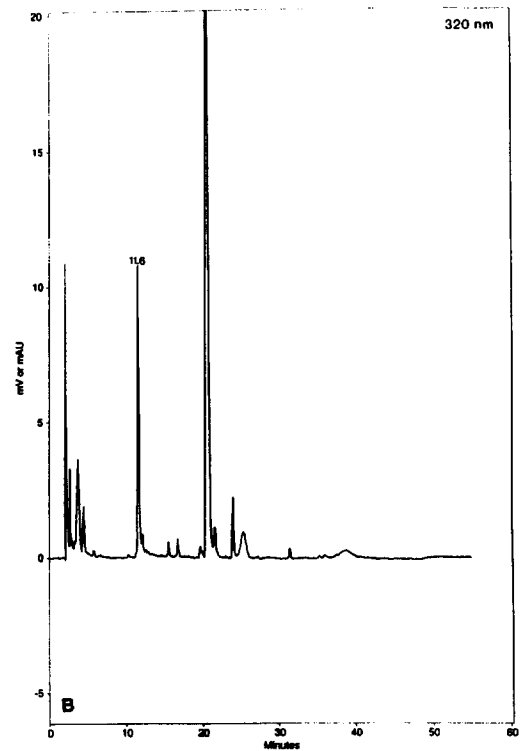
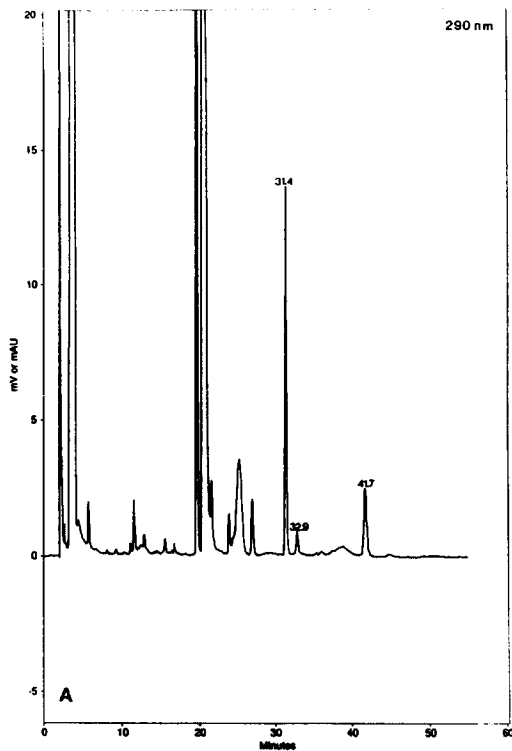


Fig. 2. Chromatograms of (methyl)thiopurine nucleosides and bases in plasma (A and B) and urine (C) of patients with ALL treated with a 24-h infusion of methotrexate ( $5 \text{ g/m}^2$ ) followed by a 24-h infusion of 6MP ( $1300 \text{ mg/m}^2$ ). (A) Plasma sample at 290 nm at the end of the 24 h 6MP infusion: 31.4' = presently, an unidentified peak, which is only present during and shortly after the infusion in plasma of patients treated with a high-dose 6MP infusion. This peak probably represents a metabolite of 6MP, but certainly is not methylthioxanthine or methylthiouric acid, and is presently being investigated for identification. 32.9' = methylmercaptapurine ( $478 \text{ nM}$ ), 41.7' = methylmercaptapurine riboside ( $3792 \text{ nM}$ ). (B) Plasma sample of (A) at 320 nm: 11.6' = 6MP ( $1363 \text{ nM}$ ). (C) Urine sample (diluted  $10\times$ ) at 342 nm of 6 h urine, collected from 6 till 12 h after start of the 6MP infusion: 5.8' = thiouric acid ( $360 \text{ } \mu\text{mol/mmol creatinine}$ ), 13.6' = thioxanthine ( $14 \text{ } \mu\text{mol/mmol creatinine}$ ).

Fig. 2 (continued).

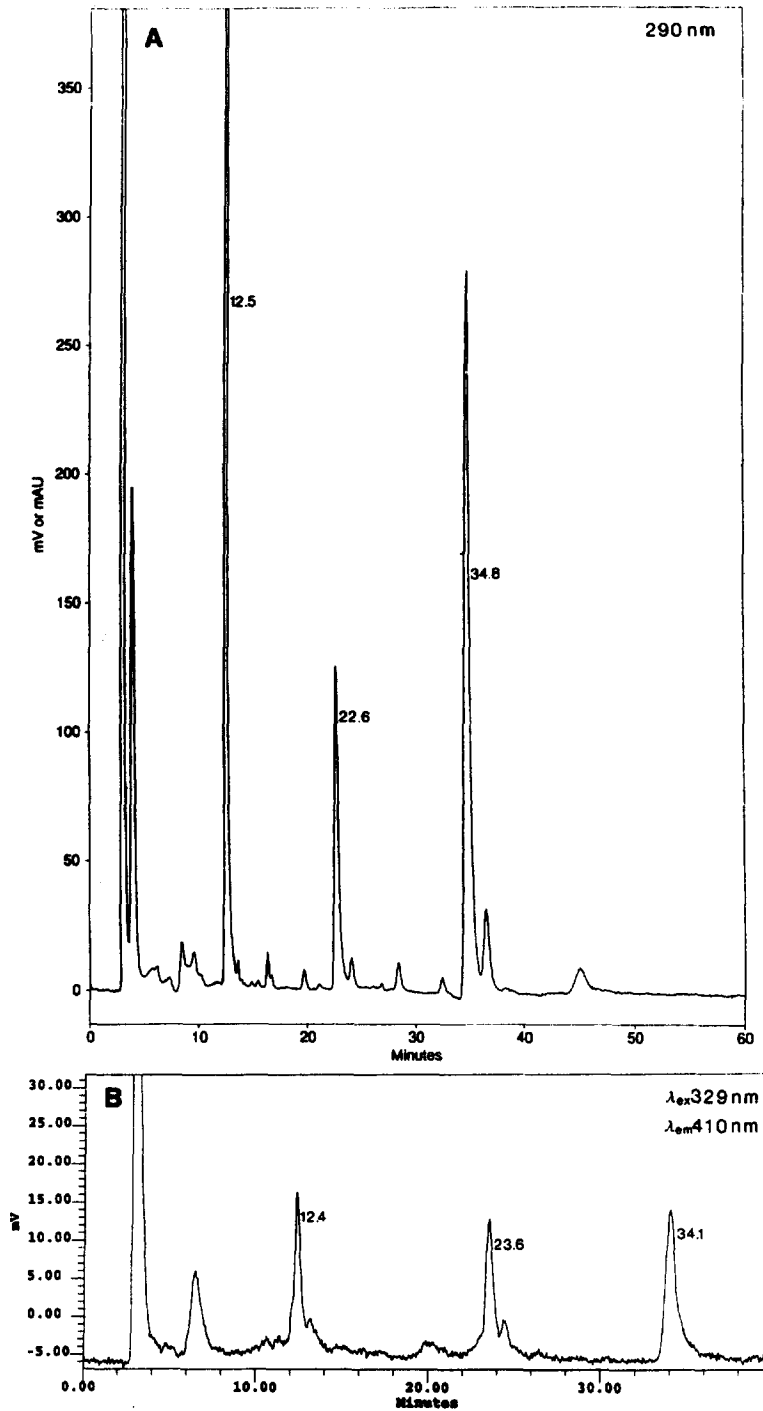


Fig. 3. Chromatograms of (methyl)thiopurine nucleotides in RBC 24 h after termination of a high-dose 6MP infusion. One patient with non-Hodgkin lymphoma (A) was treated with a high-dose 6MP ( $1300 \text{ mg/m}^2$  in 24 h) and another patient with ALL (B) was treated with a 24-h infusion of methotrexate ( $5 \text{ g/m}^2$ ) followed by a 24-h infusion of 6MP ( $1300 \text{ mg/m}^2$ ). (A) UV absorbance detection at 290 nm: 12.5' = MetIMP ( $342 \text{ pmol}/10^8 \text{ RBC}$ ), 22.6' = MetIDP ( $97 \text{ pmol}/10^8 \text{ RBC}$ ), 34.8' = MetITP ( $430 \text{ pmol}/10^8 \text{ RBC}$ ). (B) Fluorimetric detection with excitation at 329 nm and emission at 410 nm: 12.4' = tGMP ( $51 \text{ pmol}/8.10^8 \text{ RBC}$ ), 23.6' = tGDP ( $50 \text{ pmol}/8.10^8 \text{ RBC}$ ), 34.1' = tGTP ( $122 \text{ pmol}/8.10^8 \text{ RBC}$ ).

prepared from tIDP and tITP their concentrations were calculated from the areas under the peaks. Absorbance of MetIMP, determined spectrophotometrically under chromatographic conditions of MetIDP and MetITP, did not differ from these two compounds.

Detection limits for the (methyl)thiopurine nucleosides and bases were between 20–50 nM, except for thiouric acid (100 nM). Detection limits measured by UV absorption were 110 nM for tIMP, 380 nM for tGMP and 130 nM for MetIMP (11, 38 and 13 pmol per 100  $\mu$ l injection). The detection limits measured fluorimetrically were 15–35 nM for tGMP, tGDP and tGTP (1.5–3.5 pmol per injection).

### 3.3. Reproducibility

The within-day ( $n=5$ ) and the day-to-day ( $n=3$ ) coefficients of variation for 6TG, 6MP, TGR and MPR were in similar ranges as described previously (1.4–12.5%) [10]. These coefficients of variation were also determined for thioxanthine, MeMP and MeMPR. The within-day variation for these three compounds was in the range of 0.4–2.7% for concentrations above 150 nM and 5.6–11.2% for concentrations below 150 nM. The day-to-day variations were 0.1–2.7% for concentrations above 150 nM and 6.5–14.7% for concentrations below this level. MeTG was not detectable at levels below 150 nM and coefficients of variation at levels above 150 nM were 4–8.5% for within-day variation and 5.9–6.0% for day-to-day variation.

### 3.4. Recovery of (methyl)thiopurine nucleosides and bases in plasma

Standards of 6MP, thioxanthine, MPR, MeMP and MeMPR, metabolites which are produced during high-dose 6MP infusions, were added to plasma in a concentration range of 0.4–15  $\mu$ M. The recovery of 6MP was in the range of 80–98%, of thioxanthine 68–81%, of MPR 89–101%, of MeMP 75–89% and of MeMPR 68–102%.

### 3.5. Recovery of (methyl)thiopurine nucleotides

The recoveries after extraction with PCA of (Me)TIN and of tGMP added to Molt-F4 lymphoblasts in varying concentrations are indicated in Table 4. More than 89% of the standards was recovered in all cases.

Recoveries of MetIMP after extraction with PCA in RBC (31–309 pmol/ $10^8$  cells) were in the range of 87–109% and in peripheral mononuclear cells (18–180 pmol/ $10^6$  cells) in the range of 70–106%. Recovery of tIMP and tGMP after extraction with PCA in peripheral mononuclear cells (18–180 pmol/ $10^6$  cells) were 71–104% and 87–104%, respectively. TGN were poorly extracted from RBC or hemolysates with PCA.

Extraction of RBC with EDTA, methanol and methylene chloride [18] and measurement with UV absorbance detection (290–340 nm) resulted in a large peak, which was caused by the combination of DTT and EDTA and which disturbed the chromatogram from 15 to 25 min as well as the recoveries. After oxidation of the RBC extract and fluorimetric detection TGN could be reproducibly measured in RBC at levels above 20 pmol per  $10^9$  RBC and the DTT–EDTA peak was not observed under these conditions. Simultaneous measurement with UV of MeTIN in the oxidized supernatant gave poor results at levels which are clinically obtained and became only reproducible at levels above 100  $\mu$ M. Thus, the MeTIN and TGN levels in RBC have to be measured by separate runs.

Table 4  
Recovery of (methyl)thiopurine nucleotides in  $4.7 \cdot 10^6$  Molt-F4 lymphoblasts

| Standard | Concentration range (pmol/ $10^6$ cells) | Recovery (%) |
|----------|--|--------------|
| tIMP     | 51–819                                   | 93–98        |
| tIDP     | 41–866                                   | 96–99        |
| tITP     | 78–1016                                  | 97–99        |
| tGMP     | 65–720                                   | 89–98        |
| MetIMP   | 46–937                                   | 92–99        |
| MetIDP   | 62–817                                   | 92–99        |
| MetITP   | 87–874                                   | 94–99        |



#### 4. Conclusion

The two HPLC methods described here enable measurement of extracellular and intracellular (methyl)thiopurine metabolites in ALL cell lines and in patients materials. These methods are needed for preclinical [5] and clinical [25, 26] research, involving the metabolism and cytotoxic pathways of thiopurines and the relation between thiopurine metabolites and treatment outcome in childhood ALL.

The method for the measurement of extracellular thiopurine nucleosides and bases previously described [10] has been modified. The present method includes also measurement of thioxanthine, thiouric acid and methylthiopurine nucleosides and bases in the same run. The recoveries of (methyl)thiopurine nucleosides and bases from plasma with PCA are good. The recovery of 6MP from plasma with trichloroacetic acid is even better (94%) [9].

The HPLC method with UV absorbance detection at two wavelength channels for intracellular metabolites enables the measurement of the (methyl)thiopurine mono-, di- and triphosphatenucleotides in a single run. Extractions on ice with PCA result in good recoveries for (methyl)thiopurine nucleotides in lymphoblasts and peripheral mononuclear cells and for MeTIN in RBC. This extraction procedure is inadequate for TGN levels in RBC. Extraction and oxidation of TGN in RBC, according to the method of Rabel et al. [18], and measurement with a fluorescence detector enables the detection of separate TGN in RBC at low concentrations.

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